P-TYPE ATPASES IN HEALTH AND DISEASE

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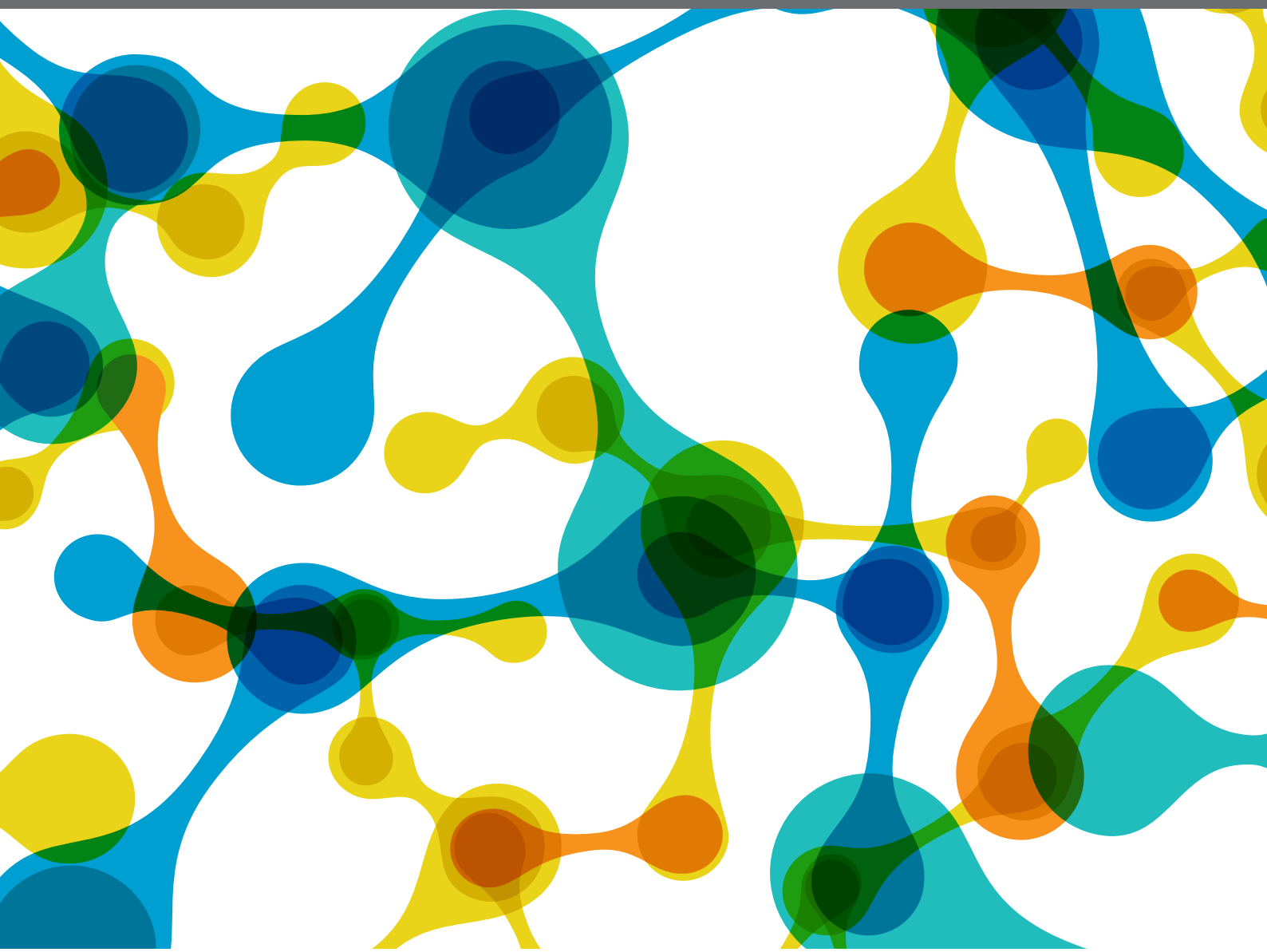

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P-TYPE ATPASES IN HEALTH AND DISEASE

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Improved Model of Proton Pump Crystal Structure Obtained by Interactive Molecular Dynamics Flexible Fitting Expands the Mechanistic Model for Proton Translocation in P-Type ATPases

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The plasma membrane H^+-ATPase is a proton pump of the P-type ATPase family and essential in plants and fungi. It extrudes protons to regulate pH and maintains a strong proton-motive force that energizes e.g., secondary uptake of nutrients. The only crystal structure of a H^+-ATPase (AHA2 from *Arabidopsis thaliana*) was reported in 2007. Here, we present an improved atomic model of AHA2, obtained by a combination of model rebuilding through interactive molecular dynamics flexible fitting (iMDFF) and structural refinement based on the original data, but using up-to-date refinement methods. More detailed map features prompted local corrections of the transmembrane domain, in particular rearrangement of transmembrane helices 7 and 8, and the cytoplasmic N- and P-domains, and the new model shows improved overall quality and reliability scores. The AHA2 structure shows similarity to the Ca^{2+}-ATPase E1 state, and provides a valuable starting point model for structural and functional analysis of proton transport mechanism of P-type H^+-ATPases. Specifically, Asp684 protonation associated with phosphorylation and occlusion of the E1P state may result from hydrogen bond interaction with Asn106. A subsequent deprotonation associated with extracellular release in the E2P state may result from an internal salt bridge formation to an Arg655 residue, which in the present E1 state is stabilized in a solvated pocket. A release mechanism based on an in-built counter-cation was also later proposed for Zn^{2+}-ATPase, for which structures have been determined in Zn^{2+} released E2P-like states with the salt bridge interaction formed.

Keywords: P-type ATPases, plasma membrane proton pump, proton gradient, *Arabidopsis thaliana* AHA2, molecular dynamics, iMDFF, crystallography, membrane transport

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INTRODUCTION

The *Arabidopsis thaliana* plasma membrane H^+-ATPase 2 (AHA2) is a member of the P_{III}-subtype of the P-type ATPase superfamily. It pumps protons out of the cell to maintain a steep electrochemical H^+ gradient and potential across the plasma membrane (Serrano et al., 1986; Blatt et al., 1987). The H^+-ATPases are of fundamental importance in plants and fungi, as well as several prokaryotes (Pedersen et al., 2014). They maintain a membrane potential at around −150 mV in plants, even down to −300 mV in fungi, control intracellular H^+ homeostasis and extracellular acidification, and potentiate secondary transporters involved in e.g., nutrient uptake (Briskin, 1990).

P_{III}-type H^+-ATPases are substantially different in sequence and function to the P_{II}-subtype ATPases such as the gastric H^+/K^+-ATPases in animals. A total of 11 isoforms of H^+-ATPases have been identified in *A. thaliana* (Harper et al., 1994), of which AHA1 and AHA2 are the most abundantly expressed in the plasma membrane. AHA1 and AHA2 can compensate for each other, while the deletion of both genes is lethal (Haruta et al., 2010). *Neurospora crassa* has only one plasma membrane H^+-ATPase (PMA), which is essential for cell growth. In *Saccharomyces cerevisiae* two isoforms (PMA1 and PMA2) are found, of which PMA1 is constitutively expressed and essential (Serrano et al., 1986), but AHA2 can compensate a PMA1 knockout (Palmgren and Christensen, 1993). Due to the important role of plasma membrane H^+-ATPases for cellular life in plants and fungi, and their significant differences to human pumps, they represent attractive targets for anti-fungal and herbicidal strategies (Schubert and Peura, 2008; Yatime et al., 2009).

Overall, the P_{III}-type plasma membrane H^+-ATPases share a similar fold with the P_{II} subfamily including Na^+/K^+-ATPases, H^+/K^+-ATPases, and Ca^{2+}-ATPases present in animal cells (Bublitz et al., 2011). Structural organization of AHA2 encompasses a cytoplasmic headpiece formed by three domains; the nucleotide (N) binding domain, the phosphorylation (P) domain, and the actuator (A) domain, as well as a membrane domain composed of 10 transmembrane segments harboring the proton binding site and the translocation pathway (**Figure 1A**; Pedersen et al., 2007). Additionally and similar to other members of the P_{III} subfamily (Mandala and Slayman, 1989; Portillo et al., 1989), AHA2 has N- and C-terminal extensions involved in auto-regulatory functions (Ekberg et al., 2010a), the latter being considerably longer and referred to as the R-domain. The first and so far only direct structure determination of a H^+-ATPase was obtained of AHA2 in 2007 from highly anisotropic, low-resolution crystallographic data (Pedersen et al., 2007). Model building and refinement with difficult data of this kind remains very challenging even today, and the 2007 model reflected this although it clearly expanded our knowledge on the spatial organization and architecture of AHA2 and afforded models on the transport mechanism.

Here, we have made use of new model-building and refinement tools to present a new, re-refined atomic model of AHA2, which can be employed for more accurate structural comparisons and models of transport. Rebuilding and re-refinement employed the new interactive molecular dynamics flexible fitting (iMDFF) approach (Croll and Andersen, 2016) in combination with phenix.refine (Adams et al., 2010) and resulted in a significantly improved model with new features emerging in the electron density maps. The revised model as well as the body of published data since 2007 invites a reiteration of the structure/function relationship, and it allows also tentative studies by molecular dynamics simulations in a lipid bilayer environment.

MATERIALS AND METHODS

Rebuilding and Refinement

Revision of the AHA2 model representing two copies (chain A and B) in the asymmetric unit of a $P2_12_12_1$ crystal form was initiated from the coordinates of Protein Data Bank entry 3B8C, via multiple rounds of rebuilding in the iMDFF environment (Croll et al., 2016), interspersed with refinement in PHENIX. Parameters for modeling of AMPPCP and DDM molecules in iMDFF were obtained using the CGENFF server (Vanommeslaeghe and MacKerell, 2012; Vanommeslaeghe et al., 2012), and for refinement in phenix.refine using phenix.elbow. The first few rounds of rebuilding/refinement were carried out using the elliptically truncated data used for a major part of the refinement/rebuilding cycles in the original analysis, but we later observed that using data reprocessed without elliptical truncation improved the stability of refinements substantially, and this was used from then on. As previously observed (Croll and Andersen, 2016), we found that the use of a TLS-only B-factor model (i.e., no individual B-factor refinement) improved the interpretability of maps, particularly during initial rounds. Worth noting, the combination of this simplified B-factor model and the strict handling of non-bonded interactions in the iMDFF environment led to an initial dramatic increase in R_{free} from 0.366 to ∼0.42 as regions making unfavorable interactions were pushed out of density, and it took several rounds of rebuilding and refinement before the R-factors started to drop below those of the original model.

In each round of rebuilding the entire structure was inspected end-to-end via localized iMDFF simulations, with a typical simulation containing 100 contiguous residues as well as the surrounding shell of residues approaching within 5 Å of these. Secondary structure was visualized by a standard cartoon overlay over the backbone atoms and updated every 50 simulation steps, while the status of residues on the Ramachandran plot was mapped to the color of the Cα atoms and updated every five steps. Small corrections such as side chain rotamers or backbone geometry were fixed on-the-fly by either simply tugging on atoms with a haptic interface or applying scripted forces to guide side chain dihedrals toward library target values. Where more substantial rearrangements were deemed necessary (e.g., rearrangement of a flexible loop or shifting the register of a secondary structure element), more localized simulations were run both to limit impact on surroundings and to provide sufficient simulation performance for interactivity. Register shifts were aided by scripted forces, applying position targets to Cα atoms to make the shift and dihedral targets to the backbone

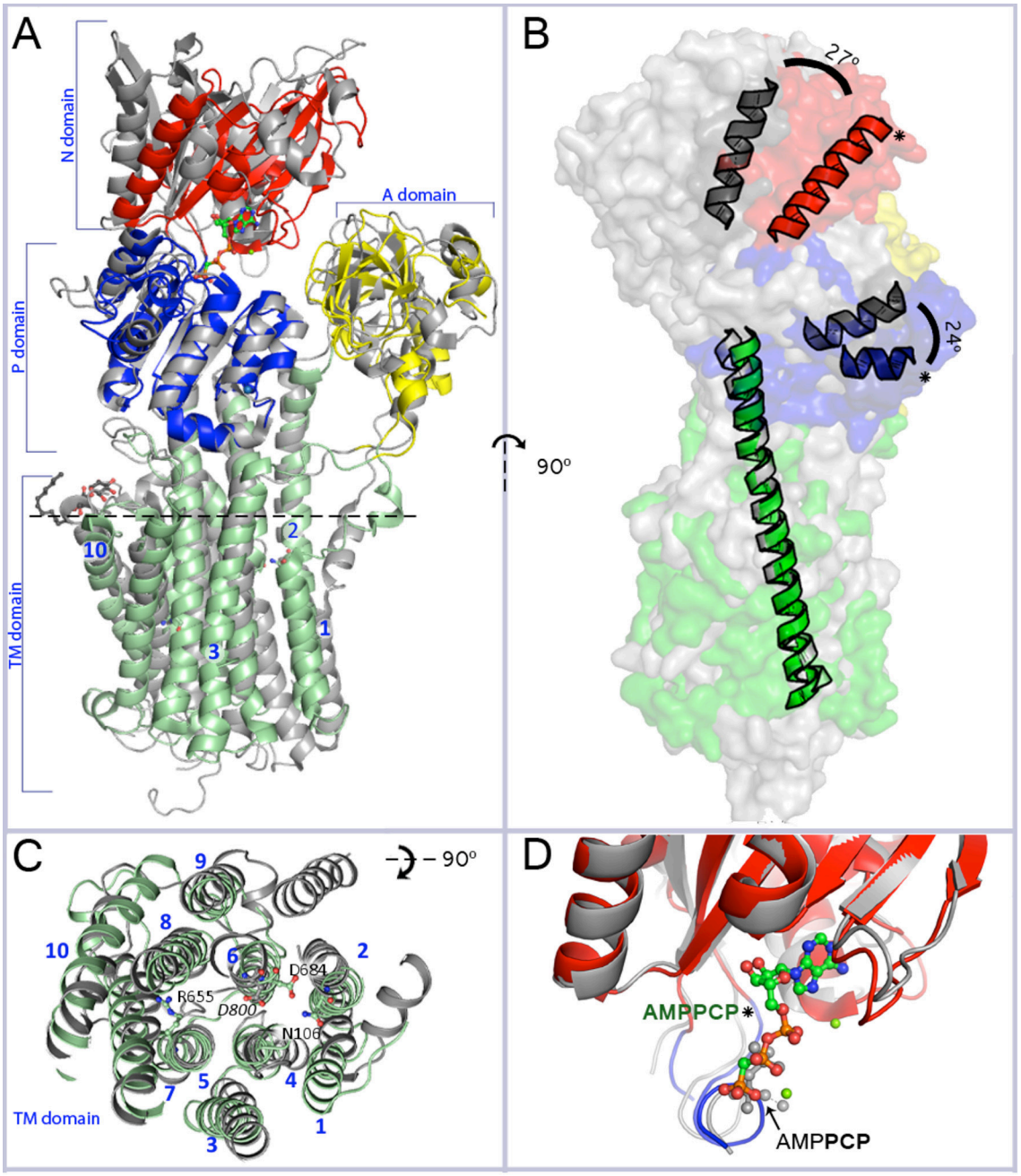

FIGURE 1 | Overall resemblance of AHA2-AMPPCP to the SERCA-SLN complex. (A) Superposition of the revised structure of AHA2-AMPPCP (domains colored as indicated) and the SERCA-SLN complex (gray; pdb id: 4H1W) both representing E1 state. The structures are superimposed on Cα position of the TM or P domains only. Mg^{2+} and K^{+} ions are shown by green and purple-blue spheres, respectively. The spatial organization of the cytoplasmic domains is similar in both E1 state structures. Two loops connecting the A-domain with TM1 and TM3, respectively, align well in AHA2 and SERCA, although for the latter TM1 is longer and kinks into a short helix parallel to the membrane. The TM3 connector forms a longer helix in the A-domain of SERCA compared to AHA2. The N-domain is moved slightly closer toward the A-domain in the SERCA structure. The adenine base part of the AMPPCP molecule is coordinated by side chains of Asp372 and Asp375 and the backbone oxygen of Ser457. The major differences between the P-domains are visible in a region between $Pro533^{AHA2}$ to $Asp559^{AHA2}$, which covers one loop and two short helixes. **(B)** Side view of the aligned pumps (superimposed on TM2–TM10 domains, r.m.s.d 3.07 Å for 159 Cα atoms) showing a tilt of the cytoplasmic headpiece of AHA2 (in colors) relatively to SERCA (gray). The N- and P-domain headpiece of AHA2 is tilted by ~25° (measured between Cα of $Ser436^{AHA2}$ and $Thr538^{SERCA}$ for the N-domain and between Cα of $Pro550^{AHA2}$ and $Pro662^{SERCA}$ for the P-domain, using Cα of $Lys625^{AHA2}$ in TM5 in both cases as an apex). Asterisks mark helices from the P- and N-domain of AHA2. **(C)** Alignment of the transmembrane region. Top view from the plane marked by dashed line at segment A. Catalytically important residues of AHA2 and conserved Asp800 of SERCA are labeled. **(D)** Superposition of the N-domain of AHA2 and SERCA-SLN (r.m.s.d 0.97 Å for 123 atoms superimposed Cα atoms), both in E1 states and showing overlapping binding of β-γ phosphates of AMPPCP. The AMPCPP molecule represents the revised AHA2 model.

φ and ψ angles to maintain secondary structure geometry. All simulations were performed under generalized Born implicit solvent conditions. During most interactive remodeling the simulation temperature was maintained at 100 K. Prior to writing coordinates for crystallographic refinement the entire structure was settled by reducing the temperature from 100 to 0 K in 20 K increments over 10–20 ps of simulation time.

Refinement in PHENIX was typically via the following protocol. A TLS-only B-factor model was first refined in 4–8 rounds with coordinates fixed. This was followed by a further 5–10 rounds of coordinate and TLS refinement with torsion-angle restraints using the input model as a reference. Reference model restraints were then released, and a further 5–10 rounds run with torsion-angle NCS restraints. In the last few rebuilding/refinement rounds, a final refinement step was added to refine individual B-factors.

Molecular Dynamics Simulation

Equilibrium simulations in an explicit membrane/water environment were carried out in NAMD (Phillips et al., 2005) using the CHARMM36 force field (Huang and MacKerell, 2013) in the NPT ensemble with periodic boundary conditions at a rate of 1 fs per time step. Van der Waals interactions were calculated every two time steps and electrostatics every four time steps. The temperature was set to 298 K and pressure to 1 atmosphere. A POPC lipid bilayer was built in VMD (Humphrey et al., 1996), and a single monomer (chain B) of the refined coordinates embedded in it. AMPPCP was handled as ATP. Phospholipid molecules with atoms overlapping protein atoms were deleted, and the resulting construct was solvated in a box of 46,503 TIP3P water molecules and neutralized with 0.15 M KCl. The system was energy minimized for 2,000 steps, and equilibrated for 1 ns with all protein atoms and the ATP fixed in space to dehydrate the membrane and allow settling of lipid around the protein. The simulation was continued for a further 3 ns with side chain atoms and ATP free to move, but with backbone atoms held by harmonic restraints. Finally, the simulation was run unrestrained for 30 ns.

Summary of Key Changes to the AHA2 Model

At a cursory glance, the structure appears similar, but the result is a noticeable improvement of quality scores over the original model (Pedersen et al., 2007), exemplified by the MolProbity score being reduced from 4.52 to 1.67 (Chen et al., 2010). Secondary structure is improved throughout, with 94% of residues now falling in the most favored regions of the Ramachandran plot. R_{free} is reduced from 36.6% (with anisotropic truncation) to 32.8% (without anisotropic truncation). Although still a quite high value we ascribe it to a large part to the anisotropic nature of the low resolution data set. All-atom root mean square deviation (r.m.s.d.) between the original and revised structure [using "align" command in PyMOL (Schrödinger)] is 1.86 Å (4648 atoms), while for Cα atoms the r.m.s.d. is 1.32 Å (569 atoms).

Perhaps the most important revision to the model is the rearrangement of transmembrane helices 7 and 8, which have been *N*-terminally shifted by 4 and 3 residues respectively, i.e., ∼1-turn (**Figure 2**). Modeling of these helices was originally complicated by poor definition of the intervening loop, no significant sequence identity to e.g., SERCA, and threading of the loop after helix 8 through a tube of strong density, which however now appears more consistent with the disaccharide head-group of a DDM detergent molecule not identified in the original maps.

For the most part density associated with the cytoplasmic domains was substantially weaker than in the transmembrane region. Single-residue register shifts were also applied to parts of the N domain (residues 414–424 and 344–364), significantly changing the adenosine pocket of the ATP binding site. We were also able to resolve a conserved K^+ binding site in the vicinity of Asp617 (Ekberg et al., 2010b) of the P-domain, and significant rearrangements were made to *N*-terminal residues 12–50 and residues 529–548 on the backside of the P domain.

STRUCTURAL AND FUNCTIONAL ANALYSIS OF AHA2

P-Type ATPase Catalytic Cycle

Conformational changes of P-type ATPases, shuttling between E1 and E2 states through E1P and E2P phosphoenzyme intermediates, are described by the Post-Albers cycle (**Figure 3**; Albers et al., 1963; Post and Sen, 1965; Pedersen and Carafoli, 1987). The resulting alternating access transport mechanism, in which the binding site of the protein is accessible only from one site of the membrane at any moment, is a prerequisite for active transport (Jardetzky, 1966). From a structural point of view the cycle is particularly well described for SERCA with overall characteristics also expected to be representative for P_{III}-ATPases.

In the E1 state of a P-type ATPase the cytoplasmic substrate (H^+ in case of AHA2, Ca^{2+} in case of SERCA) binds with high affinity at membraneous site(s). This in turn triggers rearrangement of the M1-M4 helices for robust occlusion, as well as of the A-domain to stabilize a tight approach of the N-domain with bound ATP to the P-domain with a Mg^{2+} binding site that coordinates the γ-phosphate of ATP at a conserved Asp-Lys-Thr-Gly motif (DKTG). Presence of the Mg^{2+} ion and the Lys side chain of DKTG compensate opposing negative charges of the γ-phosphate and the Asp side chain (Asp329 in AHA2) and promote phosphoryl transfer (Sørensen et al., 2004; Toyoshima et al., 2004) resulting in formation of the fully occluded, covalent aspartyl-phosphoanhydride intermediate (E1P).

The γ-phosphate transfer breaks the ATP mediated linkage between the P- and N-domain and allows the transition of the pump to the outward-facing E2P state observed for SERCA, Na^+, K^+-ATPase, and P1B-ATPases (Olesen et al., 2007; Yatime et al., 2011; Andersson et al., 2014; Wang et al., 2014), where the transported ions are extruded from low-affinity sites. The transition is caused by withdrawal of the N-domain that yields space for the A-domain to interact closely with the phosphorylated P-domain. In doing so, the A-domain rotates

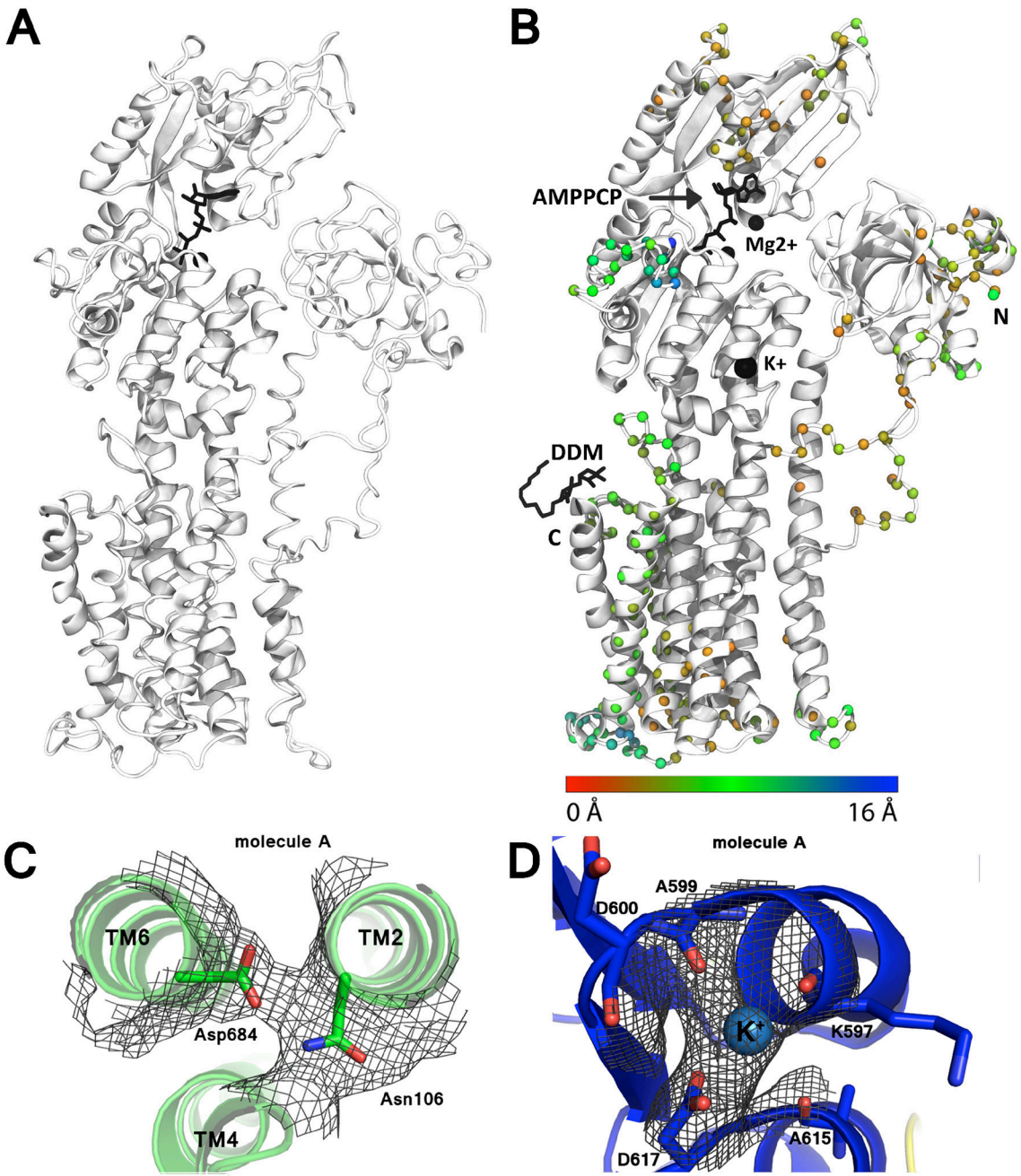

FIGURE 2 | Improvement of the AHA2 model. (A) The initial AHA2 model (pdb id: 3B8C). **(B)** Final, revised model after re-refinement (pdb id: 5KSD). Spheres mark changes in the position of the Cα atoms between the two models (coloring according to the legend below). N and C-termini are indicated; DDM indicates bound n-Dodecyl β-D-maltoside. **(C)** Electron density map contoured at 1.0 r.m.s.d for the Asn106-Asp684 pair. **(D)** Electron density map contoured at 2.0 r.m.s.d. for the K^+ ion binding site.

∼120° and places a conserved TGES motif in close interaction with the phosphorylated DKTG motif of the P-domain, which overall rotates by ∼15° relative to the membrane. The A-domain movement affects the configuration of transmembrane helices and the ion binding site(s) in the transmembrane domain, resulting in the opening of the exit pathway toward the extracellular site of the membrane.

Counterion interactions at the membraneous site stimulate reclosure of the extracellular access pathway. The occlusion induces a small rotation of the A-domain, which engages the Glu side chain of the TGES motif to catalyze the hydrolysis of the phosphorylated Asp side chain. Subsequent release of the liberated phosphate promotes further rotation of the A-domain, away from the P domain. The pump can now return to the

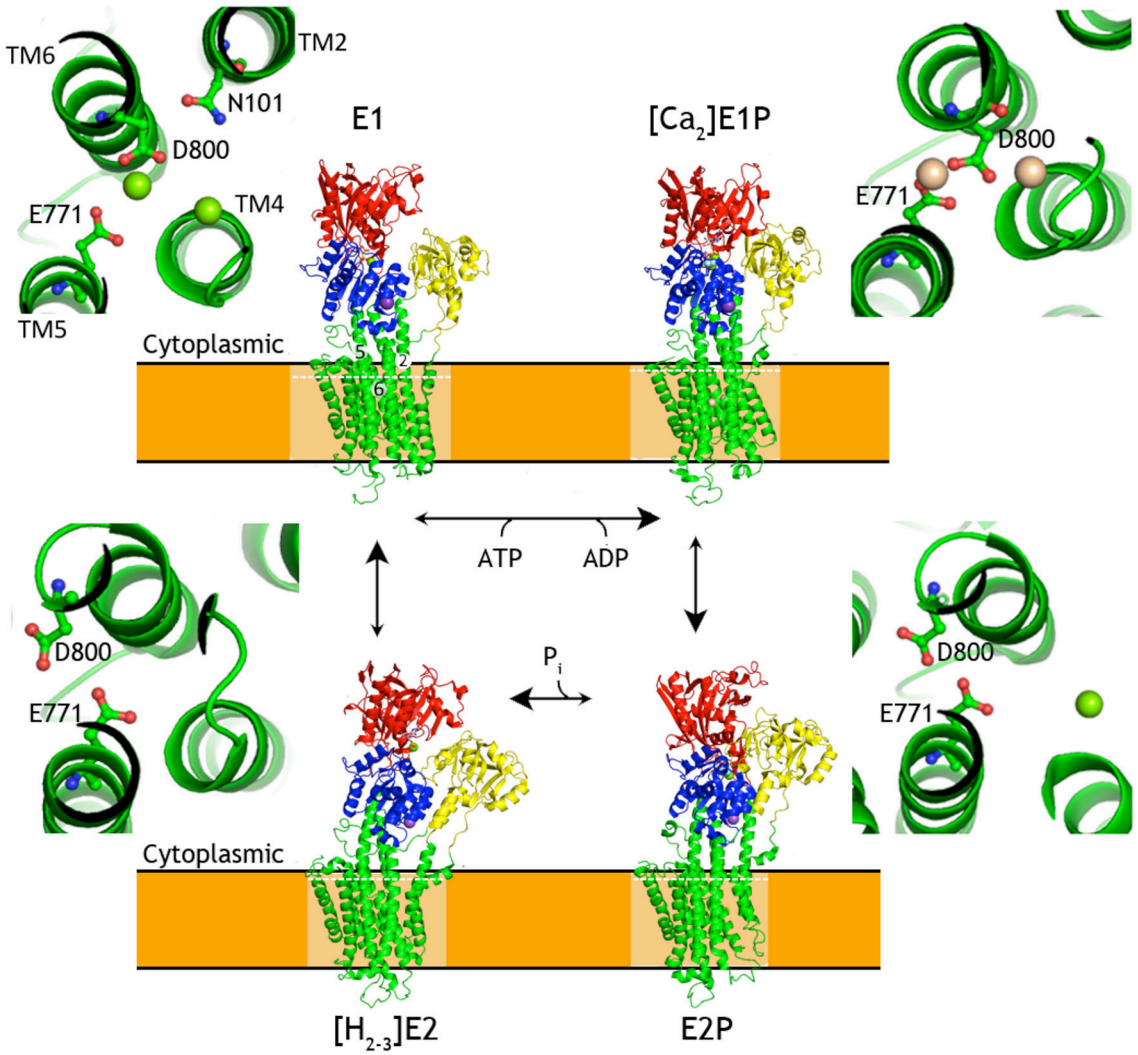

FIGURE 3 | Overview of the P-type ATPases cycle. Schematic presentation of structural changes occurring during the catalytic cycle of P-type ATPases, represented by SERCA structures. Insets show changes in relative orientation of the residues involved in formation of SERCA ion binding throughout the transport cycle. White dashed lines mark the planes corresponding to the zoomed cross-sections. Cytoplasmic domains are colored as P-domain in blue, N-domain in red, A-domain in yellow. The transmembrane domain is shown in green. Ligands and ions are shown as spheres (K^+ in purple-blue, Na^+ in purple, Ca^{2+} in wheat, Mg^{2+} in green) and ball-and-sticks representation for nucleotides and metallofluorides. SERCA1a [structures 4H1W (Winther et al., 2013), 1T5T (Sørensen et al., 2004), 3B9B (Olesen et al., 2007), 2C8K (Jensen et al., 2006)]. The structures were aligned by their TM domains on TM7-TM10.

cytoplasmically oriented E1 state along with counterion release to the cytoplasm for those P-type ATPases performing this transport also.

Overall Structure of the AHA2 E1-AMPPCP Complex

The structure of AHA2 obtained at pH 6.0 and with the ATP analog AMPPCP shows an overall typical E1 arrangement of domains, and an upright angle of the cytoplasmic headpiece relative to the membrane domain (Møller et al., 2010), although not identical to SERCA. Importantly, the E1 state observed in the structure of SERCA with sarcolipin (SLN) reported in 2013 (Toyoshima et al., 2013; Winther et al., 2013) appears to be functionally equivalent to the AHA2 state, both stabilized by AMPPCP and with the three cytoplasmic domains approaching a closed E1P conformation (**Figure 1**). Superpositioning of the highly conserved P-domain (r.m.s.d = 0.94 Å on Cα atoms) reveals an overlapping position of the N domain (**Figure 1A**), which is however noticeably smaller in AHA2 and rotated by ∼25°, when the structures are aligned by the transmembrane domains (**Figure 1B**).

Alignment of the AHA2 and SERCA E1 structures (**Figure 1A**) shows that the cytoplasmic domains of AHA2 adopt a more compact conformation around the bound AMPPCP. Details of the coordination of the nucleotide are different than previously reported for AHA2. The adenine base of AMPPCP is rotated by ∼60° resulting in suitable conformation for interactions with the N-domain via residues

Asp372 and Asp375, while Ser457 interacts with the ribose (**Figure 4**) of AMPPCP. The γ-phosphate of AMPPCP is placed ∼5 Å from Asp329 and coordinated only by P domain residues (Thr331, Thr511, Gly512) and the nearby Mg^{2+} ion, which also interacts with the β-phosphate. A second Mg^{2+} coordinates Asp372 of the N-domain.

Ion binding and occlusion at the membraneous site accompanies the formation of the catalytically competent E1P-like state (Olesen et al., 2007; Toyoshima et al., 2013; Winther et al., 2013). The transmembrane (TM) region however do not overlap when the two structures are aligned by their P-domains indicating that the SERCA and AHA2 E1 structures may have been captured at different intermediates of the E2 to E1P trajectory (**Figure 5**). The TM1 helix (see alignment in **Figure 6**) is differently placed, but shows both for AHA2 and SERCA an amphipathic association with the membrane interface (**Figure 7**). The TM segments 2–10 align with an r.m.s.d of 3.07 Å for Cα atoms. The K^+ binding site was previously visualized using AHA2 E1-AMPPCP cocrystallized with rubidium chloride (Ekberg et al., 2010b). The re-refined AHA2 structure revealed this site directly at the P domain, where the K^+ is coordinated by back bone oxygens of Lys597, Ala599, Asp600, Ala615 and by side chain oxygens of highly conserved Asp617 (**Figure 2**).

Proton Binding Site at the Conserved Asp684

Concerning proton transport and yeast PMA knock-out complementation, Asp684 of AHA2 is an indispensable, titratable residue in the TM domain (Buch-Pedersen et al., 2000). It is therefore considered the central binding site in proton transport. The superpositioning of the TM domains of AHA2 and SERCA shows a remarkable overlap of Asp684 and the critical Asp800 of SERCA that coordinates Ca^{2+} at both sites I and II in SERCA (Toyoshima et al., 2000). A cavity in AHA2 localized between TM helices 4, 5, and 6 connects Asp684, which is paired to Asn106 of TM2 in the current structure, and the Arg655 residue of TM 5 (**Figure 8**, and see below).

Some Asp684 mutants are still functional ATPases, but show no proton transport, except for the D684E mutation that also complements a yeast PMA knock-out. Remarkably, the Asp684 mutants were more than 1,000-fold less sensitive to vanadate, suggesting that they accumulate in the E1P state (Buch-Pedersen and Palmgren, 2003) as also supported by proteolytic cleavage analysis (Buch-Pedersen et al., 2000). In other words, an occluded structure is likely reachable to support phosphorylation of Asp684 mutated forms, but no proton release mechanism will subsequently stimulate turn-over of the phosphoenzyme that therefore accumulates in E1P.

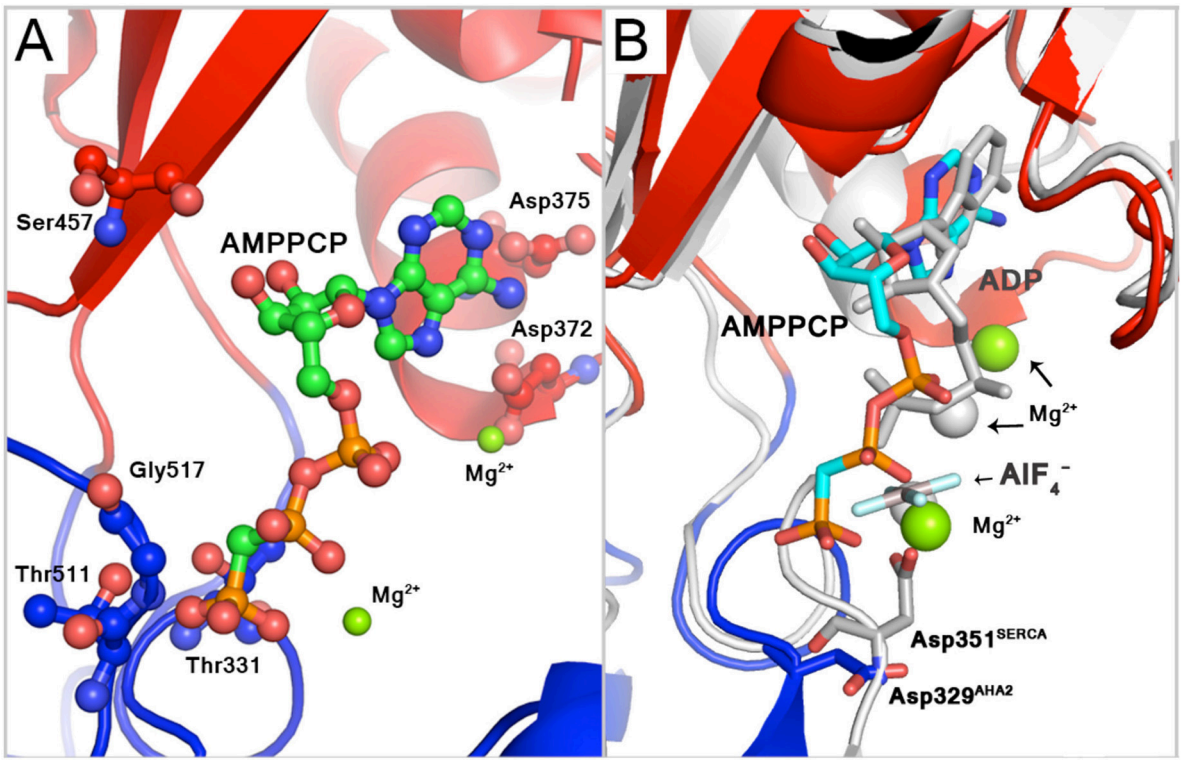

FIGURE 4 | Binding of AMPPCP. (A) Binding of the phosphate groups of AMPPCP includes two Mg^{2+} ion, side chains of Thr511 and Thr331 and backbone atoms of Gly512 and Thr331. The adenine base can interact with side chains of Asp375 and Asp372, the latter of which also interacts with the Mg^{2+} ion that coordinates α-phosphate group. The ribose ring interacts with the backbone carbonyl of Ser457. **(B)** AHA2 binding of AMPPCP (green carbons) compared to ADP-AlF_4^- (gray) binding to SERCA in the E1P-like state, pdb id: 1T5T (Sørensen et al., 2004). Mg^{2+}—green spheres in AHA2, gray spheres in SERCA.

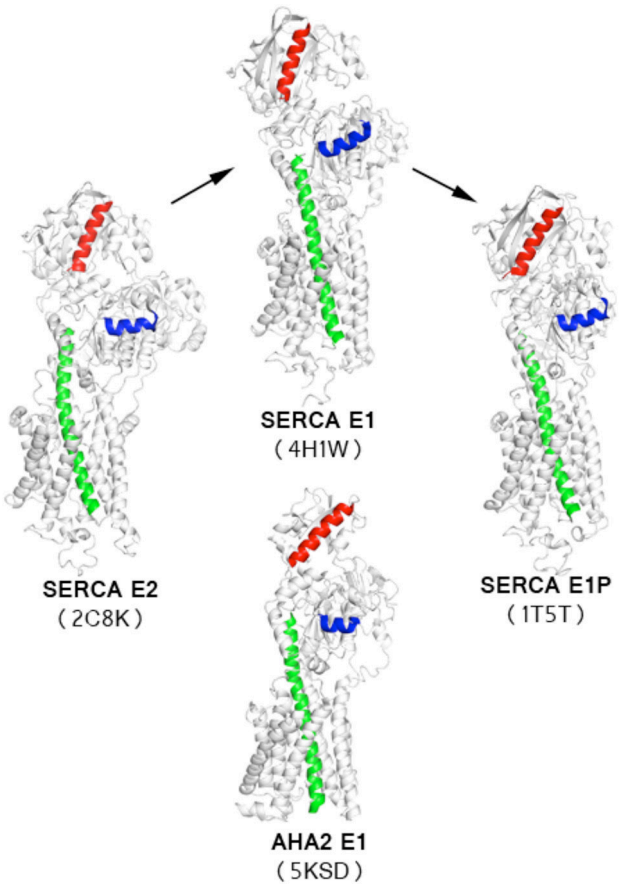

FIGURE 5 | Comparison of AHA2-AMPPCP model with available SERCA structures. Orientation of a reference helix from the P-domain (blue) shows that the AHA2-E1 structure adopts an intermediate state of the transition between SERCA-E2 and SERCA-E1 states. Structures were aligned using helices TM5-TM9. TM5 marked in green. Corresponding helices from the N-domain are marked in red.

Proton Occlusion at the Asp684-Asn106 Pair

An important role of Asn106 was apparent from the structure of AHA2 (Pedersen et al., 2007). It is conserved in all P_{III}-type H^+-ATPases and positioned close to a large intramembraneous cavity (**Figure 9**), where it pairs with Asp684. The proximity of the two residues is compatible with formation of a neutral hydrogen-bonded pair between a protonated Asp684 and Asn106 as a basis for a stable proton binding site associated with the occluded E1P state (**Figure 8**). Similarly, occlusion of the Asp684-Asn106 pair is likely to increase the pKa of Asp684 and therefore stabilize protonation (Buch-Pedersen et al., 2009). The important functional role of Asn106 was further highlighted by mutational studies (Ekberg et al., 2013). Various point mutations (N106A, N106D, N106K, N106Q, and N106T) could complement a PMA1 knockout and maintain a membrane potential. Kinetic characterization of the purified mutant proteins confirmed their ability to hydrolyze ATP and transport protons, however at reduced rates as compared to the WT pump and with an acidic shift in the pH dependence as indeed to be expected from reduced stabilization of the protonated Asp684. Notably, a three-fold increased vanadate sensitivity of the N106D mutant suggested that relative to the wildtype it is shifted toward an outward-oriented E2 state (Ekberg et al., 2013).

PROTON ENTRY PATHWAY

Associated with E1 function, a proposed cytoplasmic proton entry pathway is located at the N-terminal part of the membrane domain at the cytoplasmic interface. A smaller cavity is evident above the ^{TM2}Asn106-^{TM6}Asp684 pair, and is defined by residues of helices TM2, 4, 5, 6, and 8, including two negatively charged glutamates (Glu113 and 114 of TM2) that may both attract protons and repel negatively charged lipids from blocking the cavity. MD simulations based on the improved model suggest a "U"-shaped solvent tunnel between TM1, 2, 4, and 6 and support the earlier proposal (Pedersen et al., 2007) that solvent enters the cavity from the cytoplasmic environment and thereby provides transmission of a proton to the Asp684-Asn106 pair.

The N-terminal end of TM1 is in close proximity to the cavity and the proposed solvent tunnel. TM1 is significantly shorter compared to the other helices with Pro68 (conserved among P_{III}-type members; Axelsen and Palmgren, 1998) defining the N-terminal starting point. The preceding A/TM1 linker is poorly defined in the crystal structure. Two positively charged lysine residues (Lys57 and Lys60) may interact with head-groups of the phospholipid membrane. Indeed, MD simulations show this region to partition into the membrane interface through an amphipathic helix for residues 56–64 and to induce a local depression in the membrane that may facilitate the solvent access to the Asp684-Asn106 pair in the current E1 state (**Figure 9**). A similarly short and kinked TM1 helix is also seen in SERCA, and for the SLN bound E1 structure of SERCA it is as well immersed into the membrane and may facilitate Ca^{2+} entry, described as a "sliding door mechanism" (Winther et al., 2013). A similar TM1 structure is observed for Na^+,K^+-ATPase (Morth et al., 2007) and a role in Na^+ entry proposed (Einholm et al., 2007; Laursen et al., 2009). The heavy-metal transporting P_{1B}-ATPases show a different N-terminal topology with a heavy-metal binding domain and two additional N-terminal transmembrane segments MA and MB, but here again the MB helix is short and kinked and has been implicated in the mechanism of heavy metal entry for the membraneous site (Gourdon et al., 2011; Wang et al., 2014). A kinked (near-) N-terminal helix distorting the cytoplasmic membrane interface therefore appears as a general mediator of substrate entry in P-type ATPases.

Proton Transport Pathway through the Membraneous Cavity

Proton release is connected to the E2P state, which is generally characterized by low ATP and E1 substrate affinity. The proposed transport mechanism with the Asp684 side chain functioning as a titratable proton acceptor/donor site raises the question of how protons are then extruded in an outward-open E2P form? Next to Asp684, toward the extracellular side, we observe a large

FIGURE 6 | Sequence alignment of AHA2, SERCA, PMA1, ZntA. Secondary structure markings (below the aligned sequences) are based on AHA2 crystal structure with tubes for α-helix, arrows–β-strand, and lines for coil. The alignment was performed with MUSCLE (Edgar, 2004), using CLC Workbench (Genomics Workbench 7.7).[1] and accession numbers: AHA2–P19456; PMA1–P05030; SERCA1a–P04191; ZntA–Q3YW59.

[1]https://www.qiagenbioinformatics.com/

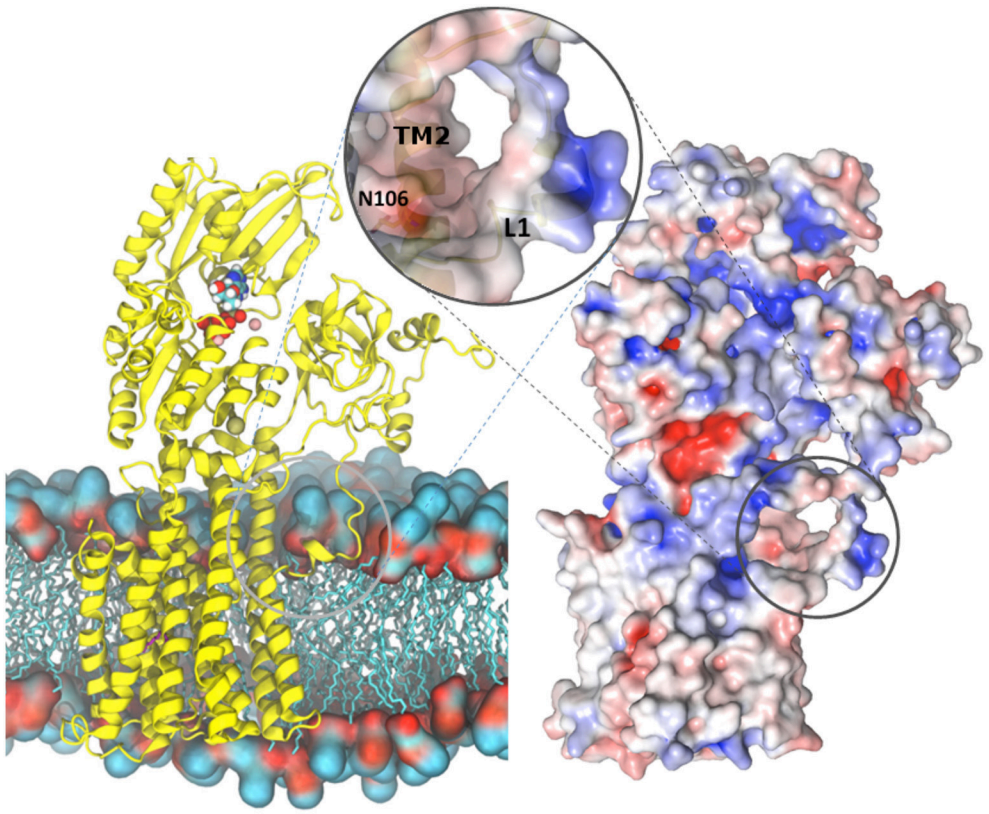

FIGURE 7 | Overview of the AHA2-AMPPCP structure. (Left) AHA2 (yellow cartoon) modeled into a lipid bilayer. Ions and AMPPCP are shown by sphere representation. The circle zooms onto a negatively charged pocket formed between TM1 and TM2 in a close proximity of the Asn106-Asp684 pair. **(Right)** Overall surface electrostatic potential of AHA2 ± 5 K_bT/e_c indicated in blue (positive) and red (negative) mapped on a surface representation. Negative electrostatic charge comes from two Glu residues situated in helix 2. The unstructured loop connecting the A domain and TM1 carries positive charges (blue color), which may interact with lipid head-groups.

intramembranous cavity of ∼265 Å^3 formed between helices TM4–TM6. This cavity is mostly due to the unwound structure of the TM4 helix at conserved Pro286 and Pro290 residues (Bukrinsky et al., 2001), and a bulged structure at the Asp684 position contributes as well. A similar deformation of the TM4 helix is present in e.g., SERCA, Na^+,K^+-ATPase, CopA, and ZntA, yet in a very different context of metal cation coordination. Backbone carbonyl and amide groups from AHA2 residues Ile282, Gly283 and Ile285 along with Gly284, Pro286 and side chains of conserved Tyr645, Tyr648, Tyr653, and Arg655 of TM5 and Asn683 of TM6 define the cavity surface, which therefore appears surprisingly polar. Ile282 has been implicated in proton translocation (Fraysse et al., 2005). Our MD simulations further suggest a role of Ile282 and Tyr653 in allowing water molecules to penetrate the cavity (**Figure 9**).

Homology modeling of the E2P state suggested an important role for Arg655 in TM helix 5 approaching toward the Asp684 and Asn106 pair to stimulate deprotonation. Also the E2P modeling suggests that the water-filled cavity merges into a then deep, solvated extrusion pathway leading from Asp684 and pass Arg655 to the extracellular environment (**Figure 8**). Arg655 is conserved among all plant PM H^+-ATPases and corresponds to a His residue of similar functionality in fungi (see below). The equivalent residue in SERCA is the Ca^{2+} binding Glu771 (**Figure 6**), which indeed faces the extracellular pathway in the E2P state. The exact conformation and interactions of the positively charged Arg655 side chain in AHA2 cannot be reliably defined at the current resolution, albeit its proximity effect on Asp684 deprotonation and its interaction with the water-filled cavity appears quite likely and as a simple model of function.

The role of Arg655 in the catalytic cycle of AHA2 has been investigated by extensive mutagenesis and functional assays (Buch-Pedersen and Palmgren, 2003). Out of three prepared mutants (R655K, R655A, R655D) the R655K mutant was reported to support growth at a WT level in a PMA1 knockout yeast strain, while R655A and R655D could not complement at all. ATP affinity was unaltered for all mutant (confirming proper folding and preserved functionality of the N-domain), and the pH-dependence of the enzyme was maintained indicating preserved function of proton binding. However, the hydrolytic activities of the mutant pumps were significantly reduced. Vanadate insensitivity and enzyme phosphorylation levels indicated an accumulation in the E1P state (Buch-Pedersen and Palmgren, 2003) in full agreement with a proposed role in

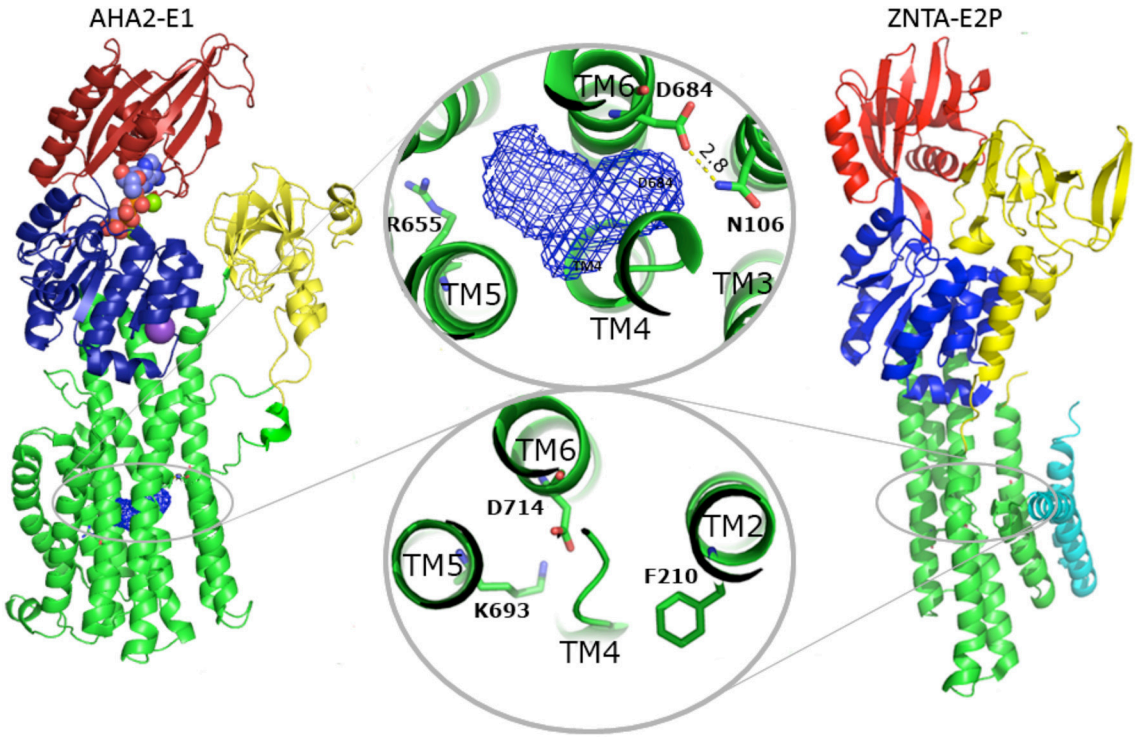

FIGURE 8 | Comparison between AHA2 and Zn^{2+}-ATPase. Despite different overall structure, the TM domains of AHA2 and the *Shigella sonnei* Zn^{2+}-ATPase ZntA exhibit significant resemblance regarding the distribution of charged residues in the TM helices associated with transport. In both cases the TM helix 2 harbors a residue involved in closure of the ligand pathway from the cytoplasmic site. $Phe210^{ZntA}$serves as a gating residue, while overlapping $Asn106^{AHA2}$ is implicated in stabilization of the occluded, protonated Asp684. The Asp side chains placed in both cases in TM6 ($Asp684^{AHA2}$ and $Asp714^{ZntA}$) likely fulfill similar roles. Zinc binding in ZntA most likely involves Asp714 (and two conserved cysteines of TM4, not shown), while zinc release and the phosphorylation is stabilized by interactions with the conserved Lys693 of TM5. In AHA2 the equivalent Asp684 is essential for transport and most likely becomes deprotonated by interaction with Arg655 of TM5 along with E2P dephosphorylation.

proton release. Double mutants of the Asp684 and Arg655 sites showed that only R655K/D684E was able, to some extent, to sustain yeast growth in a PMA knockout.

Importantly, a similar mechanism has been proposed for the bacterial Zn^{2+}-ATPase where Zn^{2+} release in the E2P state could be facilitated by the positively charged K693 residue, which indeed stabilizes the Zn^{2+} binding Asp714 as a built-in counter ion (Wang et al., 2014) in the Zn^{2+}-free E2 Pi state (**Figure 8**). Localization of this lysine at the exit of the ion pathway suggests that it too can block re-entry, in this case of extracellular Zn^{2+} ions to the transmembrane domain (Wang et al., 2014). Functional experiments have failed to identify any counter-transported ligand for the fungal and plant proton pumps supporting the model of Arg655 as a built-in counterion (Pedersen et al., 2007). In the fungal proton pump, which sustains even stronger polarization of the membrane potential than the plant protein (Blatt et al., 1987), the position corresponding to the Arg655 is occupied by His701 (yeast PMA1 numbering) and in close proximity to Arg649 located next to the water-filled cavity. Such clustered arrangement of positively charged residues at the proton release pathway may explain the remarkably steep membrane potentials that can be attained in fungal cells through PMA activity.

Comparison to other Proton Transporters

The functional role of Arg655 and Asp684 in the AHA2 proton transport mechanism is similar in concept to many other proton transport proteins such as bacteriorhodopsin (Pebay-Peyroula et al., 1997; Luecke et al., 1998) and F-/V-type ATPases (Hutcheon et al., 2001; Fillingame and Dmitriev, 2002) where an Arg-dependent pKa shift of a carboxylic acid side chain facilitates the proton transfer (Buch-Pedersen et al., 2009). Localization of Arg655 at the exit pathway of the proposed proton transport channel suggests that it can also function as a built-in counter ion that facilitates E2P occlusion and dephosphorylation and as a positively charged block for extracellular proton re-entry (Pedersen et al., 2007). The proposed behavior of the Asp684 and Arg655 residues in AHA2 resembles mechanisms utilized by other proton transporters. In case of F_1F_0-ATPase, the proposed gating of periplasmic H^+ is based on periodic formation of a salt bridge at the interface of the transmembrane *a* subunit and a rotor-like *c* subunit.

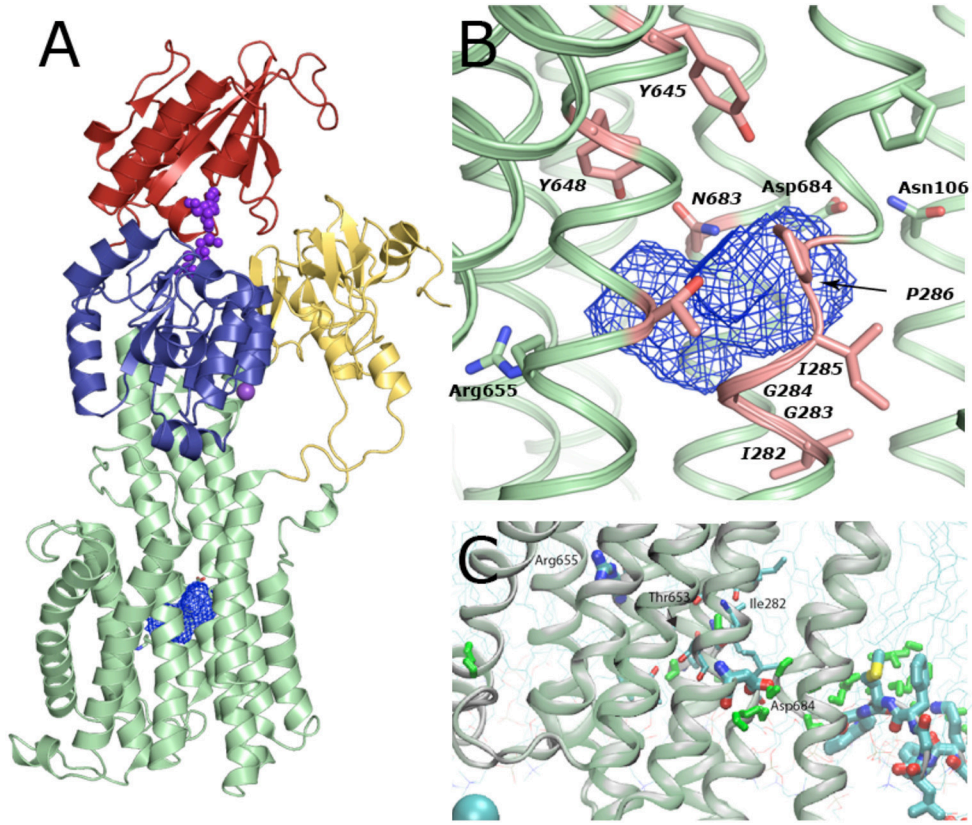

FIGURE 9 | Solvent cavity in the TM domain of AHA2. (A) Overview on the cavity location within the TM domain. The cavity likely encloses 8–10 bound water molecules in this conformation of the pump. **(B)** Zoom-in of the cavity viewed in the plane of the membrane. The cavity is formed by backbone atoms of Ile282, Gly283, and Ile285 along with Gly 284, Pro286 and side chains of conserved Tyr645, Tyr648, Tyr653, and Arg655 of TM5 and Asn683 of TM6. **(C)** Molecular Dynamics simulations indicate that a continuous string of water molecules (shown in green) can reach from the cytoplasm to the Asp684 residue, the proposed proton binding site, i.e., an open proton translocation pathway, which must close later in the functional cycle, most likely along with E1P formation. Two stable water molecules reached almost half way through the membrane into the TM domain where they can interact with Ile282, Thr653 and backbone oxygen of Ala649, and Cys247, next to the central solvent cavity.

The relative position of the involved residues, *a*Arg210 and *c*Asp61, promotes either a formation of the salt bridge which facilitates a proton release to the cytoplasmic half-channel of the ATP synthase, or, when the salt bridge is broken due to movement of the *a* subunit triggered by acidification, allow for protonation of an available *c*Asp61 residue (Dong and Fillingame, 2010).

A mechanism similar to the function of the Asn106-Asp684 pair observed in AHA2 was proposed for the *Escherichia coli* ClCec Cl^-/H^+ antiporter and cytochrome C oxidase. Based on a crystal structure of the former protein, a Glu148 residue, located at the beginning of the translocation pathway, was suggested to serve as a gate for the ions, changing its side chain conformation in response to e.g., pH (Dutzler et al., 2003; Accardi and Miller, 2004). In cytochrome c oxidase a proton pathway has a form of a ∼25 Å long cavity, made by polar residues and several ordered water molecules. From the crystal structures it was noticed that an Asn139 residue affects formation the integrity of a water chain that supports proton translocation (Iwata et al., 1995; Tsukihara et al., 1996; Svensson-Ek et al., 2002; Qin et al., 2006). Free-energy simulations visualized a metastable rotamer state where the residue changes the side chain conformation and opens the channel to form a functional ion translocation pathway (Henry et al., 2009).

CONCLUSIONS

The application of iMDFF environment in refinement of low-resolution protein structures was successfully reported in studies on Human Insulin Receptor Ectodomain (Croll et al., 2016), and we have applied it here to the original, highly anisotropic 3.6 Å resolution crystallographic data obtained from AHA2 crystals (Pedersen et al., 2007). Structural changes of the revised AHA2 model includes a local rearrangement of transmembrane helices 7 and 8, where ∼1-turn N-terminal register shift is observed, and local changes at the nucleotide binding pocket of the N-domain. Additionally, improvement in the model quality allowed for inclusion of a conserved K^+-binding site located at the P-domain.

The improved quality of the model provides a more confident basis of the proposed H^+ transport mechanism utilized by P_{III}-type ATPases. The proton translocation pathway, which centers on earlier identified residues Asn106, Asp684, and Arg655, begins at the cytoplasmic side of the TM domain from where protons are delivered to the Asn-Asp pair via a solvent tunnel located between TM1, 2, 4, and 6. Solvent accessibility of the proton entrance is obtained by a characteristically short, helical structure of TM1. Furthermore, the angle between the P-domain and the membrane maybe important for the function of this entry pathway. Based on modeling of E1P-E2P conformational changes, protons are likely transported via a large solvent-filled cavity that merges with an exit pathway toward the extracellular side of the membrane. Arg655, proximal to the cavity stimulates deprotonation of Asp684 and proton release, serving as a built-in counter ion required for E2P closure and dephosphorylation and the E2-E1 transition of the pump.

The AHA2 structure reveals mechanistic concepts that can also be recognized in other transmembrane proton/cation transport systems, yet future structures of E2 states will be required to fully elucidate functional transitions of P_{III}-type H^+-ATPases. Interestingly, single-molecule studies of AHA2 function have been introduced and suggest also that inactive states and protein-mediated proton leaks must be considered as part of the functional cycle (Veshaguri et al., 2016), thus many structural and mechanistic questions remain to be addressed to get a full insight into the inner workings of the plasma-membrane proton pump.

AUTHOR NOTE

We dedicate this paper to the memory of the late Carolyn Slayman, a pioneer of the plasma-membrane proton pump field.

ENDNOTE

The re-refined coordinates are available in the Protein Data Bank through accession number 5KSD.

AUTHOR CONTRIBUTIONS

TC performed new refinement, assisted by BP and PN. DF drafted the paper and performed structural analysis. BP and PN supervised the project.

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The Apical Localization of Na^+, K^+-ATPase in Cultured Human Retinal Pigment Epithelial Cells Depends on Expression of the β_2 Subunit

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Na^+, K^+-ATPase, or the Na^+ pump, is a key component in the maintenance of the epithelial phenotype. In most epithelia, the pump is located in the basolateral domain. Studies from our laboratory have shown that the β_1 subunit of Na^+, K^+-ATPase plays an important role in this mechanism because homotypic β_1-β_1 interactions between neighboring cells stabilize the pump in the lateral membrane. However, in the retinal pigment epithelium (RPE), the Na^+ pump is located in the apical domain. The mechanism of polarization in this epithelium is unclear. We hypothesized that the apical polarization of the pump in RPE cells depends on the expression of its β_2 subunit. ARPE-19 cells cultured for up to 8 weeks on inserts did not polarize, and Na^+, K^+-ATPase was expressed in the basolateral membrane. In the presence of insulin, transferrin and selenic acid (ITS), ARPE-19 cells cultured for 4 weeks acquired an RPE phenotype, and the Na^+ pump was visible in the apical domain. Under these conditions, Western blot analysis was employed to detect the β_2 isoform and immunofluorescence analysis revealed an apparent apical distribution of the β_2 subunit. qPCR results showed a time-dependent increase in the level of β_2 isoform mRNA, suggesting regulation at the transcriptional level. Moreover, silencing the expression of the β_2 isoform in ARPE-19 cells resulted in a decrease in the apical localization of the pump, as assessed by the mislocalization of the α_2 subunit in that domain. Our results demonstrate that the apical polarization of Na^+, K^+-ATPase in RPE cells depends on the expression of the β_2 subunit.

Keywords: Na^+, K^+-ATPase, retinal pigment epithelium, apical polarity, ARPE-19, AMOG/β_2, re-morphogenesis

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INTRODUCTION

Na^+, K^+-ATPase, or the Na^+ pump, is the principal transporter in eukaryotic cells that sustains a non-equilibrium distribution of Na^+ and K^+ ions across the plasma membrane (Kaplan, 2002). Na^+, K^+-ATPase is a heterodimer that consists primarily of α and β subunits. The α subunit has a molecular mass of 110 kDa and is responsible for the catalytic functions of the enzyme

Abbreviations: ITS (mixture of insulin, transferrin and selenic acid); RPE (retinal pigment epithelium).

(Ohtsubo et al., 1990). The β subunit is a glycoprotein with a molecular mass of 35 kDa and is indispensable for the structural stabilization and functional maturation of the holoenzyme (Geering et al., 1989; Ackermann and Geering, 1990) and the transport of the α subunit to the plasma membrane (Noguchi et al., 1987; Martin-Vasallo et al., 1989). Ion transport requires the participation of both α and β subunits (Fambrough, 1988; Martin-Vasallo et al., 1989). There are four distinct isoforms of the α subunit (α_1, α_2, α_3, and α_4) and three isoforms of the β subunit (β_1, β_2, and β_3) that are tissue-specific in their expression (Fambrough, 1988; Cortas et al., 1991; Blanco and Mercer, 1998). Finally, there is a small γ subunit that belongs to the FXYD family of proteins that modulates Na^+, K^+-ATPase activity (Cortas et al., 1991).

The establishment of cell surface polarity for most membrane proteins in epithelia implicates sorting signals that are encoded in their amino acid sequence (Sweadner et al., 2000; Rodriguez-Boulan et al., 2005), trafficking routes that involve apical or basolateral recycling endosomes (Weisz and Rodriguez-Boulan, 2009), and interactions with epithelial-specific protein complexes such as AP-1B and clathrin, which may be regulated by small GTPases (Ellis et al., 2006; Gonzalez and Rodriguez-Boulan, 2009; Weisz and Rodriguez-Boulan, 2009). Na^+, K^+-ATPase is polarized and directed toward the basolateral membrane of most epithelial cells (Deborde et al., 2008) and, more specifically, at cell borders facing the intercellular space (Contreras et al., 1995; Cereijido et al., 2001). In epithelial cells, newly synthesized Na^+, K^+-ATPase is delivered directly to the basolateral membrane (Contreras et al., 1989; Shoshani et al., 2005). Although it is clear that the α_1 subunit carries the information for the basolateral targeting of Na^+, K^+-ATPase in typical epithelia (Mays et al., 1995), efforts to identify an amino acid sequence that functions as a basolateral polarity signal in the α_1 subunit have been unsuccessful (Dunbar et al., 2000). In the target membrane domain, the asymmetric distribution of Na^+, K^+-ATPase is reinforced by selective retention through binding to the ankyrin-fodrin cytoskeleton (Hammerton et al., 1991; Muth et al., 1998). Several lines of evidence have demonstrated that the β_1 subunit anchors the pump at the lateral borders of epithelial cells through homotypic β_1-β_1 interactions, provided the neighboring cells express an identical β_1 subunit (Contreras et al., 1995; Shoshani et al., 2005). Recent studies have further shown that the adhesive properties of the β_1 subunit play a principal role in the basolateral localization of the pump (Vagin et al., 2006; Padilla-Benavides et al., 2010). However, in the choroid plexus epithelium (Wright, 1972), cockroach salivary gland epithelium (Just and Walz, 1994) and retinal pigment epithelium (RPE; Gundersen et al., 1991), Na^+, K^+-ATPase is expressed in the apical membrane.

The RPE makes up the outmost layer of the retina and has many supporting functions that are fundamental for the survival of photoreceptors. The RPE forms the outer hemato-retinal barrier and regulates the volume and chemical composition of the subretinal space. Na^+, K^+-ATPase is vital for several RPE cell functions, such as the vectorial transport of ions and solutes from the choroid to the photoreceptors and the reestablishment of Na^+ and K^+ gradients required for the photoreceptor dark current, synaptic activity, action potentials, and transmitter uptake in the subretinal space (Miller and Steinberg, 1979). RPE cells are distinctive in that they contain apical Na^+, K^+-ATPase (Miller and Steinberg, 1979; Gundersen et al., 1991). Nevertheless, depending on the RPE preparation studied, apical expression can be lost (Geisen et al., 2006) or accompanied by basolateral expression (Okami et al., 1990; Hu et al., 1994; Marrs et al., 1995). Despite many years of investigation, the sorting signals and mechanisms that mediate the apical polarization of Na^+, K^+-ATPase remain poorly understood (Cereijido et al., 2012).

The present work focuses on the intriguing mechanism underlying the polarity of the Na^+ pump in the RPE. Because the β_1 subunit plays a key role in the basolateral localization of the pump in classic epithelia, we anticipated that β subunit isoforms may be crucial elements in explaining the apical localization of the pump in the RPE. In this context, it is worth recalling that a role for the β subunit, particularly the β_2 isoform, in the apical polarization of Na^+, K^+-ATPase has been suggested in previous studies (Wilson et al., 2000; Vagin et al., 2005). In the present study, we examined the hypothesis that the apical targeting of Na^+, K^+-ATPase in RPE cells depends on the expression of the β_2 subunit.

MATERIALS AND METHODS

Reagents and Antibodies

The following reagents were used: DMEM, F12, PBS, and FBS (GIBCO Cat. 12100-061, Cat. 21700-026, Cat. 21300-058, and Cat. A15-751), the antibiotics penicillin and streptomycin (10,000 U/μg/ml, *In vitro*, A-01), laminin (SIGMA-ALDRICH Cat. L2020), ITS (a mixture of insulin, human transferrin and selenic acid, BD Biosciences Cat. 354352), Protease Inhibitor Mix (GE Healthcare, Cat. 80-6501-23), a chemiluminescent detection system (ECL Plus; Amersham Biosciences Cat. RPN2132), Lipofectamine 2000 (Invitrogen, Cat. 11668-019), an siRNA Labeling Kit-Cy3 (Ambion by Life Technologies Cat. AM1632), Sp1 siRNA (Sta. Cruz Cat. sc-29488), siRNA β_1 and β_2 (FlexiTube siRNA QIAGEN: SI04284966, SI04249098, SI04173134, SI03149909, SI04273003, SI04138162, SI04274543, SI04284014), the Light Cycler-Fast Start DNAMaster SYBR Green I Kit (Roche, (Applied Biosystems, 4309159), and BCA protein assay reagent (Thermo Scientific, 23224 and 23223).

The following antibodies were used: anti-Na^+, K^+-ATPase α_1 subunit (IF: Novus NB300-146), anti-α_2 Na^+, K^+-ATPase (Thermo Scientific, PA5-25725), anti-Na^+, K^+-ATPase β_1 subunit (IF: Bio Reagents Cat. No. MA3-93; WB: Novus 464.8), anti-Na^+, K^+-ATPase β_2 subunit (WB: Transduction Laboratories Cat. No. BD610915, IF: Biorbit orb10952 and Creative Biolabs MOB-3916z), anti-Na^+, K^+-ATPase β_3 subunit (Transduction Laboratories Cat. No. BD610992), anti-Ezrin (Sigma E1281), anti-β-catenin (Invitrogen 13-8400), anti-N-cadherin (IF: ZYMED Cat. No. 333900), anti-CD147 (Bioscience No. Cat. 555961), Alexa 488- or 594-conjugated donkey anti-mouse or anti-rabbit IgG (Invitrogen, A11094, A21207, A21203, and A21202), TO-PRO (Invitrogen, T3605), Hoechst (Invitrogen H21491), peroxidase-conjugated anti-mouse and anti-rabbit antibodies (Zymed California, Cat. 62-6520 and 62-6120), and FITC-phalloidin (Sigma Chemical).

Cell Culture

The ARPE-19 cell line (ATCC CRL-2302) was originally obtained from a spontaneously transformed human RPE primary culture. We only used cells from the 5th to the 20th passages, when the cultures grew rapidly and formed cobblestone monolayers. Cells were maintained in 6-cm-diameter culture dishes in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (FSB; 10%), penicillin (100 U/ml) and streptomycin (100 μg/ml). The cultures were incubated in an atmosphere of 95% air with 5% CO_2 at 37°C. The cells were propagated on 6.5-mm or 24-mm-diameter Millicell Hanging Cell Culture Inserts (0.4 μm pore) (Transwell Corning Incorporated cat. 3450 and 3470) that had been previously coated with laminin (10 μg/ml) and were maintained in 1:1 Dulbecco's Modified Eagle's Medium and Ham's F-12 medium (DMEM:F-12) supplemented with FBS (10%) for 1 week. For the remaining time, they were supplemented with FBS (1%) and ITS until a polarized monolayer was formed (4–6 weeks). These culture conditions were defined as re-morphogenic conditions.

Transepithelial Resistance (TER)

The degree of tight junction (TJ) sealing to ionic solutes was assessed by measuring the TER of cells grown for 6 weeks on transwell-permeable supports using an epithelial volt-ohmmeter (EVOM; World Precision Instruments Inc., Sarasota, FL). All measurements were performed at room temperature. Final values were obtained by subtracting the resistance of the bathing solution and the empty insert, and the results are expressed as the mean ± SE in ohms times centimeters squared ($\Omega \bullet cm^2$).

Immunofluorescence (IF), Immunocytochemistry and Confocal Microscopy

IF assay were performed using monolayers grown on 6.5-mm transwells covered with laminin that were washed with PBS (phosphate-buffered saline) and fixed with ice-cold methanol for 10 min. The cells were then soaked in blocking solution (PBS containing 3% BSA) for 1 h at 37°C or overnight at 4°C. Then, cells were incubated with the primary antibodies for 60 min at 37°C, washed quickly 7 times with PBS, and then incubated with the secondary antibodies for 45 min at 37°C. All antibodies were diluted in blocking solution, and the following secondary antibodies were used: Alexa 488- or 594-conjugated donkey anti-mouse or anti-rabbit IgG. Nuclei were counterstained with TO-PRO dye and then washed twice. To detect filamentous actin, the cells were fixed in paraformaldehyde and labeled with FITC-phalloidin.

Human eye preparations were obtained from the ophthalmic pathology service at the "Dr. Luis Sánchez Bulnes" APEC hospital in Mexico City. Paraffin-embedded eyes without lesions in their fundus were selected from the service compendium, and 5-μm sections were mounted on slides. For Na^+, K^+-ATPase assessment, β_2 subunit-stained sections were deparaffinized and rehydrated using a series of incubations with xylene (2–5 min and 1:1 xylene/ethanol) and ethanol (2 min each in 100, 95, 80, and 70% ethanol), followed by three washes in PBS or water. The samples were bleached via incubation in 0.25% $KMnO_4$ and PBS-Ca^{2+} for 30 min. After three washes with PBS-Ca^{2+}, the samples were incubated in 1% oxalic acid and washed again. Antigen retrieval was accomplished via incubation in 0.05% trypsin for 10–30 min at 37°C. Sections were permeabilized using 0.25% Triton X-100 and 1% FBS for 30 min and then blocked with 0.25% Triton X-100 and 10% FBS for 90–120 min. The samples were incubated with primary antibodies (against CD147 and the α2 and β_2 subunits of Na^+, K^+-ATPase) in permeabilization solution overnight at 4°C. The next day, the samples were washed three times and incubated with a secondary antibody for 1 h at 37°C. The samples were washed twice, and the nuclei were counterstained with DAPI.

Confocal microscopy was performed using a Leica laser-scanning confocal microscope (Leica TCS SP2 or TCS SP8). Data acquisition and analysis were performed with the LCS Leica software and ImageJ® software from the National Institutes of Health (Bethesda, MD), respectively. The relative fluorescence intensity was quantified using ImageJ 1.43u software.

Western Blot (WB) Analysis

All extraction steps were performed at 4°C. To detect the protein levels of the Na^+, K^+-ATPase subunits, monolayers grown on 24-mm transwells covered with laminin were lysed in a buffer containing 40 mM Tris (pH 7.6), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 0.5% Na^+ deoxycholate, 0.2% SDS, and protease inhibitors (Complete, Mini). The extract was sonicated for 30 s and centrifuged at 15,000 × g in a microfuge for 15 min. The supernatant was recovered, and the protein content was measured using BCA protein assay reagent following the manufacturer's instructions. Thirty micrograms of protein from each condition were separated via 10% SDS-PAGE and immunoblotted with the indicated primary antibodies, followed by species-appropriate peroxidase-conjugated secondary antibodies, which were imaged using a chemiluminescence detection system. The immunoblots were quantified via densitometry using ImageJ 1.43u software.

Steady State Surface Biotinylation Assay

ARPE-19 cells were maintained for 4 weeks in a culture on polyester transwell inserts as described above. Cell monolayers were biotinylated with 1 mg/ml of EZ-Link Sulfo-NHS-SS-Biotin (Thermo scientific, 21331). After quenching the biotinylation reaction, the cells were washed and then lysed, and the membranes were solubilized by incubating them with 200 μl of PBS (pH 8.0) with 1% Triton X-100 and protease inhibitors. Cell lysates were clarified via centrifugation (15,000 × g for 10 min). Samples containing 50 μl of supernatant mixed with SDS-containing sample buffer were loaded into SDS-PAGE gels to determine the total ARPE-19 protein in the supernatant (input). To isolate biotinylated proteins, the rest of each supernatant was incubated with 100 μl of streptavidin-agarose beads (Gibco, 5942SA) in a total volume of 150 μl of lysis buffer overnight at 4°C with continuous rotation. The bead-adherent complexes were washed 6 times [PBS (pH 8.0), 1% Triton X-100 and 150 mM NaCl]. Next, the proteins were eluted from the beads via incubation in SDS-PAGE sample buffer for 5 min at 80°C,

separated in SDS-PAGE gels and analyzed via WB analysis using primary antibodies against human Na^+, K^+-ATPase β subunits and human N-cadherin.

Transfection of siRNA

ARPE-19 cells were cultured for 4 weeks and incubated for 48 h with a mixture of Lipofectamine 2000 and 278 ng/μl of siRNA for the β_1 subunit, 700 ng/μl of siRNA for the β_2 subunit or 75 pmoles/μl of siRNA for Sp1, as indicated by the manufacturer. Thereafter, the transfection medium was removed, and the cells were processed for IF or WB analysis to estimate the silencing efficiency. siRNA for the Na^+, K^+-ATPase β_2 subunit and for Sp1 were pre-labeled with Cy3 using the siRNA Labeling Kit-Cy3 according to the manufacturer's protocol.

Relative mRNA Quantification via qPCR

Real-time PCR was performed with a Light Cycler 2.0 system (Roche) using the Light Cycler-Fast Start DNA Master SYBR Green I Kit (Applied Biosystems). We used the following sets of primers: β_2 subunit forward: GAGCTTCGTTCCACAGCTTC and reverse: CCCACCAAACCGTCTAGAAA; β_1 subunit forward: AGGCGTACGGTGAGAACATT and reverse: GGG AAAGATTTGTGCTTGTGA; β_3 subunit forward: TCGAGT ACTCCCCGTAACGA and reverse: AGGCTCTGGTTGAGG GACTT; α_1 forward: GAAGCAAGACGTCCTGGAAT and reverse: TTTCAGTCTTTCCGGGTGTT; α_2 forward: CTACCC TGTTGCTTTGGCTTTC and reverse: TGAGGGACCTTAGC GGGAGA; and GAPDH forward: ACGGCACAGTCAAGG CTGAG and reverse: CAGCATCACCCCATTTGATGTTGG. PCRs were performed using 45 cycles that included the following steps: 30 s of denaturation at 95°C, a 30-s annealing phase at 60°C, and a 30-s template-dependent elongation phase at 72°C. The amplification of each DNA template was performed in at least three experiments with two technical replicates in the same PCR run. The differential gene expression of the investigated genes was calculated as the ratio normalized to the expression

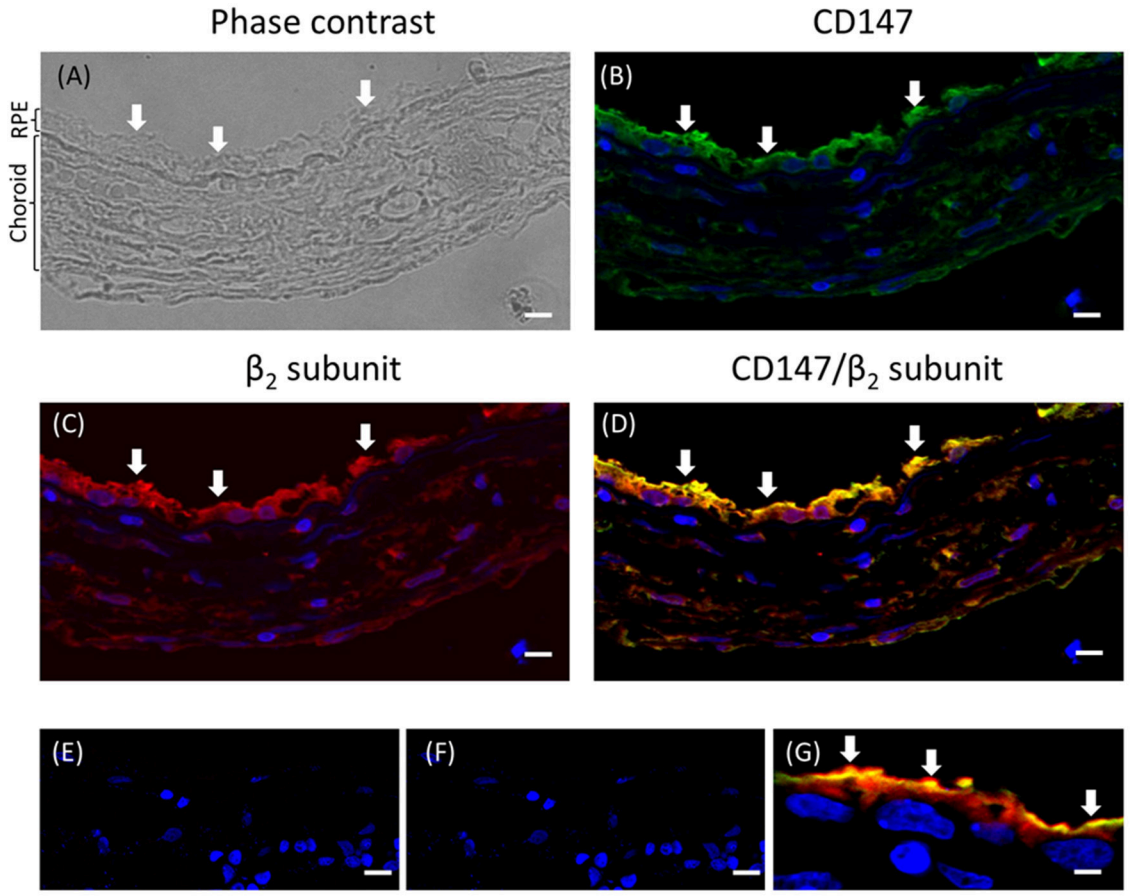

FIGURE 1 | Immunofluorescence of adult human eye *in situ*. (A) A phase-contrast image of the human eye section studied. The RPE layer and the choroid are indicated. The retina is already detached in the paraffin block. This section was co-stained for CD147 **(B)** and the β_2 subunit of the Na^+, K^+-ATPase **(C)** using Alexa 488- and Alexa 594-conjugated donkey anti-rabbit and anti-mouse IgG secondary antibodies, respectively. The merged image showing co-localization at the apical domain is in **(D)**. Panels **(E,F)** show similar sections treated only with the fluorescent secondary antibody (anti-rabbit and anti-mouse IgG, respectively) as negative controls. Panel **(G)** shows a higher-magnification image from a different field of the same preparation. All the preparations were counterstained with DAPI (blue). Arrows indicate the apical domain of RPE cells. Scale bars are 40 μm in **(A–F)** and 10 μm in **(G)**.

of the GAPDH gene. The data were analyzed using the equation described by Livak (Livak and Schmittgen, 2001; amount of target $= 2^{-\Delta\Delta CT}$).

Statistical Analysis

GraphPad Prism version 4.00 software was used for all statistical analyses. The data are presented as the mean ± SEM. Statistical significance was determined using a one-tailed, non-parametric *t*-test. $P \leq 0.05$ were considered significant.

RESULTS

The β_2-subunit of Na^+, K^+-ATPase is Expressed at the Apical Domain of the RPE in the Eye

To test the hypothesis that the apical targeting of Na^+, K^+-ATPase in RPE cells involves the expression of the β_2 subunit, we first analyzed the expression of the β_2 isoform at the apical membrane of the RPE in the eye. As shown in sections from human eye (**Figure 1**), co-localization at the apical domain was observed using anti-β_2 antibody and anti-CD147 antibody (basigin or cluster of differentiation 147, the accessory subunit of monocarboxylate transporters; 35). Thus, our data suggest that the apical Na^+, K^+-ATPase expressed in human RPE includes the β_2 isoform.

ARPE-19 Cells are Suitable for Studying the Mechanism Underlying the Polarity of Na^+, K^+-ATPase in the RPE

To further test our hypothesis, we chose human ARPE-19 cells as a model. ARPE-19 cells are fibroblast-like when cultured on inserts and go through a process of re-morphogenesis that lasts 6–8 weeks (Dunn et al., 1996). This period probably reflects the time required to up-regulate the expression of genes associated with differentiated RPE cells and is needed to develop the molecular machinery involved in membrane protein localization in RPE cells. First, we analyzed the polarized expression of Na^+, K^+-ATPase in ARPE-19 cells. In ARPE-19 cells cultured up to 4 weeks on transwell inserts covered with laminin, immunofluorescent staining of actin using rhodamine phalloidin showed flat cells with stress fibers and very few circumferential actin microfilament bundles (**Figure 2A**). The expression of

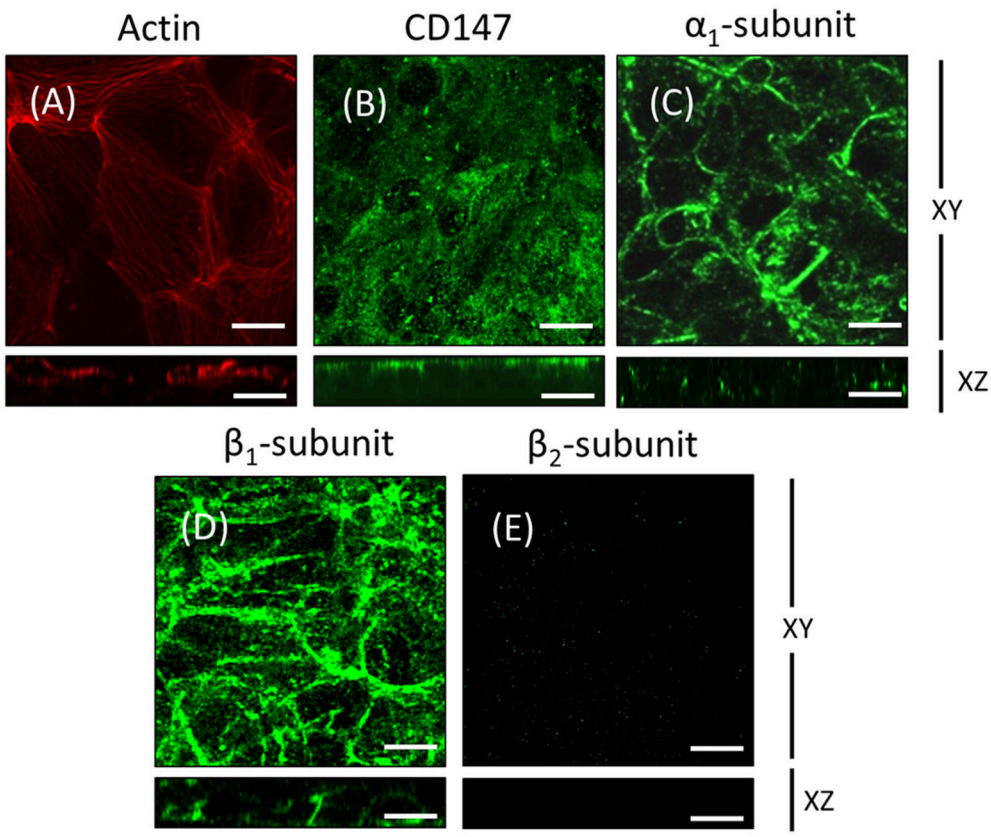

FIGURE 2 | ARPE-19 cells cultured on transwell inserts for 4 weeks are not completely polarized. ARPE-19 cells were cultured up to 4 weeks on transwell inserts covered with laminin. The immunofluorescence image in **(A)** shows actin localization using rhodamine phalloidin. The cells are flat with stress fibers and very little circumferential actin microfilament bundles. The expression of CD147, a RPE marker, was detected using a specific antibody in the apical membrane domain **(B)**. The expression of Na^+, K^+-ATPase using anti-α_1 **(C)** and anti-β_1 antibodies **(D)** was observed mostly at the basolateral membrane. Immunofluorescence detection with anti-human β_2 antibody revealed a very weak signal **(E)**. Scale bar: 10 μm.

CD147, was detected in the apical membrane domain using a specific antibody (**Figure 2B**). The expression of Na^+, K^+-ATPase using anti-α_1 and β_1 antibodies was mostly observed in the basolateral membrane (**Figures 2C,D**). Furthermore, using anti-human β_2 antibody, a very weak signal was detected (**Figure 2E**). Under these culture conditions, we were unable to study the mechanism underlying the apical polarization of the Na^+ pump. Thus, we decided to add ITS, which has been reported to epithelialize ARPE-19 cells (Luo et al., 2006). We then examined whether this supplement resulted in the apical localization of Na^+, K^+-ATPase. As shown in **Figure 3**, after 4 weeks of culturing, cells cultivated in the presence of ITS were epithelial-like in shape, with a circumferential actin microfilament bundle and occasional stress fibers (**Figure 3A**). As expected for RPE cells, the expression of molecular markers such as β-catenin and N-cadherin was observed in the lateral membrane (**Figures 3B,C**), and CD147 was observed at the apical and basolateral membrane (**Figure 3D**). Ezrin, a membrane-organizing phosphoprotein that tethers actin microfilaments to cell membrane proteins, is an apical polarization marker in the RPE (Kivelä et al., 2000). As shown in **Figure 3E**, after 4 weeks of culturing in the presence of ITS, ezrin was localized at the apical membrane in a pattern suggesting the formation of microvilli. The expression of Na^+, K^+-ATPase assessed using anti-α_1 and anti-β_1 antibodies (**Figures 3F,G**) was mainly observed in the basolateral domain, although β_1 expression was also observed in the apical domain. An apical pattern was observed using anti-β_2 antibody (**Figure 3H**). TJ formation was evaluated based on measurement of the TER of the monolayers. As depicted in **Figure 3I**, the TER was stabilized at 4 weeks, with an average value of 80 Ω•cm^2, which is a characteristic value reported in these cells (Dunn et al., 1996; Luo et al., 2006). Hence, we considered that under these conditions (designated as re-morphogenic conditions and detailed in the Methods), it would be feasible to perform experiments addressing the intriguing issue of the "reversed" apical polarization of Na^+, K^+-ATPase in RPE cells.

Expression of the α_2 and β_2 Isoforms is Up-regulated during Re-morphogenesis

To characterize the expression of Na^+, K^+-ATPase under re-morphogenic conditions, we analyzed the mRNA and protein expression levels of various isoforms of the α and β subunits. The applied antibodies were carefully chosen to ensure that they were specific for the designated isoform. As shown in **Figure 4A**, ARPE-19 cells cultured in the presence of ITS expressed the three β subunits (β_1, β_2, β_3). Remarkably, these findings are the first evidence of β_3 isoform expression in ARPE-19 cells. We then evaluated changes in the amount of mRNA for the α and β isoforms over time via qPCR. As shown in **Figures 4B,C**, mRNAs corresponding to all five studied isoforms (α_{1-2}, β_{1-3}) were expressed in ARPE-19 cells, and their relative amounts increased during re-morphogenesis. As shown in **Figure 4B**, although the relative amounts of β_1 and β_2 mRNA increased up to the sixth

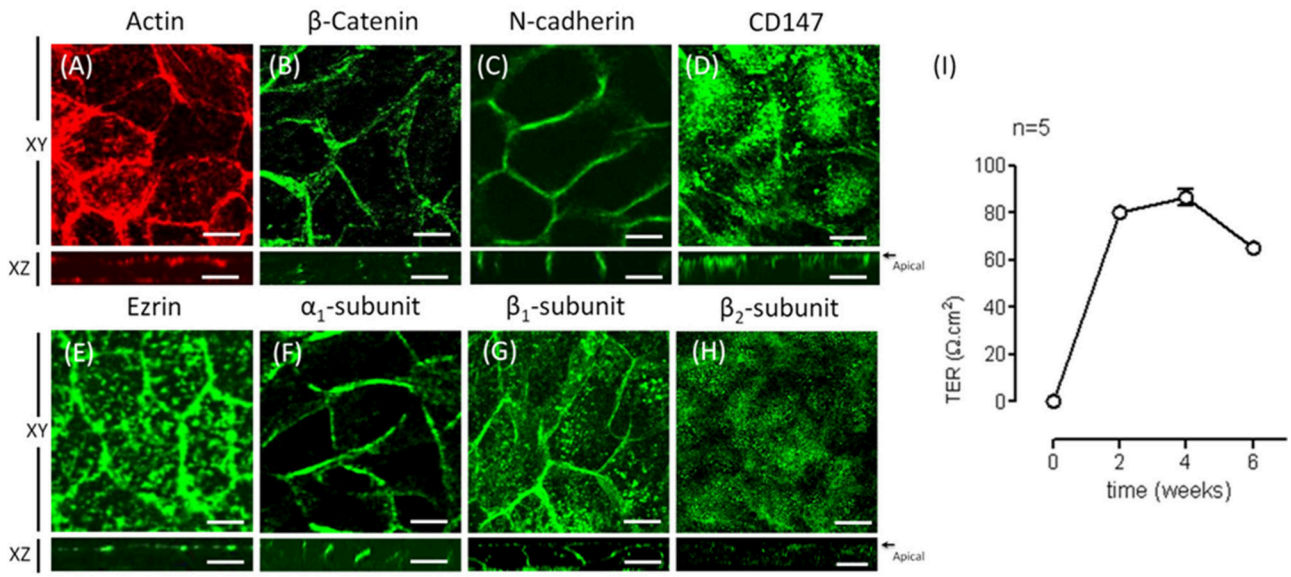

FIGURE 3 | Re-morphogenesis of ARPE-19 cells treated with ITS. (A) The photomicrographs show ARPE-19 cells cultured for 4 weeks with ITS and stained for actin with Texas Red-X phalloidin. Note the tight intercellular contact, epithelial-like cellular shape, and predominantly peripheral cortical actin staining. Confocal immunofluorescence images of the same culture conditions showing β-catenin **(B)** and N-cadherin **(C)** at the lateral membrane, CD147 **(D)** mostly in the apical domain and ezrin **(E)** in the apical domain. **(F)** The immunofluorescence image shows immunostaining of the α_1 subunit of Na^+, K^+-ATPase at the lateral membrane and more precisely, as observed in the XZ image, at the cell-cell contacts. **(G)** The β_1 subunit is observed at the cell border and at the apical domain; the XZ image confirms both basolateral and apical staining. **(H)** The β_2 subunit is observed mainly at the apical domain; the XZ image confirms apical staining. Scale bar: 10 μm. **(I)** Quantitative analysis of the transepithelial resistance (TER) of ARPE-19 cells during re-morphogenesis is depicted. Note a stable TER of ~80 Ω cm^2 between the second and fourth week in culture.

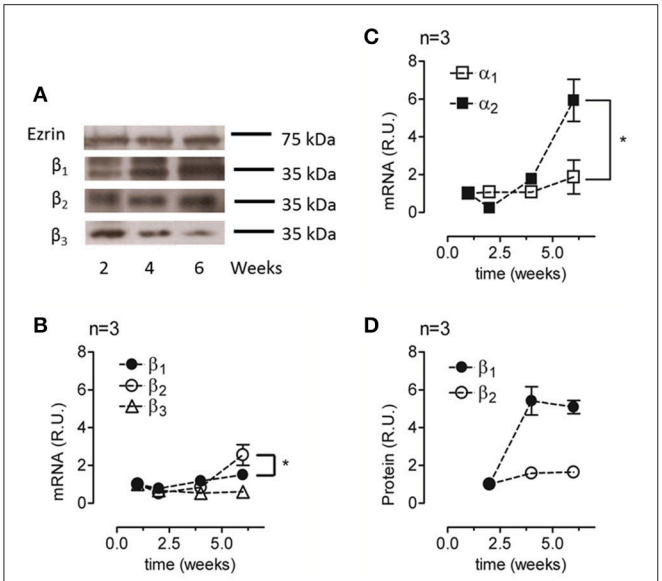

FIGURE 4 | Analyses of the relative amounts of α and β isoforms in ARPE-19 cells during re-morphogenesis. (A) Western blot analysis of the lysates of ARPE-19 cells cultured for the indicated time under conditions established for re-morphogenesis. The upper part of the blot was probed with an antibody against ezrin (as a loading control), whereas the lower part was probed with antibodies against the β_1, β_2, and β_3 subunits. Western blot analyses of all samples were conducted on the same day. A non-relevant lane between 2 and 4 weeks in the ezrin blot was eliminated; therefore, it appears to be discontinued. The blots represent three different experiments. Total mRNA was extracted at weeks 1, 2, 4, and 6 of culture and analyzed via qPCR. The relative mRNA levels of the β_1, β_2, and β_3 subunits **(B)** and of the α_1 and α_2 subunits **(C)** are illustrated. The amount of mRNA was normalized to that detected in the first week. **(D)** Quantification of β_1 and β_2 subunit expression via densitometry in three independent experiments normalized to the loading control. Densitometry results were also corrected for the film light level whenever the background level was different. Error bars represent the mean ± SEM of three independent experiments. Significant changes are indicated by an asterisk ($P < 0.05$, non-parametric *t*-test).

week, the mRNA levels of β_2 increased to a significantly higher value than those of β_1. However, the amount of β_3 mRNA remained relatively constant during this time period (**Figure 4B**). Therefore, we did not study this isoform further. Nevertheless, while the amount of α_1 mRNA was doubled, the expression of α_2 increased 6-fold (**Figure 4C**). When analyzing the amounts of total protein of the β_1 and β_2 isoforms, we observed an increase over time, reaching maximal expression at 6 weeks. Altogether, **Figure 4** shows that during re-morphogenesis, the expression of the β_2 subunit was up-regulated, resulting in increasing amounts of both mRNA and protein. This suggests transcriptional level regulation.

The Transcription Factor Sp1 is Involved in Regulating the Expression of the β_2 Subunit in ARPE-19 Cells

During re-morphogenesis, expression of the α_2 and β_2 isoforms is up-regulated, increasing both their mRNA and protein levels. The transcription factor specificity protein 1 (Sp1) binds GC-rich motifs and regulates gene expression through protein–protein interactions (Shull et al., 1989; Samson and Wong, 2002). Based on previous works by Kawakami et al. (1990, 1992) and Avila et al. (1998) that reported that Sp1 enhances the promoter activity of the β_2 subunit in rat neuroblastoma, in rat embryo cell lines and in human lymphocytes, we suspected that Sp1 could be at least one of the factors regulating this process. Therefore, we explored whether Sp1 was involved in the up-regulation of the β_2 subunit during the re-morphogenesis of ARPE-19 cells. As shown in **Figure 5A**, the relative amount of Sp1, as estimated via WB analysis, was slightly changed during re-morphogenesis. IF images in **Figure 5B** show that Sp1 was expressed in the nuclei of ARPE-19 cells cultured for 4 weeks in the presence of ITS. The silencing of Sp1 by siRNAs specific for human Sp1 in ARPE-19 cells (cultured for 4 weeks) reduced the total protein level, as estimated from the WBs, by ∼40% (**Figure 5C**). This partial silencing corresponds to the IF image of the silenced cells in **Figure 5D**. The arrowheads indicate cells in which the expression of Sp1 (in green) was not observed, although these were still surrounded by cells that did express Sp1 in their nuclei. We anticipated that if Sp1 was involved in β_2 transcription, Sp1 silencing would also reduce the amount of the β_2 subunit in ARPE-19 cells. As shown in **Figure 5E**, the total amount of β_2 estimated via WB analysis was reduced by ∼50% in Sp1-silenced cells. Correspondingly, the IF image of β_2 subunit expression (**Figure 5F**) shows zones in the Sp1-silenced monolayer with low fluorescence signal (indicated by arrowheads). These data suggest that the transcription factor Sp1 is probably involved in regulating the expression of the β_2 subunit in ARPE-19 cells.

Apical Expression of Na^+, K^+-ATPase in ARPE-19 Cells during Re-morphogenesis is Correlated with the Expression of the α_2 and β_2 Isoforms

The WB and qPCR results in **Figure 4** show that ARPE-19 cells expressed all three β isoforms and at least two α isoforms. We therefore proceeded to analyze the polarized distribution of the different α and β isoforms in these cells. As observed from the confocal IF analysis (**Figure 6**) and in contrast to the non-polarized distribution of the β_1 isoform shown in **Figure 3G**, β_3 was mostly localized at the basolateral membrane and did not co-localize with ezrin (**Figure 6A**). Therefore, we did not study the role of this isoform in the apical localization of the pump in RPE. The β_2 subunit was distributed in a typical dotted pattern that suggested an apical localization (**Figure 6B**). The β_1 subunit was distributed in both the basolateral and apical domains (**Figure 6C**). Nonetheless, the apparent apical pattern was not homogenous, presenting a mosaicism that has been previously reported for RPE cells (Burke, 2008). Because the β subunit must associate with the α subunit to reach the plasma membrane, we analyzed the α isoform accompanying the β_2 subunit in ARPE-19 cells using an IF assay. **Figure 6D** shows that α_1 was present at the basolateral membrane and was clearly excluded from the apical domain marked by ezrin. However,

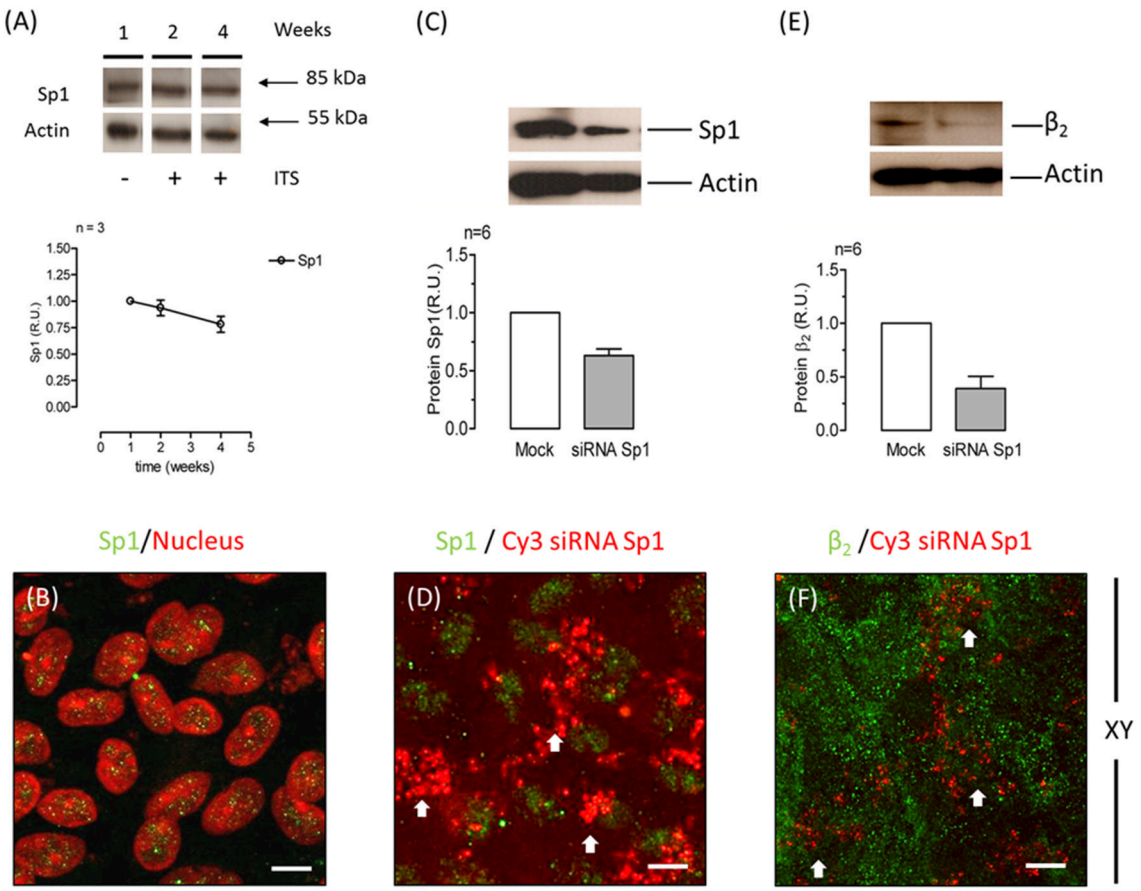

FIGURE 5 | The transcription factor Sp1 is involved in β2 subunit regulation in ARPE-19 cells. (A) Western blot analysis showing the expression of Sp1 over 4 weeks of culturing (upper panel). Actin was used as a loading control in all Western blots shown in this figure. Quantitative analysis of Sp1 normalized to the loading control is illustrated in the lower panel. **(B)** Immunofluorescent staining of Sp1 in ARPE-19 cells is shown in green. Counterstaining of nuclei is shown in red. Merged image shows the nuclear localization of Sp1 in ARPE-19 cells cultured for 4 weeks in the presence of ITS. Scale bar: 20 μm. **(C)** Representative Western blot and quantitative analyses of six independent experiments conducted on ARPE-19 cells treated with Sp1 siRNA are shown. Control cells were transfected without siRNA (Mock). Error bars represent the mean ± SEM. **(D)** Immunofluorescence image of ARPE-19 cells incubated with Cy3-siRNAs to silence Sp1 (red) and immunostained for Sp1 expression (green). White arrows indicate siRNA-transfected cells (identified by red fluorescence) that did not express Sp1 in the nucleus. Scale bar: 20 μm. **(E)** Representative Western blot and quantitative results of the immunodetection of the β_2 subunit in six independent experiments of Sp1 knockdown in cells (60%). **(F)** Immunofluorescence image of Sp1-silenced ARPE-19 cells stained for β_2 subunit expression.

α_2 was distributed in an apical pattern and was apparently excluded from cell-cell contacts (**Figure 6E**), very similar to the β_2 pattern. We also observed a lack of co-localization between α_1 and β_2 (**Figure 6F**), α_2 and β_1 (**Figure 6G**) and α_1 and α_2 isoforms (**Figure 6H**). Thus, the apical pump in RPE cells is most likely an α_2/β_2 complex. Although the IF distribution pattern of the β_2 subunit in polarized ARPE-19 cells suggests an apical localization, we had to confirm that the pump assembled by the β_2 isoform was actually delivered to the apical membrane domain of ARPE-19 cells. Therefore, we examined the co-localization of the β_2 and α_2 subunits with the apical marker CD147 (**Figures 7A,B**) and with the basolateral markers N-cadherin and β-catenin (**Figures 7C,D**). Images obtained via confocal microscopy (**Figure 7**) show that the β_2 subunit did not co-localize with markers of the basolateral or apical domains. However, a lack of co-localization demonstrated by IF was not sufficient to conclude that β_2 does not reside at the apical membrane. Thus, we proceeded to perform a steady-state surface biotinylation assay. ARPE-19 cells were cultured for 4 weeks with ITS on inserts. Biotin was added to both the apical and basolateral sides of the monolayer. As shown in **Figure 7E**, these cells expressed both N-cadherin and the β_2 subunit, as detected in the total cell lysate (input). N-cadherin was labeled with biotin as expected. Nevertheless, the β_2 subunit was not detected in the biotin-labeled (streptavidin-precipitated) fraction in any of the 6 experiments performed. Therefore, our results indicate that apical pumps including β_2 subunits probably did not accumulate in the apical membrane domain of ARPE-19 cells. To evaluate this possibility we used immunofluorescence assays to analyze whether α_2 and β_2 co-localize at the apical domain in sections of human eye. As shown in **Figure 8**, the α_2 and β_2 subunits co-localize in an

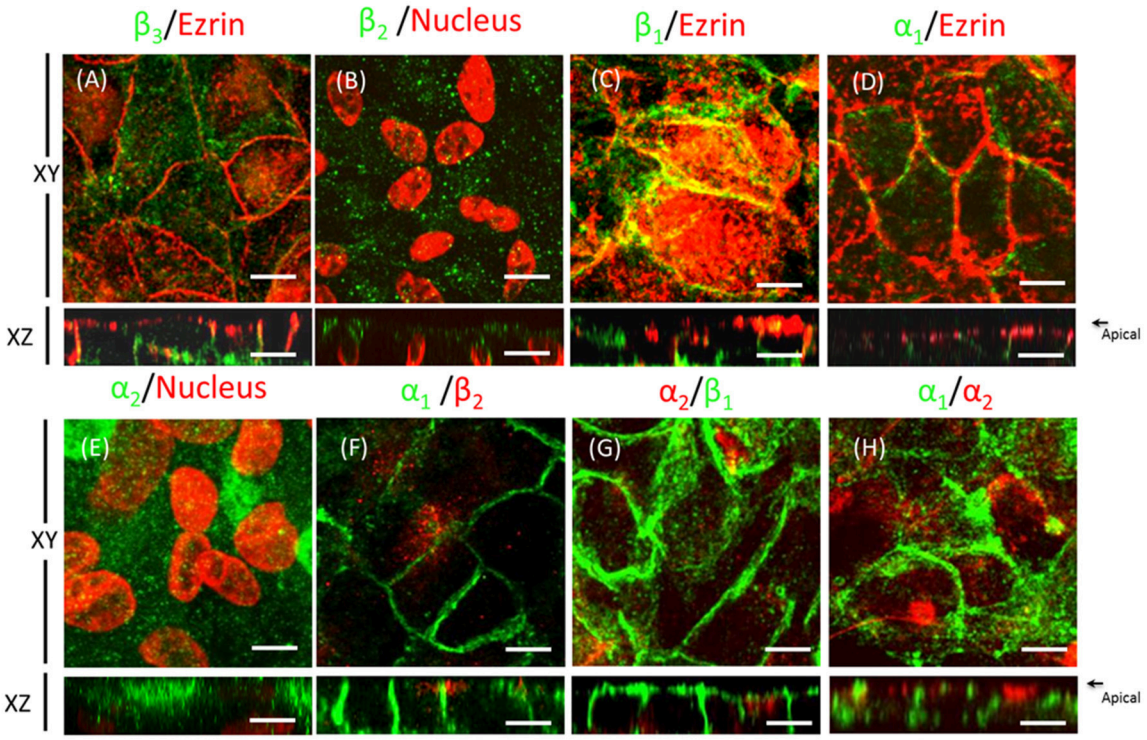

FIGURE 6 | Domain-specific distribution of the Na^+, K^+-ATPase isoforms in ARPE-19 cells. Immunofluorescence assays of ARPE-19 cells cultured for 4 weeks with ITS. **(A–E)** Fluorescence images of immunostaining with isoform-specific antibodies against the α and β subunits of Na^+, K^+-ATPase; co-staining with anti-ezrin as an apical marker **(A,C,D)** or counterstaining with propidium iodide for the detection of nuclei **(B,E)**. β_3 **(A)** and α_1 **(D)** subunit expression was detected mainly in the lateral domain. The distribution pattern of the β_2 and α_2 subunits **(B,E)** suggests an apical localization. The distribution pattern of the β_1 subunit **(C)** in both the lateral and apical domains suggests non-polarized expression. Minor co-localization with ezrin at the apical domain is observed. The α_1/β_2 **(F)**, α_2/β_1 **(G)** and α_1/α_2 subunits **(H)** do not co-localize. Scale bar: 10 μm.

apical domain. Considering that the β_2 subunit is an adhesion molecule (Gloor et al., 1990), we speculated that it did not stabilize in the plasma membrane because it could not interact with a receptor protein at the apical "lumen" of the monolayer. Hence, the apical pumps observed via IF in ARPE-19 cells may result from apical recycling of β_2 subunits accumulated in endosomes (AREs or CREs, 12). Interestingly, the β_1 isoform was clearly detected when the biotin-labeled fraction of the ARPE-19 cells was blotted for the β_1 subunit (data not shown), suggesting that the β_1 isoform had a slower turnover in the plasma membrane and therefore was detectable in a steady-state analysis. However, these findings do not preclude the involvement of the β_2 subunit in the apical polarization of Na^+, K^+-ATPase.

Silencing the Expression of the β_2 Isoform in Mature ARPE-19 Cells Decreases the Apical Localization of Na^+, K^+-ATPase

To further examine the dependence of the apical sorting of the Na^+ pump on the expression of the β_2 isoform, we knocked down the β_2 isoform using siRNAs. As shown in **Figure 9A**, the expression of β_2 mRNA in ARPE-19 cells treated with siRNAs specific for human β_2 decreased by 60%. Meanwhile, the relative amount of α_2 mRNA was also reduced. Nevertheless, the mRNA levels of the α_1 and β_1 isoforms were sustained at the same levels observed in the non-silenced cells (mock). The WB results for the total cell lysates (**Figure 9B**) show that the overall amount of β_2 in siRNA-treated monolayers of ARPE-19 cells was only slightly decreased. This can be explained by the fact that ARPE-19 monolayers have a low index of proliferation and therefore a low rate of protein recycling. Although the amount of mRNAs is significantly reduced there is always a high amount of remnant proteins in the cells. Nevertheless, the IF images in **Figure 10B** show areas in the monolayer in which silencing was apparently effective because the fluorescence signal due to β_2 expression in green was faint. At the same time, the apical expression of the α_2 subunit (**Figure 10D**) also seemed to be reduced. The localization of β_1 subunits changed substantially, displaying more apical rather than lateral distribution (**Figures 10E,F**). The lateral distribution of α_1 subunit, as shown in **Figures 6D,F**, **10G**, was also altered, showing a mixed distribution in the apical and lateral domains. The WB and IF results in **Figures 9**, **10** show a partial silencing. The siRNA-transfected monolayer still contained cells expressing the α_2 and β_2 subunits at the apical membrane, Therefore, it was difficult to determine whether the apical localization of the pump was indeed altered. Hence, we knocked down the monolayer with siRNAs that were pre-labeled

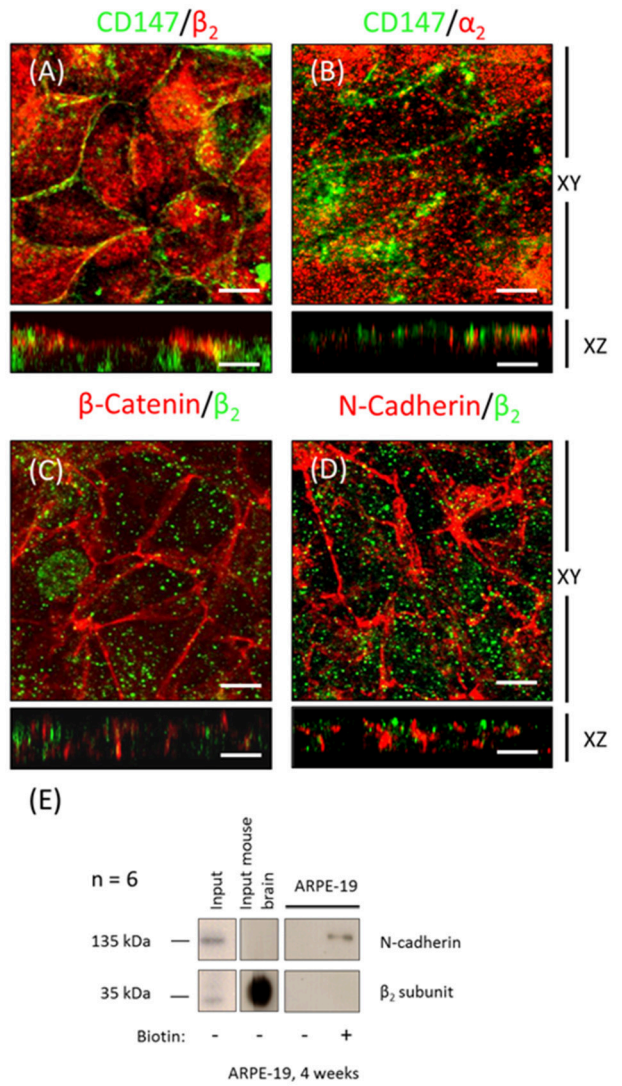

FIGURE 7 | The β_2 subunit in ARPE-19 cells does not stabilizes at the apical plasma membrane domain. Confocal images of the β_2 and α_2 subunits (in red) were analyzed for co-localization with CD147 (green), an apical marker **(A,B)**. Co-localization of the basolateral markers β-catenin and N-cadherin (in red) with the β_2 subunits (in green) are shown in **(C,D)**. Although an apparently apical pattern is observed for the Na^+, K^+-ATPase subunits β_2 and α_2, they do not co-localize with either apical or lateral markers. Scale bar: 10 μm. Representative Western blot of six independent experiments conducted in ARPE-19 cells for steady state surface labeling with biotin is presented in **(E)**. Notice the lack of a β_2 subunit band in the biotin labeled lane.

with the fluorochrome Cy3 because it helped us identify probable silenced cells in a partially silenced monolayer. As shown in **Figures 11A,B**, the monolayer was not completely silenced, and β_2-subunit staining was still observed at the apical domain in non-silenced cells (green fluorescence spots). Nevertheless, as shown in **Figure 11B**, expression of the β_2 subunit in silenced cells that were stained with Cy3 (in red) is clearly weaker (white arrows), suggesting effective silencing in these cells. In **Figure 11C**, we measured the green fluorescence intensity of the β_2 subunit in Cy3-positive cells. The average expression in these cells is reduced by 75%, indicating effective silencing. Expression of the α_2 subunit was also monitored in silenced cells. As illustrated in **Figures 11D,E**, the apical localization of the α_2 subunit was conserved. Nevertheless, measuring the fluorescence intensity of the α_2 subunit in Cy3-positive cells revealed a 40% decrease in α_2 subunit expression (**Figure 11F**). Our silencing results support the notion that the apical sorting of Na^+, K^+-ATPase in ARPE-19 cells during re-morphogenesis depends on the expression of the β_2 isoform and its association with the α_2 subunit.

DISCUSSION

RPE cultures appear to have a limited ability to reiterate epithelialization and undergo phenotypic maturation, a process described as re-morphogenesis by Burke (2008). In the present study, we first had to establish conditions that supported the re-morphogenesis of ARPE-19 cells *in vitro*. The appearance of Na^+, K^+-ATPase in the apical domain of ARPE-19 cells reflects the maturation and differentiation of the monolayer (Burke et al., 2000; Kannan et al., 2006; Sonoda et al., 2010). In this study, we addressed one aspect of the apical polarization mechanism: identifying the isoform that may contain the apical information (signal) necessary for the apical sorting of Na^+, K^+-ATPase in the RPE. Our observations indicate that the α_2 and β_2 isoforms are rarely detected in non-mature ARPE-19 cells (**Figure 2**) and constitute the apical pump in polarized RPE cells (**Figure 8**). We showed that the apical sorting of Na^+, K^+-ATPase in ARPE-19 cells correlates with the expression of the β_2 subunit (**Figure 6**), a finding that is consistent with the observations in fixed eye sections (**Figure 1**). We also showed that during the process of re-morphogenesis, the expression of the α_2 and β_2 isoforms was up regulated (**Figure 4**) and that Sp1 is probably involved in that regulation (**Figure 5**). Although in the eye, the pumps composed of α_2 and β_2 subunits were localized in the apical membrane domain (**Figure 8**), they did not accumulate in the apical membrane in cultured ARPE-19 cells (**Figure 7**) but were probably retained in a sub-apical compartment. The increase in mRNA was much more pronounced than that of the extracted protein. Accordingly, silencing of the Na^+, K^+-ATPase β_2 subunit by siRNA resulted in a decrease in the apical localization of Na^+, K^+-ATPase in knocked-down cells (**Figures 10**, **11**) but without a clear change in α_1/β_1 localization.

ARPE-19 Cells As a Model for Studying Polarity in the RPE

The RPE forms the outer blood-retinal barrier that regulates the movement of solutes between the capillaries of the choroid and the photoreceptor layer of the retina. Although human fetal RPE (hfRPE) primary cultures are considered the best model for exploring the polarity and trafficking mechanisms in RPE (Lehmann et al., 2014), we have no access to primary cultures of hfRPE. Therefore, we used the cell line ARPE-19, which was obtained from a spontaneously transformed human

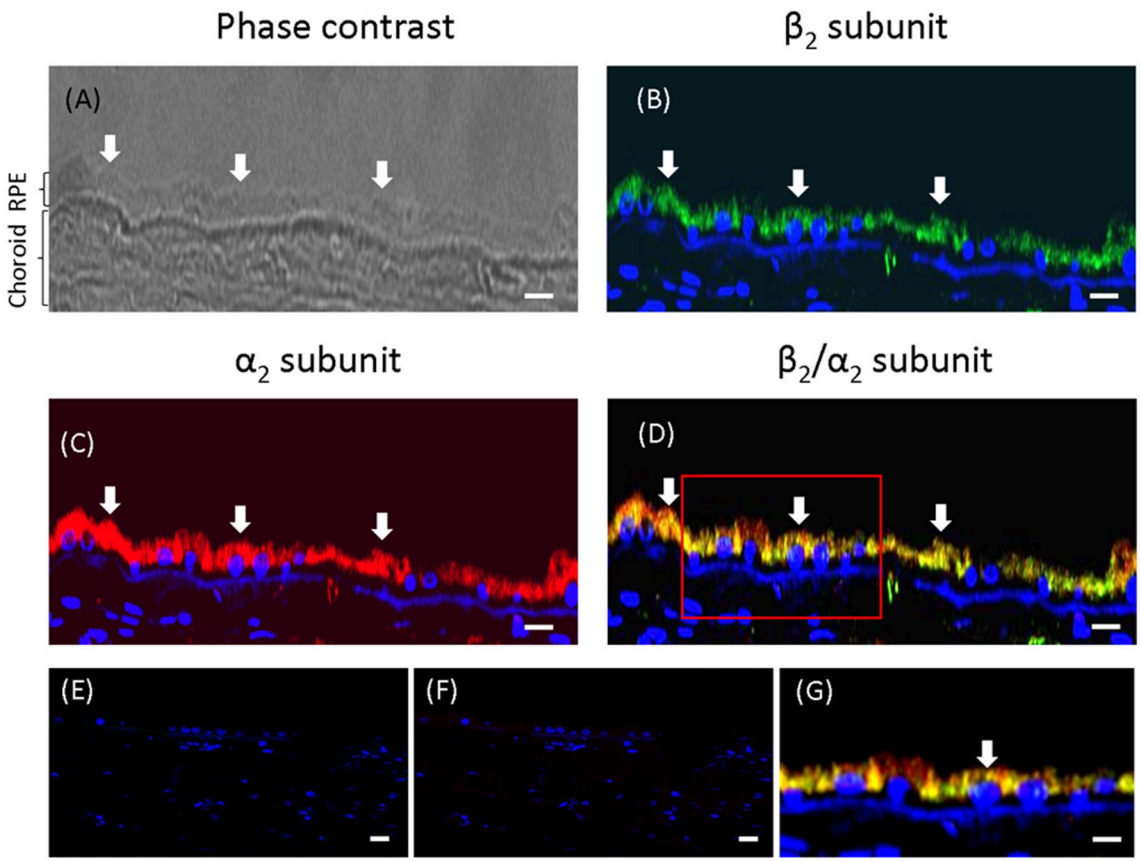

FIGURE 8 | Co-localization of the α_2 and β_2 subunits in adult human eye. (A) A phase-contrast image of the human eye section studied. The RPE layer and the choroid are indicated. This section was co-stained for the β_2 **(B)** and α_2 subunits **(C)** of Na^+, K^+-ATPase using Alexa 488- and Alexa 594-conjugated donkey anti-rabbit and anti-mouse IgG secondary antibodies, respectively. The merged image showing co-localization at the apical domain is in **(D)**. Panels **(E,F)** show images of similar sections treated only with the secondary fluorescent antibody (anti-rabbit and anti-mouse, IgG, respectively) as a negative control. Panel **(G)** shows the field indicated by the square in **(D)** at higher magnification. All the preparations were counterstained with DAPI (blue). Arrows indicate the apical domain of RPE cells. Scale bars are 25 μm in **(A–D)**, 40 μm in **(E,F)** and 30 μm in **(G)**.

RPE primary culture (Dunn et al., 1996). ARPE-19 shows acceptable conservation of polarity and barrier function for studies of protein trafficking. The main advantages of ARPE-19 cells are its normal karyotype, relatively fast proliferation rate, and maintenance of several RPE-specific characters (Dunn et al., 1996). Lehmann et al. (2014) mention that in ARPE-19 cells, "the trafficking machinery is likely different from RPE *in situ* because the Na^+, K^+-ATPase was reported to be basolateral in ARPE-19 cells." Based on our experiments, we suggest using greater precision when considering Na^+, K^+-ATPase polarity and discussing specific dimer compositions: $\alpha_1\beta_1$ or $\alpha_2\beta_2$. Thus, our data are consistent with the findings of Ahmado et al. (2011) with respect to the basolateral distribution of $\alpha_1\beta_1$. Surprisingly, several studies do report an apical localization of the Na^+ pump when using anti-α_1 antibodies in ARPE-19 cells. Nevertheless, different authors define distinct patterns of localization based on IF images as apical (Geisen et al., 2006; Kannan et al., 2006). It is well documented that both primary cultures and cell lines tend to lose the RPE-specific properties with consecutive passages. The disruption of cell-cell adhesion induces an EMT, resulting in a loss of the RPE phenotype that can become irreversible (Grisanti and Guidry, 1995; Gallagher-Colombo et al., 2010; Tamiya et al., 2010; Adijanto et al., 2012). Accordingly, we suggest that $\alpha_1\beta_1$ is the default dimer expressed and is sorted primarily to the basolateral membrane domain in non-differentiated ARPE-19 cells. During re-morphogenesis, only some ARPE-19 cells epithelialize to achieve a RPE phenotype, while others remain in a mesenchymal state. Here, we applied culture conditions that augmented the proportion of well-differentiated cells but still failed to obtain a fully differentiated cell population. Under these improved conditions, the expression of the $\alpha_2\beta_2$ dimer was up-regulated, and after 4 weeks, there was a large proportion of cells with this dimer localized in a pattern resembling an apical distribution. Evidently, the $\alpha_2\beta_2$ dimer was absent from the basolateral domain. The apparent apical localization probably depends on the maturation and differentiation of the apical trafficking machinery, which was also only partially achieved.

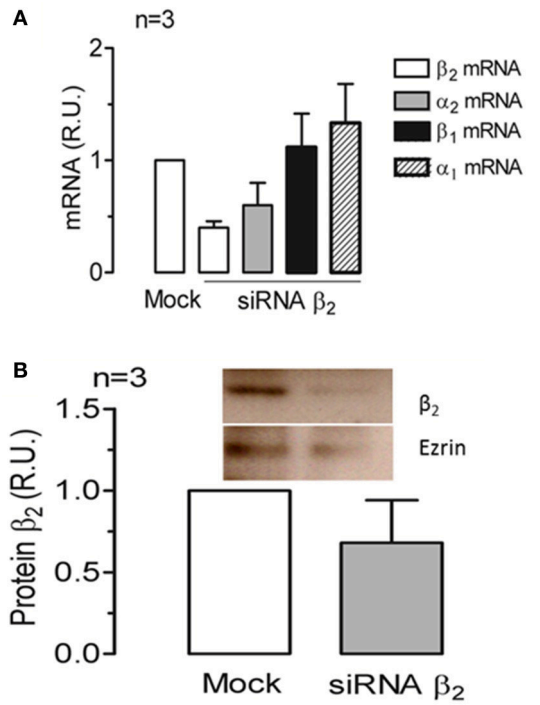

FIGURE 9 | siRNA silencing of the human Na^+, K^+-ATPase β_2 isoform in mature ARPE-19 cells. ARPE-19 cells cultured for 4 weeks with ITS were knocked down using siRNAs against the human β_2 subunit. **(A)** mRNA levels of the α_1, α_2, β_1, and β_2 subunits were assessed in three independent experiments in ARPE-19 cells transfected with β_2 siRNA or without any siRNA (Mock) via qPCR. The extent of β_2 mRNA silencing was ~60%. **(B)** Western blot and quantitative results from silenced ARPE-19 cells treated as in **(A)** are presented. The amount of protein corresponding to the β_2 subunit was normalized to the level detected in the mock-transfected cells. The extent of β_2 protein silencing was ~40%.

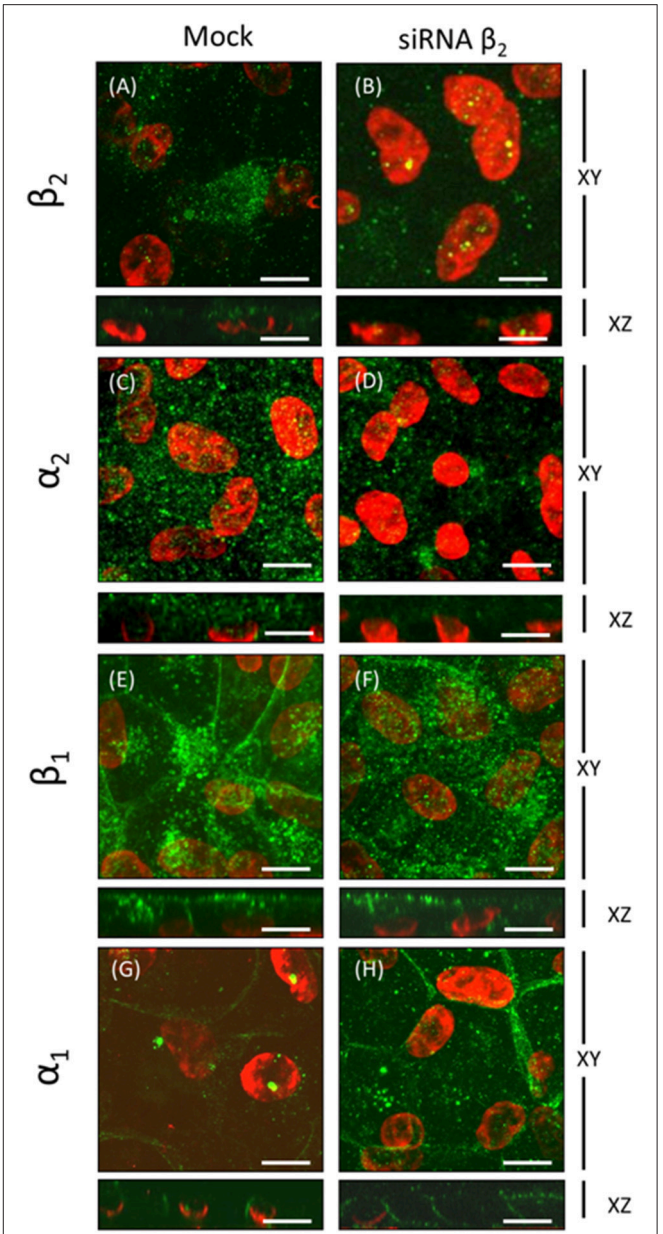

FIGURE 10 | Immunofluorescence analyses of ARPE-19 cells silenced with siRNA against the human β_2 isoform. ARPE-19 cells cultured for 4 weeks were transfected with or without siRNA specific to the human β_2 isoform, as shown in **Figure 8**. Confocal images of silenced (right panels) or not-silenced (left panels) cells immunostained for specific α and β isoforms are displayed. Apical expression of the β_2 and α_2 isoforms in mock-transfected cells is apparent in **(A,C)**. Mislocalization and reductions in the fluorescence intensity of both β_2 and α_2 subunits are observed in silenced cells **(B,D)**. Basolateral and apical expression of the β_1 isoform **(E)** is generally maintained in β_2-silenced cells. However, in the presented field, β_1 has a noted apical distribution pattern **(F)**. The mainly basolateral pattern of the α_1 isoform **(G,H)** is maintained in silenced cells. Scale bar: 10 μm.

The Transcription Factor Sp1 Expressed in ARPE-19 Cells is Probably Involved in Regulating the Expression of the β_2-Subunit

During re-morphogenesis, the mRNA and protein expressions of the α_2 and β_2 isoforms are up-regulated. It is conceivable that this long-range up-regulation suggests transcriptional regulation and thus the participation of transcription factors. Shull et al. (1989) and Ikeda et al. (1993) observed that Sp1 also activates the α_2 promoter in rat and human skeletal myoblasts. Together, these data suggest that the transcription factor Sp1 is involved in the up-regulation of α_2 and β_2. Our observations (**Figure 5**) support these previous findings. Recent evidence points to a role for Sp1 in regulating the transcription of genes in response to extracellular signals such as insulin (Therien and Blostein, 2000). Hence, the addition of insulin (a component of the ITS mixture) to the culture medium could activate Sp1, promoting Na^+, K^+-ATPase expression via binding to positive regulatory *cis*-acting elements on the Na^+, K^+-ATPase β_2 gene (Takeyasu and Kawakami, 1989; Sweadner et al., 2000; Tanos and Rodriguez-Boulan, 2008). Nevertheless, additional experiments are needed to clarify the mechanism of β_2 regulation via Sp1 in ARPE-19 cells.

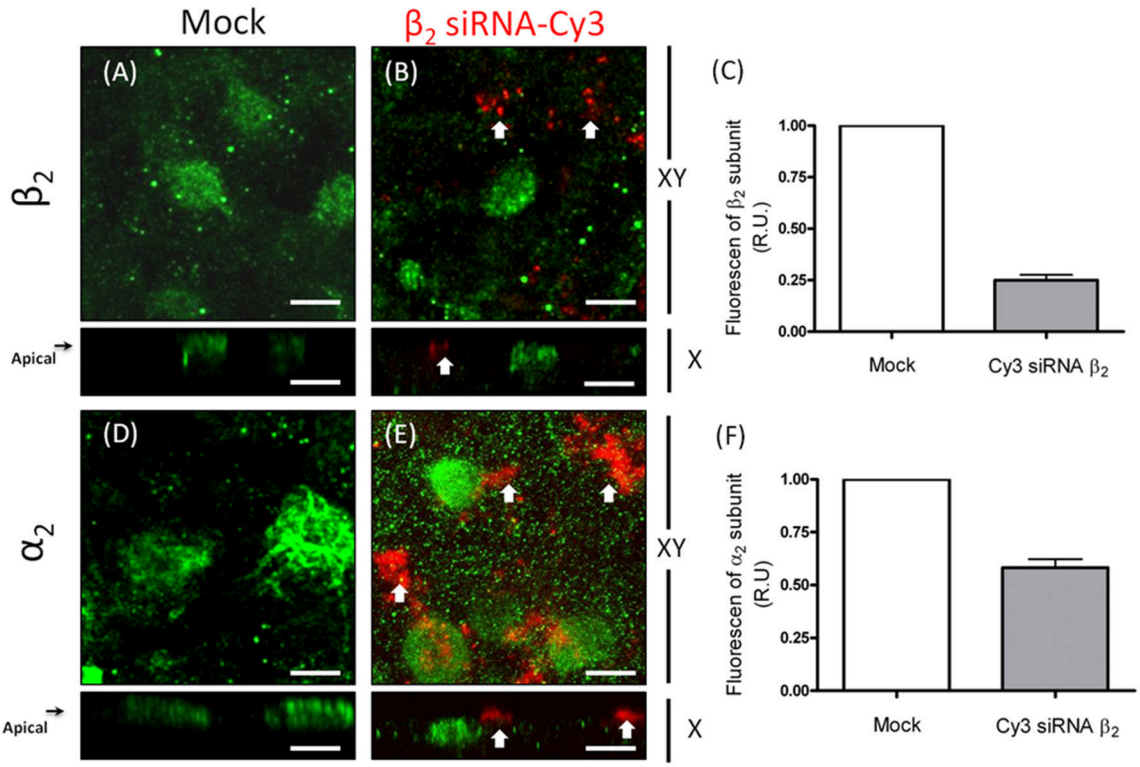

FIGURE 11 | Silencing of the β_2 subunit in ARPE-19 cells affects the apical distribution of Na^+, K^+-ATPase. The transfected siRNAs for the β_2 isoform are labeled with Cy3. Red fluorescent spots thus identify transfected cells. Confocal images of mock-transfected cells in which the β_2 and α_2 subunits are immunostained **(A,D)**, respectively show an apical distribution pattern for both subunits. Images of silenced cells show an apical expression of the β_2 **(B)** and α_2 **(E)** subunits (green) in non-transfected cells. Arrows in **(B)** indicate the lack of β_2 expression in the apical domain of siRNA-transfected cells. The absence of α_2 immunostaining in the apical domain is also indicated by arrows in panel **(E)**, implying the mislocalization of Na^+, K^+-ATPase from the apical domain. The fluorescence intensity of β_2 **(C)** and α_2 subunits **(F)** in silenced cells was measured in 10 different fields, and the mean ± SEM is shown. Scale bar: 10 μm.

The Apparent Apical Polarization of the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ Dimers in ARPE-19 Cultures

In classic epithelia, the mechanism underlying the basolateral polarization of Na^+, K^+-ATPase is related to the expression of the α_1 and β_1 subunits. Nevertheless, published reports on the isomer-specific composition of Na^+, K^+-ATPase in the RPE are somewhat confusing. Because the RPE originates from the neuroepithelium of the optic vesicle, we hypothesized that it would express the neuronal AMOG/β_2 isoform, which was supported by our IF experiments depicted in **Figures 1**, **8**. Nonetheless, the literature includes both consistent and contradictory reports. (a) An analysis of human RPE mRNA revealed the expression of the α_1, β_1 and β_2 isoforms but not the α_2 isoform (Ruiz et al., 1995, 1996). (b) The distribution of all subunits examined revealed that α_1 and β_1 were the predominant isoforms expressed in mouse and rat RPE, while the β_2 isoform was detected in photoreceptors, bipolar cells and Müller glia but not in the RPE (Wetzel et al., 1999). (c) Most studies using RPE cells *in vitro* have utilized anti-α_1 and anti-β_1 antibodies for immunodetection of Na^+, K^+-ATPase (Miller and Steinberg, 1979; Rizzolo and Zhou, 1995; Burke et al., 2000; Kannan et al., 2006). Our observations suggest that in non-polarized ARPE-19 cells, the ubiquitous $\alpha_1\beta_1$ dimer is the default housekeeping Na^+ pump essential for all living cells. This dimer likely uses the non-differentiated trafficking mechanism to arrive at the plasma membrane and is then stabilized and enriched in cell-cell contacts due to β_1-β_1 *trans* interactions between neighboring cells. Considering that the β_2 subunit is an adhesion molecule, in the eye, it would interact with a heterotypic adhesion protein localized on the outer segment of the photoreceptor membrane, maintaining the complex at the apical domain. Our images taken from human eye sections (**Figure 8**) support this assumption. Accordingly, it is plausible that Na^+, K^+-ATPase is only detected at the apical domain of cultured RPE cells under very specific conditions (Hu et al., 1994; Marrs et al., 1995; Rizzolo and Zhou, 1995; Kannan et al., 2006; Sonoda et al., 2010) because of the lack of an interaction of RPE cells with photoreceptors in cultures. However, in various RPE models, Na^+, K^+-ATPase is observed in the apical domain, even in the absence of contact with the retina. Nevertheless, no previous studies have confirmed these observations via co-staining with apical markers or surface biotinylation. Evidently, the absence of photoreceptors on the apical side of cultured RPE cells does not mean that the Na^+ pump is not being sorted and delivered to that domain, but

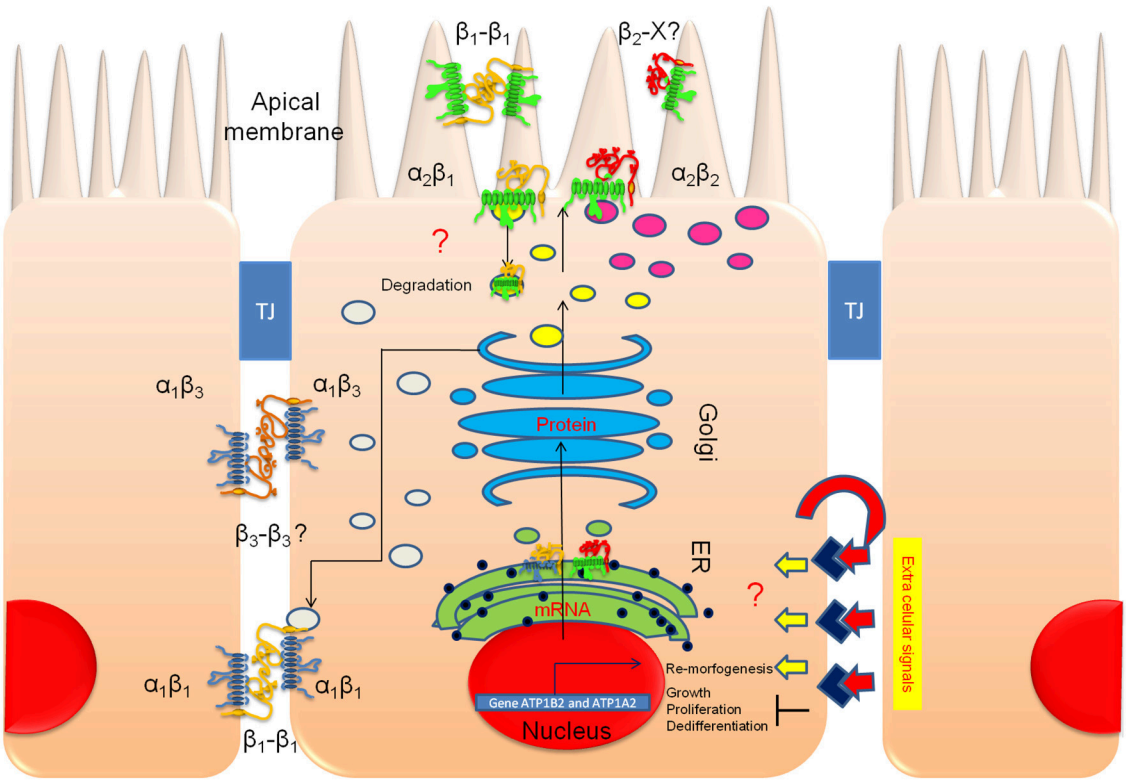

FIGURE 12 | The apical polarization of Na^+, K^+-ATPase in polarized ARPE-19 cells is regulated by the β_2 subunit. An illustration of polarized ARPE-19 cells cultured for 4 weeks on permeable inserts in the presence of ITS is depicted; the cells are relatively tall, express RPE markers and form adjacent tight junctions (TJs). Our model proposes that extracellular signals trigger transduction pathways that activate re-morphogenesis. In non-polarized ARPE-19 cells, a basolateral targeting mechanism carries $\alpha_1\beta_1$ and $\alpha_1\beta_3$ dimers to the lateral membrane (gray vesicles). The β_1-β_1 (and perhaps β_3-β_3) trans-interaction between neighboring cells stabilizes and retains these dimers at the lateral membrane domain for housekeeping. Upon the triggering of re-morphogenesis, a concurrent apical targeting mechanism is activated by the association of the α_2 and β_2 subunits. $\alpha_2\beta_2$ is delivered to the apical domain (magenta vesicles). In cultures, this complex probably does not stabilize at the apical plasma membrane but accumulates in a sub-apical compartment. *In situ*, the pump is inserted in the apical membrane domain, where it is probably stabilized by heterotypic trans interaction(s) of the β_2 subunit with adhesion proteins on the outer segments of the photoreceptor (β_2-X).

it implies that the $\alpha_1\beta_1$ dimer, observed mostly using anti-α_1 antibody, is actually a non-polarized pump that is directed to all membrane domains, including the apical one (**Figure 6** and Hu et al., 1994; Sonoda et al., 2010; Kannan et al., 2006). Our silencing experiments support this notion, as silencing the β_2 isoform diminished the apical localization of the$\alpha_2\beta_2$ dimer but not that of $\alpha_1\beta_1$ (**Figure 10**).

Apical/Basolateral Sorting of Multimeric Membrane Proteins is an Intricate Mechanism

Current models of apical/basolateral sorting mechanisms in epithelia are mostly based on evidence obtained for monomeric proteins (TfR, LDLR, and FcR; Matter et al., 1994; Gan et al., 2002; Perez Bay et al., 2014). Corresponding models for multimeric proteins, such as Na^+, K^+-ATPase, have not been clearly established. It is accepted that polarized proteins carry apical or basolateral sorting signals and that in some cases, two or more opposing signals co-exist in the same protein (Philp et al., 2011). In the case of Na^+, K^+-ATPase, the α_1 subunit contains an unidentified dominant basolateral signal. However, it has been established that the β subunits also contain sorting information that is recessive relative to the basolateral signal of α_1. In particular, the N-glycosylation of the β_2 subunit functions as an apical sorting signal (Vagin et al., 2005). As shown by Castorino et al. (2011) for the sorting signals of CD147, the same sorting signal can be interpreted in different ways in distinct cell types. This observation justifies investigating the role of the apical sorting signal of the β_2 subunit in ARPE cells. Our present observations in polarized ARPE-19 cells, summarized in **Figure 12**, partially clarify some of the complicated and confusing data presented in the literature, as illustrated by the following points. (a) Na^+, K^+-ATPase evaluated based on the expression of the β_1 isoform was detected in both the basolateral and apical domains, consistent with the findings of Hu et al. (1994). (b) The predominant combination at the apical membrane domain of polarized ARPE-19 cells is the $\alpha_2\beta_2$ combination, which is consistent with the concept that the α_2 isoform is the preferred binding partner of the β_2 isoform in

assembling the Na^+, K^+-ATPase in different tissues (Lin et al., 2005; Harada et al., 2006; Tokhtaeva et al., 2012) and with the co-localization of $\alpha_2\beta_2$ in human eye sections shown in this work. (c) Polarized ARPE-19 monolayers express Na^+, K^+-ATPase subunits in a membrane domain-specific pattern: α_1 is detected only at the basolateral domain, α_2 is present only in the apical domain (as seen in **Figure 6**), β_1 subunits are localized in both the basolateral and apical domains, β_2 subunits are preferentially localized in the apical domain, and β_3 subunits are exclusively localized in the basolateral domain. Together, these results suggest that $\alpha_1\beta_1$ and $\alpha_1\beta_3$ are the basolateral combinations. It is unclear whether apical β_1 is a mislocalized $\alpha_1\beta_1$ dimer or a non-preferential combination with α_2. It is most likely an $\alpha_2\beta_1$ dimer because we did not detect α_1 subunits in the apical domain (**Figure 4**). (d) IF assays in human eye sections (**Figures 1**, **8**) reveal apical staining of the RPE using antibodies specific to α_2 and β_2 isoforms. These data indicate that the apical Na^+, K^+-ATPase in RPE cells includes the $\alpha_2\beta_2$ dimer. Thus, a comprehensive analysis of the sorting machinery and trafficking routes that direct the $\alpha_2\beta_2$ complex to the apical domain in polarized ARPE-19 cells must be performed in future studies.

AUTHOR CONTRIBUTIONS

JL designed and performed the experiments, analyzed the data, made the figures and wrote the manuscript. JB, MR, and TL performed experiments. RG and JB designed the experiments and revised the manuscript. LS designed the study, analyzed the data and wrote the manuscript.

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Regulation of Cardiac Remodeling by Cardiac Na^+/K^+-ATPase Isoforms

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Cardiac remodeling occurs after cardiac pressure/volume overload or myocardial injury during the development of heart failure and is a determinant of heart failure. Preventing or reversing remodeling is a goal of heart failure therapy. Human cardiomyocyte Na^+/K^+-ATPase has multiple α isoforms (1–3). The expression of the α subunit of the Na^+/K^+-ATPase is often altered in hypertrophic and failing hearts. The mechanisms are unclear. There are limited data from human cardiomyocytes. Abundant evidences from rodents show that Na^+/K^+-ATPase regulates cardiac contractility, cell signaling, hypertrophy and fibrosis. The α1 isoform of the Na^+/K^+-ATPase is the ubiquitous isoform and possesses both pumping and signaling functions. The α2 isoform of the Na^+/K^+-ATPase regulates intracellular Ca^{2+} signaling, contractility and pathological hypertrophy. The α3 isoform of the Na^+/K^+-ATPase may also be a target for cardiac hypertrophy. Restoration of cardiac Na^+/K^+-ATPase expression may be an effective approach for prevention of cardiac remodeling. In this article, we will overview: (1) the distribution and function of isoform specific Na^+/K^+-ATPase in the cardiomyocytes. (2) the role of cardiac Na^+/K^+-ATPase in the regulation of cell signaling, contractility, cardiac hypertrophy and fibrosis *in vitro* and *in vivo*. Selective targeting of cardiac Na^+/K^+-ATPase isoform may offer a new target for the prevention of cardiac remodeling.

Keywords: cardiac remodeling, Na^+/K^+-ATPase, isoform, hypertrophy, fibrosis, cardiomyocyte, ouabain

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INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the United States, contributing to more than a third of deaths annually. Heart failure is a complex clinical syndrome resulting from structural or functional alterations in the heart which render it unable to meet the body's need for blood. In the United States, the risk of developing heart failure is 20% for the population aged 40 and over (Writing Committee et al., 2013). One in 9 deaths in 2009 included heart failure as contributing cause. Although the clinical management for heart failure has improved, mortality rates remain at ∼50% within 5 years of diagnosis.

Cardiac remodeling occurs after cardiac pressure or volume overload or ischemic injury during the development of heart failure, and is a crucial factor in the prognosis of heart failure (Rizzello et al., 2009). Preventing or reversing remodeling is a goal of heart failure treatment (Konstam et al., 2011). Currently, beta-blockers and angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) are the first line of treatment in heart failure patients. Although several risk factors (hypertension, diabetes and coronary artery disease, etc.) have been identified, there are no effective strategies to prevent cardiac remodeling and heart failure.

Digoxin is the only FDA-approved cardiac glycoside. It can be beneficial in mild or moderate heart failure patients with reduced ejection fraction. Several clinical trials have shown that digoxin treatment improves symptoms and modestly reduces the combined risk of death and hospitalization (Writing Committee et al., 2013). Besides the known inotropy, effects of cardiac glycosides on cardiac remodeling have a long history. In 1933, Christian (1933) advocated the prophylactic use of digitalis to retard cardiac enlargement in heart disease patients without heart failure. In 1965, Williams and Braunwald (1965) presented the first experimental evidence that supports this proposal. Rats, subjected to suprarenal aortic constriction and treated with daily non-toxic doses of digitoxin prior to and following aortic constriction, exhibited less myocardial hypertrophy and a lower incidence rate of fatal heart failure than those subjected to aortic constriction but not treated with digitoxin. On the other hand, digoxin is reported to increase the mortality in chronic renal failure patients on dialysis (Chan et al., 2010). Infusion of cardiac glycosides causes reactive oxygen species stress (Charlemagne et al., 1994) and stimulates renal and cardiac fibrosis in animals with experimental renal injury (Kennedy et al., 2006; Elkareh et al., 2007; Fedorova et al., 2009).

The Na^+/K^+-ATPase is the only known receptor for cardiac glycosides (Shattock et al., 2015). It is unclear whether cardiac glycosides regulate cardiac remodeling and whether they can prevent or promote cardiac remodeling. The inconsistencies of the effect of cardiac remodeling may be due to the complexity of multiple isoforms of the α subunit and different binding properties of cardiac glycosides.

The Na^+/K^+-ATPase is an integrated membrane protein, which hydrolyzes ATP for the energy of the coupled active transport of Na^+ and K^+. It belongs to the P-type family of ATPase and consists of two non-covalently linked α and β subunits that are essential for ion pumping (Sweadner, 1989; Blanco and Mercer, 1998; Kaplan, 2002). The FXYD1 protein, i.e., phospholemman (PLM), is the third subunit in the heart that regulates the function of the enzyme (Geering, 2005). There are 3 isoforms of the α subunit (α1, α2, and α3) and 2 isoforms of the β subunits in the heart. The human heart expresses all 3 isoforms of the α subunit (Sweadner et al., 1994). Monkey and dog heart have roughly equal amount of α1 and either α2 or α3. Sheep and chicken heart only have the α1 isoform (Sweadner et al., 1994). The α1 isoform is ubiquitously present in the heart of all species. In the recent literature, a large amount of results came from rats and mice. In rodents, adult cardiomyocytes express mainly two isoforms of Na^+/K^+-ATPase α subunit, α1 (∼75%) and α2 (≤25%), which have low and high affinities for ouabain, respectively (Sweadner et al., 1994; Bers et al., 2003; Verdonck et al., 2003).

In addition, different cardiac glycosides, e.g., ouabain and digoxin, have the same binding sites on the Na^+/K^+-ATPase although slightly different contacts may be made owing to the different sugar backbones (Laursen et al., 2015). Katz A. et al. have reported that glycosylation of cardiac glycosides contributes to human Na^+/K^+-ATPase isoform selectivity (Katz et al., 2010). At least in the case of digoxin, there is up to four-fold preference for α2 or α3 over the α1 isoform (Laursen et al., 2015). Those reports suggest that targeting different isoforms through modification of chemical structure of cardiac glycosides is a possible approach.

In this article, we will review the structural and enzymatic differences of α isoforms of the Na^+/K^+-ATPase in cardiomyocytes and the role of α isoforms of the Na^+/K^+-ATPase in cardiac hypertrophy and fibrosis.

ENZYMATIC ACTIVITY, DISTRIBUTION, AND FUNCTION OF Na^+/K^+-ATPase ISOFORMS IN CARDIOMYOCYTES

The Na^+/K^+-ATPase (or sodium pump) was discovered in 1957 (Skou, 1957). It is a key enzyme in human cardiac myocytes (density up to 10^7 molecules per cell) (Lelievr et al., 2001). This enzyme plays an important role in maintaining the cellular Na^+ and K^+ ion gradient, regulates cell volume, and enables the Na^+-coupled transport of a multitude of nutrients and other ions across the cell membrane. Under normal conditions, the electrochemical potential gradient for Na^+ ions, which the enzyme maintains, is one of the driving forces of Na^+/Ca^{2+} exchanger to extrude intracellular Ca^{2+}. In the classical ion pumping view of the Na^+/K^+-ATPase, when cardiac glycosides bind to the enzyme, they inhibit the active Na^+ efflux and increase intracellular Ca^{2+} through Na^+/Ca^{2+} exchanger. As a result, cardiac glycosides increase cardiac contractility.

The α subunit of the Na^+/K^+-ATPase is considered as the catalytic subunit and has ATP, cardiac glycosides, and other ligand binding sites. The β subunit is essential for the assembly of a functional enzyme (McDonough et al., 1990). There are multiple isoforms of each subunit with tissue and species specificities, and variations among the sensitivities of the isoforms to cardiac glycosides. In human cardiomyocytes, α1β1, α2β1, α3β1 are expressed in all regions (LA, RA, LV, RV, and S), while there is very low β2 expression in certain regions only (Wang et al., 1996; Schwinger et al., 2003). As judged by the sensitivities of Na^+/K^+-ATPase activity to ouabain (IC 50), the K_D values (measured by ^3H ouabain binding), and by a biphasic ouabain dissociation process, at least two functionally active Na^+/K^+-ATPase isoforms coexist in normal human hearts (Lelievr et al., 2001): The IC 50 values are 7.0 ± 2.5 nM and 81 ± 11 nM; the K_D values in the presence of 10 mM $[K^+]$ are 17.6 ± 6 nM and 125 ± 25 nM; the dissociation rate constants are 360×10^{-4} min^{-1} and 42×10^{-4} min^{-1} (Lelievr et al., 2001).

There are observed kinetic differences (e.g., Km values for Na^+, K^+) among these isoforms, but their subtlety makes them an unlikely basis for physiological significance. Instead, recent work suggests that the major functional distinction among the isoforms is their interaction with regulatory proteins (Pressley et al., 2005). Isoform specific region among the isoforms are the NH2 terminus, the extracellular ouabain binding site, and the cytoplasmaic region between amino acids 403 and 503 (Blanco and Mercer, 1998). Moreover, the isoform-specific effects of modulatory proteins such as protein kinase C seem to originate within two regions of structural divergence: the amino terminus and the 11 residues near the center of the alpha subunit of the isoform-specific region (Blanco and Mercer, 1998; Pressley

et al., 2005). The comparative protein model using human α sequencing based on pig α1 shows that there are very few isoform differences in the transmembrane and in the regions interacting with β and γ subunits; conversely, large clusters of isoform differences map at surface-exposed regions of the A- and N-domains (Morth et al., 2009). The structure difference may lead to isoform-specific cell signaling. Another example is that, in trafficking, α1 is recruited to the membrane by adaptor protein 1 via Tyr 255, a residue not conserved in other isoforms (Cinelli et al., 2008).

Besides its ion pumping function, the Na^+/K^+-ATPase also serves as a scaffold protein interacting with neighboring proteins and facilitates multiple cell signaling events. As shown in **Figure 1**, Na^+/K^+-ATPase signaling mainly has two parallel pathways, one is the EGFR/Src/ERK pathway and another is the phosphoinositide 3-kinase (PI3K) α/Akt/β-GSK/mTOR pathway. To be sure there is some debate regarding the nature of the interaction between the Na^+/K^+-ATPase and Src (Weigand et al., 2012; Clifford and Kaplan, 2013; Yosef et al., 2016). Ouabain indeed does activate Src in myocytes (Haas et al., 2000; Mohammadi et al., 2001, 2003; Xie and Askari, 2002; Liu et al., 2003, 2004) and several lines of evidence support the finding that the Na^+/K^+-ATPase and Src do interact and induced by ouabain or high salt by immunoprecipitation assay in cardiomyocytes, breast cancer cells, and primary pig proximal tubular cells and LLC-PK1 cells (Mohammadi et al., 2003; Kometiani et al., 2005; Liu et al., 2011; Yan et al., 2013). However, the mechanisms of the interaction between the Na^+/K^+-ATPase and Src are unclear. The mechanistic analysis on living cells are required to clarify the complicated network in cells. Thus, some of the conflicting results regarding the interaction between the Na^+/K^+-ATPase and Src may be related not only to cell specificity and specific *in vitro* conditions with detergent-treated membrane purified sodium pump.

The α1 and α2 isoforms play different roles in cardiomyocyte function. There is ample evidence of α1 isoform signaling (Xie and Askari, 2002; Bossuyt et al., 2009; Han et al., 2009; Shattock et al., 2015; Stanley et al., 2015) while no direct evidence is shown on the α2 isoform signaling in cardiomyocytes. Based on different affinities of α1 and α2 for ouabain in mice, and the cardiomyocytes detubulation, Berry et al. (2007) found that α1 is the predominant current conductor, contributing 88% of total recordable current $I_{\text{total}-\text{pump}}$. Although $I_{\alpha 1}$ density predominates over $I_{\alpha 2}$ in both the surface sarcolemmal membrane (SSL) and T-tubules, the difference of current density between α2 and α1 is markedly decreased in the T-tubule. It was reported that α1 is uniformly distributed between SSL and T-tubules, while α2 is ∼5 times more concentrated in T-tubules; strongly suggesting a different role of two isoforms in cardiomyocytes. A similar distribution pattern of two isoforms was recapitulated in rat cardiomyocytes (Despa and Bers, 2007; Swift et al., 2007). NCX and L-type Ca^{2+} channels are also enriched in T-tubules. Both α1 and α2 were shown to be functionally and physically coupled with

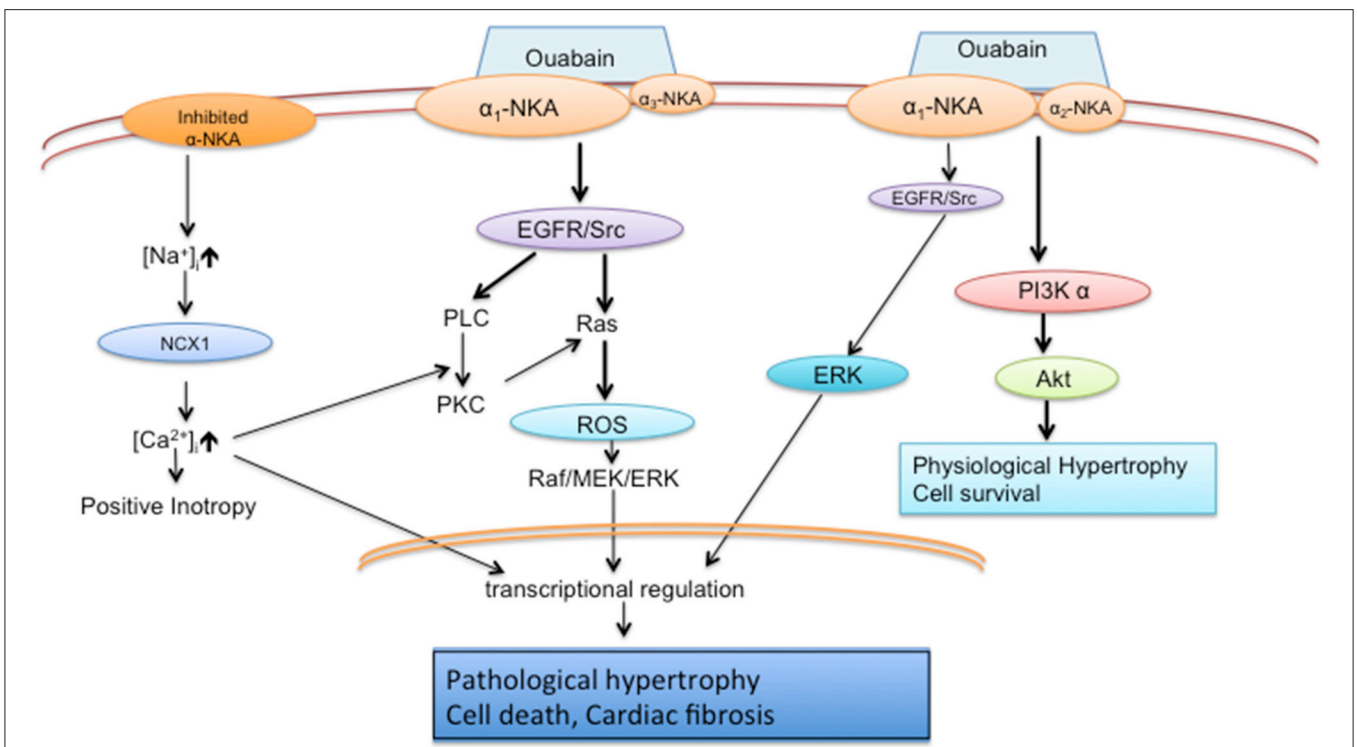

FIGURE 1 | Schematic diagram of Na^+/K^+-ATPase pumping and signaling functions in cardiomyocytes (Liu et al., 2006, 2007; Wu et al., 2015). Inhibited pump alters local $[Na^+]_i$ and induces myocytes contractility; Major effect of ouabain signaling is Src/Ras/ROS/ERK cascade in α1/ α3 neonatal cardiomyocytes; Major effect of ouabain signaling is PI3Kα /Akt pathway in α1/ α2 adult cardiomyocytes.

NCX in cardiomyocytes; however, they differ in their effects on intracellular Ca^{2+} through regulating $[Na]_i$ (Yamamoto et al., 2005). The α1 isoform regulates global $[Na]_i$, while α2 controls local $[Na]_i$. α2 isoform is reported to regulate calcium (James et al., 1999), preferentially modulate Ca^{2+} transients and sarcoplasmic reticulum Ca^{2+} (Despa et al., 2012). Recent reports have shown that the α2 isoform of the Na^+/K^+-ATPase is inactive during the resting potential in adult rat cardiomyocytes and primarily affects calcium handling during systole (Stanley et al., 2015). This suggests that the α2 isoform of Na^+/K^+-ATPase is a specific voltage-dependent isoform (Stanley et al., 2015).

Adrenergic signaling may affect Na^+/K^+-ATPase activity. This effect is stimulated by PKA and/or PKC and phosphorylation of FXYD1 (phospholemman, PLM) in hearts (Bers and Despa, 2009). Cardiac ischemia induces PKA-dependent phosphorylation of PLM and activates α1 Na^+/K^+-ATPase activity but not α2 in rats (Fuller et al., 2004). Interestingly, α-adrenergic agonist increases α2 specific Na^+/K^+-ATPase activity in guinea-pig cardiomyocytes (Gao et al., 1999). Bers and colleagues clearly showed that β-adrenergic signaling stimulates α1 Na^+/K^+-ATPase activity, but not α2 activity on mouse cardiomyocytes (Bossuyt et al., 2009). Similarly, forskolin (activating cAMP/PKA, the downstream of β-adrenergic signaling) also specifically activates α1 Na^+/K^+-ATPase activity in guinea-pig cardiomyocytes (Gao et al., 1999; Silverman et al., 2005). Furthermore, the combination of β- and α -adrenergic signaling in the heart somehow leads to dramatic reduction of α2 but not α1 (Sjogren et al., 2016). While these effects of β-adrenergic stimulation have been documented in rodent cardiac myocytes, it is less clear how this may translate to human cardiac myocytes especially in the setting of heart failure where reductions in α1 and α2 Na^+/K^+-ATPase have been noted.

ALTERATIONS OF Na^+/K^+-ATPase ISOFORMS IN CARDIAC HYPERTROPHY

Isoforms of the cardiac Na^+/K^+-ATPase play different roles in cardiac hypertrophy (Huang et al., 1997a; Kometiani et al., 1998; Xie and Askari, 2002; Bai et al., 2013). Most data shown in the literature are in rodent. While there are α1 and α2 isoforms in adult cardiomyocytes, α1 and α3 isoforms are expressed in neonatal cardiomyocytes.

In cultured neonatal cardiomyocytes, hypertrophic stimuli that mimic pressure overload induces reduced Na^+/K^+-ATPase activity and the regression of α3 mRNA and protein without the alteration of α1 mRNA and protein (Huang et al., 1997b).

Ouabain activates phosphoinositide 3-kinase (PI3K) α /Akt/β-GSK/mTOR and lead to physiological hypertrophy in cultured adult cardiomyocytes (Liu et al., 2007; Bai et al., 2013; Wu et al., 2015). It is featured different from pathological hypertrophy as no increase the influx of Ca^{2+} (Bai et al., 2013) and hypertrophic markers (ANP and BNP) in hypertrophic myocytes (Bai et al., 2013; Wu et al., 2015).

Regression of the ouabain-sensitive isoform α2 is the marker associated with cardiac hypertrophy *in vivo* (Book et al., 1994; Charlemagne et al., 1994; Wu et al., 2015), although several reports have demonstrated that the ouabain-resistant isoform α1 is also downregulated in cardiac remodeling (Norgaard et al., 1988; Semb et al., 1998; Borlak and Thum, 2003; Zwadlo and Borlak, 2005; Kennedy et al., 2006). The α2 isoform mRNA and protein are decreased during hypertrophy of the left ventricle, e.g., in pressure-overload (Book et al., 1994; Ruiz-Opazo et al., 1997; Rindler et al., 2013), isoprenaline-induced cardiac hypertrophy (Baek and Weiss, 2005), myocardial infarction (Book et al., 1994), and uremic cardiomyopathy (Kennedy et al., 2006). Alteration of the α2 isoform of the Na^+/K^+-ATPase may be a mechanism for pressure overload-induced transcriptional response (Ruiz-Opazo et al., 1997). This downregulation of the α2 isoform attenuates the control of Na^+/Ca^{2+} exchanger (NCX) activity and reduces the capability to extrude Ca^{2+} from cardiomyocytes (Swift et al., 2008). In failing hearts, the α2 isoform are correlated to increases Ca^{2+} cycling (Liu and O'Rourke, 2008) and disorganized T-tubule network in cardiomyocytes (Swift et al., 2008). However, the cause-and-consequence of down-regulation of α2 in cardiac remodeling is unclear.

It is interested to know if the compensation between the isoforms and interaction among the isoforms and other proteins would be true in human heart. In $\alpha1^{+/-}$ heterozygote mice, cardiac α2 was increased 50%. In $\alpha2^{+/-}$ heterozygous mice, α1 was not changed but NCX was dramatically increased (Yamamoto et al., 2005). Another example is Ankyrin-B. Ankyrin-B is a universal cell membrane adaptor protein. It may be the scaffold protein for the interaction between Na^+/K^+-ATPase and NCX. Reduced T-tubular α1 and α2 were shown in the mice with heterozygous knockdown of Ankyrin-B (Mohler et al., 2003).

Overexpression of cardiac-specific α2 but not α1 (Correll et al., 2014) protects the heart from pressure overload induced cardiac hypertrophy, fibrosis, and cardiac dysfunction, suggesting that α2 regulates cardiac pathological hypertrophy. Na^+/K^+-ATPase α2 overexpression does not block TAC-induced pro-hypertrophic signaling pathways, such as previously established Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and nuclear factor of activated T cells (NFAT) (Correll et al., 2014), but its impact on NCX1 is sufficient to improve cardiac function during the cardiac remodeling. The possible mechanisms may be because overexpression of α2 decreases PLM levels and phosphorylation. PLM is an inhibitor of Na^+/K^+-ATPase activity. Although both α1 and α2 isoforms directly couple to NCX1, α2 isoform is much more enriched in T-tubules and partial inhibition of α2 but not α1 can increase Ca^{2+} transients suggesting α2 isoform is responsible for regulating NCX1 to control intracellular Ca^{2+}. $[Na^+]$ curve of Na^+/K^+-ATPase activity in overexpressed α2 myocytes shifted to left compared to control and α1 overexpression suggesting α2 has a higher affinity and it may be due to less regulated by PLM. Intracellular Na^+ plays a crucial role in contractility and cardiac remodeling in failing hearts because lower intracellular Na^+ results in less damage to mitochondria and reduction in ATP production (Pieske et al., 2002; Pogwizd et al., 2003).

Cardiac-specific α2 isoform knockout mice are able to survive and have no change in baseline cardiac function (Correll et al., 2014). Reversing the sensitivities of the α1 and α2 isoform

to ouabain causes more severe hypertrophy and fibrosis by pressure-overload (Wansapura et al., 2011). These findings indicate that α1 and α2 isoforms play distinct roles in regulating cardiac remodeling.

To be sure the relative insensitivity of rodent Na^+/K^+-ATPase compared to human is well known and is an important methodological limitation in studies that examine Na^+/K^+-ATPase interactions with cardiac glycosides, such as those referenced above. Thus, these studies need to be interpreted in light of this important limitation and care needs to be given to extrapolating their relevance to humans, especially as these differences may be exacerbated in the heart.

OUABAIN INFUSION IN CARDIAC REMODELING

Infusion of ouabain (15 μg/kg/day × 18 weeks) doubles plasma "ouabain-like" immune-reactive material from 0.3 to 0.7 nM and induces hypertension as well as cardiac and renal hypertrophy in rats (Ferrandi et al., 2004). Other studies (Manunta et al., 1994, 2000; Huang and Leenen, 1999; Rossoni et al., 2002) also showed that infusion of ouabain (25–30 μg/kg/day) for 5 weeks induces hypertension in rats. Rats are much more sensitive to hypertension compared to mice via ouabain. However, other researchers stated that cardiac hypertrophy in rats by ouabain is independent of hypertension (Jiang et al., 2007).

Conversely, ouabain does not induce cardiac hypertrophy in mice; moreover, ouabain is protective against pathological hypertrophy. Infusion of ouabain (300 μg/kg/day) induces hypertension in mice and results in 3.3 nM of "ouabain-like" immune-reactive material (Dostanic et al., 2005). Infusion of ouabain (50 μg/kg/day × 4 weeks) does not induce mouse hypertension and cardiac hypertrophy *in vivo* (Wu et al., 2015). Others (Dostanic et al., 2005) also reported that the repeated daily administration of 100 μg/kg of ouabain to mice resulted in no significant change (the range of 0.75–0.87 nM) in serum of "ouabain-like" immune-reactive material and no effect on systolic blood pressure. The minimal dose of ouabain causing positive inotropic effect is noted to be 40 nM in isolated perfused heart (Dostanic et al., 2003). Infusion of ouabain (50 μg/kg/day) with an implantable osmotic pump in mice for the first 4 weeks starting 1 day after transverse aortic constriction (TAC) prevents pressure-overload-induced cardiac hypertrophy (Wu et al., 2015). The prophylactic effect of sub-inotropic and sub-nanomolar dose of ouabain is associated with activation of PI3Kα (Wu et al., 2015). Ouabain also attenuates TAC-induced reduction of the α2 Na^+/K^+-ATPase. These results demonstrate the regression of α2 Na^+/K^+-ATPase in cardiac hypertrophy and suggest that preservation of the α2 Na^+/K^+-ATPase improves cardiac function and prevents cardiac hypertrophy. These data provide experimental evidence that ouabain can be beneficial to stage A [at high risk for heart failure (HF) but without structural heart disease or symptoms of HF] and B (structural heart disease but without signs or symptoms of HF) patients but not the stage C (structural heart disease with prior or current symptoms of HF) and D (refractory HF requiring specialized interventions) patients [according to the American Heart Association (AHA) and American College Cardiology Foundation (ACCF) guideline (Writing Committee et al., 2013)].

Intriguingly, recent findings by Neubig and colleagues (Sjogren et al., 2012, 2016) have shown that by screening several thousand compounds, digitalis drugs (including ouabain and digoxin) are able to stabilize RGS2 protein, a molecular brake for overdriven Gq signaling in the diseased heart. More interestingly, they further proved the stabilization of RGS2 protein by very low concentration of digoxin (2 μg/kg/day, 7 days) protects heart from injury in mice (Sjogren et al., 2016). However, it is unclear that the impact of digitalis drugs on RGS is direct or indirect via Na^+/K^+-ATPase.

OXIDATIVE STRESS, ENDOGENOUS CARDIOTONIC STEROIDS, AND CARDIAC FIBROSIS

Ouabain activates membrane receptor tyrosine kinase and Src/Ras and results in increase of mitochondrial reactive oxidase species (ROS) in cardiomyocytes (Liu et al., 2000, 2006). Ouabain-induced ROS is independent of the changes of intracellular calcium and sodium (Xie et al., 1999; Liu et al., 2000, 2006). ROS are not contributed to the positive inotropic effect of ouabain, but a ROS-dependent pathway is involved in ouabain-induced hypertrophy (Xie et al., 1999), and contributes to gene transcriptional regulation of hypertrophy (Liu et al., 2000; Liu and Xie, 2010).

Since "endogenous ouabain" in human was first reported in 1991 (Hamlyn et al., 1991), many research groups have isolated Na^+/K^+-ATPase ligands and identified them as "ouabain-," "digoxin-," "marinobufagenin (MBG)-," and "telocinobufagin"-like steroids (Hamlyn, 2014) and collectively referred to as "cardiotonic steroids" (Bagrov et al., 2009). Several clinical and experimental studies have reported that endogenous Na^+/K^+-ATPase ligands act as natriuretic hormones and are elevated in cardiovascular and renal diseases (Kolmakova et al., 2011; Kennedy et al., 2015). "Endogenous ouabain" in the immediate postoperative period was strongly indicative of a more severe cardiac disease and predicts mortality in heart failure patients undergoing elective cardiac surgery (Simonini et al., 2015). There are debates and inconsistencies within this field. For example, conversely, other research groups were not able to detect plasma endogenous ouabain in any conditions (Baecher et al., 2014; Lewis et al., 2014). Technical problems on the measurement of these "endogenous" steroids need to be resolved in the near future.

The hypothesis proposed by Blaustein (2014) is that endogenous ouabain (EO) has similar short term effect but different long term effect than that of digoxin. Long-term effects of EO cause hypertension and heart failure (Blaustein, 2014). Fedorova and coworkers demonstrated that in response to salt loading, transient increases in brain EO, stimulates adrenocortical MBG via an angiotensin I receptor pathway resulting in renal sodium pump inhibition and elevation in blood pressure (Fedorova et al., 2005). Nevertheless, Endogenous

MBG affects different signaling pathways and functions from ouabain in the heart. Plasma MBG is elevated in uremic cardiomyopathy in rats (Haller et al., 2012), as well as in the left anterior descending (LAD) ligation model of heart failure in mice (Fedorova et al., 2015a; Kennedy et al., 2015). Elevated plasma MBG is correlated with higher blood pressure, especially in salt sensitive men (Fedorova et al., 2015b). Moreover, MBG stimulates collagen synthesis and cardiac fibrosis (Elkareh et al., 2007; Kennedy et al., 2015; Drummond et al., 2016). Infusion of MBG to mice increases nitrative stress and cardiac fibrosis (Fedorova et al., 2015a; Kennedy et al., 2015), while monoclonal antibodies against MBG are able to reverse cardiac fibrosis in uremic cardiomyopathy (Haller et al., 2012). MBG (100 nM) also resulted in a two-fold rise in collagen-1 in cultured rat aortic smooth muscle cells and a marked reduction in the vasorelaxation following endothelin-1 stimulated constriction in the aortic rings (Fedorova et al., 2015a). Elevated MBG levels are associated with worsened right ventricular function even after controlling for age, sex, diabetes mellitus, and ischemic pathogenesis in humans (Drummond et al., 2016). Clearly, MBG exhibits at least some of its effects via the α1 isoform as infusion of MBG causes cardiomyocyte death and dilated cardiomyopathy in α1 knockdown mice (Liu et al., 2012).

SUMMARY

The Na^+/K^+-ATPase α isoforms play an important role in the regulation of cardiac remodeling. A schematic diagram that visually summarizes the reviewed results and conclusion is presented to assist readers in efficiently seeing the proposed interaction effects in **Figure 1**. The α1 isoform regulates cell growth and survival pathways, ROS generation, hypertrophy, and cardiac fibrosis. While the α2 isoform has known functions in the regulation of calcium, contractility and hypertrophy on cardiomyocytes, further work will be necessary to delineate any signal transduction role beyond these known functions. While human cardiomyocytes contain three α isoforms, much work remains to determine their function in the healthy and diseased heart and their potential contribution to cardiac remodeling. The Na^+/K^+-ATPase α isoforms are positioned as promising therapeutic targets that can be exploited in both the prevention and treatment of heart failure. As such, future mechanistic work investigating the contribution of specific isoforms of the Na^+/K^+-ATPase will not only advance our understanding of cardiac remodeling but may also provide insight into novel treatment strategies for patients with heart failure.

AUTHOR CONTRIBUTIONS

LL designed, wrote and revised the article; JW wrote the article; DK wrote and revised the article.

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REVIEW
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"Oxygen Sensing" by Na,K-ATPase: These Miraculous Thiols

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Control over the Na,K-ATPase function plays a central role in adaptation of the organisms to hypoxic and anoxic conditions. As the enzyme itself does not possess O_2 binding sites its "oxygen-sensitivity" is mediated by a variety of redox-sensitive modifications including S-glutathionylation, S-nitrosylation, and redox-sensitive phosphorylation. This is an overview of the current knowledge on the plethora of molecular mechanisms tuning the activity of the ATP-consuming Na,K-ATPase to the cellular metabolic activity. Recent findings suggest that oxygen-derived free radicals and H_2O_2, NO, and oxidized glutathione are the signaling messengers that make the Na,K-ATPase "oxygen-sensitive." This very ancient signaling pathway targeting thiols of all three subunits of the Na,K-ATPase as well as redox-sensitive kinases sustains the enzyme activity at the "optimal" level avoiding terminal ATP depletion and maintaining the transmembrane ion gradients in cells of anoxia-tolerant species. We acknowledge the complexity of the underlying processes as we characterize the sources of reactive oxygen and nitrogen species production in hypoxic cells, and identify their targets, the reactive thiol groups which, upon modification, impact the enzyme activity. Structured accordingly, this review presents a summary on (i) the sources of free radical production in hypoxic cells, (ii) localization of regulatory thiols within the Na,K-ATPase and the role reversible thiol modifications play in responses of the enzyme to a variety of stimuli (hypoxia, receptors' activation) (iii) redox-sensitive regulatory phosphorylation, and (iv) the role of fine modulation of the Na,K-ATPase function in survival success under hypoxic conditions. The co-authors attempted to cover all the contradictions and standing hypotheses in the field and propose the possible future developments in this dynamic area of research, the importance of which is hard to overestimate. Better understanding of the processes underlying successful adaptation strategies will make it possible to harness them and use for treatment of patients with stroke and myocardial infarction, sleep apnoea and high altitude pulmonary oedema, and those undergoing surgical interventions associated with the interruption of blood perfusion.

Keywords: Sodium-Potassium-Exchanging ATPase, redox regulation, thiols, hypoxia, S-glutathionylation, S-nitrosylation

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INTRODUCTION. OXYGEN, AND OXYGEN SENSING FROM EVOLUTIONARY AND MODERN PERSPECTIVES

Oxygen, Redox State, Ions, Energy, and Na,K-ATPase

Sustaining of life is a process requiring high energy costs. Energy production in living systems utilizes transmembrane electrochemical gradients, those for ions and redox equivalents. No gradients can be sustained without a membrane, so membranes are the key elements of any living cell since the first proto-cell, the last universal common ancestor (LUCA), that existed more than 3 billion years ago on our planet (Sousa et al., 2013). As life evolved, more specialized compartments were formed within cells, each surrounded by its own membrane. Modern plasma membrane uses the energy of ATP produced by aerobic or anaerobic pathways to generate transmembrane Na^+/K^+ and Ca^{2+} gradients. One member of the P-type ATPases family, Na,K-ATPase, transforms the energy of phosphate bonds within the ATP to the energy of transmembrane Na/K gradient that is used to support the excitability of neurons and myocytes, control of intracellular Ca^{2+} levels and pH, intake of amino acids and fuel, and for sensing and signaling (Blanco and Mercer, 1998; Therien and Blostein, 2000; Blanco, 2005; Geering, 2006; Toyoshima et al., 2011; Reinhard et al., 2013; Shattock et al., 2015). It uses 1 ATP molecule to exchange 3 intracellular Na^+ ions for 2 extracellular K^+ ions per cycle. The number of cycles varies between 1500 and 10,000 per min (Liang et al., 2007) and the corresponding energy expenditure ranges between 20% of total energy consumption in non-excitable cells to 75% in excitable tissues under hypoxic conditions (Buck and Hochachka, 1993; Erecinska and Silver, 2001). Being a major sink for ATP under hypoxic conditions, Na,K-ATPase is capable of "sensing" the changes in O_2 availability and adjusting its activity to the rates of ATP production (Bogdanova et al., 2006). This review summarizes the progress in our understanding of the mechanisms utilized by the Na,K-ATPase for "O_2 sensing." Recent developments in of the field of free radical biology and medicine have provided decisive clues for dissection of these mechanisms and the role that protein thiols play in it.

Reactive Oxygen (ROS) and Nitrogen (RNS) Species As Signal Messengers

Oxygen is a key component of the cell energy production machinery driving Cambrian explosion ~500 Mio years ago. It plays a role of the final acceptor of the mitochondrial electron transport chain (ETC), which is coupled to ATP production by the H^+-dependent reversible mitochondrial ATPase in the mitochondrial oxidative phosphorylation (OXPHOS) system. Many other metabolic reactions in the cell also use oxygen as a necessary component. Thus, cells have developed systems that can sense fluctuations in oxygen concentration and perform different adaptations, and there are several links between hypoxia sensing and redox reactions that we briefly explore here.

Oxygen produces reactive oxygen species (ROS) by successive one-electron reductions (**Figure 1**). Superoxide anion ($O_2^{\bullet-}$) is the first ROS formed from O_2; its half-life is as short as 2×10^5 $M^{-1}s^{-1}$ (Kalyanaraman, 2013), what makes $O_2^{\bullet-}$ particularly difficult to detect. It is not a powerful oxidant, and its effect on protein thiols is mainly limited to disruption of iron-sulfur clusters; in this regard, mitochondrial aconitase inactivation has been a classical hallmark of mitochondrial superoxide production (Fridovich, 1995; Hausladen and Fridovich, 1996). It dismutates spontaneously or by the action of cytosolic or mitochondrial superoxide dismutases (Cu,Zn-SOD or Mn-SOD respectively), producing a reduced form, hydrogen peroxide (H_2O_2), and an oxidized form, water (H_2O). Hydrogen peroxide half-life is quite greater than that of its predecessor, 10^3–10^4 $M^{-1}s^{-1}$ (Kalyanaraman, 2013), it can cross biological membranes and oxidize thiols within Cys residues in proteins. The latter capacity has been a useful tool for nature to design molecular sensors of H_2O_2; likewise several laboratories have developed different fluorescent proteins capable of detecting H_2O_2 through reversible oxidation of critical, sensitive thiols in their structure (Hanson et al., 2004; Belousov et al., 2006; Ermakova et al., 2014). All together, these features make H_2O_2 the most easily detectable ROS and the best known. A one-electron reduction of hydrogen peroxide forms hydroxyl radical ($^{\bullet}OH$), a reaction that can be catalyzed by the oxidation of Fe^{2+} into Fe^{3+} in the so-called Fenton reaction (Kalyanaraman, 2013). $^{\bullet}OH$ is the most reactive and probably the most toxic free radical due to the initiation of radical chain reactions. It is worth mentioning the very fast reaction of superoxide with nitric oxide ($NO^{\bullet}$) to form peroxynitrite ($ONOO^-$), with a rate comparable to that of diffusion, $4–6 \times 10^9$ $M^{-1}s^{-1}$ (Kalyanaraman, 2013). Although peroxynitrite (one of the reactive nitrogen species, RNS) is well known as an inducer of tyrosine nitration, it is a peroxide and as such it is a very effective two-electron oxidant of thiols.

ROS/RNS and Hypoxia

Since ROS are chemical derivatives of O_2, hypoxia would lower the production of ROS due to the law of mass action, i.e., as there is a lower oxygen concentration, one would expect to observe a decrease in ROS production. However, cell systems are often more complicated than the basic chemical systems in a test tube with multiple players and reactions involved in production and scavenging of ROS and RNS. Apart of thermodynamic and kinetic restrains, compartmentalization within the cell and species/cell type-specific settings affect the capacity of electron donors to react with the oxygen molecules and time course of these reactions. Indeed, there has been a long-standing controversy in the field, as there are a number of observations reporting lower ROS production in hypoxia (Hagen et al., 2003; Acker et al., 2006; Chua et al., 2010; Fernandez-Aguera et al., 2015) but also the opposite (Chandel et al., 1998, 2000; Guzy and Schumacker, 2006), what has been called the paradoxical ROS increase in hypoxia (Turrens, 2003; Guzy and Schumacker, 2006). This controversy emerged from the differences in experimental design including timing of hypoxic exposure prior to ROS detection, cell type, tissue or organism, as well as from the methodological diversity in ROS detection. For example, two groups detecting $O_2^{\bullet-}$ in cells using superoxide-sensing probe (dihydroethidium) and microscopy reported the

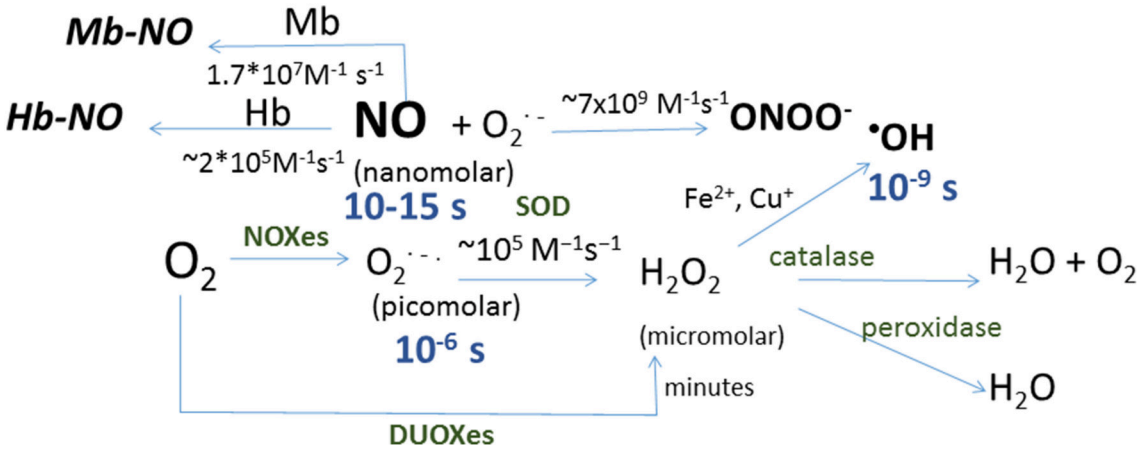

FIGURE 1 | Schematic representation of reactions in which reactive oxygen and nitrogen species are formed. Shown in blue are the half-life for each species and in brackets the concentration range for each species in biological systems, and in green the enzymes catalyzing the corresponding reactions: dual oxidizes (DOUXes), superoxide dismutases (SOD). Under the arrows are the rate constants for the reactions shown. Myoglobin (Mb) and hemoglobin (Hb) are shown as sinks for NO.

opposing findings (Quintero et al., 2006; Chua et al., 2010). The only decisive difference was the time of hypoxic exposure. Detection of $O_2^{\bullet-}$ in cells following 3 h of hypoxia showed no changes in superoxide levels compared to the normoxic control (Chua et al., 2010), while facilitation of $O_2^{\bullet-}$ production was observed during the first hour of hypoxia (Quintero et al., 2006). A recent report reconciled this apparent controversy, as it showed that production of mitochondrial superoxide is upregulated exclusively during the first minutes of hypoxia and its production decreases afterwards, in what has been called a superoxide burst in hypoxia (Hernansanz-Agustin et al., 2014).

Several sources have been proposed to be the origin of ROS in hypoxia. One of the first enzymes implicated in ROS generation in hypoxia was NADPH oxidase, which plays a role in hypoxic pulmonary vasoconstriction (HPV) (Marshall et al., 1996). Together with this, xanthine oxidase was also shown to produce ROS in hypoxia in neurons, contributing to cell death in ischemic conditions (Abramov et al., 2007).

Last but not least, mitochondria-derived ROS have been also shown to increase in hypoxia (Chandel et al., 1998, 2000; Hernansanz-Agustin et al., 2014), are necessary for ROS production by NADPH oxidase in hypoxic pulmonary vasoconstriction (Moreno et al., 2014) and are prior to the xanthine oxidase-derived ROS (Abramov et al., 2007). The source of electron leakage giving rise to an excessive production of $O_2^{\bullet-}$ remains a matter of intensive investigation. Listed below are some considerations regarding the causes and possible mechanisms of hypoxia-inducible $O_2^{\bullet-}$ burst in the mitochondria.

ROS and RNS Production in Hypoxic Mitochondria

Mitochondrial electron transport chain (mtETC) consists of four protein complexes within the mitochondrial inner membrane. Complexes I and II oxidize electron carriers NADH and $FADH_2$, and transfer the electrons to complex III by means of ubiquinone. Complex III reduces cytochrome *c*, which transports the electrons to complex IV and reduces O_2 to H_2O. These activities of complexes I, III, and IV are coupled to the pumping of H^+ across the mitochondrial inner membrane, thus creating an electrochemical gradient. The oxidative phosphorylation system (OXPHOS) includes a fifth complex, complex V or ATP synthase that transforms the energy of proton gradient to phosphorylation of ADP to ATP (Mitchell, 1961; Mitchell and Moyle, 1967).

It is suggested that ROS generation occurs mainly at the level of complex I, but also at complex III (Turrens, 2003; Murphy, 2009; Drose and Brandt, 2012). FMN group within complex I oxidizes NADH into NAD^+ and transfers electrons to a series of Fe-S clusters which, in turn, reduce ubiquinone into ubiquinol (Berrisford and Sazanov, 2009; Hunte et al., 2010; Zickermann et al., 2015) in the so-called forward electron transport. On the other hand, at high mitochondrial transmembrane potential $\Delta\Psi$mt or in the presence of the complex II substrate succinate, complex I can accept electrons from ubiquinol and reduce NAD^+ into NADH at the level of flavin mononucleotide FMN, together with the intrusion of H^+ in a process called reverse electron transport (RET) (Vinogradov and Grivennikova, 2005, 2016; Drose and Brandt, 2012). In both forward and reverse transport there is a leakage of electrons giving rise to $O_2^{\bullet-}$ production. Inhibitors targeting the ubiquinone-binding site (e.g., rotenone; Pryde and Hirst, 2011) increase the leakage in the forward transport, but inhibit it in RET. It is also reported in the presence of high concentrations of succinate, condition associated with progression of ischemia-reperfusion injury (Drose and Brandt, 2012; Chouchani et al., 2014). RET also takes place at elevated $\Delta\Psi$mt (Korshunov et al., 1997; Drose and Brandt, 2012). $O_2^{\bullet-}$ produced by complex I will be released toward the mitochondrial matrix where it is dismutated by Mn-SOD (SOD2) to H_2O_2. The latter is further detoxified by GSH-Glutaredoxin2 system

within the matrix (Drose et al., 2014). Complex III can also give rise to $O_2^{\bullet-}$ production along with oxidation of ubiquinol to ubiquinone known as Q cycle (Boveris et al., 1976; Cadenas et al., 1977). This process includes formation of semiquinone as an intermediate step (Trumpower, 1990), and this step becomes rate-limiting at high $\Delta\Psi$mt or in the presence of the complex III-Qi site inhibitor Antimycin A. In case of electron leakage from complex III $O_2^{\bullet-}$ is accumulating within the intermembrane space. After dismutation by Cu,Zn-SOD (SOD1) the resulting H_2O_2 is extruded from the mitochondria into the cytosol through porin (Drose et al., 2014) and then detoxified by catalase and GSH/glutaredoxin 2 systems. Free radicals and H_2O_2 originating from these two complexes most likely serve as local signaling messengers.

Evidence obtained using transgenic animal models suggest different mitochondrial complexes of the mtETC are involved in generation of superoxide anion participating in redox signaling. Knocking down Rieske iron-sulfur protein (RISP) within the complex III indicates the key role of electron transport by this complex for HIF-1α stabilization under hypoxic conditions (Brunelle et al., 2005; Guzy et al., 2005; Mansfield et al., 2005). Silencing of the NDUFS2 protein expression within complex I compromised hypoxia-induced ROS production and arterial chemoreception *in vivo* (Fernandez-Aguera et al., 2015). However, none of these studies explained the paradox of ROS production in hypoxia or addressed the mechanism of their production. Our recent data point to the key roles of complex I deactivation and Na^+/Ca^{2+} exchange through the mitochondrial Na^+/Ca^{2+} exchanger in the hypoxic superoxide production (Hernansanz-Agustín et al., manuscript submitted).

CO, H_2S, and NO production are most likely supported within the mitochondria by heme oxygenase 1 (Ryter et al., 2006), mitochondrial NO synthase (Ghafourifar and Sen, 2007), and 3-mercaptopyruvate sulfurtransferase (Li et al., 2011). All gasses were shown to protect tissues from irreversible suppression of respiratory capacity of mitochondria during ischemia-reperfusion injury (Elrod et al., 2007). In addition to blocking complex III H_2S is also capable of direct binding to the heme group of complex IV (Cooper and Brown, 2008). Supplementation of H_2S has been shown to inhibit HIF-1α stabilization in hypoxic, but not in anoxic conditions, which is probably related to its capacity of inhibiting mitochondrial respiration (Kai et al., 2012). Systemic administration of sulfide was shown to sustain hibernation state (Blackstone et al., 2005). Nitrite causes inhibition of complex I by S-nitrosylation (reviewed in Martinez-Ruiz et al., 2011) and decreases free radical generation in tissues exposed to ischemia-reperfusion (Shiva et al., 2007).

Genetic adaptation to hypoxia via stabilization of the alpha subunit of hypoxia-inducible factors (HIF1α and HIF2α), has been suggested to require mitochondrial ETC $O_2^{\bullet-}$ production (Chandel et al., 1998, 2000). However, how ROS act over HIFα subunits or its degrading enzymes, the prolyl-hydroxylases (EGLNs), is still unknown (Kaelin and Ratcliffe, 2008). More recently, mitochondrial complex I and ROS production have been shown to play a key role in acute oxygen sensing by carotid body (Fernandez-Aguera et al., 2015). Such increase in ROS production depolarizes glomus cells through inhibition of K^+ channels and increase of cytosolic Ca^{2+} by extracellular Ca^{2+} influx. Both stabilization of HIF-α subunits and inhibition of K^+ channels could be influenced by ROS through oxidation of thiols. Indeed, redox balance is also modified in hypoxia since ROS reversibly oxidize thiols in the cytosolic compartment of cells which, in turn, could have a role in cell signaling and survival during hypoxia (Izquierdo-Alvarez et al., 2012). Superoxide anion generation by mitochondria upon decrease in oxygenation below "normoxic values" as well as decline in NO production by neuronal and inducible NO synthases that have low affinity for O_2 (K_d 2–5 kPa; Dweik, 2005) result in an increase in oxidized glutathione in hypoxic heart tissues of animals that are hypoxia-sensitive, but not in hypoxia-resistant ones (Petrushanko et al., 2012; Yakushev et al., 2012). Oxidized glutathione joins the reactions of non-enzymatic dithiol exchange in which S-glutathionylated adducts of thiol residues are formed in multiple proteins including the Na,K-ATPase. For mitochondrial ROS/RNS to be regulators of the Na,K-ATPase function under hypoxic conditions, the ROS generators should be co-localized with the ATPase. The existence of membrane-bound pool of mitochondria has been confirmed (Westermann, 2015). However, the role of ROS and RNS produced by the mitochondria in control of the Na,K-ATPase activity, amplifying and complementing the signals generated as NADPH oxidases, xanthine oxidase and other free radical generators, awaits further investigation.

Therefore, mitochondria-derived ROS produced in the first minutes of hypoxia (Hernansanz-Agustin et al., 2014), may be a primary and necessary event in redox signaling in hypoxia, leading to the activation of multiple redox processes. All these events converge to targeted thiol oxidation and development of acute adaptive response (Izquierdo-Alvarez et al., 2012).

Both mitochondria and Na,K-ATPase are corner-stones in control of metabolic state of hypoxic tissue. The intimate relation between them remains to be unraveled as it does not seem to be limited to the production and consumption of ATP alone.

VERSATILITY OF OXYGEN SENSING. MULTIPLE SIGNALS-MULTIPLE TARGETS—MULTIPLE RESPONSES—MULTIPLE OUTCOMES

Changes in O_2 availability occurring in the course if evolution trigger multiple responses in every cell of any living organism on this planet (Holland, 2006). These responses are species- and cell type-specific. Diversity in responses matches the oxygen levels in the tissue under normoxic conditions (Bogdanova et al., 2006) and is associated with adaptations some species developed to survive hypoxic periods (Hochachka et al., 1999). A single protein, such as the Na,K-ATPase, may demonstrate essential hypoxia-insensitivity (Yakushev et al., 2012), maintain maximal activity within a narrow window of O_2 concentrations (Petrushanko et al., 2007), or show linear dose-dependence of O_2 concentration in the environment (Bogdanova et al., 2005;

Yakushev et al., 2012) depending on the species and cell type. These responses also vary depending on the duration and severity of hypoxia (Dada et al., 2003; Fuller et al., 2003; Bogdanova et al., 2006; Petrushanko et al., 2007, 2015; Yakushev et al., 2012).

Patterns of response correlate with the changes in ROS/RNS and NO production in the cells and the corresponding shifts in redox state. Apart of the regulatory thiols within the Na,K-ATPase changes in the enzyme activity are in part mediated by its phosphorylation by multiple redox-sensitive kinases and phosphatases (Devarie-Baez et al., 2016). These versatile signaling messengers as well as the variability in free radical scavenging systems make responses to hypoxia dependent on location of free radical generators and speciation of the messengers (free radical- and gaseous-based). The mechanisms of oxygen-inducible regulation are largely restricted to the oxidative modifications of thiols (formation of mixed di-thiols with glutathione, sulfide or other protein thiols, S-nitrosylation, or proline/threonine carbonylation (Yan et al., 2013). These changes occur in multiple proteins at the same time. Fine-tuning of protein complexes is accomplished translating into the protection against hypoxia or reperfusion injury at the tissue and organism levels (Yan, 2014).

OXYGEN AND REDOX-SENSITIVITY OF THE NA,K-ATPase

Na,K-ATPase and Its Thiols

Sodium potassium pump is formed by the 100–113 kDa catalytic α subunit, the regulatory obligatory 55 kDa β subunit and the tissue-specific regulatory proteins of 7–11 kDa belonging to the FXYD family (**Figure 2A**; Blanco and Mercer, 1998; Blanco, 2005; Geering, 2006; Toyoshima et al., 2011). Furthermore, Na,K-ATPase serves as a "docking station" for multiple other proteins of those other ion transporters, receptors, cytoskeletal proteins and members of signaling proteins are known (Reinhard et al., 2013). Each Na,K-ATPase subunit type contains cysteine residues. The only reduced thiol within the beta subunit is localized at the edge to the membrane surface, diving into and out of the membrane during the pumping cycle (White et al., 2009; Bibert et al., 2011). Muscle-specific FXYD subunit pospholemman (PLM) has two thiols (Bibert et al., 2011). The catalytic α subunit is the largest of the three subunits, forms the ATP binding site and binding sites for ions and transport pore, and has 23–24 thiols depending on the isoform (Bogdanova et al., 2006). Several of these thiols are considered to be the targets of regulatory reversible thiol modifications. These modifications make the Na,K-ATPase function extremely sensitive to the changes in redox state and oxygen availability (chronic and acute hypoxia).

Irreversible Oxidation of Thiols

Oxidative stress represents an imbalance between an augmented production of pro-oxidative equivalents (mostly ROS and RNS) and/or their detoxification rate by the so-called antioxidant systems (Sies, 2015). Under these circumstances oxidants attack reduced protein thiols turning them into sulfinic or sulfonic acids. Irreversible oxidation induced in the Na,K-ATPase isolated from kidneys by its exposure to 20 mM H_2O_2 results in a decrease in V_{max} to a half and inability to form oligomeric complexes (Kurella et al., 1999). Sensitivity to oxidation varied for the Na,K-ATPase isozymes, those from the heart and the brain (astrocytes) formed by the α2 isoform of the catalytic subunit being more susceptible for oxidation than the ubiquitously expressed α1 isozyme (Xie et al., 1995). ATP was protecting the enzyme from irreversible oxidation (Xu et al., 1997). Depletion of cytoplasmic and mitochondrial GSH or exposure of cerebellar granule cells to hyperoxia also caused suppression of the enzyme activity associated with massive free radical burst (Petrushanko et al., 2006, 2007).

As reviewed below terminal irreversible oxidation of the enzyme has two more consequences. It makes the regulatory thiols inaccessible for reversible thiol modifications and therefore renders the enzyme insensitive to redox changes and alterations in oxygen levels (Petrushanko et al., 2012). Irreversible oxidation primes the proteins to degradation (Thevenod and Friedmann, 1999). In the lungs severe hypoxia (1.5 kPa) promotes ubiquitination and lysosomal degradation of the alpha1-isozyme of the Na,K-ATPase by phosphorylation of the catalytic subunit at Ser18 by PKCzeta (redox-sensitive kinase containing cysteine clusters; Dada et al., 2003, 2007; Dada and Sznajder, 2003).

S-nitrosylation of Thiols

S-nitrosylation (and S-nitrosation, for nomenclature see Heinrich et al., 2013) is a common thiol modification in biological systems (Martinez-Ruiz and Lamas, 2004; Martinez-Ruiz et al., 2013). Nitric oxide is unable to directly interact with reduced thiols. Several mechanisms have been described for nitrosothiol formation, which do not require the presence of specific enzymes that catalyze these reactions (Martinez-Ruiz et al., 2013).

Direct reaction of NO to nitrosylate a cysteine thiyl radical ($P\text{-}S^{\bullet}$) whenever the latter has been formed.

$$P\text{-}S^{\bullet} + {}^{\bullet}NO \rightarrow P\text{-}S\text{-}NO \quad (1)$$

This is a fast reaction may compete with NO binding to the heme of soluble guanylate cyclase sGC heme (Madej et al., 2008; reviewed in Smith and Marletta, 2012). However, its biological significance is limited to those proteins in which the thiyl radical could be formed when NO is being produced.

NO autooxidation can also lead to S-nitrosylation, via the formation of N_2O_3:

$$2\,NO + O_2 \rightarrow 2\,NO_2 \quad (2)$$

$$NO_2 + NO \rightarrow N_2O_3 \quad (3)$$

$$N_2O_3 + RSH \rightarrow RSNO + H^+ + NO_2^- \quad (4)$$

The rate-limiting reaction is the formation of NO_2, so it requires a very high NO concentration for producing a significant amount of S-nitrosylation. This high NO concentration can be achieved in the proximity of NO sources, mainly NOS. Indeed, the reaction is 30-fold faster in hydrophobic environments (Moller et al., 2007), suggestion that it can be favored in regions close to cell membranes, where NOS enzymes localize.

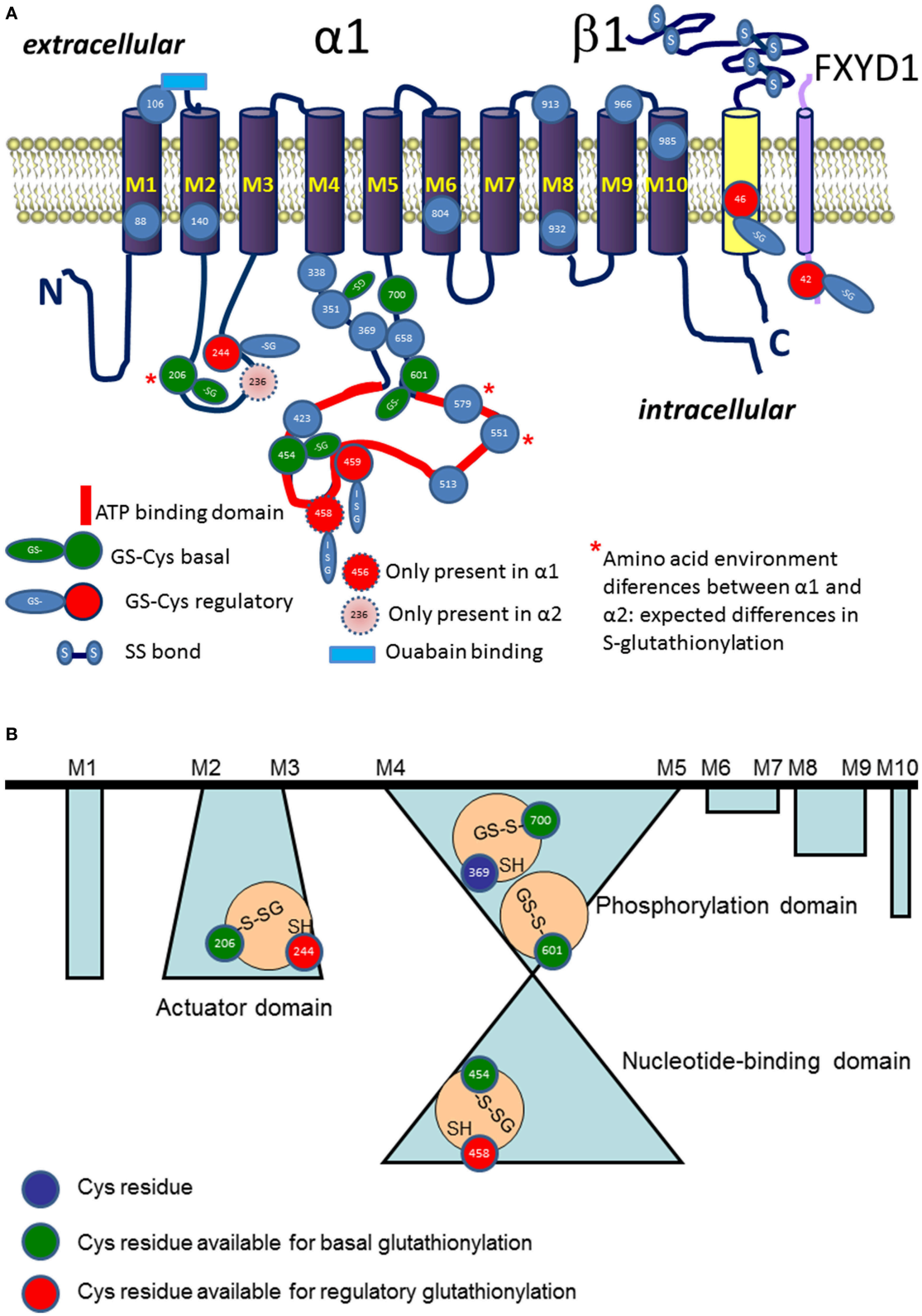

FIGURE 2 | Schematic representation of localization S-glutathionylated cysteine residues in α (in blue), β (in yellow), and phospholemman (FXYD, in cyan) subunits (A). Regulatory S-glutathionylation sites are shown in red. Basal S-glutathionylation is shown in green. ATP binding site is highlighted in red. Blue rectangle depicts ouabain binding site. Stars highlight the Cys residues with differences in pK between the α1 and α2 isoform (for details see **Table 1**). **(B)** Shows schematically the cavities with trapped S-glutathionylated Cys residues inaccessible for de-glutathionylation without detergents and representing "redox memory."

Transnitrosylation in reactions involving modified cysteine residues, including S-nitrosoglutathione, has emerged as an effective and regulated mechanism forming this modifications (Nakamura and Lipton, 2013; Kohr et al., 2014):

$$\mathrm{RSNO} + \mathrm{R'SH} \rightarrow \mathrm{RSH} + \mathrm{R'SNO}\ (\mathrm{trans-nitrosylation,\ nitrosylated\ glutathione\ may\ be\ involved}) \quad (5)$$

Interaction of peroxynitrite with cysteine thiol residues does not give S-nitrosylated products directly but causes oxidation of thiols to sulfenic acid that in turn can form mixed disulfides, in particular with glutathione (see S-glutathionylation below; Alvarez and Radi, 2003):

$$\begin{aligned} \mathrm{RS^-} + \mathrm{ONOOH} &\rightarrow \mathrm{RSOH} + \mathrm{NO_2^-} \\ \mathrm{RSO^-} + \mathrm{GSH} &\rightarrow \mathrm{RSSG} + \mathrm{OH^-} \end{aligned} \quad (6)$$

Denitrosylation mainly involves two mechanisms (Smith and Marletta, 2012). GSNO concentration is controlled by enzymatically-catalyzed NADH (for GSNO reductase (GSNOR) or NADPH-dependent (carbonyl reductase 1, CBR1) reduction to GSH. Since trans-nitrosylation of thiols involves GSNO (reaction 5), this process may be considered as rate-limiting in S-nitrosylation of proteins. This is true for the Na,K-ATPase, as increase in the levels of GSNO induces the second trans-nitrosylation step and formation of S-glutathionylated adducts of the α (Li et al., 2001; Petrushanko et al., 2015) and β (Liu et al., 2013) subunits of the enzyme. Thioredoxin catalyzes denitrosylation of multiple targets by trans-nitrosylation of its active cysteines Cys32 and 35.

Hypoxia alters the number of S-nitrosylated cysteines in numerous proteins. In endothelial cells with high levels of eNOS (NOSIII) multiple targets get S-nitrosylated under hypoxic conditions. This NO synthase has high affinity to O_2 (Kd 0.29 kPa, Dweik, 2005) and can therefore support NO production at low O_2 levels. At the same time in heart tissue, where NOSI and NOSII with low O_2 affinity are dominating (Kd 23–35 kPa, Dweik, 2005) hypoxia is associated with rapid cessation of NO production and decrease in S-nitrosylation of cysteine residues of the α subunit of the Na,K-ATPase (Yakushev et al., 2012). NO and S-nitrosylation were shown to protect thiols from irreversible oxidation and S-glutathionylation, and allow the enzyme to maintain high activity in the brain and heart (Petrushanko et al., 2007; Yakushev et al., 2012). Irreversible oxidation makes regulatory thiols inaccessible for S-nitrosylation. This may explain the fact that the sensitivity of the Na,K-ATPase to NO was lost in a mouse model of amyotrophic lateral sclerosis (Ellis et al., 2003).

S-glutathionylation of Thiols: Chemistry

S-glutathionylation (also referred to as S-glutathiolation), the formation of a mixed disulfide between a protein cysteine and that of glutathione. This thiol modification is produced in a number of chemical reactions (Klatt and Lamas, 2000; Dalle-Donne et al., 2007, 2008; Martinez-Ruiz and Lamas, 2007; Mieyal et al., 2008; Bachi et al., 2013).

Reactions resulting in formation of S-glutathionylated protein adducts include classical thiol disulfide exchange (reaction 7) that does not require any catalysis, and several mechanisms requiring prior oxidative modification of the thiol residues (either the protein thiol or cysteine residue of GSH, reactions 8–13).

$$\mathrm{P\text{-}S^-} + \mathrm{GSSG} \rightarrow \mathrm{P\text{-}SSG} + \mathrm{GS^-} \quad (7)$$

Reactions priming thiols to S-glutathionylation include prior formation of thiyl radicals with O_2 as an electron acceptor (reactions 8–9), oxidation of thiols to sulphenic acid (reaction 10–11) or to nitrosothiol (reactions 12–13)

$$\mathrm{P-S^\bullet} + \mathrm{GS^-} + \mathrm{O_2} \rightarrow \mathrm{P\text{-}SSG} + \mathrm{O_2^{\bullet -}} \quad (8)$$

$$\mathrm{P-S^-} + \mathrm{GS^\bullet} + \mathrm{O_2} \rightarrow \mathrm{P\text{-}SSG} + \mathrm{O_2^{\bullet -}}\ (\mathrm{catalyzed\ by\ glutaredoxin}) \quad (9)$$

$$\mathrm{P\text{-}SOH} + \mathrm{GSH} \rightarrow \mathrm{P\text{-}SSG} + \mathrm{H_2O} \quad (10)$$

$$\mathrm{P\text{-}SH} + \mathrm{GSOH} \rightarrow \mathrm{P\text{-}SSG} + \mathrm{H_2O} \quad (11)$$

$$\mathrm{P\text{-}SNO} + \mathrm{GSH} \rightarrow \mathrm{P\text{-}SSG} + \mathrm{HNO} \quad (12)$$

$$\mathrm{P\text{-}SH} + \mathrm{GSNO} \rightarrow \mathrm{P\text{-}SSG} + \mathrm{HNO} \quad (13)$$

S-glutathionylation of thiols in hypoxic cells is triggered by a local shift in GSSG/GSH ratio toward more GSSG (Petrushanko et al., 2012; Yakushev et al., 2012). On the other hand, oxidative stress following superoxide anion burst in hypoxic mitochondria, will promote oxidation of thiols to sulfenic acid priming them to S-glutathionylation via reaction 10.

Reversibility of S-glutathionylation is supported. Glutaredoxin1 and 2 may catalyze de-glutathionylation or glutathionylation reactions depending on the redox environment (NAD(P)H:NAD(P), GSH:GSSG, NO availability; Mieyal et al., 2008). Not all thiols in the proteins are accessible for S-glutathionylation. Whereas glutaredoxin1 is active in the cytosol and inter-membrane space, whereas glutaredoxin 2 is localized in the mitochondrial matrix, controlling the state of protein thiols in these compartments (Allen and Mieyal, 2012). Further enzymes capable to catalyze de-glutathionylation reaction include sulfiredoxin (Lei et al., 2008), glutathione transferase P (Townsend et al., 2009), and glutathione transferase omega 1 (Menon and Board, 2013).

Not all thiols are equally accessible for S-glutathionylation. Specific conditions that have to be fulfilled for the thiol to become glutathionylated are discussed below.

Localization of the Groups Favoring Glutathionylation

The likelihood for a given thiol to undergo S-glutathionylation is defined by several factors: (i) dissociation constant pKa of a thiol (ii) microenvironment (amino acid composition in vicinity to the cysteine residue), (iii) accessibility of the group and steric restrictions, and (iv) redox potential of a thiol (Nagy, 2013). Nucleophilic substitution reaction in which S-glutathionylated adduct is formed involves interaction of a thiolate anion with a GSSG molecule (Allen and Mieyal, 2012). Thiol therefore has to be deprotonated to join this reaction. The average pKa of majority of cysteine residues is 8.5. Thus, probability of deprotonation of cysteine residues under physiological conditions is relatively

low making S-glutathionylation selective (Dalle-Donne et al., 2009). Reduction in pKa of distinct thiols is supported by the positive charge of three flanking amino acids such as arginine, lysine, or histidine (Allen and Mieyal, 2012; Sun et al., 2013; Zhao et al., 2015). Proximity of negatively charged amino acids to a thiol on the contrary compromises interaction with GSSG. Protein sequence analysis for the α1 subunit of the Na,K-ATPase revealed that cysteines 204, 452, 599, and 698 (corresponding to the Cys 206, 454, 601, and 700 in **Figure 2**) are prone to S-glutathionylation (Mitkevich et al., 2016).

Steric restriction and localization of cysteine residue within the protein sequence are yet other parameters that should be taken into consideration when predicting S-glutathionylation sites (Pineda-Molina et al., 2001). Hydrophilicity of GSSG suggests that the residues should be exposed to aqueous phase as the reaction with GSSG occurs. Furthermore, the exposed surface of thiol should be sufficient for docking of GSSG to form mixed dithiols (Sun et al., 2013). "Basal S-glutathionylation" of cysteines that are buried within the protein, but are S-glutathionylated, represents a special case and is discussed below.

Further restrictions limiting the number of S-glutathionylated residues are related to the redox state of a thiol. For most proteins GSH:GSSG ratio should decrease from 100:1 to 1:1 to achieve S-glutathionylation of 50% of thiols (Allen and Mieyal, 2012). Very few proteins, such as c-Jun may be glutathionylated within physiological range of half-cell redox potential for GSH:GSSG couple (Klatt et al., 1999; Allen and Mieyal, 2012). Na,K-ATPase is one of these few proteins as interaction of the cysteine residues within its catalytic α subunit with GSSG can be observed as GSSG reaches 150 μM levels in the presence of 1.5 mM GSH (Petrushanko et al., 2012; Yakushev et al., 2012).

S-glutathionylation and Targets in the Na,K-ATPase

Alpha Subunit of the Enzyme

Cysteines of all three types of subunits forming Na,K-ATPase undergo S-glutathionylation. Catalytic α subunit contains 23 or more (depending on the isoform) cysteine residues (**Figure 2A**). Fifteen out of 23 Cys residues of the α1 isoform are localized within the cytosolic loops and are easily accessible for interaction with cytosolic GSSG. Out of seven cysteine residues of the regulatory β subunit 6 are forming S-S bonds with each other and only one remaining cysteine possesses a thiol accessible for S-glutathionylation (White et al., 2009). Tissue-specific FXYD subunits also contain 1–2 cysteines of which at least one undergoes S-glutathionylation (Bibert et al., 2011).

Regulatory S-glutathionylation Cysteine residues that were found S-glutathionylated within the α1 subunit isolated from duck salt gland were classified as targets for regulatory or basal S-glutathionylation (Petrushanko et al., 2012). Whereas basal S-glutathionylation is not associated with the changes in the enzyme hydrolytic activity, binding of glutathione to the regulatory cysteine residues causes the enzyme's complete inactivation that can be reversed upon de-glutathionylation (Petrushanko et al., 2012; Yakushev et al., 2012). Regulatory S-glutathionylation could be accomplished by incubation of the purified enzyme or membrane fraction with GSSG indicating that regulatory thiols were accessible for classical thiol disulfide exchange (reaction 5). Similar response was obtained for the α1 subunit within the broad range of tissues and cell type (cell line, salt gland, kidneys, heart tissue) and species (duck, rabbit, pig, mouse, rat, Spalax mole rat, trout, and human; Petrushanko et al., 2012, 2015; Yakushev et al., 2012; Juel, 2014; Xianyu et al., 2014; Juel et al., 2015; Mitkevich et al., 2016). Apart of α1, S-glutathionylation of the α2 isozyme was shown in heart and skeletal muscle. Moreover, α2 isozyme appeared to be more sensitive to GSSG-inducible inhibition in rat heart (Petrushanko et al., 2012; Xianyu et al., 2014; Juel, 2016). This observation is in agreement with the report of Xie on exceptionally high susceptibility of the α2 subunit to oxidation (Xie et al., 1995) and suggests that Cys residue(s) within this isoform are more accessible for oxidation (e.g., Cys 579 in **Table 1**). As can be seen from the **Table 1**, microenvironment for two cysteine residues (Cys 206 and 579) within the cytosolic loops of the α2 subunit favors S-glutathionylation as non-charged amino acids present in the α1 isoform are substituted by positively charged ones facilitating thereby deprotonation of the thiols. Furthermore, α2 subunit harbors the extra cysteine (Cys 236) in the actuator domain and lacks the Cys458 (present in the α1). Thus, although the number of cysteines does not differ between the α1 and α2 subunits, their location, pK and the ability to become S-glutathionylated show clear isoform-specificity.

Localization of the sites of regulatory S-glutathionylation was identified by means of mass spectrometry (Petrushanko et al., 2012). Three of them, Cys 454, 456, and 459 are proximal to the ATP binding site whereas Cys 244 is localized in the small cytosolic loop that may approach the ATP binding pocket in E2 conformation (Bogdanova et al., 2006). Binding of glutathione to the regulatory sites displaces adenine nucleotides from interaction with the enzyme (Petrushanko et al., 2012). In turn, GSSG cannot block the enzyme's hydrolytic activity in the presence of ATP or ADP (Petrushanko et al., 2012; Xianyu et al., 2014). ATP was showing maximal "protective effect" compared to the other nucleotides (Xianyu et al., 2014). Interaction with ATP (but not with the other nucleotides) brings the nucleotide-binding domain of the α subunit closer to the phosphorylation domain (E1-closed state) shielding thiols of the small and large cytosolic loops from attack by GSSG or oxidants (Petrushanko et al., 2014) and making them less prone to irreversible oxidation (Xie et al., 1995; Xu et al., 1997).

Increase in S-glutathionylation of the α subunit was triggered by hypoxia associated with mild oxidative stress and modest ATP depletion in rat heart and SC1 cell line derived from mouse fibroblasts (Petrushanko et al., 2012, 2015; Yakushev et al., 2012). Inhibition of the Na,K-ATPase and induction of S-glutathionylation could be mimicked by the modulation of intracellular redox state by exposure of cells to glutathione derivatives et-GSH, GSNO, GSSG, or depletion of the intracellular GSH (Petrushanko et al., 2006, 2015). S-glutathionylation of the α subunit cannot be sustained under anoxic conditions. This thiol modification is induced by 0.2% O_2

TABLE 1 | Analysis of flanking amino acids' composition of selected thiols in the α1 and α2 subunits (rat sequences).

Number of Cys	Sequence fragment	Substitutions α1-> α2	Comments
206 α1 α2	NGCKV HGCKV .****	N->H	Asp-> His (neutral to positive in α2)
236 α1 α2	NIAFF NICFF **.**	A->C	Cys present in α2 and missing in α 1 isoform
458 α1 α2	EVCCGS ELSCGS *:.***	V->L C->S	Cys missing in the α2 isoform and present in α 1 Val->Leu (neutral)
513 α1 α2	DFCSS DFCST ****:	S->T	Neutral substitutions
551 α1 α2	GFCHL GFCQL ***:*	H->Q	His->Gln (positive to neutral in α2)
579 α1 α2	NLCFV KLCFV :****	N->K	Asp->Lys (neutral to positive in α2)
932 α1 α2	VICKT IICKT :****	V->I	Neutral exchange

Data shown in the table are obtained as a result of sequence alignment (Clustal O(1.2.1)) for the Na,K-ATPase α1(P06685) and α2 (P06686) subunits' sequences for rat (Rattus norvegicus), published in UniProt-Knowledgebase (Swiss-Prot collection of sequences, http://www.uniprot.org/uniprot). Included into the table are the amino acids flanking the cysteine residues for which the isoform-specific differences (substitution, omission) have been observed. Asterisks stand for the conserved amino acids whereas dots and semicolon indicate the substitutions. Cysteines are color-coded in blue and the substituted amino acids in yellow.

but less pronounced at 0.05% O_2 in mouse fibroblast-derived cell line (Petrushanko et al., 2015).

Basal S-glutationylation of the α subunit

Basal S-glutathionylation of the α subunit of the Na,K-ATPase is not associated with the changes in the enzyme function. 15 cysteine residues facing the cytosol are accessible for S-glutathionylation. Treatment of the cell lysates, membrane fractions or purified active Na,K-ATPase preparations with reducing agents (e.g., by Dithiotreitol or Tris(2-carboethyl)-phosphine, TCEP) could not completely remove glutathione bound to the α subunit's thiols (Mitkevich et al., 2016). Complete de-glutathionylation could only be achieved under conditions supporting partial denaturation of the protein (8 M urea and 8% SDS). De-glutathionylation of the cysteines inaccessible for reducing agents in the protein retaining its native structure was associated with substantial loss of the enzyme activity due to unfolding. The only chance these Cys residues shielded from GSSG and reducing agents have to acquire S-glutathionylation is before the folding was completed (**Figure 2B**). This implies that S-glutathionylation of certain Cys residues is a co-translational rather than post-translational modification and it may be required for correct protein folding. Detailed analysis of the X-ray structures of the porcine α1 subunit-containing enzyme (PBD codes: 3B8E, 3KDP, 3WGU, 3WGV, 4HYT) revealed a number of isolated cavities with unresolved electron density next to the Cys residues (numbering as in **Figure 2B**) Cys 206–Cys 244; Cys 454–Cys 458–Cys 459; Cys 700–Cys 369, Cys 601 (Mitkevich et al., 2016).

These regions of relatively high residual electron density that cannot be explained by the presence of water are sufficient in size to home glutathione. However, so far no X-ray structures of the actual catalytic subunit with S-glutathionylated cysteine adduct was reported. Detection of glutathionylated residues in crystal structures of proteins is not impossible (Srinivasan et al., 2014), but much less common that detection of these modifications by means of mass spectrometry. One of the technical approaches to use for identification of S-glutathionylated cysteine residues is tracking for glutathione localisation using analysis of unresolved density next to the cysteine residues. This approach was used for identification of glutathione bound to the ABC transporter Atm1 (Srinivasan et al., 2014). In each cavity of the α subunit only one Cys residue was reported to be S-glutathionylated (basal type of S-glutathionylation, **Figure 2B**, Mitkevich et al., 2016). These findings imply that Cys454 is located in a cavity and, hence, cannot be a regulatory thiol as suggested earlier (Petrushanko et al., 2012). Its basal S-glutathionylation will contribute to the

protein folding instead. Out of the Cys206-Cys244 couple Cys 206 is capable of basal S-glutathionylation whereas Cys 244 carries a regulatory thiol group. In contrast to regulatory S-glutathionylation observed within minutes after the drop in O_2 availability, increase in basal S-glutathionylation is only observed after 72 h of hypoxic exposure (Mitkevich et al., 2016). This kinetics correlate with the onset of *de novo* protein synthesis rather than acute alterations in thiol state.

Moreover, as this modification is retained during the life-span of a protein it represents "redox memory" that reflects the cellular redox state at the moment of synthesis of this molecule. Thus, this "redox memory" may represent the process of adaptation to the alterations in redox state occurring in particular in response to deoxygenation. The fact that basal S-glutathionylation levels differ in muscle fibers being higher in oxidative fibers compared to the glycolytic fiber type suggests that it is likely to be associated with the metabolic state of the tissue (Juel, 2014).

S-glutathionylation of Beta Subunit

A single reduced thiol group in β subunit of the Na,K-ATPase, Cys 46 (**Figure 2A**), is the one that may undergo reversible post-translational modifications. S-glutathionylation of this cysteine residue was reported for all three muscle types (skeletal, smooth, and heart muscles; Figtree et al., 2009; Liu et al., 2013; Juel et al., 2015). Since interaction of glutathione with Cys 46 results in down-regulation of the enzyme function this residue is a site of regulatory S-glutathionylation (Figtree et al., 2009). Inhibitory effect is achieved due to the weakening of the interaction between α and β subunits upon S-glutathionylation (Figtree et al., 2009). S-glutathionylation of β subunit does not inactivate the enzyme completely (Figtree et al., 2009). Unfortunately, the studies in which S-glutathionylation of the β subunit was detected were not presenting the information on the degree of S-glutathionylation of the α subunit, making it impossible to discriminate between the impacts of these two processes into the enzyme activity regulation (Liu et al., 2013).

In contrast to the regulatory cysteines of the catalytic α subunit that are readily interacting with GSSG Cys 46 does not join reaction of classical thiol disulfide exchange with GSSG (Petrushanko et al., 2012). This may be attributed to the localization of this Cys residue. According to the X-ray structure it is buried within the membrane in E2 2K+Pi conformation (Ogawa et al., 2009) and is only accessible for S-glutathionylation in E1ATP and E1Na(3) conformation (Liu et al., 2012). Furthermore, Cys 46 may become accessible or S-glutathionylation upon the loss of association between the α and β subunits (Garcia et al., 2015). Ouabain stabilizes the enzyme in E2 conformation and compromises S-glutathionylation of β subunit (Liu et al., 2012). De-glutathionylation can be achieved by treatment of the S-glutathionylated enzyme with Glutaredoxin.

Localization of the regulatory cysteine defines conditions required for its S-glutathionylation. It only occurs in the presence of peroxynitrite and hence involves reactions 12 and 13. Physiological and pathophysiological role of this form of regulatory thiol modification in the heart has been intensively investigated. Stimulation of $O_2^{\bullet-}$ production by NADPH oxidases, that co-localize and co-immunoprecipitate with the Na,K-ATPase, induces S-glutathionylation of the β subunit at Cys46 (Liu et al., 2013). The stimulation of NADPH oxidases could be triggered by their phosphorylation by PKC produced upon activation of β1/β2 adrenoceptors or treatment of the myocardium with angiotensin II. Furthermore, increase in S-glutathionylation of the β subunit was reported in the infarcted area in sheep heart (Figtree et al., 2009). Similar effect was achieved by exposure of cardiomyocytes to the activator of adenylate cyclase forskolin and the following activation of PKCε (White et al., 2010) or direct administration of ONOO- (Figtree et al., 2009). Scavenging of $O_2^{\bullet-}$ using superoxide dismutase on the contrary abolishes S-glutathionylation of the β subunit. Similar effect may be achieved by the stimulation of sGC that interfered with phosphorylation and activation of NADPH oxidases (Chia et al., 2015). Activation of β3 adrenoreceptor is a physiological stimulus decreasing S-glutathionylation of the Cys 46 (Bundgaard et al., 2010). These differential responses are easily explained based on the chemistry of S-glutathionylation of thiols. Deoxygenation is associated with suppression in production of NO by NOS1 and NOS2 as well as in reduction of activity of NADPH oxidases Kd of which for O_2 is within 2 kPa range. Thus, decrease in O_2 levels below this threshold reduces production of peroxynitrite and decreases S-glutathionylation of Cys 46 in β subunit. Uncoupling of oxidative phosphorylation and $O_2^{\bullet-}$ burst in the mitochondria on the contrary will facilitate GSSG production in the cytosol and induce S-glutathionylation of regulatory cysteines within the cytosolic loops of the catalytic α subunit.

S-glutathionylation of FXYD Subunits

Tissue-specific FXYD proteins associate with Na,K-ATPase αβ complex stabilizing it and modulating the enzyme activity (Geering, 2006). These modulatory subunits also contain 1 or 2 cysteine residues that undergo reversible thiol modifications. Most of the information on the role of these modifications in control of Na,K-ATPase function was obtained for the cardiac-specific FXYD1 subunit also known as phospholemman (PLM). It contains 2 Cys residues (C1 corresponding to Cys40 and C2 corresponding to Cys42 in human FXYD1 protein, **Figure 2A**), and S-glutathionylation of the C2 was shown to correlate reciprocally with the availability of glutathionylated form of the β subunit (Bibert et al., 2011). Localization of C2 and mechanisms of induction of S-glutathionylation of PLM are most likely shared with the β subunit of the Na,K-ATPase and involve peroxynitrite. Interaction of PLM with glutathione may be triggered by forskolin and prevented by exposure to superoxide dismutase. De-glutathionylation is also catalyzed by glutaredoxin1 or treatment with DTT. Thus, both PLM and β subunits lack cysteines that are not accessible for de-glutathionylation (redox memory). Physiological stimuli increasing the level of PLM S-glutathionylation include angiotensin II and infarction (Bibert et al., 2011).

The importance of the amino acids flanking the cysteine residue in control of accessibility of it for S-glutathionylation was emphasized as the amount of glutathionylated adducts was compared for several members of FXYD family. C2 cysteines

were found to be prone to S-glutathionylation in FXYD2 (renal) and FXYD7 (brain-derived) (Geering, 2006; Bibert et al., 2011). Phospholemman (PLM, FXYD1) interacts with both catalytic and regulatory subunits of the Na,K-ATPase. Thus, interaction between α and β subunits and the enzyme function is affected by S-glutathionylation of the FXYD1. Furthermore, the reciprocal regulation of S-glutathionylation of the β and FXYD subunits suggests that FXYD may reverse the inhibitory action mediated by binding of glutathione to the regulatory β subunit on the Na,K-ATPase. It remains unclear if glutathione is transferred from the Cys46 on to the β subunit to the C2 residue in reaction of thiol disulfide exchange of the FXYD protein or if S-glutathionylation of the FXYD protein makes Cys46 inaccessible for S-glutathionylation.

H_2S and the Na,K-ATPase

Hypoxic exposure was shown to cause a rapid increase in H_2S levels in a number of cells including chemoreceptory glomus cells (Peng et al., 2010; Olson, 2015; Yuan et al., 2015). Direct interaction of H_2S with the Na,K-ATPase has not been reported. However, exposure of renal tubular epithelial cells to H_2S causes internalization of the Na,K-ATPase in complex with other proteins. This inhibitory response of the pump is triggered by sulfhydrolation of cysteines 797 and 798 at the epidermal growth factor receptos (EGFRs) (Ge et al., 2014).

COORDINATED REGULATION OF REDOX-SENSITIVE PROTEIN NETWORKS BY REVERSIBLE THIOL MODIFICATIONS

S-glutathionylation and S-nitrosylation are altering the function of numerous proteins that form redox sensitive networks (Sun et al., 2006; Dalle-Donne et al., 2009). Protein clusters forming these networks are the ones controlling metabolism, ion transport, cell fate and cycle, adaptation, and others. This type of free radical-based signaling is of key importance in responses to hypoxia and reoxygenation mediating acute and long-term tissue-specific coordination of function of multiple proteins. S-glutathionylation of the α subunit of the Na,K-ATPase occurs among with alterations in thiol state of other proteins. The most obvious protein partners that undergo S-glutathionylation are the adjacent β and FXYD subunits. Thiol disulfide exchange in which PLM most likely acquires glutathione from the Cys 46 subunit of the β subunit is suggested to cause dissociation of the FXYD subunit of from the Na,K-ATPase as it does not co-precipitate with the α subunit (Bibert et al., 2011). As PLM is a regulatory subunit that is shared between the Na,K-ATPase and the Na/Ca exchanger, it was suggested that S-glutathionylation as well as its redox-sensitive phosphorylation of it impacts activity of both transporters (Silverman et al., 2005; Cheung et al., 2007; Zhang et al., 2011). Calcium handling in the heart is intimately linked to the Na^+ levels (Shattock et al., 2015) and, hence to the activity of the α2 isozyme of the Na,K-ATPase that is particularly prone to inhibition by GSSG (James et al., 1999; Petrushanko et al., 2012). Apart of the Na/Ca exchanger reversible thiol modifications are known to control the activity of L-type Ca^{2+} channel, RyR2 ryanodine receptors, SERCA2a Ca^{2+} pump and multiple other ion transporters (Sun et al., 2006; Bull et al., 2008; Lancel et al., 2009; Donoso et al., 2011).

These coordinated redox-driven changes in activity of multiple ion transporters may support cytoprotection and survival of the cell/tissue/organism or promote death under hypoxic conditions (see Section Regulation of the Na,K-ATPase Activity by Cardiotonic Steroids).

OXYGEN-SENSITIVE PHOSPHORYLATION

Regulatory phosphorylation is known to control the activity, sensitivity to the cardiotonic steroids, and availability of the Na,K-ATPase on the plasma membrane. Changes in phosphorylation state of the enzyme were reported in response to hypoxic challenge and for a long time believed to be the only mechanism involved in "channel arrest" response of the Na,K-ATPase in hibernating animals (MacDonald and Storey, 1999; Ramnanan and Storey, 2006; McMullen and Storey, 2008). At present "channel arrest" responses seem to be more versatile, and yet triggered by a single mechanism of regulation, in this case not only the Na,K-ATPase itself, but also the redox-sensitive kinases, namely, reversible thiol modifications of regulatory cysteine residues or action of gasotransmitters. Phosphorylation sites at the α, β and FXYD subunits are schematically presented in **Figure 3**.

Protein Kinase C (PKC)

Hypoxic exposure of alveolar epithelial cells was shown to trigger actively controlled internalization of the Na,K-ATPase in the form of clathrin-coated vesicles (Dada et al., 2003). This process was induced by phosphorylation of the α subunit of the Na,K-ATPase by at atypical PKC isozyme.

Novel and classical PKC isoforms are also capable to alter activity of the Na,K-ATPase in various cell types. Classical (conventional α, β1 and β2, and γ isoforms) and novel isoforms PKC δ, ε, η, θ, and μ may be activated diacylglycerol (DAG), phorbol esthers and phosphatidylserine and are sensitive to the changes in Ca^{2+} in the cell. Atypical PKCι and ξ retain the sensitivity to phosphatidylserine but are Ca^{2+}-insensitive (**Figure 3**; Newton, 1995; Parker and Murray-Rust, 2004).

Catalytic core of PKC contains critical cysteine-rich motifs that are conserved in all PKC isozymes making all proteins of this class redox-sensitive (Newton, 1995). This motif is duplicated in classical and novel PKC isoforms, whereas in atypical PKCs only one repeat is found. Oxidation of these cysteines causes transient activation of the enzyme but makes it unable to interact with DAG and phorbol esters making it Ca^{2+}- and DAG-insensitive (Gopalakrishna and Anderson, 1989). S-glutathionylation of these regulatory thiols is inactivating α, βI, βII, γ, δ, ε, and ζ isoforms of PKC. Formation of glutathionylated adducts occurs within physiological range of GSH concentrations (0.5–10 mM) and results in the enzyme inactivation (Ward et al., 1998, 2000; Chu et al., 2001). In addition, PKC may undergo S-nitrosylation that was shown to suppress activity of PKCα (Choi et al., 2011). Depletion of intracellular GSH by exposure to conjugating agent

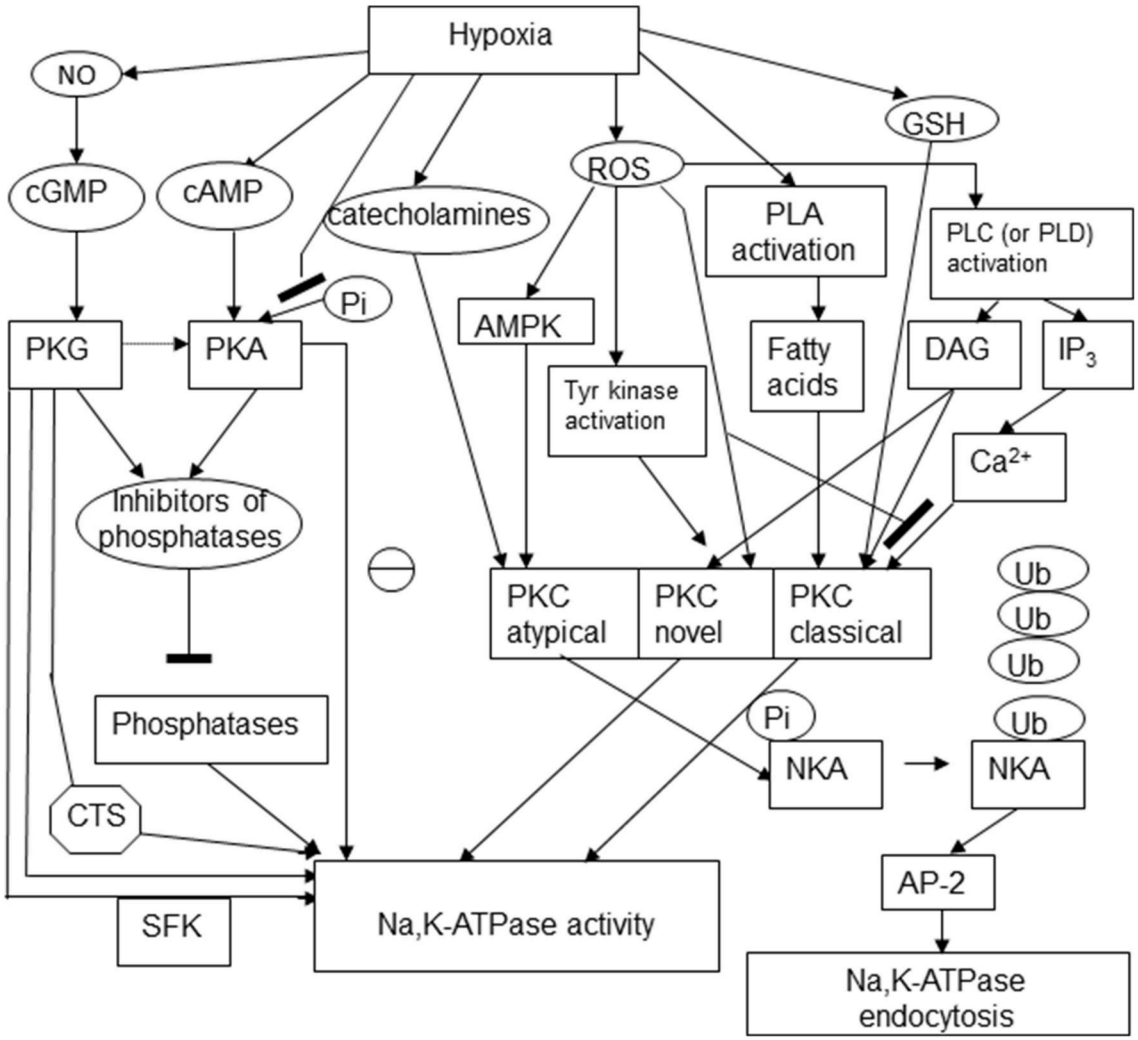

FIGURE 3 | Schematic representation of the multiple pathways involved in regulatory phosphorylation of the Na,K-ATPase under hypoxic conditions. The following abbreviations were used in the scheme: cyclic adenosine and guanosine monophosphates (cGMP and cAMP), protein kinases C, A, and G (PKC, PKA, PKG), cardiotonic steroids (CTS), Src kinase family (SFK), AMP-activated protein kinase (AMPK), phospholipase C (PLC), diacylglycerol (DAG), inositol 3-phosphate (IP3), ubiquitin (Ub), NKA (Na,K-ATPase), adaptor protein 2 (AP-2).

diethylmaleimide or inhibition of *de novo* GSH production by L-buthionine-S,R-sulfoximine results in reduction in activity of conventional PKC isoforms (α, βI, and βII) by 35–50%, along activation of the novel isoforms (δ and ε) (Domenicotti et al., 2000). This response of the PKCs to the changes in redox state will induce endocytosis of the Na,K-ATPase (**Figure 3**).

PKC phosphorylates the catalytic α1 subunit at Ser11 and 18 (rat sequence) at the N-terminus of the protein that forms the actuator domain. Ser18 that is considered as a major phosphorylation site is available for phosphorylation in the E2 conformation (Feschenko and Sweadner, 1997). In the E1 conformation the N-terminus is translocated approaching the small cytosolic loop that shields the phosphorylation site from interaction with the kinase (Feschenko et al., 1997; Segall et al., 2003). The analogs of Ser18 and Ser11 are missing in the α2 isoform of the catalytic subunit (compare sequences A24639 for α1 and B24639 for α2 subunit in PubMed protein sequence library http://www.ncbi.nlm.nih.gov) making this isozyme PKC-insensitive. Phosphorylation efficiency may also vary depending on the PKC isoform. Conventional isoforms are more efficient in phosphorylation of the α1 isoform of the pump isolated from rat retinal cells than the novel isoforms δ and ε (Kazanietz et al., 2001). Phosphorylation of the α subunit by PKC may have stimulatory of inhibitory effects on the Na,K-ATPase depending on the cell type (Therien and Blostein, 2000). It is suggested that phosphorylation modulates interaction of the enzyme with other proteins or trigger its internalization (Feschenko and Sweadner, 1997). For example, activation of the atypical isoforms of PKC ξ triggers internalization of the Na,K-ATPase in alveolar epithelial cells under hypoxic conditions (Dada and Sznajder, 2003). This effect is initiated by the free radical burst in the

mitochondria that is followed by the activation by the 5′-AMP-activated protein kinase (AMPK) that is phosphorylated at Thr172. The activated AMPK phosphorylates PKCξ at Thr410 and its translocation to the plasma membrane (Gusarova et al., 2009). Upon translocation PKC phosphorylates Na,K-ATPase at the Ser18 residue in the N-terminus of the α1 subunit. Endocytosis of the α1-containing isozyme of the ATPase is precluded by ubiquitination of the lysine residues next to the Ser18 (Dada et al., 2007; Lecuona et al., 2007). Ubiquitination makes the enzyme recognizable for the mu2 subunit of the adaptor protein that binds to the Tyr527 of the α1 subunit initiating its endocytosis in clathrin-coated vesicle is initiated with the following degradation of the Na,K-ATPase (Chen et al., 2006; Lecuona et al., 2007).

PLM is one more target of phosphorylation by PKCϵ at Ser36 and Ser 68. Phosphorylation it stimulated by Ca^{2+} and is NO-dependent (Pavlovic et al., 2013). Ischemia was associated with facilitation of phosphorylation of PLM, its de-attachment from the αβ complex and increase in the Na,K-ATPase hydrolytic activity in sarcoplasmic membrane fraction (Fuller et al., 2003, 2009).

Protein Kinase G (PKG)

Hypoxia and reoxygenation result in the alterations in NO, CO, and H_2S production (see above). All gasotransmitters are capable of activation of sGC that in turn triggers activation of cGMP-dependent protein kinase PKC (PKG) (Therien and Blostein, 2000; Chen et al., 2015). Similar to that for PKC, phosphorylation by PKG was reported to have diverse effects on the Na,K-ATPase activity (Therien and Blostein, 2000). It remains unclear if PKG can directly access the phosphorylation site within the α subunit, as it could only be phosphorylated in the presence of detergents in the purified protein preparation (Fotis et al., 1999; Beltowski et al., 2003). However, treatment of yolk-free homogenates of Xenopus oocytes with cGMP results in phosphorylation and activation of the Na,K-ATPase in the absence of detergents (Fotis et al., 1999). It may reflect the indirect action of PKG on the ATPase via the suppression of dephosphorylation. Yet one more report on the activation of the α2/3 isozymes of the Na,K-ATPase in the central nervous system involves activation of cGMP-PKG pathway following the stimulation with glutamate (Munhoz et al., 2005; Scavone et al., 2005). On the other hand, supplementation of NO donors was shown to inhibit the Na,K-ATPase in nonpigmented epithelial cells of porcine eyes that is associated with activation of PKG (Shahidullah and Delamere, 2006). This effect involves PKG-driven activation of src-family kinases (Shahidullah et al., 2014).

Apart of the alterations in the enzyme activity, stimulation of the cGMP-PKG pathway may affect binding of cardiotonic steroids to the Na,K-ATPase. Increases the sensitivity of the renotubular Na,K-ATPase to marinobufagenin triggered by atrial natriuretic peptide is mediated by the cGMP-PKG signaling cascade (Fedorova et al., 2012).

Protein Kinase A (PKA)

Cyclic AMP-sensitive protein kinase (PKA) is a redox-sensitive enzyme containing regulatory thiols (Brennan et al., 2006). In the absence of cAMP the enzyme exists in the inactive tetrameric state and the regulatory Cys199 within the C subunits is inaccessible for regulatory S-glutathionylation (Humphries et al., 2002). Binding of glutathione to this cysteine residue inactivates the kinase and enhances dephosphorylation (Humphries et al., 2005). Vector of the changes in activity of PKA in response to deoxygenation is very much dependent on the cell type and on the activity of G-protein coupled receptors signaling via cAMP-PKA transduction pathway (Jiang et al., 2011).

PKA phosphorylates the catalytic α subunit at Ser938 (rat α1 sequence) within the cytosolic M8-M9 loop. This loop may interact with the M10, C-terminus and the third Na^+ binding site. In line with that phosphorylation at Ser938 decreases affinity of this Na^+ binding site to Na^+ and thereby suppresses the enzyme function (Einholm et al., 2016).

In the purified Na,K-ATPase protein preparation this target is accessible for phosphorylation in the presence of detergent (Feschenko and Sweadner, 1994; Lutz et al., 1996). In intact COS cells phosphorylation of the α subunit at Ser943 is triggered by β-adrenergic stimulation with the following inhibition of hydrolytic and transport activity of the Na,K-ATPase (Cheng et al., 1997).

Activation of cAMP-PKA-dependent pathways in NRK-52E and L6 cell lines may suppress activity of PKC and reduce phosphorylation of the Na,K-ATPase at the PKC binding sites (Feschenko et al., 2000). This cross-talk may result from the close proximity of the PKG binding site to that of PKC (Kruger et al., 2003).

Along with PKC, PKA may catalyze phosphorylation of PLM at Ser68 (Fuller et al., 2004, 2009) in response to ischemia or β adrenergic stimulation (Despa et al., 2005) causing release of the inhibitory action of association of the FXYD1 subunit with the Na,K-ATPase.

REGULATION OF THE NA,K-ATPASE ACTIVITY BY CARDIOTONIC STEROIDS

Cardenolides and bufadienolides are the two classes of endogenous cardiotonic steroids that serve as hormones selectively interacting with Na,K-ATPase. These compounds are produced by midbrain and adrenocortical cells, and released into the circulation in sub-micromolar concentrations in response to various stimuli such as angiotensin II, acetylcholine, vasopressin, catecholamines, and hypoxic exposure (Bagrov et al., 2009). Interaction of these very low doses of endogenous inhibitors with the Na,K-ATPase does not compromise the transmembrane Na^+ gradients, but induces activation of Src kinase and formation of protein complex in which Src kinase and Na,K-ATPase associate with epidermal growth factor receptor (EGRF) and initiate several signaling cascades (Li and Xie, 2009). Signaling modalities depend on the type of cardiotonic steroid and its dose (Dvela et al., 2007). At the molecular level these differences in physiological action of cardenolide ouabain and bufadienolide marinobufagenin are reflected by the specific pattern of conformational changes unique for each steroid

upon binding to the purified Na,K-ATPase enzyme (Klimanova et al., 2015). Affinity of the Na,K-ATPase to ouabain is maximal in E2P conformation (in this conformation binding constant for ouabain exceeds that for marinobufagenin by 17-fold), whereas marinobufagenin does not discriminate between the E1 and E2 conformations when binding to the enzyme (Klimanova et al., 2015).

Notably, substitution of Cys 244 by Ala makes cells ouabain-intolerant (Shi et al., 2000). This cysteine is a target of regulatory S-glutathionylation (Petrushanko et al., 2012). Furthermore, binding of cardiotonic steroids fixes the enzyme in distinct conformation depending on the type of cardiotonic steroids (Klimanova et al., 2015). Thus, binding of cardiotonic steroids may alter susceptibility of cysteine residues to S-glutathionylation and, *vice versa*, reversible thiol modifications may possibly alter sensitivity of the enzyme to cardiotonic steroids. This hypothesis awaits further investigations.

Local (renal) or systemic reduction in oxygen availability was shown to trigger release of cardiotonic steroids (bufadienolides or endogenous ouabain) into the circulation in rodents and humans (Zhao et al., 1995; Bagrov et al., 1998; De Angelis and Haupert, 1998; Tian et al., 2010). Apart of the induction of cytoprotective signaling pathway, endocytosis of Na,K-ATPase will support reduction of ATP consumption by hypoxic tissue (De Angelis and Haupert, 1998). Protective effect of distinct cardiotonic steroids (low doses oleandrin for the brain and ouabain or the heart) was reported for the brain and heart exposed to ischemia-reperfusion (Pasdois et al., 2007; Van Kanegan et al., 2014). Binding of ouabain in low doses to the ATPase protects from toxicity of other cardiotonic steroids (Nesher et al., 2010). Whereas micormolar doses of cardiotonic steroids trigger Ca^{2+} overload and promote apoptosis (Winnicka et al., 2007), nanomolar doses of some of these compounds stimulate proliferation (Winnicka et al., 2010). This anti-apoptotic action may be reflect the changes in affinity of the Na,K-ATPase-ouabain complex to other proteins interacting with the Na,K-ATPase such as PKC, BAX, and Bcl-2 (Lauf et al., 2015). Release of them from the enzyme into the cytosol protects the cells from apoptosis.

On the other hand endogenous ouabain and marinobufagenin act as powerful vasoconstrictors actively participating in development of hypertension (Bagrov and Fedorova, 1998), and have a potential to cause Ca^{2+} overload secondary to the inhibition of the Na^+ extrusion from hypoxic tissue (Schwinger et al., 1999). At the moment, we clearly know too little about the mechanisms of cytoprotection by cardiotonic steroids to use them effectively as therapeutic agents avoiding deadly side-effects (Washam et al., 2015).

THE ROLE OF ACUTE REGULATION OF THE NA,K-ATPase IN ADAPTATION OR IRREPARABLE DAMAGE AT LOW O_2 LEVELS

As shown above, the capacity of the Na,K-ATPase to sense and respond to the changes in oxygen availability is immensely diverse with multiple signaling cascades implicated in fine-tuning of the enzyme's activity apart and before terminal ATP depletion is reached. The choice of signaling mechanism as well as the resulting vector and amplitude of the change in the Na,K-ATPase activity depends on the cell type, severity and duration of hypoxia, and is rather species-specific. Strictly speaking, we cannot refer to Na,K-ATPase as the "oxygen-sensitive" protein as all the processes involved in its responding to hypoxia are mediated by secondary products of O_2 transformation to either gaseous messengers or products of O_2 reduction.

Several types of response to hypoxia include (i) inhibition by thiol modifications (ii) modulation of the enzyme function/availability at the membrane due to the changes in phosphorylation, and (iii) interaction with nanomolar doses of endogenous cardiotonic steroids.

Diversity of regulatory pathways allows for fine dose-dependent regulation of the enzyme within minutes after the alteration in O_2 availability. Based on the current knowledge the following scheme of responses may be suggested (**Figure 4**). Following gradual deoxygenation NO availability in cells expressing NOS1 and NOS2 becomes limited whereas $O_2^{\bullet-}$ production by NADPH oxidases is maintained while pO_2 is above 2–3 kPa (K_d for NOXes $\sim$ 2 kPa) (Pacher et al., 2007). This is not the case for tissues in which NOS3 isoform of NO synthase with high affinity for O_2 prevails (such as vascular endothelial cells). These conditions most likely favor S-glutathionylation of β subunit that is associated with activation of superoxide production by NOXes (White et al., 2009) and α subunits of the Na,K-ATPase due to the gradual accumulation of GSSG (Petrushanko et al., 2012). Further decrease in oxygenation to 1–2 kPa supports uncoupling of electron transfer and triggers mitochondrial $O_2^{\bullet-}$ production and accumulation of H_2O_2 in the cytosol. Oxidation of GSSG becomes more pronounced and complete inactivation of the enzyme may be achieved as soon as ATP levels decrease below 50 μM (Petrushanko et al., 2012). Further decrease in O_2 to 0.2 kPa reduces S-glutathionylation and renders regulatory thiols within the α subunit oxidized to sulfinic and sulfonic acid making the enzyme insensitive to the changes in GSSG and $ONOO^-$ (Petrushanko et al., 2015). Phosphorylation may support or reduce the probability of complete reversible inactivation by S-glutathionylation of the regulatory cysteines by shifting the equilibrium between the E1 and E2 conformation of the enzyme (see Section Oxygen-Sensitive Phosphorylation).

Survival under hypoxic conditions implies that Na^+ uptake particularly high in excitable tissues is balanced with equally efficient extrusion of Na^+ by the pump (Hylland et al., 1997; Nilsson, 2001). Inhibition of the Na,K-ATPase is deadly when not synchronized with the closing of the cation channels and cessation of activity of the animal (channel arrest) (Buck and Hochachka, 1993; Hochachka et al., 1996; Hylland et al., 1997; Silver et al., 1997; Nilsson, 2001; Ross et al., 2006; Wilkie et al., 2008; Dave et al., 2009). If the Na,K-ATPase is suppressed and the channels are not "arrested," survival is limited to the time of Na^+/K^+ gradients dissipation in the brain and in the heart (Hylland et al., 1996, 1997; Silver

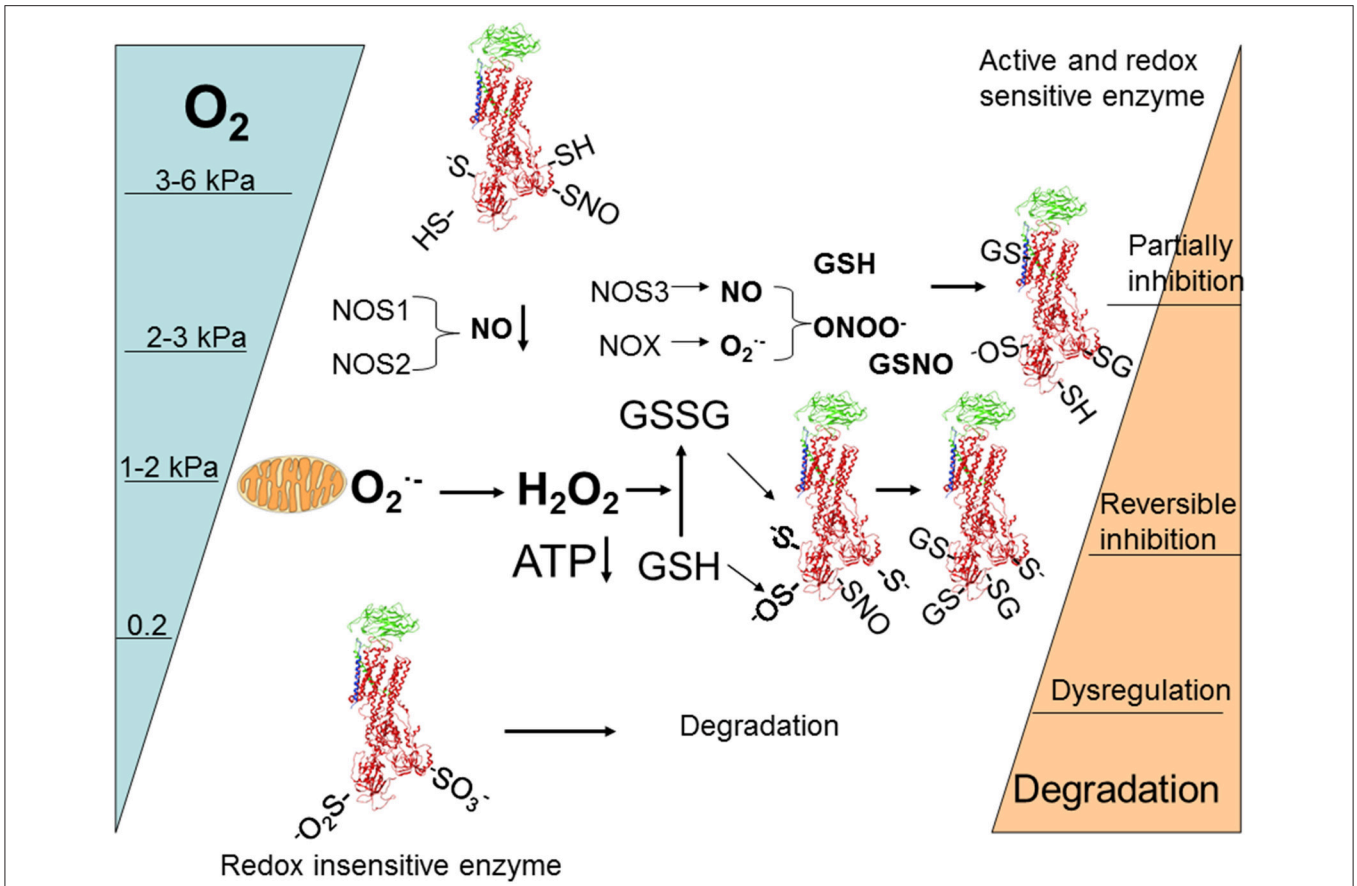

FIGURE 4 | Schematic representation of O_2-induced regulation of activity and abundance of Na,K-ATPase in majority of cells and tissues (for details see the Section The role of acute regulation of the Na,K-ATPase in adaptation or irreparable damage at low O_2 of levels of the review).

et al., 1997; Nilsson, 2001). It is tempting to assume that the alterations in free radical production and reversible thiol modifications are involved in the simultaneous regulation of multiple ion transporting systems coupling their activity to the mitochondrial function and ATP availability. However, data providing direct support for this hypothesis are currently missing.

The pilot studies suggest that preservation of redox state in hypoxic heart is sufficient to maintain the Na,K-ATPase activity in Spalax mole rate and trout supporting activity of both species under conditions of critical O_2 shortage for at least 20 min (Yakushev et al., 2012). S-nitrosylation of the regulatory cysteine residues in the α subunit may also prevent S-glutathionylation and the enzyme inactivation by GSSG in myocardial membranes (Petrushanko et al., 2012). Whether this is the case for some or all anoxia-tolerant species (e.g., Hylland et al., 1997; Nilsson, 2001; Ross et al., 2006; Dave et al., 2009), remains to be clarified.

Maintenance of the transmembrane Na^+ gradients does not only support neuronal function and heart contractility. It also regulates intracellular Ca^{2+} by controlling the activity of Na/Ca exchanger and that of voltage-gated Ca^{2+} channels. Ca^{2+} transport pathways are by themselves targets regulated by reversible thiol modifications in hypoxic cells (Lehotsky et al., 2002; Wang and Zheng, 2010).

Recent data revealed the existence of intimate link between the activity of the Na,K-ATPase and the gene expression in hypoxic cells and tissues. Two factors that were recently suggested to impact the gene expression under hypoxic conditions include the shift in transmembrane Na/K gradients (Koltsova et al., 2014) and the alteration in HIF1α levels upon binding of the nanomolar concentrations of cardiotonic steroids to their binding site within the Na,K-ATPase (Zhang et al., 2008; Cao et al., 2014). Decrease in HIF1α in hypoxic cells (1% O_2) in the presence of <50 nM ouabain is not caused by its degradation but rather by a drop in protein synthesis (Zhang et al., 2008), most likely at the level of translation (Cao et al., 2014). Alterations of HIF1α availability by cardiac glycosides contributes to the modulation of long-term hypoxic responses of the organism. For example digoxin treatment prevents remodeling of pulmonary vasculature underlying development of hypoxic pulmonary hypertension (Abud et al., 2012). It is known that signaling cascades initiated by binding of low doses of cardiotonic steroids to the Na,K-ATPase include modulation of intracellular Ca^{2+} levels and free radical production as well as activation of several kinases. Molecular mechanisms linking

these processes to the regulation of HIF1α translation remain unclear.

AUTHOR CONTRIBUTIONS

AB is the author the general schematics of review, she contributed to all the sections and assembling of the review. AM, PH were contributing to the sections Introduction. Oxygen, and Oxygen Sensing from Evolutionary and Modern Perspectives, Versatility of oxygen sensing. Multiple Signals-Multiple Targets—Multiple Responses—Multiple Outcomes, and Oxygen and Redox-Sensitivity of the Na,K-ATPase. IP contribution were the sections Oxygen and Redox-Sensitivity of the Na,K-ATPase, Coordinated Regulation of Redox-Sensitive Protein Networks by Reversible Thiol, Oxygen-Sensitive Phosphorylation, and Regulation of the Na,K-ATPase Activity by Cardiotonic Steroids. All co-authors discussed the topics and writing the review. All of them agree with the final text.

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REVIEW
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Effects of Age on Na^+,K^+-ATPase Expression in Human and Rodent Skeletal Muscle

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The maintenance of transmembrane Na^+ and K^+ concentration gradients and membrane potential is vital for the production of force in skeletal muscle. In aging an inability to maintain ion regulation and membrane potential would have adverse consequences on the capacity for performing repeated muscle contractions, which are critical for everyday activities and functional independence. This short review focusses on the effects of aging on one major and vital component affecting muscle Na^+ and K^+ concentrations, membrane potential and excitability in skeletal muscle, the Na^+,K^+-ATPase (Na^+,K^+-pump, NKA) protein. The review examines the effects of age on NKA in both human and rodent models and highlights a distant lack of research in NKA with aging. In rodents, the muscle NKA measured by [^{3}H]ouabain binding site content, declines with advanced age from peak values in early life. In human skeletal muscle, however, there appears to be no age effect on [^{3}H]ouabain binding site content in physically active older adults between 55 and 76 years compared to those aged between 18 and 30 years of age. Analysis of the NKA isoforms reveal differential changes with age in fiber-types in both rat and humans. The data show considerable disparities, suggesting different regulation of NKA isoforms between rodents and humans. Finally we review the importance of physical activity on NKA content in older humans. Findings suggest that physical activity levels of an individual may have a greater effect on regulating the NKA content in skeletal muscle rather than aging *per se*, at least up until 80 years of age.

Keywords: age, Na^+K^+-pump, single fiber, [^{3}H]ouabain

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IMPLICATIONS OF IMPAIRED SKELETAL MUSCLE ION REGULATION IN AGING

A fundamental factor underpinning skeletal muscle contractile function is the maintenance of membrane excitability, which is heavily dependent on transmembrane sodium (Na^+), potassium (K^+), and chloride (Cl^-) gradients and conductance's via their effects on muscle membrane potential (E_m) (Hodgkin and Horowicz, 1959). The intramuscular regulation of these ions is important in both the development of and preservation against muscle fatigue (Sejersted and Sjøgaard, 2000; McKenna et al., 2008). Therefore, any disturbances in muscle ion regulation with aging are likely to impact adversely on cellular excitability and the capacity to undertake repeated muscle contractions, thereby affecting the capability to successfully complete simple daily tasks, and thus on quality of life. Preservation of muscle mass and function is critical in older individuals, due their greater risk of falls, and the consequential effects, that include ongoing physical disability,

declines in physical and mental health and in social isolation for the individual (Stel et al., 2004). This short review focusses on the effects of aging on one major component of ion regulation in skeletal muscle, the Na^+,K^+-ATPase (Na^+,K^+-pump, NKA) protein in skeletal muscle, which is vital for the regulation of transmembrane Na^+ and K^+ concentration gradients, E_m and excitability in skeletal muscle cells (Clausen, 2003b). This review does not cover the acute activation and regulation of the NKA, for this we direct readers to excellent reviews (Clausen, 2003b; Pirkmajer and Chibalin, 2016).

Na^+,K^+-ATPase (NKA) IN SKELETAL MUSCLE

In healthy young adults, the skeletal muscle NKA content is typically around 250–350 pmol.g wet weight^{-1} (Clausen, 2013). Whilst neural tissue contains a higher NKA content per unit mass, skeletal muscle represents the largest pool of NKA in the body due to the very large muscle mass; thus a human weighing 70 kg is estimated to have approximately 8.4 μmol of NKA (Clausen, 2013). The NKA comprises alpha (α) and beta (β) subunits, which together constitute a functional αβ heterodimer (Clausen, 2003b; Green, 2004). The NKA α subunit (∼100–112 kDa) contains binding sites for Na^+, K^+, and Mg^{2+} ions as well as phosphate and ATP and typically undergoes both phosphorylation and oxidation (Clausen, 2003b; Lingrel et al., 2003; McKenna et al., 2006). The β subunit (∼35–55 kDa) is glycosylated and is necessary for the structural maturation of the α subunit, localisation of the NKA heterodimer to the sarcolemma and regulation of NKA activity (Cougnon et al., 2002). Each isoform is encoded by separate genes including four α isoforms (α_1, α_2, α_3, α_4), and three β isoforms (β_1, β_2, β_3) (Blanco and Mercer, 1998). The expression of these isoforms differs across tissues, which suggests a varying function and regulation of each isoform (McDonough et al., 2002). An additional regulatory (γ) subunit associated with NKA activity, known as the FXYD family of proteins comprising seven isoforms (Geering, 2005); the predominant FXYD isoform in skeletal muscle is FXYD1, or phospholemman (Bibert et al., 2008).

To understand aging effects on NKA in muscle, it is first important to understand distributions of NKA isoforms in specific fiber types. In rat skeletal muscle, based on experiments using isoform-specific antibodies, most studies reveal that the NKA α_1 isoform has a similar abundance in oxidative and glycolytic muscles (Hundal et al., 1993; Thompson and McDonough, 1996; Ng et al., 2003; Fowles et al., 2004; Zhang et al., 2006; Kristensen and Juel, 2010; Ingwersen et al., 2011). Similarly, the α_2 is abundant in both oxidative and glycolytic muscles in the rat (Thompson and McDonough, 1996; Fowles et al., 2004; Kristensen and Juel, 2010; Ingwersen et al., 2011). In contrast, rat skeletal muscle exhibits distinct differences in β_1 and β_2 expression between fiber types, with an almost exclusive expression of β_1 in muscles rich in slow twitch fibers, whereas the β_2 is more abundant in fast twitch fibers (Hundal et al., 1993; Thompson and McDonough, 1996; Fowles et al., 2004; Zhang et al., 2006). The β_3 isoform in rat muscle was found to be similarly abundant in red and white gastrocnemius muscles (Ng et al., 2003).

In human skeletal muscle, all isoforms except the α_4 isoform are expressed (Murphy et al., 2004) and recent studies indicate that muscle fiber type NKA expression differs considerably from that in the rat. In human muscle, an initial study found no fiber-type specific differences abundance for the α_1, β_1, or FXYD1, but revealed a greater abundance of α_2 in Type II fibers, with no measures of the other NKA isoforms (Thomassen et al., 2013). Two subsequent studies from our group reported no fiber-type specific abundance in the α_1, α_2, and β_1 isoforms, but a greater abundance of the α_3 and β_2 in Type II fibers (Wyckelsma et al., 2015, 2016). The FXYD1 protein was not measured in these latter studies.

The differing abundances of the NKA isoforms between fiber-types in rat muscle and to a lesser extent in human muscle, raises the important issue of understanding what the functional roles of different isoforms are in skeletal muscle. However, our understanding of the specific functions of the NKA isoforms within skeletal muscle is incomplete. Several genetically modified mouse models have been developed to address this, including mice with either one copy of either the α_1 ($\alpha_1^{+/-}$) or α_2 ($\alpha_2^{+/-}$) gene knocked out, leaving the mice with one-half of either the α_1 or α_2 isoform compared to wild-type (WT) mice (He et al., 2001), or a skeletal muscle-specific α_2 gene knock out (sk$\alpha2^{-/-}$; Radzyukevich et al., 2012). The extensor digitorum longus (EDL) muscles from $\alpha_1^{+/-}$ mice showed reduced muscle force compared with WT mice, while the $\alpha_2^{+/-}$ mice had improved force (He et al., 2001). When a fatigue protocol was implemented the $\alpha_2^{+/-}$ mouse EDL muscle fatigued at a faster rate than the $\alpha_1^{+/-}$ and WT (He et al., 2001). It was suggested the improved force in basal (i.e., non-fatigued) conditions in $\alpha_2^{+/-}$ mice could have been explained by altered Ca^{2+} handling in these mice (He et al., 2001). In sk$\alpha2^{-/-}$ mice, the resting membrane potential of the EDL was not different to WT mice (Radzyukevich et al., 2012); however, the sk$\alpha2^{-/-}$ muscle showed a rapid decline in the maximum twitch and tetanic forces and fatigued earlier during a treadmill running test compared to WT mice (Radzyukevich et al., 2012). These studies now establish, at least in murine skeletal muscle, that the NKA α_1 plays an important role in Na^+/K^+ exchange and E_m regulation during basal conditions, presumably in a "house-keeping" role, whilst the α_2 isoform plays a key role during muscle contractions. The α_3 is the least abundant of the NKA α isoforms in rat skeletal muscle (Blanco and Mercer, 1998), but its role in skeletal muscle has not yet been established. The α_3 isoform is the major NKA α isoform expressed in neurones, with NKA α_3 mutations linked with dystonia-parkinsonism and alternating hemiplegia of childhood (Heinzen et al., 2014). An investigation with α_3 haplo-insufficiency in mice found that while they could compete physically with WT mice, they were slower in cognitive challenges, such as navigating a water maze (Moseley et al., 2007). Thus, further research is required to determine the functional significance of NKA α_3 in skeletal muscle.

The overall amount of β isoforms present in a muscle is also important for NKA activity. Muscles expressing NKA α:β isoforms in a ratio of 1:2 had a higher NKA activity than those with a 1:1 ratio, with activity measured by the K^+-dependent 3-O- methylfluorescein phosphate activity assay (Lavoie et al., 1997). The alpha-beta heterodimer with a β_1 isoform has a higher affinity to Na^+ and lower affinity for K^+ compared to β_2, independent of the α isoform paired with (Crambert et al., 2000). Whilst the expression of β_1 and β_2 isoforms differs between muscle fiber types in the rat, the specific roles of the different β isoforms in skeletal muscle NKA regulation contractile have not yet been defined.

The NKA in muscle is adaptable with disease and physical activity. In humans, declines in skeletal muscle [^{3}H]ouabain binding site content have been reported with various diseases including muscular dystrophy (Desnuelle et al., 1982), McArdles disease (Haller et al., 1998), and liver cirrhosis (Aagaard et al., 2002), as well as injury and associated inactivity (Leivseth and Reikerås, 1994; Perry et al., 2015). In contrast, physical training increases muscle [^{3}H]ouabain binding site content and is also typically associated with improved exercise performance (McKenna et al., 1996; Clausen, 2013). Given this malleability in muscle NKA and the link with muscular performance, any effects of age on the NKA are of considerable interest.

AGE ASSOCIATED ALTERATIONS TO NKA WHOLE MUSCLE CONTENT MEASURED VIA [^{3}H]OUABAIN BINDING

The widely accepted method for quantification of the total number of functional NKA in skeletal muscle is through measurement of the [^{3}H]ouabain binding site content (Clausen, 2003a). The procedure is performed on small pieces of whole muscle samples (typically between 10 and 20 mg) and is based on the high affinity binding of cardiac glycosides to the α subunit of the NKA, with a stoichiometry of 1:1 (Hansen, 1984). By incubation of muscle samples in tritiated ouabain and counting of β particles via liquid scintillation, it is possible to quantify the NKA content in molar units, typically expressed as NKA in pmol·g wet weight^{-1} (Hansen and Clausen, 1988). In human skeletal muscle, the standard [^{3}H]ouabain binding assay detects each of the three α isoforms (Wang et al., 2001) and thus this is a measure of NKA content; thus in the article these terms can be read interchangeably. This contrasts rat muscle, where only the α_2 isoform is detected at the concentration of ouabain used, due to its much higher affinity than the other α isoforms (Hansen, 2001), although this is clearly the most dominantly expressed of the α isoforms (Hansen, 2001). Thus, when referring to rat skeletal muscle the [^{3}H]ouabain binding site content is referred rather than the term NKA content which is incorrect.

Few studies have investigated age effects on skeletal muscle [^{3}H]ouabain binding site content. From the limited available literature, rodent muscle shows a clear age-dependent effect, although by far the greatest changes are a large upregulation that early occur early in life. In rats, soleus muscle [^{3}H]ouabain binding site content increased four-fold from birth up until 1 year of age, which was then followed by a 50–70% decline over the following 2–20 months (Kjeldsen et al., 1984). In rats, soleus muscle exhibited a 58% decrease in [^{3}H]ouabain binding site content from rats aged between 28 and 85 days (Kjeldsen et al., 1982). Mice showed less prominent changes in muscle [^{3}H]ouabain binding site content than rats. From the first week of life [^{3}H]ouabain binding site content increased from around 300 to 800 pmol.g wet weight^{-1} by the 4th week of life, this was followed by a 25% decline which plateaued over the subsequent 4 weeks (Kjeldsen et al., 1984).

There are no lifespan time-course studies investigating chronological changes in human muscle NKA. Several studies utilizing a cross-sectional design have compared muscle NKA content in young healthy participants with a mean age of ∼24 years vs. older adults with a mean age of ∼68 years (Klitgaard and Clausen, 1989; McKenna et al., 2012; Wyckelsma et al., 2016). The earliest study reported a non-significant (14% lower) difference in NKA content in older compared to the younger adults (Klitgaard and Clausen, 1989). Two recent studies from our group reported no difference in NKA content between young and older adults (McKenna et al., 2012; Wyckelsma et al., 2016). These studies are therefore consistent in finding no significant difference in [^{3}H]ouabain binding site content between young

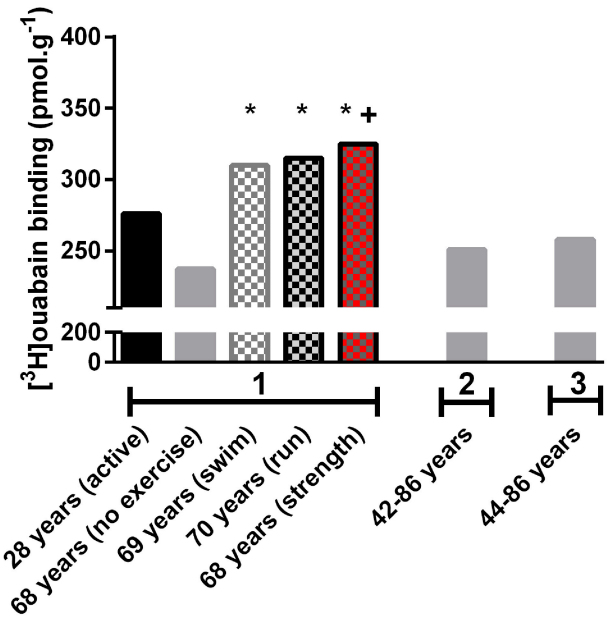

FIGURE 1 | Human skeletal muscle [^{3}H]ouabain binding with aging and physical activity from studies conducted in the Clausen laboratory (Aarhus, Denmark). Data from early papers investigating [^{3}H]ouabain binding in older adults from different studies measured in the same laboratory to ensure comparisons are made utilizing the same methodology. 1. Indicates data from (Klitgaard and Clausen, 1989), 2. from (Dørup et al., 1988a), and 3. from (Dørup et al., 1988b). Data from 2 to 3 were from healthy control subjects in studies undertaking comparison against clinical populations. These clinical population data have not been included in this figure. *Different to 68 years (no exercise), $^+$ different to young (active), $p < 0.05$.

and older adults. However, an interesting observation from another recent study was that NKA content was 26% lower in older adults aged between 69 and 81 years compared to those aged from 55 to 68 years (Perry et al., 2013). This suggested that an age associated reduction in muscle NKA content in humans might only be apparent at more advanced ages. To investigate this possible age effect on NKA content further, we analyzed all of the [^{3}H]ouabain binding site content data in aging-related research collected in healthy, older participants in our laboratory over the past 4 years (McKenna et al., 2012; Perry et al., 2013; Wyckelsma et al., 2016). The first comparison revealed no difference in NKA content between younger adults aged 18–30 years ($n = 32$) compared to those aged from 55 to 76 years ($n = 25$; 348.3 ± 82.2 vs. 361.8 ± 59.0 pmol.g wet weight^{-1}, Young vs. Old, respectively, $p = 0.53$), confirming the above conclusion (**Figure 2**). Comparison between adults after dividing older adults in subgroups based on decades lived, from 18 to 30 ($n = 32$), 55–59 ($n = 3$), 60–69 ($n = 11$), and 70–76 years of age ($n = 11$), also revealed no differences with age (**Figure 2**). This suggests that the decline in [^{3}H]ouabain binding reported previously in the oldest age category (Perry et al., 2013) may be related to their chronic physical activity levels rather than their age. Older participants in one of the studies reported similar physical activity levels to the healthy young controls, despite the intensities of activities likely differing considerably between the groups (Wyckelsma et al., 2016). This may suggest that preserving some level of physical activity might be the important factor in the maintenance of skeletal muscle NKA with age in humans. Thus, whilst studies in rat and murine muscle suggest that aging is associated with a moderate decline in [^{3}H]ouabain binding site content, there is no evidence that this occurs in human skeletal muscle. However, since we have no analyses of muscle [^{3}H]ouabain binding in healthy adults greater than 80 years, we cannot exclude the possibility that a decline may occur beyond this age.

AGE ASSOCIATED ALTERATIONS TO NKA ISOFORMS

Despite the [^{3}H]ouabain binding site content being the gold standard for measuring NKA content in human muscle, this method (using standard concentration of ouabain) cannot differentiate between the individual α isoforms. Hence researchers are unable to identify which of the three specific α isoforms might have changed with a particular intervention, condition or with aging. Given the possibility of differing roles and fiber-type specificity of the individual isoforms, it is therefore necessary to also investigate the isoform abundances in muscle. This has typically been conducted via western blotting, although one study utilized immunohistochemistry (IHC) to analyze differences in NKA isoforms in aged rat muscle (Zhang et al., 2006). The advantages of IHC are that it allows detection of the cellular localization of specific isoforms and can provide an indication of the fiber-type specificity of a protein. However, disadvantages include the inability to quantify the abundance of protein and the problem of non-specific binding of antibodies.

Few studies have investigated the impacts of aging on NKA isoforms in rat muscle, and the use of different ages of rats and analytical techniques (e.g., western blotting vs. IHC) makes direct comparisons between studies difficult; it is therefore not surprising that findings are inconsistent (Sun et al., 1999; Ng et al., 2003; Zhang et al., 2006). Nonetheless, these studies have identified that aging in rats is associated with skeletal muscle NKA isoform changes that comprise an increased α_1, either no change or a decline in α_2, an increase or no change in β_1, decreased β_2, and an increases β_3, as summarized in **Table 1**. These findings from advanced aged in rodent muscle suggests there may be more NKA $\alpha_1\beta_1$ heterodimers present in aged skeletal muscle (Sun et al., 1999; Ng et al., 2003). The $\alpha_1\beta_1$ has a higher Na^+ affinity in resting muscle (Crambert et al., 2000), and it has been hypothesized that aged rat muscle may

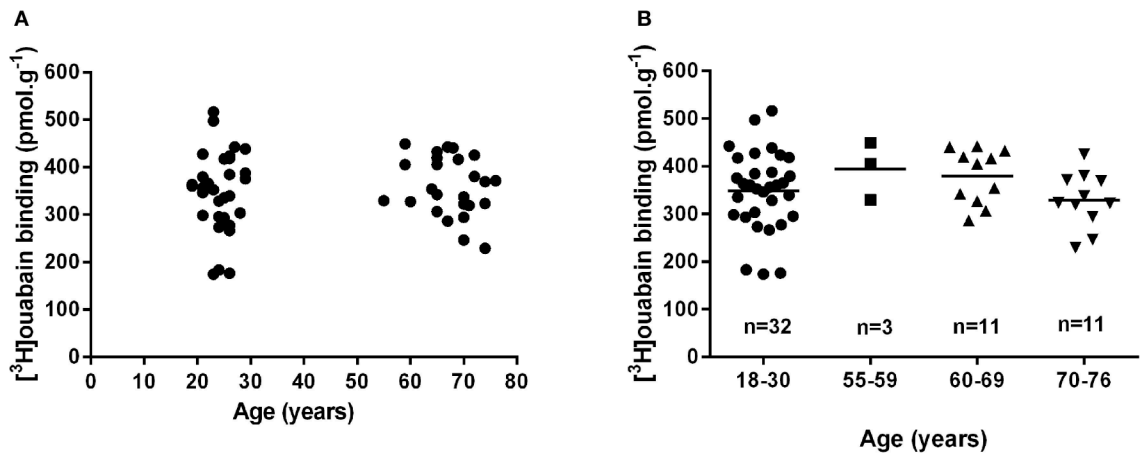

FIGURE 2 | Muscle [^{3}H]ouabain binding does not change with age in human skeletal muscle. Muscle [^{3}H]ouabain binding site content collated from data collected on healthy young and healthy older adults from the McKenna research group between 2012 and 2016. **(A)** Shows the data combined into two discrete age groups and analyzed by unpaired *t*-test ($p = 0.53$). **(B)** Shows all data plotted into relevant decades of life analyzed by one-way ANOVA ($p = 0.30$); the mean of each group is also shown as a horizontal line.

TABLE 1 | Effects of age on NKA isoform abundances in skeletal muscle.

Ref	Species	Age (months/years)	*N*	Technique (normalization)	Muscle Measured	NKA Isoform					
						α_1	α_2	α_3	β_1	β_2	β_3
1	Rat	Young (6 months)	5	Western blot	EDL						
			6		Sol	30 > 6	18 < 6		–		
		Adult (18 months)			RG	30 > 6,18	18, 30 < 6		30 > 6,18	30 < 6	
			7		WG	30 > 6,18	18, 30 < 6		30 > 6,18	30,18< 6, 30 < 18	
		Old (30 months)									
2	Rat	Young (16 months)	NR	Western blot	EDL	NS	NS		NS		
					Sol						
		Old (29 months)			RG	↑	NS		NS	↓	↑
					WG	↑	NS		NS	↓	↑
3	Rat	Young (6 months)	12-15	IHC	RG	↑ *	↓ *		↑ *	↓ *	↑ *
					WG	↑ *	↓ *		↑ *	↓ *	↑ *
		Old (30 months)									
4	Human	Old 66.8 ± 6.4	17	Western blot (GAPDH)	VL	–	↓ 24%	–	–	–	–
		Young 23.9 ± 2.2	16								
5	Human	Old 69.4 ± 3.5	17	Western blot (Calibration Curve)	VL	–	–	–	–	–	↑ 250%
		Young 25.5 ± 2.8	14		VL Type I fibers	↑ 71%	–	–	–	–	↑ 96%
					VL Type II fibers	–	–	↓ 47%	–	↓ 85%	↑ 285%

Reference 1, Sun et al., 1999; 2, Ng et al., 2003; 3, Zhang et al., 2006; 4, McKenna et al., 2012; 5, Wyckelsma et al., 2016.

*Age, mean ± SD. Symbols: ↓, denotes decrease; –, no change; ↑, increase, data in parentheses denotes % difference between groups. ↑ * not quantitative but increased compared to young ↓ * not quantitative but decreased compared to young. NR not reported, NS not significant.*

Muscles: EDL, Extensor Digitorum Longus; RG, Red Gastrocnemius; WG, White Gastrocnemius; VL, Vastus Lateralis.

have increased K^+ and Na^+ fluxes with aging, due to reduced amount of caveolin-3 located in the transverse tubular system of aged mice (Barrientos et al., 2015). This is consistent with the important housekeeping role of the α_1 isoform in Na^+/K^+ exchange. From the above findings and also the reduction of $\alpha_2\beta_2$ in aged isoforms in rat, it seems reasonable to suggest in aged rats, that the increased abundance of the $\alpha_1\beta_1$ isoforms may be compensatory for these losses. It was also shown in red gastrocnemius muscle, that α_1 was less phosphorylated in aged rats (Zhang and Ng, 2007), although the functional implications of this are not yet understood.

Research into the NKA isoform abundances with age in humans is sparse. The initial study by our group investigated NKA isoform abundance in human vastus lateralis muscle homogenates and found a 24% decrease in α_2 and 23% decrease in β_3 isoform abundances in older compared to young adults, without change in the other NKA isoforms (McKenna et al., 2012). As there was no difference in [^{3}H]ouabain binding site content, it was suggested that the decrease in α_2 abundance might reflect an increased fiber membrane density due to the smaller fibers in aged muscle (McKenna et al., 2012). A later study revealed a decrease with age in the abundance of several proteins typically used as housekeeping proteins, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Vigelsø et al., 2015). The GAPDH protein was used in the above study to normalize NKA western blots (McKenna et al., 2012). We therefore re-examined the effects of age on NKA isoform abundances in muscle homogenates, but normalized against total protein rather than GAPDH and also introduced a calibration curve for normalization (Murphy and Lamb, 2013; Wyckelsma et al., 2016). With this improved method in our subsequent study, we found no difference in the abundance in the α_2 in aged muscle, which was consistent with the lack of difference in [^{3}H]ouabain binding site content. We also observed no differences in other NKA isoforms, apart from a large (∼250%) increase in β_3 isoform abundance (Wyckelsma et al., 2016). This suggests that aging may have quite different effects on the abundance of NKA isoforms in rat and human skeletal muscle.

Since human skeletal muscle comprises a mixed muscle fiber type population, analysis of protein changes in a muscle homogenate might mask changes with aging that occur in different muscle fiber types. We therefore also undertook analysis of NKA isoforms from single fiber segments from biopsies

obtained from elderly humans (Wyckelsma et al., 2016). This study firstly showed a lack of fiber-type specificity for any of the six NKA isoforms, with isoforms similarly abundant in Type I and II fibers (Wyckelsma et al., 2016). When isoform abundance was compared between young and older adults in a given fiber type, a number of differences were identified. Compared to young adults, the NKA α_3 and β_2 isoforms were both lower by ∼47% and ∼85%, respectively, in Type II fibers of the older adults, whilst there was a ∼71% greater abundance of α_1 in Type I fibers in aged muscle compared to young. The NKA β_3 was greater in aged muscle in both Type I (∼96%) and II (∼285%) fibers, reflective of β_3 changes seen in the whole muscle homogenate (Wyckelsma et al., 2016).

The greater α_1 and β_3 isoforms, and lesser β_2 isoforms in aged human single fiber segments, showed some similarities with rodent studies (see **Table 1**). However, no age-associated decreases in the α_2 or β_1 have been reported in studies with humans, contrary to some animal studies (Sun et al., 1999). Direct comparison between these studies is difficult due to varying methodological techniques, as well as is the different ages of mice studied. Previous studies utilized mice aged 29 (Ng et al., 2003), and 30 months (Sun et al., 1999; Zhang et al., 2006). Since 13.8 days for a rat is approximately equivalent to 1 year for a human, mice aged 29–30 months would be equivalent to a human aged 75–80 years (Sengupta, 2013). In human physiological research it is quite difficult to recruit older participants of quite advanced age that are in good health, have limited ongoing pharmacological treatments, as well as are willing to undergo invasive procedures, such as muscle sampling. Furthermore, those that do volunteer may already tend to be reasonably active, which might in itself influence expression of NKA α isoforms (Perry et al., 2013).

In human muscle, the increase in the abundance of α_1 which was commonly seen in rodent has been observed, but no measures of phosphorylation have been investigated. Interestingly all studies with rodents report decreased β_2 in white and red gastrocnemius muscle (Sun et al., 1999; Ng et al., 2003; Zhang et al., 2006) and also with humans in Type II fibers (Wyckelsma et al., 2016). The role of β_2 is unknown in skeletal muscle. It is important to explore the role the β_2 isoform plays in NKA enzymatic activity and excitability, especially in Type II fibers, since Type II fibers undergo a loss of specific force in aged compared to compared to young adults (Lamboley et al., 2015). Whilst FXYD1 phosphorylation has been measured in human muscle (Thomassen et al., 2013, 2016), and increased with exercise in aged rats (Reis et al., 2005), the effects of aging have not yet been investigated in aged humans.

CHRONIC REGULATION OF NKA WITH EXERCISE TRAINING IN THE AGED

In young adults, physical activity is known to upregulate the NKA content in skeletal muscle (McKenna et al., 1996; Clausen, 2003b), but little is known about training effects on muscle NKA in the aged. The study by Klitgaard and Clausen (1989), compared [^{3}H]ouabain binding site content in healthy but sedentary older and younger adults with three cohorts of active older adults, who had undertaken 12–17 years of regular physical training. The active groups participated in either running, swimming or resistance training and had considerably greater [^{3}H]ouabain binding site content than the sedentary older adults (**Figure 1**). Furthermore, it was found that the resistance trained older adults had a greater [^{3}H]ouabain site content compared to untrained young controls (Klitgaard and Clausen, 1989).

In rats, exercise training further increased the α_1 isoform, which was already upregulated with age, as measured by western blotting and appeared to reverse the age associated alterations of the NKA β_3 isoform (Ng et al., 2003) (**Table 2**). Additional training adaptations included increases in the α_2 and β_1 in rat EDL, red and white gastrocnemius muscle (Ng et al., 2003). Another study in aged rats found upregulation of FXYD1 in EDL, red and white gastrocnemius muscle with exercise training (Reis et al., 2005) (**Table 2**). There were also tendencies for the co-immunoprecipitation of FXYD1 to the α_1 which tended to decrease with training and the α_2 tended to increase the co-immunoprecipitation of FXYD1 with training (Reis et al., 2005). However, this study was conducted in only 3 rats, which they suggested may have resulted in a Type II error. The effects of longitudinal training in older adults is yet to be published.

TABLE 2 | Comparison of NKA isoform responses to training in aged rat and human.

Ref	Species	Age	*N*	Training	Technique	Muscle Measured	NKA Isoform						FXYD1
							α_1	α_2	α_3	β_1	β_2	β_3	
1	Rat	29 Months	12–15	13–14 weeks motorized treadmill running	Western blot	EDL	↑	↑	↑	–	↓	–	
						RG	↑	↑	↑	–	↓		
						WG		↑	↑	–			
2	Rat	29 months	3	13–14 weeks motorized treadmill running	Western blot	EDL							↑
						RG							↑
						WG							↑

1, Ng et al., 2003; 2, Reis et al., 2005. Blank space indicated not measured, ↑ increased ↓ decreased – no change.
Muscles: EDL, Extensor Digitorum Longus; RG, Red Gastrocnemius; WG, White Gastrocnemius; VL, Vastus Lateralis.

PERSPECTIVES

The review highlights the lack of research in aging and NKA regulation in skeletal muscle, with many aspects of NKA regulation still required to be explored. It further demonstrates that changes that might be observed with age in rodent muscles cannot be anticipated to also occur in humans and the reasons for this are unclear. This highlights the importance of conducting studies in human muscle. Such research is particularly important given the vital role of NKA in regulation muscle excitability and function. The use of skeletal muscle knockout mice provide excellent models to determine function of the specific NKA isoforms in skeletal muscle. Future research could consider the effects of aging utilizing these knockout mice to investigate muscle NKA, contractility and function, and including with α_3, and β_{1-3} isoform knock out models. Until the roles of the NKA isoforms in skeletal muscle are more fully understood, the functional translation of findings in rodent to human muscle physiology can only be speculative. Further research should in addition explore the acute activation of the skeletal muscle NKA in aged muscle and its regulation, as well as the impacts on muscle E_m, excitability and the link to function during repeated contractions. Finally, any future work with NKA regulation in aged human skeletal muscle should include measures in single fiber segments to ensure that any fiber-type specific responses are not masked.

AUTHOR CONTRIBUTIONS

VW and MM drafted, edited and approved the final version of the manuscript.

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REVIEW
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P4-ATPases as Phospholipid Flippases—Structure, Function, and Enigmas

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P4-ATPases comprise a family of P-type ATPases that actively transport or flip phospholipids across cell membranes. This generates and maintains membrane lipid asymmetry, a property essential for a wide variety of cellular processes such as vesicle budding and trafficking, cell signaling, blood coagulation, apoptosis, bile and cholesterol homeostasis, and neuronal cell survival. Some P4-ATPases transport phosphatidylserine and phosphatidylethanolamine across the plasma membrane or intracellular membranes whereas other P4-ATPases are specific for phosphatidylcholine. The importance of P4-ATPases is highlighted by the finding that genetic defects in two P4-ATPases ATP8A2 and ATP8B1 are associated with severe human disorders. Recent studies have provided insight into how P4-ATPases translocate phospholipids across membranes. P4-ATPases form a phosphorylated intermediate at the aspartate of the P-type ATPase signature sequence, and dephosphorylation is activated by the lipid substrate being flipped from the exoplasmic to the cytoplasmic leaflet similar to the activation of dephosphorylation of Na^+/K^+-ATPase by exoplasmic K^+. How the phospholipid is translocated can be understood in terms of a peripheral hydrophobic gate pathway between transmembrane helices M1, M3, M4, and M6. This pathway, which partially overlaps with the suggested pathway for migration of Ca^{2+} in the opposite direction in the Ca^{2+}-ATPase, is wider than the latter, thereby accommodating the phospholipid head group. The head group is propelled along against its concentration gradient with the hydrocarbon chains projecting out into the lipid phase by movement of an isoleucine located at the position corresponding to an ion binding glutamate in the Ca^{2+}- and Na^+/K^+-ATPases. Hence, the P4-ATPase mechanism is quite similar to the mechanism of these ion pumps, where the glutamate translocates the ions by moving like a pump rod. The accessory subunit CDC50 may be located in close association with the exoplasmic entrance of the suggested pathway, and possibly promotes the binding of the lipid substrate. This review focuses on properties of mammalian and yeast P4-ATPases for which most mechanistic insight is available. However, the structure, function and enigmas associated with mammalian and yeast P4-ATPases most likely extend to P4-ATPases of plants and other organisms.

Keywords: P-type ATPases, P4-ATPases, ATP8A2, CDC50, flippases, membrane asymmetry, phospholipid transport

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INTRODUCTION

Non-random distribution of lipids across the lipid bilayer is a characteristic feature of most eukaryotic cell membranes. This asymmetrical distribution is most evident in the plasma membrane where phosphatidylcholine (PC), sphingomyelin, and glycolipids are enriched on the external or exoplasmic leaflet of the membranes and phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) are primarily confined to the cytoplasmic leaflet (Zachowski, 1993; Lenoir et al., 2007). Cholesterol, a major lipid of the plasma membrane, can move freely between the two leaflets, but is likely to be more concentrated on the exoplasmic leaflet due to its affinity for sphingomyelin and glycosphingolipids. Membranes of other subcellular organelles including the trans-Golgi network (TGN), secretory vesicles, and endosomes show a similar asymmetrical distribution of lipids. Membrane lipid asymmetry plays a crucial role in numerous cellular processes that take place at the plasma membrane and intracellular membranes including cell and organelle shape determination and dynamics, vesicle budding and trafficking, membrane protein regulation, membrane stability and impermeability, cell signaling, neurite extension, blood coagulation, apoptosis, fertilization, and bile and cholesterol homeostasis, among others.

The amphipathic nature of phospholipids impedes their transverse movement or flip-flop across the hydrophobic lipid bilayer. The half time for phospholipid flip-flop in protein-free liposomes typically exceeds several hours. However, the movement of lipids across biological membranes is greatly accelerated by membrane proteins generally called flippases (Pomorski and Menon, 2006). Three families of proteins, scramblases, ATP-binding cassette (ABC) transporters, and P4-ATPases, have been implicated in the transverse movement of lipids across membranes. Scramblases are energy-independent, bidirectional transporters that dissipate membrane asymmetry (Williamson, 2015). Ca^{2+}-activated scramblases play an important role in exposing PS on the surface of cells to initiate such cellular processes as blood coagulation and apoptosis. Some scramblases such as the G-protein coupled receptor rhodopsin are constitutively active thereby randomizing transmembrane lipid distribution in specialized membranes such as photoreceptor discs (Menon et al., 2011). ABC transporters and P4-ATPases use the energy from ATP hydrolysis to transport specific lipids across membranes against their concentration gradient, thereby establishing and maintaining lipid asymmetry in biological membranes (Coleman et al., 2013; Lopez-Marques et al., 2014). Most ABC transporters translocate lipids from the cytoplasmic to the exoplasmic leaflet of membranes and are often called floppases. Some mammalian ABC transporters, however, can transport phospholipids in the opposite direction (Quazi and Molday, 2013). P4-ATPases are a subfamily of P-type ATPases that transport specific phospholipids from the exoplasmic to the cytoplasmic leaflet of membranes to generate and maintain membrane lipid asymmetry (Tang et al., 1996; Coleman et al., 2009; Zhou and Graham, 2009).

A number of excellent reviews have focused on the molecular properties of lipid flippases and their role in cellular processes (Sebastian et al., 2012; Coleman et al., 2013; Lopez-Marques et al., 2013, 2014; Hankins et al., 2015; Williamson, 2015; Montigny et al., 2016). In this review we discuss recent studies on the lipid substrate specificity and transport mechanism of P4-ATPases and their role in cell physiology and disease with emphasis on mammalian and yeast P4-ATPases. We also present an overview of recent site-directed mutagenesis and molecular modeling studies that provide insight into the possible pathways used by P4-ATPases to translocate phospholipids across membranes. Finally, we discuss similarities in the structure and transport mechanisms of P4-ATPase lipid pumps and P2-ATPase ion pumps.

P-TYPE-ATPases

P-type ATPases constitute a family of membrane proteins that utilize the energy from ATP hydrolysis to transport ions and lipids across biological membranes (Palmgren and Nissen, 2011). These pumps are found in prokaryotes, archaea, and eukaryotes and are distinguished from other ATP-dependent transporters by the presence of a conserved aspartic acid residue that undergoes transient phosphorylation during the ATP cycle (Pedersen and Carafoli, 1987). On the basis of phylogenetic analysis, P-type ATPases have been organized into five main classes (P1-P5 ATPases) (Palmgren and Axelsen, 1998). The first three classes have been further divided into subclasses based on sequence similarity and ion transport specificity. P1A-ATPases are only found in some bacteria and transport K^+; P1B-ATPases transport heavy metal ions such as Ag^+, Zn^{2+}, Cd^{2+}, and Cu^{2+}; P2A ATPases (sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) and secretory pathway Ca^{2+}-ATPase (SPCA)) and P2B ATPases (plasma membrane Ca^{2+}-ATPase (PMCA)) transport Ca^{2+} and Mn^{2+}; P2C-ATPases transport monovalent ions and include Na^+/K^+-ATPases and H^+/K^+-ATPases; P2D-ATPases are found in fungi and transport Na^+; P3A-ATPases transport H^+; and P3B ATPases transport Mg^{2+}. P4-ATPases are unique in that they transport or flip phospholipids across membranes. The substrates for P5-ATPases have yet to be determined, but they are predicted to play an important role in the endosomal-lysosomal system and have been implicated in Parkinson's disease (Schultheis et al., 2004; Cohen et al., 2013; De La Hera et al., 2013).

P4-ATPases

P4-ATPases are only found in eukaryotes. The human genome encodes 14 P4-ATPases that are organized into five classes with each class having multiple members (**Table 1**, **Figure 1A**): Class 1a (ATP8A1, ATP8A2); Class 1b (ATP8B1, ATP8B2, ATP8B3, ATP8B4); Class 2 (ATP9A, ATP9B); Class 5 (ATP10A, ATP10B, ATP10D); and Class 6 (ATP11A, ATP11B, ATP11C) (Paulusma and Elferink, 2010; Van Der Mark et al., 2013). In contrast, yeast (*Saccharomyces cerevisiae*) contains five P4-ATPases (Drs2p, Neo1p, Dnf1p, Dnf2p, Dnf3P).

All P4-ATPases consist of a large polypeptide with a molecular mass of approximately 120 kDa which is composed of four main domains (**Figure 1B**). Based on amino acid sequence

TABLE 1 | Mammalian P_4-ATPases.

Class	P_4-ATPase Complex (α/β)	Substrate	Expression	Disease/Disorder	References
Class 1a	ATP8A1/CDC50A	PS >PE	Ubiquitous, high in skeletal muscle, thyroid, spinal cord	Defective hippocampus-dependent learning (mice)	Levano et al., 2012; Kato et al., 2013; Lee et al., 2015
	ATP8A2/CDC50A	PS > PE	High in brain, retina, testis, spinal cord	Cerebellar Ataxia, Mental Retardation and Disequilibrium Syndrome (CAMRQ) (humans) Neurological degeneration, spinal, axonal degeneration; retinal degeneration; hearing loss (mice)	Coleman et al., 2009, 2014; Onat et al., 2012; Zhu et al., 2012; Vestergaard et al., 2014
Class 1b	ATP8B1/CDC50A ATP8B1/CDC50B	PC (PS?)	Ubiquitous, high in small intestine, pancreas	Progressive Familial Intrahepatic Cholestasis (PFIC); Benign Recurrent Intrahepatic Cholestasis (BRIC) (human) Intrahepatic cholestasis, hearing loss, (mice)	Bull et al., 1998; Eppens et al., 2001; Paulusma et al., 2006; Stapelbroek et al., 2009; Folmer et al., 2009b; Takatsu et al., 2014
	ATP8B2/CDC50A ATP8B2/CDC50B	PC	Ubiquitous	Unknown	Takatsu et al., 2014
	ATP8B3/??	PS?	Testis	Abnormal sperm-egg interactions	Wang et al., 2004; Gong et al., 2009
	ATP8B4/CDC50A ATP8B4/CDC50B?	Unknown	Moderate levels throughout brain	Alzheimer disease?	Li et al., 2008; Van Der Velden et al., 2010b
	ATP8B5 (FetA)	Unknown	Testis	Defects in sperm capacitation	Xu et al., 2009
Class 2	ATP9A	Unknown	Ubiquitous, high in brain, pancreas	Unknown	Takatsu et al., 2011; Ansari et al., 2015
	ATP9B	Unknown	Ubiquitous, high in testis	Unknown	Takatsu et al., 2011
Class 5	ATP10A/CDC50A	PC	High in brain, pancreas, kidney, lung	Obesity, type 2 diabetes, insulin resistance	Dhar et al., 2004, 2006; Naito et al., 2015
	ATP10B/CDC50A	Unknown	Low expression, brain	Unknown	
	ATP10D/CDC50A	Unknown	High in placenta, low kidney, undetectable in other major organs	Obesity; hyperinsulinemia	Flamant et al., 2003; Takatsu et al., 2011
Class 6	ATP11A/CDC50A	PS > PE	Ubiquitous, moderate levels in liver, skeletal muscle, ovary	Marker for metastasis in colorectal cancer (human)	Miyoshi et al., 2010; Takatsu et al., 2014
	ATP11B/CDC50A	PS > PE	Ubiquitous, high levels in kidney, testis, ovary	Unknown	
	ATP11C/CDC50A	PS > PE	Ubiquitous, high liver, pancreas, heart	Impaired B lymphocyte differentiation, cholestasis, hepatocarcinoma; anemia, altered erythrocyte shape (mice)	Siggs et al., 2011b;Takatsu et al., 2014; Yabas et al., 2014

alignment (**Figure 2**), P4-ATPases possess three cytoplasmic domains involved in the ATPase catalytic cycle similar to Ca^{2+}-ATPase and Na^+,K^+-ATPase: the nucleotide or N-domain binds ATP; the phosphorylation or P-domain contains the aspartic acid (D) residue within the conserved DKTGT motif that undergoes transient phosphorylation; and the actuator or A-domain has the DGET (TGES in Ca^{2+}-ATPase and Na^+/K^+-ATPase) motif that facilitates the dephosphorylation of the phosphorylated intermediate (Lenoir et al., 2009; Coleman et al., 2012). The membrane or M-domain serves as the pathway for translocation of lipid substrates across cell membranes and is predicted to contain 10 transmembrane segments (M1-M10, **Figure 2**) analogous to the Ca^{2+}-ATPase and the Na^+/K^+-ATPase (Maclennan et al., 1985; Toyoshima et al., 2000; Morth

et al., 2007). Relatively short segments join the transmembrane segments on the exoplasmic side of the membrane, whereas large domains and segments connect some of the transmembrane segments on the cytoplasmic side (**Figures 1**, **2**). Recent studies suggest that transmembrane segments M1–M6 form the principal unit for transport of phospholipids across membranes with M7–M10 playing a supporting role (Baldridge and Graham, 2012; Vestergaard et al., 2014). P4-ATPases also contain regulatory domains that modulate the transport activity and targeting domains that participate in the trafficking of the P4-ATPases to their preferred subcellular membranes. These domains are present, at least in part, along the cytoplasmic C-terminal and N-terminal segments of P4-ATPases, but to date are poorly characterized for mammalian P4-ATPases (Takatsu et al., 2011; Zhou et al., 2013).

CDC50–ACCESSORY SUBUNIT

P4-ATPases with the exception of ATP9A and ATP9B assemble with an accessory or β-subunit to form a heteromeric complex, most likely a heterodimer. The β-subunit belongs to the CDC50/LEM3 family of evolutionary conserved proteins present in all eukaryotes (Katoh and Katoh, 2004; Saito et al., 2004). There are three CDC50 proteins (CDC50A, CDC50B, and CDC50C, also known as TMEM30A, TMEM30B, and TMEM30C) in humans and three in yeast (Cdc50p, Lem3p, Crf1p). Since there are more P4-ATPases (α-subunits) than CDC50 proteins (β-subunits), many P4-ATPases associate with the same CDC50 protein to form the heteromeric complex (Bryde et al., 2010).

The interaction of mammalian P4-ATPases with specific CDC50 proteins has been studied primarily by heterologous protein co-expression and co-immunoprecipitation (Paulusma et al., 2008; Bryde et al., 2010; Van Der Velden et al., 2010b; Coleman and Molday, 2011; Takatsu et al., 2011, 2014; Naito et al., 2015). These studies indicate that most mammalian P4-ATPases use CDC50A as their β-subunit. These include ATP8A1, ATP8A2, ATP11A, ATP11B, ATP11C, ATP10A, ATP10B, and ATP10D. Several P4-ATPases including ATP8B1, ATP8B2, and ATP8B4 can associate with either CDC50A or CDC50B. The physiological consequence of this dual specificity, however, remains to be determined. CDC50C is predominantly expressed in testes (Osada et al., 2007), but to date it has not been shown to associate with any P4-ATPase. Although, heterologous overexpression provides useful information on the interaction of P4-ATPases with specific CDC50 proteins, the presence of such complexes needs to be confirmed in physiologically relevant cells and tissues. In one study ATP8A2 was found to interact with CDC50A in photoreceptors in support of heterologous cell expression and immunoprecipitation studies (Coleman et al., 2009; Coleman and Molday, 2011).

CDC50 proteins are relatively small membrane glycoproteins containing approximately 350 amino acids. They consist of two transmembrane segments joined by a large exoplasmic domain with two or more N-linked oligosaccharide chains that contribute to the stability of the protein (Coleman and Molday, 2011; Garcia-Sanchez et al., 2014; **Figure 1B**). CDC50 proteins typically migrate on SDS gels as a broad band with an approximate molecular mass of 50 kDa due to the highly heterogeneous nature of the oligosaccharide chains. The exoplasmic domain also contains four conserved cysteine residues that stabilize the structure through the formation of two disulfide bonds (Puts et al., 2012). Relatively, short N-terminal and C-terminal segments are exposed on the cytoplasmic side of the membrane.

Heterologous expression studies point to the crucial role of CDC50 in protein folding and formation of a functionally active P4-ATPase complex (Paulusma et al., 2008; Bryde et al., 2010; Van Der Velden et al., 2010b; Coleman and Molday, 2011). In the absence of CDC50 expression, P4-ATPases fail to exit the ER and are incapable of undergoing phosphorylation or phospholipid-dependent ATP hydrolysis (Lopez-Marques et al., 2010; Coleman and Molday, 2011; Puts et al., 2012). Likewise, in the absence of P4-ATPase expression, CDC50A is retained in the ER of cells. However, co-expression of P4-ATPases and CDC50 results in a P4-ATPase-CDC50 complex that can exit the ER and function as an active phospholipid flippase (Coleman and Molday, 2011). CDC50 proteins do not play a direct role in the targeting of P4-ATPases to their preferred subcellular location once they exit the ER in multicellular organisms (Lopez-Marques et al., 2010).

Several studies suggest that CDC50 proteins participate in ATP-dependent phospholipid transport. The affinity of yeast Drs2p for Cdc50p fluctuates during the catalytic reaction cycle with the highest affinity observed for Drs2p in the E2P state with a bound phospholipid substrate (Lenoir et al., 2009). Phosphorylation of Drs2p, ATP8B1, and ATP8B2 are dependent on interaction with their CDC50 subunit (Lenoir et al., 2009; Bryde et al., 2010). Studies using mammalian CDC50A/CDC50B chimeric proteins further indicate that both the transmembrane and exoplasmic domains of CDC50A are necessary for the binding CDC50A to ATP8A2 and the formation of an active complex (Coleman and Molday, 2011). The N-terminal cytoplasmic domain of CDC50A also modulates the kinetics of ATP8A2 ATPase activity.

Certain mutations in the transmembrane segments of ATP8A2 decrease glycosylation of CDC50 without significantly affecting the functional properties of the complex and seem to define a groove between M1, M3, and M4 of ATP8A2 as a possible location of CDC50 interactions (Vestergaard et al., 2015; see later text in relation to **Figure 6**). Altogether, the available information indicates that CDC50 accessory proteins contact P4-ATPases at multiple sites to promote correct protein folding, exit of the P4-ATPase complex from the ER, and formation of a functionally active flippase complex. Hence, CDC50 proteins appear to play a role reminiscent of the β-subunits of Na^+/K^+-ATPases (Puts and Holthuis, 2009). Because E2P is the state, in which the P4-ATPase is supposed to bind its phospholipid substrate from the exoplasmic membrane leaflet (see reaction cycle in **Figure 3B**), the preferential interaction of the CDC50 protein with E2P (Lenoir et al., 2009) is consistent with participation of CDC50 in substrate loading analogous to the role of the β-subunit of Na^+/K^+-ATPase in K^+ binding (Shinoda et al., 2009). Nevertheless, CDC50 and the β-subunit of Na^+/K^+-ATPase differ with respect to the number of transmembrane segments (two for CDC50 and only one for the Na^+/K^+-ATPase

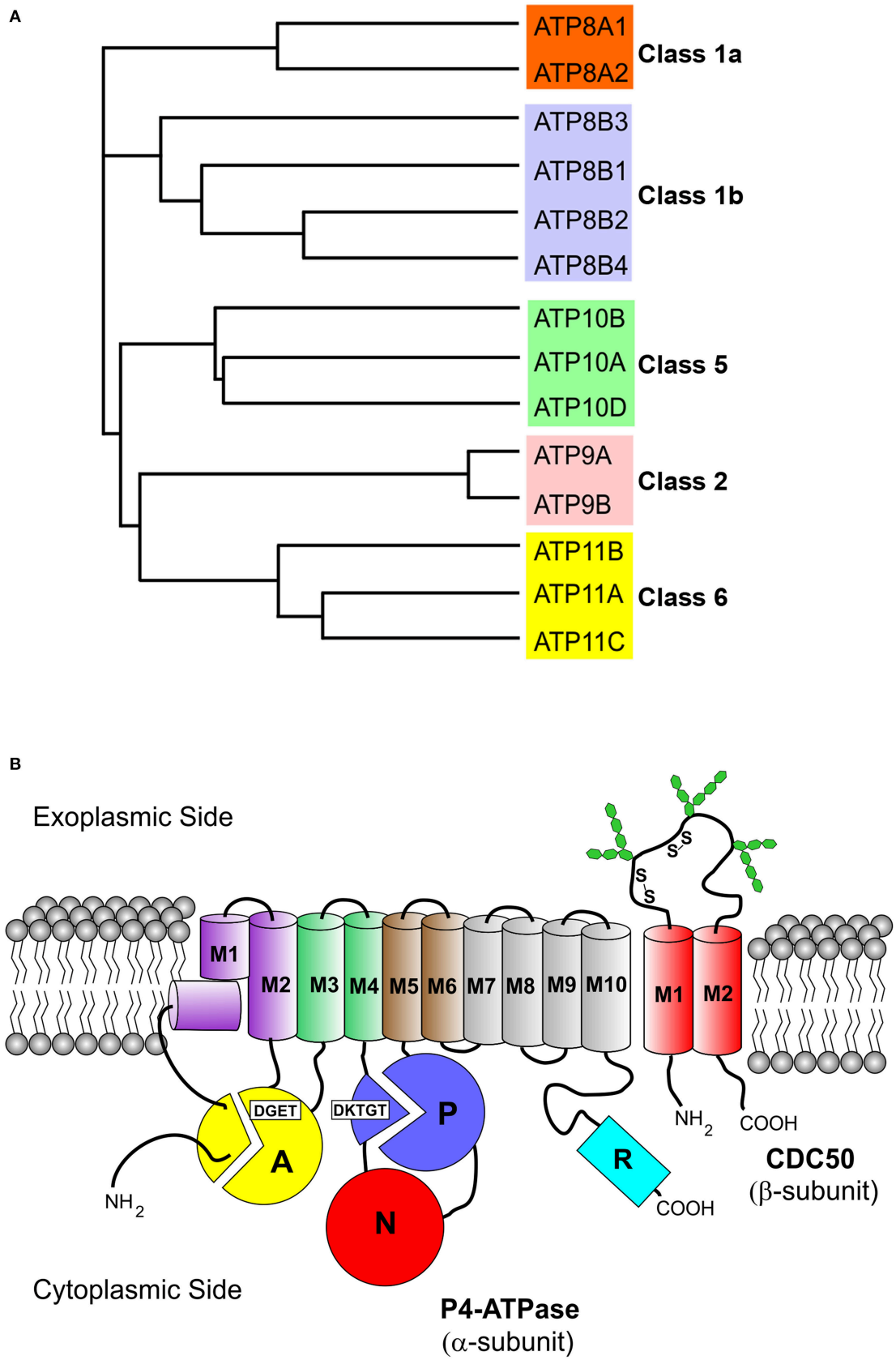

FIGURE 1 | Continued

FIGURE 1 | Continued
Phylogenetic analysis and membrane topology of P4-ATPases. (A) The sequences of the 14 human P4-ATPases were aligned using the Clustal Omega multi-sequence alignment program for generation of the phylogenetic tree. Protein sequences are from the following accession numbers: ATP8A1 (Q9Y2Q0); ATP8A2 (Q9NTI2); ATP8B1 (O43520); ATP8B2 (P98198); ATP8B3 (O60423); ATP8B4 (Q8TF62); ATP9A (O75110); ATP9B (O43861); ATP10A (O60312); ATP10B (O94823); ATP10D (Q9P241); ATP11A (P98196); ATP11B (Q9Y2G3); ATP11C (Q8NB49); **(B)** The cytoplasmic A ("actuator"), N (nucleotide binding), and P (phosphorylation) domains of the α-subunit are shown as colored circles. Conserved motifs involved in phosphorylation (DKTGT) and dephosphorylation (DGET) are indicated for the respective domains. The 10 transmembrane helices of the α-subunit are represented by cylinders (M1 kinked), M1–M2 purple, M3–M4 green, M5–M6 brown, and M7–M10 gray. The two transmembrane helices of the β-subunit (CDC50 protein) are shown as red cylinders. Glycosylation and disulfide bridges of the exoplasmic domain of the β-subunit are also indicated. The C-terminal cytoplasmic extension of the α-subunit furthermore contains a regulatory domain shown as cyan cylinder (R domain).

β-subunit), the phylogenetic origin, and apparently also the α-subunit contact sites (M7 and M10 for the Na^+/K^+-ATPase; a groove between M1, M3, and M4 for ATP8A2).

Class 2 P4-ATPases (ATP9A and ATP9B in mammals and Neo1p in yeast) do not appear to use CDC50 proteins as β-subunits. Both ATP9A and ATP9B exit the ER and localize to their respective cell membranes in the absence of CDC50 proteins (Takatsu et al., 2011). Neo1p is the only yeast P4-ATPase that is lethal when its gene is deleted pointing to its essential role in yeast survival (Prezant et al., 1996; Hua et al., 2002). Neo1p forms a complex with Ysl2p, a guanine nucleotide exchange factor for Arl1p that functions in vesicle trafficking within the Golgi/endosomal system (Wicky et al., 2004; Barbosa et al., 2010). The function of ATP9A and ATP9B is not known and it remains to be determined if these P4-ATPases require an accessory subunit or associated protein for activity and whether they transport phospholipids across membranes.

DISTRIBUTION OF P4-ATPases IN CELLS

The subcellular localization of mammalian P4-ATPases has been mostly studied by immunofluorescence microscopy and transport of fluorescently labeled phospholipids into cells overexpressing the protein complex. Although, there is some variation possibly due to the cell type used or extent of overexpression, in general several P4-ATPases including ATP11C, ATP11A, ATP10A, ATP10D, ATP8B1, ATP8B2, and ATP8B4 in association with their β-subunit preferentially localize to the plasma membrane of cells where they transport specific phospholipids from the extracellular to the cytoplasmic leaflet (Van Der Velden et al., 2010b; Takatsu et al., 2011, 2014; Naito et al., 2015). Of these P4-ATPases, ATP11C has been widely studied and shown to play a critical role in the internalization of PS thereby preventing its exposure on cell surfaces (Yabas et al., 2011, 2014; Segawa et al., 2014). The P4-ATPases ATP8A1, ATP8A2, ATP10B, and ATP11B are largely confined to Golgi/recycling endosomal system when overexpressed in culture cells (Coleman and Molday, 2011; Takatsu et al., 2011; Lee et al., 2015). However, in some studies, several of these P4-ATPases have been reported to be also present on the plasma membrane of cells (Segawa et al., 2016). ATP8A2 has been shown to transport fluorescently labeled PS into cells and prevent exposure of PS on cell surfaces. ATP8A1 localizes to the plasma membrane of erythrocytes (Soupene and Kuypers, 2006). Taken together, these studies indicate that P4-ATPases can cycle between the Golgi/endosomal recycling system and the plasma membrane, but preferentially localize to either the plasma membrane or Golgi-recycling endosome network depending on the specific P4-ATPase.

The subcellular localization of the Class 2 P4-ATPases, ATP9A and ATP9B, has also been studied by overexpression in Hela cells (Takatsu et al., 2011). ATP9A localizes to early/recycling endosomes and the trans-Golgi network (TGN), whereas ATP9B localizes exclusively to the TGN. A chimeric ATP9 protein in which the N-terminal cytoplasmic region of ATP9A was replaced with the corresponding region of ATP9B localized exclusively to the TGN indicating that a segment within the N-terminal cytoplasmic region of ATP9 proteins is responsible for subcellular localization. ATP9A is highly expressed in neural and pancreatic tissue whereas ATP9B is more ubiquitously expressed. Recently, ATP9A has been reported to be present in human and rat pancreatic islets and based on membrane fractionation appears concentrated in the plasma membrane (Ansari et al., 2015). ATP9A, like other P4-ATPases may cycle between the TGN and plasma membrane of cells.

It should be noted, however, that protein overexpression can result in abnormal localization in cells. Accordingly, it will be important to examine the distribution of endogenous P4-ATPases in physiologically relevant tissues and cells. A set of highly specific antibodies to the various P4-ATPases would be particularly valuable to probe their distribution in various cells and tissues.

PHOSPHOLIPID SUBSTRATE SPECIFICITY

The substrate specificities for only a few mammalian P4-ATPases have been determined at a biochemical level with most being specific for the aminophospholipids, PS and PE (**Table 1**). Historically, aminophospholipid translocase (APLT) activity was first found in red blood cells and bovine chromaffin granules (Moriyama and Nelson, 1988; Morrot et al., 1990; Zimmerman and Daleke, 1993; Auland et al., 1994; Tang et al., 1996). The membrane protein responsible for this activity was identified as ATPaseII (now known as ATP8A1) (Mouro et al., 1999; Paterson et al., 2006). Expression in insect cells and yeast confirmed the activation of the ATPase activity of ATP8A1 by PS and more moderate activation by PE (Ding et al., 2000; Paterson et al., 2006; Soupene et al., 2008). More recently, ATP8A1 expressed and purified from HEK293 cells and reconstituted into liposomes has been confirmed to flip PS and to a lesser

FIGURE 2 | Amino acid sequence alignment of P4-ATPases and P-type ion pumps. Sequences of two P4-ATPases, bovine ATP8A2 (UniProt: C7EXK4) and human ATP8B1 (UniProt: O43520), are shown aligned with sequences of the P2- and P3-ATPases shark Na^+/K^+-ATPase (UniProt: Q4H132, PDB: 2ZXE), plant AHA2 H^+-ATPase (UniProt: P19456, PDB: 3B8C), and sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) isoform 1a (UniProt: P04191, PDB: 3B9B). Letter colors *(Continued)*

FIGURE 2 | Continued
denote positively (blue) or negatively (red) charged, polar (green), or hydrophobic (black) residues. Membrane parts of transmembrane helices of SERCA identified in the crystal structure are indicated by gray shading with helix numbering and indication of exoplasmic loops under the sequence. Arrowheads with residue number above the bATP8A2 sequence indicate some of the residues that are mentioned in the text or shown in the figures. Arrowheads below the SERCA sequence indicate residues of SERCA or the Na^+/K^+-ATPase involved in binding the ions transported by these ATPases. This alignment was based on a previously described multiple sequence alignment (Vestergaard et al., 2014).

extent PE across membranes (Lee et al., 2015). While ATP8A1 is widely expressed in different mammalian tissues, the second member of the ATP8A subgroup, ATP8A2, is most highly expressed in retina, brain, spinal cord, and testes (Coleman et al., 2009; Cacciagli et al., 2010; Zhu et al., 2012). ATP8A2 has been purified from retinal photoreceptor cells and transfected HEK293 cells for analysis of its ATPase and phospholipid flippase activity (Coleman et al., 2009, 2012; Coleman and Molday, 2011). Like ATP8A1, ATP8A2 preferentially transports PS across membranes, but can also uses PE as a substrate. Functional complementation experiments further show that ATP8A2 can rescue defects in ATP8A1-depleted cells indicating that ATP8A1 and ATP8A2 share overlapping functional activities (Lee et al., 2015).

While the PS substrate specificity of the ATP8A subclass of P4-ATPases has been confirmed by multiple investigations, the results with respect to the ATP8B proteins have been less definitive. ATP8B1 has been implicated in hepatic disorders such as progressive familial intrahepatic cholestasis (PFIC) and benign recurrent intrahepatic cholestasis (BRIC) (Bull et al., 1998; Eppens et al., 2001). As a result, it has been one of the most studied of the mammalian P4-ATPases. Initial evidence based on increased biliary excretion of PS in ATP8B1 deficient mice, plasma membrane uptake of fluorescent PS, and higher exposure of PS on the outer plasma membrane leaflet in the absence of an active flippase complex implicated ATP8B1 in the flipping of PS across the plasma membrane (Paulusma et al., 2006, 2008). But the results presented in a more recent study point toward PC as being the primary transport substrate of ATP8B1 (Takatsu et al., 2014). The same study also found that ATP8B1 mediated uptake of a NBD-labeled PC analog could be negated by concomitant expression of ABCB4, an ABC transporter that mediates ATP-dependent transport of phospholipids to the extracellular leaflet of plasma membranes. The substrate specificity of the other ATP8B members has not been studied at a biochemical level. Cell based studies, however, suggest that ATP8B2 like ATP8B1 transports PC (Takatsu et al., 2014). ATP8B3 has been implicated in the transport of PS, but the substrate specificity for this flippase requires further study (Wang et al., 2004; Gong et al., 2009). ATP8B5 is present in mice but not humans, and has been implicated in spermatogenesis, but the phospholipid substrate remains to be determined (Xu et al., 2009).

The structural basis for the reported difference in substrate specificity between Class 1a and Class1b P4-ATPases has not been elucidated. Although, the amino acid sequences of the transmembrane segments of ATP8A2 and ATP8B1 only exhibit 33% sequence identity, most of the positively charged residues in ATP8A2 that may interact preferentially with the net negatively charged head group of PS are conserved in ATP8B1 (**Figure 2**, Naito et al., 2015). Subtle structural differences of the α subunits, matching the translocation pathway with the size and hydrogen bonding pattern, rather than the net charge of the head group, could be decisive for the substrate specificity, which might also depend on differences in the interaction of the α-subunit with the CDC50 protein. It is of note that most of the amino acid residues with a suggested role in determining the respective PS and PC specificities of the yeast flippases Drs2p and Dnf1p (Baldridge and Graham, 2012, 2013) are not conserved in the mammalian P4-ATPases with corresponding substrate specificity (Naito et al., 2015).

The substrate specificity of ATP11A and ATP11C has also been investigated (Takatsu et al., 2014). These P4-ATPases, like ATP8A1 and ATP8A2, flip PS as their primary substrate confirming earlier results showing that ATP11C is involved in PS translocation into B-lymphocytes and other immune cells (Yabas et al., 2011, 2016; Segawa et al., 2014). Substrate specificity of ATP10A has recently been reported (Naito et al., 2015). Exogenously expressed ATP10A localizes to the plasma membrane in HeLa cells and produces a significant increase in the uptake of fluorescent NBD-PC, but not other phospholipids. This internalization of PC was not observed in cells expressing an ATPase deficient form of ATP10A.

It needs to be pointed out that aside from ATP8A1 and ATP8A2 reports about the transport substrates of most other mammalian P4-ATPases are based on the ability of cells expressing these proteins to ingest fluorescent phospholipid analogs. This experimental setup is open to the introduction of some confusion regarding the exact nature of the substrate specificity of flippases as seen with the yeast plasma membrane flippases. Greater clarity in this regard is expected to evolve in the future from biochemical characterization of purified proteins.

P4-ATPases expressed in the yeast, *Saccharomyces cerevisiae*, historically have been the most well characterized members of the P4-ATPase family. Drs2p, one of the first phospholipid flippases to be identified, localizes to the TGN and endosomes, where it transports PS and PE from the lumenal to the cytoplasmic leaflet of membranes. Characterization of Drs2p flippase activity was initially based on the results of experiments which looked at the transport of fluorescent (NBD)-labeled PS and PE analogs across TGN membranes purified from yeast strains expressing either the wild type or a temperature sensitive Drs2p mutant (Natarajan et al., 2004). These results were confirmed in direct flippase assays using purified and reconstituted Drs2p (Zhou and Graham, 2009). The TGN membrane shows expression of another P4-ATPase, Dnf3p, which is also involved in transmembrane transport of PS and PE as determined from the loss of asymmetric distribution of fluorescent analogs in the secretory vesicles of dnf3Δ cells (Alder-Baerens et al., 2006). Loss

of both Drs2p and Dnf3p dissipates the asymmetric distribution of aminophospholipids in the membranes of TGN and secretory vesicles. Recent mutational analysis has revealed the ability of Drs2p to distinguish between the mono- and di-acylated phospholipid substrate molecules (Baldridge et al., 2013). On the other hand, the two yeast flippases that localize to the plasma membrane, Dnf1p and Dnf2p, have been implicated in the flipping of PE and PC (Pomorski et al., 2003; Stevens et al., 2008). Although, the initial study had suggested that these two proteins might have a role in maintaining the asymmetric distribution of PS in the plasma membrane, it was subsequently shown that they are not essential for this function. In fact, the study by Stevens et al. (2008) found that net internalization of PS increases in dnf1Δdnf2Δ yeast strain, probably due to the enhanced activity of other flippases.

REGULATION OF P4-ATPase ACTIVITY

The mechanism of P4-ATPase regulation has been examined most thoroughly in yeast. The activity of Drs2p is regulated by multiple mechanisms depending on the particular endomembrane system. The C-terminal domain of Drs2p contains a segment of basic amino acids (*RMKKQR*) that has sequence homology to a split PH domain (Natarajan et al., 2009). Drs2p interacts with the phosphatidylinositol-4-phosphate (PI4P) through this domain and this interaction is required for PS flippase activity (Natarajan et al., 2009). The PI4P binding domain in the C-terminal domain of Drs2p overlaps with the binding site of another regulatory element of flippase activity, the Arf-GEF protein Gea2p (Chantalat et al., 2004; Natarajan et al., 2009). As in the case of PI4P, the activity of Drs2p is abolished in the absence of interactions with Gea2p. The requirement for binding of these two regulatory molecules for activation of Drs2p is removed upon cleavage of the C-terminal domain of the protein (Zhou et al., 2013). It has been proposed that the C-terminal domain of Drs2p acts as an auto-inhibitory domain by interfering with the catalytic domains of the protein. Binding of either PI4P or Gea2p disengages the auto-inhibitory domain from the catalytic domain resulting in Drs2p flippase activity. Along with binding to Gea2p at the C-terminal domain, Drs2p also binds to Arl1p at the N-terminal domain. Formation of this trimeric complex is required for flippase activity and for recruitment of cargo during vesicle formation at the TGN (Tsai et al., 2013). While Drs2p interacts with Gea2p at the TGN, it interacts with the F-box protein Rcy1p at an overlapping domain in early endosomes. This interaction is required for the recycling of cargo between endosomes and the TGN (Hanamatsu et al., 2014). The early endosome to TGN retrieval pathway also includes the interaction between Drs2p and Arf-GAP protein Gcs1p (Sakane et al., 2006). The two different interactions of Drs2p with Rcy1 and Gcs1 at the early endosomes may be required for the recruitment of different types of adaptor molecules during the formation of recycling vesicles destined to different membranes.

Apart from the binding of regulatory molecules, the activity of P4-ATPases is also regulated by phosphorylation and sphingolipid concentration. The activity of yeast plasma membrane localizing P4-ATPases, Dnf1p and Dnf2p, requires phosphorylation by kinase proteins, Fpk1 and Fpk2 (Nakano et al., 2008). Deletion mutants for both Fpk1 and Fpk2 (*fpk1Δfpk2Δ*) eliminated the plasma membrane uptake of NBD-labeled phospholipids though the flippases localized normally to the membrane. The activity of Fpk1 and Fpk2 is further regulated by the kinase, Ypk1p. Hence, Ypk1-catalyzed phosphorylation of Fpk1/2 eliminates the activity of these proteins, and the activity of Ypk1 in turn is inhibited due to the phosphorylation by Fpk1/2 proteins. The activity of Fpk1/2 proteins is stimulated by presence of sphingolipids in the membrane. Thus, membrane sphingolipids positively modulate the flippase activity of Dnf1p and Dnf2p (Roelants et al., 2010). Apart from Ypk1, the flippase activity of Dnf1p and Dnf2p is also negatively controlled by Gin4p which phosphorylates Fpk1 to suppress its activity (Roelants et al., 2015).

Little is known about the regulation of mammalian P4-ATPase flippase activity. From limited studies, we know that the flippase activities of ATP11A and ATP11C are down regulated by exposure to calcium with an IC50 in the 100–200 μM range (Segawa et al., 2016). The activity of these two P4-ATPases has also been shown to be suppressed by caspase mediated cleavage. It is hypothesized that the aminophosopholipid translocation activity at the plasma membrane is inactivated by this caspase route during apoptotic exposure of PS (Segawa et al., 2014, 2016). The effect of other regulatory mechanisms including kinase-catalyzed phosphorylation, calcium sensor proteins, and lipids remains to be investigated.

ROLE OF P4-ATPases IN CELL PHYSIOLOGY

P4-ATPases play a critical role in many cellular processes associated with the plasma membrane and intracellular membranes. A key function of P4-ATPases is to establish and maintain phospholipid asymmetry across membranes. This is most evident for P4-ATPases that utilize PS and PE as substrates. These P4-ATPases including ATP11 and ATP8 subclasses ensure that the extracellular leaflet of the plasma membrane is devoid of PS since exposure of this phospholipid on cell surfaces is an "eat me" signal for ingestion by phagocytic cells. PS and PE specific P4-ATPases also ensure that the cytoplasmic leaflet of membranes is highly enriched in these aminophospholipids. This enables cytoskeletal components, adaptor proteins and enzymes to bind to these membranes where they can function in any of a variety of processes such as cell signaling pathways, enzyme regulation, metabolism, vesicle trafficking and cell motility (Newton and Keranen, 1994; Huang et al., 2011; Lee et al., 2015). The role of PC specific P4-ATPases is less clear, but the active transport of PC to the cytoplasmic leaflet may be important for maintaining the integrity of specialized membranes and/or recruitment of specific PC binding proteins to the cytoplasmic surface under defined conditions.

Although, the main function of P4-ATPases is to actively transport phospholipids across membranes, it is possible that

some cellular functions of P4-ATPases are ATP-independent (Van Der Velden et al., 2010a). Other P-type ATPases including Na^+/K^+ ATPases are known to bind intracellular and extracellular proteins and hence serve as a scaffold for protein-protein interactions distinct from their ATP-dependent ion transport function (Xie and Cai, 2003; Liang et al., 2007; Molday et al., 2007). ATP-independent functions of P4-ATPases have not been explored in detail, but it is plausible that some P4-ATPases may serve as scaffolds for cell signaling or vesicle trafficking through protein-protein interactions. An overview of some key cellular functions of some mammalian P4-ATPases is given below.

PS-ASYMMETRY IN BLOOD CELLS

The erythrocyte plasma membrane has been most extensively studied and shown to exhibit a high degree of aminophospholipid asymmetry (Daleke and Huestis, 1989). Early studies showed that ATP8A1 is present in mature erythrocytes and as a result this P4-ATPase was thought to be crucial in restricting PS to the inner leaflet of erythrocytes (Soupene and Kuypers, 2006). However, PS was not exposed on the surface of erythrocytes of ATP8A1-deficient mice and the shape of erythrocytes appeared normal suggesting that increased expression of other P4-ATPases including ATP8A2 may compensate for the absence of ATP8A1 (Levano et al., 2012). Exposure of PS on the external surface of hippocampal neurons of ATP8A1 knockout mice, however, was observed, which correlates with impaired hippocampus-dependent learning. These studies suggest that some cells express other P4-ATPases that can compensate for the loss of ATP8A1 whereas other cells such as hippocampal neurons require ATP8A1 to maintain PS asymmetry.

Studies of ATP11C-deficient mice have provided strong evidence for a crucial role of ATP11C in establishing PS asymmetry in developing erythrocytes (Yabas et al., 2014). ATP11C-deficient mice were found to display reduced uptake of fluorescently labeled PS by erythroblasts but not mature erythrocytes. However, circulating erythrocytes accumulated PS on their surface, displayed abnormal shape characteristic of stomatocytosis, and had a shortened lifespan. These studies indicate that ATP11C is critical in generating and maintaining PS asymmetry during erythropoiesis, a process that is important in generating normal mature erythrocytes. The high content of PS and PE on the cytoplasmic leaflet of erythrocytes may be important for the interaction of the spectrin-ankyrin cytoskeletal network with the plasma membrane required for maintaining the normal shape of erythrocytes whereas the absence of PS on the exoplasmic leaflet of erythrocytes is important in maintaining the normal lifespan of these cells. ATP11C mediated internalization of PS has also been shown to be crucial for differentiation of B lymphocytes (Siggs et al., 2011a; Yabas et al., 2011).

PS asymmetry is also important in blood coagulation (Lentz, 2003). In this case retention of PS on the cytoplasmic leaflet of platelets and other blood cells prevents blood coagulation from occurring (Lentz, 2003; Lhermusier et al., 2011). This asymmetry is established by one or more active P4-ATPases and an inactive scramblase. In a proteomic screen, several P4-ATPases have been identified in human platelets including ATP8A1, ATP8B2, ATP11A-C, and ATP9B (Lewandrowski et al., 2009). Whether all or only selective P4-ATPases contribute to PS asymmetry remains to be determined. Blood coagulation is initiated when the bidirectional scramblase TMEM16F is activated by an increase in the intracellular Ca^{2+} concentration leading to PS exposure on platelet cell surfaces (Suzuki et al., 2010; Lhermusier et al., 2011; Malvezzi et al., 2013; Brunner et al., 2014). As part of this process, P4-ATPases may also be inhibited directly or indirectly by an increase in intracellular Ca^{2+}, although this remains to be determined. Coagulation factors including Factor V can bind to the surface exposed PS through their discoidin domains to initiate the blood coagulation cascade. Loss-of-function mutations in TMEM16F have been shown to cause Scott syndrome, a severe bleeding disorder supporting the role of this scramblase in PS exposure and the blood coagulation process (Lhermusier et al., 2011).

PS-ASYMMETRY IN APOPTOSIS

Apoptosis is another well-established process involving the interplay of P4-ATPases and scramblases (Segawa et al., 2014; Hankins et al., 2015). The exposure of PS on the surface of cells is an "eat me" signal for phagocytic cells to engulf the apoptotic cell. Xkr8, a member of the XK family of membrane proteins, has been implicated as the scramblase that exposes PS on apoptotic cells for engulfment by macrophages (Suzuki et al., 2013). ATP11C plays a key role in restricting PS to the inner leaflet of the plasma membrane of normal cells. Cleavage of ATP11C at multiple caspase sites can inactivate ATP11C thereby facilitating apoptosis. Mutations in ATP11C that prevent caspase cleavage, but retain PS transport activity, do not expose PS on the surface of cells and these cells are not ingested by macrophage (Segawa et al., 2014). However, ATP11C is not the only P4-ATPase which is important in preventing PS exposure on cell surfaces. In some cells ATP11A with a similar substrate specificity and plasma membrane localization as ATP11C can compensate for the loss of ATP11C and prevent exposure of PS on cell surfaces (Segawa et al., 2016). Like ATP11C, ATP11A has a number of caspase cleavage sites. These studies suggest that the activation of the scramblase together with inhibition of P4-ATPases may be required for exposure of PS on cells as a signal for apoptosis.

P4-ATPases IMPLICATED IN INTRACELLULAR VESICLE TRAFFICKING

In addition to playing a role in establishing phospholipid asymmetry in the plasma membrane, P4-ATPases also play a crucial role in generating phospholipid asymmetry in intracellular membranes, a process linked to vesicle trafficking in cells. Most membrane lipids are synthesized on the cytoplasmic side of the ER and redistributed symmetrically across the membrane by scramblases. Vesicles budding from

the ER fuse with the Golgi complex. Lipid asymmetry is induced in the TGN through the action of active flippases and floppases. Most information on the role of P4-ATPases in vesicle trafficking comes from studies of the yeast P4-ATPase Drs2p by Graham and colleagues (Sebastian et al., 2012). Drs2p is localized to the TGN where it flips PS and PE to the cytoplasmic leaflet to generate aminophospholipid asymmetry (Hua et al., 2002; Natarajan et al., 2004). The flippase activity of Drs2p is thought to initiate membrane curvature by increasing PS and PE content on the cytoplasmic leaflet at the expense of the lumenal leaflet leading to membrane curvature as initially proposed in the coupled bilayer hypothesis of Sheetz and Singer (1974) and Xu et al. (2013). Membrane curvature together with the increase in the negatively-charged PS recruits Arf-GTPase-activating protein Gcs1 and clathrin coat proteins to the cytoplasmic leaflet as part of the vesicular transport process between the TGN and early endosomes (Sebastian et al., 2012).

P4-ATPases have also been implicated in vesicle budding and trafficking in other eukaryotic cells although the mechanism is poorly understood. ATP8A2 is crucial for axon elongation in PC12 cells and hippocampal neurons suggesting a role of this P4-ATPase in vesicle trafficking required for neurite extension (Xu et al., 2012). Similarly, ATP8A2 deficient mice show a striking loss in the length of photoreceptor outer segments suggesting that a decrease in vesicle trafficking between the inner and outer segment of these cells occurs in the absence of ATP8A2 (Coleman et al., 2014). More recently, Lee et al. have shown that ATP8A1-catalyzed flipping of PS in recycling endosomes is important in the recruitment of EHD1 to the cytoplasmic leaflet of recycling endosomes and membrane fission (Lee et al., 2015). Finally, ATP8A1 has been associated with cell migration (Kato et al., 2013).

ROLE OF P4-ATPases IN HUMAN DISEASE

To date, two P4-ATPases, ATP8B1 and ATP8A2, have been directly associated with human genetic disorders. Mutations in the *ATP8B1* are responsible for two related liver diseases, known as benign recurrent intrahepatic cholestasis type 1 (BRIC1) and the more severe form progressive familial intrahepatic cholestasis type 1 (PFIC1) (Bull et al., 1998). These early onset diseases are characterized by impaired bile flow or cholestasis. Affected individuals can also experience hearing loss and are more susceptible to pneumonia (Stapelbroek et al., 2009; Ray et al., 2010). ATP8B1 has been localized to the canalicular membrane where it is thought to function as a phospholipid flippase to maintain the integrity of the membrane (Folmer et al., 2009b). This is supported in studies defining the effect of ATP8B1 knockdown on cultured rat hepatocytes (Cai et al., 2009). Loss in ATP8B1 resulted in normal ABCB4 expression and localization to the canalicular membrane, but impaired bile secretion and aberrant microvilli morphology. In Caco-2 cells, loss in ATP8B1 expression also causes significant reduction in microvilli supporting a role of ATP8B1 in maintaining the apical membrane structure. The effect of missense mutations associated with PFIC1 and BRIC1 on the stability of ATP8B1 and its interaction with CDC50A was studied in transfected CHO cells (Folmer et al., 2009b). The mutants were found to be less stable than the wild-type proteins and show a loss or decreased interaction with CDC50A. Furthermore, the mutants were not detected on the canalicular membrane of WIF-B cells. The impaired targeting to the plasma membrane of ATP8B1 caused by the most frequent ATP8B1 disease mutation in European patients, I661T, was recently shown to be rescued by compounds known to be efficient correctors of CFTR misfolding in cystic fibrosis, thus pointing to a possible therapeutic strategy (Van Der Woerd et al., 2016). The mechanism by which loss in ATP8B1 leads to cholestasis is poorly understood, but it has been proposed that increased PS on the luminal leaflet on canalicular membranes due to the loss in ATP8B1 flippase activity results in abnormal lipid packing (Folmer et al., 2009a). This in turn makes the outer leaflet of the canalicular membrane susceptible to bile salt mediated extraction of cholesterol and phospholipids resulting in reduced activity of ABCB11, a major bile salt exporter that has also been linked to PFIC2 (Strautnieks et al., 1998). The recent studies suggesting that ATP8B1 is a PC and not a PS flippase, however, leaves this model open to further investigations. It is possible that deficiency in PC transport to the inner membrane of canalicular membranes due to mutations in ATP8B1 could compromise the stability of the membrane to bile salts. More studies are needed to further define the role of ATP8B1 in cholestasis.

Genetic defects in ATP8A2 have been reported in two families. One individual with mental retardation and hypotonia had a balanced translocation of chromosomes 10 and 13 which disrupted the coding sequence of the *ATP8A2* gene (Cacciagli et al., 2010). More recently, a missense mutation (I376M) was identified as the cause of a rare neurodegenerative disease known as cerebellar ataxia, mental retardation, and dysequilibrium syndrome (CAMRQ) in a consanguineous family from Turkey (Onat et al., 2012). The I376M mutation is present in a conserved region of the M4 transmembrane segment of ATP8A2. Biochemical characterization confirmed that this missense mutation results in the loss in ATP8A2 PS flippase activity, as further discussed below (Vestergaard et al., 2014). Two ATP8A2-deficient mouse models, wabbler-lethal (*wl*) mouse and ATP8A2 knockout mouse, show similar neurological abnormalities including body tremors and ataxia resulting from distal axonal degeneration of the spinal cord (Zhu et al., 2012; Coleman et al., 2014). The *wl* mouse has a deletion in exon 22 of *Atp8a2* resulting in the expression of an inactive protein (Zhu et al., 2012). In addition to these characteristics, ATP8A2 deficient mice exhibit a loss in visual and auditory function and degeneration of photoreceptor and spiral ganglion cells (Coleman et al., 2014). These studies indicate that ATP8A2 plays an essential role in the function and survival of many neuronal cells, possibly by contributing to vesicle trafficking in axons and related neurological structures.

More limited information is available on the role of other P4-ATPases in human diseases (Van Der Mark et al., 2013). ATP10A has been implicated in the control of insulin-mediated glucose uptake in mouse adipose tissue and skeletal muscle

and is considered as a risk factor for Type 2 diabetes in African-Americans (Dhar et al., 2004, 2006). ATP11A has been suggested to be a predictive marker for prognosis of colorectal cancer (Miyoshi et al., 2010). ATP11C-deficient mice have been shown to exhibit multiple abnormalities including loss in B cell development, hyperbilirubinemia, hepatocellular carcinoma, and anemia (Siggs et al., 2011a,b; Yabas et al., 2011). It remains to be determined if loss of function mutations in human ATP11C cause similar disorders. ATP8B4 has been implicated in Alzheimer's disease based on its chromosomal localization and analysis of single-nucleotide polymorphisms (Li et al., 2008).

STRUCTURAL AND MECHANISTIC FEATURES OF P4-ATPases

Reaction Cycle of P4-ATPases

The predicted domain structure and topology together with conserved motifs for phosphorylation and dephosphorylation (**Figures 1**, **2**) argue in favor of a flippase mechanism related to the well-known mechanism of the P2-ATPase ion pumps Ca^{2+}-ATPase and Na^+/K^+-ATPase. Indeed, like the P2-ATPases and, presumably, all other P-type ATPases, the P4-ATPases are phosphorylated in a vanadate-sensitive manner by ATP at the conserved P-domain aspartate (Ding et al., 2000; Lenoir et al., 2009; Bryde et al., 2010; Coleman et al., 2012). The reaction cycle has been examined in some detail for ATP8A2 (Coleman et al., 2012), showing that the phosphoenzyme exists in E1P and E2P states, and that the dephosphorylation of E2P is activated by the specific phospholipid substrates PS and PE, but not by PC (**Figure 3A**). The latter finding is reminiscent of the effect of K^+ binding from the external side to the Na^+/K^+-ATPase (Post et al., 1972). The K^+ activation of dephosphorylation is one of the key features of the coupling mechanism of the Na^+/K^+-ATPase that allows K^+ translocation from the external side to the internal side of the plasma membrane (**Figure 3C**). The specific phospholipid substrate apparently acts on the P4-ATPase in a similar way, promoting dephosphorylation in association with the translocation of the lipid from the exoplasmic to the cytoplasmic leaflet of the lipid bilayer (**Figure 3B**). Approximately 50-fold higher ATPase activity is observed for ATP8A2 in lipid vesicles containing 10% PS/90% PC as compared with 100% PC, where the ATPase activity is diminutive, and with PE the activation is also substantial (Coleman et al., 2009, 2012; Coleman and Molday, 2011). Crystal structures of the P2-ATPase ion pumps Na^+/K^+-ATPase and Ca^{2+}-ATPase have shown how the conserved glutamate of the A-domain TGES motif (in P4-ATPases DGET) is brought into the correct position for catalyzing the hydrolysis of the aspartyl phosphoryl bond in the P-domain. This unique event consists of a 90° rotation of the A-domain in relation to the E1P–E2P transition followed by an additional, smaller change of conformation elicited by the binding of the ion to be transported from the exoplasm toward the cytoplasm (Toyoshima, 2009; Palmgren and Nissen, 2011). Similar structural changes are expected to result in dephosphorylation of P4-ATPases. Accordingly, it was demonstrated for ATP8A2 that mutation of the conserved A-domain glutamate blocks dephosphorylation (**Figure 3A**; Coleman et al., 2012). It is noteworthy that the structural information from the P2-ATPases shows that the catalytic site at which dephosphorylation occurs is located some 50 Å from the membrane site at which K^+ binds to promote the dephosphorylation. In P4-ATPases the corresponding distance is likely no less, with the catalytic site being composed of the cytoplasmic A-, P-, and N-domains, and the lipid substrate coming from the exoplasmic leaflet, thus indicating that the signal transduction must take place through a conformational change reaching over long distance.

Kinetic Difference between ATP8A2 and Drs2p

Dephosphorylation of the yeast homolog of ATP8A2, Drs2p, is also accelerated by PS, but to a lesser extent than the dephosphorylation of ATP8A2 and only in the simultaneous presence of phosphatidylinositol-4-phosphate binding to the C-terminal regulatory domain (Jacquot et al., 2012). This observation is in accordance with the evidence mentioned above that Drs2p requires such interaction for PS flippase activity, thus being autoinhibited by the C-terminus in the absence of the activator. The C-terminus likely interferes with the optimal positioning of the A-domain for catalysis of dephosphorylation (Montigny et al., 2016). However, even in the presence of phosphatidylinositol-4-phosphate the PS-activated ATP hydrolysis and dephosphorylation rates of Drs2p are 1–2 orders of magnitude lower than those of ATP8A2 (Jacquot et al., 2012). The ATP hydrolysis and dephosphorylation rates determined for ATP8A2 are comparable to the rates seen for Ca^{2+}-ATPase and Na^+,K^+-ATPase (Coleman et al., 2012). It has been suggested that the high catalytic turnover rate of ATP8A2 is needed particularly in the retinal membranes housing ATP8A2, due to rapid dissipation of lipid asymmetry by the floppase activity of the retinal protein opsin (Jacquot et al., 2012). However, ATP8A2 is expressed not only in the retina, but all over the brain, where it is indispensable, as evidenced by the CAMRQ syndrome resulting from a single missense mutation of ATP8A2. Even in the presence of its known activator Drs2p appears more autoinhibited than ATP8A2. Worth noting in this connection is the fact that there is only poor conservation between ATP8A2 and Drs2p of the C-terminal tail including the putative RMKKQR phosphatidylinositol-4-phosphate-binding site (Jacquot et al., 2012). More information that possibly can be obtained by studying the consequences of exchanging their C-termini is needed to resolve this issue. The coupling between ATP hydrolysis and lipid flipping should also be considered. The stoichiometry of PS molecules flipped per ATP molecule hydrolyzed has not been determined with confidence, one reason being that the ATPase activity is rather low with the NBD-labeled PS used for measurement of transport. It is thus an open question

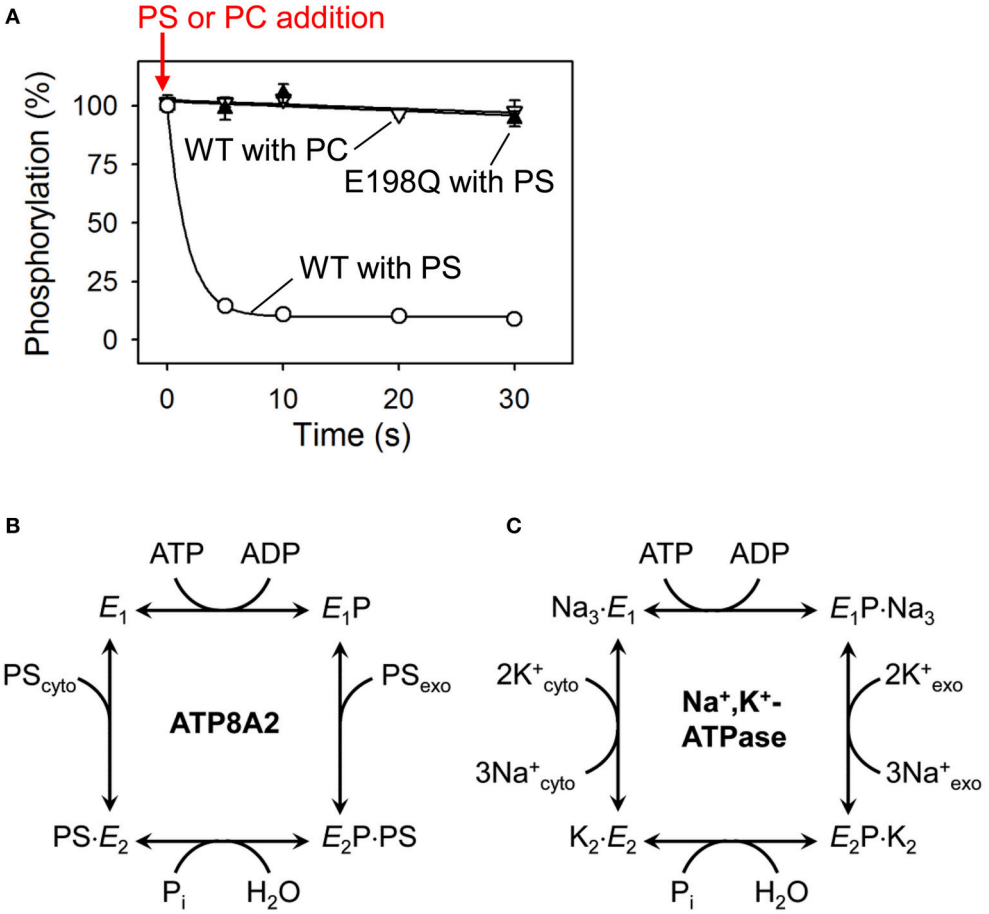

FIGURE 3 | Dephosphorylation of P4-ATPases and Na^+/K^+-ATPase is activated by binding of the transported substrate. (A) Experimental data (Coleman et al., 2012) showing the accelerating effect of addition of phosphatidylserine (PS) on dephosphorylation of ATP8A2 (open circles). No dephosphorylation occurs when phosphatidylcholine (PC) is added (open triangles). The PS-induced dephosphorylation is blocked by the E198Q mutation replacing the glutamate in the DGET motif of the A domain with glutamine (filled triangles). **(B)** Reaction cycle of P4-ATPases proposed on the basis of the finding illustrated in A together with additional analysis of the conformations of the phosphoenzyme intermediate of ATP8A2 (Coleman et al., 2012). **(C)** Classic Post-Albers model for the reaction cycle of Na^+,K^+-ATPase (Post et al., 1972) illustrating that the activation of dephosphorylation of the P4-ATPase by PS (coming from the exoplasmic lipid bilayer leaflet) shown in **(A,B)** is analogous to the activation of dephosphorylation of Na^+,K^+-ATPase by K^+ (coming from the exoplasmic medium). Na^+ binding from the cytoplasm is required for phosphorylation of Na^+,K^+-ATPase, whereas no known ion or other substrate needs to bind to the P4-ATPase to allow phosphorylation (see text for more explanation).

whether ATP8A2 hydrolyzes more ATP per lipid flipped than Drs2p.

Phosphorylation of P4-ATPases Does Not Need Triggering by Ions Being Countertransported

In the Na^+/K^+-ATPase and the Ca^{2+}-ATPase, the binding of the ion being transported from the cytoplasmic to the exoplasmic side triggers the phosphorylation from ATP (**Figure 3C**). It is therefore relevant to ask whether the analogy between P4-ATPase and P2-ATPase catalytic mechanisms further implies that the binding of another specific substrate transported in the reverse direction of the known lipid substrates, from the cytoplasmic to the exoplasmic side, is needed to activate the phosphorylation of the P4-ATPase from ATP. This question has been addressed by examining the effects of various ions including Na^+, K^+, Cl^-, SO_4^{2-}, Ca^{2+}, N-methyl-D-glucamine, acetate, and H^+ on the phosphorylation of ATP8A2 from ATP, with the result that none of these ions promote phosphorylation at a significantly higher rate than the others (Coleman et al., 2012). Similar results were obtained for Drs2p in a study that tested in addition to the ions mentioned above, Co^{2+}, Zn^{2+}, La^{3+}, carbonyl cyanide, m-chlorophenylhydrazone, oxalate, and arsenate, in all cases without finding appreciable effects on phosphorylation (Jacquot et al., 2012). Perhaps the most likely candidate for a non-lipid substrate transported by the P4-ATPases toward the exoplasm would be H^+, but rapid kinetic analysis of the pH dependence of the phosphorylation rate showed no significant variation in the interval from pH 6.5 to pH 9.0, thus indicating that if proton

binding at a specific site (supposedly followed by transport) really were required for activation of the phosphorylation reaction, then the proton affinity of this site would be unusually high with deprotonation occurring only above pH 9.0 (Coleman et al., 2012). The conclusion of these studies is therefore that presently there are no ions known as activators of the phosphorylation of P4-ATPases except for Mg^{2+}, which is required as catalyst at the catalytic site as for other P-type ATPases. The apparent affinity for Mg^{2+} activation of phosphorylation is in the micromolar range and very similar to that pertaining to Na^+/K^+-ATPase (Coleman et al., 2012).

Pathway for Phospholipid Movement Studied by Mutagenesis and Structural Modeling

Giant Substrate Problem

While the catalytic mechanism of ATP hydrolysis by P4-ATPases appears similar to that of the P2-type ATPases, these ion pumps might be less suitable models for the P4-ATPases when it comes to understanding the handling of the lipid substrate. The size of the ions being transported by the P2-ATPases is only 2–3 Å in diameter, allowing occlusion of the ions in a narrow space between the transmembrane helices M4, M5, M6, and M8 during their passage across the membrane. Phospholipids are much larger (the hydrocarbon chains are approximately 20 Å long and the head group 5 Å), taking up space corresponding to half the membrane bilayer thickness, and must reorient during the flipping to keep the hydrophilic head group at the bilayer surface. The large size of the lipid substrate and the complex movement it has to undergo make it hard to imagine a common transport mechanism for ions and lipids. Solving this enigma, the so-called "giant substrate problem," is a most essential challenge of P4-ATPase research. Because flippase activity is retained following purification and reconstitution of ATP8A2 together with its accessory CDC50A subunit in lipid vesicles (Coleman et al., 2009, 2012; Coleman and Molday, 2011), there is no doubt that these are the only protein components required for the movement of the lipid and its coupling with ATP hydrolysis, thus providing a sound basis for using site-directed mutagenesis to search for residues interacting with the lipid substrate during the flipping. Mutational effects on the apparent affinity for the lipid substrate can be assessed from the PS or PE concentration dependences of the ATPase activity and/or the dephosphorylation step. PS or PE is added at various concentrations to the ATP8A2-CDC50A complex maintained solubilized in detergent and PC. This procedure first pinpointed the lysine K873 as critical for the interaction with the lipid substrate (Coleman et al., 2009; Coleman and Molday, 2011; Coleman et al., 2012). The K873A and K873E mutants showed conspicuous reductions of the apparent affinity for PS in ATPase activity studies as well as in studies of the PS-activated dephosphorylation, whereas a more moderate reduction was seen for K873R, thus indicating a role of the positive charge of the side chain, which might be to interact with a negative charge of the phospholipid head group. Because the binding of PE was disturbed as well by the mutation, the suggested interaction would implicate the phosphate part of the head group common to both lipids (Coleman et al., 2012). K873 is located in the M5 transmembrane helix at exactly the position corresponding to a serine in Na^+/K^+-ATPase that is a key residue in the coordination of K^+ during the transport (Blostein et al., 1997; Pedersen et al., 1998; Shinoda et al., 2009). Moreover, significant effects were observed for mutation of the adjacent N874, which corresponds to an asparagine that participates in the coordination of K^+ in Na^+/K^+-ATPase and Ca^{2+} in the Ca^{2+}-ATPase (Coleman et al., 2012), see the sequence alignment in **Figure 2**. Thus, the similarity between the P4-ATPase and the P2-ATPases clearly reaches beyond the mechanism of ATP hydrolysis, managed by the cytoplasmic domains, to the events taking place in the membranous part of the protein.

Would it really be feasible that the giant phospholipid substrate is translocated through a pathway in the protein that involves occlusion between the transmembrane helices M4, M5, M6, and M8 similar to the occlusion of ions being translocated by the Na^+/K^+-ATPase and the Ca^{2+}-ATPase? After all, this possibility might not be totally unrealistic, because the crystal structure of another related P-type ATPase ion pump, the plant H^+-ATPase AHA2 (P3-ATPase), unlike the crystal structures of the Na^+/K^+-ATPase and the Ca^{2+}-ATPase, shows the presence of a large central cavity containing water (Pedersen et al., 2007). The existence of this cavity is possibly the consequence of the absence in the H^+-ATPase transmembrane helices of most of the negatively charged residues that in Na^+/K^+-ATPase and Ca^{2+}-ATPase cross-link the helices through the binding of the transported cations. These carboxylic acid residues are also absent in the P4-ATPases, being replaced by hydrophobic residues in several cases (**Figure 2**). Hence, as long as structural details of P4-ATPases in the membrane region remain unknown, the option of a central cavity that might accommodate a lipid temporarily during the flipping needs to be taken into consideration. This hypothesis has also been referred to as a "canonical binding site" (Baldridge and Graham, 2013), because the lipid being flipped would be located similarly to the occluded ions in the "classic" ion pumps.

Possible Peripheral Pathway

The alternative, and at the moment perhaps most realistic, transport model is one in which a peripheral pathway in the protein allows for flipping of the phospholipid in such a way that only the polar head group interacts directly with the protein. This would allow the hydrocarbon tail to slide through the hydrocarbon layer of the lipid bilayer surrounding the protein, sometimes referred to as the "credit card model" because the interaction of the protein exclusively with the head group is comparable to a credit card payment terminal, which touches only the magnet stripe or chip of the card. The relevance of such a peripheral pathway type of model is underscored by the recent publication of the crystal structure of a scramblase, TMEM16 (Brunner et al., 2014). The scramblase equilibrates PS and PE phospholipids between the two bilayer leaflets and contains at its lipid bilayer exposed surface an 8–11 Å wide crevice spanning the entire membrane. This crevice is formed by transmembrane helices and is twisted like a spiral staircase with one side exposed

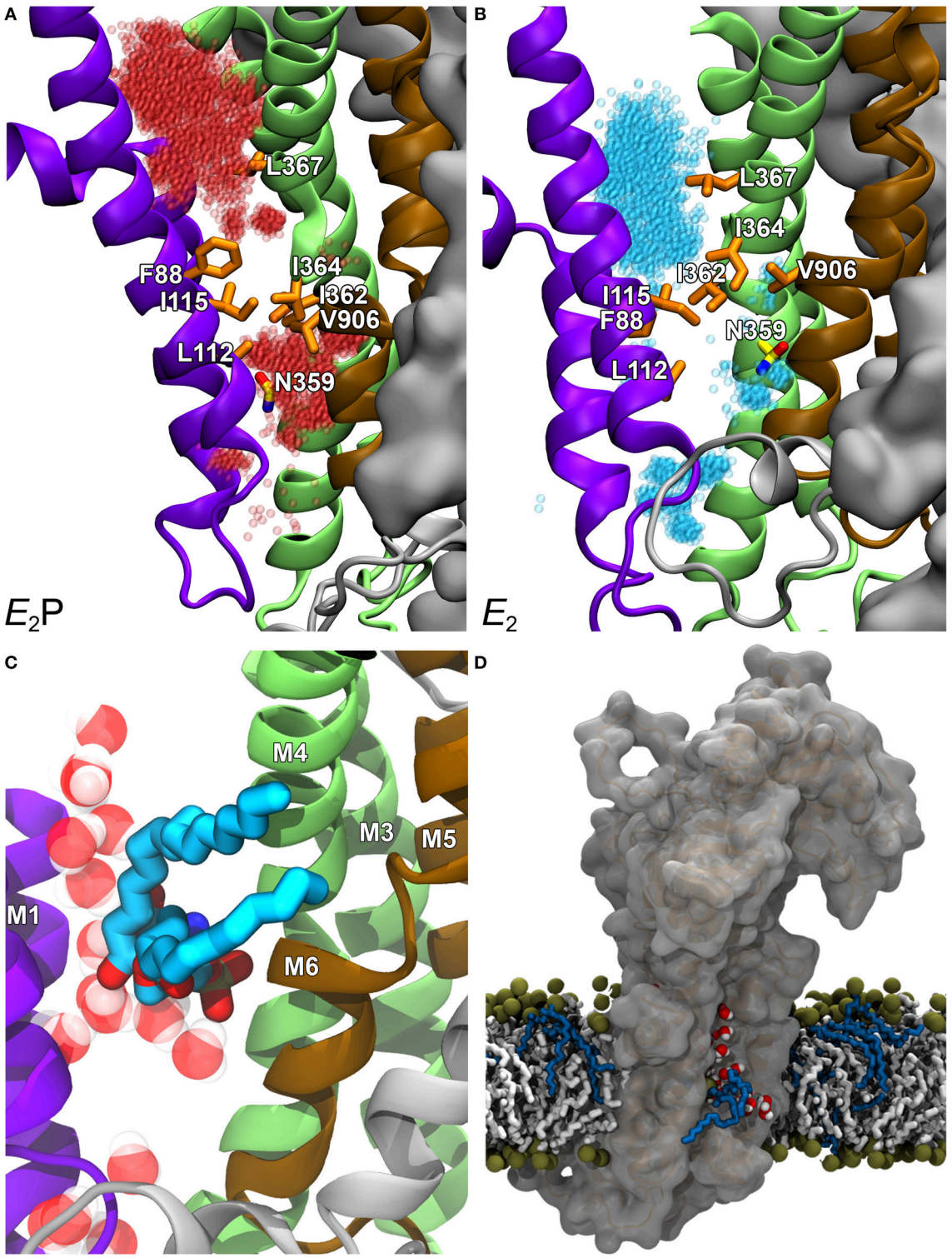

FIGURE 4 | Structural model of ATP8A2 in E_2P and E_2 states revealing the hydrophobic gate pathway. The model was constructed on the basis of the alignment of ATP8A2 with SERCA1a shown in **Figure 2** and the crystal structures of SERCA1a in E_2P and E_2 states supplemented with refinement of the membrane domain by molecular dynamics simulation, opening up a groove between M1, M2, M4, and M6 (Vestergaard et al., 2014). In all panels, the cytoplasmic side is up. **(A,B)** Refined models of E_2P and E_2 states are shown in cartoon with helices M1–M2 in purple, M3–M4 in green, M5–M6 in brown, and M7–M10 as molecular isosurface representation in gray. Selected hydrophobic side chains including I364 are shown in licorice representation with carbons in orange for hydrophobic residues and in yellow for N359, nitrogen in blue, and oxygen in red. Water molecules in groove are red transparent spheres in the E_2P model and cyan in the E_2

(Continued)

FIGURE 4 | Continued
model. **(C)** Metadynamics simulations were used to steer a phosphatidylserine molecule into the groove of the E_2 model. **(D)** Visualization of credit card transport hypothesis. E_2 model in surface representation embedded in the phospholipid membrane, where brown spheres indicate phosphate parts of the phospholipid head groups and hydrocarbon chains are shown in white (PC) or blue (PS) licorice representation. The hydrophilic head group of a PS molecule is being moved upwards along the groove, partly solvated by water molecules (oxygen red, hydrogen white). The blue hydrocarbon chains of the lipid project into the membrane lipid phase.

to the membrane. Its surface is strongly hydrophilic, thus being able to accommodate the head group of the phospholipid being flipped, with the hydrocarbon tail extending into the surrounding lipid bilayer in accordance with the "credit card model."

Non-canonical Peripheral Pathway Model

A clue to where in the P4-ATPase structure a peripheral pathway might be located was first obtained in studies of the yeast P4-ATPases Dnf1p and Drs2p (Baldridge and Graham, 2012, 2013). The different lipid substrate specificities of these flippases (PC and PS, respectively) allowed identification of amino acid residues with a role in defining the substrate specificity by examining chimeras and point mutants in which residues had been exchanged between Dnf1p and Drs2p. Critical residues were pinpointed in the membrane region, particularly a M3 asparagine (N550 of Dnf1p corresponding to N302 in bovine ATP8A2) and a M4 phenylalanine/tyrosine (Y618 of Dnf1p corresponding to L367 in bovine ATP8A2), as well as in M1 near its exoplasmic end. Supposedly residues contributing to define the selectivity for PC versus PS would be near a pathway for translocation of the phospholipid head group, and the locations of the pinpointed residues are not consistent with a central, canonical binding site. Consideration of a structural model of Dnf1p based on the crystal structure of the Ca^{2+}-ATPase in the E1 state led to the conclusion that these residues face the surrounding lipid phase in accordance with a peripheral translocation pathway involving mainly M1, M3, and M4, which was denoted as the "non-canonical pathway" (Baldridge and Graham, 2012, 2013).

Hydrophobic Gate Peripheral Pathway Model

More recently, a slightly different location of a putative peripheral pathway was suggested by structural modeling prompted by mutagenesis results for other residues in M4, particularly the isoleucine in the middle of M4 (Vestergaard et al., 2014). This isoleucine is part of the PISL motif (**Figure 2**), which is conserved among P4-ATPases. Significantly, the isoleucine is located exactly at the position corresponding to the glutamate in the corresponding PEGL motif of the Ca^{2+}-ATPase and the Na^+/K^+-ATPase. Like P4-ATPases, P3-ATPases such as the AHA2 mentioned above have either isoleucine or valine at the corresponding position (**Figure 2**). On the basis of mutagenesis work in combination with structural information from crystallization, the glutamate present in the Na^+/K^+-ATPase and the Ca^{2+}-ATPase is known to be a key element in the transport mechanism. It works as a gating residue at the ion binding pocket and furthermore directly binds and moves the ions undergoing transport, being alternately exposed toward the two sides of the membrane during the pump cycle. Hence, in these P2-ATPase ion pumps M4 moves vertically like a pump rod, allowing delivery to the exoplasm of Na^+ or Ca^{2+} bound at the M4 glutamate from the cytoplasmic side, or delivery to the cytoplasm of K^+/H^+ bound at the same glutamate from the exoplasmic side (Vilsen and Andersen, 1998; Olesen et al., 2007; Toyoshima, 2009). It was therefore intriguing to learn that a missense mutation replacing this isoleucine (I376 in human ATP8A2) with methionine had been identified as the first (and so far only) disease-causing mutation in ATP8A2, giving rise to CAMRQ (Onat et al., 2012). When expressed in mammalian cell culture the equivalent mutation in bovine ATP8A2, I364M, disrupted flipping of fluorescent NBD-labeled PS and reduced ATPase activity to very low levels, although wild type-like expression levels were retained. Analyses of the lipid substrate concentration dependence of the overall and partial reactions of the enzyme cycle in ATP8A2 mutants with various replacements of this isoleucine led to the conclusion that during the lipid flipping the PS head group passes near I364, and I364 is critical to the release of the lipid into the cytosolic leaflet (Vestergaard et al., 2014).

To examine the exact role of I364, in the absence of a crystal structure of a P4-ATPase, homology-based models of ATP8A2 were built for E2P and E2 (the two states that interact with the lipid substrate, cf. **Figure 3B**), starting from a multiple sequence alignment and the high-resolution crystal structures of the Ca^{2+}-ATPase in these conformations. The models were subsequently refined by molecular dynamics simulation, which relaxed the original ATP8A2 homology model structures to structures where a groove between the M1, M2, M4, and M6 transmembrane helices had opened to a wider state, thus suggesting that this region, now deviating significantly from that of the P2-ATPase ion pumps, might be directly involved in lipid transport by the P4-ATPases (Vestergaard et al., 2014). Substantial amounts of water entered the groove during the molecular dynamics refinement, gathering in two distinct pockets placed in the exoplasmic and cytoplasmic ends of the groove, separated by a cluster of hydrophobic residues that included I364 (**Figures 4A,B**). This hydrated groove has a size appropriate for accommodation of the PS head group and is located rather peripherally in the protein, thus allowing the hydrocarbon tail of the lipid to project out into the surrounding membrane bilayer lipid during the flipping (**Figures 4C,D**), exactly as proposed for the scramblase TMEM16 described above. In the P4-ATPase, the phospholipid head group in the groove would be solvated by the water in the groove, thereby aiding the movement of the head group. An important feature of the ATP8A2 E2P and E2 structural models is moreover that I364 moves during the transition between these states, thereby apparently functioning as a "hydrophobic gate" propelling the water in the groove with the solvated phospholipid head

group toward the cytoplasmic side against the concentration gradient.

Mutational analysis further implicated adjacent hydrophobic residues including I115 (of M2) as part of the gate (Vestergaard et al., 2014). The polar residue N359 (of M4) appears to be involved in the recognition of the lipid substrate entering the groove from the exoplasmic side (**Figures 4A,B, 5**). These findings suggest a plausible phospholipid translocation mechanism that is both distinct from and related to the well-characterized P2-ATPase ion transport mechanisms. In lipid pumps as well as ion pumps, M4 plays a central role as the pump rod generating the movement. In fact, the less wide groove present between M1, M2, M4, and M6 in the Ca^{2+}-ATPase also seems to contain water and has been proposed to be the pathway taken by Ca^{2+} migrating from the cytoplasmic side to the Ca^{2+} occlusion pocket (Musgaard et al., 2012), thus overlapping partially with the hydrophobic gate pathway proposed for ATP8A2 (Vestergaard et al., 2014). In a recent review (Montigny et al., 2016) it was furthermore pointed out that the most conserved of the two Ca^{2+} binding sites found in Ca^{2+}-ATPases is the one involving the glutamate in M4 corresponding to I364 of ATP8A2, denoted site II in SERCA, implying that Ca^{2+}-ATPases like PMCA and SPCA that only transport one Ca^{2+} in each cycle do not possess a site corresponding to site I of SERCA, and therefore do not use residues in M5 and M8 for Ca^{2+} binding. Hence, the hydrophobic gate pathway must be very similar to the transport pathway used by the one-site Ca^{2+}-ATPases and possibly also other P-type ATPases pumping a single ion per cycle (Montigny et al., 2016). As previously alluded to in discussing the relation between P2- and P4-ATPases (Lenoir et al., 2007; Baldridge et al., 2013), the Ca^{2+}-ATPase E2 crystal structure shows the presence of a phospholipid molecule approximately corresponding to the cytoplasmic exit of the hydrophobic gate pathway (top of **Figure 5**). In the P4-ATPases, this primordial phospholipid-binding site might have evolved into a site playing a role in the lipid transport. Worth mentioning in relation to the conservation of the structure and basic mechanism between P2- and P4-ATPases is also the proline adjacent to the isoleucine in the PISL motif (**Figure 2**). This proline is conserved in all P-type ATPases, and in ATP8A2 the P363A mutation abrogated expression of the protein in HEK cells, suggesting that the local unwinding of the M4 helix near PISL caused by the presence of the proline is crucial to proper folding and processing of the protein (Vestergaard et al., 2014).

Merge of Structural Models Resulting from ATP8A2 and Yeast Flippase Studies of the Lipid Transport Pathway

The structural locations of the various proposed P4-ATPase lipid translocation pathways are summarized in **Figure 6**: the peripheral pathway associated with the hydrophobic gate (between M1, M2, M4, and M6; Vestergaard et al., 2014); the central pathway implying occlusion of the lipid between M4, M5, M6, and M8 similar to the occlusion of ions in the two-sites Ca^{2+}-ATPases and the Na^+,K^+-ATPase; and the non-canonical pathway between M1, M3, and M4 (Baldridge and Graham, 2012, 2013). It is of note that in the structural model of Vestergaard et al. (2014), the M4 residue L367, which is equivalent to Y618 shown to be important for lipid head group selectivity in yeast Dnf1p (Baldridge and Graham, 2012, 2013), points toward the hydrophobic gate pathway, being located one helix turn above I364 (**Figure 5**). Likewise, the M2 residues A119 and E123 at positions equivalent to yeast Dnf1 residues T254 and D258 shown to couple with the M4 Y618 to confer lipid head group selectivity (Baldridge et al., 2013), seem in the structural model of Vestergaard et al. (2014) to be associated with the cytoplasmic exit of the hydrophobic gate pathway (**Figure 5**). On the other hand, N302, equivalent to the asparagine N550 also pinpointed as critical for head group selectivity in the yeast flippase studies (Baldridge and Graham, 2013), is too distant

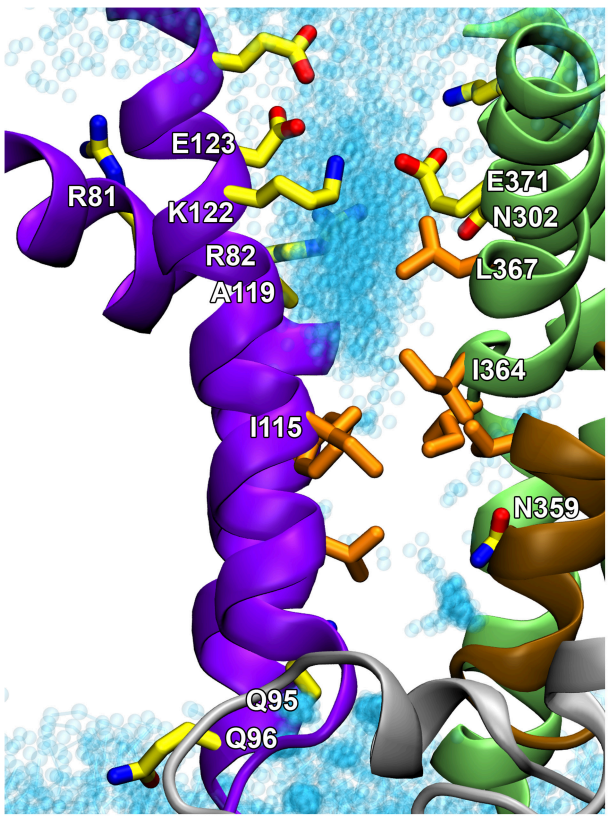

FIGURE 5 | Proposed hydrophobic gate pathway in side view with indication of residues that might play a role in the recognition and translocation of the phospholipid. The E2 structural model of the ATP8A2 membrane domain (Vestergaard et al., 2014) is visualized with the cytoplasmic side up. Selected residues, some of which are mentioned in the text, are shown in licorice representation with carbons in orange for hydrophobic residues and in yellow for polar/charged residues. Water molecules in the groove are shown as cyan transparent spheres. I364 and I115 are key residues in the hydrophobic gate. N359 is important for the affinity for the lipid substrate (Vestergaard et al., 2014). Q95, Q96, A119, E123, N302, and L367 are located at positions corresponding to the yeast Dnf1 flippase residues G230, A231, T254, D258, N550, and Y618, which have been shown to be crucial to the specific lipid selectivity of this flippase (Baldridge and Graham, 2012, 2013).

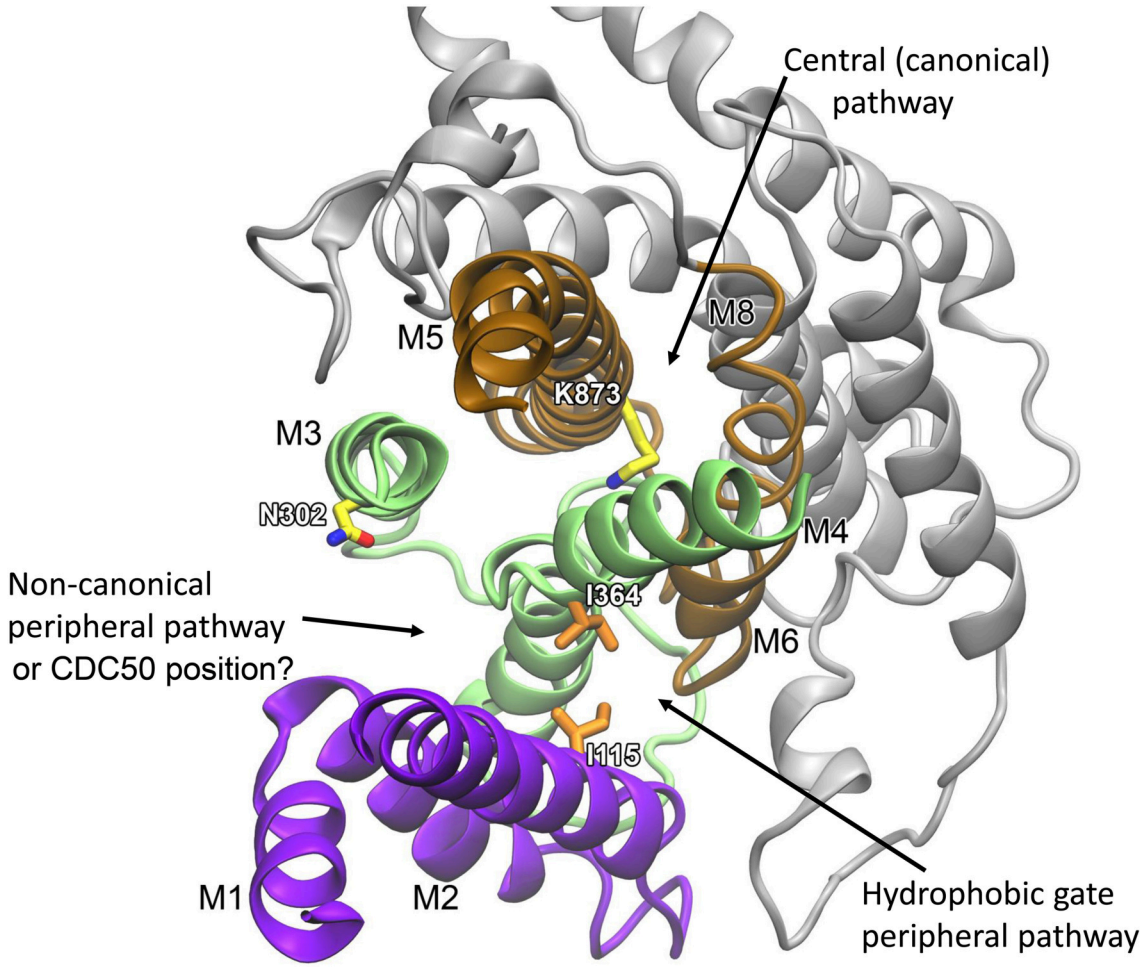

FIGURE 6 | Overview of the locations of three proposed phospholipid transport pathways and possible location of CDC50. The refined membrane domain of the E2 structural model of ATP8A2 (Vestergaard et al., 2014) is visualized as viewed from the cytoplasmic side. Helices M1–M2 are shown in purple, M3–M4 in green, M5–M6 in brown, and M7–M10 in gray. Selected residues mentioned in the text are shown in licorice representation with carbons in orange for hydrophobic residues and in yellow for polar/charged residues. Recent evidence suggests a location of the CDC50 protein corresponding to the non-canonical peripheral pathway (Vestergaard et al., 2015).

from the hydrophobic gate pathway to be directly involved (**Figures 5**, **6**).

It is clear that in addition to the hydrophobic gate, residues on its cytoplasmic side pointing toward the proposed translocation pathway contribute to determining the affinity for the lipid substrate. The yeast flippase studies have furthermore identified two residues near the exoplasmic end of M1 that are important for lipid substrate recognition (Baldridge and Graham, 2013). At the equivalent positions of ATP8A2 are Q95 and Q96 shown in **Figure 5**. These residues might be associated with the exoplasmic entrance to the hydrophobic gate pathway. The separation of the residues critical to lipid substrate recognition in two clusters, one near the exoplasm and the other near the cytoplasm, observed in the yeast flippase studies, led to the view that there are two gates, an entry gate, and an exit gate (Baldridge and Graham, 2013). In fact the studies of ATP8A2 indicate that there are residues critical to the interaction with the lipid substrate all along the hydrophobic gate pathway. It is, however, not clear why residues near the cytoplasmic exit of the putative translocation pathway should be as critical for the lipid substrate recognition as the residues at the inlet. One possibility is that residues near the exit form bonds (note in **Figure 5** that K122 and E371 are very close, probably forming a salt bridge) required to position the helices correctly for entry and recognition of the lipid substrate at the other end of the groove.

Role of the M5 Lysine

In light of the proposal of the hydrophobic gate pathway one might wonder about the role of the more centrally placed critical lysine K873 of transmembrane helix M5 discussed above. In our refined models, K873 is situated on the opposite side of M4 with respect to the hydrophobic cluster including I364 and does not point into the suggested transport pathway (**Figure 6**). K873 is closely surrounded by polar residues with which it

may form hydrogen bonds, including N905 located at the position corresponding to an ion binding asparagine/aspartate of Ca^{2+}-ATPase and Na^+/K^+-ATPase (**Figure 2**), and this part of the models was most stable during refinement. K873 might therefore acts as a stabilizer of the central part of the structure, aiding the correct placement of the dynamic regions including M4 and the hydrophobic gate.

Structural Location of the CDC50 Protein

The "dark horse" in the modeling studies, so far not taken properly into consideration, is the CDC50 protein (the β-subunit). Recently, screening of 141 point mutations of the ATP8A2 catalytic subunit pinpointed six mutations that indirectly affect the glycosylation of the CDC50 protein, suggesting the possibility of a defect in the structural interaction between the two subunits of the complex (Vestergaard et al., 2015). Interestingly, in our structural model four of the six residues implicated are situated above each other almost along a line at the periphery of the ATP8A2 protein, pointing toward the putative location of the non-canonical pathway, between M1, M3, and M4 (**Figure 6**). Hence, one or both transmembrane helices of CDC50 might actually be located corresponding to the non-canonical pathway, which would place the CDC50 exoplasmic domain in close proximity to the putative exoplasmic entrance of the hydrophobic gate pathway, thereby enabling CDC50 to directly promote substrate binding in the hydrophobic gate pathway.

CONCLUSIONS AND FUTURE PERSPECTIVES

Cell-based and biochemical studies from a number of research groups have convincingly shown that P4-ATPases actively flip phospholipids from the exoplasmic to the cytoplasmic leaflet of the plasma membrane and intracellular membranes. Like yeast P4-ATPases, some members of the mammalian P4-ATPase family actively transport the PS and PE to generate and maintain lipid asymmetry, whereas other members appear to be specific for PC. Cell culture and animal studies in which specific P4-ATPases are overexpressed or knocked down support the importance of these mammalian P4-ATPases in generating and maintaining lipid asymmetry for such key cellular processes as vesicle budding and trafficking, cell migration, neurite extension and cell survival. Although, significant progress has been made, a number of questions and areas of investigation remain. The distribution of P4-ATPases in native tissues and cells and their role in various biochemical pathways need to be studied in more detail. The substrate specificity of several mammalian P4-ATPases including ATP9A and ATP9B remain to be determined. Whether these proteins require an auxiliary protein to complete the flippase complex also remains to be determined. The mechanisms for regulation and subcellular targeting of mammalian P4-ATPases have yet to be examined in detail. We still know relatively little about the individual CDC50 proteins and their role in lipid flippase activity. Molecular modeling together with site-directed mutagenesis has provided important insight into how P4-ATPases may recognize and pump specific phospholipid substrates across membranes. Although, the lipid substrates of P4-ATPases are "giant" in comparison with the ions translocated by ion pumps, similarities between lipid and ion pumps of P-type greatly outnumber their differences. Not only are the mechanisms for ATP hydrolysis the same, but even the handling of the lipid substrate has much in common with the pumping of ions, and the suggested transport pathways of P-type lipid pumps and ion pumps appear to overlap to some extent. The hydrophobic gate pathway hypothesis should be validated by additional mutational studies, with the aim of identifying the determinants of the specific substrate selectivity of the P4-ATPases. For example, is there a "selectivity gate" at the exoplasmic entrance of the pathway, perhaps involving the CDC50 protein? A full understanding of the mechanism will require determination of the structure of a P4-ATPase with its bound lipid substrate. The highly purified ATP8A2 preparation recently developed seems optimal for such studies.

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REVIEW
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Carbonylation Modification Regulates Na/K-ATPase Signaling and Salt Sensitivity: A Review and a Hypothesis

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Na/K-ATPase signaling has been implicated in different physiological and pathophysiological conditions. Accumulating evidence indicates that oxidative stress not only regulates the Na/K-ATPase enzymatic activity, but also regulates its signaling and other functions. While cardiotonic steroids (CTS)-induced increase in reactive oxygen species (ROS) generation is an intermediate step in CTS-mediated Na/K-ATPase signaling, increase in ROS alone also stimulates Na/K-ATPase signaling. Based on literature and our observations, we hypothesize that ROS have biphasic effects on Na/K-ATPase signaling, transcellular sodium transport, and urinary sodium excretion. Oxidative modulation, in particular site specific carbonylation of the Na/K-ATPase α1 subunit, is a critical step in proximal tubular Na/K-ATPase signaling and decreased transcellular sodium transport leading to increases in urinary sodium excretion. However, once this system is overstimulated, the signaling, and associated changes in sodium excretion are blunted. This review aims to evaluate ROS-mediated carbonylation of the Na/K-ATPase, and its potential role in the regulation of pump signaling and sodium reabsorption in the renal proximal tubule (RPT).

Keywords: Na/K-ATPase, ROS, protein carbonylation, signaling

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Accumulating evidence suggests that excessive dietary salt intake may play a role in the pathogenesis of hypertension, with more pronounced effects seen in salt-sensitive patients (Calhoun et al., 2008). Consequently, modest restriction of dietary salt and diuretic therapy are often recommended for treatment of resistant hypertension, particularly within the salt-sensitive sub-group (He and MacGregor, 2004; Calhoun et al., 2008). Long-term blood pressure (BP) regulation is highly associated with renal sodium handling (Guyton, 1991). Recent studies observe the CTS-activated Na/K-ATPase signaling pathway to contribute to RPT sodium handling and salt sensitivity (Buckalew, 2005; Meneton et al., 2005; Schoner and Scheiner-Bobis, 2007, 2008; Bagrov and Shapiro, 2008; Fedorova et al., 2010; Liu and Xie, 2010). Various intercellular and extracellular functions are regulated by the signaling function of the Na/K-ATPase. Discussion of Na/K-ATPase signaling and the downstream physiological and pathophysiological implications can be found in several references (Bertorello and Sznajder, 2005; Buckalew, 2005; Aperia, 2007; Liu and Shapiro, 2007; Schoner and Scheiner-Bobis, 2007, 2008; Bagrov and Shapiro, 2008; Bagrov et al., 2009; Blaustein et al., 2009; Li and Xie, 2009; Fedorova et al., 2010; Liu and Xie, 2010). Based on our recent observations, we focus on the effect of oxidative (carbonylation) modification of Na/K-ATPase and sodium handling in RPTs.

PROTEIN CARBONYLATION AND CELL SIGNALING

Biologically, electron reduction of oxygen (O_2) leads to generation of ROS including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO'). ROS is able to oxidize various types of biological molecules including proteins, lipids, and DNA, leading to their functional changes. Through Fenton's reaction, H_2O_2 (generated by O_2^- or via other mechanisms) is reduced to HO' by coupling oxidation of reduced ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}). This metal-catalyzed oxidation (MCO) process oxidizes proteins by introducing carbonyl groups (such as aldehydes, ketones, or lactams) into the side chains of certain amino acids (such as proline, arginine, lysine and threonine; Stadtman and Berlett, 1991; Stadtman and Levine, 2000; Nyström, 2005). Unlike this direct (primary) carbonylation, indirect (secondary) carbonylation on lysine, cysteine, and histidine can occur by reactive carbonyl compounds generated from other types of oxidation, such as lipid and carbohydrate oxidation via Michael addition reactions and formation of Schiff bases. Protein carbonylation is a well-recognized marker of oxidative stress because of its stability, its effect on protein functions, and its link to various biological and pathological conditions. Oxidative stress has been implicated in the aging process, various conditions like ischemia-reperfusion and hyperoxia, and various human diseases like Alzheimer's disease, chronic lung disease, chronic renal failure, diabetes, and sepsis (Stadtman and Levine, 2000; Dalle-Donne et al., 2003, 2006a,b). Since the Fenton reaction involves the conversion of H_2O_2 to $HO^{\bullet}$, any specie of ROS with H_2O_2 as an intermediate and/or end product may stimulate the reaction. In biological systems, H_2O_2 is one of the most common end products of most ROS generating systems.

Oxidative modification of protein, reversible and irreversible, dynamically regulates protein structure, function, and trafficking, as well as cellular signaling and function (Go and Jones, 2013). Direct protein carbonylation is very stable and "chemically" irreversible (Stadtman and Berlett, 1991; Nyström, 2005). Recent studies from the Suzuki laboratory have demonstrated the role of the carbonylation/decarbonylation process in ROS signal transduction in which thiol groups were responsible for decarbonylation via enzymatic processes, likely through thioredoxin reductase (Wong et al., 2008, 2010, 2012, 2013).

CTS, THE NA/K-ATPASE, AND RENAL SODIUM HANDLING

CTS, also known as endogenous digitalis-like substances, include plant-derived glycosides and vertebrate-derived aglycones (Schoner and Scheiner-Bobis, 2007, 2008). Although, the production and secretion of endogenous CTS are not completely understood, they appear to be regulated by angiotensin II and adrenocorticotropic hormone (Hamlyn et al., 1991; Laredo et al., 1997; Schoner and Scheiner-Bobis, 2007; Bagrov et al., 2009). CTS are present in measurable amounts under normal physiological conditions, and are elevated under a number of pathological states. Different species of endogenous CTS show variations in kinetics and tissue action in response to salt loading in both animal models and in human hypertensive patients (Haddy and Pamnani, 1998; Fedorova et al., 2005; Manunta et al., 2006; Schoner and Scheiner-Bobis, 2007, 2008).

The Na/K-ATPase belongs to the P-type ATPase family and consists of two non-covalently linked α and β subunits. Several α and β isoforms, expressed in a tissue-specific manner, have been identified and functionally characterized (Sweadner, 1989; Blanco and Mercer, 1998; Kaplan, 2002; Sanchez et al., 2006). The α1 subunit contains multiple structural motifs that interact with soluble, membrane and structural proteins (Jordan et al., 1995; Beggah and Geering, 1997; Feschenko et al., 1997; Zhang et al., 1998, 2006a; Yudowski et al., 2000; Lee et al., 2001; Xie and Cai, 2003; Barwe et al., 2005; Song et al., 2006; Tian et al., 2006). Binding to these proteins not only regulates the ion pumping function of the enzyme, but it also conveys signal transducing functions to the Na/K-ATPase (Xie and Cai, 2003; Kaplan, 2005; Kaunitz, 2006; Schoner and Scheiner-Bobis, 2007; Li and Xie, 2009).

It has been hypothesized for years that increases in endogenous CTS enhance natriuresis and diuresis by direct inhibition of renal tubular Na/K-ATPase, leading to reduced renal reabsorption of filtered sodium (Blaustein, 1977; Haddy et al., 1979; de Wardener and Clarkson, 1985). The first unequivocal demonstration of ouabain-like substance in human plasma was reported 25 years ago (Hamlyn et al., 1991). *In vivo* experiments suggest the essential role of endogenous CTS in modulating renal sodium excretion and BP with different approaches. First, administration of some (e.g., ouabain) but not all CTS induces natriuresis (Foulkes et al., 1992; Yates and McDougall, 1995). Second, in transgenic mice expressing ouabain-sensitive Na/K-ATPase α1 subunit, both acute salt load and ouabain infusion augment natriuretic responses, which may be inhibited by administration of an anti-digoxin antibody fragment (Dostanic-Larson et al., 2005; Loreaux et al., 2008). Third, immune-neutralization of endogenous CTS prevents CTS mediated natriuretic and vasoconstrictor effects (Fedorova et al., 2001, 2002; Bagrov and Shapiro, 2008; Nesher et al., 2009). Fourth, administration of the ouabain-antagonist, rostafuroxin (previously PST 2238) not only prevents ouabain induced Na/K-ATPase signaling, but also prevents ouabain-induced increase in BP (Ferrandi et al., 2004). Finally, in humans, high salt intake increases circulating endogenous CTS (Manunta et al., 2006; Anderson et al., 2008; Bagrov and Shapiro, 2008). Increased CTS excretion is directly linked to enhanced RPT-mediated fractional Na+ excretion, but inversely related to age and to age-dependent increase in salt-sensitivity (Anderson et al., 2008).

THE NA/K-ATPASE SIGNALING AND SALT SENSITIVITY

Although historical focus has largely been on the direct inhibition of the Na/K-ATPase ion-exchange activity and sodium reabsorption in RPTs by CTS, this does not appear to be the predominant mechanism for several reasons. In contrast,

the newly appreciated signaling function of Na/K-ATPase has been widely confirmed and provides a realistic, mechanistic framework that we will discuss further. We have observed that the renal Na/K-ATPase and its signaling play a key role in regulating renal sodium handling (Liu et al., 2002, 2004, 2005, 2011; Periyasamy et al., 2005; Oweis et al., 2006; Cai et al., 2008; Yan et al., 2013).

Decreases in basolateral Na/K-ATPase activity alone do not appear sufficient to decrease net sodium reabsorption across the renal tubular epithelium. In porcine RPT LLC-PK1 cells, ouabain activates the Na/K-ATPase signaling pathways and consequently redistributes the basolateral Na/K-ATPase and the apical sodium/hydrogen exchanger isoform 3 (NHE3) in a coordinated manner; this leads to symmetrical reduction of cell surface Na/K-ATPase and NHE3 expression, and ultimately decreases net transcellular sodium transport (Liu et al., 2002, 2004, 2005; Oweis et al., 2006; Cai et al., 2008; **Figure 1**). In this experimental model, the concentrations of ouabain used *in vitro* were chosen to mimic the concentrations of CTS seen *in vivo* with salt loading. No significant acute change in intracellular Na^+ concentration was observed (Cai et al., 2008), further suggesting the coordination of the downregulation of both apical and basolateral sodium transporters. This Na/K-ATPase signaling mediated regulation of renal tubular epithelial ion transporters was additionally confirmed in *in vivo* studies (Periyasamy et al., 2005; Liu et al., 2011).

The Dahl salt-resistant (R) and salt-sensitive (S) strains were developed from Sprague Dawley rat strain by selective breeding, depending on the resistance or susceptibility to the hypertensive effects of high dietary sodium (Dahl et al., 1962). In these strains, sodium handling within the RPT is an essential determinant of their different BP responses (Dahl et al., 1974; Rapp, 1982; Rapp and Dene, 1985; Mokry and Cuppen, 2008). At the cost of elevated systolic BP, Dahl S rats rid excess sodium primarily via pressure-natriuresis. In contrast, Dahl R rats counterbalance salt loading via significant reduction of renal sodium reabsorption without increasing BP. *In vivo* studies indicate that impaired RPT Na/K-ATPase signaling appears to be causative of experimental Dahl salt-sensitivity (Liu et al., 2011). Specifically, in Dahl R rats (Jr strain), a high salt diet (2% NaCl for 7 days) and exposure to ouabain activates RPT Na/K-ATPase signaling and stimulates coordinated redistribution of Na/K-ATPase and NHE3, resulting with increases in renal sodium excretion. However, this does not occur in age- and gender-matched Dahl S rats (Jr strain; Liu et al., 2011). At present, we do not have a simple explanation for this occurrence. First, the α1 subunit is essentially the only α isoform expressed in RPTs (Blanco and Mercer, 1998; Summa et al., 2004) and genes coding α1 subunit and NHE3 (in rat chromosomes 1 and 2, respectively) are not located in identified and proposed BP quantitative trait loci (Joe, 2006). Second, there is no difference in α1 gene (Atp1a1) coding (Mokry and Cuppen, 2008), α1 ouabain-sensitivity (Nishi et al., 1993), and α1 expression (Liu et al., 2011) between these two strains. Third, acute salt-loading increases circulating CTS (ouabain and MBG) in both S and R rats (Fedorova et al., 2000). These observations suggest that there must be resistance to CTS signaling in the Dahl S rat, a phenomenon that we only partially understand and will discuss further below.

ROS AND THE NA/K-ATPASE SIGNALING

It is well established that an increase in oxidative stress occurs in many forms of experimental hypertension (Kitiyakara et al., 2003; Touyz, 2004; Wilcox, 2005; Vaziri and Rodriguez-Iturbe, 2006; Welch, 2006). We and others have observed that a high salt diet stimulates endogenous CTS release and ROS generation within the RPT (Moe et al., 1991; Yang et al., 2008; Panico et al., 2009; McDonough, 2010; Banday and Lokhandwala, 2011; Liu et al., 2011). The increases in ROS (Meng et al., 2002; Kitiyakara et al., 2003; Taylor et al., 2006) regulate physiological processes including renal tubular ion transport, fluid reabsorption, and sodium excretion (Moe et al., 1991; Zhang et al., 2002; Garvin and Ortiz, 2003; Han et al., 2005; Yang et al., 2008; Panico et al., 2009; Wang et al., 2009; Banday and Lokhandwala, 2011; Liu et al., 2011; Schreck and O'Connor, 2011). In particular, increases in ROS regulate the activity and cellular distribution of the basolateral Na/K-ATPase as well as the apical NHE3 and sodium/glucose cotransporter, at least under normal circumstances (Moe et al., 1991; Fisher et al., 2001; Silva and Soares-da-Silva, 2007; Yang et al., 2007, 2008; Panico et al., 2009; Crajoinas et al., 2010; Johns et al., 2010; Liu et al., 2011). In our *in vitro* studies with LLC-PK1 cells, we have observed that ouabain stimulates generation of ROS which is critical in CTS-mediated Na/K-ATPase signaling, transporter trafficking, and $^{22}Na^+$ flux (Yan et al., 2013). Pre-treatment with higher doses, but not a low dose, of anti-oxidant N-acetyl-L-cysteine (NAC) attenuated the effect of ouabain on c-Src activation and transcellular $^{22}Na^+$ flux, suggesting a role of basal physiological redox status in the initiation of ouabain induced Na/K-ATPase signaling. This is analogous to the observation that the Na/K-ATPase activity is redox-sensitive with an "optimal redox potential range" (Petrushanko et al., 2006). While CTS stimulates ROS generation and Na/K-ATPase signaling in different *in vitro* and *in vivo* models (Xie et al., 1999; Liu et al., 2000, 2006; Tian et al., 2003; Kennedy et al., 2006a,b; Elkareh et al., 2007), glucose oxidase-induced H_2O_2 alone also stimulates Na/K-ATPase signaling, promotes Na/K-ATPase endocytosis, and inhibits active transcellular $^{22}Na^+$ transport (Liu et al., 2006; Yan et al., 2013). The phenomenon of redox-sensitivity of the Na/K-ATPase has been demonstrated within many animal species, tissues, and cell types. Oxidative modification can affect Na/K-ATPase activity through different mechanisms. For example, S-glutathionylation is the formation of a mixed disulphide (cysteine-S-S-glutathione) between cysteine-SH with glutathione-SH or thiol-disulfide exchange. S-glutathionylation of cysteine residue(s) of the Na/K-ATPase α subunit can block the intracellular ATP-binding site, leading to inhibition of its enzymatic activity (Petrushanko et al., 2012). Ouabain-induced S-glutathionylation of cysteine of the Na/K-ATPase β1 subunit, a process affected by Na/K-ATPase conformational poise (Liu et al., 2012), reduces α1/β1 association and enzymatic activity by stabilizing the enzyme in an E2-prone conformation

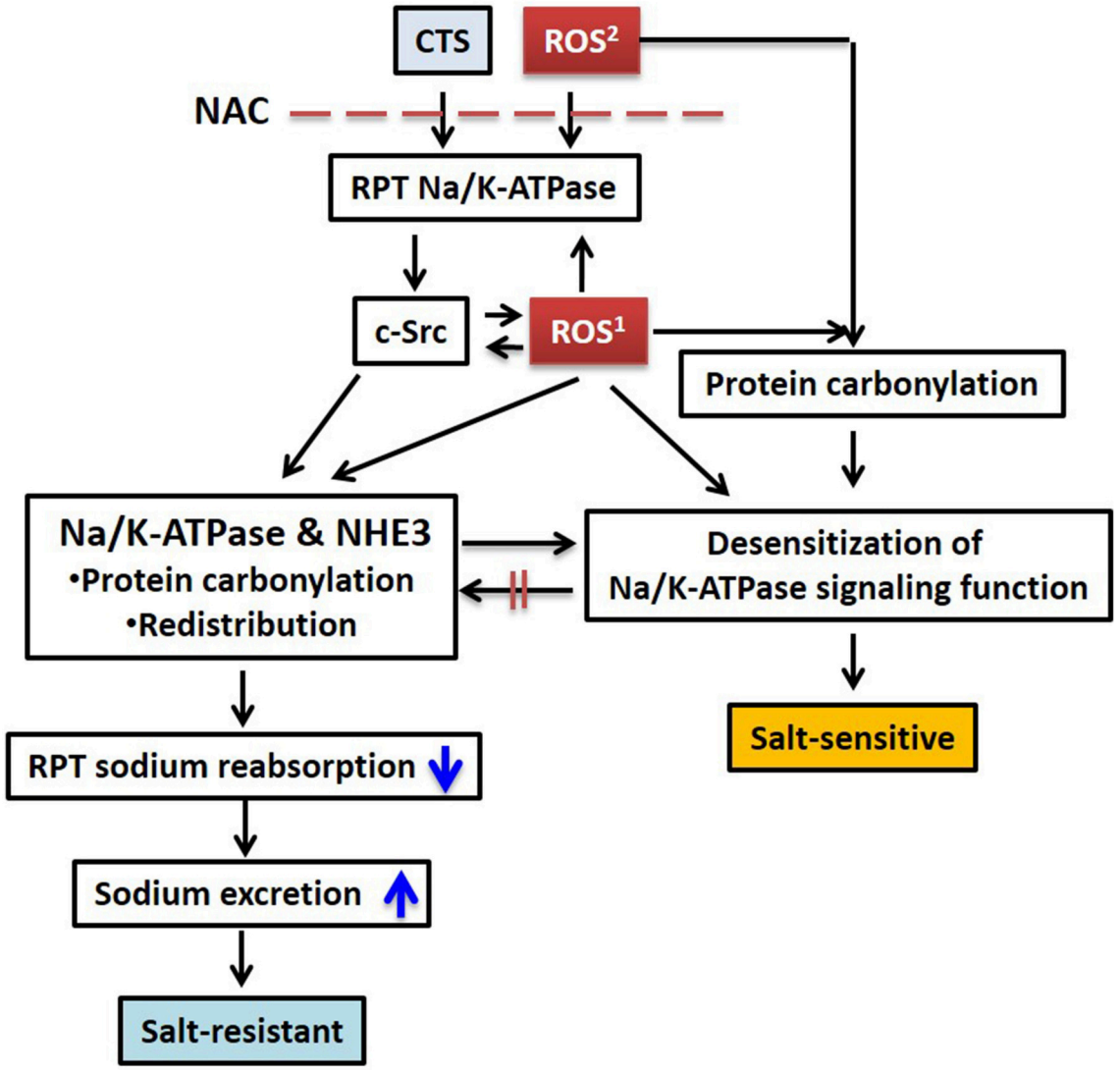

FIGURE 1 | Schematic illustration of CTS and ROS mediated sodium handling in RPT and its relation to salt sensitivity. CTS induced intracellular ROS generation and extracellularly generated ROS stimulates Na/K-ATPase/c-Src signaling, protein carbonylation, and transporter redistribution. Pretreatment with NAC blocks this process. ROS1, CTS induced intracellular ROS generation through Na/K-ATPase signaling; ROS2, extracellular ROS generated by glucose oxidase or other stimuli. Please also see **Figure 2**. RPT, renal proximal tubule; NAC, N-acetyl-L-cysteine.

(Figtree et al., 2009). Oxidants and oxidative modification of the Na/K-ATPase can lead to functional changes (Kim and Akera, 1987; Xie et al., 1990; Huang et al., 1992; Mense et al., 1997; Thevenod and Friedmann, 1999; Zhang et al., 2002; Ellis et al., 2003; Bogdanova et al., 2006; Liu et al., 2006; Reifenberger et al., 2008; Blaustein et al., 2009; Figtree et al., 2009; White et al., 2009; Bibert et al., 2011; Figtree et al., 2012; Petrushanko et al., 2012; Soares-da-Silva, 2012) and formation of Na/K-ATPase oligomeric structure (Dobrota et al., 1999). As partner of Na/K-ATPase signaling, tyrosine kinase c-Src and lipid rafts (including caveolae structural component caveolins) are also redox-sensitive and critical in redox signaling platform formation (Seshiah et al., 2002; Zuo et al., 2005; Touyz, 2006; Zhang et al., 2006b; Han et al., 2008). This suggests a redox-sensitive Na/K-ATPase signaling and its possible role in ROS regulation.

Both ouabain and glucose oxidase-induced H2O2 stimulate Na/K-ATPase signaling and neutralization of the increase in ROS attenuated ouabain-induced effects (Xie et al., 1999; Liu et al., 2000, 2006; Tian et al., 2003; Kennedy et al., 2006a; Elkareh et al., 2007; Yan et al., 2013; Wang et al., 2014). We further observed that both ouabain and glucose oxidase-induced H2O2 stimulate direct protein carbonylation of Pro222 and Thr224 residues of the Na/K-ATPase α1 subunit (α1 carbonylation) in LLC-PK1 cells (Yan et al., 2013). The Pro222 and Thr224 are located in peptide 211VDNSSLTGESEPQTR225 [UniProtKB/Swiss-Prot No P05024 (AT1A1_PIG)]. While the α1 subunit is highly conserved amongst human, pig, rat, and mouse (the homology

is over 98.5%), the identified peptide is 100% identical amongst these four species (**Table 1**). This peptide is located in the actuator (A) domain of α1 subunit, and Pro222/Thr224 are highly exposed and facing the nucleotide binding (N) domain of the α1 subunit. Upon ouabain binding, Na/K-ATPase undergoes conformational changes, in which the A domain is rotated to the N domain. Structure-function analysis indicates that these conformational changes may affect binding of the α1 subunit to signaling molecules such as c-Src and PI3K (Yatime et al., 2011). In addition, the peptide also contains the TGES motif that is the anchor of A domain rotation (Yatime et al., 2011). In immunoprecipitated α1 subunit, both ouabain and glucose oxidase do not induce formation of advanced glycation end products (AGEs) adducts. Like ouabain, glucose oxidase is able to activate Na/K-ATPase signaling, leading to reduction of transcellular $^{22}Na^{+}$ transport.

Recent studies suggest that, in biological systems, protein carbonylation is reversible (decarbonylation) and may function as regulatory mechanism of cell signaling (Wong et al., 2008, 2010, 2012, 2013). We also observed a decarbonylation mechanism, which apparently reverses the carbonylation of the Na/K-ATPase α1 subunit induced by CTS (Yan et al., 2013). Removal of ouabain from the culture medium clearly reverses ouabain-mediated carbonylation; inhibition of *de novo* protein synthesis as well as degradation pathways through lysosome and proteasome does not affect this decarbonylation, which is still poorly understood. It is possible that carbonylation modification might stabilize the Na/K-ATPase in a certain conformational status favoring ouabain binding to the Na/K-ATPase α1 subunit and ouabain-Na/K-ATPase signaling, as seen in S-glutathionylation of cysteine residue(s) of the Na/K-ATPase (Figtree et al., 2009; Petrushanko et al., 2012). Nevertheless, the underlying mechanism might be physiologically significant since the carbonylation/decarbonylation process could be an important regulator of the RPT Na/K-ATPase signaling and sodium handling.

OXIDATIVE (CARBONYLATION) MODIFICATION AND SALT SENSITIVITY, A HYPOTHESIS

Based on our data and literatures, we propose that carbonylation modification of RPT Na/K-ATPase α1 subunit has biphasic effects. (1) Physiological and controllable α1 carbonylation stimulates Na/K-ATPase signaling and sodium excretion, rendering salt resistance (**Figure 2A**) whereas (2) prolonged exposure to oxidant stress leads to overstimulated α1 carbonylation and desensitized Na/K-ATPase signaling, effecting salt sensitivity (**Figure 2B**). First, Dahl S rats show considerably higher basal levels of oxidative stress than R rats, and high salt diets increase renal oxidative stresses that contribute to salt-sensitive hypertension (Meng et al., 2002; Kitiyakara et al., 2003; Taylor et al., 2006). Second, while high salt diets increase

TABLE 1 | Partial alignment of α1 subunit of human, pig, rat, and mouse.

SP|P05023|ATA1_HUMAN	211	CKVDNSSLTGESEP^{224}QT^{226}RSPDFTNENPLETR	240
SP|P05024|ATA1_PIG	209	CKVDNSSLTGESEP^{222}QT^{224}RSPDFTNENPLETR	238
SP|P06685|ATA1_RAT	211	CKVDNSSLTGESEP^{224}QT^{226}RSPDFTNENPLETR	240
SP|Q8VDN2|ATA1_MOUSE	211	CKVDNSSLTGESEP^{224}QT^{226}RSPDFTNENPLETR	240

Pro and Thr are shown in red.

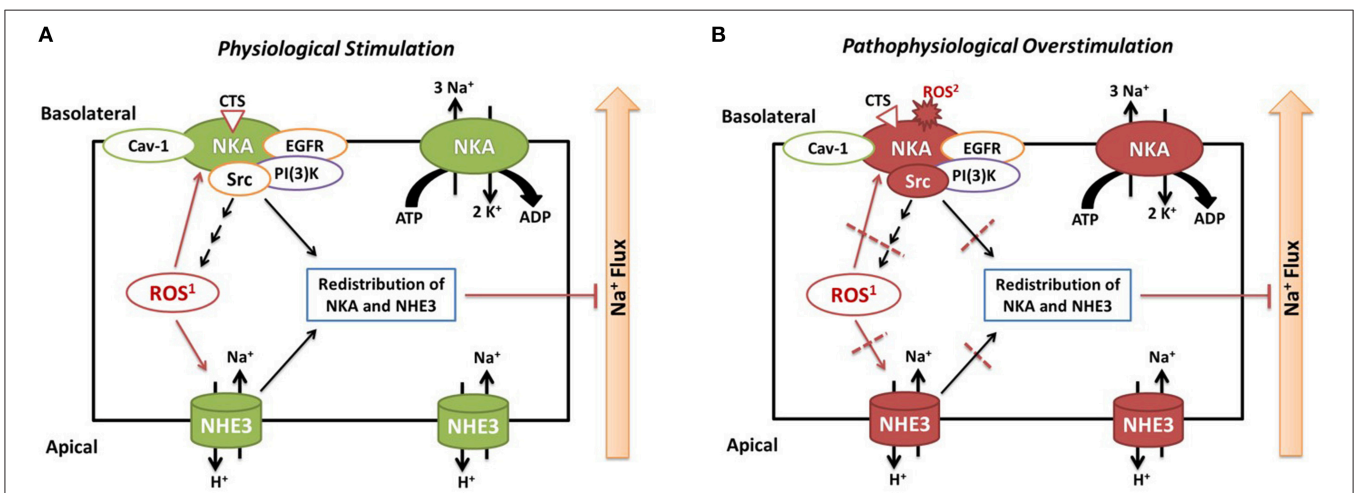

FIGURE 2 | Illustrated hypothesis of α1 carbonylation on Na/K-ATPase signaling and sodium handling. Panel **(A)** shows events under physiological stimulation and Panel **(B)** shows events under pathophysiological overstimulation. ROS1, CTS induced intracellular ROS generation through Na/K-ATPase signaling; ROS2, extracellular ROS generated by glucose oxidase or other stimuli.

circulating CTS, we have observed that a high salt diet (HS, 2% NaCl for 7 days) stimulates the Na/K-ATPase signaling in isolated RPTs from Dahl R but not S rats (i.e., impaired Na/K-ATPase signaling in S rats; Liu et al., 2011). Third, in RPT LLC-PK1 cells, CTS- and H_2O_2-mediated redox-sensitive Na/K-ATPase signaling and α1 carbonylation is involved in this signaling process, in a feed-forwarding mechanism (Yan et al., 2013). Fourth, high but not low concentration of NAC is able to prevent α1 carbonylation and Na/K-ATPase signaling (Yan et al., 2013). Even though it is still not clear of the carbonylation/decarbonylation process, it is reasonable to postulate that prolonged excessive α1 carbonylation (by CTS and/or other factors) might overcome the decarbonylation capacity, leading to desensitization or termination of the Na/K-ATPase signaling function. This is reminiscent of the observations in clinical trials using antioxidant supplements. The beneficial effect of antioxidant supplements is controversial and not seen in most clinical trials with administration of antioxidant supplements (reviewed in Touyz, 2004; Munzel et al., 2010). Low doses of antioxidant supplementation may be ineffective, but high doses may be even dangerous since excess antioxidants might become pro-oxidants if they cannot promptly be reduced in the anti-oxidant chain (Huang et al., 2006). It appears that the balance of the redox status, within a physiological range, may be critical in order to maintain beneficial ROS signaling.

PERSPECTIVE

The Na/K-ATPase has recently emerged as a therapeutic target (Aperia, 2007; Yatime et al., 2009). A clearer understanding of the mechanisms whereby a CTS-ROS-Na/K-ATPase signaling axis counterbalancing salt retention would have major pathophysiological and therapeutic implications, and further explain the progressive impairment of renal sodium handling under excessive oxidative stresses such as hypertension, aging, obesity, and diabetes. Impairment of coordinated regulation of the basolateral Na/K-ATPase and the apical NHE3 antiporter is implicated in salt-sensitive BP changes. Furthermore, recent evidence suggests both the Na/K-ATPase and, its adjacent signaling counterpart, c-Src to be redox-sensitive. Although carbonylation modification of the Na/K-ATPase is involved in the Na/K-ATPase signaling, a more thorough mechanistic understanding is necessary. Some pertinent questions remain to be resolved, such as the possible effect of carbonylation on CTS binding affinity, Na/K-ATPase conformational change, mechanisms of carbonylation/decarbonylation, and the destiny of the carbonylated Na/K-ATPase.

AUTHOR CONTRIBUTIONS

PS, RM, YY, JS, and JL discussed the topic and wrote the manuscript. YY, JS, and JL reviewed and commended on the manuscript. PS and RM did the final edit.

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ATP1A2 Mutations in Migraine: Seeing through the Facets of an Ion Pump onto the Neurobiology of Disease

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Mutations in four genes have been identified in familial hemiplegic migraine (FHM), from which *CACNA1A* (FHM type 1) and *SCN1A* (FHM type 3) code for neuronal voltage-gated calcium or sodium channels, respectively, while *ATP1A2* (FHM type 2) encodes the α_2 isoform of the Na^+,K^+-ATPase's catalytic subunit, thus classifying FHM primarily as an ion channel/ion transporter pathology. FHM type 4 is attributed to mutations in the *PRRT2* gene, which encodes a proline-rich transmembrane protein of as yet unknown function. The Na^+,K^+-ATPase maintains the physiological gradients for Na^+ and K^+ ions and is, therefore, critical for the activity of ion channels and transporters involved neuronal excitability, neurotransmitter uptake or Ca^{2+} signaling. Strikingly diverse functional abnormalities have been identified for disease-linked ATP1A2 mutations which frequently lead to changes in the enzyme's voltage-dependent properties, kinetics, or apparent cation affinities, but some mutations are truly deleterious for enzyme function and thus cause full haploinsufficiency. Here, we summarize structural and functional data about the Na^+,K^+-ATPase available to date and an overview is provided about the particular properties of the α_2 isoform that explain its physiological relevance in electrically excitable tissues. In addition, current concepts about the neurobiology of migraine, the correlations between primary brain dysfunction and mechanisms of headache pain generation are described, together with insights gained recently from modeling approaches in computational neuroscience. Then, a survey is given about ATP1A2 mutations implicated in migraine cases as documented in the literature with focus on mutations that were described to completely destroy enzyme function, or lead to misfolded or mistargeted protein in particular model cell lines. We also discuss whether or not there are correlations between these most severe mutational effects and clinical phenotypes. Finally, perspectives for future research on the implications of Na^+,K^+-ATPase mutations in human pathologies are presented.

Keywords: familial hemiplegic migraine, Na^+,K^+-ATPase, human ATP1A2, neuronal hyperexcitability, protein expression, protein stability, protein targeting, structure-function studies

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Na^+,K^+-ATPase IN FAMILIAL HEMIPLEGIC MIGRAINE AND OTHER INHERITED DISEASES

Migraine is a particularly disabling pathology with a high cost for human society. The "Atlas of Headache Disorders and Resources in the World 2011" issued by the WHO reported that about 10% of the world's population suffer from migraine, with three times more women affected than men, causing 190 million days lost from work every year in Europe alone, which ranks migraine in the fourth place among neurological disorders with an estimated annual cost of about 116 billion Euros.

Migraine frequently appears with perceptional or somatosensory disturbances, which are called "aura" symptoms. These can include alterations in the field of vision (scotoma), flashes, strange smells or sounds, but also tingling, numbness or partial paresis. Familial Hemiplegic Migraine (FHM) is an autosomal dominantly inherited form of migraine with aura (MA, as opposed to migraine without aura, MO), in which the accompanying aura symptom of the typical half-sided headache is transient motor weakness (hemiparesis) that is frequently accompanied by other cortical symptoms. Four FHM types have been identified by human geneticists, from which FHM type1 (FHM1) and type 3 (FHM3) affect the genes coding for the neuronal voltage-gated P/Q-type calcium channel's α-subunit (*CACNA1A*) (Ophoff et al., 1996) or the neuronal voltage-gated sodium channel's α-subunit (*SCN1A*) (Dichgans et al., 2005), respectively. FHM type 2 (FHM2) is caused by mutations in the *ATP1A2* gene (De Fusco et al., 2003), which encodes the isoform 2 of the human Na^+,K^+-ATPase's large catalytic α-subunit, which in the adult central nervous system (CNS) is mainly expressed in astrocytes. Recently, a fourth FHM gene, *PRRT2*, has been identified (Riant et al., 2012), which encodes a proline-rich transmembrane protein of still unknown function. The PPTR2 protein was suggested to interact with the synaptosomal-associated protein 25 (SNAP-25), a t-SNARE protein, which accounts for the specificity and execution of synaptic vesicle fusion with the plasma membrane (Rizo and Südhof, 2002).

Hemiplegic migraine and MA/MO also occur as a comorbidity in proximal renal tubular acidosis (pRTA) patients carrying certain homozygous mutations in the *SLC4A4* gene (encoding the Na^+-HCO_3^- cotransporter NBCe1), in which mutations in the other known FHM-related genes were ruled out (Suzuki et al., 2010). The NBCe1B splice variant is expressed in several tissues including brain, and its transport activity in astrocytes is thought to modulate neuronal excitability by regulating local pH (Chesler, 2003) suggesting that also defective pH regulation in the brain may be a susceptibility factor in hemiplegic and other types of migraine.

The Na^+,K^+-ATPase belongs to the large family of P-type ATPases (Axelsen and Palmgren, 1998). The minimal unit is composed of a large catalytic α-subunit (∼1020 amino acids, see Section Functional Insights Gained from Structural Studies) and a smaller, ancillary β-subunit (∼300 amino acids, one transmembrane domain (TM) with a heavily glycosylated ectodomain). The β-subunit is a mandatory feature of K^+-countertransporting P_{2C}-type ATPases, which assists in proper folding, assembly and targeting of the holoenzyme (Jaunin et al., 1993), and modulates cation affinities (Crambert et al., 2000). According to molecular modeling studies, the particular β-isoform serves in tuning the pump depending on its individual tilt angle (Hilbers et al., 2016) by differentially stabilizing the $E_1P(3Na^+)$ state. There is a still unresolved controversy about the existence of higher oligomeric states (see Donnet et al., 2001; Clarke, 2009; Shattock et al., 2015; and references therein), which, if true, would allow for speculations about possible dominant-negative effects in the heterozygous state of affected patients. Based on earlier biochemical evidence (Forbush et al., 1978), a third, auxiliary γ-subunit was identified (66 amino acids, one TM) (Mercer et al., 1993), which belongs to the class of FXYD-domain containing ion transport regulator proteins (Sweadner and Rael, 2000) and is now classified as FXYD2. The FXYD family, named after the invariant amino acid motif FXYD, comprises seven members in humans (FXYD1, or phospholemman; FXYD2, or Na^+,K^+-ATPase γ-subunit; FXYD3, or Mat-8; FXYD4, or corticosteroid hormone-induced factor, CHIF; FXYD5, or "related to ion channel", RIC, also termed dysadherin; FXYD6, or phosphohippolin; FXYD7), from which all but FXYD6 were shown to associate with Na^+,K^+-ATPase α/β-complexes and exerted distinct effects on pump function (see reviews by Garty and Karlish, 2006; Geering, 2006). Since the various FXYD isoforms have different tissue distribution and functional effects, with prominent expression in electrically excitable or fluid- and solute-transporting tissues, these proteins act as tissue-specific modulators of Na^+,K^+-ATPase in order to fine-tune its kinetic properties according to the tissue's requirements or physiological state. In the brain, FXYD1, -6, and -7 are the most abundant isoforms (Garty and Karlish, 2006).

Four α-isoforms exist in humans, from which α_1 is ubiquitously expressed and therefore the most indispensable isoform for cellular ion homeostasis, volume regulation, excitability etc. The α_2-isoform (ATP1A2) is particularly high expressed in skeletal muscle (SM), but also heart and vascular smooth muscle (VSM) express it. In the adult central nervous system (CNS), α_2 is mainly found in astrocytes, whereas α_3 (ATP1A3) is neuronal-specific, and α_4 has only been found in testis (spermatozoa) (see Blanco et al., 1999; Larsen et al., 2014; Shattock et al., 2015 and references therein). Two other types of human inherited diseases have been linked to α-subunit isoforms. Mutations in the *ATP1A3* gene cause Rapid Dystonia Parkinsonism (RDP, DYT12) (de Carvalho Aguiar et al., 2004), as well as Alternating Hemiplegia of Childhood (AHC) (Heinzen et al., 2012). While the *ATP1A1* gene for the ubiquitously expressed α_1-isoform is regarded as a susceptibility locus in human essential hypertension (Glorioso et al., 2001), congenital mutations in the gene have not been described so far. However, somatic *ATP1A1* mutations were detected in aldosterone-producing adenomas (APA) and secondary hypertension (Azizan et al., 2013; Beuschlein et al., 2013). Functional studies of the ATP1A1 mutants showed loss of pump activity, strongly reduced K^+ affinity and augmented inward proton leak currents (see next section) at physiological potentials

and concentrations of Na^+ and K^+ (Azizan et al., 2013). Furthermore, abnormal depolarization was observed in primary adrenal adenoma cells (Beuschlein et al., 2013), which was suggested to be the consequence of enhanced inflow of protons rather than being caused by reduced Na^+,K^+ pumping (Azizan et al., 2013). This may indirectly lead to enhanced Ca^{2+} signaling and, consequently, enhanced aldosterone output (Beuschlein et al., 2013). Another syndrome, renal hypomagnesemia type 2 (HOMG2), an autosomal dominant pathology with isolated renal magnesium loss, is linked to mutations in the γ-subunit's *FXYD2* gene. Evidence has accumulated that *FXYD2* mutations cause misrouting of the Na^+,K^+-ATPase, which would lead to loss of plasma membrane protein in the kidney (Meij et al., 2000).

At least indirectly associated with Na^+,K^+-ATPase is Long QT Syndrome type 4 (LQT4), a type of cardiac arrythmia with a prolonged QT-phase in electrocardiograms (Lu and Kass, 2010) that frequently causes cardiac fibrillation and sudden death. Unlike other LQT types, LQT4 does not relate to genes of cardiac ion channels, but affects the *ANK2* gene encoding ankyrin-B. Ankyrin-B serves as a scaffold protein responsible for proper targeting of Na^+,K^+-ATPase, the Na^+,Ca^{2+}-exchanger and the InsP3 receptor to T-tubules/sarcoplasmic reticulum microdomains in cardiac muscle cells (Mohler et al., 2005). Thus, not only molecular function, but also cellular processing must be considered in the neurobiology of disease.

FHM2 cases frequently share comorbidity with other neurological disorders, such as seizures, AHC and even epilepsy (Supplementary Table 1). This overlap is intriguing, since all these phenomena are linked to deficient regulation of the cortical excitatory/inhibitory balance (Pietrobon and Moskowitz, 2013). Most of the aura symptoms are caused by the phenomenon of Cortical Spreading Depression (CSD) (Leão, 1944) or CSD-like events that are characterized by a spreading front of excitation, which is followed by a long-lasting depression (Moskowitz et al., 2004). Whereas CSD spreads slowly over the neocortex, epilepsy is characterized by rapidly circulating waves of neuronal hyperexcitation. This pathophysiological overlap raises the question, which parameters determine the evolution of such a highly non-linear excitable system as the neocortex into one or the other hyperexcitation pattern (Ullah et al., 2015).

Na^+,K^+-ATPase: FUNCTION AND STRUCTURE

Functional Properties of the Na^+,K^+-ATPase

The Na^+,K^+-ATPase is an electrogenic, primary active transporter protein, which energizes the membrane of all animal cells with the characteristic electrochemical gradients for Na^+ and K^+ ions. These gradients are pivotal for the activity of secondary active transporters such as the Na^+,Ca^{2+}-exchanger (NCX), neurotransmitter uptake transporters or voltage-gated Na^+ and K^+ channels involved in electrical excitability. The mechanism of function is generally expressed in the form of the Post-Albers scheme (Albers, 1967; Post et al., 1972), as shown in **Figure 1**. In each reaction cycle, the Na^+,K^+-ATPase transports three Na^+ ions out of and two K^+ ions into the cell upon hydrolysis of one ATP molecule. The enzyme undergoes cyclic interconversions between two principal conformations, E_1 and E_2 and phosphorylated intermediates thereof, E_1P and E_2P, in which a phosphate group from ATP is covalently attached to a critical aspartate residue within the S**D**KTGTLT motif (see next section about structural details). Upon binding of three Na^+ ions from the intracellular side in the ATP-bound E_1 conformation, the phosphorylated intermediate with three occluded Na^+ ions, $E_1P(3Na^+)$, is formed. This is followed by a conformational change to the $E_2P(3Na^+)$ conformation, from which Na^+ ions are extracellularly released. Because of the increased affinity for K^+ in this configuration, two K^+ ions bind subsequently from the extracellular side, which triggers dephosphorylation and occlusion of two K^+ ions in the $E_2(2K^+)$ state. After another conformational change to $E_1(2K^+)$, the K^+ ions dissociate into the cytoplasm, a process that is speeded up by ATP binding.

Also included in **Figure 1** are inhibitors of the Na^+ pump. Ouabain (or g-strophanthin) belongs to the family of cardiotonic steroids, from which digitalis (g-strophantidin) from the red foxglove *Purpurea officinalis* was in medicinal use against dropsy and cardiac insufficiency for centuries (Withering, 1785). Ouabain is used to isolate Na^+,K^+-ATPase functional activity in cells or tissue preparations and was a prerequisite for the identification of the "sodium- and potassium-transporting adenoside triphosphatase" by Chemistry Nobel Prize awardee Jens Christian Skou (Skou, 1957). The compound arrests the Na^+ pump with high affinity (with IC_{50} or K_D values in the range of tens of nM for human Na^+,K^+-ATPase α_1-, α_2-, or α_3-subunits in complex with human β_1-subunit, (see Katz et al., 2010; Weigand et al., 2014a) and references therein) by binding from the extracellular side to the E_2P conformation, and this interaction competes with extracellular K^+ binding. In contrast, (ortho-)vanadate (VO_4^-) blocks the pump from the intracellular side with nanomolar affinity. With its similarity to the phosphate (PO_4^{3-}) anion and its trigonal bipyramidal structure, it serves as a transition state analog for the hydrolytic dephosphorylation of the phosphointermediate. Vanadate arrests the Na^+ pump in the $E_2P(3Na^+)$ form (Glynn, 1985). Oligomycin, a macrolide antibiotic from *Streptomyces*, also inhibits the Na^+,K^+-ATPase (Glynn, 1985) by promoting the occlusion of Na^+ ions in the E_1P form, and decreases the rate of Na^+ release from the phosphoenzyme, thereby inhibiting the $E_1P \rightarrow E_2P$ interconversion (Glynn, 1985; Skou, 1990).

The sequential translocation of Na^+ and K^+ ions requires strict cation specificity of the phosphorylation and dephosphorylation reactions, mutual changes of the apparent affinities for Na^+ and K^+ and alternating access of the cation binding sites to the extra- and intracellular medium. Active transport by an ion pump also requires the operation of two "occlusion gates" (indicated by a blue and red bar in **Figure 1**) shielding the bound cations from the extra- or intracellular medium, which must never be open simultaneously (Gadsby, 2009). According to the $3Na^+/2K^+$ stoichiometry, the Na^+,K^+-ATPase produces an outward movement of one positive charge per cycle and generates a pump current. The major electrogenic event has been shown to take place during extracellular release (or reverse binding) of Na^+ (Fendler et al., 1985; Gadsby et al.,

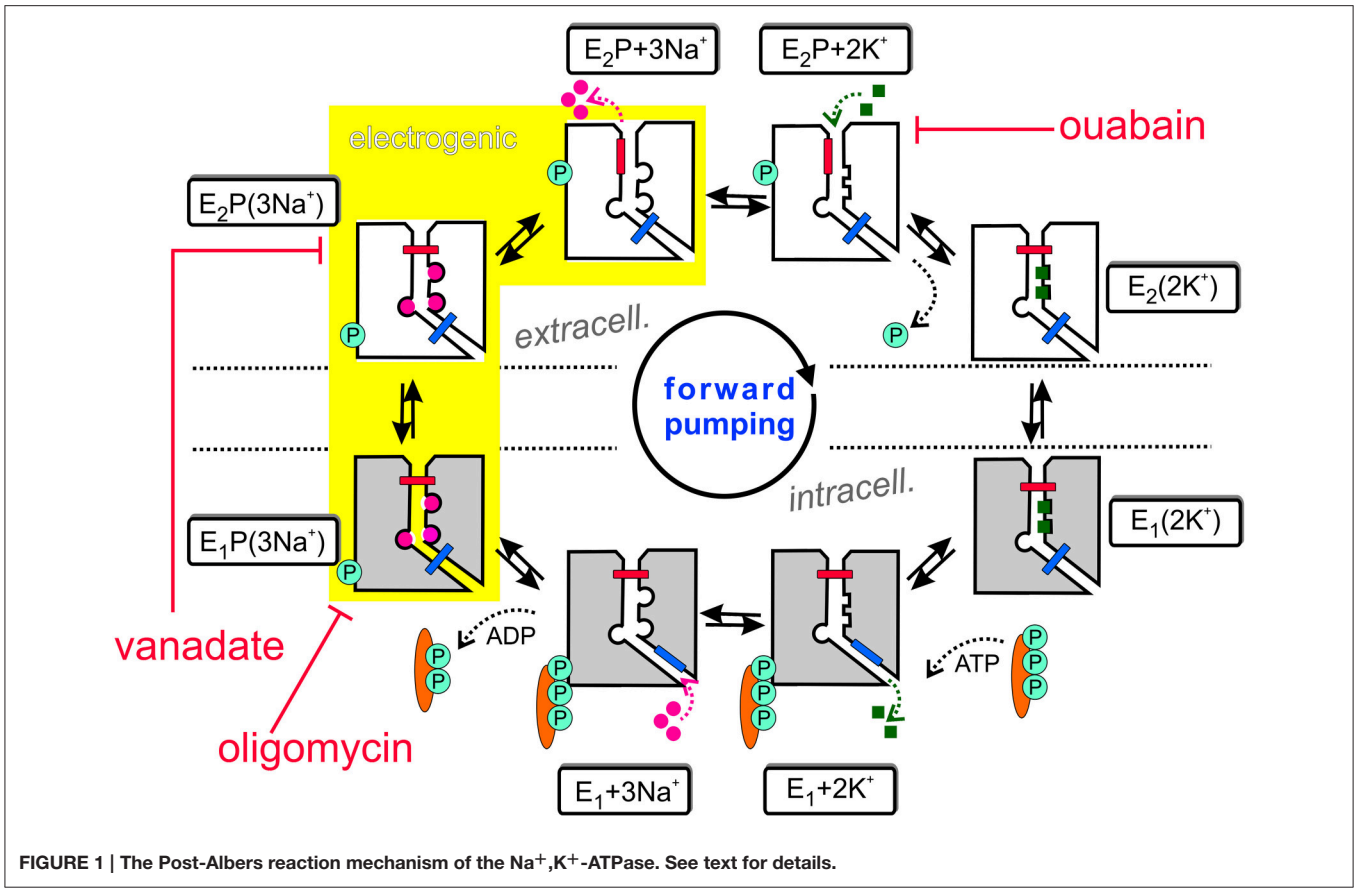

FIGURE 1 | The Post-Albers reaction mechanism of the Na^+,K^+-ATPase. See text for details.

1985; Nakao and Gadsby, 1986; Gadsby and Nakao, 1989; Rakowski et al., 1991; Rakowski, 1993; Wuddel and Apell, 1995) within a sequence of coupled partial reactions, which is underlaid in yellow in **Figure 1**. Electrogenicity arises from passage of Na^+ ions through a narrow, high-field "access channel" to/from the extracellular space (Läuger, 1979; Gadsby et al., 1993; Hilgemann, 1994; Sagar and Rakowski, 1994; Rakowski et al., 1997; Holmgren et al., 2000; Holmgren and Rakowski, 2006). This term, combined with the notion of its "fractional depth", is frequently used to denote that an ion passes a certain fraction of the transmembrane electric field in order to reach or exit from its binding site. In the same way as the existence of a positive slope of the stationary current-voltage (I-V) curve in the negative voltage range indicates electrogenic extracellular Na^+ release (or reverse binding) (Nakao and Gadsby, 1989), the negative slope in the I-V curve at positive voltages (Rakowski et al., 1991) suggests that also K^+ ions bind within an extracellular access channel, albeit of smaller fractional depth (see Section Electrophysiological Assays: The Two-Electrode Voltage Clamp for more details). There might well be other steps in the catalytic cycle, which contribute to the total electrogenicity of the Na^+ pump, such as intracellular Na^+ binding, as shown by Pintschovius et al. (1999) as well as Apell and Karlish (2001).

Electrophysiology has elucidated another functional detail of the Na^+,K^+-ATPase, namely the ouabain-sensitive "leak currents", which were first observed at negative voltages in the absence of extracellular Na^+ and K^+, and are augmented by extracellular acidification. Initially reported by Rakowski et al. (1991) and later investigated in more detail by Efthymiadis et al. (1993), Wang and Horisberger (1995), Rettinger (1996) and Li et al. (2006), this property has for a long time been merely recognized as a footnote in Na^+ pump research, until structural and functional evidence highlighted the critical role of protons in the transport cycle, as indicated by mutations that interfere with a C-terminal pathway for protons (see Section Functional Insights Gained from Structural Studies) to access the cation binding pocket (Morth et al., 2007; Poulsen et al., 2010). Recently, it was demonstrated that the proton leak inward current is a property inherent to Na^+,K^+-ATPase that also flows at physiological K^+ and Na^+ concentrations and membrane potentials (Mitchell et al., 2014; Vedovato and Gadsby, 2014), even in native cells, i.e., in the presence of the pump's normal regulatory subunit phospholemman, which is prevalent in cardiac tissue (Mitchell et al., 2014). Vedovato and Gadsby concluded that inward proton leak exploits the reversibility of a subset of conformational changes associated with extracellular Na^+ release from the phosphorylated enzyme. Although such a back-step of phosphorylated Na^+,K^+-ATPase that enables proton import is not required for completion of the $3Na^+/2K^+$ transport cycle, it readily occurs during Na^+,K^+ transport when external K^+

ion binding and occlusion are retarded, and it occurs more frequently when the probability for extracellular proton access is increased by acidification (Vedovato and Gadsby, 2014). The protons presumably pass through the Na^+-selective binding site III (which may be in fact two sites, see Section Functional Insights Gained from Structural Studies) via the carboxylates of Glu-958 to Asp-930 *via* the intervening hydroxyl of Tyr-775 (ATP1A2 numbering), which is distinct from the principal pathway of the Na^+ and K^+ ions passing through binding site II. From the simultaneous occurrence of Na^+,K^+ exchange and H^+ import during the same conformational cycle of a single molecule, the Na^+,K^+-ATPase classifies as a hybrid transporter, although the physiological or pathophysiological significance of pump–mediated proton inflow still has to be clarified (Vedovato and Gadsby, 2014). By performing experiments over an expanded range of extracellular pH (pH_o) values and meticulous ion competition assays, Mitchell et al. delineated a previously unrecognized inhibitory action of extracellular protons on the leak current, which occurs in addition to the pH_o-induced leak stimulation (Mitchell et al., 2014). Based on the strong voltage dependence of the pH_o-induced leak stimulation, these authors concluded that protons leak through the site responsible for strong Na_o- and voltage-dependent inhibition of pump current, which must be site III, in agreement with (Vedovato and Gadsby, 2014). The pH_o-dependent inhibition of the leak, although profoundly enhanced by decreasing pH_o, was nevertheless only weakly voltage-dependent, similar to the inhibition by Na_o^+ or extracellular K^+ binding, suggesting that inhibition of the leak current by external cations (H^+, Na^+ or K^+) requires binding to sites I and II that are not responsible for the Na^+,K^+-ATPase's voltage dependence (Mitchell et al., 2014).

The inward current was found to be augmented by mutations at the Na^+,K^+-ATPase's C-terminus (Yaragatupalli et al., 2009; Meier et al., 2010; Poulsen et al., 2010; Vedovato and Gadsby, 2010; Paulsen et al., 2012), and sometimes further enhanced with increasing extracellular [Na^+], which conforms with an earlier proposal that Na^+ ions might flow along what has been referred to as the Na^+,K^+-ATPase "leak" pathway, a process that is promoted by extracellular protons (Vasilyev et al., 2004). The studied C-terminal mutations frequently correlate with drastic decreases in the apparent affinity for extracellular and intracellular Na^+ (Toustrup-Jensen et al., 2009, 2014), because C-terminal mutations interfere with Na^+ binding site III.

Whether or not Na^+,K^+ pump-mediated proton uptake plays a physiological or pathological role is still unclear. Vedovato and Gadsby pointed out, that already at physiological pH_o, and even more so at decreased pH_o in a pathophysiological situation, the Na^+,K^+ pump will import one or several protons downhill in each ATPase transport cycle. Thus, proton inward leak might significantly accompany Na^+,K^+ pumping at the normal negative resting potentials of neurons as well as cardiac and skeletal muscle cells, if extracytoplasmic pH became sufficiently low, as e.g., during vigorous muscle exercise, or in cardiac or cerebral ischemia, since Na^+,K^+-ATPase densities in muscle, nerve, and heart can be very high ($\geq 1000\,\mu m^2$). It is also conceivable that the known limitation of H^+-ATPase-mediated acidification of early endosomes by endocytosed Na^+,K^+-ATPase might be the direct consequence of Na^+,K^+ pump-mediated flow of protons from the endosome lumen to the cytoplasm (Vedovato and Gadsby, 2014). The importance of cortical pH regulation is underlined by the involvement of certain homozygous mutations in the NBCe1 Na^+-HCO_3^- cotransporter, which is expressed in astrocytes, in pRTA patients that additionally suffer from hemiplegic migraine (Suzuki et al., 2010). Glial cell depolarization usually results from elevated extracellular [K^+], which causes glial cell acid secretion via inward electrogenic Na^+-HCO_3^- cotransport, resulting in depolarization-induced alkalosis (DIA) in the cytoplasm of glial cells (Chesler, 2003). The extracellular acidosis that occurs simultaneously to DIA makes the surrounding neuronal cells less excitable because excitatory NMDA receptors are blocked by protons (Suzuki et al., 2010). Therefore, extracellular acidosis suppresses neuronal excitability, and the reduction of DIA due to enhanced proton inward transport by mutant glial Na^+,K^+-ATPase could also create a positive feedback loop of increased neuronal activity leading to further NMDA-mediated neuronal hyperactivity, depolarization of brain cells, and CSD (Suzuki et al., 2010). Although increased proton leak has only been reported for the R937P mutation in ATP1A2 (Poulsen et al., 2010), this may not be an isolated observation, since no other ATP1A2 allele has so far been scrutinized for altered proton leak.

Functional Insights Gained from Structural Studies

For the Na^+,K^+-ATPase, high-resolution structures of different reaction cycle intermediates are available, which provided an atomic-level understanding of active cation transport coupled to enzymatic catalysis. Structures include the Rb^+-occluded E_2P-like conformation $[Rb_2^+]E_2{\cdot}MgF_4^{2-}$ (Morth et al., 2007), which was further refined in the $E_2{\cdot}2K^+{\cdot}P_i$ (also stabilized by MgF_4^{2-}) structure that revealed the full arrangement of the β-subunit (Shinoda et al., 2009), the low-affinity ouabain- and $2K^+$-bound $E_2{\cdot}2K^+{\cdot}P_i$ (also stabilized by MgF_4^{2-}) structure (Ogawa et al., 2009), the high-affinity ouabain-bound E_2P-like state with Mg^{2+} bound to the cation binding pocket (Ogawa et al., 2009), the Na^+-bound $E_1{\cdot}(AlF_4^-){\cdot}ADP{\cdot}3Na^+$ structure (stabilized with AlF_4^-) of an intermediate preceding the Na^+-occluded $E_1P(3Na^+)$ state (Kanai et al., 2013), and a comparable $[Na_3^+]E_1P$-ADP state in complex with AlF_4^-, with Na^+ saturation stabilized by oligomycin (Nyblom et al., 2013). These structural data, together with the wealth of intermediate structures determined for the related SERCA Ca^{2+}-ATPase from sarcoendoplasmic reticulum (Olesen et al., 2007), provide a comprehensive concept of the catalytic mechanism carried out by the Na^+ pump.

Figure 2 shows the arrangement of the α-, β- and γ-subunit of the Na^+,K^+-ATPase in the $2Rb^+$-bound E_2P-like conformation (Morth et al., 2007). Several distinct domains can be distinguished on the α-subunit, with 10 TM segments forming the TM domain harboring the coordination sites for cations and the central TM5 helix, which extends into the central P (phosphorylation) domain. The P domain harbors the P-type ATPase consensus motif ^{373}S**D**KTGTLT^{380} with the intermediately phosphorylated Asp-374 (all numbering refers to

human ATP1A2), which is surrounded by the ^{612}MVTGD^{616} and ^{713}DG(V/M)ND^{717} motifs and the critical Lys-605. Nucleotide binding occurs in the N domain, and the A (actuator) domain serves as an anchor for the movement of the N domain by performing a hinge-like movement during the conformational cycle to bring the conserved ^{217}TGES^{220} motif into close proximity with ^{612}MVTGD^{616} to expedite dephosphorylation. Movement of the A domain also entails a piston-like movement of TM helices 1 and 2. The crystal structures also highlighted a particularly crucial arrangement of the enzyme's C-terminal sequence ^{1013}WVEKETYY^{1020}. The first part of this motif assumes an α-helical structure accommodated between the TM of the β-subunit and the TM7 and TM10 helices, and the two terminal tyrosines project into a binding pocket between TM7, TM8, and TM5, with the terminal Tyr-1020 interacting with Lys-770 on TM5 and Arg-937 in the loop connecting TM8 and TM9 (Morth et al., 2007). Deletion of the terminal KETYY sequence resulted in a 26-fold reduction of Na^+ affinity, reminiscent of the effect of mutations of putative Na^+ coordinating residues. This has prompted investigations of the functional importance of C-terminal residues by studying deletions, mutations of the terminal tyrosines, or C-terminal extensions. These studies showed that alterations in the C-terminal sequence entail drastic decreases in Na^+ affinity and enhance the propensity of the mutant pumps to permit inward proton leak (Yaragatupalli et al., 2009; Meier et al., 2010; Poulsen et al., 2010; Vedovato and Gadsby, 2010, 2014; Paulsen et al., 2012). This has given rise to the concept that the C-terminal pathway occupied by the terminal tyrosines defines a mandatory access route for intracellular protons to the Na^+ binding site III to bring about stoichiometric $3Na^+/2K^+$ transport, in which Arg-937 and Asp-923 play a critical role (Poulsen et al., 2010).

From the two "common" cation binding sites, site I is made up from five oxygen atoms (from Thr-776 main chain, and from Ser-779, Asn-780 (all TM5), and Asp-808 (TM6) side chains), and one water molecule, whereas site II is coordinated by the main chain carbonyls of Val-327, Ala-328, and Val-330 (all TM4) and the side chain oxygens of Asn-780, Glu-783 (TM5) and Asp-808 (TM6), and possibly Glu-332 (TM4) (Shinoda et al., 2009). Of note, the TM5 helix is unwound at Asn-780 to create sufficient space for cation coordination. The unwinding is due to Pro-782; both amino acids reside in the highly conserved ^{779}SNIPE^{783} motif. In the two E_1·P·ADP·$3Na^+$-like structures, the geometry for Na^+ coordination at the two "common" sites is essentially the same, with similar TM5 helix unwinding. Regarding the location of Na^+ binding site III, the structure by Kanai et al. (2013) identified two slightly different locations in the two protomers of the crystallographic unit, and the study by Nyblom et al. (2013) had to resolve a similar ambiguity by proposing two sites, IIIa and IIIb, from which site IIIa is surrounded by Glu-958, Tyr-775, and Thr-811, and site IIIb adjacent to Thr-778, Gln-858, Gln-927, and Asp-930, with a sufficiently clear electron density to identify IIIb as primary Na^+ binding site III. Mutational analysis and electrophysiology suggested that site IIIa is a transient Na^+ binding site during extracellular Na^+ release. The latter was also inferred from proton leak currents of mutant enzymes (see below), which showed that site IIIa mutations (Y775F, Q958A) permitted leak currents in the absence of extracellular Na^+ but with Na^+ closing the leak, whereas site IIIb mutations (D930E and Q858N) permitted leak currents with and without Na^+. This gave rise to the concept that protonation of Asp-930 is associated with voltage-dependent release of Na^+ from site IIIb via site IIIa to the extracellular space. The proton leak current can occur under conditions when either no Na^+ ions are bound or when only the two common sites are occupied by Na^+ ions (Nyblom et al., 2013), or, as later shown, by protons (Mitchell et al., 2014). Then, site IIIa is accessible to an extracellular proton, which upon application of negative voltage can move via site IIIb to the cytoplasm, thus making site IIIa accessible for an extracellular proton again, which eventually leads to a sustained inward proton leak current. This notion was corroborated and refined by two comprehensive electrophysiological studies (Mitchell et al., 2014; Vedovato and Gadsby, 2014).

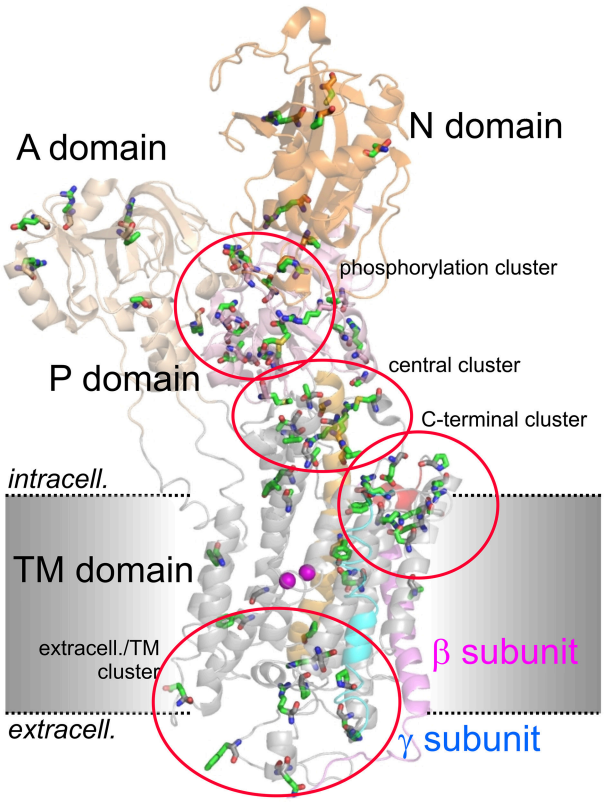

FIGURE 2 | Structure of the Na^+,K^+-ATPase (PDB code 3B8E; Morth et al., 2007) and location of migraine-associated ATP1A2 mutations. The domains of the cytoplasmic part are A (actuator; light orange), N (nucleotide binding; orange) and P (phosphorylation; pink) domain, transmembrane helices are depicted in gray, except for the about 70 Å-long central TM5 helix (orange). The β- and γ-subunits are shown in magenta and blue, respectively, two Rb^+ ions in the cation binding pocket are shown as purple spheres. Amino acids mutated in migraine cases as listed in Supplementary Table 1 are shown in stick representation. More than 80% of mutations fall into four clusters, one around the catalytic P domain, one in a central region between P and TM domain, one within the extracellularly-facing part of the TM domain, and one around the enzyme's C-terminus.

THE HEADACHE OF ATP1A2 MUTATIONS: NEUROBIOLOGY OF MIGRAINE

How can Na^+,K^+-ATPase dysfunction be linked to migraine? In the case of *CACNA1A* and *SCN1A* mutations, functional disturbances are typically gain-of-function effects, and mutated channels, e.g., exhibit an abnormal residual activity in the inactivated state, which leads to prolonged cation influx into neurons, thus causing, *inter alia*, depolarization and lowering of the activation threshold. Whether ATP1A2 mutations in FHM2 classify as gain- or loss-of-function phenomena is not that clear-cut. While many mutations abolish or largely reduce Na^+,K^+ pumping implying loss-of-function, others are characterized by subtle changes in voltage dependence (Supplementary Table 1). In these cases, a shift of the pump current's I-V curve may entail loss-of-function in a particular voltage range, but gain-of-function in another. From the viewpoint of Na^+,K^+-ATPase cation transport, a first clue is obtained from the fact that hyperkalemia is a known CSD and migraine trigger. Since the transporter in neurons or astrocytes removes extracellular K^+, dysfunction of the pump can lead to elevated extracellular K^+ levels, which cause depolarization and reduce the activation threshold. But also Na^+ extrusion by the enzyme is critical, since, e.g., the activity of secondary active transporters depends on the Na^+ gradient across the membrane. First, the Na^+,Ca^{2+}-exchanger (NCX1) utilizes Na^+ import for the extrusion of Ca^{2+}, and failure to efficiently remove cytosolic Ca^{2+} during neuronal activity leads to Ca^{2+} accumulation in intracellular stores, from which it is then more heavily released during an action potential, and again, hyperexcitability is the outcome. Second, excitatory neurotransmitter uptake transporters (EAATs) also couple to the Na^+ gradient, and failure to remove neurotransmitters such as glutamate from the synaptic cleft again results in sustained hyperexcitability (see **Figure 3**).

It is generally accepted that cortical spreading depression (CSD) is the neurophysiological correlate of migraine aura (Pietrobon and Striessnig, 2003). Therefore, neurobiologists split up the question concerning the pathogenic mechanism of migraine into two parts: (1) What is the relation of ATP1A2 mutations to CSD, and (2), what are the links between CSD and cortical pain perception? Although the existence of a "migraine generator" in the brainstem has not been completely ruled out (Pietrobon and Moskowitz, 2013), there is currently no doubt, that CSD plays a central role in the pathophysiology of migraine.

From ATP1A2 Mutations to CSD: The Glutamatergic Hypothesis

From clinical studies, it is known that neurophysiological abnormalities in sensory information processing (which are most intense 12–24 h before a migraine attack during the premonitory phase, but disappear a few hours before or during the attack) change in intensity in temporal relation to the migraine episode. This suggests that some intrinsic mechanism in the brains of migraineurs progressively increases the dysfunction in central information processing and the susceptibility to a migraine trigger. These mechanisms may lead to the premonitory symptoms and, above a certain threshold of cortical dysfunction in response to migraine triggers, may eventually ignite CSD. Depending on the study, the cortex of migraineurs is hyperexcitable as a consequence of either enhanced excitation or reduced inhibition, or is hypoexcitable, or has a lower preactivation level. Thus, also here, rather than merely hypo- or hyperexcitability, defective regulation of cortical excitability and the consequently reduced ability to maintain the cortical excitatory/inhibitory (E/I) balance appears to underlie abnormal sensory processing (Pietrobon and Moskowitz, 2013, 2014).

The analysis of experimental CSD in FHM knockin mouse models suggests that CSD is a key migraine trigger, since both FHM1 and FHM2 knockin mice showed a lower electrical stimulation threshold for CSD induction and faster CSD propagation (van den Maagdenberg et al., 2004, 2010; Leo et al., 2011). In FHM1 knockin mice carrying the mild R192Q mutation or the severe S218L mutation in CACNA1A, the strength of CSD facilitation as well as the severity of the subsequent neurological motor deficits and the propensity of CSD to propagate into subcortical structures correlated with the strength of the gain-of-function of the CACNA1A channel and the severity of the clinical phenotype (van den Maagdenberg et al., 2004, 2010). Interestingly, the velocity of propagation and the frequency of CSDs elicited by local application of high [K^+] were larger in female than in male FHM1 mouse mutants, in correspondence with the higher migraine prevalence of females (Eikermann-Haerter et al., 2009). However, such gender differences were not found in FHM2 knockin mice carrying the (heterozygous) W887R mutation in ATP1A2 (Leo et al., 2011). More importantly, the analysis of cortical synaptic transmission in FHM1 knockin mice revealed differential effects of FHM1 mutations at excitatory and inhibitory synapses: Excitatory synaptic transmission on cortical pyramidal cells was enhanced as a consequence of increased action potential-evoked Ca^{2+} influx and increased glutamate release, and enhanced short-term synaptic depression during trains of action potentials was observed. In contrast, inhibitory neurotransmission at cortical fast spiking interneuron synapses was not altered in FHM1 knockin mice, although being initiated by P/Q-type Ca^{2+} channels as well (Tottene et al., 2009). Although these considerations were restricted to specific cortical subcircuits, the differential effect of FHM1 mutations on excitatory and inhibitory neurotransmission may produce hyperexcitation in certain brain conditions, but may leave the excitatory/inhibitory balance intact in others, consistent with the episodic nature of the disease (Pietrobon and Moskowitz, 2013). The gain-of-function of glutamate release at synapses of cortical pyramidal cells can explain the facilitation of experimental CSD in FHM1 knockin mice, which supports a model of CSD initiation, in which CACNA1A-dependent release of glutamate from cortical pyramidal cell synapses and activation of NMDA receptors play a key role in the positive feedback cycle that ignites CSD (Pietrobon and Moskowitz, 2013). It was suggested that excessive NMDA receptor-mediated glutamatergic transmission following impaired clearance of glutamate by astrocytic processes surrounding glutamatergic synapses (the "tripartite synapse" see

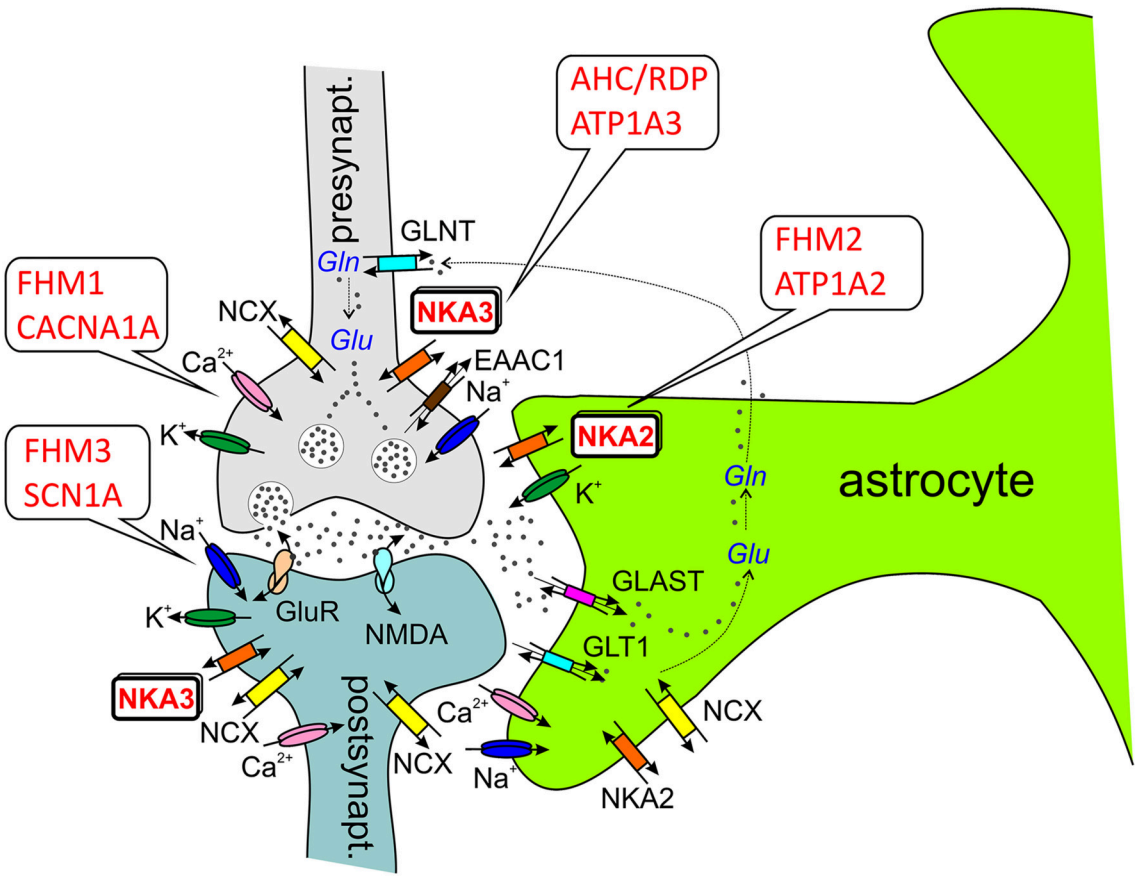

FIGURE 3 | ATP1A2 and ATP1A3 in the tripartite synapse (Perea et al., 2009). Most glutamatergic synapses are in contact with astrocytic processes, which express a high density of excitatory amino acid transporters (EAATs, e.g., GLT1, GLAST), which are crucial for the synaptic glutamate (Glu) clearance. In astrocytes, glutamate is converted to glutamine by glutamine synthetase as part of glutamate recycling. Ion channels involved in electrical excitation are indicated by the respective cations, Na^+, K^+, and Ca^{2+}. Abbreviations: GLAST (EAAT1), glutamate/aspartate transporter; GLT1 (EAAT2), glutamate transporter; GLNT, glutamine transporter; GluR, (metabotropic) glutamate receptor; NKA2/3, Na^+, K^+-ATPase α_2-α_3-subunit; NMDA, ionotropic glutamate receptor; NCX, Na^+,Ca^{2+}-exchanger; Gln, glutamine.

Figure 3), where the α_2 Na^+,K^+-ATPase is functionally coupled to glutamate transporters (Cholet et al., 2002; Rose et al., 2009), may underlie the enhanced CSD susceptibility in the FHM2 mouse model (Leo et al., 2011). This has given rise to the "glutamatergic" hypothesis. In the nervous system, the Na^+,K^+-ATPase α_2-isoform is expressed primarily in neurons during embryonic development and at birth, but almost exclusively in astrocytes in the adult (Moseley et al., 2003). Whereas neurons express α_1- and α_3-subunits (with distinctly different subcellular localization pattern, Juhaszova and Blaustein, 1997), astrocytes express α_1- and α_2-subunits, and studies on primary cultured rat astrocytes suggest, that the contribution of α_2 to extracellular K^+ clearance is about 30% of total Na^+,K^+-ATPase activity (Larsen et al., 2014). However, α_1 and α_2 are differentially distributed in astrocytes. Whereas α_1 is evenly present at the plasma membrane, α_2 rather shows a reticular distribution, like the Na^+,Ca^{2+}-exchanger NCX1, where it may play an important role in Ca^{2+} signaling (Juhaszova and Blaustein, 1997; see Section ATP1A2 and Ca^{2+} Signaling), and it was found to be heavily present in glial leaflets surrounding dendritic spines and axo-dendritic synapses, where it colocalizes with GLAST and GLT-1 glutamate transporters (Cholet et al., 2002). This functional link to glutamate transporters in astrocytic processes surrounding glutamatergic synapses suggests specific roles in the regulation of glutamate clearance (Pietrobon, 2007; Pietrobon and Moskowitz, 2013). However, ATP1A2 could have a crucial role in extracellular K^+ clearance by astrocytes (Larsen et al., 2014), similar to VSM (DiFranco et al., 2015) and cardiomyocytes (Stanley et al., 2015). Due to the stronger voltage-dependent inhibition of α_2 pumps (which is even augmented for the glial α_2/β_2 subunit composition compared to α_1/β_1 pumps) and the lower extracellular K^+ affinity, α_2 pumps are essentially inactive at the normal, negative resting potential and at normal extracellular [K^+], whereas it will be fully activated during cell depolarization and elevated K^+. This provides a substantial reserve pumping activity for K^+ clearance during strong cortical activity (Larsen et al., 2014). Yet, although ATP1A2 is a key player in K^+ clearance, this aspect has been considered less important for the pathophysiology of migraine

because the duration of the CSD was not prolonged in the FHM2 mouse model (Leo et al., 2011). These findings collectively suggest that ATP1A2 mutations in migraine primarily cause a disorder of glutamatergic neurotransmission with defective regulation of the E/I balance in the brain (Pietrobon and Moskowitz, 2013).

From CSD to Trigeminovascular Nociception: The Neuroinflammatory Hypothesis

CSD can be triggered by local elevations of extracellular $[K^+]$ as a consequence of the hyperactivity of neuronal circuits in the cerebral cortex. Of note, CSD is a slowly propagating wave of strong neuronal and glial depolarization accompanied by depression of electroencephalographic (EEG) activity and by a large increase in extracellular $[K^+]$ (Pietrobon and Striessnig, 2003). To explain pain generation, the so-called vascular theory prevailed in the past, which proposed that abnormal dilation of meningeal and/or extracranial arteries causes pain, since these are the only cerebral structures endowed with primary pain receptors. However, clinical and experimental evidence rendered this hypothesis implausible, since vasodilation is neither necessary nor sufficient to cause migraine pain (Pietrobon and Striessnig, 2003). It currently emerges that the migraine headache depends on the activation and sensitization of trigeminal nociceptors that innervate the meninges and their large blood vessels. Mediators of noxious pain, such as protons, nitric oxide, arachidonic acid, and serotonin, besides glutamate and other neurotransmitters, are released during CSD. The neuroinflammatory hypothesis suggests that these substances may activate trigeminal nociceptors innervating blood vessels in the *pia mater*, and, via axon collaterals, dural trigeminal afferents and/or may slowly access the meningeal afferents after disruption of the blood-brain barrier, thus eventually activating central trigeminovascular neurons in the trigeminocervical complex. Activation of the meningeal afferents leads to release of proinflammatory vasoactive neuropeptides, e.g., the calcitonin gene-related peptide (CGRP). These processes may promote "sterile" neurogenic inflammation in the *dura mater* and sustain the activation or sensitization of the trigeminovascular afferents (Pietrobon and Striessnig, 2003).

ATP1A2 and Ca^{2+} Signaling

The concerted action between Na^+,K^+-ATPase and NCX1 is particularly important, since α_2- and α_3-isoforms (but not α_1) were found to co-immunoprecipitate with NCX1 in rat brain membrane preparations. This, together with co-localization studies by immunocytochemistry suggested that plasma membrane microdomains containing NCX1 and Na^+ pumps with α_2- or α_3-subunits in neurons and astrocytes form Ca^{2+} signaling complexes with plasma membrane-subjacent "junctional" endoplasmic reticulum (jER) microdomains containing ryanodine receptors and sarco/endoplasmic reticulum Ca^{2+}-ATPase (Lencesova et al., 2004), as found previously for the junctional sarcoplasmic reticulum (jSR) microdomains in VSM cells as well as astrocytes (Juhaszova and Blaustein, 1997). In line with this notion, it was shown on primary cultured astrocytes from wild-type $\alpha_2(+/+)$, knockout $\alpha_2(-/-)$, and $\alpha_2(+/-)$ heterozygous mouse fetuses that graded loss of ATP1A2 activity successively increases Ca^{2+} signaling (Golovina et al., 2003). The typical volume of such a jS/ER or "PLasmERosome" (Blaustein and Golovina, 2001) compartment is sub-femtoliter in size, in which 1000 ions already account for micromolar concentrations. As a consequence, a 50% loss in Na^+ pump activity may indeed be a matter of concern already on short time scales.

Beyond the Glutamatergic Hypothesis: Cell Volume as a Control Parameter?

One aspect attracting the attention of neurophysiologists is the importance of cell volume changes that inevitably accompany the massive changes in ion concentrations during seizures, spreading depression, or anoxic depolarization. During CSD, extracellular $[K^+]$ increases to 30–60 mM, but extracellular $[Na^+]$ and $[Cl^-]$ decrease to 50–70 mM (Pietrobon and Moskowitz, 2014). The concomitant massive uptake of Na^+ and Cl^- by the Na^+,K^+,$2Cl^-$ transporter (NKCC1) of astrocytes and simultaneous water uptake through aquaporins leads to astrocyte swelling (Larsen et al., 2014). In this respect, it is interesting to note that CSD can be optically monitored in cortical preparations by measuring the so-called intrinsic optical signal (IOS), a neuroimaging technique that measures cortical reflectance changes with high temporal and spatial resolution. The parameters, which the IOS is sensitive to, are changes in light scattering, blood volume, oxy-/deoxyhemoglobin balance and cytochrome oxidation (Ba et al., 2002). Light scattering (monitored at 850 nm) is particularly sensitive to cell volume changes, and this signal component coincides with the electrical signal of the spreading wavefront. New modeling tools and concepts in computational neuroscience have recently identified cell volume as a critical control parameter that separates CSD from seizures as well as other types of spreading depolarization (Wei et al., 2014; Ullah et al., 2015). These approaches use stunningly sophisticated models of neuronal excitability based on a Hodgkin-Huxley-type framework of differential equations that includes the activity of Na^+,K^+ ion pumps (Cressman et al., 2009; Ullah et al., 2009), conservation of particles and charge, and accounts for the energy required to restore ionic gradients (Dahlem et al., 2013; Ullah et al., 2015). According to Larsen et al. (2014), K^+-induced swelling of astrocytes is mediated by NKCC1, but NKCC1 does not contribute to extracellular K^+ clearance, an activity exclusively spared for α_2-containing Na^+,K^+-ATPase. Moreover, these authors found indications that glial Na^+,K^+-ATPase acts to dampen cell swelling during clearance of stimulus induced $[K^+]$. So the action of glial Na^+,K^+-ATPase could be four-fold: (1) direct astrocytic K^+ buffering and clearance, (2) glutamate clearance (indirect *via* glutamate transporters), (3) osmolyte transport to dampen cell swelling, and (4) direct repolarizing activity due to electrogenic charge transport. Given the emphasis that computational neuroscientists currently place on cell volume as a control parameter discriminating between seizures and CSD

(Ullah et al., 2015), it will be rewarding to study the relation between Na^+ pump dysfunction and cell volume regulation in the CNS more closely by experiment and theory.

Computational neuroscientists outlined that the premonitory symptoms of a migraine attack, such as the abnormal responses to food, stress, or light, instead of mistaking them as trigger factors themselves, should rather be considered as indicators of a systemic transition at a culminating point that follows some universal pattern, which is determined by dynamic network biomarkers (DNBs) (Dahlem et al., 2013, 2014). In contrast to traditional biomarkers (e.g., biochemical substances) that are statically enhanced or increased in the pathological state, DNBs are dynamical features of biological networks (also substances or, in general, signals), which, though highly fluctuating, are strongly correlated only during the premonitory phase. Such DNBs characterizing the premonitory period are known for lung injury disease, liver and lymphoma cancer, but still need to be identified for migraine. However, their identification could help to develop strategies for early therapeutic intervention. A new scientific discipline, translational computational neuroscience, which still needs to be inaugurated by close interactions between clinicians, experimentalists and theoreticians, may fuse dynamical systems theory with control theory in order to drive innovations in therapeutic brain stimulation to treat neurological diseases based on theoretical concepts (Dahlem et al., 2013).

THE WEAL AND WOE OF HAVING Na^+,K^+-ATPase α_2-SUBUNITS IN CERTAIN TISSUES

What is the advantage of having different Na^+,K^+-ATPase α-isoforms expressed in a tissue-specific manner, and which other pathophysiological effects could be expected in the case of α_2-subunit haploinsufficiency besides the migraine phenotype? Whereas the α_1-isoform is ubiquitously expressed and most indispensable for the organism, the α_2-isoform is expressed mainly in heart, skeletal, and vascular smooth muscle, brain, lung, and adipocytes. The α_3-isoform occurs mainly in neurons and ovaries, as well as in developing hearts of rat and in adult human heart and in white blood cells, and α_4 is found in sperm, where it is required for sperm motility (see Lingrel, 2010, and references therein). The possibility to coassemble with three β-isoforms and up to seven FXYD proteins adds up further complexity. The tissue and subcellular distribution of the α_2-isoform, in particular its selective expression in electrically excitable cells, or the cells that surround them, or in VSM suggests that α_2 could also modulate excitability and contractility in heart and skeletal muscle as well as in the vasculature (Radzyukevich et al., 2004). The functional properties that distinguish α_2- from α_1-containing Na^+ pumps define the importance of this isoform in muscle (DiFranco et al., 2015; Stanley et al., 2015) and astrocytes (see Section From ATP1A2 Mutations to CSD: The Glutamatergic Hypothesis). Stanley et al. showed recently that the unusually steep voltage dependence of ion transport of α_2-containing Na^+ pumps in the range of physiological potentials, which is even exacerbated by assembly with β_2, provides a strong reservoir of pumping activity during the cardiac action potential, while keeping it inactive at normal resting potentials. In addition, as earlier found by Han et al. (2009), it was demonstrated that α_2 pumps in cardiomyocytes (Stanley et al., 2015) and skeletal muscle (DiFranco et al., 2015) have reduced affinity for extracellular K^+, allowing them to be readily stimulated by physiological rises in K_o^+ occurring under exercise. The distinct (cardiomyocytes) and almost exclusive (skeletal muscle) localization of the α_2-isoform to T-tubules (see DiFranco et al., 2015; Stanley et al., 2015, and references therein), which are highly diffusion-restricted spaces where K_o^+ may rise to tens of millimolar during muscle activity (DiFranco et al., 2015), further supports the notion that α_2 provides a safety net for K_o^+ clearance and Na^+ extrusion that is only recruited on request.

Should cardiac, skeletal muscle or vasculature deficits accompany the phenotype of ATP1A2 haploinsufficiency? In the heart, the low (about 10–15%) overall proportion of the α_2-isoform and the presence of potentially compensating α_1 in T-tubules may alleviate loss of α_2 activity. Results from transgenic animals seem to depend on the knockout strategy. James et al. investigated a global germline deletion of one copy of α_2 (heterozygous $\alpha_2(+/-)$ knockout mice) and observed cardiac hypercontractility as a result of increased Ca^{2+} transients during the contractile cycle, in accordance with the proposed role of α_2 in cardiac inotropy (James et al., 1999). Later, Rindler et al. generated mice with tissue-targeted knockout of α_2 that resulted in more than 90% loss of α_2 exclusively in the cardiovascular system (Rindler et al., 2011) or in the heart (Rindler et al., 2013). These authors found cardiac and vascular contractility unaltered. Similar contrasting results were obtained regarding the effects of α_2 knockouts in the vasculature, in which the ratio of α_2 to α_1 is 30%/70% (Shelly et al., 2004). Whereas heterozygous $\alpha_2(+/-)$ knockout mice had elevated systolic blood pressure, increased myogenic tone and arterial contractility (Shelly et al., 2004; Rindler et al., 2011), the cardiovascular knockout model showed normal basal blood pressure and vascular contractility suggesting that expression of α_2 in cardiac myocytes and vascular smooth muscle is not involved in the regulation of basal blood pressure. Possibly, α_2 in another cell type might be responsible for the hypertension observed in global $\alpha_2(+/-)$ mice (Rindler et al., 2011). In rare cases, vascular abnormalities coincide with hemiplegic migraine, such as pulmonary arterial hypertension (Montani et al., 2013) and reversible cerebral vasoconstriction (Hermann et al., 2013).

The situation should definitely be different in skeletal muscle, in which α_2 comprises nearly 90% of total α-subunit content (He et al., 2001) and is almost exclusively present in T-tubules (DiFranco et al., 2015). However, the α_2-isoform does not set resting ion gradients (He et al., 2001) or the resting potential (Radzyukevich et al., 2004, 2013) in skeletal muscle, the canonical roles of the Na^+,K^+-ATPase in most other cell types, due to its profound voltage-dependent inhibition at the about −90 mV resting potential in skeletal muscle and its lower K^+ affinity compared to α_1. In accordance, the α_1-isoform localized to the surface sarcolemma provides up to 75% of the basal Na^+,K^+ transport needed to stabilize ion gradients and membrane potential at rest. However, α_1 operates at the upper edge of

its regulatory range for activation by K^+, and the tremendous transport capacity of α_2 pumps needs to be recruited during exercise. Skeletal muscle also differs from other excitable tissues because its resting potential is set by ClC chloride channels (Pedersen et al., 2016), as illustrated by the hyperexcitability of skeletal muscle in myotonia congenita, a muscle disease resulting from loss-of-function mutations in the *ClC-1* gene (Koch et al., 1992). Also for skeletal muscle effects, studies on transgenic mice are not fully conclusive. Whereas increased isometric force, hypercontractility and increased fatique was observed in isolated skeletal muscle from heterozygous $\alpha_2(+/-)$ mice (He et al., 2001), the $\alpha_2(+/-)$ animals themselves did not fatique faster than wild-type animals (Moseley et al., 2007). Increased fatique was observed in skeletal muscle-targeted $\alpha_2(+/-)$ knockout animals and muscle preparations derived thereof (Radzyukevich et al., 2013). Of note, a 2.5-fold upregulation of α_1 was observed in these skeletal muscle $\alpha_2(-/-)$ knockout animals, and even some presence of α_1 in T-tubules was observed suggesting that some partial compensation by α_1 could take place (Radzyukevich et al., 2013). Since the largest effects were observed in the severe skeletal muscle knockout system but not in the global $\alpha_2(+/-)$ heterozygotes, it seems plausible that the vast capacity of α_2 to cope with tremendous physiological load increases during physical exercise may keep sufficient reserve at hand that 50% loss in α_2 may not entail an additional skeletal muscle phenotype in FHM2.

METHODS FOR EXPERIMENTAL ASSESSMENT OF Na^+,K^+-ATPase FUNCTION

Ouabain Survival Assays

For the investigation of functional consequences of FHM2 mutations, the so-called ouabain survival assays on HeLa cells are most frequently applied (Bassi et al., 2004; de Vries et al., 2007). The mutations are introduced into an ouabain-resistant ATP1A2 backbone carrying the Q116R/N127D double mutation in the TM1-2 loop, which produces IC_{50} values in the 100 micromolar range (Price and Lingrel, 1988). HeLa cells are either transiently or stably transfected and put under micromolar ouabain stress, which is sufficient to block the endogenous Na^+ pumps, so that only cells expressing an ATP1A2 mutant construct with sufficient residual activity can survive. The heterozygous state of patients can be mimicked by co-transfecting equal amounts of wild-type and mutant ATP12A2 DNA in order to address possible dominant-negative effects (De Fusco et al., 2003). The cell line for transfection should be carefully chosen as highlighted by the differences observed from ouabain survival assays on COS7 and HeLa cells for the W887R mutation (De Fusco et al., 2003; Leo et al., 2011). Whereas the mutant protein did not confer cell survival in both cell lines, (De Fusco et al., 2003) reported normal cellular distribution and plasma membrane expression in COS7 cells, but (Leo et al., 2011) found drastically reduced protein level and a mainly intracellular distribution in HeLa cells suggesting that COS7 cells might be prone to saturation artifacts, a common threat in transient transfection studies.

Biochemical Assays

Molecular function can be addressed by biochemical assays, which requires recombinant protein production in appropriate expression hosts such as stably transfected mammalian cells (Toustrup-Jensen et al., 2014), or insect cells (e.g., Sf9 derived from the fall armyworm *Spodoptera frugiperda*, Weigand et al., 2014b). Yeast cells (*Pichia pastoris*) have also been used (Cohen et al., 2005), but this host has not yet been employed for the study of FHM2 mutations. Subsequently, the whole set of biochemical techniques can be performed (see Glynn, 1985; Kaplan, 2002, for reviews), such as the "classical" ATPase (Skou, 1957) or phosphorylation assays, in which also the effects of different Na^+ and K^+ concentrations, the steady-state phosphoenzyme level, conformational preference, ouabain binding and sensitivity, and vanadate sensitivity can be determined. The maximum phosphorylation levels obtained in the presence of oligomycin are used to measure the concentration of active enzyme sites, which, together with ATP hydrolysis rates, are used to determine the maximum turnover number (Glynn, 1985; Vilsen, 1995), thus yielding a thorough characterization of enzymatic properties.

Electrophysiological Assays: The Two-Electrode Voltage Clamp

The cation transport function of the Na^+,K^+-ATPase as such can only be investigated in intact cells, which maintain the extra-/intracellular sidedness of substrate access and allow for the application of a membrane (or more general: electrochemical) potential load on cation transport. Na^+,K^+ transport and the most significant properties relating to the electrogenicity of the Na^+ pump are determined by electrophysiology, mostly by applying the two-electrode voltage clamp technique on oocytes from the frog *Xenopus laevis* (Stühmer and Parekh, 1995). This technique allows one to study the dependence of Na^+,K^+ pump currents on extracellular [K^+], [Na^+], ouabain and voltage, so that the complex interplay between Na^+ and K^+ at the externally facing cation binding sites can be analyzed.

Typical Na^+,K^+ pump currents, as can be recorded with the two-electrode voltage clamp on *Xenopus laevis* oocytes, are shown in **Figure 4A** together with the voltage and extracellular [K^+] dependence (**Figure 4B**), from which $K_{0.5}(K^+)$ values for half-maximal pump current stimulation can be determined (**Figure 4C**), which symbolize the voltage dependence of the enzyme's apparent affinity for extracellular K^+.

In the absence of extracellular K^+ and at high extracellular [Na^+], the enzyme is restricted to the partial reaction sequence underlaid in yellow in **Figure 1** that accounts for ouabain-sensitive "transient" currents in response to voltage pulses (**Figure 5A**). Extracellular release of Na^+ ions occurs in three distinct steps, from which only the deocclusion and release of the first Na^+ ion is major electrogenic (Holmgren et al., 2000). This step is rate-limited by the preceding (presumably non-electrogenic) $E_1P \rightarrow E_2P$ conformational change and cation deocclusion, which gives rise to the startling observation that the forward rate constant for Na^+ release is not voltage-dependent (see the flat progression of rate constants in **Figure 5B** at positive voltages). However, in the reverse direction, the reverse binding

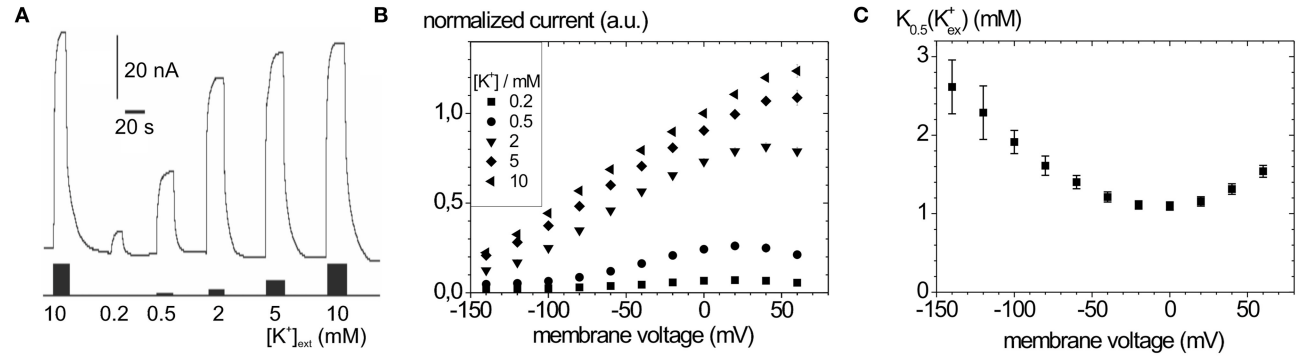

FIGURE 4 | Stationary Na^+/K^+ pump currents of the Na^+,K^+-ATPase (human ATP1A2) from two-electrode voltage clamp experiments on *X. laevis* oocytes. (A) Pump currents in response to different extracellular $[K^+]$ at −30 mV holding potential, **(B)** I-V curves for different $[K^+]_{ext}$ at high $[Na^+]_{ext}$ (100 mM). **(C)** $K_{0.5}(K^+ext)$ values from fits of a Hill function to the $[K^+]$ dependent pump current amplitudes at different potentials from **(B)**.

of Na^+ ions from the extracellular side induced by negative voltage pulses occurs with a strongly voltage-dependent rate constant (see the steeply rising rate constants in **Figure 5B** at negative voltages). Following release of the first Na^+ ion, the high-field access channel to the other Na^+ occlusion sites is restructured such that the exit of the remaining two Na^+ ions contributes only little to the overall electrogenicity. In terms of the "access channel" model, positive voltage pulses drive Na^+ ions extracellularly out of the access channel resulting in ouabain-sensitive "transient" currents with positive polarity. Conversely, negative voltage pulses promote the reverse binding of Na^+ ions to the binding sites in E_2P and induce negative transient currents (**Figure 5A**). Due to the electrogenicity of reverse binding of extracellular Na^+, the occupancy of the Na^+ binding sites is controlled by $[Na^+]_o$ and voltage. The amount of charge moved in response to a certain voltage step *Q(V)* follows a characteristic, sigmoidal, Boltzmann-type function (see **Figure 5C**):

$$Q(V) = Q_{\min} + \frac{Q_{\max} - Q_{\min}}{1 + e^{-\frac{z_q \cdot F}{R \cdot T}(V - V_{0.5})}} \quad (1)$$

which is used to fit the experimentally obtained *Q(V)* curves. Here, $Q_{\min}$ and $Q_{\max}$ are the saturation values of *Q(V)*, *F* is the Faraday constant, *R* the molar gas constant, *T* the absolute temperature in K, *V* the membrane voltage, and z_q the slope factor or equivalent charge. This distribution is centered at a half-maximal voltage ($V_{0.5}$), at which 50% of Na^+ binding sites of the pump molecules are occupied. Since the Na^+ uptake/release steps are kinetically coupled to the $E_1P \longleftrightarrow E_2P$ conformational transition, 50% of the enzyme molecules are in E_2P and 50% are in E_1P at this stage. Thus, the $V_{0.5}$ value at a given $[Na^+]_o$ is a characteristic parameter for each Na^+,K^+-ATPase isozyme (or mutant), and since $V_{0.5}$ shifts with the extracellular $[Na^+]$, changes in $V_{0.5}$ induced by mutations indicate changes in the apparent affinity for Na^+_o. Positive shifts of $V_{0.5}$ indicate an increased apparent Na^+ affinity and *vice versa*.

The equivalent charge z_q indicates the electrogenicity of the Na^+ transport step, i.e., which fraction of the transmembrane field is "sensed" by a unitary charge moved or, conversely, which fraction of a charge encounters the full transmembrane field during an elementary charge-moving event.

Of late, the ouabain-sensitive leak currents are investigated by electrophysiology as well, since mutations interfering with a C-terminal access pathway for protons drastically affect cation affinities (Morth et al., 2007; Poulsen et al., 2010). Thus, especially C-terminal mutations identified in FHM2 and AHC have been scrutinized for suspicious leak current activity (Poulsen et al., 2010; Li et al., 2015). As an example, the leak currents of the Na^+ pump that occur upon deletion of the two C-terminal tyrosines of the α-subunit are shown in **Figure 6**. **Figure 6A** shows ouabain-sensitive transient currents of the ATP1A2-ΔYY mutant in the absence of extracellular K^+ and $[Na^+]_o = 100$ mM (Meier et al., 2010). Compared to the transient currents of the WT enzyme (**Figure 5A**), the "ON" transient currents of the mutant do not decay to zero at negative voltages, but a steady inward current results, which is augmented with increasing $[Na^+]_o$ (**Figure 6B**). This phenomenon was also observed by Yaragatupalli et al. (2009), Poulsen et al. (2010) and Vedovato and Gadsby (2010).

ATP1A2 MUTATIONS CORRELATED WITH CLINICAL MIGRAINE CASES

To date, about 81 *ATP1A2* mutations have been reported in migraine-correlated neurological disease cases in the literature. A complete list is provided in Supplementary Table 1 together with information about the diagnosed diseases, the location of the mutated residues within the structure of the Na^+,K^+-ATPase (see **Figure 2**) and, if available, a brief summary of functional consequences of the mutations. The vast majority (about 60) of the mutations were classified as FHM. Moreover, about 25 mutations were diagnosed in sporadic cases of hemiplegic migraine, SHM, (with overlap in the case of G815R, R908Q, P979L, which were identified in different unrelated pedigrees or individuals) showing that mutations in the *ATP1A2* gene locus substantially account for *de novo* mutations causing hemiplegic migraine. About 10% of *ATP1A2* mutations were identified

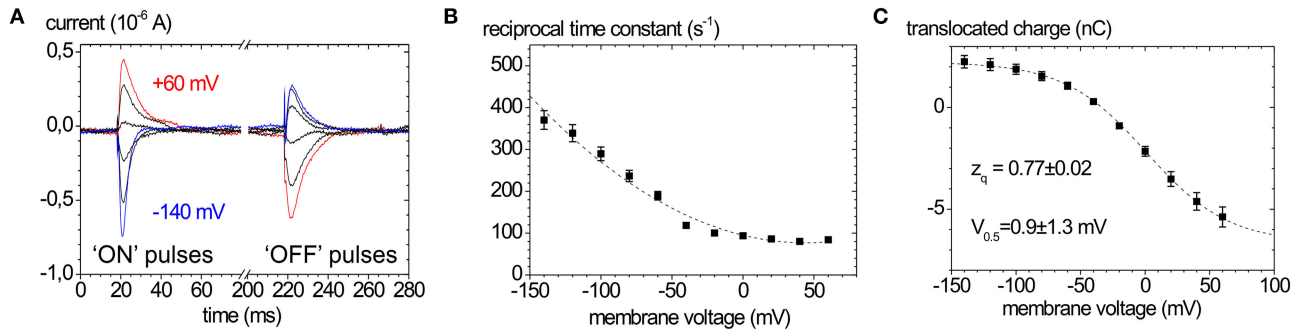

FIGURE 5 | Properties of ouabain-sensitive transient currents of the Na^+,K^+-ATPase. (A) Transient currents evoked by pulses from −30 mV to voltages between +60 and −140 mV in −40 mV decrements measured on human ATP1A2 in two-electrode voltage clamp experiments on *X. laevis* oocytes ("ON" currents), and from pulses back to −30 mV ("OFF" pulses). **(B)** Reciprocal time constants from fits of a single exponential function to the current traces in **(A). (C)** *Q(V)* distribution from the improper integrals of the transient current signals ("OFF" pulses) from **(A)** with the parameters obtained from fits of a Boltzmann-type function to the data. The *Q(V)* distribution from "ON" transient currents would be obtained by multiplying the above curve with (−1).

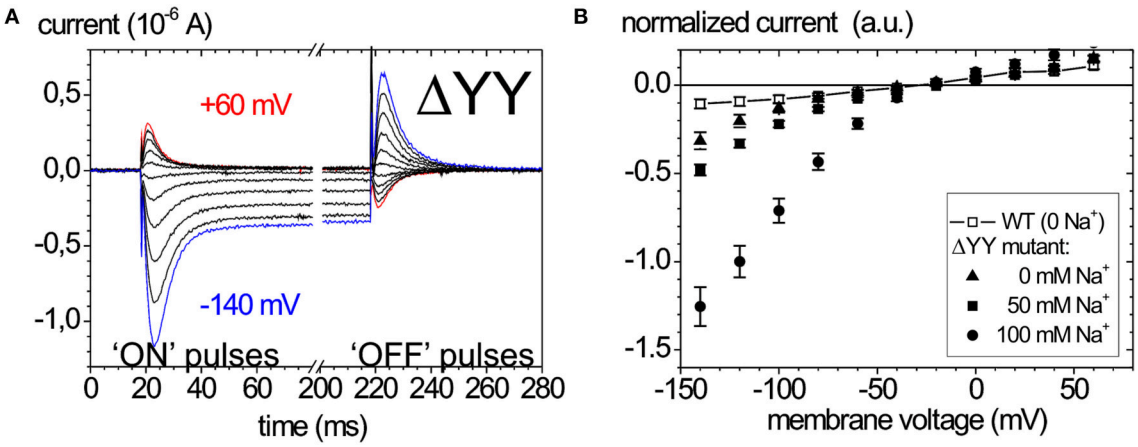

FIGURE 6 | Leak currents of the Na^+,K^+-ATPase upon mutation of the α-subunit's C-terminus. (A) Ouabain-sensitive transient currents measured at $[K^+]_o = 0$, $[Na^+]_o = 100$ mM (pH 7.4) upon pulses from −30 mV to voltages between +60 and −140 mV in −40 mV decrements for mutant ATP1A2-ΔYY in two-electrode voltage clamp experiments on *X. laevis* oocytes ("ON" currents), and from pulses back to −30 mV ("OFF" pulses). **(B)** $[Na^+]_o$ dependence of the current-voltage curves of the steady-state inward leak currents (closed symbols), the leak currents of the WT enzyme at zero $[Na^+]$ are shown as open squares.

in migraine with or without aura (MA/MO) indicating that the gene might also be a susceptibility locus for common forms of migraine (Todt et al., 2005). An overlap with epilepsy or seizures has been noted in about 15% of cases. Of note, two ATP1A2 mutations were identified in patients with AHC, such as the I589T mutation reported in an atypical case of AHC (Al-Bulushi et al., 2014), and T378M, which was found in two families, either correlated with FHM (Bassi et al., 2004) or with AHC (Swoboda et al., 2004). Other pathologies associated with *ATP1A2* mutations were sensorineural hearing loss (V191M, Oh et al., 2015), basilar migraine (R548H, Ambrosini et al., 2005), benign familial infantile convulsions (BFIC; R689Q, Vanmolkot et al., 2003), generalized epilepsy with febrile seizures (GEFS+; G874S, Costa et al., 2014), pulmonary arterial hypertension (S940L, Montani et al., 2013) and reversible cerebral vasoconstriction (P979L Hermann et al., 2013).

Within the Na^+,K^+-ATPase crystal structure, more than 80% of mutations fall into four spatially distinct clusters, one around the catalytic P domain, one in a central region between P and TM domain, one within the extracellular-facing part of the TM domain, and one around the enzyme's C-terminus (**Figure 2**), which are all regions of critical importance for function. About 75% of the reported ATP1A2 mutations have been scrutinized for function at different levels of experimental sophistication (Supplementary Table 1).

Functional Studies: Mildly and Severely Deleterious ATP1A2 Mutations

Among the ATP1A2 mutations studied so far, some stand out because only mild consequences were observed (Supplementary Table 1). These include mutations at the enzyme's N-terminus, Y9N (SHM) and R51H (MO), which behaved similar to WT in biochemical studies on Sf9 cell membrane preparations (Swarts et al., 2013). A clue for the physiological consequences can be inferred from Song et al. (2006), who showed that ATP1A2 and ATP1A3 share an N-terminal targeting sequence

within amino acids 1–90, which is apparently responsible for localization to jS/ER compartments in primary cultured mouse astrocytes. It remains to be clarified whether the N-terminal mutations of ATP1A2 interfere with targeting in order to decide whether or not these mutations are rare missense variants without pathogenic effects. The same accounts for E174K (MO), which was inconspicious in electrophysiological experiments on *Xenopus* oocytes and showed only mildly reduced activity in Sf9 cell preparations (Todt et al., 2005; Swarts et al., 2013), although the mutation inverts the charge of a highly conserved residue in the A domain, which might form a salt bridge with Lys-432 in the N domain important for inter-domain interactions. Very similar observations were reported for the E902K (FHM) mutation, which showed electrophysiological properties like the WT enzyme (Spiller and Friedrich, 2014), but reduced ATPase activity and ouabain binding in Sf9 cell membrane preparations (Swarts et al., 2013). Also for K1003E (SHM+seizures) and R1007W (FHM), only mild consequences were reported from electrophysiology on *Xenopus* oocytes (Pisano et al., 2013; Spiller and Friedrich, 2014), despite the charge inverting/neutralizing effect.

At the other end of the functional spectrum, mutations that lead to a premature stop codon, a frame shift or a deletion/insertion are expected to cause severe functional disruptions. These include K95del (FHM & epilepsy), F305del (SHM), R834X (FHM), del(K935-S940)insI (FHM), S966fs (FHM), L944del (SHM+focal seizures), and Y1009X (SHM), which all classify as familial or *de novo* mutations causing hemiplegic migraine, frequently with severe accompanying symptoms and, as far as functional studies are available, lead to complete loss of function and/or loss of plasma membrane targeting.

The remaining mutations, which entail loss or drastic reduction of activity, are depicted in **Figure 7**. These mutations mostly locate to the P domain and the extracellular TM domain cluster, with only a few others in the N domain (T415M, C515Y, R548H) and two in the central cluster (L764P, R937P). Mutation G301R (identified in two FHM kindreds) showed slightly reduced plasma membrane expression but no pumping activity in the *Xenopus* oocyte system, indicating reduced protein stability and complete loss-of-function (Tavraz et al., 2009). In HeLa cells, however, the mutant showed strongly reduced cell viability in ouabain survival assays, no plasma membrane expression and no protein detectable in Western blots (Santoro et al., 2011). The structure-destabilizing and functional disruption effect can be attributed to the insertion of a bulky, charged side chain within the center of the block of TM helices close to one of the common cation binding sites.

T376M (FHM) and T378N (FHM, AHC) affect two threonines in the ^{373}S**D**K<u>T</u>G<u>T</u>LT^{380} motif around the intermediately phosphorylated Asp-374. Although the mutant proteins were expressed similar to the WT enzyme in *Xenopus* oocytes (T376M, Tavraz et al., 2008) and HeLa cells (T378N, Bassi et al., 2004), no pump activity or cell survival was observed. T415M (FHM) and C515Y (MA) affect two highly conserved residues at the periphery of the N domain, for which effects on function are difficult to infer. Nevertheless, the T415M mutant did not confer cell viability in ouabain survival assays despite normal protein level (Vanmolkot et al., 2007), and the C515Y mutation entailed strongly reduced pump currents and ATPase activity in *Xenopus* oocytes (Todt et al., 2005). Inter-domain interactions are also a critical concern for the R584H (basilar migraine/MA) and R548C (FHM) mutations, since Arg-548 likely forms a salt bridge with Glu-221 that stabilizes the interaction between the A and N domains in the E_2P conformation, but probably forms another salt bridge to the β-phosphate of ATP. Although ATP affinity was not changed for mutant enzymes prepared from Sf9 cells, strongly reduced ATPase activities were observed (Swarts et al., 2013) indicating that the structure-coordinating effect of the salt bridge might be critical.

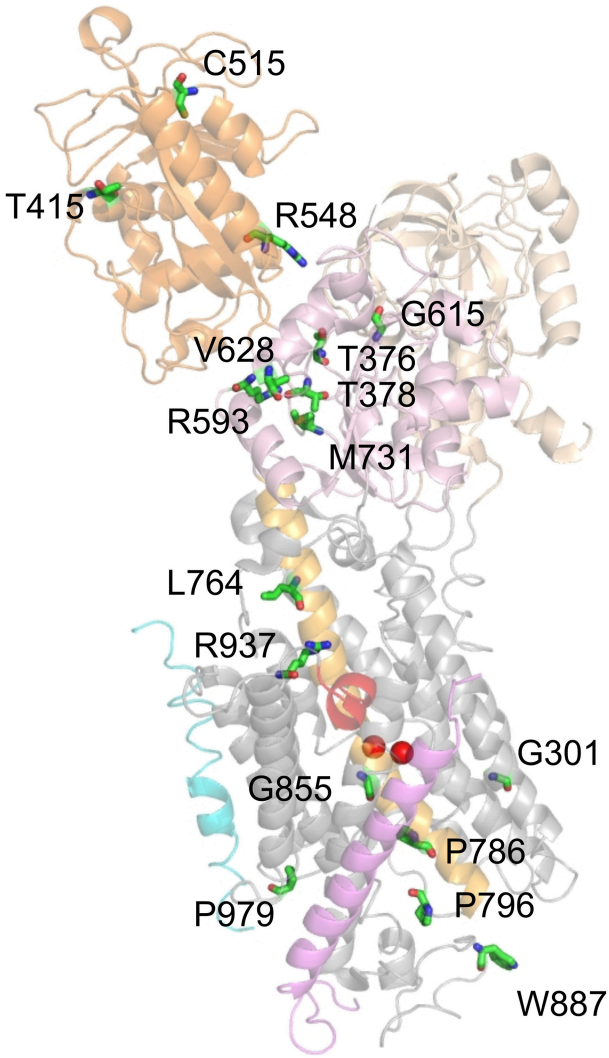

FIGURE 7 | Location of ATP1A2 mutations with most severe consequences on function. Mutated amino acids, for which functional consequences are described in the text, are shown in stick representation. Mutant P979L was shown to be fully functional in the *Xenopus* oocyte system, whereas temperature-dependent effects on protein stability and plasma membrane targeting were observed in mammalian cells (Tavraz et al., 2008, 2009).

Mutations R593W and V628M were both identified in FHM and showed strongly reduced cell viability in ouabain survival assays with normal protein level (Vanmolkot et al., 2006a), and R593W exhibited strongly reduced ATPase activity and reduced phosphoenzyme level in COS-1 cell membranes (Schack et al., 2012). Arg-593 is located at the border of the P domain, directly after two prolines that form the hinge between the P and N domains. The residue is within hydrogen bonding distance of the backbone carbonyls of Gly-377 and Thr-378, which are located in the same loop as Asp-374 and Thr-376 indicating that the steric clash resulting from insertion of the tryptophan might disturb phosphorylation (Vanmolkot et al., 2006a; Schack et al., 2012). Val-628 resides in the P domain and is located at the border of the short, conserved P3 helix that together with the seven-stranded parallel β-sheet forms the typical Rossmann fold implicated in nucleotide binding suggesting an impact on catalytic activity (Schack et al., 2012). The G615R mutation, which was identified in FHM with particularly severe accompanying symptoms and in a patient with SHM, affects the glycine in the critical ^{612}MVTGD^{616} structure motif in the P domain, which rationalizes that the mutation has been found to be deleterious for cell viability (Vanmolkot et al., 2006b).

The M731T mutation was functionally assessed by several groups (Capendeguy and Horisberger, 2004; Segall et al., 2005; Schack et al., 2012), with the most stringent study carried out on the human ATP1A2 enzyme (Schack et al., 2012), whereas the other two studied rat ATP1A2 (Segall et al., 2005) or ATP1A1 from *Bufo marinus* (Capendeguy and Horisberger, 2004). All studies converged on the notion of strongly reduced ATPase activity or loss of pump function. For efficient phosphorylation, the loop between β-sheet 6 and helix P7 of the Rossmann fold, in which Met-731 is located, has to be strained upon Mg^{2+} binding. Thus, the role of Met-731 may be to reduce the mobility of this loop to ensure the strain. Met-731 is also flanked by Arg-593 (see above), and the mutation might prevent proper bending of the P domain (Schack et al., 2012).

Mutations L764P and W887R were identified in the first report on FHM2 (De Fusco et al., 2003). L764P inserts a structure-breaking proline into the central helix TM5, which connects the phosphorylation site to the cation-binding pocket. The P786L (SHM) mutation also affects a residue on the extracellularly-oriented part of the TM5 helix. In accordance with the critical role of TM5, all studies investigating the effects of the TM5 mutations showed severe or complete loss of catalytic activity (Koenderink et al., 2005; Swarts et al., 2013) or cell survival (De Fusco et al., 2003; de Vries et al., 2007). The ^{796}P796S^{799} (FHM) mutation, which affects a proline within the conserved PLPL turn connecting TM5 and TM6, also did not support cell survival (Castro et al., 2008) or ATPase activity in Sf9 cell membranes (Weigand et al., 2014b).

The W887R mutant also did not confer cell survival (De Fusco et al., 2003), showed no pump currents (Capendeguy and Horisberger, 2004; Koenderink et al., 2005) and strongly reduced Rb^+ uptake, despite normal plasma membrane expression in *Xenopus* oocytes (Koenderink et al., 2005). From the structure, it is difficult to rationalize a loss of functional activity by this mutation. Trp-887 is located within the TM7-8 loop important for interaction with the β-subunit, but is not directly involved (Nyblom et al., 2013). If the interaction with the β-subunit were disrupted, a defect in membrane insertion and protein folding accompanying biosynthesis in the ER could be expected, with consequently reduced plasma membrane targeting, but this was not observed in the expression systems studied. Furthermore, the defective ouabain binding of the W887R mutant (Koenderink et al., 2005) is puzzling since Trp-887 was not implicated in ouabain binding. However, it was found in a mouse model for FHM2 that the mutant ATP1A2 protein was hardly detectable in the brain of homozygous ATP1A2(W887R/W887R) mutants and strongly reduced in ATP1A2(+/W887R) heterozygous mutants (Leo et al., 2011). In transfected HeLa cells, these authors also found profound protein loss, likely as the consequence of endoplasmic reticulum retention and subsequent proteasomal degradation. This finding contrasts with observations from the aforementioned study on transfected COS7 cells (De Fusco et al., 2003) indicating that it is important to avoid saturation effects in cell line used for transfection (Leo et al., 2011).

Mutation G855R (FHM+febrile seizures) did not confer cell survival (de Vries et al., 2009) or pump currents in the *Xenopus* oocyte system (Spiller and Friedrich, 2014), which in the latter system coincided with strongly reduced plasma membrane protein level. Gly-855 resides within TM7, a helix forming multiple contacts to the TM of the β-subunit (Nyblom et al., 2013), but Gly-855 is not directly involved in helix-helix contacts. However, due to the close packing of the helices, the introduction of a bulky side chain can indirectly affect the interaction with the β-subunit, which appears likely since the defective plasma membrane expression suggests improper folding and premature degradation.

Mutation R937P (FHM) did not show pump currents or Rb^+ uptake in an initial study on *Xenopus* oocytes (Tavraz et al., 2008), and no ATPase activity in Sf9 cell membranes (Weigand et al., 2014b). However, based on the knowledge about the involvement of Arg-937 in coordinating the α-subunit's C-terminus, its link to Na^+ binding site III and the reported effects on Na^+ affinity (Toustrup-Jensen et al., 2009), it could be shown by electrophysiology that the mutation caused enhanced proton "leak" currents and a drastic negative shift of the *Q(V)* curve from transient currents (Poulsen et al., 2010), in line with a drastically reduced apparent affinity for extracellular Na^+. In effect, the R937P mutant still retains some essential properties of the Na^+ pump, albeit with severely changed voltage dependence and enhanced proton leak, but the properties of the mutant's Na^+, K^+ pump currents have still to be elucidated. R937P is so far the only FHM2 mutation that shows increased proton leak currents. Work by the Artigas and Gadsby labs showed that pump-mediated proton current is an intrinsic property of Na^+,K^+-ATPase (Mitchell et al., 2014; Vedovato and Gadsby, 2014) at physiological K^+ and Na^+ concentrations and resting potentials suggesting that this "hybrid" transporter function may well have been exploited by nature for some physiological purpose. It still remains to be established whether Na^+,K^+-ATPase-mediated proton uptake plays any physiological or pathological role (see Section Functional Properties of the Na^+,K^+-ATPase).

Mutation P979L Entails Temperature-Dependent Protein Instability and Mistargeting

P979L is a particularly interesting mutation, which was reported to cause FHM2 in one of the earliest accounts on the genetic background of the disease (Jurkat-Rott et al., 2004), and, later, also in a case of SHM (Hermann et al., 2013). Comorbidities were serious, since the first report listed particularly severe attacks accompanied by recurrent coma and tonic-clonic seizures, whereas the SHM patient suffered from prolonged aura phase and severe reversible cerebral vasoconstriction. Within the crystal structure, Pro-979 is located within the TM9-10 loop and the protein backbone kink induced by the proline may be important for protein folding. However, when assayed by electrophysiology in *Xenopus* oocytes, all functional parameters were identical to those of the WT enzyme, with normal total and plasma membrane protein levels (Tavraz et al., 2008). However, in surface biotinylation assays on transfected HEK293FT cells, it was later found that the amount of P979L protein was strongly reduced, when the cells were incubated at 37°C, but not upon cell incubation at 28°C (Tavraz et al., 2009). This discrepancy highlights the influence of the model cell system used. *Xenopus* oocytes are kept at 18–20°C, a temperature range, which is advantageous for protein folding, whereas mammalian cells are grown at 37°C, where protein instability might lead to misfolding and degradation. The situation is reminiscent of observations on the epithelial CFTR Cl^- channel mutated in cystic fibrosis (CF). The most common CF mutation, the ΔF508 deletion, leads to rapid degradation of the protein before exiting the ER in mammalian cells (kept at 37°C), while the mutant protein could be functionally expressed in *Xenopus* oocytes. It could be shown that this effect is temperature-sensitive since protein degradation can be rescued at permissive temperature (Denning et al., 1992).

SUMMARY AND PERSPECTIVES

The detailed atomic-scale understanding of ion transport and catalysis of the Na^+,K^+-ATPase provided by the wealth of structural data together with the multi-modal efforts of numerous experimentalists have provided a rather stringent concept of the effects of migraine-associated ATP1A2 mutations on molecular function. While the spectrum of functional disruptions matches the complexity of the Na^+ pump's inner workings and the enzyme's even more complex integration into cellular signaling networks, the notion emerges that loss or change of any kind of functional parameter, including the seemingly subtle changes in voltage dependence could be of pathophysiological relevance. This is because for any of these alterations, pathophysiological conditions are conceivable that render these changes critical for controlling the excitability of electrically excitable tissues. Two seemingly self-evident but oversimplifying conclusions should be avoided: First, changes in Na^+ pump function brought about by disease-related mutations do not converge on a simple loss-of-function concept. While this is certainly true for mutations abolishing Na^+,K^+ pumping, a change in voltage dependence may entail loss-of-function in a particular voltage range, but gain-of-function in another. Furthermore, given the controversy about whether proton leak currents are part of the Na^+ pump's physiological spectrum of functions, both, reduced proton leaks (loss-of-function, as discussed for the E815K mutation in ATP1A3 in AHC) as well as increased proton leaks (gain-of-function, as discussed for somatic ATP1A1 mutations in APA) could be causative for one or the other pathophysiological state. Second, the CSD phenomenon is not simply the consequence of either cortical hypo- or hyperexcitability. Rather, it emerges that the reduced ability to dynamically maintain the *cortical excitatory/inhibitory balance* and the failure to prevent excessive increases in cortical excitation mechanistically explain the abnormal sensory processing in migraineurs.

While it is commonly accepted that functional changes in FHM-related genes including those in *ATP1A2* converge on CSD as the neurophysiological correlate of migraine aura, the link to the most disabling condition of the disease, the throbbing migraine pain, is still a matter of debate. Currently, the view emerges that CSD can cause sustained activation of meningeal nociceptors and central trigeminovascular neurons to initiate the headache mechanisms in a process termed sterile meningeal inflammation. Moreover, the physiological control parameters discriminating between the propagation speeds of rapid (epileptic seizures) or slow (CSD) waves of cortical hyperexcitation need to be investigated in more detail to identify critical network parameters for or by *in silico* modeling of excitable biological matter. In this context, knowing the parameters of dysfunction of mutated ion pumps and channels of the CNS may help to identify the elements that count. The remarkable progress achieved by computational neuroscientists who have just recently put forward the idea that cell volume regulation is critical for determining whether an excitable tissue may evolve into seizures or spreading depression, provides novel hypotheses to be tested by experiment. This should encourage synergistic, cross-disciplinary collaborations between researchers studying excitable matter on the clinical, *in vivo*, *in vitro,* and *in silico* level.

In order to classify an ATP1A2 mutation as causative for a disease, at least the simplest test should be performed, an ouabain survival assay in mammalian cells. For the assessment of enzyme catalysis, biochemical studies on protein preparations from mammalian cell lines or Sf9 cells provide exhaustive tools of characterization. However, elucidation of the most essential physiological function, electrogenic Na^+,K^+ transport, requires dedicated electrophysiological assays on intact cells. For this purpose, the *Xenopus* oocyte expression system is a versatile tool, since it permits electrophysiology as well as cation uptake assays, and some means of fundamental protein biochemistry. Whereas electrophysiology on oocytes is indispensible for researchers interested in understanding Na^+ pump function in molecular detail, the *Xenopus* system is less physiological when it comes to effects on protein expression, stability or targeting, as the example of the P979L mutant and others noted in this work have shown. Thus, as a rule of thumb, the observed functional consequences should ideally be cross-checked in at least two different cell systems in order to avoid both false-positive and false-negative conclusions.

AUTHOR CONTRIBUTIONS

TF and NT performed literature searches, TF prepared figures and all authors wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2016.00239

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Insights into the Pathology of the α_3 Na^+/K^+-ATPase Ion Pump in Neurological Disorders; Lessons from Animal Models

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The transmembrane Na^+-/K^+ ATPase is located at the plasma membrane of all mammalian cells. The Na^+-/K^+ ATPase utilizes energy from ATP hydrolysis to extrude three Na^+ cations and import two K^+ cations into the cell. The minimum constellation for an active Na^+-/K^+ ATPase is one alpha (α) and one beta (β) subunit. Mammals express four α isoforms (α_{1-4}), encoded by the *ATP1A1-4* genes, respectively. The α_1 isoform is ubiquitously expressed in the adult central nervous system (CNS) whereas α_2 primarily is expressed in astrocytes and α_3 in neurons. Na^+ and K^+ are the principal ions involved in action potential propagation during neuronal depolarization. The α_1 and α_3 Na^+-/K^+ ATPases are therefore prime candidates for restoring neuronal membrane potential after depolarization and for maintaining neuronal excitability. The α_3 isoform has approximately four-fold lower Na^+ affinity compared to α_1 and is specifically required for rapid restoration of large transient increases in $[Na^+]_i$. Conditions associated with α_3 deficiency are therefore likely aggravated by suprathreshold neuronal activity. The α_3 isoform been suggested to support re-uptake of neurotransmitters. These processes are required for normal brain activity, and in fact autosomal dominant *de novo* mutations in *ATP1A3* encoding the α_3 isoform has been found to cause the three neurological diseases Rapid Onset Dystonia Parkinsonism (RDP), Alternating Hemiplegia of Childhood (AHC), and Cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss (CAPOS). All three diseases cause acute onset of neurological symptoms, but the predominant neurological manifestations differ with particularly early onset of hemiplegic/dystonic episodes and mental decline in AHC, ataxic encephalopathy and impairment of vision and hearing in CAPOS syndrome and late onset of dystonia/parkinsonism in RDP. Several mouse models have been generated to study the *in vivo* consequences of *Atp1a3* modulation. The different mice show varying degrees of hyperactivity, gait problems, and learning disability as well as stress-induced seizures. With the advent of several *Atp1a3*-gene or chemically modified animal models that closely phenocopy many aspects of the human disorders, we will be able to reach a much better understanding of the etiology of RDP, AHC, and CAPOS syndrome.

Keywords: α_3 sodium ion pump, neurons, rapid onset dystonia parkinsonism (RDP), alternating hemiplegia of childhood (AHC) and cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss (CAPOS), mouse models

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THE NA$^+$/K$^+$ ATPases: EXPRESSION AND FUNCTION

The Na$^+$/K$^+$ ATPases are transmembrane ion-pumps located at the plasma membrane of all mammalian cells. With each pump cycle, the Na$^+$/K$^+$ ATPases utilize the energy from hydrolysis of one adenosine triphosphate (ATP) to extrude three Na$^+$ ions and import two K$^+$ ions into the cell. The minimum constellation of an active pump consists of an alpha (α) and a beta (β) subunit (Kaplan, 2002; Bublitz et al., 2011; Palmgren and Nissen, 2011). The α subunit is responsible for the catalytic and pharmacological properties (Blanco et al., 1994) whereas the β and optional γ subunits may have regulatory functions (Jaisser et al., 1994; Béguin et al., 1997; Hilbers et al., 2016).

Mammals express four Na$^+$/K$^+$ ATPase α isoforms (α_{1-4}) of which α_{1-3} are expressed in the CNS. While α_1 is expressed ubiquitously and considered to maintain housekeeping cellular functions, the α_2 isoform is expressed primarily in astrocytes and developing neurons and α_3 isoform is restricted to neurons (McGrail et al., 1991; Bøttger et al., 2011). Thus, the cell type-specific distribution of the α isoforms suggests that each isoform has a particular function. Several suggestions have been made to elaborate on the specific role of the α_3 isoform in neurons (Reviewed in Dobretsov and Stimers, 2005). Although the α_3 isoform is expressed in many CNS neurons, several neuronal subsets lack the expression of this isoform (Hieber et al., 1991; McGrail et al., 1991; Bøttger et al., 2011). The ongoing hypothesis is thus that while the ubiquitously expressed α_1 isoform maintains neuronal housekeeping functions, the α_3 isoform serves as a reserve pump that only becomes activated when the intracellular Na$^+$ concentration $[Na^+]_i$ is high, e.g., after repeated action potentials (Brodsky and Guidotti, 1990; Jewell and Lingrel, 1991; Munzer et al., 1994; Blanco et al., 1995; Zahler et al., 1997; Monteith and Blaustein, 1998; Crambert et al., 2000), supported by the fact that the kinetics between the different isoform favors this scenario (Reviewed in Dobretsov and Stimers, 2005).

NEURONAL ACTIVITY AND α_3

In the CNS, the Na$^+$ and K$^+$ gradients across the plasma membrane are essential for regulating neuronal excitability, and for multiple cellular functions, including cell volume regulation and Na$^+$-coupled secondary transport. The distinguishing feature of α_3 Na$^+$/K$^+$-ATPases is their several-fold lower affinity for activation by cytoplasmic Na$^+$ compared to that of α_1 Na$^+$/K$^+$-ATPases (Crambert et al., 2000). In rapidly firing neurons, therefore, when action potentials increase the intracellular Na$^+$ concentration, $[Na^+]_i$, beyond saturating levels of the "housekeeping" α_1 Na$^+$/K$^+$-ATPases, activation of α_3 Na$^+$/K$^+$-ATPases continues to increase as $[Na^+]_i$ rise. The α_3 isoform thus protects neurons against catastrophic elevation of $[Na^+]_i$ (Dobretsov and Stimers, 2005), and also of $[Ca^{2+}]_i$ (because Na$^+$/Ca^{2+} exchange is weakened) and general loss of the Na$^+$ electrochemical gradient. As the α_3 isoform is detected in several basal ganglia neuronal subsets (McGrail et al., 1991; Bøttger et al., 2011), reduced α_3 activity may therefore interfere with reuptake of neurotransmitters such as glutamate, γ-aminobutyric acid (GABA) and dopamine (Kristensen et al., 2011), in those neurons.

The neurotransmitter transporters (NTTs) use ion gradients for the active transport, generally by co-transport of Na$^+$ and Cl$^-$. As an example, the glycine transporters are functionally coupled to the Na$^+$ electrochemical gradient, which is actively generated and maintained by the Na$^+$/K$^+$-ATPases. The glycine transporter, GlyT2 cotransports three Na$^+$ and one Cl$^-$ for every glycine (López-Corcuera et al., 1998), generating large rises in $[Na^+]_i$ that must be efficiently reduced by the Na$^+$/K$^+$-ATPase to preserve ion homeostasis, which is absolutely necessary for synaptic transmission and neuronal excitability. The α_3 co-localize and interacts with GlyT at the synapse in spinal cord neurons (de Juan-Sanz et al., 2013). As GlyT belongs to the solute carrier 6 (SLC6) family of highly homologous NTTs, it is possible that the transporter for dopamine, norepinephrine, serotonin, and GABA can affected by Na$^+$/K$^+$-ATPase activity loss (Kristensen et al., 2011). It is well-documented that dopamine increases the activity of the Na$^+$/K$^+$-ATPase in an organ-specific manner (Reviewed in Zhang et al., 2013). Moreover, evidence of direct interaction between Na$^+$/K$^+$-ATPase and dopamine receptors has also been reported (Hazelwood et al., 2008). Dopamine modulates the Na$^+$/K$^+$-ATPase by affecting both dopamine and other hormones. It has been shown that the D2 receptor stimulates striatal Na$^+$/K$^+$-ATPase activity after a short-term morphine treatment, in contrast to a long-term morphine treatment that inhibited striatal Na$^+$/K$^+$-ATPase activity (Wu et al., 2007).

Failure of the Na$^+$/K$^+$-ATPase to maintain Na$^+$ and K$^+$ gradients will cause a decrease in Na$^+$ and K$^+$ currents (through voltage-dependent channels). This will lead to a decrease in the membrane potential, action potential and most likely a loss of neuronal excitability. This will further affect sodium-coupled co- and counter-transport. Moreover, it can also affect Na$^+$/H$^+$ and Na$^+$/Ca^{2+} exchange, essential for cellular functions, and could lead to increased intracellular Ca^{2+} and acidification. Not surprisingly, mutations in the *ATP1A3* gene have been associated with neurological disorders.

ATP1A3-RELATED DISEASES

Mutations in the *ATP1A3* gene are associated with three related rare neurological disorders, Rapid-onset Dystonia-Parkinsonism (RDP) (de Carvalho Aguiar et al., 2004), Alternating Hemiplegia of Childhood (AHC) (Heinzen et al., 2012; Rosewich et al., 2012), and recently, Cerebellar ataxia, Areflexia, Pes cavus, Optic atrophy, and Sensorineural hearing loss (CAPOS) syndrome (Demos et al., 2014).

Presently 12 *ATP1A3* missense mutations have been associated with RDP (Heinzen et al., 2014). Classical RDP patients typically develop stress-induced permanent dystonia and Parkinsonism in late adolescence or early adulthood. Other 59 different *ATP1A3 de novo* missense mutations are associated with AHC (Heinzen et al., 2014; Rosewich et al.,

2014; Sasaki et al., 2014; Ulate-Campos et al., 2014; Yang et al., 2014; Panagiotakaki et al., 2015; Viollet et al., 2015). AHC is characterized by onset of hemiplegic/quadriplegic episodes within 18 months of birth. Other, more variable neurological abnormalities of AHC include choreathosis, ataxia, developmental delays, seizures, and high incidence of neuropsychiatric disorders (Demos et al., 2014).

Recently, the mutations causing RDP and AHC were mapped according to their amino acid position in the α_3 isoform showing their location and the number of patients identified that harbors these mutations (Heinzen et al., 2014).

So far, only a single missense mutation in *ATP1A3* is associated with CAPOS syndrome (Demos et al., 2014; Heimer et al., 2015). CAPOS patients show onset of symptoms at the age of 6 months to 5 years. CAPOS syndrome is characterized by episodes of ataxic encephalopathy, weakness, and loss of hearing and sight (Brashear et al., 2014).

Interestingly, a recent report identified a G316S mutation in the α_3 isoform associated with Adult Rapid-onset Ataxia (Sweadner et al., 2016). The clinical examination noted most RDP symptoms except dystonia.

Thus, it appears that the *ATP1A3*-related disorders arise from autosomal dominant mutations with variable penetrance (Demos et al., 2014). Affected patients typically present in the context of an acute onset of paroxysmal, episodic neurological symptoms that may include hemiplegia, dystonia, ataxia, or seizures. Some symptoms may persist after resolution, such as neurodevelopmental delays, attention deficits, hyperactivity, trunk instability, dystonia, or ataxia (Mikati et al., 2000; Shafer et al., 2005; Panagiotakaki et al., 2010; Heinzen et al., 2012, 2014; Sweney et al., 2015). Although the *ATP1A3*-related neurological disorders are considered clinically distinct, the phenotypic spectrum of each disease continues to expand (Heinzen et al., 2014; Rosewich et al., 2014; Sweney et al., 2015). In support, there have recently been identified patients with intermediate, non-classical symptoms (Sasaki et al., 2013; Termsarasab et al., 2015).

GENOTYPE-PHENOTYPE—AFFECT PROTEIN FUNCTION—CAUSE SYMPTOMS

A recent case study of 35 AHC patients showed relatively mild symptoms in patients carrying the D801N mutation, whereas the E815K mutation was associated with far more severe symptoms (Sasaki et al., 2014). In fact, the same *ATP1A3* mutation may result in quite different time of onset and disease progression (Dobyns et al., 1993; Oblak et al., 2014), emphasizing that other factors, such as genetic background and epigenetic regulation play a large role in disease penetrance.

Recent functional studies suggest that most RDP mutations do not reach the cell surface (Heinzen et al., 2012). In contrast, the majority of AHC mutant proteins exert dominant negative effects on the wild type protein at the cell surface (Clapcote et al., 2009; Li et al., 2015), thus explaining the more severe phenotypes associated with AHC. The majority of AHC mutations (>70%) are located within the transmembrane helices of the Na^+/K^+-ATPase protein (Heinzen et al., 2012). Homology modeling of the two most common mutations, D801N and E815K suggested different mutational consequences: In the D801N mutation, a positive dipole was formed, which through electrostatic repulsion directly affected passage of K^+ ions (Kirshenbaum et al., 2013). In contrast, the E815K mutation prevented inward H^+ currents through the Na^+/K^+-ATPase. Intracellular H^+ currents through the Na^+/K^+-ATPase were recently identified (Vedovato and Gadsby, 2014) and are known regulators of neuronal excitability (Takahashi and Copenhagen, 1996), thus suggesting a correlation between severity and loss of H^+ transport. The significance of this discovery however remains to be determined *in vivo*.

ATP1A3-MODIFIED MOUSE MODELS

Four genetically modified mouse models targeting the *Atp1a3* gene has been reported, and have been extensively used to study *in vivo* functions of the α_3 isoform (**Table 1**). A detailed comparison of mouse models looking at clinical features in AHC and *Atp1a3* mouse models, as well as behavioral testing has been reported (Hunanyan et al., 2015).

The *Atp1a3*$^{tm1Ling/+}$ was produced by introducing a single base pair mutation in intron 4 ($\alpha_3^{+/KOI4}$), causing an aberrant splicing of *Atp1a3,* effectively knocking out the allele (Moseley et al., 2007). The $\alpha_3^{+/\Delta E2-6}$ was generated by an eGFP-*Atp1a3* gene replacement strategy, knocking out one *Atp1a3* allele ($\alpha_3^{+/\Delta E2-6}$) (Ikeda et al., 2013). The *Myshkin* mouse ($\alpha_3^{+/I810N}$) (Clapcote et al., 2009) expressed the I810N AHC mutation (Panagiotakaki et al., 2015). The Mashlool mouse ($\alpha_3^{+/D801N}$, $Mashl^{+/-}$) (Hunanyan et al., 2015) was generated to study the effects of the most common mutation in AHC, D801N (Heinzen et al., 2012).

The cardiotonic steroids (CTS) constitute a group of organic compounds that show high binding affinity toward Na^+/K^+-ATPases and inhibit the catalytic activity by stabilizing the enzyme's phosphorylated E2-P state (Yatime et al., 2011; Laursen et al., 2013). CTS such as ouabain have been used for more than 50 years to study the function of Na^+/K^+-ATPases *in vitro* and in acute animal models. An ouabain-perfused mouse model presumably targeting the α_3 isoform in cerebellum and basal ganglia (Calderon et al., 2011) supplements the findings summarized in **Table 1**.

BASIC CHARACTERIZATION

Hippocampal lysates showed a significant reduction in α_3 protein expression in the $\alpha_3^{+/KOI4}$ mice and a compensatory upregulation of α_1 expression but not of α_2 (Moseley et al., 2007). In contrast, whole brain lysates from the *Myshkin* mice (Myk/+ and Myk/Myk) showed no changes in protein expression levels of the α_1, α_2, and α_3 isoforms relative to wild type, suggesting that the *Myshkin* mutation is a functional null allele of the *Atp1a3* gene, which encodes a normally expressed, but inactive enzyme, as revealed by reduced total ATPase activity (Clapcote et al., 2009). In support of this, the authors observed normal plasma

TABLE 1 | *Atp1a3* gene modified animals.

Mouse model[*a]	*Atp1a3* genetic alteration	Major behavioral observations	References
Atp1a3$^{tm1Ling/+}$ ($\alpha_3^{+/KOI4}$)	Deletion targeting intron 4	Increased locomotor activity	Moseley et al., 2007; DeAndrade et al., 2011; Kirshenbaum et al., 2011b
		Increased methamphetamine response	
		Intact grip strength	
		Spatial learning impairment	
		• Stress-induced symptoms • Motor deficits • Sensory system defects • Impaired novel object recognition • Despair • Anhedonia • Increased anxiety • Reduced learning and memory • Reduced sociability	
$\alpha_3^{+/\Delta E2-6}$	Deletion targeting exon 2–6	Increased locomotor activity	Ikeda et al., 2013; Sugimoto et al., 2014
		Increased dystonic response to intracerebellar kainate injections	
		Enhanced inhibitory neurotransmission	
		Intact grip strength	
		Enhanced motor balance	
		Stress-induced motor deficits	
Myshkin ($\alpha_3^{+/I810N}$)	Single nucleotide substitution causing a single amino acid substitution (I810N)	Increased locomotor activity	Clapcote et al., 2009; Kirshenbaum et al., 2011a, 2012, 2013
		Increased methamphetamine response	
		Spontaneous epileptic seizures	
		Neuronal hyperexcitability	
		Reduced learning and memory	
		Ataxia	
		Mania-like behavior	
Mashl$^{+/-}$ ($\alpha_3^{+/D801N}$) (Mashlool)	Single nucleotide substitution causing a single amino acid substitution (D801N)	Increased locomotor activity	Hunanyan et al., 2015
		Spontaneous epileptic seizures	
		Neuronal hyperexcitability	
		Reduced learning and memory	
		Ataxia	
		Dystonia	
		Hemiplegia	

[*a] *Two Atp1a3 knock-out (KO) and two knock-in (KI) mice have been described. For all four models, only heterozygous animals are viable after birth.*

membrane localization of the *Myskhin* α_3 isoform expressed in COS cells with only small amounts retained in the ER (Clapcote et al., 2009).

AHC patients tend to be smaller and weigh less—presumably due to eating difficulties (Neville and Ninan, 2007; Panagiotakaki et al., 2010). Although abilities to chew and swallow have not been addressed for any of the mouse models, it is interesting that the *Myshkin* and male Mashl$^{+/-}$ mice were reported to have smaller body sizes (Clapcote et al., 2009; Hunanyan et al., 2015). *Myshkin* mice crossed with mice expressing a bacterial artificial chromosome expressing wild type *Atp1a3* (*Atp1a3* BAC) regained approximately 80% of wild type total Na^+/K^+-ATPase activity and showed normal body size, thus supporting the theory that Na^+/K^+-ATPase activity loss correlates with symptoms (Clapcote et al., 2009).

EFFECTS ON MOTOR FUNCTION, BALANCE, AND COORDINATION

The motor dysfunction in RDP and AHC i.e., ataxia, dystonia, unsteady gait, and tip-toing are reflected in motor function,

balance problems, and gait disturbances (Brashear et al., 1997; Linazasoro et al., 2002; Svetel et al., 2010).

Combined, the *Atp1a3*$^{tm1Ling/+}$ and $\alpha_3^{+/\Delta E2-6}$ mouse models displayed mild motor symptoms, whereas the *Myshkin* and the Mashl$^{+/-}$ mice recapitulated a much broader spectrum of *ATP1A3*-disease related symptoms. Motor tests showed these defects were caused by dyscoordination rather than lack of muscle strength as grip strength was intact (Kirshenbaum et al., 2013; Hunanyan et al., 2015), and thus corresponds to reports of AHC patients.

Adult *Atp1a3*$^{tm1Ling/+}$ and $\alpha_3^{+/\Delta E2-6}$ mice displayed normal motor function when tested on the balance beam and accelerated rotarod (DeAndrade et al., 2011; Sugimoto et al., 2014). However, restraint caused a stress-induced deterioration of motor performance of mice on the balance beam and rotarod and a significant drop in ATPase activity from 85 to 67% (DeAndrade et al., 2011).

Similarly, restraint stress significantly reduced the hanging times of the $\alpha_3^{+/\Delta E2-6}$ mice (Sugimoto et al., 2014). In contrast to *Atp1a3*$^{tm1Ling/+}$ and $\alpha_3^{+/\Delta E2-6}$ mice, distinct motor symptoms were apparent in naïve *Myshkin* and the Mashl$^{+/-}$ mice: Both strains showed ataxia and tremor on the balance beam but only the *Myshkin* mice performed poorly on the accelerated rotarod (Kirshenbaum et al., 2013; Hunanyan et al., 2015).

Both *Myshkin* and the Mashl$^{+/-}$ mouse strains showed abnormal stride length (Kirshenbaum et al., 2013; Hunanyan et al., 2015). A similar phenotype was induced by restraint stress in the $\alpha_3^{+/\Delta E2-6}$mice (Sugimoto et al., 2014).

THE CEREBELLUM-BASAL GANGLIA CIRCUITRY

Dystonia, Ataxia, and Parkinsonism

Dystonia, ataxia, and Parkinsonism are core symptoms of RDP. A recent study showed that dystonia and ataxia could be reproduced by partially blocking the Na^+/K^+-ATPase in the cerebellum of mice using ouabain whereas disruption of the basal ganglia circuit caused Parkinsonism i.e., rigidity, akinesia, tremor, and hunched posture (Calderon et al., 2011). The symptoms induced by ouabain perfusion were shown to develop over several days and to be highly dependent on the ouabain concentration used. Interestingly, mice perfused with low concentrations of ouabain into the cerebellum and basal ganglia developed symptoms corresponding to high concentration of ouabain when subjected to stress. Thus, reflecting the stress-sensitive nature of *ATP1A3*-related diseases.

Cerebellar dysfunction has been observed in several rodent dystonia strains (Lorden et al., 1992; LeDoux and Lorden, 1998; Richter et al., 1998; Campbell et al., 1999; Fremont et al., 2014) and in patients suffering from AHC (Saito et al., 1998; Yamashita et al., 2005; Sasaki et al., 2009) and RDP (Oblak et al., 2014; Liu et al., 2015).

The $\alpha_3^{+/\Delta E2-6}$ mice showed prolonged dystonic periods after intracerebellar kainate injections (Ikeda et al., 2013). Despite reduced motor performance of both naïve *Myshkin* and the Mashl$^{+/-}$ mice, only the Mash$^{+/-}$ mice were reported to develop stress-induced generalized dystonia (Hunanyan et al., 2015). Mild dystonia is common among AHC patients carrying the Mashl$^{+/-}$ equivalent D801N mutation (Panagiotakaki et al., 2015), whereas only one of two AHC patients known to carry the *Myskhin*, I810N mutation, showed dystonia (Yang et al., 2014; Panagiotakaki et al., 2015). Presently, it is therefore not possible to determine if the differences observed can be attributed specifically to the *ATP1A3* mutation or to genetic background.

The deep cerebellar nuclei (DCN) integrate inhibitory signals from GABAergic Purkinje neurons and excitatory glutamatergic mossy fibers and climbing fiber pathways and constitute the majority output fibers from the cerebellum. The DCN connects to premotor and motor nuclei in the basal ganglia (Faull, 1978; Faull and Carman, 1978). Abnormal output from the DCN will have profound effects on motor function. The Purkinje neurons are the main output to the DCN and play a central role in DCN signal integration and overall cerebellar function.

Purkinje neurons are characterized by a high Na^+ channel density and short spike duration during which large amounts of Na^+ ions enter the neurons, emphasizing the requirement for functional a Na^+/K^+-ATPase (Llinás and Sugimori, 1980; Raman and Bean, 1997; Carter and Bean, 2009). Cerebellar Purkinje neurons only express the Na^+/K^+-ATPase α_3 isoform (Peng et al., 1997) and are therefore particularly sensitive to *ATP1A3* mutations. Furthermore, in developing $\alpha_3^{+/\Delta E2-6}$mice, reduced Na^+/K^+-ATPase α_3 expression caused a build-up of $[Na^+]_i$ and $[Ca^{2+}]_i$ in the terminals of molecular-layer interneurons resulting in enhanced inhibition of Purkinje neurons (Ikeda et al., 2013). This observation is potentially very interesting, as it offers a possible mechanism to explain how Na^+/K^+-ATPase perturbations may affect neuronal plasticity and motor learning.

Although further experiments are required to determine the extent of this observation, it is interesting, that cortical neurons isolated from the *Myshkin* mice showed increased $[Ca^{2+}]_i$ as well (Kirshenbaum et al., 2011a). This observation could imply that other neuronal populations may be affected. Indeed, Na^+/K^+-ATPase α_3 showed co-localization with markers for several populations of GABAergic neurons in the rodent and human brain (Bøttger et al., 2011; Ikeda et al., 2013; Paciorkowski et al., 2015), suggesting these populations to be sensitive to *ATP1A3* perturbations as well. Supporting GABA dysfunction, hippocampal neurons from the *Myshkin* and Mashl$^{+/-}$ mice showed increased excitability in response to high frequency stimulation (Clapcote et al., 2009; Hunanyan et al., 2015).

HEMIPLEGIA

Hemiplegia is specific to AHC (Sweney et al., 2015). The mechanism driving this symptom is poorly understood. Single-photon emission computed tomography (SPECT) scans of AHC patients showed malperfusion leading up to or during events in several cases (Kanazawa et al., 1991; Zupanc et al., 1991; Aminian et al., 1993; Wong et al., 1993). However, there have also been examples, where this phenotype was absent (Sweney et al., 2009; Sasaki et al., 2011). Supporting a vascular phenotype, the vasodilator, flunarizine is currently among the most widely

used prophylaxis of hemiplegia in AHC (Casaer and Azou, 1984; Bourgeois et al., 1993; Mikati et al., 2000). So far, there has been no investigation of this mechanism in the current animal models. Of the four mouse models, only the Mashl$^{+/-}$ mice were reported to develop stress-induced episodes of hemiplegia and quadriplegia (Hunanyan et al., 2015).

NEUROPSYCHIATRIC SYMPTOMS—MANIA

Previous studies have shown intracerebroventricular (ICV) injections of ouabain in high concentrations to be CNS stimulatory and convulsive (Doggett and Spencer, 1971; Davidson et al., 1978; Corazzi et al., 1985; Haglund and Schwartzkroin, 1990; Lees et al., 1990; Yu, 2003). In rats, this approach was used to induce mania (El-Mallakh et al., 2003; Riegel et al., 2009). Episodes mimicking manic periods of bipolar mood disorder are common among AHC patients. Especially the manic episodes are particularly detrimental, as the children are at high risk of injury (Personal communications, see Acknowledgement).

There is a strong link in literature between Na^+/K^+-ATPase dysfunction and bipolar mood disorder (el-Mallakh and Wyatt, 1995). Bipolar patients showed reduced Na^+/K^+-ATPase *ATP1A2* gene expression in isolated erythrocytes (Hokin-Neaverson and Jefferson, 1989a,b; Looney and el-Mallakh, 1997) and in the temporal cortex (Rose et al., 1998) and reduced Na^+/K^+-ATPase *ATP1A3* expression in prefrontal cortex (Tochigi et al., 2008).

A prominent feature of manic episodes is hyperactivity. Although direct comparison of hyperactivity levels is impossible, all *Atp1a3* mouse models showed open field hyperlocomotion (Kirshenbaum et al., 2011a; Ikeda et al., 2013; Hunanyan et al., 2015).

Exploration-based anxiety tests revealed all *Atp1a3* mouse strains to be less anxious, to have increased impulsivity and risk-taking and a reduced habituation, all of which are symptoms of mania (Moseley et al., 2007; Kirshenbaum et al., 2011a; Ikeda et al., 2013; Hunanyan et al., 2015; Termsarasab et al., 2015).

In further support of mania-like behavior, the *Myshkin* mice showed changes to circadian rhythm (Kirshenbaum et al., 2011a). These symptoms, along with hyperactivity were reversed by treating the *Myshkin* mice with the mood stabilizers, lithium and valproate (Kirshenbaum et al., 2011a).

ICV injection of ouabain in rats caused phosphorylation of ERK and AKT in the hippocampus (Ruktanonchai et al., 1998; Kim et al., 2008; Yu et al., 2010). Similar increases in phosphorylated ERK and AKT were observed in the *Myshkin* mice. Both signaling pathways have been implicated in the control of behavioral excitement in rodents (Prickaerts et al., 2006; Creson et al., 2009; Engel et al., 2009; Ackermann et al., 2010), making them potential targets for future mood stabilizers. Correspondingly, open field hyperactivity and open arm visits were reduced after administration of the ERK inhibitor, SL327 (Kirshenbaum et al., 2011a). The *Atp1a3*-BAC transgenic *Myshkin* showed a Na^+/K^+-ATPase activity increase from 58 to 74% relative to wild type levels and a partial normalization of AKT phosphorylation (Clapcote et al., 2009; Kirshenbaum et al., 2011a). Correspondingly, treating the mice with the ouabain inhibitor, rostafuroxin, had a normalizing effect on hyperlocomotion (Kirshenbaum et al., 2011a).

Although the manic phase seems most prevalent in all mouse models, there have been examples of the *Atp1a3*$^{tm1Ling/+}$ mice being able to recapitulate the depression-like symptoms of bipolar disorder. When subjected to a chronic variable stress protocol, the mice showed prominent symptoms of anxiety, including reduced exploration of open areas and attention deficits during novel object and sociability tests. At this point, the mice showed a Na^+/K^+-ATPase activity of 67% relative to wild type levels (Kirshenbaum et al., 2011b). Interestingly, these symptoms did not occur in wild type mice, suggesting that *ATP1A3* mutations may increase vulnerability to stress.

EPILEPSY

Epilepsy and bipolar disorder share a common pathophysiology (Mazza et al., 2007) and is often comorbid in human patients (Mula et al., 2010). According to a recent study, approximately half of all AHC patients experience at least one epileptic seizure (Panagiotakaki et al., 2010).

Reduced Na^+/K^+-ATPase activity has been reported in genetic animal models of epilepsy and in hippocampal tissue from epileptic patients (Brines et al., 1995; Fernandes et al., 1996; Vaillend et al., 2002) and have been proposed as a causal factor in myoclonus epilepsy and ragged red fibers disease, a rare form of inherited epilepsy (McNamara, 1994). Also, Na^+/K^+-ATPase activity was decreased in the brain of rodents after chemical induction of seizures using the convulsant, pentylenetetrazol (Schneider Oliveira et al., 2004; Marquezan et al., 2013). Recently, two children with catastrophic early life epilepsy were shown to carry novel *ATP1A3* mutations (Paciorkowski et al., 2015).

As described, ouabain is convulsive when administered to rodents intraventricularly in sufficiently high concentrations (Doggett and Spencer, 1971; Davidson et al., 1978; Corazzi et al., 1985; Haglund and Schwartzkroin, 1990; Lees et al., 1990; Yu, 2003; Alonso et al., 2013).

Several mechanisms can explain why neurons are vulnerable to *ATP1A3* insults. Seizures are often associated with a loss in metabolic energy (Araujo et al., 2014). Na^+/K^+-ATPases are highly sensitive to such perturbations, as they require approximately 50% of the energy available to the brain under normal circumstances (Attwell and Laughlin, 2001). Reduced Na^+/K^+-ATPase activity may cause hyperexcitability due to increased $[K^+]_o$ and membrane depolarization (Haglund and Schwartzkroin, 1990; Somjen, 2002). Further effects arise from the post-tetanic buildup of $[Na^+]_i$ (Azarias et al., 2013) and the following inhibition of the Ca^{2+}/Na^+ exchanger causing accumulation of $[Ca^{2+}]_i$ and subsequent effects on gene transcription (Lyons and West, 2011), neurotransmitter release (Neher and Sakaba, 2008), and synaptic plasticity (Zucker, 1999).

The *Myshkin* mice showed epileptic seizures and neuronal hyperexcitability (Clapcote et al., 2009). Supporting the

correlation between Na^+/K^+-ATPase α_3 activity and seizure resistance, the epileptic seizures did not occur in *Atp1a3*-BAC transgenic *Myshkin* mice (Clapcote et al., 2009).

Furthermore, the link between *ATP1A3*-disease mutations and epilepsy was observed in a Chinese 12-year old boy with the I810N mutation, who was reported to have AHC with developmental delay and epilepsy (Yang et al., 2014).

The Mashl$^{+/-}$ mice showed flurothyl-indiced seizures, focal epileptogenesis (via kindling) and demonstrated spontaneous recurrent seizures and neuronal excitability (Hunanyan et al., 2015). Similar predisposition to epileptogenesis has been observed in humans affected by AHC.

There have been no reports of epilepsy in either of the *Atp1a3*$^{tm1Ling/+}$ and $\alpha_3^{+/\Delta E2-6}$ mouse models (naïve or stressed), although the $\alpha_3^{+/\Delta E2-6}$ mice showed increased sensitivity to cerebellar kainate injections (Ikeda et al., 2013).

Epilepsy is associated with cognitive decline in human patients (Bergen, 2006). Correspondingly, signs of hippocampal necrosis and glial activation were initially reported for the *Myshkin* mice maintained on the 129S1/SvImJ strain background. Hippocampal pathology disappeared once the mice were crossed into the C57BL/6NCr strain (Kirshenbaum et al., 2011a). Also, once maintained in the C57BL/6NCr strain for 20 generations, the *Myshkin* mouse strain no longer developed spontaneous seizures (Kirshenbaum et al., 2011a). This observation parallels previous publications identifying the 129S1/SvImJ and C57BL/6 strains as relatively resistant to kainate-induced seizures whereas only the C57BL/6 strain was resistant to kainate-induced cell death (Schauwecker, 2002; McLin and Steward, 2006).

MEMORY

In a comprehensive report of 157 AHC patients, mental retardation was recorded in at least 92% of the cases (Panagiotakaki et al., 2010). Likewise, cognitive decline has been described in RDP patients (Cook et al., 2014).

Overall, naïve *Myshkin* and Mashl$^{+/-}$ mice displayed poor memory performance, whereas the performance of the *Atp1a3*$^{tm1Ling/+}$ and $\alpha_3^{+/\Delta E2-6}$ mice to some degree was dependent on stress. The *Atp1a3*$^{tm1Ling/}$ mice thus showed no learning in locating a hidden platform in the Morris water maze (Moseley et al., 2007). In contrast the *Atp1a3*$^{tm1Ling/+}$ mice performed normally in a novel object recognition test, but showed significantly worse performance after subjection to a CVS protocol (Kirshenbaum et al., 2011b).

The *Myshkin* mice performed significantly worse in contextual- and cued-dependent fear conditioning tests. In contrast, Mashl$^{+/-}$ mice showed impaired novel object recognition, but intact cued-dependent fear memory (Hunanyan et al., 2015).

The dorsal part of the hippocampus plays a central role in learning and spatial memory, whereas the ventral hippocampus primarily regulates emotional and motivated behaviors through interaction with the amygdala (Fanselow and Dong, 2010). Accordingly, ouabain injection into these brain regions caused impairments in spatial learning (Zhan et al., 2004) and fear-dependent memory, respectively (Mizumori et al., 1987).

This could suggest a difference in the performance of the ventral hippocampus between the two *Myshkin* and Masl$^{+/-}$ strains. However, the strong hyperactivity of the both strains may have interfered with the readout of the conditioning tests, as the tests rely on the ability to suppress movement. Furthermore, the *Myshkin* mice showed reduced learning in a conditioned taste aversion test, suggesting also hippocampus-independent memory functions were affected (Reilly et al., 1993; Purves et al., 1995).

Due to a high voltage dependency and a high permeability for Ca^{2+}, the N-methyl-D-aspartate receptors (NMDR) are important for triggering several different forms of synaptic plasticity, including long term potentiation and long-term depression (Cull-Candy et al., 2001). The *Atp1a3*$^{tm1Ling/+}$ mice showed reduced hippocampal expression of the NMDA NR1 subunit (Moseley et al., 2007), suggesting increased neuronal activity (Kvajo et al., 2004). Interestingly, NR1 expression was unaffected in the *Myshkin* mice (Clapcote et al., 2009). This observation is unexpected, as the *Myshkin* mice developed spontaneous seizures. However, direct comparison is not possible due to the FVB background of the *Myshkin* mice.

EFFECTS ON NEUROTRANSMITTER HOMEOSTASIS AND CIRCUITRY

As previously exemplified, decrease in Na^+/K^+-ATPase activity is associated with neuronal hyperexcitability and the release of neurotransmitters. Particularly dopamine, serotonin, and norepinephrine are involved in regulating movement and behavior (Perona et al., 2008).

In support of a dopamine phenotype, all naïve *Atp1a3* mouse models displayed hyperlocomotion in the open field test, which was further induced by amphetamine (Moseley et al., 2007; Kirshenbaum et al., 2011a). High Performance Liquid Chromatography (HPLC) analysis showed no changes in striatal levels of dopamine, serotonin, or their metabolites in the *Atp1a3*$^{tm1Ling/+}$ mice (DeAndrade et al., 2011). In cerebrospinal fluid (CSF) samples obtained from two RDP patients, low levels of the dopamine metabolite, homovanilic acid, was reported (Brashear et al., 1998). However, this observation remains to be confirmed as a diagnostic criterion for RDP. A recent study reported normal CSF neurotransmitter levels in AHC patients (Fons et al., 2012).

ADDITIONAL NEUROLOGICAL SYMPTOMS

Some ATP1A3-disease mutation related patients show additional neurological symptoms that range from mild limb cramping sometimes decades before developing RDP (Brashear et al., 2007) to dysfunction of the autonomic nervous system with cardiac repolarization problems (Novy et al., 2014; Jaffer et al., 2015) excessive or lack of perspiration, skin discoloration, gastrointestinal problems and changes in body temperature

leading up to or during attacks in AHC patients (Mikati et al., 2000; Fons et al., 2012).

The *Myshkin* mice showed increased systolic and diastolic blood pressure, but normal heart rate (Kirshenbaum et al., 2013). Most likely, this effect is related to *ATP1A3* expression in cardiomyocytes (Zahler et al., 1993). The Mashl$^{+/-}$ mice and stressed female *Atp1a3*$^{tm1Ling/+}$ mice showed delayed temperature response (DeAndrade et al., 2011; Hunanyan et al., 2015), suggesting impaired thermoception. The α_3 Na$^+$/K$^+$-ATPase expression was recently reported in dorsal root ganglion γ motor neurons located in the spinal cord of mice (Edwards et al., 2013). It is therefore very likely to have implications for motor control also. Somatosensory evoked potentials (SEP) during the interictal period showed abnormal recovery cycle in a recent case report of seven AHC patients, suggesting multilevel somatosensory system hyperexcitability (Vollono et al., 2014). Sensory abnormalities have been proposed to play a role in the pathophysiology of dystonia as the basal ganglia and other motor areas are heavily connected to the somatosensory system (Tinazzi et al., 2003). Future studies may elucidate if a similar interaction affects the pathophysiology of *ATP1A3*-related diseases.

SUMMARY

The current *Atp1a3* mouse models recapitulate to a large part the symptoms of RDP and AHC.

Through the collaborative efforts of the *ATP1A3*-disease research community, it has recently been possible to carry out several studies on relatively large patient groups. Such studies continue to be invaluable not only in the search for common denominators but also for establishing the animal models for the ATP1A3-diseases.

Previous studies using CTS to study Na$^+$/K$^+$-ATPase function suggest a strong correlation between reduced Na$^+$/K$^+$-ATPase activity and severity of symptoms. The present *Atp1a3* mouse models seem to support this as the *Atp1a3*$^{tm1Ling/+}$ and $\alpha_3^{+/\Delta E2-6}$ mice (with mild Na$^+$/K$^+$-ATPase reduction) showed relatively mild symptoms whereas the *Myshkin* and Mashl$^{+/-}$ models carrying AHC mutations (with larger Na$^+$/K$^+$-ATPase reduction), recapitulated most of the key phenotypes. As a proof of concept, most symptoms of the *Myshkin* mouse were rescued by increasing the Na$^+$/K$^+$-ATPase activity. Future experiments will be required to establish if similar approaches can be translated into a possible treatment.

Animal models represent valuable tools to study the pathology of human diseases. The biological questions can be assessed by different model system, depending on the nature of the study. Different advantages and disadvantages for designing and generating mouse models. Knock-out mouse models represent loss-of-function studies of a gene, while knock-in allows to explore the consequences of a single amino acid mutation inroduced into the genome. Both knock-out and knock-in models are can be designed as conditional models, allowing to knock-out, or introduce a mutation (knock-in) in single cell populations or organs, using the Cre LoxP system (Branda and Dymecki, 2004). Blocking Na$^+$/K$^+$-ATPase activity by infusion of ouabain (Fremont et al., 2014) or RNAi tools (Fremont et al., 2015) into specific brain areas represent a another model to investigate the function in specific brain areas in rodent models.

The highly variable nature of *ATP1A3*-disease related symptoms are becoming increasingly apparent. Despite recent advances in elucidating the etiology of individual *ATP1A3* mutations, large variations are reported even for patients with the same mutation. It is very likely that genetic background, epigenetic as well as environmental factors play a central role in disease penetrance.

AUTHOR CONTRIBUTIONS

TH and KL discussed, outlined and co-wrote the review.

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REVIEW
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The Influence of Na^+, K^+-ATPase on Glutamate Signaling in Neurodegenerative Diseases and Senescence

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Decreased Na^+, K^+-ATPase (NKA) activity causes energy deficiency, which is commonly observed in neurodegenerative diseases. The NKA is constituted of three subunits: α, β, and γ, with four distinct isoforms of the catalytic α subunit (α_{1-4}). Genetic mutations in the *ATP1A2* gene and *ATP1A3* gene, encoding the α_2 and α_3 subunit isoforms, respectively can cause distinct neurological disorders, concurrent to impaired NKA activity. Within the central nervous system (CNS), the α_2 isoform is expressed mostly in glial cells and the α_3 isoform is neuron-specific. Mutations in *ATP1A2* gene can result in familial hemiplegic migraine (FHM2), while mutations in the *ATP1A3* gene can cause Rapid-onset dystonia-Parkinsonism (RDP) and alternating hemiplegia of childhood (AHC), as well as the cerebellar ataxia, areflexia, pescavus, optic atrophy and sensorineural hearing loss (CAPOS) syndrome. Data indicates that the central glutamatergic system is affected by mutations in the α_2 isoform, however further investigations are required to establish a connection to mutations in the α_3 isoform, especially given the diagnostic confusion and overlap with glutamate transporter disease. The age-related decline in brain $\alpha_{2/3}$ activity may arise from changes in the cyclic guanosine monophosphate (cGMP) and cGMP-dependent protein kinase (PKG) pathway. Glutamate, through nitric oxide synthase (NOS), cGMP and PKG, stimulates brain $\alpha_{2/3}$ activity, with the glutamatergic N-methyl-D-aspartate (NMDA) receptor cascade able to drive an adaptive, neuroprotective response to inflammatory and challenging stimuli, including amyloid-β. Here we review the NKA, both as an ion pump as well as a receptor that interacts with NMDA, including the role of NKA subunits mutations. Failure of the NKA-associated adaptive response mechanisms may render neurons more susceptible to degeneration over the course of aging.

Keywords: Na^+, K^+-ATPase, glutamate, aging, *ATP1A2* and *ATP1A3* mutations, neurodegenerative diseases

INTRODUCTION

Na^+, K^+-ATPase

In 1957, the Danish physician Jens C. Skou discovered the mechanism behind active ion transport in homogenates of leg nerve from shore crabs: a Mg^{2+}-dependent ATPase stimulated by Na^+ and K^+, speculated to be located at the plasma membrane (Skou, 1957). In the same year that Skou published his ATPase work, Robert L. Post and Philip Jolly showed that interdependent active Na^+ efflux and K^+ influx followed an electrogenic stoichiometry of 3:2 (Post and Jolly, 1957), an exchange previously shown to be blocked by the cardiotonic steroid (CTS) isolated from plant species of the genus *Strophanthus*, strophantin, widely known as ouabain (OUA) (Schatzmann, 1953; Schatzmann and Witt, 1954). In 1960, Post and colleagues demonstrated the presence of Na^+, K^+-ATPase (NKA) in the plasma membrane of human red blood cells (Post et al., 1960),including its transient phosphorylation by ATP on an aspartyl residue (Post et al., 1965; Post and Kume, 1973). Subsequent work indicated a feed-forward reaction, whereby as Na^+ binds to the enzyme, it stimulates a Mg^{2+}-dependent phosphorylation via ATP, while K^+ binding facilitates dephosphorylation, giving a transition of two conformations named E1 and E2, respectively (Post et al., 1972). Later work made it clear that activity of this molecular motor is vital for cell integrity, given its maintenance of the osmotic balance and its powerful role in cell homeostasis, including preserving and contributing to the resting membrane potential, electrolyte constitution of the cerebrospinal fluid (CSF) and influencing the cellular and/or transcellular transport of other ions, energy substrates and neurotransmitters (Blanco and Mercer, 1998; Skou, 1998; Blanco, 2005).

The enzyme is composed of two distinct polypeptides (Kyte, 1971), with equimolar stoichiometry (Craig and Kyte, 1980). The larger one is the α subunit, with an amino acid sequence (>1010 amino acids) that was first deduced from sheep kidney complementary DNA in 1985 by Jerry Lingrel's group (Shull et al., 1985). The α subunit has a mass of 110–112 kDa, with 10 transmembrane domains, mostly with protein resides in the cytoplasm side and with less than 10% exposed to the extracellular milieu (Blanco, 2005). The α subunit is also known as the catalytic or functional subunit, since it contains the binding sites for Na^+ and K^+, as well as for CTS. The α subunit is subject to transient phosphorylation and breaks down Mg^{2+}-ATPase (Kyte, 1975; Blanco, 2005). The smaller β subunit is a type II (one transmembrane domain with intracellular N-terminal) glycoprotein of 302–304 amino acids that noncovalently binds to the α subunit (Shull et al., 1985). Although the α subunit can display independent ATP catalysis (Blanco et al., 1994), it is only when it is associated with the β subunit that normal pumping activity is achieved. The β subunit also influences the NKA kinetic characteristics, assisting the correct assembly and membrane delivery of the enzyme (chaperone function), as well as helping in cell adhesion and cell polarity (Blanco, 2005; Geering, 2008; Cereijido et al., 2012; Vagin et al., 2012). A third partner comprises the αβ protomer (formerly termed the γ subunit) (Forbush et al., 1978). This type I (one transmembrane domain with intracellular C-terminal) protein is small—around 65 amino acids, with a mass of 7 kDa—and belongs to the FXYD family of ion transport regulators. FXYD represents the signature motif found in all 7 members. FXYD2 is the original γ subunit, although all can interact with NKA (Geering, 2008). However, they are not necessary for enzymatic activity, modulating, usually decreasing, pump affinity for cations (Geering, 2006, 2008).The electrophoresis technique has unveiled puzzling pharmacological data that demonstrate differences in the potency and affinity of CTS for NKA, when NKA is derived from different tissues and species (Akera et al., 1972; Tobin and Brody, 1972; De Pover and Godfraind, 1976, 1979; Marks and Seeds, 1978; Noel and Godfraind, 1984). By the end of 1970's, Kathlyn Sweadner demonstrated in brain preparations of several mammalian species that two protein bands of slightly different molecular weights could be clearly defined, with the biochemical characteristics of NKA (Sweadner, 1979). The kidney α subunit co-migrated with the brain lower migrating (lighter) band in gel and was designated α (now α1), with the other brain band, heavier and not present in kidney, designated α(+) (Sweadner and Gilkeson, 1985). A third form was revealed from rat brain cDNA sequence analysis and named αIII (now α3), a major form in the central nervous system (CNS) (Shull et al., 1986; Schneider et al., 1988; Sweadner, 1989). α(+) was indeed a mixture of α2 and α3. Years later, a fourth α isoform was discovered and characterized (Shamraj and Lingrel, 1994; Woo et al., 1999; Blanco et al., 2000). All isoforms are encoded by different genes, have a high degree of sequence homology and are expressed in a cell/tissue-specific manner (Broude et al., 1989; Mobasheri et al., 2000; Blanco, 2005): α1 is expressed in all mammalian tissue, being virtually the only α isoform expressed in the kidney across species; α2 is found in striated and smooth muscles, adipose tissue, nervous tissue and some other tissues; α3 and α4 have a more restricted distribution, with the former being primarily found in nervous tissue, more specifically in neurons, where it may be considered a brain neuronal marker (Dobretsov and Stimers, 2005), and the latter is only expressed in the midpiece of the sperm, where it is important for sperm motility (Sanchez et al., 2006).

A Pump or a Receptor: The Janus Face of an ATPase

NKA has been classically conceptualized as a pump, with its specific inhibition by CTS that increases intracellular Na^+ and Ca^{2+} concentrations leading to cell ionic imbalance (Akera and Brody, 1977). This was challenged by a series of elegant studies headed by Zi-Jian Xie and Amir Askari 20 years ago. These researchers observed that OUA induced myocyte hypertrophy by activating proto-oncogenes and late response genes, with effects mediated by the Ras-Raf-ERK1/2-pathway (Peng et al., 1996; Huang L. et al., 1997; Kometiani et al., 1998). This was preceded by EGFR transactivation through the nonreceptor Tyr kinase, Src, steps not dependent on Ca^{2+}(Haas et al., 2000). Such signal transducer pump effects arise from activity in restricted areas of the plasma membrane, within a subset of lipid rafts known as caveolae (Liu et al., 2003; Wang et al.,

2004; Quintas et al., 2010). NKA (E1 state) can interact with Src at the kinase and SH3 domain with OUA, by arresting the E2 conformation of the ATPase, unleashing the kinase domain of Src, thereby inducing protein-protein signaling (Liang et al., 2006; Tian et al., 2006; Ye et al., 2013). As such, NKA-Src forms a binary receptor that transduces signals after CTS binding.

After the first reports on the novel signaling role of NKA, a number of different intracellular pathways involved in distinct cell fates has been unveiled, confirming NKA as more than an ion pump (Nesher et al., 2007; Cai et al., 2008; Riganti et al., 2011; Arnaud-Batista et al., 2012; Lucas et al., 2012) and helping to clarify how endogenous CTS can produce their effects *in vivo*, even at very low concentrations (Xie and Xie, 2005; Aperia et al., 2016).

NKA Mutations and Diseases

Considering the importance of NKA in basic cellular functions, it has been suggested that NKA mutations affecting α1 (*ATP1A1*), α2 (*ATP1A2*), and α3 (*ATP1A3*) genes can contribute to the pathogenesis of several CNS diseases.

ATP1A1 Mutations

Mutation in the *ATP1A1* gene leads to primary aldosteronism (Azizan et al., 2013), which is the main cause of secondary hypertension. Aldosterone production is elevated and non-suppressible by sodium loading (Duan and Mete, 2015). The mutation causes a decrease in NKA activity and in K^+ affinity, consequently leading to an inappropriate cellular depolarization (Beuschlein et al., 2013). Primary aldosteronism can also be caused by mutations in ATP2B3 (Ca^{2+}-ATPase), CACNA1D (Cav1.3), and KCNJ5 (K^+ channel) (Azizan et al., 2013; Zennaro et al., 2015). In a study with 474 patients, *ATP1A1* mutation was found in 5.3% of the sample, although the relationship between the disorder and the *ATP1A1* mutation was only discovered recently, requiring further investigations as to the mechanism involved (Fernandes-Rosa et al., 2014).

ATP1A3 Mutations

ATP1A3 is only expressed in CNS neurons, mostly in the cerebellum and basal ganglia, key structures in the regulation of a range of functions, including motor activity, memory and spatial learning. The *ATP1A3* mutations are common in the conserved transmembrane or N-terminus domain of NKA and are related to rare disorders, such as rapid-onset dystonia-parkinsonism (RDP), alternating hemiplegia of childhood (AHC), and cerebellar ataxia, areflexia, pescavus, optic atrophy, and sensorineural hearing loss (CAPOS) syndrome. Although having many common features, these three diseases have quite distinct phenotypes (Sweney et al., 2015).

RDP (or DYT12) is a type of dystonia, being classed as a hyperkinetic movement disorder. RDP onset can be highly variable, occurring from 18 months to 55 years, suggesting considerable heterogeneity in its pathophysiology (Sweney et al., 2015). The main features are involuntary muscle contractions, abnormal posture and repetitive movements. RDP was first linked to *ATP1A3* mutations by De Carvalho Aguiar and colleagues in 2004 (de Carvalho Aguiar et al., 2004), having an autosomal dominant inheritance. However, this disorder can also be sporadic or not related to any mutation in *ATP13A* (Kabakci et al., 2005). Although some RDP symptoms resemble Parkinson's disease, with both disorders showing evidence of abnormal CSF dopamine metabolites, RDP patients are unresponsive to deep brain stimulation (Charlesworth et al., 2013) or to L-DOPA treatment (Asmus and Gasser, 2010). RDP differentiation from Parkinson's disease is based on: triggering by physical or emotional stress, abrupt onset, bulbar involvement and normal computed tomography in the striatum (Zanotti-Fregonara et al., 2008; Asmus and Gasser, 2010).

Twelve mutations are associated with RDP, each being related to different severity levels. RDP treatment is only symptomatic, mostly utilizing benzodiazepines (Sweney et al., 2015). The T613M mutation is the most common and with the most severe outcome. Psychiatric conditions, such as bipolar disorder and anxiety, seem to be related to RDP (Barbano et al., 2012). A growing variety of clinical presentations have been reported in association with these mutations, including episodes of flaccidity and lack of motion for hours leading to stiffness (Anselm et al., 2009) or delayed motor development and hypotonia that lead to a uncoordinated gait, as well as speech and swallowing difficulties in R756H and D823N mutations.

Recent studies on a family with RDP, where only women present with a symptomatic phenotype, indicate a new mutation that causes a deletion (c.443_445delGAG, p.Ser148del). A male member from the same family also carries the p.Ser148del mutation, but he does not have any symptoms, suggesting a reduced penetrance of this mutation (Wilcox et al., 2015). RDP patients are still commonly misdiagnosed, indicating the need to incorporate new features in order to make an appropriate diagnosis (Brashear et al., 2012).

The onset of motor symptoms in RDP patients occurs usually by 25 years of age, with initial symptoms occurring in the upper body. Following initial diagnosis, patients have been shown to have poor learning, memory and psychomotor speed vs. controls (Cook et al., 2014). This would seem to arise from α3 being highly expressed in all nuclei of the basal ganglia and cerebellum, which are important regions for the control of movement, and in the hippocampus, which is crucial to spatial learning and memory (Oblak et al., 2014). Experiments utilizing *in vitro* and *in vivo* models have investigated the alterations driven by these mutations, including the role of impaired NKA activity. A C-terminus protein mutation decreases Na^+ affinity, although no abnormality in biogenesis or plasma membrane targeting was found. Also, a decreased survival in these neuronal cells was observed when they were treated with OUA (Blanco-Arias et al., 2009).

Other mutations were also tested in COS7 cells, with no phosphorylation from ATP and low Na^+ affinity being detected in most mutations. Consequently, increased intracellular Na^+ and decreased extracellular K^+ were observed. Thus, the affinity of Na^+ may be an important concern in RDP (Toustrup-Jensen et al., 2014) (**Figure 1**). In mutated α3 mice (Het mice), stress exposure was capable of causing motor and balance deficits characteristic of RDP (DeAndrade et al., 2011). These authors

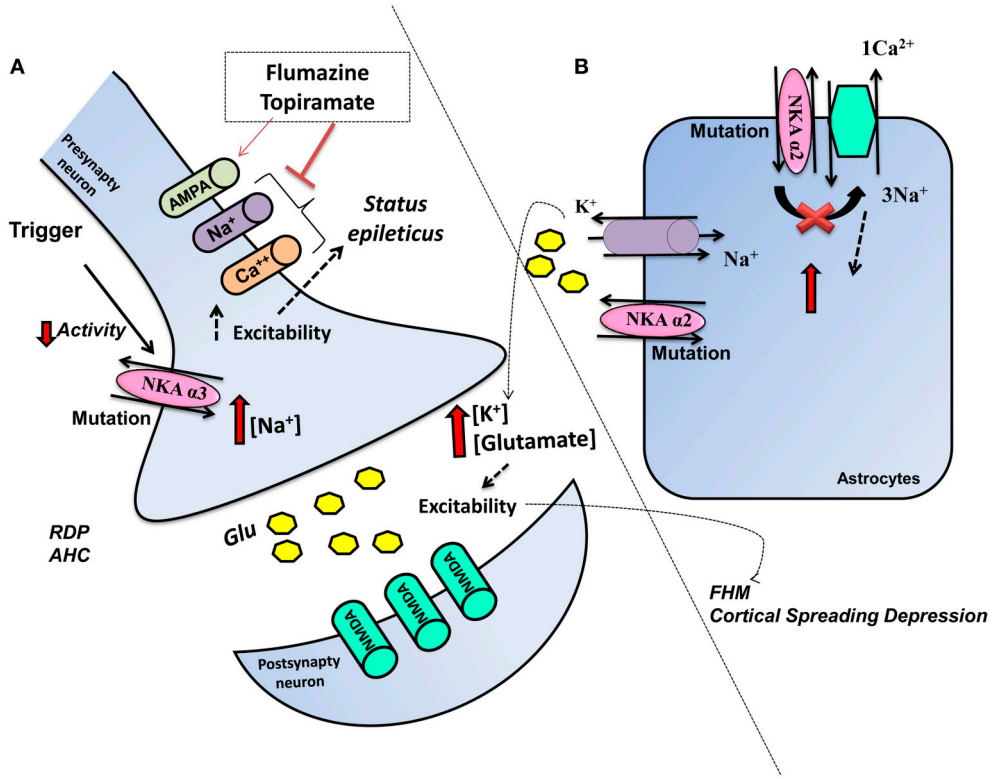

FIGURE 1 | Schematic representation of the influence of *ATP1A2* mutations in glia and *ATP1A3* mutations in neurons on glutamatergic system activity. Mutations cause a dysfunctional NKA activity, **(A)** *ATP1A3* mutation in neurons decreases the NKA activity and increases intracellular Na^+, which increases the cellular excitability, thereby affecting neuronal functions. Flumarazine and topiramate could be used as treatment in some cases. **(B)** *ATP1A2* in astrocytes results in change of metabolism, more K^+, and glutamate in the extracellular space and rendering the CNS more vulnerable to migraine, seizures, and neurodegenerative process.

also showed significant alterations in monoamine metabolism and thermal sensitivity in this model (DeAndrade et al., 2011).

AHC is a neurodevelopmental disorder described initially in 1971 by Verret and Steele (1971). However, it was only in 2012 that AHC was discovered to be a heterozygous *ATP1A3 de novo* mutation (Heinzen et al., 2012; Rosewich et al., 2014). AHC patients present with an onset before the age of 18 months and following an environmental or physiological stimulus, which is also present in RDP. AHC patients present with motor delay and attacks of hemiplegia, as well as cognitive and intellectual deficits (Sweney et al., 2015). AHC disorder can be difficult to differential diagnosis especially in relation to familial hemiplegic migraine (FHM) and glutamate transport disease (Jen et al., 2005). Sleep can stop AHC attacks, although even after sleep, attacks can return. Avoidance of the possible triggers is also important to prophylaxis, whilst antiepileptic drugs should only be used in patients showing *status epilepticus* (Rosewich et al., 2014; Paciorkowski et al., 2015). Flunarizine is a Ca^{2+} and Na^+ channel blocker, which was developed to treat migraine and is currently the main AHC treatment, although the mechanism of action is unknown. Similarly, topiramate's mode of efficacy is unknown and may include its inhibition of 2-amino-3-(3-hydroxyl-5-methyl-4-isoxazolyl) propionic acid (AMPA) receptors and carbonic anhydrase, thereby lowering extracellular pH (Hoei-Hansen Et al., 2014; Yang et al., 2014) (**Figure 1**).

Another strategy used in AHC patients is the ketogenic diet/modified Atkins diet, given that a family with the p.Asp923Asn mutation was initially misdiagnosed as GLUT1DS (glucose transporter deficiency syndrome), with 15 months of diet management leading to no further attacks. A ketogenic diet provides an alternative energy source for the brain and may decrease neuroexcitability (Roubergue et al., 2015). In some cases, when a patient has two mutations in SLC2A1 and *ATP1A3*, flunarizine provides an incomplete response, with concurrent use of a ketogenic diet improving the utility of flunarizine (Ulate-Campos et al., 2014). A patient with c.2401G>A; p.D801N mutation failed to respond to all the treatment strategies and only the use of a corticosteroid achieved complete remission (Wong and Kwong, 2015).

In Japan, the more severe phenotype arising from the E815K mutation was found to be more common than in other countries, occurring in 50% of investigated patients. The more severe phenotype evident in these mutations includes: earlier age of onset, more epileptic episodes and increased motor impairment (Ishii et al., 2013). Usually patients with no detectable mutation have less symptomatology (Hoei-Hansen Et al., 2014), whilst an early onset of AHC symptoms seems

positively correlated with a more severe clinical course (Viollet et al., 2015).

A Danish study showed bilateral episodes to be common, with hemiplegic episodes not always alternating. Consequently, the term AHC may not be the most appropriate (Hoei-Hansen Et al., 2014). A recent study of 155 patients showed 34 mutations to be related to AHC, with at least one being detected in 85% of patients. The three major mutations were found p.Glu815Lys, p.Asp801Asn, and p.Gly947Arg, with the first having the most severe phenotype, as indicated by greater intellectual impairment and motor disability. In contrast, p.Asp801Asn presents with a milder phenotype, whilst p.Gly947Arg appears to have the most favorable prognosis, in comparison to other mutations (Panagiotakaki et al., 2015).

Some mutations show a symptomatology that indicates a combination of RDP and AHC, including c.2600G>A, D923N and R756 (Rosewich et al., 2014). The E277K and D923N mutations have been described in both (Boelman et al., 2014). The difference between RDP and AHC may be due to different regions and cells affected by the mutations in ATP13A, with differential impacts arising from effects within a developmental context (Ozelius, 2012). Patients with epilepsy had an earlier onset, although no relationship with a specific mutation (Panagiotakaki et al., 2015). AHC patients have electrocardiogram and repolarization abnormalities compared to people with epilepsy, contributing to episodes of collapse unrelated to seizures, as well as to some premature deaths (Novy et al., 2014; Jaffer et al., 2015). Activated platelets from AHC patients have functional and structural abnormalities, with 93 proteins related to metabolism being differentially expressed in comparison to healthy controls. Cathepsin B was also more highly expressed, indicating an important role of apoptotic pathway activation in driving mutation-associated cell death (Di Michele et al., 2013). An AHC patient showing a mutation in SLC2A1 p.Gly18Arg, which leads to the loss of GLUT1 expression, was also diagnosed with hemiplegic migraine, which can be related to increases in cortex glutamate levels. As such, glutamate levels should also be investigated in mutation-associated AHC patients (Weller et al., 2015).

Mutations of *ATP1A3* that can be evident in AHC patients, have been tested in Sf9 cells. OUA binding, NKA activity and phosphorylation were measured for each mutant and were found to be abnormal in I274N, E815K, and G947R mutations. Some mutations showed normal bind to OUA, although no NKA activity or phosphorylation were found. Such differences may be associated with phenotype severity (Weigand et al., 2014). In a murine AHC model, the D801N mutant mouse (Mashlool, Mashl+/), a similar symptomatology is found as that evident in AHC patients, including abnormalities in gait and motor coordination, as well as spontaneous recurrent seizures and hemiplegia. The study of hippocampal slices showed increased excitability, which contributes to abnormal plasticity and a predisposition to more spontaneous seizures (Hunanyan et al., 2015).

Another animal model, the Myshkin (Myk) model, carries the I810N mutation, which presents with a reduced threshold for hippocampal seizures and neuronal loss. Although α3 is normally expressed, NKA activity is reduced with a consequent reduction in thalamocortical functional connectivity (Clapcote et al., 2009; Kirshenbaum et al., 2013). This murine model is also used as a model of bipolar disorder, with symptoms being improved by melatonin and physical exercise (Kirshenbaum et al., 2014). An α3 mutation is evident in the Atp1a3tm1Ling murine model, leading to a reduction in hippocampal α3 expression, thereby lowering NKA activity. Dystonia is only induced in this model after kainate injection into the cerebellum (Ikeda et al., 2013).

In 1996, CAPOS syndrome was described, with only the c.2452G> mutation in *ATP1A3* being found in four families studied. This is a gain-of-function mutation, which differs from the loss mutations evident in RDP and AHC (Demos et al., 2014). CAPOS is a rare syndrome associated with febrile illness, with each patient differing in regard to severity and outcome (Sweney et al., 2015). Recently, CAPOS syndrome has been shown to cause hemiplegic migraine in a single case study (Potic et al., 2015).

ATP1A2 Mutations

Migraine is a brain disorder that affects over 10% of the population, characterized by a headache associated with neurological and autonomic symptoms. Moreover, it frequently occurs as a pulsating and unilateral headache, usually followed by phono/photophobia and nausea (Russell et al., 1994). Migraine is thought to be initiated by brain dysregulation, leading to activation and increased vulnerability of the trigeminovascular system, mainly the trigeminal nociceptive afferents innervating the meninges (Goadsby et al., 2002; Pietrobon and Striessnig, 2003; Pietrobon, 2005). Migraine disorders may be preceded, or not, by neurological symptoms that are often visual, and can therefore be classified as migraine with or without aura (Pietrobon and Striessnig, 2003; Pietrobon, 2005). Clinical studies indicate that migraine aura is related with a wave of cortical spreading depression (CSD). CSD is a strong wave of short duration of neuronal and glial cell depolarization, which extends over the cortex and is accompanied by long-lasting depression of neuronal activity (Lauritzen, 1994; Cutrer et al., 1998; Bowyer et al., 2001; Hadjikhani et al., 2001). Studies have shown a relationship between CSD and migraine aura in humans and animal models and its importance to trigeminal activation (Russell et al., 1994; Bowyer et al., 2001; Hadjikhani et al., 2001; Bolay et al., 2002; Goadsby, 2002; Dalkara et al., 2006; Zhang et al., 2010).

FHM is a special autosomal dominant subtype of migraine with aura, characterized by some degree of motor frailty or paralysis, often hemiparesis (Thomsen et al., 2002). Furthermore, severe cases have been associated with symptoms such as progressive cerebellar ataxia, coma, fever, and/or epileptic seizures (Ducros et al., 1997). FHM is a genetically heterogeneous disease, in which mutations can occur in the ion transportation genes *CACNA1A*, *ATP1A2,* and *SCN1A* (Rogawski, 2008), promoting the different types of familial migraines, FHM1, FHM2 and FHM3 (Russell et al., 1994; Pierelli et al., 2006; Deprez et al., 2008). FHM1 is induced by mutations in *CACNA1A* at chromosome 19p13, which encodes the Cav2.1 (P/Q-type) (Ophoff et al., 1996). FHM2 is occasioned by mutations in the *ATP1A2* at chromosome 1q23 (De Fusco et al., 2003). The

last gene is *SCN1A*, encoding the α1 subunit of the neuronal sodium channel, the mutation in which leads to FHM3 (Dichgans et al., 2005). In this review, we describe only mutations in *ATP1A2*, as well as the role of glutamate in the development of FHM. FHM was the first human disease linked to mutations of the α2 catalytic subunit, which was first demonstrated in two Italian families (De Fusco et al., 2003). There is also a linkage between sporadic hemiplegic migraine (SHM) and mutations in α2 (Thomsen and Olesen, 2004; Kirchmann et al., 2006), with other brain disorders also associated with α2 mutations.

In the mouse, α2 is expressed in cardiac and skeletal muscle throughout life. However, in neurons, *Atp1a2* is only expressed during embryonic development (Moseley et al., 2003), being present in astrocytes and meningeal tissues in adulthood (McGrail et al., 1991; Watts et al., 1991; Peng et al., 1997). During neuronal activity, NKA is important for removing K^+ from the synaptic cleft. Moreover, it is fundamental for the re-uptake of released glutamate from the extracellular space, since active transport of glutamate into neurons and astrocytes is regulated by Na^+ and K^+ gradients, emphasizing the importance of NKA in synaptic regulation (Ransom et al., 2000; D'Ambrosio et al., 2002; Jorgensen et al., 2003). *Atp1a2*-deficient embryos have impaired neurotransmitter clearance, as well as potentiated neural excitation and cell death, specifically in the amygdala and piriform cortex. Consequently, heterozygous adult mice show increased levels of anxiety/fear behaviors, concurrent to raised levels of c-Fos and increased neuronal activity in the piriform cortex and amygdala after fear stimuli. This indicates a role for α2 in regulating not only neural activity (Ikeda et al., 2003), but also affective processing, including in the role of the amygdala in cortex development.

Kawakami et al. (2005) reported that heterozygous *Atp1a2* mice, $\alpha_2^{+/-}KOE_{21}$, show spontaneous epileptic seizures. In addition, their data demonstrated neuronal apoptosis in the piriform cortex and amygdala (Ikeda et al., 2003), being a possible explanation as to the manifestation of epilepsy in 20% of FHM2/SHM families (Bianchin et al., 2010). These epileptic seizures arise when neurons situated in temporal lobe regions, including the amygdala, piriform cortex and hippocampus become hyperexcitable (Aroniadou-Anderjaska et al., 2008). However, histological changes were not observed in the piriform cortex and amygdala in the brains of $\alpha_2^{-/-}KOE_{21}$ knockout mice (Ikeda et al., 2004). A colocalization of the NKA α2 with NCX occurs in astrocyte cultures, suggesting a central NKA role in the modulation of intracellular Ca^{2+} of the endoplasmic reticulum (Juhaszova and Blaustein, 1997). In addition, high levels of intracellular and intra-endoplasmic reticulum Ca^{2+} ions were detected in cultured astrocytes from *Atp1a2*-deficient mice (Golovina et al., 2003), linking NKA α2 to the manifold consequences of Ca^{2+} dysregulation.

Mutation in astrocyte NKA α2 facilitates both neuronal paroxysmal depolarizing shifts and hyper-excitability, triggering seizures as well as CSD induction and propagation in FHM/SHM patients (Pietrobon, 2005). Bolay et al. (2002), using an animal model of migraine, observed that CSD activates a N-methyl-D-aspartate (NMDA)-dependent pathway leading to an activation of nociceptive trigeminal afferents in the meninges, resulting in headache. Additionally, a specific role for NKA in glutamate clearance during synaptic transmission has also been established, with NKA being stimulated by glutamate in astrocyte cultures (Pellerin and Magistretti, 1997). Furthermore, a colocalization and functional coupling between NKA and glutamate transporters has also been demonstrated (Rose et al., 2009), with α2 having a similar perisynaptic glial localization to the glutamate transporters GLAST and GLT-1in the rat somatosensory cortex (Cholet et al., 2002; Rose et al., 2009). As such, inefficient astrocyte glutamate reuptake resulting in increased cortical excitatory neurotransmission, specifically as driven by NMDA receptor-dependent transmission during high-frequency action potential sequences, may enhance the sensibility to CSD in FHM2 (Tzingounis and Wadiche, 2007). Wider ionic dysregulation may also be evident, with the increase of extracellular K^+ levels resulting from the impaired clearance by NKA, and an increase in the intracellular Na^+ resulting in an elevation in the intracellular Ca^{2+} level through the NCX (Pietrobon, 2005), contributing to wider central disruption. Moreover, recent data indicates that impaired glutamate clearance by astrocytes, and involving the α2, is an important molecular mechanism of FHM2 (**Figure 1**). This *in vitro* study, in a hippocampal mixed astrocyte and neuronal culture derived from homozygous $\alpha_2^{G301R/G301R}$ E17 embryonic mice, demonstrated reduced glutamate uptake due to the loss of functional NKAα2 in astrocytes. Interestingly, *in vivo* data in the same study showed a relationship between the glutamate system and the female sex hormone in psychiatric manifestations of FHM2. Furthermore, higher glutamate levels were evident in different brain regions from adult female $\alpha_2^{+/G301R}$ mice, compared to male mice (Bøttger et al., 2016).

In support of a role for glutamatergic dysregulation in migraine, MTDH, a regulator of glutamate transporters, associates with the frequent form of migraine with aura. Furthermore, a mutation in the SLCA3 gene that encodes the glial excitatory amino acid transporter type 1 (EAAT1) has also been found, leading to neuronal hyper-excitability and consequent hemiplegia, as well as seizures and episodic ataxia, which arise as a consequence of impaired glutamate uptake (Jen et al., 2005).

Recent studies have demonstrated a link between long-term memory formation in the rat hippocampus and the astrocyte-neuron lactate transport (Suzuki et al., 2011). Furthermore, there is a relationship between NKA and the astrocyte specific glutamate-dependent transport of lactate (Kleene et al., 2007). This interesting finding may contribute to the pathophysiological processes underlying language disorder, cognitive impairment and mental retardation evident in FHM2/SHM patients (Le Pira et al., 2004; Kalaydjian et al., 2007). As such, evidence suggests that the central glutamatergic system has an important role in driving the consequences of *ATP1A2* mutations and should also be more explored in *ATP13A* mutations, due to the diagnostic confusion and overlap with glutamate transporter diseases.

Glutamatergic Signaling: a Brief Introduction

Glutamate is an amino acid, which is considered the main central excitatory neurotransmitter, being highly abundant in the majority of animals, especially in the cortex, hippocampus and caudate nucleus (reviewed in Erecinska and Silver, 1990). Glutamate's cellular actions are mediated through the activation of metabotropic and ionotropic receptors. The ionotropic receptors are divided according to their affinities for specific agonists, namely NMDA, AMPA, and kainate (McLennan, 1981). The glutamatergic metabotropic receptors were first investigated in late 1980's (Nicoletti et al., 1986). There are eight types of metabotropic receptors divided into three groups (Groups I, II, and III) depending on DNA sequence and the signaling pathway they activate (reviewed in Wieronska et al., 2016).

Glutamate has many roles, including being part of intermediary metabolism in the brain (Krebs, 1935; Berl et al., 1961) as well as acting as a precursor for different compounds, such as the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Roberts and Frankel, 1950). By acting through metabotropic and/or ionotropic receptors, glutamate is also an important element in a variety of core processes such as learning and memory, partly by regulating synaptic plasticity *via* long-term potentiation (LTP) (Bliss and Collingridge, 1993; Vickery et al., 1997), long-term depression (LTD) (Dudek and Bear, 1992; Yokoi et al., 1996; Nicoll et al., 1998; Dong et al., 2013) or both (Manahan-Vaughan, 1997; Huang L. Q. et al., 1997; Wang et al., 2015). The hippocampus and cortex are the major brain regions investigated in studies of cognition, with these regions also showing the highest concentrations of brain glutamate. Glutamate is crucial to many other brain processes, including motor behavior (Conquet et al., 1994; Pekhletski et al., 1996; Coesmans et al., 2003; Talpalar and Kiehn, 2010; Ohtani et al., 2014; Guimaraes et al., 2015), as well as in brain development (Deisseroth et al., 2004; Poulsen et al., 2005; DeSilva et al., 2012; Yamasaki et al., 2014; Jantzie et al., 2015).

Abnormalities in glutamate function can arise from alterations in a variety of factors, including: glutamate transporters and glutamatergic receptors, as well as glutamate metabolism and synthesis. Also, changes in the levels of glutamate's precursor, glutamine, may drive alterations in the extracellular glutamate concentration. Inflammation is linked to a host of medical conditions, with effects linked to sustained glutamate elevations (Takaki et al., 2012) or changes in glutamatergic receptor expression (Drouin-Ouellet et al., 2011). Increased levels of glutamate contribute to inflammatory process, as indicated by the efficacy of glutamatergic receptor antagonist in reducing inflammation (Bonnet et al., 2015).

Lack of glutamate could lead to memory deficits, given that LTP in some brain areas is NMDA receptor-dependent (Malenka and Bear, 2004). However, excess glutamate can be excitotoxic to neurons. A number of studies show a strong correlation between abnormal glutamatergic signaling pathway and neurodegenerative/psychiatric diseases, including Alzheimer's disease (Takahashi et al., 2015; Haas et al., 2016), Parkinson's disease (Alvarsson et al., 2015; Morin et al., 2015), Huntington's disease (Behrens et al., 2002; Estrada-Sánchez et al., 2009), amyotrophic lateral sclerosis (Plaitakis et al., 1988; Veyrat-Durebex et al., 2015), epilepsy (Wong et al., 2015), ischemia (Dhami et al., 2013), migraine (Peres et al., 2004; Campos et al., 2013), schizophrenia (Spangaro et al., 2012; Matsuno et al., 2015), depression (Berman et al., 2000; Duric et al., 2013; Peng et al., 2016), addiction (Aitta-aho et al., 2012; Perry et al., 2016), and autism spectrum disorder (Bristot Silvestrin et al., 2013).

NKA and Glutamatergic Signaling

In the mammalian CNS, astrocytes are the most abundant glial cells. Astrocytes show two different NKA α isoforms, α1 and α2, with α1 being ubiquitously expressed (Geering, 2008). Under physiological conditions, during excitatory synaptic activity, astrocytes are responsible for glutamate uptake from the synaptic space, keeping extracellular levels of glutamate at nanomolar concentrations, and thereby avoiding the harmful consequences of glutamate receptor over-stimulation (Rothstein et al., 1996; Herman and Jahr, 2007; Kirischuk et al., 2012; Illarionova et al., 2014; Zhang et al., 2016). Astrocytes are therefore considered significant regulators of glutamatergic synaptic transmission (Amara and Fontana, 2002; Zhang et al., 2016). The glial glutamate transporters (GluTs) that mediate glutamate uptake rely on the NKA electrochemical gradient (Levy et al., 1998; Anderson and Swanson, 2000; Chatton et al., 2000; Kanner, 2006). The family of excitatory amino acid transporters (EAAT) are composed of the glutamate transporters, with GLAST (glutamate/aspartate transporter or EAAT1) and GLT-1 (glutamate transporter 1, or EAAT2) being considered the most important (Tanaka, 2000; Zhang et al., 2016) and the predominant type of GluT in adult astrocytes (Rothstein et al., 1996; Dunlop, 2006; Matos et al., 2013). As such, an alteration in the NKA ionic and electrochemical gradient is intimately associated with astrocyte regulation of glutamatergic activity.

In the cell, ATP depletion can lead to a reversal (Longuemare et al., 1999), or inhibition, of glutamate uptake (Sheean et al., 2013; Shan et al., 2014), as shown by inhibitory doses of OUA, which impairs glutamate transport (Pellerin and Magistretti, 1997; Rose et al., 2009; Genda et al., 2011) and leads to the redistribution and clustering of GluTs (Nakagawa et al., 2008; Nguyen et al., 2010). Some studies indicate a co-localization and physical interaction between the α2 subunit and GluTs (Cholet et al., 2002; Rose et al., 2009; Genda et al., 2011; Matos et al., 2013; Illarionova et al., 2014), suggesting NKA regulation of GluTs by direct contact, as well as *via* the electrochemical gradient.

Elevated glutamate concentrations (up to 50 μM) may stimulate NKA activity (Gegelashvili et al., 2007). This was observed in a study of human fetal astrocytes, which showed that during synaptic activity, when glutamate is elevated, there is an increase in cell membrane expression of the NKA FXYD2/γ subunit and GLAST, suggesting a possible interaction between them (Gegelashvili et al., 2007; Nguyen et al., 2010; Zhang et al., 2016). Furthermore, the inhibition of Src in forebrain synaptosomes with PP2 or SU6656, as well as the inhibition of NKA by OUA, both decreased glutamate transport activity to the same level (Rose et al., 2009), suggesting the existence of a

possible protein complex formed between NKA/Src/GluT (Rose et al., 2009). Moreover, adenosine can modulate glutamate uptake through activation of astrocyte adenosine A_{2A} receptors (A_{2A}Rs), which can interact with the α2 isoform in the adult mouse brain, leading to the inhibition of astrocyte glutamate uptake (Matos et al., 2013). This data strengthens the idea of a macromolecular complex in astrocytic membranes encompassing A_2ARs, α2 and GLT-I (Matos et al., 2013; Reinhard et al., 2013), with parallels in neurons. In 2009, Rose and colleagues showed that in the rat hippocampus GLT-1b isoform is co-expressed with α2 in astrocytes, whereas GLT-1a isoform was co-expressed with α3, a neuronal specific isoform (Chen et al., 2004; Bassan et al., 2008; Furness et al., 2008; González-González et al., 2008; Rose et al., 2009; Petr et al., 2015).

Notwithstanding the existence of a well described physical interaction between α2 and GluTs (Cholet et al., 2002; Porras et al., 2008; Rose et al., 2009; Genda et al., 2011; Bauer et al., 2012; Matos et al., 2013; Rose and Chatton, 2016), the substantial Na^+ influx that is driven into astrocytes during synaptic activity has been related to a possible functional interaction, whereby astrocytes increase the local availability of metabolic substrates (Rose and Chatton, 2016). During glutamate stimulation, there is a dramatic reduction in neuronal ATP mediated by NMDA receptor activation followed by an increase in NKA activity (Foo et al., 2012).

In the 1990's, Inoue and Matsui observed an increase in K^+ uptake by α2 and α3 after stimulation of rat embryonic neurons with glutamate (100 μM) (Inoue and Matsui, 1990). Similar results were observed some years later in Wistar adult rat slices (Nathanson et al., 1995; Munhoz et al., 2005) by Munhoz et al. (2005), which described a pathway whereby glutamate stimulation of NMDA receptor-NOS leads to increased NKA activity, possibly due to increased cyclic GMP (cGMP) synthesis. Glutamate-induced Ca^{2+} influx leads to the activation of neuronal nitric oxide synthase (nNOS) and increased production of nitric oxide (NO). NO, by activating soluble guanylylcyclase (sGC) enzyme, increases concentrations of the second messenger cGMP, thereby up-regulating cGMP-dependent protein kinase (PKG). PKG modulates NKA, altering the activity of NKA isoforms, both centrally and peripherally (McKee et al., 1994; de Oliveira Elias et al., 1999; Munhoz et al., 2005; Scavone et al., 2005). Accordingly, a strong correspondence occurs between pharmacologically induced cGMP and NKA modulation in the cerebellum, reinforcing the role of the glutamate-NO-cGMP pathway in NKA regulation (Nathanson et al., 1995).

In 1999, Inoue and colleagues showed that when primary neuronal cultures from rat embryos were treated with three typical agonists of ionotropic glutamate receptors, namely kainic acid (KA), AMPA and NMDA, a remarkable increase in the activity of α2 and α3 isoforms and a slight decreased activity of α1 was observed, suggesting that glutamate effects on NKA may be mainly mediated by ionotropic glutamate receptors (Inoue et al., 1999; Kawamoto et al., 2012), with the neuronal NKA isoforms differing in physiological functions and playing a crucial role in restoring ion gradients after neuronal excitation (Inoue et al., 1999). Meanwhile, Brines and Robbins (1992) observed that prolonged inhibition of α2 and α3 could lead to the potentiation of glutamate neurotoxicity, with NKA activity also regulating the cell surface expression and turnover of AMPA receptors (Zhang et al., 2009). Interestingly, when primary cultured cerebellar neurons were treated with 0.1 μM glutamate a 37% increase in NKA activity was observed, while a concentration of 100 μM glutamate was able to increase NKA activity by 85%. However, when pre-treated with the NMDA receptor antagonist, MK-801, NKA activity increase was abrogated, suggesting that glutamate-driven NKA activation is mediated by NMDA receptors (Marcaida et al., 1996). Once NMDA receptors are activated, the subsequent Ca^{2+} influx can activate calcineurin, a calcium/calmodulin-dependent protein phosphatase (Marcaida et al., 1996; Rambo et al., 2012; Unoki et al., 2012; de Lores Arnaiz and Bersier, 2014), which, through NKA dephosphorylation, restores NKA enzyme activity (Bertorello et al., 1991; Aperia et al., 1992; Marcaida et al., 1996). It is well proven that protein kinase C (PKC) phosphorylates the α subunit, leading to a decrease in its enzyme activity (Bertorello and Aperia, 1989; Fukuda et al., 1991; Beguin et al., 1994; Marcaida et al., 1996; Cheng et al., 1997; Chibalin et al., 1998; Nishi et al., 1999; Taub et al., 2010; Liu et al., 2016). Calcineurin can desensitize NMDA receptors (Lieberman and Mody, 1994) *via* the C-terminus of the NR2A subunit (Krupp et al., 2002) and regulates calmodulin effects, reducing the open time of the channel (Rycroft and Gibb, 2004).

During learning processes PKC is active and phosphorylates some Src family kinases (SFK), which in turn induces tyrosine phosphorylation of NMDA receptors, leading to a rapid up-regulation of NMDA receptor membrane expression (Groveman et al., 2012). Some studies have proposed that Src protein forms a complex with the NMDA receptor through the binding of its SH2 domain with the N-terminal region of postsynaptic density protein 95 (PSD-95). Furthermore, PSD-95 seems to negatively regulate Src (Kalia et al., 2006; Groveman et al., 2012). Indeed, co-activation of mGluR5 and the NMDA receptor with low concentrations of their agonists (CHPG and NMDA, respectively), in rat hippocampal slices, leads to the phosphorylation of NR2B (Tyr1472) by Src kinase (Sarantis et al., 2015), in turn increasing NMDA-mediated excitability.

It is important to note that NKA acts as a specific receptor for CTS. The interplay between the NKA and NMDA receptors is proposed to arise from the initial administration of CTS leading to conformational changes that enhance NMDA subunit expression as a compensatory mechanism (de Lores Arnaiz and Bersier, 2014). OUA is also able to regulate NMDA binding and receptor activity in the adult rat brain (Reines et al., 2001, 2004; Bersier and Rodríguez de Lores Arnaiz, 2009), in a concentration-dependent manner (Bersier and Rodríguez de Lores Arnaiz, 2009). Akkuratov et al. (2015), in rat cerebellar neurons, showed that both α1 and α3 subunits can functionally interact with NMDA receptors whereas Sibarov and colleagues demonstrated by patch-clamp and Ca^{2+} imaging experiments that rat cortical neurons treated with 30 μM NMDA or KA with 1 nM OUA or digoxin for 4 or 6 h, were protected from apoptosis (Sibarov et al., 2012; Abushik et al., 2013). Similar protection was observed *in vivo* (Golden and Martin, 2006). One possible explanation is that

an up-regulation of the plasma membrane NCX leads to more Ca^{2+} extrusion preventing the increase of sEPSC frequency, which is typically found in excitotoxicity process. This up-regulation of NCX activity seems to be due to an interaction between NKA and NCX (Sibarov et al., 2012).

Furthermore, a study published in 2013, in primary cerebellar cell culture treated with OUA in different concentrations (1, 10, and 100 mM) for different times (1, 2, and 4 h), suggested nuclear factor-kappaB (NF-κB) activation in a time and concentration-dependent manner. However, this effect was not observed when neurons were pre-treated with MK-801, PP1 (Src-family tyrosine kinase inhibitor), manumycin A (farnesyltransferase inhibitor), and PD98059 (mitogen-activated protein kinase (MAPK) inhibitor). As such, this study described a pathway whereby OUA activates NF-κB through the NMDA receptor-Src-Ras-MAPK pathways (de Sá Lima et al., 2013). Strong evidence indicates that NMDA can activate NF-κB in the rat hippocampus (Lipsky et al., 2001; Kawamoto et al., 2012), with intra-hippocampal injection of 1 μM NMDA 1 h before the injection of 10 nM OUA potentiating NF-κB activity (Kawamoto et al., 2012). This activation was reduced when cells were pre-treated with an NMDA receptor antagonist (MK-801), suggesting that NF-κB activation by OUA, in part, arises from NMDA receptor modulation (Kawamoto et al., 2012). Overall, the above results indicate that OUA, through the action of NKA as a pump or as a receptor, can modulate many signals important in neurotransmission.

NKA-Glutamatergic Signaling: Aging and Neurodegenerative Diseases

Several studies have shown age-related modifications in the NMDA receptor-NO-cGMP cascade in the CNS (Peterson and Cotman, 1989; Tamaru et al., 1991; Wenk et al., 1991; Vallebuona and Raiteri, 1995; Wardas et al., 1997; Ossowska et al., 2001; Chepkova et al., 2015). In addition, the aging process is associated with a progressive decline in NKA activity, both centrally and peripherally (Scavone et al., 2000; Kawamoto et al., 2008; Vasconcelos et al., 2015). In the CNS, this reduction is due to the modulation of α2 and α3 isoforms with no change in α1 and Mg^{2+}-ATPase activities, as reported in the rat cerebellum and hippocampus (Kawamoto et al., 2008; Vasconcelos et al., 2015). As no differences in the α2 and α3 protein levels are evident, the age-related decrease in NKA activity is linked to modifications in glutamate-cGMP-PKG pathway (Kawamoto et al., 2008). The age-related failure of NKA modulation by glutamate through cGMP-PKG signaling may be connected to ionic disturbances during senescence, which may predispose to neurodegenerative diseases (Scavone et al., 2005). Interestingly, while inflammation, closely associated with aging and neurodegeneration, worsens aging-associated glutamate signaling and NKA defects, intermittent fasting counteracts this effect (Vasconcelos et al., 2015) (**Figure 2**).

DNA repair defects (Garinis et al., 2008; Schumacher et al., 2008; Végh et al., 2012), and consequent mitochondrial dysfunction (Zahn et al., 2007; Yankner et al., 2008), are considered important causes of aging and, in turn, with neurodegenerative disorders (Lin and Beal, 2006; Johri and Beal, 2012). Likewise, ROS-induced mitochondria impairment may contribute to the etiology of neurodegenerative disorders (Lin and Beal, 2006). Compromised mitochondria lead to impaired energy supply, producing less ATP and even more free radicals, as a consequence of inefficient oxidative phosphorylation, which, in turn, can directly or indirectly affect NKA activity (Nathanson et al., 1995). In support of this, a number of studies show the α subunit to be sensitive to free radical attack (Kim and Akera, 1987; Mense et al., 1997; Xie and Xie, 2005), with the oxidized α subunit being degraded (Zolotarjova et al., 1994; Thevenod and Friedmann, 1999). Furthermore, age-related increases in the levels of the free radical superoxide (O_2^-) lead to $ONOO^-$ formation *via* reaction with the NO generated by glutamate signaling (Wink et al., 1996). $ONOO^-$ also impairs NKA activity (Boldyrev et al., 1997; Sato et al., 1997; Muriel and Sandoval, 2000; Kocak-Toker et al., 2005; Reifenberger et al., 2008). NKA inhibition also increases neuronal susceptibility to glutamate excitotoxicity, at least in part by reducing the Na^+ gradient necessary for glutamate transport and clearance from the synapse cleft, leading to a positive feedback loop (Lees et al., 1990; Brines and Robbins, 1992). Both cGMP and PKG are well-established as important mediators of LTP, which NKA also modulates (Stevens and Wang, 1993; Zhuo et al., 1994; Nathanson et al., 1995). Moreover, NKA impairment downregulates the synaptic AMPA receptor, contributing to synaptic transmission defects, and consequently cognitive decline (Zhang et al., 2009). Since cognitive impairment is a hallmark of aging and neurodegenerative diseases, NKA-mediated glutamate signaling dysfunction may be an important contributory factor to neurodegenerative disorders (Végh et al., 2012).

EAAT downregulation has been associated with many neurodegenerative diseases (Sheldon and Robinson, 2007). As described above, the resulting elevation in glutamate levels in the synapse cleft and consequent excitotoxicity may interfere in NKA functioning by increasing NO release and free-radical production (Dawson et al., 1991).

Glutamatergic signaling dysfunction and its associated consequences are critical contributors to the pathophysiological underpinnings of Alzheimer's disease (AD) (Lewerenz and Maher, 2015). AD is the most frequent form of dementia, being characterized by memory and cognitive impairments. The AD brain shows two classical changes: increased Aβ peptide deposition in senile plaques; and tau hyperphosphorylation that leads to neurofibrillary tangles (Khachaturian, 1985; Mattson, 2004). AD is also associated with increased ROS production (Butterfield et al., 2001), compromised glutamate clearance (Lauderback et al., 2001; Vitvitsky et al., 2012) and increased immune-inflammatory activity, in association with raised levels of glutamate, which regulates tryptophan catabolites (Maes and Anderson, 2016).

ATPase activities play a key role in protecting neurons from excitotoxic insults (Hattori et al., 1998). Hattori and colleagues showed reduced NKA activity in postmortem AD patients' brains (77.2% of the controls) with no change in Mg^{2+}-ATPase activities (Hattori et al., 1998). The protein levels of alpha and beta subunits were also decreased in AD brains (Hattori et al.,

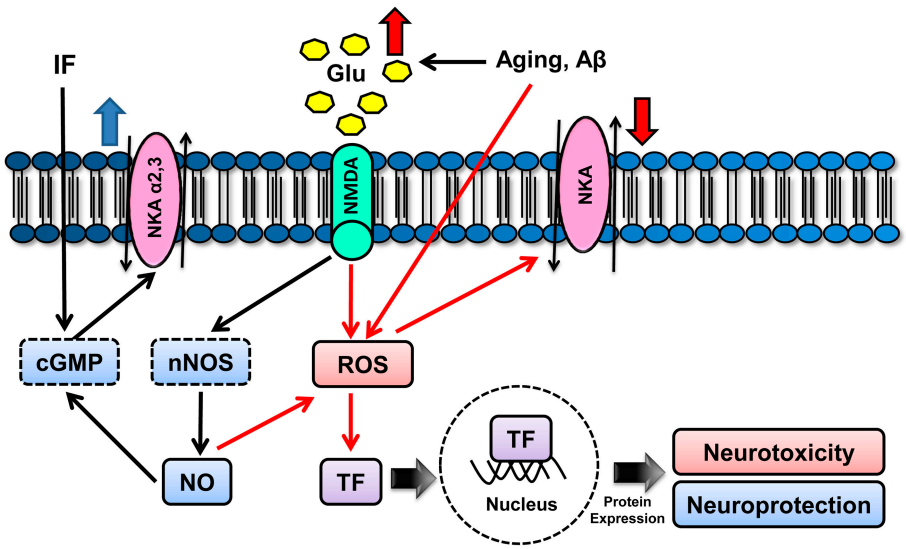

FIGURE 2 | Schematic model for molecular mechanisms underlying aging modulatory effects on NKA isoforms. Aging can either increase the production of ROS, such as superoxide radical and hydrogen peroxide, or induce NO• release by impairing Ca^{2+} homeostasis and subsequently increasing intracellular Ca^{2+} (nNOS-mediated NO• production). NO• is a free radical and can generate peroxynitrite, which may cause neurotoxicity by lipid peroxidation, mitochondria disruption, mutations of DNA and proteins, apoptosis and impairment of α1 activity. Strategies that induce NMDA activation can also activate the cGMP pathway, which, in turn, may lead to neuroprotective signaling, partly by upregulating α2 and α3 (cGMP, cyclic GMP; GLU, glutamate; NKA, Na^+,K^+-ATPase).

1998). However, although this study showed a clear reduction of NKA activity in the frontal lobes of AD brains (Hattori et al., 1998), a previous study found no change in NKA enzyme activity in 4 AD brains vs. 5 control brains (Liguri et al., 1990).

In an *in vitro* study, the acute administration of $A\beta_{25-35}$ or $A\beta_{1-40}$ oligomers in rat hippocampal neurons resulted in lower NKA activity (Mark et al., 1995), suggesting a role of Aβ toxicity in the NKA activity impairment. Also, a disruption in NKA activity leads to Na^+ intracellular accumulation, increasing the influx of calcium through voltage-dependent calcium channels (Mark et al., 1995), suggesting an NKA role in AD cellular toxicity and apoptosis (Hattori et al., 1998). In an AD model, the APP+PS1 transgenic mouse, a decrease in hippocampal NKA activity and protein levels is also evident (Dickey et al., 2005), further implicating a role for Aβ regulation of NKA in AD.

Supporting the NKA dysfunction in AD, Vitvitsky and colleagues showed an imbalance of Na^+ and K^+ ion concentrations in both postmortem AD brain tissue and *in vitro* primary astrocytes treated with $A\beta_{25-35}$ or $A\beta_{1-40}$peptides (Vitvitsky et al., 2012). The electrochemical Na^+ gradient maintained by NKA is the driving force for glutamate re-uptake by EAATs (Rose et al., 2009). In the astrocyte cell culture, ion homeostasis disruption was associated with reduced levels of NKA and Na^+-dependent EAATs (Vitvitsky et al., 2012). Furthermore, homocysteine, a protein involved in many CNS disorders, including Parkinson's disease (Kuhn et al., 1998) and AD (Gallucci et al., 2004), was shown to reduce NKA activity and consequently impair glutamate reuptake in the rat hippocampus (Machado et al., 2011). Hence, NKA impairment associated with glutamate signaling imbalance may contribute to the pathophysiology of AD and other neurodegenerative diseases by significantly deregulating membrane transport, brain electrophysiological activity and other important cellular processes (Vitvitsky et al., 2012).Huntington's disease (HD) is another neurodegenerative disorder associated with cognitive decline and synapse impairment (DiFiglia et al., 1995; Smith et al., 2005; Rozas et al., 2010; Nithianantharajah and Hannan, 2013; Valencia et al., 2013). Many studies show glutamate signaling to be defective in HD, which is proposed to play a role in its pathophysiology (Coyle and Schwarcz, 1976; Beal et al., 1986; Zeron et al., 2002; Shehadeh et al., 2006). In $Hdh^{140Q/140Q}$, a knock-in mouse model of HD, a reduction of NKA and glutamate transporters, VGlut1 and Vglut2, by more than 30% is evident at 12 months vs. controls (Valencia et al., 2010, 2013).

Amyotrophic lateral sclerosis (ALS) is an age-related fatal disease characterized by progressive motoneuron degeneration. The loss of Cu/Zn superoxide dismutase 1 (SOD1) activity, an antioxidant enzyme, following increased oxidative stress, is widely thought to have a role in the etiology and/or course of ALS (Julien, 2001). Other possible contributing factors include increases in iNOS (Almer et al., 1999), glutamate excitotoxicity due to EAATs dysfunction (Rothstein et al., 1995; Bruijn et al., 1997; Trotti et al., 1999) and mitochondrial defect (Beal, 1995). Importantly, Ellis et al. showed that the NO-sGC-cGMP pathway was significantly impaired in the transgenic SOD1 ALS murine model, with this cascade being unable to modulate NKA activity (Ellis et al., 2003). Furthermore, in this study, a marked reduction in NKA activity accompanied by a decrease in the protein levels of the α subunits was evident. As to whether such changes are causal or a consequence of other pathophysiological processes in ALS requires further investigation (Ellis et al., 2003).

CONCLUSIONS AND FUTURE PERSPECTIVE

NKA is present in the membranes of most eukaryotic cells and acts as ion pump. NKA can be modulated by hormones and neurotransmitters, allowing these factors to regulate NKA's diverse important effects, including in the regulation of neurotransmission. NKA can also act as a receptor for steroids, as typified by the effects of OUA, with this being another mechanism whereby it contributes to the regulation of many essential cellular functions, centrally and peripherally. Changes in NKA activity, as occurs in aging as well as when arising from mutations, will play a role in a host of CNS diseases, partly via deficits in energy and glutamatergic regulation. Central energy deficiency has been proposed to be a key factor in many, currently poorly managed, neurodegenerative diseases, which are linked to changes in brain metabolism and increased levels of apoptosis. Given that NKA is fundamental for optimizing central synaptic functioning, as well as energy regulation, its further study, including its environmental, epigenetic and genetic regulation, are likely to be important in the development of new pharmacological and non-pharmaceutical treatments for a host of medical conditions that are currently poorly managed, as well as for the study and regulation of aging *per se*.

AUTHOR CONTRIBUTIONS

Conceived and designed the manuscript: EK, LQ, and CS. Wrote the manuscript: AO, AV, PK, JL, and LQ have contributed equally to this work. Final revision: LQ, EK, CS, AO, PK, AV, JL.

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REVIEW
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Specialized Functional Diversity and Interactions of the Na,K-ATPase

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Na,K-ATPase is a protein ubiquitously expressed in the plasma membrane of all animal cells and vitally essential for their functions. A specialized functional diversity of the Na,K-ATPase isozymes is provided by molecular heterogeneity, distinct subcellular localizations, and functional interactions with molecular environment. Studies over the last decades clearly demonstrated complex and isoform-specific reciprocal functional interactions between the Na,K-ATPase and neighboring proteins and lipids. These interactions are enabled by a spatially restricted ion homeostasis, direct protein-protein/lipid interactions, and protein kinase signaling pathways. In addition to its "classical" function in ion translocation, the Na,K-ATPase is now considered as one of the most important signaling molecules in neuronal, epithelial, skeletal, cardiac and vascular tissues. Accordingly, the Na,K-ATPase forms specialized sub-cellular multimolecular microdomains which act as receptors to circulating endogenous cardiotonic steroids (CTS) triggering a number of signaling pathways. Changes in these endogenous cardiotonic steroid levels and initiated signaling responses have significant adaptive values for tissues and whole organisms under numerous physiological and pathophysiological conditions. This review discusses recent progress in the studies of functional interactions between the Na,K-ATPase and molecular microenvironment, the Na,K-ATPase-dependent signaling pathways and their significance for diversity of cell function.

Keywords: Na, K-ATPase molecular heterogeneity, subcellular microdomains, cardiotonic steroids, signaling pathways, blood pressure, cell survival

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INTRODUCTION

The Na,K-ATPase is "an enzyme of life" because of its essential role in cell life and death. The Na,K-ATPase is an ubiquitous membrane transport protein responsible for establishing and maintaining high K^+ and low Na^+ concentrations in the cytoplasm (Skou, 1957). This ion translocation activity underlies the resting membrane potential, excitability, and provides the driving force for secondary ion transport. Ionic homeostasis maintained by the Na,K-ATPase is critical for numerous cellular functions and processes, including cell growth, differentiation, migration, contraction, secretion, and volume regulation. The list of these cellular tasks is constantly growing. Na,K-ATPase varies in its molecular organization, exhibiting distinct properties, and localization that suggests a specialized functional diversity (Sweadner, 1989; Blanco and Mercer, 1998; Mobasheri et al., 2000; Geering, 2008; Li and Langhans, 2015). Our current knowledge suggests highly complex and isoform-specific reciprocal functional interactions and signaling between the Na,K-ATPase and neighboring proteins and lipids.

Na,K-ATPase is able to form multimolecular complexes where it participates as scaffolding protein in formation of specialized sub-cellular microdomains or microcompartments (Xie and Askari, 2002; Schoner and Scheiner-Bobis, 2007; Rajasekaran et al., 2008; Reinhard et al., 2013; Krivoi, 2014).

The extracellular loops of Na,K-ATPase catalytic α subunit form unique highly specific binding site for cardiotonic steroids (CTS) and their circulating endogenous analogs (Bagrov et al., 2009; Ogawa et al., 2009; Lingrel, 2010; Laursen et al., 2013). Physiological significance of CTS binding site is still under debate. It remains uncertain whether it affects numerous cellular functions by inhibiting enzymatic activity that leads to modulation of ion homeostasis or by conformational changes of the α subunit and initiation of a signal transduction. The role of the Na,K-ATPase CTS binding site suggests its involvement in regulation of diverse cellular functions, including synaptic and neural processes (Lichtstein and Rosen, 2001; Goldstein et al., 2006, 2011; Song et al., 2013), cell survival and neuroprotection (Golden and Martin, 2006; Dvela et al., 2012; Sibarov et al., 2012; Dvela-Levitt et al., 2014), muscle contraction (Dostanic-Larson et al., 2005; Radzyukevich et al., 2009), intercellular communications (Matchkov et al., 2007, 2012), and gene expression (Xiao et al., 2002; Kulikov et al., 2007; Orlov and Hamet, 2015; Blanco and Venugopal, 2016). Knowledge on the role of Na,K-ATPase and endogenous CTS in intracellular signaling opens new perspectives for modulation of cell function under normal and pathological conditions. The present review focuses on the isoform-specific functions of the Na,K-ATPase and specialized interactions with molecular environment which underlie a variety of the Na,K-ATPase-dependent regulatory mechanisms.

MOLECULAR DIVERSITY OF Na,K-ATPase

A molecular heterogeneity of the same functional protein is one of the well-documented principles in cell biology (Le Novère et al., 2002; Massoulié, 2002; Drabkina and Krivoi, 2004; Markov et al., 2015). It includes the heterogeneity in structure and diversity in function of the Na,K-ATPase (Sweadner, 1989; Blanco and Mercer, 1998; Mobasheri et al., 2000; Geering, 2008; Li and Langhans, 2015).

Na,K-ATPase is a P-type ATPase, a protein vital for cellular function and ubiquitously expressed in the plasma membrane of all animal cells. An enzymatic activity of the Na,K-ATPase provides an electrical excitability and driving force for many other transmembrane transports. An ion translocation, described by Post-Albers reaction, suggests cyclic transitions of the Na,K-ATPase between two principal conformational states, E1 and E2 which selectively bind three Na^+ ions or two K^+ ions, respectively. Each cycle uses the energy from hydrolysis of one ATP molecule. This active ion transport generates an additional negative (electrogenic) membrane potential due to the net outward transfer of one positive charge per transport cycle (Sperelakis, 2001; Dobretsov and Stimers, 2005).

Minimal functional unit of the Na,K-ATPase is a heteromeric complex consisting of a large α catalytic (∼110 kDa) and smaller β glycoprotein (∼31.5 kDa) subunits. The α subunit is responsible for ion transport. This subunit has 10 transmembrane domains which contain binding sites for Na^+ ions on the extracellular loops and for K^+ ions and ATP on the intracellular loops (Blanco and Mercer, 1998; Mobasheri et al., 2000). The β subunit is a single-transmembrane protein which is required for enzymatic activity and modulates the enzyme affinity to Na^+ and K^+ ions. It also functions as chaperone targeting the α subunit to the plasma membrane and plays an important role in cell adhesion (Liu and Askari, 2006; Liu et al., 2011; Tokhtaeva et al., 2012). In some tissues a small single-transmembrane protein of FXYD family (∼7 kDa) has been found to be associated with the functional Na,K-ATPase α/β complexes and known to modulate enzymatic activity (Sweadner and Rael, 2000; Garty and Karlish, 2006; Geering, 2008; Pavlovic et al., 2013; Arystarkhova, 2016). Four isoforms of the α subunit and three isoforms of the β subunit are expressed in a cell- and tissue-specific manner providing wide molecular diversity of the Na,K-ATPase (Blanco and Mercer, 1998; Mobasheri et al., 2000; Mijatovic et al., 2007; Li and Langhans, 2015). Seven proteins of the FXYD family provide additional diversity to these assemblies (Sweadner and Rael, 2000; Garty and Karlish, 2006; Geering, 2008; Pavlovic et al., 2013; Arystarkhova, 2016).

It is generally accepted that the ubiquitous α1 isoform plays main "housekeeping" role while the other isoforms are expressed in a cell-specific manner. In some tissues, e.g., erythrocytes, kidney epithelia and liver, the α1 isoform is the only isoform expressed, while the majority of other cell types co-expressed other α isoforms serving additional regulatory functions that often are poorly understood. Thus, the α2 isoform is principally expressed in skeletal, cardiac and smooth muscles as well as in glial cells while the α3 isoform is characteristic for neuronal tissues (Blanco and Mercer, 1998; Mobasheri et al., 2000; Dobretsov and Stimers, 2005; Krivoi, 2012; Li and Langhans, 2015). The α4 isoform has been found only in testis (Woo et al., 2000).

CARDIOTONIC STEROID BINDING SITE OF THE Na,K-ATPase AND ENDOGENOUS INHIBITORS

All known Na,K-ATPase isozymes contain specific receptor for inhibitors collectively known as CTS—compounds characterized by a steroid nucleus. Several plants are shown to contain CTS such as ouabain, digoxin, digitoxin, and proscillaridin A (Mijatovic et al., 2007; Bagrov et al., 2009). CTS are also found in animal species and occur mainly in toads, e.g., marinobufagenin isolated from the skin of *Bufo marinus* (Bagrov et al., 2009). CTS are lethal in high concentrations while in low concentrations they (particularly, digoxin, and digitoxin) are widely used as positive inotropic agents (Gheorghiade et al., 2004).

The specific binding site for CTS is formed by an extracellular region between M1–M2, M5–M6, and M7–M8 transmembrane domains of the Na,K-ATPase α subunit (Mijatovic et al., 2007; Bagrov et al., 2009; Ogawa et al., 2009; Lingrel, 2010; Sandtner et al., 2011; Laursen et al., 2013). CTS molecules bind and

stabilize the Na,K-ATPase in E2 conformation inhibiting the transport activity of the enzyme. Isoforms of the α subunit Na,K-ATPase differ in their sensitivity to ouabain, a CTS found in plants and animal tissues, with greatest difference in rodents. In rodents, the α1 isozyme is relatively resistant to ouabain (the IC_{50} values for inhibition of the Na,K-ATPase are between tens to hundreds micromolar), while the α2, α3, and α4 isozymes are two-four orders of magnitude more sensitive (Dobretsov and Stimers, 2005; Lingrel, 2010). The sensitivity to ouabain is determined by two amino acids at the positions 111 and 122 in transmembrane domains M1–M2. Genetic manipulations substituting these amino acids in mice produced mice with various combinations of the α1 and α2 isozyme sensitivities to ouabain and to study the physiological role of the CTS binding site (Lingrel, 2010).

Knowledge on the Na,K-ATPase sensitivity to ouabain and other CTS is of a great importance. Notably, apart from rodents, the α1 Na,K-ATPase isozyme in rabbit, pig, dog, sheep, guinea pig, and human is relatively sensitive to ouabain (see for review: Blanco and Mercer, 1998; Dobretsov and Stimers, 2005; Mijatovic et al., 2007; Lingrel, 2010). Some studies in humans showed high and similar affinity of α1, α2, and α3 isozymes for cardiac glycosides with ouabain binding constants in nanomolar concentration range (Wang et al., 2001). Moderate selectivity between human α-subunit isoforms was also shown. By contrast, digoxin and a number of other CTS demonstrated lower affinities and more significant selectivity compared to ouabain (Crambert et al., 2000; Katz et al., 2010; Cherniavsky Lev et al., 2015). The reasons for this differentiated selectivity remain to be elucidated.

The presence of an endogenous ouabain-like compounds was suggested almost 40 years ago (Haddy and Overbeck, 1976; Blaustein, 1977) and endogenous ouabain was later purified from human blood plasma (Hamlyn et al., 1991). Several facts point toward ouabain being a hormone synthesized and secreted by the adrenal cortex. First, ouabain has been found in high concentrations in the adrenal cortex (Hamlyn et al., 1991; Blaustein, 1993; Hamlyn, 1998; Li et al., 1998). Second, bovine adrenocortical cells have been shown to secrete ouabain in amounts greater than their storage capacity under *in vitro* conditions (Laredo et al., 1994, 1995). Third, the concentration of ouabain in adrenal venous blood is significantly higher than in arterial plasma (Boulanger et al., 1993). Moreover, adrenal cortex tumors have been characterized by overproduction and secretion of ouabain (Komiyama et al., 1999). Consistently, administration of anti-ouabain antibodies to rats produces adrenal cortex enlargement, further implicating the adrenal gland as a source of ouabain (Nesher et al., 2009).

Ouabain is believed to be synthesized in zona glomerulosa cells of the adrenal cortex, as other adrenal steroids and the synthesis involves a rate-limiting side chain cleavage of cholesterol (Laredo et al., 1995). Hydroxycholesterol, pregnenalone, and progesterone have been shown to increase the secretion of ouabain, possibly acting as precursors in its biosynthetic pathway (Hamlyn et al., 1998; Lichtstein et al., 1998). Moreover, the synthesis of ouabain follows a similar pathway as aldosterone (Hamlyn et al., 2003). However, the exact mechanisms and precursors involved in ouabain biosynthesis are still unclear. Also hypothalamus has been suggested to synthesize ouabain or ouabain-like compound(s) (Li et al., 1998) which may play a central neuromodulatory role leading to excitation of the central sympathoexcitatory pathways (Blaustein et al., 2012).

In addition to ouabain, structurally similar digoxin, marinobufogenin and a number of other digitalis-like compounds were further identified endogenously (Lichtstein and Rosen, 2001; Schoner and Scheiner-Bobis, 2007; Bagrov et al., 2009). Endogenous ouabain and marinobufagenin are well-studied. Their concentration in blood plasma and cerebro-spinal fluid of mammals varies at subnanomolar range. Elevated level of endogenous CTS has been found under physiological (e.g., strenuous exercise, newborn infants) and pathophysiological (e.g., congestive heart failure, hypertension, chronic renal failure, preeclampsia, and affective disorders) conditions suggesting the role for these compounds for modulation of body function and in pathologies (Lichtstein and Rosen, 2001; Schoner, 2002; Dobretsov and Stimers, 2005; Bagrov et al., 2009).

SUBCELLULAR COMPARTMENTALIZATION OF THE Na,K-ATPase FUNCTION

Subcellular compartmentalization is one of the basic principles of intracellular organization (Saks et al., 2009). Particularly diffusion restriction between cytosolic bulk and spatially limited submembrane space is enabled in local microdomains. In cardiac cell, the α1 Na,K-ATPase isozyme is relatively uniformly distributed between external sarcolemma and T-tubular membranes, while the α2 isozyme is concentrated in T-tubules with a preferential localization close to the junctional sarcoplasmic reticulum (SR) (Shattock et al., 2015). In addition, the Na,K-ATPase forms large membrane macromolecular complexes with Na^+,Ca^{2+} exchanger (NCX), and ATP-sensitive K^+ (K_{ATP}) channels, coordinated by ankyrin-B (Li et al., 2010). The K_{ATP} channels are densely expressed at the places where T-tubules create membrane junctions with the SR (Alekseev et al., 2012). Being co-localized with the Na,K-ATPase, K_{ATP} channels open in response to increased energy utilization accompanied by the local ATP depletion within a diffusion-restricted submembrane space. This adjusts cardiac cell electrogenesis and excitability across a wide range of workloads (Kabakov, 1998; Alekseev et al., 2012). Similar close functional interaction between the α2 Na,K-ATPase isozyme and the K_{ATP} channels was shown in vascular smooth muscle cells (Glavind-Kristensen et al., 2004; Matchkov et al., 2007).

In skeletal muscle cells, the α1 and α2 Na,K-ATPase isozymes also have distinct distributions and membrane localization. The α1 isozyme comprises up to 40% of total Na,K-ATPase and is expressed only on the outer plasma membrane. The α2 isozyme comprises 60–80% of total Na,K-ATPase content (Orlowski and Lingrel, 1988; He et al., 2001) and the majority of the α2 isozyme is expressed in the interior transverse tubule membranes, with smaller pools localized to the end-plate membrane and surface caveolae (Williams et al., 2001; Cougnon et al., 2002; Heiny et al., 2010; Kristensen and Juel, 2010). Diffusion alone is not sufficient

to remove the excitation-related K^+ load in the T-tubules, the concentration of which, reaches tens of millimolar (Sejersted and Sjogaard, 2000; Clausen, 2013). The existence of two α isozymes of the Na,K-ATPase with distinct locations and K^+ affinities was proposed (DiFranco et al., 2015). The α1 isozyme with relatively high K^+ affinity mediates most of the basal Na^+ and K^+ ion transport and plays a major role in setting resting transmembrane ion gradients and resting membrane potential. Located in T-tubules α2 isozyme with apparently low K^+ affinity operates substantially below its maximum capacity in the resting muscles, but its activity can rapidly increase during membrane excitation and K^+ accumulation. This helps to maintain muscle excitability, contraction, and oppose fatigue.

The Na,K-ATPase containing membrane microdomains were shown to be associated to the "junctional" SR and include the NCX as one of the key functional players (Moore et al., 1993; Juhaszova and Blaustein, 1997; Golovina et al., 2003; Lynch et al., 2008). These microdomains were shown in a variety of cell types including neurons, glia, and myocytes. Other membrane proteins were also shown to co-localize in these microdomains, e.g., diverse plasma membrane Ca^{2+} channels, sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), sarcoplasmic reticulum IP_3, and ryanodine receptors (Blaustein and Golovina, 2001; Blaustein, 2013). In these specialized microdomains, called "PLasmERosome," the Na,K-ATPase and its molecular environment function cooperatively to regulate locally the intracellular Ca^{2+} signaling (Blaustein and Golovina, 2001; Blaustein, 2013). The plasma membrane and SR components appear to be linked through the cytoskeletal spectrin network and adaptor protein, ankyrin 2 (Lencesova et al., 2004). These microdomains specifically contained ouabain-sensitive α2 or α3 Na,K-ATPase isozymes. The N-terminal sequence targets and tethers the α2 Na,K-ATPase isozyme to its specific localization in the plasma membrane (Song et al., 2006).

Restriction of Na^+ and Ca^{2+} diffusion enables the appearance of concentration gradients between these restricted spaces and the bulk cytosol. Interestingly, the α2 Na,K-ATPase isozyme have much lower affinities for Na^+ than the α1 isozyme (Zahler et al., 1997). This suggests that intracellular Na^+ will rise more in the restricted spaces controlled by the α2 Na,K-ATPase isozyme than the global intracellular Na^+ which is under α1 isozyme control. Thus, these α2-isozyme-associated-microdomains are well-organized to control the local Na^+ electrochemical gradient which can influence Ca^{2+} homeostasis via the co-localized NCX isoform 1 (Golovina et al., 2003; Lynch et al., 2008). This links cellular Ca^{2+} concentration to Na^+ concentration; a spatially restricted rise in Na^+ will lead to a localized elevation of intracellular Ca^{2+}. Such interactions in ion metabolism do not only control local Ca^{2+} but also affect global intracellular Ca^{2+} via modulation of the SR load. This interaction generally explains the well-known potentiating effect of ouabain on vascular contraction (Miriel et al., 1999; Iwamoto et al., 2004; Zhang et al., 2005, 2010; Blaustein and Wier, 2007).

Alterations in the Na,K-ATPase activity will change the cellular Ca^{2+} homeostasis and enhance loading of intracellular Ca^{2+} stores (Golovina et al., 2003). This has normally been considered a result of the elevation in the intracellular Na^+ concentration slowing the clearance of Ca^{2+} by NCX and, therefore, allowing extra Ca^{2+} pumped into the stores (see above). The reduction of α2 isozyme activity (either by knocking it down or by pharmacological inhibition) should therefore, be associated with elevated contractility of vascular smooth muscle cells, although this is not always the case. It has been previously reported that, in contrast to the effect of ouabain, a transient siRNA-induced downregulation of the α2 Na,K-ATPase isozyme suppresses contractile responses of rat mesenteric small arteries (Matchkov et al., 2012). Although ouabain increased sensitivity to the contractile stimuli in control arteries, it had no effect on the α2-isozyme-downregulated arteries. Surprisingly, the reduced expression of α2 isozyme was associated with higher intracellular Ca^{2+} concentration but suppressed contractile response of the arterial wall (Matchkov et al., 2012). This suggests that downregulation of the α2 Na,K-ATPase isozyme led to reduction in Ca^{2+}-sensitivity of vascular smooth muscle cell contractile machinery.

These surprising findings might seem to be contrasting to other reports where small arteries from α2-isozyme-knockout mice showed an increased myogenic tone (Iwamoto et al., 2004; Shelly et al., 2004; Dostanic et al., 2005; Zhang et al., 2005). However, the α2-isozyme-downregulated arteries also had increased myogenic contraction possibly due to elevated basal intracellular Ca^{2+} which might be a consequence of coordinated reduction in the NCX expression (Matchkov et al., 2012). Stimulation with agonists was, however, less effective in the α2-isozyme-downregulated arteries where both sensitization to Ca^{2+} and Ca^{2+} release through the IP_3 receptors were suppressed. The reason for this compromised agonist-induced Ca^{2+} sensitivity in the arteries with reduced α2 Na,K-ATPase isozyme expression is not known but it suggests a more complex mechanism for the control of smooth muscle contractility by the Na,K-ATPase than the modulation of intracellular Ca^{2+} concentration via membrane potential (Mulvany et al., 1984; Aalkjaer and Mulvany, 1985) and ion homeostasis (Golovina et al., 2003; Lynch et al., 2008).

A direct interaction of the α Na,K-ATPase subunit N-terminus with IP_3 receptor has been established suggesting that ouabain-induced conformational changes in the α subunit can directly liberate Ca^{2+} from intracellular depot (Aizman et al., 2001; Zhang et al., 2006; Tian and Xie, 2008). Importantly, ouabain-evoked Ca^{2+} signaling can not only affect the contractility of cardiac and smooth muscles but also regulates via the Ca^{2+}-sensitive transcription factors protein expression, cell proliferation, and differentiation (Aizman et al., 2001; Fontana et al., 2013).

In membrane microdomains the Na,K-ATPase is shown to be organized together with interacting proteins in signalosome (Aydemir-Koksoy et al., 2001; Wang et al., 2004; Efendiev et al., 2005; Tian et al., 2006) restricted to caveolae (Wang et al., 2004; Liu and Askari, 2006; Schoner and Scheiner-Bobis, 2007; Tian and Xie, 2008; Liu et al., 2011; Morrill et al., 2012). Caveolin is a protein essential for caveolae formation and direct Na,K-ATPase/caveolin interaction was previously shown (Wang et al., 2004; Cai et al., 2008; Heiny et al., 2010; Morrill et al., 2012). Cholesterol-rich membrane microdomains, i.e., lipid

rafts and caveolae, are nanoscale assemblies of sphingolipid, cholesterol, and proteins that form platforms for subcellular signaling and trafficking (Razani et al., 2002; Lingwood and Simons, 2010; Harvey and Calaghan, 2012; Sebastiao et al., 2013). The formation of cholesterol rich lipid microdomains is important for Na,K-ATPase targeting and regulation and the reciprocal interactions between the Na,K-ATPase and cholesterol were proposed (Cornelius, 2008; Chen et al., 2011; Kapri-Pardes et al., 2011; Haviv et al., 2013; Cornelius et al., 2015).

THE Na,K-ATPase/SRC SIGNALING PATHWAY

In the functional signalosome the Na,K-ATPase has been suggested to interact and regulate protein kinases as well as function as scaffold protein for receptors and effectors (Li and Xie, 2009). The experimental findings during the last decade suggest that the Na,K-ATPase can function as an important signal transducer (Aizman and Aperia, 2003; Li et al., 2009; Liu and Xie, 2010). Indeed, two functionally separate pools of the Na,K-ATPase have been suggested to be engaged in the "classic" ion transport and cellular activities other than ion pumping (Xie et al., 2015).

The unconventional non-pumping Na,K-ATPase resides in restricted membrane microdomains, where it directly interacts with protein kinases, ion channels, and transporters (Xie, 2003; Xie and Cai, 2003). Thus, it has been shown that the central loop of Na,K-ATPase interacts with phospholipase C-γ (PLCγ) and the N-terminus binds to IP_3 receptors (Yuan et al., 2005). This signalosome comprises also several anchoring proteins, Src kinase and has been shown to be an important modulator of intracellular Ca^{2+} signal (Haas et al., 2000; Liu et al., 2000). Ouabain can act through this signalosome in two synergistic manners (Yuan et al., 2005). First, it can force PLCγ and IP_3 receptors into close proximity and facilitate the signal transmission. Second, an activation of the Na,K-ATPase-associated Src could lead to tyrosine phosphorylation of both PLCγ and the IP_3 receptors that will sensitize the receptor to IP_3 produced by PLCγ. In this term, the Na,K-ATPase-Src interaction is important not only for ouabain signaling but also for many other agonist-induced intracellular responses that involve IP_3 signaling and tyrosine phosphorylation in general. Binding of ouabain to the Na,K-ATPase releases Src kinase that can affect intracellular Ca^{2+} as well as modulate other signaling pathways including gene expression (Xie and Cai, 2003).

Although there is some controversy regarding an interaction between the Na,K-ATPase and Src kinase, an activation of Src kinase by phosphorylation is a well-established response to submicromolar concentrations of ouabain. Several studies suggest that the Na,K-ATPase-associated Src kinase specifically activated by ouabain (Haas et al., 2000; Liang et al., 2006; Tian et al., 2006; Li et al., 2009; Lai et al., 2013; Ye et al., 2013; Banerjee et al., 2015). This signaling microdomain model disagreed with other studies suggesting that ouabain-induced Src kinase activation is a result of the ATP-sparing effect of the Na,K-ATPase inhibitor on these two enzymes competing for ATP (Weigand et al., 2012; Gable et al., 2014). However, some skeletal muscle studies showed that submicromolar and micromolar concentrations of ouabain do not affect the global intracellular ATP/ADP ratio while significant phosphorylation of Src kinase and its activation were observed (Kotova et al., 2006a,b). Nevertheless, these studies addressed the global ATP/ADP ratio, and there is a possibility for spatially restricted changes in the concentrations. Accordingly, it has been shown that the Na,K-ATPase-dependent Src kinase activity is maintained in cells expressing a non-pumping mutant of rat α1 isoform (Liang et al., 2006). The involvement of Na,K-ATPase in signaling cascade does not exclude a role for its ion pumping function in ouabain-induced effects. Moreover, since intracellular Na^+ ions regulate the conformation of the Na,K-ATPase (e.g., the E1 state), it is possible that changes in intracellular Na^+ concentration could also regulate the formation of the Na,K-ATPase/Src complex, and thus cellular Src activity (Li et al., 2009). This Na,K-ATPase-dependent Src kinase signaling is hypothesized to modulate arterial contraction and blood pressure as discussed below.

Na,K-ATPase AND HYPERTENSION

The correlation between circulating ouabain and blood pressure was suggested almost 35 years ago (Hamlyn et al., 1982). Almost 50% of patients with uncomplicated essential hypertension have been reported to have elevated endogenous ouabain (Rossi et al., 1995). In accordance with hemodynamic background of hypertension characterized by an increase in peripheral resistance and unchanged cardiac output, plasma ouabain level correlates positively with elevated peripheral resistance and left ventricular hypertrophy, but not with cardiac output (Manunta et al., 1999; Pierdomenico et al., 2001). Also other endogenous CTS correlate with blood pressure, e.g., urinary marinobufagenin level increases with elevated blood pressure and aortic stiffness in patients (Jablonski et al., 2013).

These human studies received further strong experimental support from several animal models of ouabain-dependent hypertension. Chronic administration of ouabain, leading to an increase of its plasma concentration to the level observed in essential hypertension, produced hypertension in rats (Yuan et al., 1993; Manunta et al., 2000; Pulgar et al., 2013). This ouabain-induced hypertension was associated with elevated peripheral vascular resistance that is a result of inward arterial structural remodeling (Briones et al., 2006) and increased contractility of the resistance arteries (Pulgar et al., 2013). Endogenous ouabain-like inhibitor of the Na,K-ATPase was also implicated in pulmonary hypertension (Janssens et al., 1993). Plasma ouabain level is elevated in several other rodent models of hypertension, including DOCA-salt, reduced renal mass, Milan hypertensive rats, Dahl S rats on high-salt diet, and adrenocorticotropic hormone induced hypertension (for review see: Blaustein et al., 2012).

Inhibition of endogenous ouabain action by systemic administration of ouabain antagonist rostafuroxin or digibind (an antibody to endogenous ouabain) lowers blood pressure and even prevents hypertension in the high-ouabain hypertension models (Dostanic-Larson et al., 2005; Manunta et al., 2006). Finally, knock-in of ouabain-resistant mutation of the α2

Na,K-ATPase isozyme prevents ouabain-induced hypertension (Dostanic et al., 2005; Dostanic-Larson et al., 2005; Lorenz et al., 2008). This indicates the importance of the α2 isozyme in pathogenesis of hypertension. The importance of the α1 Na,K-ATPase isozyme was, however, also suggested. Thus, blood pressure was shown to correlate with the expression of either α1 or α2 isozyme (Pritchard et al., 2007). However, these two isozymes have coordinated expression profiles, where overexpression of one isozyme increased the expression of another. This makes it therefore difficult to distinguish their specific roles, although it had been concluded that the α2 Na,K-ATPase isozyme seems to play more significant role (Pritchard et al., 2007).

Presently, there is no generally accepted molecular mechanism which could explain how the inhibition of Na,K-ATPase leads to an elevation of blood pressure. The situation is further complicated by the fact that not all CTS have a similar effect on blood pressure. Thus, in contrast to ouabain, digoxin does not raise blood pressure and has even antihypertensive action in ouabain-dependent hypertension models (Manunta et al., 2000; Zulian et al., 2013). Nevertheless, all known "classic" CTS inhibit the Na,K-ATPase pumping activity and exert vasotonic effects *in vitro*; however, digoxin-like steroids can antagonize the vasotonic effects of ouabain-like steroids, and vice versa (Song et al., 2014). This phenomenon of ouabain-digoxin antagonism is not unique for blood vessels and is also characteristic for glutamate-induced Ca^{2+}-transients in primary cultured hippocampal neurons (Song et al., 2014).

Ouabain-digoxin antagonism might be a result of functional selectivity or biased signaling of the Na,K-ATPase as it is known for some G-protein-coupled receptors (Kenakin, 2011; Kenakin and Christopoulos, 2013). Thus, the possibility for different conformational changes of the Na,K-ATPase upon a binding of different CTS has been hypothesized to be the reason for different functional effects in spite of their uniform inhibitory action. However, recent crystal structure analyses of the high-affinity Na,K-ATPase-ouabain and -digoxin complexes do not support this possibility, although they do not exclude it (Laursen et al., 2013, 2015).

It has been suggested that the Na,K-ATPase functions as a tetraprotomer (Hah et al., 1985) where single CTS blocks all pumping activity but digoxin-like steroids are able to reactivate the ouabain-inhibited tetraprotomers via de-oligomerization (Song et al., 2014). This antagonism is shown for resistance arteries *in vitro*. However, ouabain-like steroids elevate blood pressure while digoxin-like steroids do not and even antagonize the effect of ouabain (Manunta et al., 2000; Zulian et al., 2013). Thus, only one direction of ouabain-digoxin antagonism can be seen *in vivo* in contrast to *in vitro* experiments (Song et al., 2014). Low constitutive level of endogenous ouabain was implicated in this inconsistency.

MODULATION OF ARTERIAL CONTRACTILITY BY THE Na,K-ATPase

In vitro studies suggest that many of the functional and structural alterations in arteries from hypertensive animals could be consequences of elevated plasma ouabain (Blaustein et al., 2012). The Na,K-ATPase has a significant role in regulation of vascular tone and contractility, and therefore has been proposed to modulate peripheral vascular resistance and blood pressure (Blaustein and Wier, 2007). Two α-isozymes of the Na,K-ATPase are expressed in vascular smooth muscles, where the α1 Na,K-ATPase isozyme is homogeneously distributed over the cell membrane while the α2 isozyme has a spatially restricted distribution (Lee et al., 2006; Matchkov, 2010).

Activation of the Na,K-ATPase results in membrane hyperpolarization and vascular relaxation while its inhibition causes membrane depolarization and vascular contraction. Inhibition of the Na,K-ATPase by ouabain in a broad concentration range between 0.01 μM and 1 mM had no effect on resting vascular tone while it significantly potentiated the agonist-induced contraction (Aalkjaer and Mulvany, 1985). Accordingly, ouabain produced acute and transient (within 10 min) concentration-dependent depolarization of smooth muscles in both resting and agonist-stimulated arteries (Nilsson and Mulvany, 1981; Mulvany et al., 1982; Aalkjaer and Mulvany, 1985). At the same time, a prolonged exposure to micromolar concentrations of ouabain suppressed arterial contractility (Nilsson et al., 2001). The reason for these short-term potentiating and long-term depressive effects of arterial contraction is unknown. It has been suggested that under physiological conditions ouabain-induced depolarization and the following voltage-dependent Ca^{2+} influx play an important role in the potentiation of arterial contraction, although the role of intracellular Na^{+} for the NCX modulation should not be underestimated (Mulvany et al., 1984; Aalkjaer and Mulvany, 1985). Simultaneous analyses of contraction and intracellular Ca^{2+} concentration changes demonstrated also that the long-term depressive effect of ouabain on arterial contraction was due to desensitization of smooth muscle contractile machinery to Ca^{2+} ions (Nilsson et al., 2001).

The discussed above hypothesis about Na,K-ATPase-dependent Src kinase signaling is mostly based on the studies with epithelial cells (Aizman and Aperia, 2003; Yuan et al., 2005; Li et al., 2009; Liu and Xie, 2010; Lai et al., 2013). However, previous study showed opposite effects of two Na,K-ATPase inhibitors, ouabain and digoxin, on blood pressure (Manunta et al., 2000). It has been suggested that although both of inhibitors block pumping activity of the Na,K-ATPase, only ouabain elevates intracellular Ca^{2+} through an activation of the Src kinase (Zulian et al., 2013). Digoxin, which is unable to activate Src kinase, fails to potentiate smooth muscle contraction. Thus, this strongly suggests the importance of the Na,K-ATPase-Src signaling pathway in regulation of arterial tone and suggests that hypertensive action of endogenous ouabain-like steroids is mediated via Src kinase signaling. This is in accordance with the functional study on isolated arterial segments showing the importance of Src signaling for vascular tone control (Toma et al., 1995).

Digoxin-related synthetic steroid, rostafuroxin (Quadri et al., 1997) does not affect pumping activity of the Na,K-ATPase (Ferrari et al., 1998) and has itself no effect on the arterial tone (Zhang et al., 2005). It antagonizes, however, the vasotonic action of ouabain-like CTS (Song et al., 2014) and hypertension

associated with an elevation of endogenous ouabain level (Ferrari, 2010). This antihypertensive effect of rostafuroxin has been associated with suppression of ouabain-induced Src-kinase-dependent signaling pathway (Wenceslau and Rossoni, 2014).

The importance of the α1 Na,K-ATPase isozyme for initiation of the Src kinase signaling has been shown (Xie et al., 2015), although whether this is also the case for vascular smooth muscle cells remains to be identified. The specific Src-kinase-dependent pathways upon ouabain binding by the Na,K-ATPase remain to be elucidated but this signaling was shown in several studies with smooth muscle cells in culture (Haas et al., 2000, 2002; Liu et al., 2004). Activation of Src kinase triggers Src-dependent phosphorylation of epidermal growth factor receptor and an activation of Ras/MAPK (mitogen-activated protein kinase) cascade (Haas et al., 2000, 2002) as well as numerous other signaling pathways important for vascular smooth muscle function and phenotype (for review: MacKay and Knock, 2015). Src kinases in smooth muscles were shown to be involved in reactive oxygen species signaling (Akhand et al., 1999; Giannoni et al., 2005; Knock and Ward, 2011), G-protein-coupled receptor stimulations (Luttrell and Luttrell, 2004), tyrosine phosphorylation of transient receptor potential channels (Kawasaki et al., 2006), voltage-gated Ca^{2+} channels (Wijetunge et al., 2000; Gui et al., 2010) and K^+ channels (Alioua et al., 2002; Sung et al., 2013), modulation of Rho pathways (Guilluy et al., 2010; Gadepalli et al., 2012) and myosin phosphatase activity (Velasco et al., 2002). These signaling pathways will affect intracellular Ca^{2+} concentration and sensitization of contractile machinery to Ca^{2+}, modulate proliferation and apoptosis suggesting their role in vascular repair and remodeling.

OTHER Na,K-ATPase DEPENDENT SIGNALING PATHWAYS IN THE VASCULAR WALL

In addition to the Src kinase signaling, the importance of which still needs to be validated for the vasculature, the Na,K-ATPase has been shown to interact in the arterial wall with several other signaling pathways. Thus, the Na,K-ATPase associates with salt-inducible kinase 1 (SIK1), a sucrose-non-fermenting-like isoform of the 5′-adenosine monophosphate-activated protein kinase (AMPK) family (Sjostrom et al., 2007). A Ca^{2+}/calmodulin-dependent activation of SIK1 results in the de-phosphorylation of the α subunit Na,K-ATPase and an increase its catalytic activity. This pathway is shown to be activated by high salt intake in both kidneys (Sjostrom et al., 2007) and human vascular smooth muscle cells (Popov et al., 2011). Interestingly, a single nucleotide polymorphism of SIK1 has associated with low blood pressure and decreased left ventricle mass suggesting the importance of this signaling for blood pressure control (Popov et al., 2011).

Glutathionylation of β1 subunit of the Na,K-ATPase is an important pathway to modulate Na,K-ATPase activity by physiological and pathophysiological stimuli. Thus, angiotensin II has previously been shown to inhibit the Na,K-ATPase in vascular smooth muscle cells via NADPH oxidase-dependent glutathionylation of β1 subunit suggesting the involvement of this pathway in elevation of arterial tone and angiotensin-induced hypertension (Liu et al., 2013). Importantly, this action was antagonized by FXYD proteins showing their important vascular protective role under oxidative stress (Liu et al., 2013). The glutathionylation pathway has also been shown to play an important role in agonist-induced inhibition of the Na,K-ATPase activity in smooth muscle cells (Dey et al., 2013). An antagonistic action of FXYD1 protein, phospholemman (PLM) was associated with de-glutathionylation of the Na,K-ATPase and has been suggested to be modulated via protein kinase C (PKC) phosphorylation (Dey et al., 2012). This PKC mediated signaling stimulates the Na,K-ATPase turnover without affecting affinity for Na^+. In pulmonary artery wall, PKC was also implemented in Na,K-ATPase inhibition by HETE-20, a cytochrome P-450 metabolite of arachidonic acid (Singh et al., 2012). This action explains a moderate potentiation of vascular tone by arachidonic acid. Finally, PKC is involved in modulation of the α2 Na,K-ATPase isozyme upon adrenoceptor stimulation while the α1 Na,K-ATPase isozyme is regulated by β-adrenoceptor-dependent protein kinase A signaling (Gao et al., 1999).

CONTROL OF INTERCELLULAR COUPLING BY THE Na,K-ATPase

Na,K-ATPase is involved in modulation of vascular tone by endothelium (Edwards et al., 1998; Dora and Garland, 2001; Wenceslau and Rossoni, 2014; Hangaard et al., 2015). Hyperpolarization of endothelial cells by chemical or mechanical excitation facilitates Ca^{2+} influx which, in this way, enhances the production of endothelium-derived relaxing factors. Besides nitric oxide (NO) and prostanoids the endothelium-dependent relaxation is mediated by an endothelium-dependent hyperpolarizing factor (EDHF) (Sandow, 2004; Edwards et al., 2010). Vasodilator effects of EDHF are strongly associated with the subjacent smooth muscle cell hyperpolarization but its nature remains controversial. It is, however, well-established that EDHF is critically dependent on endothelial Ca^{2+}-activated K^+ channels, $K_{Ca}2.3$ and $K_{Ca}3.1$ (Coleman et al., 2004; Sandow, 2004). Strong experimental data indicates also the significance of myoendothelial gap junctions (MEGJs) and the Na,K-ATPase in EDHF (Edwards et al., 1998; de Wit et al., 2006; Dora et al., 2008; Hangaard et al., 2015).

The heterogeneous nature of signals could be the reason for different EDHF properties depending on the type of vascular bed and experimental conditions. Thus, in rat mesenteric arteries EDHF can only in part be explained by MEGJs signaling (Edwards et al., 1998). It has been suggested that under these conditions K^+ efflux through the Ca^{2+}-activated intermittent conductance K^+ channels (e.g., $K_{Ca}3.1$) increases near myoendothelial projections local K^+ concentration (K^+ "cloud") which, in turn, activates on the Na,K-ATPase in smooth muscle cell membrane (and inward-rectifying K^+ channels) providing hyperpolarization of the subjacent smooth muscle cells. Which catalytic subunit of the Na,K-ATPase is important for this signal is under debate but pharmacological profile (Dora

and Garland, 2001; Dora et al., 2008) and a high extracellular K^+ saturation (McCarron and Halpern, 1990; Blanco and Mercer, 1998) suggested a major importance of the α2 Na,K-ATPase isozyme (Longden and Nelson, 2015). Moreover, it has been suggested a specific localization of the α2 Na,K-ATPase isozyme in myoendothelial projections in a close proximity with Ca^{2+}-sensing receptor (CaSR) and $K_{Ca}3.1$ (Dora et al., 2008).

It has been suggested that EDHF signals differentiate between MEGJs and K^+ "cloud" by CaSR action on the $K_{Ca}3.1$ channels via protein kinase A pathway (Dora et al., 2008; Hangaard et al., 2015). This differentiation is overlapping since the Na,K-ATPase activity has been shown to modulate intercellular coupling, including MEGJs (Martin et al., 2003; Matchkov et al., 2007, 2012). It has been shown that a spatially restricted microdomain of the Na,K-ATPase and the NCX (Matchkov et al., 2007) modulates intercellular communications between smooth muscle cells via controlling local intracellular Ca^{2+} homeostasis. Moreover, a physical interaction in the membrane microdomain between the α2 Na,K-ATPase isozyme, NCX and gap junction protein, connexin43 was shown (Matchkov, 2010). The role of α2 Na,K-ATPase isozyme was further validated by siRNA-induced downregulation and importance of this signaling for MEGJs was shown (Matchkov et al., 2012). In accordance with the suggested role of Na,K-ATPase for intercellular coupling pharmacological inhibition with ouabain and downregulation of the α2 Na,K-ATPase isozyme suppressed intercellular coupling and inhibited EDHF response in arteries (Matchkov et al., 2007, 2012).

Signaling pathway between the Na,K-ATPase and gap junctions is unclear. We suggested previously a Ca^{2+}-dependence of uncoupling action of ouabain (Matchkov et al., 2007); however, the involvement of other signaling molecules cannot be excluded. Thus, intracellular Ca^{2+} ions can modulate gap junction directly (Enkvist and McCarthy, 1994; Schirrmacher et al., 1996; Thimm et al., 2005) and via Ca^{2+}-dependent protein kinase pathways (Chuderland and Seger, 2008; Chuderland et al., 2008). Three to four types of connexins form gap junctions in the vascular wall (Gustafsson et al., 2003; Matchkov et al., 2006) but connexin43 is a suitable candidate for the regulation via Na,K-ATPase signaling. Connexin43 expressed in cultured smooth muscle cells, A7r5 (Moore et al., 1991) where this signaling is also shown and is one of the most heavily regulated gap junction proteins. It has been shown to be regulated by intracellular Ca^{2+} concentration and a broad range of intracellular signaling pathways (Lampe and Lau, 2004). Other connexin isoforms also cannot be excluded.

BI-MODAL VASCULAR EFFECTS OF OUABAIN

Importantly, vascular effects of ouabain can be subdivided to acute and chronic responses. In contrast to acute responses, chronic manipulations with the Na,K-ATPase have been shown to affect the expression of membrane proteins involved in Ca^{2+} transport, e.g., the NCX and C-type transmembrane receptor potential protein-6 (TRPC6) (Pulina et al., 2010; Matchkov et al., 2012; Chen et al., 2015). These expressional effects were suggested to be mediated by the α2 Na,K-ATPase isozyme via an initiation of protein kinase signaling cascade, including the Src kinase pathway. Accordingly, pharmacological inhibition of tyrosine phosphorylation prevented the expressional effects of chronic ouabain treatment (Zulian et al., 2013) but did not affect vascular responses to acute ouabain (Song et al., 2014). Downstream signalings from Src kinase activation, e.g., extracellular signal-regulated kinases 1/2 (Erk1/2) and p38, have been shown to modulate protein expression and affect cellular phenotype (Haas et al., 2000, 2002; Aizman and Aperia, 2003; Nguyen et al., 2007; Li et al., 2009; Wang et al., 2014, 2015). The involvement of other signaling pathways in the modulation of cellular phenotype cannot be excluded (Liu et al., 2007; Wu et al., 2013).

The structural remodeling of resistance arteries is an essential characteristic of hypertension (Heagerty et al., 1993; Mulvany, 1993, 2002). The functional link between arterial wall thickening and/or lumen narrowing and elevated level of endogenous ouabain-like steroids is unresolved. Ouabain has been shown to promote cell growth, proliferation and migration (Atkinson et al., 1983; Aydemir-Koksoy et al., 2001; Abramowitz et al., 2003; Allen et al., 2003; Liu et al., 2004, 2014; Schoner and Scheiner-Bobis, 2007). This action of ouabain has been suggested to be mediated through both changes in ion homeostasis and intracellular signaling pathways (Blaustein et al., 2012). Obviously, an identification of these pathways involved in structural remodeling is one of the central questions in future strategies of hypertension treatment; but, unfortunately, this was not in the scope of the majority of previous studies.

Na,K-ATPase AND SKELETAL MUSCLE MOTOR ACTIVITY

The Na,K-ATPase is obligatory for excitability, electrogenesis, and contractility of skeletal muscles (Sejersted and Sjogaard, 2000; Clausen, 2008, 2013) which express the α1 and α2 Na,K-ATPase isoforms (Orlowski and Lingrel, 1988). The α2 Na,K-ATPase isozyme specifically enables working muscles to maintain contraction and resist fatigue (Radzyukevich et al., 2004, 2013; Heiny et al., 2010; DiFranco et al., 2015; Kravtsova et al., 2016). Skeletal muscle activity strongly upregulates the content of Na,K-ATPase although the α1 and α2 isozymes are regulated differently (Yuan et al., 2007; Clausen, 2008; Kristensen et al., 2008; Murphy et al., 2008; Juel, 2009; Nordsborg et al., 2009). Accordingly, physical inactivity of skeletal muscle induced by functional unloading (disuse) reduces the content of Na,K-ATPase (Clausen, 2008). However, the isoform-specificity of these changes was not studied in details.

Mechanical unloading of skeletal muscle under bed rest, joint immobilization, spinal cord injury, or other forms of muscle disuse leads to loss of muscle mass and functional decline (Baldwin et al., 2013; Bodine, 2013; Brooks and Myburgh, 2014). Weightless conditions during space flight and microgravity are also known to induce similar adaptations in skeletal muscles with the largest effect seen in postural muscles such as soleus (Fitts et al., 2013). Importantly, molecular and cellular mechanisms of

disuse-induced atrophy are not completely understood (Baldwin et al., 2013; Bodine, 2013; Brooks and Myburgh, 2014).

The hindlimb suspension (HS) of rodents is a well-validated model for skeletal muscle disuse providing an insight into underlying mechanisms of disuse-induced atrophy (Thomason and Booth, 1990; Morey-Holton et al., 2005; Shenkman and Nemirovskaya, 2008; Giger et al., 2009; Baldwin et al., 2013). HS leads to progressive and marked atrophy of the postural skeletal muscles which becomes evident already after 3–7 days and is associated with dramatic remodeling (Shenkman and Nemirovskaya, 2008; Baldwin et al., 2013; Pierno et al., 2013; Ogneva et al., 2014) that include a decrease of resting membrane potential (Desaphy et al., 2001; Pierno et al., 2002; Krivoi et al., 2008; Tyapkina et al., 2009). This membrane depolarization was shown to be a result of decreased electrogenic activity of the α2 Na,K-ATPase isozyme (Krivoi et al., 2008). Moreover, it was recently shown that short-term muscle disuse (6–72 h of HS) transiently and isoform-specifically regulates the electrogenic activity, protein, and mRNA content of α2 Na,K-ATPase isozyme in rat soleus muscle (Kravtsova et al., 2015a, 2016) (**Figures 1A,B**). Importantly, electrogenic activity of the α2 Na,K-ATPase isozyme was altered by a decrease in enzyme activity rather than as a consequence of altered mRNA and protein contents or localization in the sarcolemma. The loss of α2 Na,K-ATPase electrogenic activity on extrajunctional membranes containing a majority of α2 pump cannot be compensated by increase of protein and mRNA contents observed after 12 h of HS. In contrast, a small subset of junctional α2 Na,K-ATPase demonstrated recovery (**Figure 1C**) suggesting that distinct pools of the α2 isozyme are differently regulated during HS. Importantly, acute low-intensity muscle workload restores function of both pools of the α2 Na,K-ATPase (Kravtsova et al., 2016).

These disuse-induced alterations in α2 Na,K-ATPase isozyme function and expression may involve PLM-dependent regulatory mechanism (Kravtsova et al., 2016). Muscle-specific auxiliary FXYD1 subunit, PLM is one of the most abundant phosphoproteins in skeletal muscles. PLM acts as a tissue-specific regulator of the Na,K-ATPase which suppresses enzymatic activity mostly by reducing Na^+ affinity. Phosphorylation of PLM removes this inhibition and thereby increases the Na,K-ATPase pumping activity. Thus, protein kinases A (PKA) and C alter the PLM substrate affinity or turnover in cell- and Na,K-ATPase isoform-specific manner (Geering, 2008; Bossuyt et al., 2009; Pavlovic et al., 2013). In muscular tissues PLM associates with both α1 and α2 Na,K-ATPase isozymes (Crambert et al., 2002; Reis et al., 2005; Bossuyt et al., 2009; Heiny et al., 2010; Chibalin et al., 2012) where at least 30% of them are associated with the PLM (Rasmussen et al., 2008). Although the exercise-induced regulation of PLM was previously shown (Juel, 2009), the mechanism behind it remains to be elucidated. It was shown that acute HS increased PLM phosphorylation at Ser^{63} and Ser^{68} (Kravtsova et al., 2016). This is expected to stimulate the Na,K-ATPase and might be an earlier adaptive response directed to counteract the loss of enzyme activity. At the same time an increased abundance and association of PLM with the α2 Na,K-ATPase were shown to provide an opposite inhibitory effect and the net pump inhibition was achieved (Kravtsova et al., 2016).

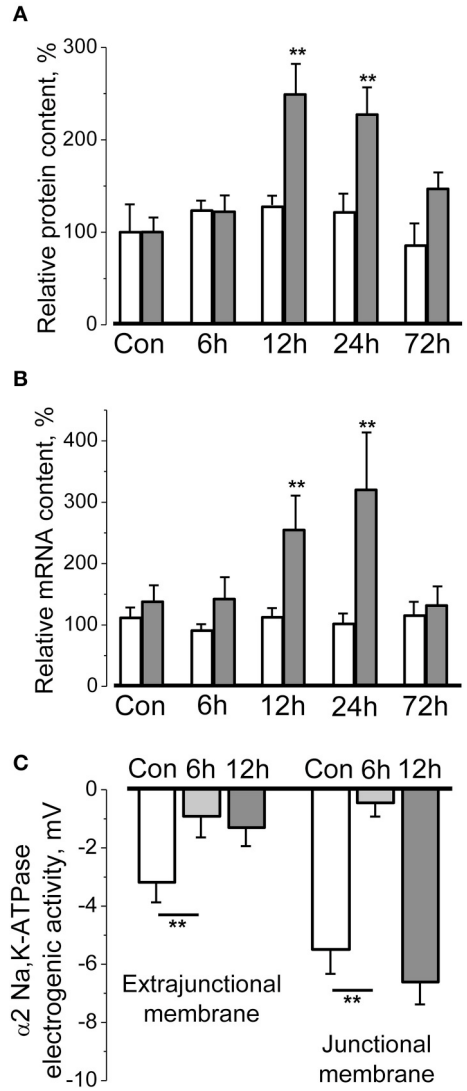

FIGURE 1 | Short-term hindlimb suspension specifically and transiently alters the α2 Na,K-ATPase protein content (A), mRNA content (B), and electrogenic activity (C) in rat soleus muscle. (A,B) Relative α1 (white bars) and α2 (gray bars) Na,K-ATPase protein and mRNA contents in the homogenates from muscles of control rats and after 6–72 h of hindlimb suspension (data normalized to the average level of expression under control conditions). **(C)** The electrogenic transport activity of the α2 Na,K-ATPase isozyme measured in the extrajunctional and junctional membrane regions of muscle fibers. $^{**}p < 0.01$ compared to corresponding control. Modified from Kravtsova et al. (2015a, 2016).

A unique role of the α2 Na,K-ATPase isozyme in the adaptations to skeletal muscle disuse is supported by the studies in humans. Thus, chronic disuse resulting from spinal cord injury (Boon et al., 2012) or knee injury (Perry et al., 2015) significantly decreased the α2 Na,K-ATPase content in human skeletal muscles. These findings raise an interesting question whether the α2 Na,K-ATPase content or activity is

regulated during other forms of disuse, e.g., sleep or treatment with muscle relaxants and anesthetics. It has been shown that electromyography (EMG) signal from soleus muscle disappears immediately after the onset of HS and remains dramatically low for several days (Ohira et al., 2002; De-Doncker et al., 2005). In contrast, different forms of periodic limb movement occurring during sleep (De Weerd et al., 2004) associate with brief EMG bursts and soleus muscle contractions (Eken, 1998). Accordingly, it was recently shown that a minimal low-intensity workload is able to restore electrogenic activity of the α2 Na,K-ATPase isozyme in soleus muscle of hindlimb-suspended rats (Kravtsova et al., 2016). These findings are in agreement with observation that limited physical activity is able to maintain abundance of the Na,K-ATPase in skeletal muscles of patients with partial spinal injury (Boon et al., 2012). Another potential experimental model to study the regulation of the Na,K-ATPase by muscle use could be hibernating animals, which overcome muscle atrophy despite prolonged disuse in dormancy.

Taken together, these results suggest that alterations specific for the α2 Na,K-ATPase precede disuse-induced skeletal muscle atrophy and indicate that different pools of this isozyme are regulated differently. Importantly, acute HS did not alter activity and content of the α1 Na,K-ATPase isozyme (**Figures 1A,B**). These findings are consistent with generally accepted hypothesis that ubiquitous α1 isozyme in skeletal muscle, as well as in other tissues, plays the main "house-keeping" role while the α2 Na,K-ATPase isozyme involved preferably in the regulation of cellular functions (Lingrel, 2010; Matchkov, 2010; Krivoi, 2012; Shattock et al., 2015). Specific regulation of the α2 Na,K-ATPase might be determined by its functional and molecular environment (Blaustein and Golovina, 2001; Lencesova et al., 2004; Song et al., 2006; Blaustein, 2013; DiFranco et al., 2015; Shattock et al., 2015) as well as by less stable than other Na,K-ATPase isozymes integration into the lipid membranes (Lifshitz et al., 2007; Kapri-Pardes et al., 2011).

INTERACTIONS BETWEEN Na,K-ATPase AND NICOTINIC ACETYLCHOLINE RECEPTOR

Inhibition of the Na,K-ATPase activity has profound effects on synaptic function associated with nerve endings membrane depolarization that stimulates release of neurotransmitters (Lichtstein and Rosen, 2001; Reich et al., 2004; Richards et al., 2007; Gulledge et al., 2013). However, it has recently become clear that the Na,K-ATPase functionally and molecularly interacts with a number of proteins and lipids to modulate synaptic, neuronal, and other cellular functions (Khatri and Man, 2013; Reinhard et al., 2013; Cornelius et al., 2015). Na,K-ATPase has been demonstrated to interact with dopamine (Hazelwood et al., 2008), AMPA (Zhang et al., 2009), δ-opioid (Deng et al., 2009), and adenosine A_{2A} (Matos et al., 2013) receptors. Moreover, functional interactions with glutamate transporters controlling glutamate uptake by astrocytes (Rose et al., 2009; Genda et al., 2011; Illarionava et al., 2014) and GlyT2 glycine transporter play an important role in glycinergic neurotransmission control (de Juan-Sanz et al., 2013).

Both the Na,K-ATPase and the nicotinic acetylcholine receptor (nAChR) are integral membrane proteins that play key roles in membrane excitation. A regulatory mechanism, where the nAChR and the Na,K-ATPase functionally interact to modulate the membrane potential, was shown in ganglion neurons (Park et al., 2010) and in skeletal muscles (Henning et al., 1994; Kragenbrink et al., 1996; Krivoi et al., 2003, 2006; Heiny et al., 2010). In ganglion neurons, micromolar concentrations of acetylcholine (ACh) induce fast depolarization through an activation of the nAChR followed by sustained hyperpolarization after ACh removal. This afterhyperpolarization is partly enabled by increase in Na^+ entry which activates the Na,K-ATPase in concentration-dependent manner. It has been suggested that this afterhyperpolarization attenuates the firing rate of post-synaptic neurons acting as an auto-regulatory mechanism for neurons excitability (Park et al., 2010).

The nAChR oscillates between resting (micromolar affinity for agonist), open or desensitized (non-conducting state with nanomolar apparent affinity for agonist) conformations (Prince and Sine, 1999; Mourot et al., 2006). High concentrations of ACh promote channel opening following by spontaneous transitions into the desensitized state. Desensitization can also occur without channel opening and is favored by prolonged exposure to low concentrations of agonist. In skeletal muscle, specific binding of nicotinic agonists to the nAChR stimulates electrogenic transport by the Na,K-ATPase causing membrane hyperpolarization. An essential role of the α2 isozyme in this response has been identified (Krivoi et al., 2003, 2006; Heiny et al., 2010). In contrast to ganglion neurons, this effect was induced by nanomolar concentrations of nicotinic agonists ($K_{0.5} \sim 30$ nM for ACh) (Krivoi et al., 2006). Importantly, stimulation of the Na,K-ATPase activity did not require ion current through the open nAChR (Heiny et al., 2010). It can be induced by the nAChR desensitization alone in the absence of nicotinic agonist and reaches saturation when the nAChR is fully desensitized. Thus, the Na,K-ATPase activation may be triggered by non-competitive blockers of the nAChR, e.g., proadifen and QX-222, which promotes the desensitized states, and suppressed by tetracaine that stabilizes resting conformation of the nAChR (Heiny et al., 2010).

The nAChR/Na,K-ATPase interaction was demonstrated in a purified membrane preparation from *Torpedo californica*, enriched by the nAChRs and the Na,K-ATPase (Krivoi et al., 2006; Heiny et al., 2010). This preparation lacks transmembrane ionic gradients and many modulatory/associative proteins and factors presented in the intact cell. It was shown that binding of nanomolar concentrations of ouabain to the Na,K-ATPase modulates specific ligand interaction with the nAChR, and vice versa, suggesting a reciprocal modulation between these two proteins (Krivoi et al., 2006). Additionally, ouabain-induced conformational changes of the Na,K-ATPase enhance conformational transition of the nAChR into a desensitized state (Heiny et al., 2010). Accordingly, these findings suggest a mechanism by which the nAChR (in desensitized state) interacts with the Na,K-ATPase and stimulates its pumping activity. Taken

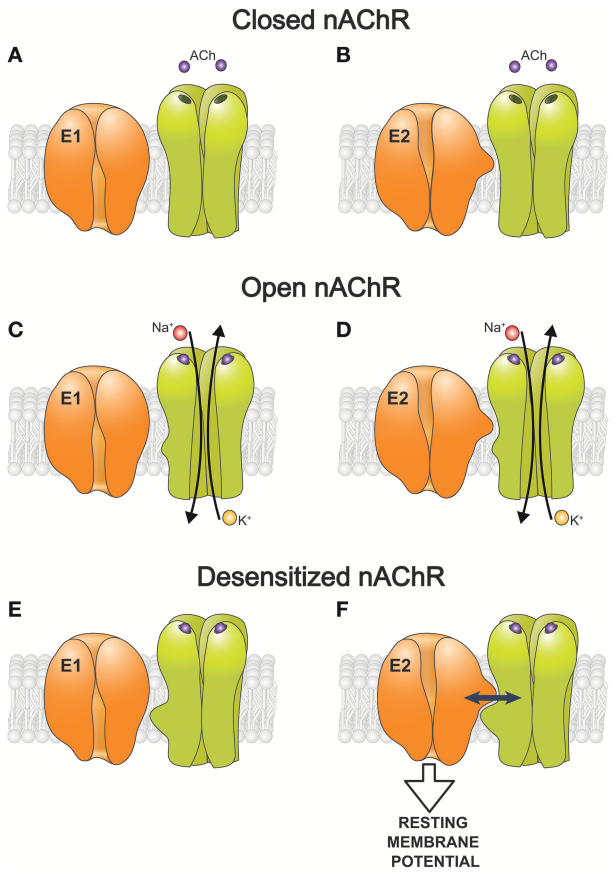

FIGURE 2 | A hypothetic scheme of the functional interaction between the nAChR and the α2 Na,K-ATPase isozyme that modulate the resting membrane potential. (A–F) Schematic representation of how the nAChR in resting (closed), open, or desensitized conformations interacts with the Na,K-ATPase in E1 **(A,C,E)** and E2 **(B,D,F)** conformations. Only the desensitized state of the nAChR and E2 conformation of the Na,K-ATPase are capable interact functionally with each other **(F)**.

into account that the binding of ouabain stabilizes the enzyme E2 conformation, it can be suggested that this conformation of the Na,K-ATPase and desensitized state of the nAChR are essential for the functional interaction between these proteins (Krivoi et al., 2006; Heiny et al., 2010; Krivoi, 2012; **Figure 2**).

Interaction between the nAChR and the Na,K-ATPase is expected to enhance muscle excitation in response to nanomolar concentrations (up to 50 nM) of non-hydrolyzed ACh which escaped hydrolysis by acetylcholinesterase, attributed to ACh released in non-quantal form (Nikolsky et al., 1994; Vyskocil et al., 2009) and remained in the synaptic cleft after nerve excitation. These nanomolar concentrations of ACh are insufficient to trigger any massive opening of the nAChR channels but it can selectively stimulate the α2 Na,K-ATPase isozyme leading to hyperpolarization of junctional membrane by ∼2–4 mV (Heiny et al., 2010; Chibalin et al., 2012). Importantly, this local hyperpolarization keeps junctional membrane at a slightly more negative potential than extrajunctional regions of the same muscle fibers. This data suggests a mechanism by which the interaction between nAChR and α2 Na,K-ATPase isozyme maintains resting potential at voltage range where the Na^+ channel inactivates slowly. This supports the excitability of junctional membrane during muscle use (Heiny et al., 2010).

This finding suggested that chronic *in vivo* exposure to nicotine, the concentration of which reaches hundreds of nanomoles during tobacco smoking (Benowitz et al., 1997) might produce long-term effects on the Na,K-ATPase and skeletal muscle electrogenesis. Experiments on rats chronically (for 21–31 days) exposed to nicotine delivered orally demonstrated that nicotine is able to modulate both α1 and α2 isozymes of the Na,K-ATPase in the diaphragm muscle. The regulatory effects include both stimulation of the α2 isozyme and inhibition of the α1 isozyme electrogenic activity leading to the net depolarizing effect. Increase in the α2 isozyme activity was accompanied with decrease in its content in the sarcolemma without change in total homogenate. The same nicotine treatment activated PKC and increased PLM phosphorylation suggested that cholinergic modulation of the Na,K-ATPase activity may utilize this regulatory pathway (Chibalin et al., 2012). Stable reciprocal interaction between the nAChR of neuronal type and the Na,K-ATPase was further confirmed in an insect nervous system (Bao et al., 2015). However, in contrast to skeletal muscle, the α2 Na,K-ATPase content decreased in homogenates of cerebral microvessels and brain tissues of rats chronically (for 14 days) exposed to nicotine using osmotic mini-pumps (Wang et al., 1994). The reasons of this contradiction are not clear; time- and use-dependence of chronic nicotine effects as well as high Ca^{2+} permeability and other features of neuronal nAChRs can be proposed.

It is established that the α2 Na,K-ATPase isozyme is enriched in end-plate membrane where it co-localizes with the nAChRs. It was also shown that the nAChRs and both α1 and α2 Na,K-ATPase isozymes co-immunoprecipitate with each other and with PLM and caveolin-3 (Heiny et al., 2010). Caveolin-3 is enriched at the neuromuscular junction (NMJ) where it co-localizes with the nAChR and promotes their clustering in the end-plate membrane. In congruence, the α subunit of nAChR has previously been shown to have a putative caveolin-binding motif (Hezel et al., 2010). Moreover, the caveolin/Na,K-ATPase interactions are also well-documented (Wang et al., 2004; Morrill et al., 2012). Since caveolin-3 is associated with caveolae in fully differentiated skeletal muscles (Galbiati et al., 2001) it suggests that the nAChR/α2 Na,K-ATPase interaction localizes in caveolae (Heiny et al., 2010). This spatially restricted complex is implemented by either direct protein-protein interaction or via additional adaptive molecular partners including lipids in the cholesterol-rich membrane microdomains, i.e., lipid rafts and caveolae. Direct molecular interactions between cholesterol and membrane receptors are shown. The role of cholesterol-rich lipid rafts as a signaling platform for the nAChRs clustering is well-established (Willmann et al., 2006; Zhu et al., 2006; Brannigan et al., 2010; Levitan et al., 2014). On the other hand, cholesterol plays an essential role in regulation of the Na,K-ATPase (Cornelius, 2008; Chen et al., 2009, 2011; Cornelius et al., 2015). It was recently shown that cholesterol chelating agent, methyl-β-cyclodextrin, eliminates local hyperpolarization of junctional

membrane in rat diaphragm muscles through a selective decrease in the α2 Na,K-ATPase isozyme electrogenic activity (Kravtsova et al., 2015b). This data suggests the involvement of cholesterol in formation and function of the nAChR/α2 Na,K-ATPase complex.

Dystrophin is a cytoskeletal protein that localizes around entire sarcolemma membrane and provides scaffolding essential for stabilization of the nAChR clusters in the NMJ. Mice lacking dystrophin (i.e., X chromosome-linked mouse mutant, MDX) causes disruption of the NMJ and de-clustering of the nAChRs (Ghedini et al., 2008; Banks et al., 2009) as well as depolarization of plasma membrane due to loss of the Na,K-ATPase activity (Miles et al., 2011). The specific involvement of the α2 Na,K-ATPase isozyme in these changes has been suggested (Kravtsova et al., 2010). However, the participation of dystrophin and other potential molecular partners, such as spectrins and ankyrins (Williams et al., 2001; Lencesova et al., 2004; Mohler et al., 2005; Doi and Iwasaki, 2008) in the formation of the nAChR/α2 Na,K-ATPase complex remains to be elucidated.

CARDIOTONIC STEROIDS AND CELL SURVIVAL

Several reports suggest that endogenous ouabain or ouabain-like compound changes the activity of α2 or α3 Na,K-ATPase isozymes that modulates glial and neuronal functions (Song et al., 2013) and may be involved in mood disorders (Lichtstein and Rosen, 2001; Goldstein et al., 2006, 2011). In addition, upon ouabain binding the Na,K-ATPase interacts with neighboring molecular environment to downstream a number of signaling intracellular pathways (Xie and Askari, 2002; Aperia, 2007; Li and Xie, 2009; Fontana et al., 2013; Reinhard et al., 2013). In different cell types, ouabain has dual effects to promote programmed cell death (Xiao et al., 2002; Kulikov et al., 2007; Blanco and Venugopal, 2016) and to protect against apoptosis (Isaev et al., 2000; Dvela et al., 2012). Nanomolar ouabain concentrations were also shown to stimulate viability and proliferation of NT2 cells, precursors for human neuronal cells, by a mechanism involving Erk1/2 activation (Dvela et al., 2012). Chronic intraperitoneal administration of low doses of ouabain significantly improves functional recovery following traumatic mouse brain injury (Dvela-Levitt et al., 2014).

Functional dysregulation of neuronal metabolism resulting from over-activation of glutamate receptors (GluRs) leads to neuronal death and underlies a variety of central nervous system disorders including stroke, neurodegenerative diseases, and spinal cord and brain injuries. The excitotoxic stress response starts with a free intracellular Ca^{2+} overload which is the most important of apoptosis (Khodorov, 2004). A similar mechanism of neuronal dysfunction and cell apoptosis is induced by micromolar ouabain (Kulikov et al., 2007; Bolshakov et al., 2012). However, some animal experimental studies have shown that low-doses of CTS provide neuroprotection against ischemia (Wang et al., 2006; Oselkin et al., 2010). This anti-apoptotic action of low ouabain was described when kainic acid and ouabain were injected into the rat brain *in vivo* (Golden and Martin, 2006).

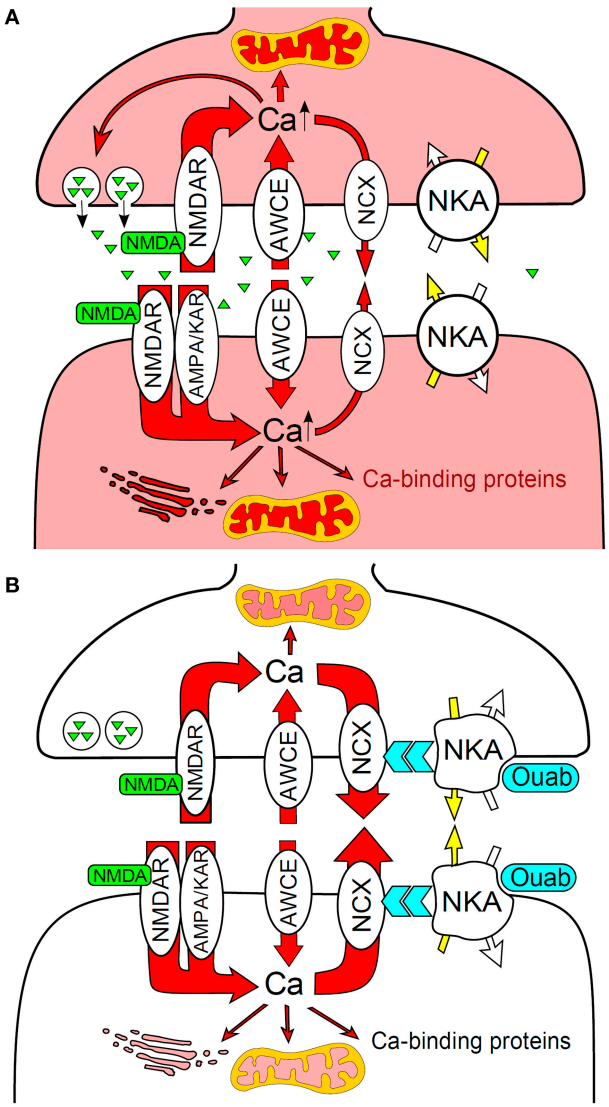

FIGURE 3 | Na,K-ATPase functionally interacts with the plasma membrane NCX to prevent Ca^{2+} overload and neuronal apoptosis during excitotoxic stress. (A) NMDA activates presynaptic and postsynaptic NMDA receptors resulted in intense Ca^{2+} entry and intracellular accumulation. **(B)** Ouabain binding to Na,K-ATPase modulates the NCX that accelerates Ca^{2+} extrusion and prevents neurons from Ca^{2+} overload. AMPA/KAR, AMPA and kainic acid receptors; AWCE, alternative to GluRs ways of Ca^{2+}entry; NKA, Na,K-ATPase. Modified from Sibarov et al. (2012).

Recently it was shown that ouabain at subnanomolar concentrations can prevent GluR agonist-induced apoptosis in primary culture of rat cortical neurons (Bolshakov et al., 2012; Sibarov et al., 2012). Apoptotic injury was prevented when the agonists were applied together with 0.1–1 nM ouabain resulting in survival of neurons in this model of excitotoxicity. Accordingly, ouabain modulated the level of anti-apoptotic protein Bcl-2, an important regulator of mitochondrial function and energy metabolism, involved in many vital cell processes (Zheng et al., 2015). Similar anti-apoptotic effects of low

ouabain doses have been shown to be associated with enhanced production of Bcl-2 in *in vivo* rat model for neurodegeneration (Golden and Martin, 2006). In cultured rat cortical neurons ouabain also prevented the increase in frequency of spontaneous excitatory postsynaptic current and the intracellular Ca^{2+} overload induced by 240-min exposure to 30 μM *N*-methyl-D-aspartate (NMDA). These effects were absent in the presence of KB-R7943, the plasma membrane NCX inhibitor (Sibarov et al., 2012). In addition, the postsynaptic epileptiform currents, reflecting periodical asynchronous glutamate release associated with elevations in intracellular Ca^{2+} concentration, were found to be suppressed by 1 nM ouabain (Sibarov et al., 2014). Ouabain was, however, found to have a bimodal effect; including anti-apoptotic action in excitotoxic stress in the concentration range from 0.1 to 1 nM, and toxic action at concentrations 10 nM–30 μM (Bolshakov et al., 2012).

It was suggested (Sibarov et al., 2012) that during excitotoxic insults ouabain accelerates Ca^{2+} extrusion from neurons via functional interaction between the Na,K-ATPase and the NCX (**Figure 3**). Since ouabain inhibits neuronal α3 Na,K-ATPase isozyme in rats at concentrations that exceed those having anti-apoptotic effects (i.e., 0.1–1 nM) (Richards et al., 2007) it can be suggested that this neuroprotective effect takes place via signaling pathways and does not directly depend on ion translocation by the Na,K-ATPase (Sibarov et al., 2012). Accordingly, circulating endogenous ouabain concentration in rat blood plasma and cerebrospinal fluid varies between 0.1 and 0.74 nM (Dobretsov and Stimers, 2005). This signaling hypothesis has been further supported by a demonstration in the crystal structure of Na,K-ATPase in high-affinity binding state for ouabain with equilibrium dissociation constant of ∼1 nM (Ogawa et al., 2009). Taken together this data suggests the novel function of the Na,K-ATPase as a neuroprotective molecule that triggers signaling pathways upon binding of endogenous ouabain or ouabain-like compounds by highly conserved binding site.

Finally, neuroprotective effects of exogenous CTS were shown *in vivo* (Golden and Martin, 2006; Wang et al., 2006; Oselkin et al., 2010). However, if endogenous CTS are already neuroprotective, then exogenously administered ouabain should have no additional effect. This opens interesting and provocative question whether the neuroprotective effects of endogenous CTS are not saturated at physiological conditions due to different properties compared to exogenous analogs. An alternative explanation suggests that different regulatory pathways are triggered and the neuroprotective effects of endogenous and exogenous CTS are not additive.

CONCLUDING REMARKS

The importance of Na,K-ATPase in various cell functions recently received new attention. It became clear that the functional role of the Na,K-ATPase can only be considered in a complex environment at the subcellular, cellular and multicellular levels where the Na,K-ATPase is structurally and functionally linked to other membrane transporters, cytoskeleton proteins and signaling molecules. We are currently only at the beginning of our understanding of these complexities. Future studies of these signalosomes, organized around specific isozymes of the Na,K-ATPase, will lead to a conceptually new view on cell physiology and will provide novel targets in treatment of several life-threatening diseases, e.g., psychiatric diseases, hypertension, and heart failure.

AUTHOR CONTRIBUTIONS

Conception and design, analysis and interpretation of data, drafting the article, article revision and approval of the final version of the manuscript: VM and IK contributed equally.

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Insights into the Pathology of the α_2-Na^+/K^+-ATPase in Neurological Disorders; Lessons from Animal Models

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A functional Na^+/K^+-ATPase consists of a catalytic α subunit and a regulatory β subunit. Four α isoforms of the Na^+/K^+-ATPase are found in mammals, each with a unique expression pattern and catalytic activity. The α_2 isoform, encoded by the *ATP1A2* gene, is primarily found in the central nervous system (CNS) and in heart-, skeletal- and smooth muscle tissues. In the CNS, the α_2 isoform is mainly expressed in glial cells. In particular, the α_2 isoform is found in astrocytes, important for astrocytic K^+ clearance and, consequently, the indirect uptake of neurotransmitters. Both processes are essential for proper brain activity, and autosomal dominantly mutations in the *ATP1A2* gene cause the neurological disorder Familial hemiplegic migraine type 2 (FHM2). FHM2 is a severe subtype of migraine with aura including temporary numbness or weakness, and affecting only one side of the body. FHM2 patients often suffer from neurological comorbidities such as seizures, sensory disturbances, cognitive impairment, and psychiatric manifestations. The functional consequences of FHM2 disease mutations leads to a partial or complete loss of function of pump activity; however, a clear phenotype-genotype correlation has yet to be elucidated. Gene-modified mouse models targeting the *Atp1a2* gene have proved instrumental in the understanding of the pathology of FHM2. Several *Atp1a2* knockout (KO) mice targeting different exons have been reported. Homozygous *Atp1a2* KO mice die shortly after birth due to respiratory malfunction resulting from abnormal Cl^- homeostasis in brainstem neurons. Heterozygous KO mice are viable, but display altered behavior and neurological deficits such as altered spatial learning, decreased motor activity and enhanced fear/anxiety compared to wild type mice. FHM2 knock-in (KI) mouse models carrying the human *in vivo* disease mutations W887R and G301R have also been reported. Both models display altered cortical spreading depression (CSD) and point to deficits in the glutamatergic system as the main underlying mechanism of FHM2.

Keywords: α_2 sodium ion pump, astrocytes, familial hemiplegic migraine type 2 (FHM2), mouse models

THE NA^+/K^+-ATPase: EXPRESSION AND FUNCTION

The Na^+/K^+-ATPase is a transmembrane ion-pump located at the plasma membrane of all mammalian cells. It's function moves three Na^+ ions out of the cell and two K^+ ions into the cell utilizing energy from ATP hydrolysis (Skou, 1957).

A functional pump consists of a catalytic α-subunit and a regulatory β-subunit (Kaplan, 2002). Four α-subunit isoforms, designated as α_1-α_4, encoded by different genes are found in mammals. Each α-subunit isoform has its own unique expression pattern and catalytic activity, which can be modulated by the β-subunit (Larsen et al., 2014; Hilbers et al., 2016). The *ATP1A1* gene encodes the $\alpha_1$isoform, which is considered to be the isoform that maintains basic cellular functions and is accordingly expressed almost ubiquitously in all tissue and cell types (Lingrel et al., 2007). The α_2 isoform, encoded by the *ATP1A2* gene, is primarily expressed in the central nervous system (CNS) and in heart-, skeletal,- and smooth muscle tissues. In the CNS, the α_2 isoform is expressed primarily in astrocytes but also in other glial cells (McGrail et al., 1991). During embryonic development, the α_2 isoform is expressed primarily in neurons, which gradually changes to the glial cell expression pattern in the adult, murine brain (McGrail et al., 1991; Cameron et al., 1994; Cholet et al., 2002; Moseley et al., 2003).

The α_3 isoform, encoded by the *ATP1A3* gene, is also expressed in the CNS and nervous tissue, but expresses specifically in the neurons of the basal ganglia, cerebellum, and hippocampus (McGrail et al., 1991; Bøttger et al., 2011; Li et al., 2013).

General Na^+/K^+-ATPase activity is crucial for multiple cellular functions such as maintaining resting membrane potential, regulating cellular volume, regulating pH, and driving the secondary active transport (Dobretsov and Stimers, 2005). Regulated and specialized pump activity is important for normal brain function. This is also reflected in the expressive relationship between the α_1, α_2, and α_3 isoforms in the CNS and the complex neurological disorders caused by mutations in the *ATP1A2* and *ATP1A3* genes (reviewed in Bøttger et al., 2012; Heinzen et al., 2014). Furthermore, increased evidence suggests roles for the Na^+/K^+-ATPase in regulating signaling pathways, such as: the membrane-associated non-receptor tyrosine kinase Src: activation of Ras/Raf/ERK1,2, phosphatidylinositol 3-kinase (PI3K): PI3K-dependent protein kinase B, phospholipase C, $(Ca_2^+)_i$ oscillations (Aperia, 2007; Schoner and Scheiner-Bobis, 2007; Liu and Xie, 2010): and gene transcription (Egr-1, Fos, Jun, Nr4a2, Hes1, and Gabre; Tupler et al., 2001).

THE α_2 ISOFORM AND ITS FUNCTION IN ASTROCYTES

Astrocytes are some of the most abundant cells in the CNS of mammals, with a ratio of 1.4 astrocytes for every neuron in the human cortex (Nedergaard et al., 2003). Through their physical properties and neuron-glial signaling pathways, they play an essential role in neuronal homeostasis through their physical properties and neuron-glial signaling pathways. During neuronal activity, a vast efflux of K^+ ions into the extracellular space occurs. The extracellular concentration of K^+ ranges from 2.5 to 3.5 mM in normal conditions but can increase to 50–80 mM under ischemic and cortical spreading depression events (Walz, 2000).

Since imbalances between extracellular K^+ and K^+ clearance can affect neuronal excitability and abnormal brain function as well as cause significant neuronal death. Even under conditions of abundant glucose supply, extracellular K^+ homeostasis must be tightly regulated to support normal brain activity (Walz, 2000).

Spatial buffering plays a critical role in the mechanism of K^+ clearance (Karwoski et al., 1989; Walz, 2000; Kofuji and Newman, 2004). This principle is based on the influx of K^+ into astrocytes though K^+ channels, with the central channel being $K_{ir}4.1$, a member of the inward rectifier-type potassium channel family. This positive influx then spreads electronically through the cytoplasm of the astrocyte and exits again as K^+ at locations distant from the active neurons (Karwoski et al., 1989; Kofuji and Newman, 2004; Macaulay and Zeuthen, 2012). In addition to $K_{ir}4.1$, both the Na^+/K^+-ATPase and the $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1) are necessary for the import and export of K^+ in the astrocytes (Galvan et al., 1979; D'Ambrosio et al., 2002; MacVicar et al., 2002; reviewed in Walz, 2000; Hertz et al., 2015). This is further described elsewhere in this special issue by MacAulay and colleagues.

In the Na^+/K^+-ATPase, the α_2 isoform combined with the β_2 subunit have K^+ affinity and voltage-sensitivity. As a result it is specifically geared to control extracellular K^+ concentration during intense neuronal firing (Larsen et al., 2014). Furthermore, the α_2 and β_2 subunit isoforms are co-expressed and appears to co-localize in astrocytes in the rodent brain (McGrail et al., 1991; Fink et al., 1996; Knapp et al., 2000; Cholet et al., 2002). Thus, the α_2/β_2 complex appears to be the main Na^+/K^+-ATPase constellation involved in the astrocytic K^+ clearance (Larsen et al., 2014).

Controlling the neurotransmitter levels present in the synapse is required for functional neuronal signaling, plasticity and neuroprotection. Removal of neurotransmitters occurs through diffusion, enzymatic degradation, and the reuptake by neurons and glial cells. The importance of astrocytic neurotransmitter uptake is highlighted in mice deficient of the astrocytic glutamate transporter excitatory amino acid transporter 2 (EAAT2), where *Eatt2*-deficiency causes lethal spontaneous seizures and increased susceptibility to acute cortical injury in mice (Tanaka et al., 1997). The α_2 isoform localizes predominately in astrocytes and other glial cells (McGrail et al., 1991; Fink et al., 1996; Knapp et al., 2000; Cholet et al., 2002) and has been shown to co-distribute with both astrocytic glutamate transporters excitatory amino acid transporter 1 (EAAT1) and EAAT2 (Cholet et al., 2002). Additionally, the α_2 isoform immunoprecipitated with both EAAT1 and EAAT2 in rat cerebellar and forebrain tissues, respectively (Rose et al., 2009). In the same study, it was shown that the ouabain-mediated inhibition of Na^+/K^+-ATPase activity had an inhibitory effect on glutamate uptake. Another study similarly found the pump combination α_2/β_2 in a protein complex with glutamate and lactate transports, thereby

sustaining glutamate-dependent lactate transport (Kleene et al., 2007). The uptake of glutamate via glutamate transporters is driven by the Na^+-gradient. Astrocytes, therefore, rely on Na^+,K^+-ATPases to pump out accumulated Na^+ (Grewer and Rauen, 2005). The direct interaction between the α_2 isoform and astrocytic glutamate transporters highlights this dependency, suggesting the existence of local regulation and crosstalk between Na^+/K^+-ATPases activity and glutamate uptake. Based on the dependency of many other neurotransmitter transporters on the Na^+-gradient, it is likely that the Na^+/K^+-ATPase is also functionally linked to these transporters. Numerous studies have shown a coupling between the α_3 subunit and neurotransmitter transport such as the glycine transporter GlyT2 (de Juan-Sanz et al., 2013).

FAMILIAL HEMIPLEGIC MIGRAINE

Familial hemiplegic migraine (FHM) is an autosomal dominant inherited migraine with aura and typically includes episodes of temporary numbness or weakness affecting one side of the body (hemiparesis). There are three recognized subtypes of FHM (FHM1-3), each caused by distinctive mutations in a specific gene. FHM Type 1, which accounts for around 50% of all FHM cases, is caused by mutations in the *CACNA1A* gene encoding the $Ca_v2.1$ P/Q voltage-dependent calcium channel (Ophoff et al., 1996). FHM Type 2 is caused by mutations in the *ATP1A2* gene (De Fusco et al., 2003) and FHM type 3 is caused by mutations in the *SCN1A* gene encoding the Na^+ channel, voltage-gated, type I, α subunit (Dichgans et al., 2005). Thus, all three types of FHM are related to alterations in ion-transport and ion-homostasis.

FHM patients in general share common migraine associated symptoms such as nausea, vomiting, and increased sensitivity to stimuli such as sound and light. In addition, diverse comorbidities have in addition been observed for each subtype and also for different mutations in a given subtype. In FHM2, observed neurological symptoms include sensory disturbance, impaired vision during aura episodes, speech difficulties, varied forms of epilepsy and seizures, developmental disabilities, cognitive impairments and cerebellar defects, which cause ataxia, dysarthria, and nystagmus. Rarer manifestations such as psychiatric disorders (depression, borderline personality, and obsessive-compulsive disorder), obesity, dystonic posturing, and anxiety have also been reported (Barrett et al., 2008; Bøttger et al., 2012; Ferrari et al., 2015).

The genetic linkage between *ATP1A2* and FHM2 was first made in 2003 (Marconi et al., 2003). In this study of two large Italian families with extensive history of FHM2, the FHM2 locus was narrowed to a 0.9 Mb region on chromosome 1q23 wherein mutations in the *ATP1A2* gene were first identified. Subsequent sequence analysis of candidate genes revealed specific FHM2-associated mutations in the *ATP1A2* gene in each of the families (De Fusco et al., 2003). Numerous new mutations in the *ATP1A2* gene have been reported post-analysis, indicating that there are more than 35 FHM2-associated mutations, spanning most of the *ATP1A2* coding region (Bøttger et al., 2012). Approximately half of the FHM2 mutations tested *in vitro* show complete loss of pump function, while a majority of the remainder showed reduced pump activity relative to normal α_2 pump activity. A small number of mutations either prevent α_2 expression or prevent the transport of α_2 to the plasma membrane (Bøttger et al., 2012).

To date, genotype-phenotype correlations have yet to be made, yet it is likely that the severity of the main symptoms and the spectra of comorbidities are linked to the effect of the mutation.

Due to the heterogeneity of human patients, however, it may be impossible to correlate genotype-phenotype by clinical patients studies. Therefore animal models are important tools for understanding the spectrum of FHM2 phenotypes and to elucidate the mechanisms behind FHM2.

GENE-MODIFIED α_2 MOUSE MODELS

Several gene-modified mouse models targeting the *Atp1a2* gene have been reported, and extensively used to study the *in vivo* functions of the α_2 isoform (**Table 1**).

Only heterozygous α_2 knock-out (KO) and knock-in (KI) mice are viable, as homozygous mice dies immediately after birth (James et al., 1999; Ikeda et al., 2003, 2004; Leo et al., 2011). These immature deaths of homozygous mice were reportedly caused by respiratory failure in $\alpha_2^{KOE2/KOE2}$ mice (Ikeda et al., 2004). The activity of respiratory motor neurons in the fourth cervical ventral root were altered in $\alpha_2^{KOE2/KOE2}$ fetuses, as

TABLE 1 | *Atp1a2* gene modified mouse models.

Mouse model*	*Atp1a2* genetic alteration	Major behavioral observations	References
$\alpha_2^{+/KOE4}$	Deletion targeting exon 4	- Enhanced fear and anxiety - Hypoactivity - Impaired spatial learning	James et al., 1999; Lingrel et al., 2007; Moseley et al., 2007
$\alpha_2^{+/KOE21}$	Deletion targeting exon 21	- Enhanced fear and anxiety	Ikeda et al., 2003
$\alpha_2^{+/KOE2}$	Deletion targeting exon 2	- Not assessed	Ikeda et al., 2004
$\alpha_2^{+/W887R}$	Single nucleotide substitution (T2763C) causing a single amino acid substitution (W887R)	- Enhanced fear and anxiety	Leo et al., 2011
$\alpha_2^{+/G301R}$	Single nucleotide substitution (G901A) causing a single amino acid substitution (G301R).	- Hypoactivity (females only) - Compulsive behaviors (females only) - Stress-induced depression	Bøttger et al., 2016

**Three Atp1a2 knock-out (KO) and two knock-in (KI) mice have been described. For all five models, only heterozygous animals are viable after birth. No models of conditional Atp1a2 KO in CNS related cells have been reported.*

the response of these neurons to electrical stimulation in the ventrolateral medulla was significantly slower in homozygous KO fetuses compared to wild type fetuses (Ikeda et al., 2004). A higher intracellular Cl^- concentration was found in these ventrolateral medulla neurons in $\alpha_2^{KOE2/KOE2}$ fetuses. Furthermore, the α_2 isoform co-immunoprecipitated with the K^+-Cl^- cotransporter KCC2, suggesting a functional coupling (Ikeda et al., 2004; Pedersen et al., 2006). This is supported by the fact that homozygous *Kcc2* KO mice also die immediately after birth due to severe motor deficits that abolish respiration (Hübner et al., 2001). Thus, homozygous $\alpha_2^{KOE2/KOE2}$ mice with a complete loss of the α_2 isoform function, exhibit severe deficits in Cl^- homeostasis in vulnerable cells including the respiratory center neurons, thereby causing abnormal neuronal activity and resulting in respiratory failure upon birth.

α_2 ISOFORM KNOCK-OUT MICE

Relative to WT littermates, heterozygous $\alpha_2^{+/KOE21}$ and $\alpha_2^{+/KOE4}$ mice showed a 50% reduction in the α_2 isoform protein levels (Ikeda et al., 2003; Moseley et al., 2007). The α_1 isoform protein levels were comparable between the two groups of mice, whereas α_3 isoform protein levels in $\alpha_2^{+/KOE4}$ mice were reduced to 80% relative to WT (Moseley et al., 2007).

Both $\alpha_2^{+/KOE4}$ and $\alpha_2^{+/KOE21}$ mice models displayed increased fear and anxiety behavior as the main abnormal behavioral phenotype (Ikeda et al., 2003; Lingrel et al., 2007; Moseley et al., 2007). Characteristic of these behavioral phenotypes, the $\alpha_2^{+/KOE21}$ mice had fewer entries and spent less time in the open arms in the elevated plus maze test (Ikeda et al., 2003). In a similar zero maze test screening for fear and anxiety, the same pattern was observed in in $\alpha_2^{+/KOE4}$ mice (Moseley et al., 2007). Another common anxiety behavioral test is the light/dark test, which consists of one illuminated room connected to one dark room. The mouse to be tested is placed in the dark room. The time of first entry into the illuminated room and all subsequent periods of time spent in this room are recorded. Compared to WT littermates, $\alpha_2^{+/KOE21}$ mice not only spent significantly less time in the illuminated room, but the latency to enter the illuminated room was also significantly higher (Ikeda et al., 2003), indicating increased fear and anxiety behavior phenotypes.

In the open field test, $\alpha_2^{+/KOE4}$ mice were found to be overall less active compared to WT mice (Moseley et al., 2007). In contrast, no altered spontaneous activity in home cage was observed for the $\alpha_2^{+/KOE21}$ mice (Ikeda et al., 2003). This suggests that the hypolocomotion observed in the open field test was induced by the enhanced stress and anxiety response in $\alpha_2^{+/KOE4}$ mice, caused by the unfamiliar and open environment of the open field setup. This correlates with the observation that $\alpha_2^{+/KOE4}$ mice spend more time in the periphery region instead of the center region during the open field test, which is a commonly used indicator of enhanced fear behavior (Moseley et al., 2007).

Utilizing the Morris water maze test, $\alpha_2^{+/KOE4}$ mice were also tested for spatial learning and memory (Moseley et al., 2007). The latency to find the hidden platform was higher for $\alpha_2^{+/KOE4}$ mice compared to WT, which normally indicates impaired learning. However, the distance traveled to reach the platform was comparable for $\alpha_2^{+/KOE4}$ and WT mice, suggesting no impairment of spatial memory (Moseley et al., 2007). Thus, the increased latency could be a consequence of the enhanced fear and anxiety related behavior observed in $\alpha_2^{+/KOE4}$ mice, as the immersion in water is highly stressful to the mouse. In support of this, the $\alpha_2^{+/KOE21}$ mice display an increased freezing time in conditioned fear stimuli, which suggests that learning and memory capability is not impaired (Ikeda et al., 2003).

The enhanced fear and anxiety behavior was investigated further in $\alpha_2^{+/KOE21}$ mice and homozygous $\alpha_2^{KOE21/KOE21}$ fetuses. It was found that homozygous $\alpha_2^{KOE21/KOE21}$ fetuses displayed selective neuronal apoptosis in the amygdala and piriform cortex, both regions involved in fear, anxiety and basic defense mechanism behavior (Davis et al., 1994; Ikeda et al., 2003). Furthermore, c-Fos expression was significantly elevated in these regions in $\alpha_2^{KOE21/KOE21}$ fetuses and also in adult $\alpha_2^{+/KOE21}$ mice after conditioned fear stimuli, which indicate neuronal hyperactivity in the areas (Ikeda et al., 2003). An increase in the excitability of amygdala output neurons increased aversive fear conditioning (Davis et al., 1994), thus the enhanced fear and anxiety behavior of α_2 KO mice probably arise from abnormal function of these brain regions.

Uptake of glutamate and GABA into crude synaptosome preparations from $\alpha_2^{KOE21/KOE21}$ fetuses was impaired compared to WT preparations and consequently, both glutamate and GABA levels were increased in brains from $\alpha_2^{KOE21/KOE21}$ fetuses relative to WT littermates (Ikeda et al., 2003). A dysfunction in the removal of neurotransmitters from synapse could be the underlying cause of neuronal hyperactivity and of the observed neurodegeneration in the amygdala and piriform cortex. This is further supported by other studies showing co-localization, molecular interaction and functional interaction between α_2 and glutamate transporters (Cholet et al., 2002; Kleene et al., 2007; Rose et al., 2009). It is reasonable to suggest that heterozygous mice also share these abnormalities, which cause the observed behavioral phenotypes in $\alpha_2^{+/KOE21}$ mice. Another plausible explanation, as observed in $\alpha_2^{KOE2/KOE2}$ fetuses, is the α_2 isoform interaction with KCC2 and altered intracellular Cl^- concentration, which appears to be the cause of respiratory failure in $\alpha_2^{-/-}$ mice (Ikeda et al., 2004; Pedersen et al., 2006). This Cl^- homeostasis deficit could result in enhanced fear and anxiety behavior for all $\alpha_2^{+/-}$ mice. Similar to the $\alpha_2^{+/KOE4}$ and $\alpha_2^{+/KOE21}$ mice, hypomorphic mice with 17% KCC2 levels display increased anxiety-like behavior in several behavioral tests, including the elevated-plus maze test (Ikeda et al., 2003; Tornberg et al., 2005; Moseley et al., 2007).

While most neurological studies involving the α_2 subunit focus on its effect in FHM2, several recent studies have also linked the Na^+/K^+-ATPase to other critical neurological disorders such as Amyotrophic lateral sclerosis (ALS), Huntington's disease and Alzheimer's disease (Acuña et al., 2013; Valencia et al., 2013; Gallardo et al., 2014; Ohnishi et al., 2015). In the ALS study, the $\alpha_2^{+/KOE4}$ model was used to study the pathology of this disease. A protein complex consisting of α_2 and α-adducin

was found enriched in cultured astrocytes expressing mutant superoxide dismutase 1 (SOD1), the most common genetic factor for ALS (Gallardo et al., 2014). Interestingly, knock down of α_2 in cultured astrocytes protected co-cultured motor neurons from degeneration, and mating $\alpha_2^{+/KOE4}$ and $SOD1^{G93A}$ mutant mice significantly reduced neurodegeneration, thereby increasing the lifespan of $\alpha_2^{+/KOE4-}$ $SOD1^{G93A}$ mice compared to $SOD1^{G93A}$ mice (Gallardo et al., 2014).

α_2 ISOFORM KNOCK-IN MICE

Western blotting for α_2 isoform protein in brain lysates showed reduced α_2 protein levels to around 60% in heterozygous adult $\alpha_2^{+/W887R}$ (Leo et al., 2011) and $\alpha_2^{+/G301R}$ mice (Bøttger et al., 2016) and to very minimal levels in homozygous fetuses of $\alpha_2^{G301R/G301R}$. Specific brain regions were additionally probed in the $\alpha_2^{+/G301R}$ mice with similar results (Bøttger et al., 2016). No alteration in the α_1 and α_3 subunit isoform protein levels was found in either of the KI mouse models (Leo et al., 2011; Bøttger et al., 2016), suggesting that these isoforms are not upregulated to compensate for the haploinsufficiency of the α_2 subunit in the $\alpha_2^{+/W887R}$ and $\alpha_2^{+/G301R}$ mice.

Mutated α_2-W887R transfected into HeLa cells was expressed but did not properly transport to the cell surface. Instead, it was found confined to the endoplasmic reticulum and proteasome inhibition with MG132 increased α_2-W887R levels significantly (Leo et al., 2011). This suggests improper folding and subsequently proteasomal degradation as the fate of α_2-W887R. However, transfection of α_2-W887R into COS7 cells, showed α_2-W887R at the plasma membrane, by both immunofluorescence staining and by subcellular fractionation (De Fusco et al., 2003). Interestingly, α_2-W887R located to the plasma membrane when expressed in *Xenopus laevis* oocytes (Koenderink et al., 2005). On measuring ion flux and ATPase activity, this W887R mutation was shown to be inactive (Koenderink et al., 2005).

In vitro studies on α_2-G301R found that this mutation did not cause significant changes in mRNA stability as mRNA levels in HeLa cells transfected with either α_2 or α_2-G301R, respectively, were comparable (Santoro et al., 2011). However, immunofluorescence staining for transfected α_2-G301R showed that the mutated version did not express properly. Thus, it was concluded that this loss of function mutation prompts deficits in folding, maturation, or intracellular trafficking of the α_2-G301R that causes it to be degraded (Santoro et al., 2011). From these studies and the reduced α_2 levels in $\alpha_2^{+/W887R}$ and $\alpha_2^{+/G301R}$ mice, it appears reasonable to consider both mutated pumps expressed but display no functional pump activity due to improper transport and/or degradation. This is an important difference to the KO models, since mutated α_2 protein (α_2-W887R and α_2-G301R) might still be able to interact with important factors such as the β subunits that could have an effect on the unmutated α_2 isoform in these models.

The phenotypic behavioral consequences of introducing the W887R mutation were assessed using a modified SHIRPA protocol (Leo et al., 2011). In this protocol, few behavioral differences were observed between $\alpha_2^{+/W887R}$ and WT mice. The only significant alteration between $\alpha_2^{+/W887R}$ mice compared to WT littermates was an elevated fear response and increased anxiety (Leo et al., 2011).

Behavioral phenotypes were likewise addressed in the $\alpha_2^{+/G301R}$ mice. During the tail suspension test, for example, the $\alpha_2^{+/G301R}$ mice exhibited depression-like behavior, such as increased immobility, compared to WT mice (Bøttger et al., 2016). In a sucrose preference test, $\alpha_2^{+/G301R}$ mice displayed stress-induced anhedonia and increased acoustic startle responses, implying abnormal levels of fear and anxiety (Bøttger et al., 2016). Female $\alpha_2^{+/G301R}$ mice were found to have additional abnormal behavioral phenotypes compared to male $\alpha_2^{+/G301R}$ mice. Unlike WT females and male $\alpha_2^{+/G301R}$ mice, $\alpha_2^{+/G301R}$ females were hypoactive in open field test (Bøttger et al., 2016). Excessive grooming behaviors were also observed in the open field, suggesting compulsive behavior in female $\alpha_2^{+/G301R}$ mice. The marble-burying test is one of the standard behavioral tests for rodents to assess repetitive compulsive-like behaviors. Female $\alpha_2^{+/G301R}$ mice were found to display obsessive compulsive disorder (OCD)-like traits as they buried significantly more marbles compared to both WT and to male $\alpha_2^{+/G301R}$ mice.

Interestingly, these female-specific phenotypes were reverted by a single progestin treatment, implying that the female hormone cycle contributes to the altered behaviors noted in $\alpha_2^{+/G301R}$ females compared to WT females and male $\alpha_2^{+/G301R}$ mice. Intriguingly, this coincides with the influence of ovarian hormones on migraines, and the higher prevalence of migraines in women compared to men (Vos et al., 2012).

N-Methyl-D-aspartic acid or N-Methyl-D-aspartate (NMDA)-receptor antagonists also reverted specific $\alpha_2^{+/G301R}$ phenotypes including hypoactivity, OCD-like behaviors and increased acoustic stimuli startle response. This indicates that the glutamatergic system is affected by the mutated α_2 isoform. In support of this, $\alpha_2^{+/G301R}$ mice had elevated glutamate levels in all major brain regions. Mixed astrocyte-neuron cultures from $\alpha_2^{G301R/G301R}$ mice displayed a significant reduction in glutamate uptake compared to similar cultures from WT mice (Bøttger et al., 2016). This further links the female sex hormone cycle and the glutamate system to the comorbid psychiatric manifestations of FHM2.

CORTICAL SPREADING DEPRESSION

A key characteristic of FHM2 is the appearance of the aura phenomenon prior to the onset of the hemiplegic migraine. It is widely accepted that cortical spreading depression (CSD) is the molecular mechanism behind aura (Lauritzen et al., 2011). CSD is a short-lasting wave of neuronal and glial cell hyperactivity followed by a wave of long-lasting depression of neuronal activity (Pietrobon and Moskowitz, 2013). During the intense neuronal activity, excess K^+ ions and glutamate are released into the extracellular space. The inefficient removal of K^+ and glutamate by astrocytes can lead to wide-reaching depolarization that depresses further neuronal firing (Lauritzen et al., 2011; Pietrobon and Moskowitz, 2013). The CSD usually

begins in the visual cortex and propagates slowly over the cortex at a rate of 2–5 mm/min (Pietrobon and Moskowitz, 2013).

In vivo CSD recordings revealed that $\alpha_2^{+/W887R}$ mice were more susceptible to CSD. This is based on a 35% decrease in induction threshold relative to WT littermates (Leo et al., 2011). Furthermore, induced CSD events in the $\alpha_2^{+/W887R}$ mice had a higher propagation velocity compared to WT littermates (Leo et al., 2011). The duration of triggered CSD events, however, was not altered between $\alpha_2^{+/W887R}$ mice and littermates (Leo et al., 2011). Electrocorticography in $\alpha_2^{+/G301R}$ mice, compared to WT littermates, revealed a prolonged recovery phase following CSD (Bøttger et al., 2016). Further CSD assays are necessary to conclude the effects of the G301R mutation on CSD.

It is difficult to compare the CSD studies of the two α_2-KI mouse models, due to differing parameter and assay conditions used in each study. Potassium acetate, for example, was used for CSD induction in the right somatosensory cortex in $\alpha_2^{+/G301R}$ mice, whereas incremental electrical current stimuli were delivered in the occipital cortex for the $\alpha_2^{+/W887R}$ mice. Moreover, the different use of anesthetics (α-chloralose versus urethane) affects the data collection, as previous studies of other anesthetics were shown to influence the CSD induction threshold and to propagate speed differently (Kudo et al., 2013). Furthermore, only male mice were used in the $\alpha_2^{+/G301R}$ study (Bøttger et al., 2016), whereas a mixed sex population was used for the $\alpha_2^{+/W887R}$ study (Leo et al., 2011). In particular, considering the female specific phenotypes of the $\alpha_2^{+/G301R}$, it appears that sex and specific sex hormones play an important role in FHM2. To sufficiently compare these two models and the CSD underlying FHM2, a comparable study using the same CSD induction method, same anesthetics and a focus on sex-specific phenotypes is required.

The CSD abnormalities observed in both FHM2 models are comparable with two FHM1 knock-in mouse models of which revealed increased susceptibility to CSD compared to WT mice. This further supports CSD as an important symptom of migraines and FHM in general (van den Maagdenberg et al., 2004, 2010). To further emphasize the importance of sex and CSD, it was found that female FHM1 KI mice were more susceptible to CSD than male KI mice (Eikermann-Haerter et al., 2009). This sex difference was abolished with ovariectomy and senescence, further solidifying the role of the female sex hormone cycle, besides genetics, in the susceptibility to CSD (Eikermann-Haerter et al., 2009).

PERSPECTIVES ON THE USE OF FHM2 MOUSE MODELS

Migraines affect nearly 15% of the world population, and while FHM2 is a relatively rare migraine form, further insight into FHM2 will lead to a broader understanding of migraines in general (Vos et al., 2012). Animal models of human genetic disorders are important tools for investigators in elucidating the molecular and physiological deficits leading to the disorder. As discussed in this review, much has already been learned from existing *Atp1a2* animal models (Ikeda et al., 2003, 2004; Lingrel et al., 2007; Moseley et al., 2007; Leo et al., 2011; Bøttger et al., 2016). The continued work with existing models and generating of new models with different FHM2 mutations will expand our understanding of FHM2. Further study of different FHM2 mouse models together with clinical descriptions of comparable FHM2 patients will shed light on any genotype-phenotype correlations, a critical aspect when studying a disorder with varying symptoms, severity, and different functional consequences of mutations.

FHM2 mouse models will be essential for drug development, drug screening and drug testing for treatment of FHM2 patients. A potential drug target for FHM2 is the Na^+/K^+-ATPase itself because lost pump function and/or decreased/altered pump activity is the main cause of FHM2 (Bøttger et al., 2012). Since all FHM2 patients are heterozygous, targeting the unmutated α_2 isoform with activators could potentially overcome this haplodeficiency. In support of this therapeutic strategy, a recent report showed that crossing a *Atp1a3* mouse model (*Myskin*) for Alternating Hemiplegia of Childhood (AHC) with a mouse model overexpressing a WT *Atp1a3* allele could rescue the mutant phenotype through increased α_3 pump activity (Kirshenbaum et al., 2016). Modulating Na^+/K^+-ATPase is also a desirable therapeutic strategy, due to this molecules' localization in the plasma membrane and well-regulated function. The mechanism of regulation is via factors (e.g., the cardiac glycoside ouabain) binding to the extracellular part of the pump.

An important aspect of *Atp1a2* deficits appears to be the resulting changes to the glutamatergic system. These changes are manifested through molecular and histological interactions between the mutant α2-subunit isoform and a glutamate transporter (i.e., EAAT1 or EAAT2) or through reduced glutamate uptake in cells with limited pump activity. Our data suggests that future studies of mouse models with elevated glutamate levels (e.g., α_2-KO and -KI mice), which exhibit reversion of key specific α_2-KI phenotypes when targeted with NMDA receptor antagonists (Cholet et al., 2002; Ikeda et al., 2003; Rose et al., 2009; Bøttger et al., 2016), might prove beneficial as a preclinical model to test drug targets within the glutamatergic system in FHM2 patients.

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REVIEW
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Managing Brain Extracellular K^+ during Neuronal Activity: The Physiological Role of the Na^+/K^+-ATPase Subunit Isoforms

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During neuronal activity in the brain, extracellular K^+ rises and is subsequently removed to prevent a widespread depolarization. One of the key players in regulating extracellular K^+ is the Na^+/K^+-ATPase, although the relative involvement and physiological impact of the different subunit isoform compositions of the Na^+/K^+-ATPase remain unresolved. The various cell types in the brain serve a certain temporal contribution in the face of network activity; astrocytes respond directly to the immediate release of K^+ from neurons, whereas the neurons themselves become the primary K^+ absorbers as activity ends. The kinetic characteristics of the catalytic α subunit isoforms of the Na^+/K^+-ATPase are, partly, determined by the accessory β subunit with which they combine. The isoform combinations expressed by astrocytes and neurons, respectively, appear to be in line with the kinetic characteristics required to fulfill their distinct physiological roles in clearance of K^+ from the extracellular space in the face of neuronal activity. Understanding the nature, impact and effects of the various Na^+/K^+-ATPase isoform combinations in K^+ management in the central nervous system might reveal insights into pathological conditions such as epilepsy, migraine, and spreading depolarization following cerebral ischemia. In addition, particular neurological diseases occur as a result of mutations in the α2- (familial hemiplegic migraine type 2) and α3 isoforms (rapid-onset dystonia parkinsonism/alternating hemiplegia of childhood). This review addresses aspects of the Na^+/K^+-ATPase in the regulation of extracellular K^+ in the central nervous system as well as the related pathophysiology. Understanding the physiological setting in non-pathological tissue would provide a better understanding of the pathological events occurring during disease.

Keywords: K^+ clearance, astrocytes, glutamate, brain ion homeostasis, extracellular space

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K^+ MANAGEMENT IN THE EXTRACELLULAR SPACE OF THE CENTRAL NERVOUS SYSTEM

Neuronal activity, in the form of propagating action potentials, results in a transient release of K^+ into the extracellular space (Frankenhaeuser and Hodgkin, 1956). Prolonged accumulation of K^+ in the extracellular space of the central nervous system (CNS) causes wide-spread depolarization of neurons and glia which results in compromised synaptic transmission, neuronal firing, and

neurotransmitter re-uptake. Consequently, it follows that extracellular K^+ must be closely managed and therefore cleared from the extracellular space following neuronal excitation. Brain extracellular K^+, $[K^+]_o$, has indeed proven to be tightly regulated under physiological conditions; from a basal level of ~3 mM, $[K^+]_o$ rarely rises above ~12 mM. This concentration is denoted as the ceiling level and more intense or longer electrical stimulation of neuronal pathways generally cannot push $[K^+]_o$ above this point (Krnjevic and Morris, 1972; Prince et al., 1973; Futamachi et al., 1974; Heinemann and Lux, 1977). However, typical physiologically occurring neuronal activity in the CNS is estimated to cause $[K^+]_o$ transients of only 0.2–0.4 mM above the baseline (Syková et al., 1974; Singer and Lux, 1975).

During neuronal activity, in which K^+ is released to the extracellular space and the neuronal structures thus lose part of their intracellular K^+, an elevation in $[K^+]_o$ is observed along with a parallel rise in intracellular $[K^+]$ in the neighboring glia cells (Ballanyi et al., 1987; Grafe and Ballanyi, 1987). The activity-dependent glial K^+ accumulation points to this cellular compartment acting as a temporal K^+ sink during neuronal release of K^+ into the extracellular space (Ballanyi et al., 1987; Grafe and Ballanyi, 1987). Shortly following termination of neuronal activity, the glial $[K^+]_i$ declines, indicative of steady glial K^+ efflux, while the neurons re-gain their $[K^+]_i$ via re-uptake into the neuronal structures (Ballanyi et al., 1987; Grafe and Ballanyi, 1987, see **Figure 1**). Surely, part of the neuronally-released K^+ simply diffuses away in the extracellular space (Gardner-Medwin, 1983) but the molecular mechanism(s) that exercise the astrocytic clearance of extracellular K^+ have been investigated and debated intensely ever since the original findings in the mid-1900s:

(I) Glial cells surrounding neurons respond directly to an increase in extracellular K^+ by a membrane depolarization (Kuffler and Nicholis, 1964; Kuffler and Potter, 1964; Orkand et al., 1966). The fact that the glial cells in essence behaved as K^+ electrodes, due to their high permeability for K^+, indicated that they might act out a protective role toward neurons in connection to K^+ by redistribution of K^+ away from the site of activity (Orkand et al., 1966). This concept of K^+ channel-mediated spatial buffering of activity-evoked K^+ release relies on local differences in K^+ equilibrium- and membrane potentials leading to Kir4.1-mediated glial K^+ influx, electrotonic transfer of K^+ through the gap junction-coupled glia syncytium and Kir4.1-mediated release at a distant site (Karwoski et al., 1989; Walz, 2000; Kofuji and Newman, 2004). Although the spatial buffer currents are well-documented, the quantitative contribution to extracellular K^+ management of this channel-mediated mechanism may be limited and is under continuous debate, see (MacAulay and Zeuthen, 2012; Larsen and MacAulay, 2014; Larsen et al., 2014). This mode of K^+ clearance will not be discussed further here.

(II) The $Na^+/K^+/2Cl^-$ cotransporter of the subtype 1 (NKCC1) is functionally expressed in cultured astrocytes in which the NKCC1 is responsible for around half of the overall cellular K^+ uptake, increasing its fractional uptake as the extracellular K^+ increases (Walz and Hertz, 1984; Larsen et al., 2014). Taken together with its low apparent K^+ affinity (~ 25 mM Larsen et al., 2014), which would allow it to increase its transport activity when faced with stimulus-evoked K^+ transients, NKCC1 has been suggested as a factor involved in clearance of K^+ from the extracellular space (Su et al., 2000; Kofuji and Newman, 2004; Hertz et al., 2015). In a recent study in rat hippocampal slices, we were nevertheless unable to demonstrate a participating role of NKCC1 in clearance of K^+ from the extracellular space of this particular brain region (Larsen et al., 2014). As negligible transcript and protein of NKCC1 is present in astrocytes *in vivo* (Plotkin et al., 1997; Clayton et al., 1998; Zhang et al., 2014) and NKCC1 is recognized to be upregulated in cultured cells of different origin (Raat et al., 1996), NKCC1 appears to be active in cultured astrocytes but not involved in stimulus-evoked K^+ clearance in the rodent hippocampus under approached physiological conditions.

(III) The Na^+/K^+-ATPase is comprised of an α and a β subunit (1:1) and drives uptake of 2 K^+ from the extracellular space in exchange for 3 Na^+ from the intracellular compartment. As this transport process translocates both ions against

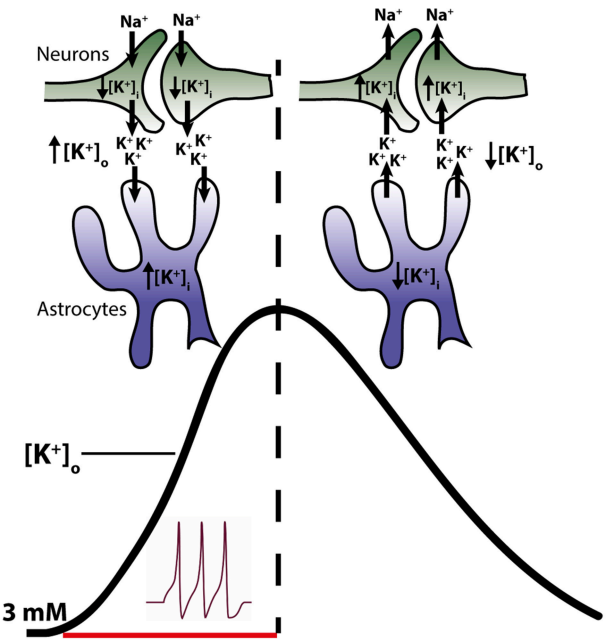

FIGURE 1 | Temporal role of astrocytes and neurons in clearance of K^+ from the extracellular space. Neuronal activity leads to a release of K^+ into the extracellular space (left side of figure) resulting in a decline in intracellular K^+ and an increase in the extracellular K^+ (black trace in figure). Astrocytes surrounding the neurons respond to the rise in $[K^+]_o$ by increased uptake of K^+ and consequently increase their $[K^+]_i$. Astrocytes thus act as K^+ sinks during the initial phase of activity. Upon cessation of stimuli, K^+ leaks out of the astrocytes, probably via Kir4.1, resulting in a reduction of astrocytic $[K^+]_i$ (right side of the figure). Meanwhile the neurons, no longer stimulated to open their voltage-gated channels, initiate reabsorbtion of their lost K^+. Thus, in this later phase, K^+ is directed from the astrocytes to the neurons.

their electrochemical gradients, the Na^+/K^+-ATPase relies on ATP hydrolysis as an integral step in its enzymatic cycle. Constant activity of the Na^+/K^+-ATPase is required to uphold the transmembrane ionic gradients, which create and maintain the membrane potential. The Na^+/K^+-ATPase is, in addition, involved in control of neuronal excitability (Haglund and Schwartzkroin, 1990; Wang et al., 2012; Gulledge et al., 2013) and has been indicated as a key contributor to management of extracellular $[K^+]$ following activity-induced K^+ release: Inhibition of the Na^+/K^+-ATPase yielded prolonged $[K^+]_o$ recovery times upon electrical stimulation in the rat optic nerve (Ransom et al., 2000) and hippocampus (D'Ambrosio et al., 2002; Larsen et al., 2014, see **Figure 2**). In support of a dominating role of astrocytes in K^+ clearance during neuronal activity, astrocytes display a maximal Na^+/K^+-ATPase transport activity (V_{max}) considerably larger than that of their neuronal counterpart (Kimelberg et al., 1978; Walz and Hertz, 1982; White et al., 1992; Hajek et al., 1996). The astrocytic Na^+/K^+-ATPase isoform combination is, in addition, of a composition that allows astrocytes to increase their Na^+/K^+-ATPase activity as a function of elevated extracellular $[K^+]$ (Grisar et al., 1980; Walz and Hertz, 1982; Hajek et al., 1996; Larsen et al., 2014) and thus allows astrocytes to respond specifically to the K^+ transients observed in connection with neuronal activity. As different isoforms and/or subunit compositions of the Na^+/K^+-ATPase, each with distinct kinetic characteristics, are expressed in different cellular structures of the CNS, one may speculate whether these combinations are assigned specific roles in regard to management of activity-induced extracellular K^+ transients.

This review aims at presenting the CNS localization, role, and physiological impact of the different subunit isoform combinations of the Na^+/K^+-ATPase in the management of extracellular K^+ in the brain.

LOCALIZATION OF THE Na^+/K^+-ATPase ISOFORMS IN NEURONS AND ASTROCYTES

Four isoforms of the α subunit of the Na^+/K^+-ATPase have been cloned (α1-α4) and three isoforms of the β subunit (β1-β3) (Blanco, 2005). Of these cloned isoforms, only α1-α3 and β1-β3 have been detected in the mammalian brain (McGrail et al., 1991; Cameron et al., 1994; Martín-Vasallo et al., 2000) although their quantity and cellular distribution remains unsettled and may depend on the brain region. The general picture that emerges reveals mRNA transcripts of α1 and β1 in both neurons and astrocytes while α2 and β2 are detected in astrocytes, and α3 exclusively in neuronal structures (Watts et al., 1991; Li et al., 2013; Zhang et al., 2014, see **Figure 3**). β3 transcript is prevalent in oligodendrocytes but negligible in astrocytes and neurons (Zhang et al., 2014). The total Na^+/K^+-ATPase α subunit mRNA increases about 10-fold from the fetal to adult stage with the α3 transcript reaching maximal and adult levels around post-natal day 7 which is only achieved for α1 and α2 around post-natal day 25 (Orlowski and Lingrel, 1988). The isoform expression pattern obtained at the mRNA level is more or less reflected at the protein level: α1 and β1 are detected in neurons and astrocytes, α2 and β2 predominantly in astrocytes and α3 in neurons (McGrail and Sweadner, 1986; McGrail et al., 1991; Cameron et al., 1994; Cholet et al., 2002; Richards et al., 2007). Alternative expression patterns have been reported which may be due to distinct regional differences in various brain structures and/or developmental stages, subpopulations

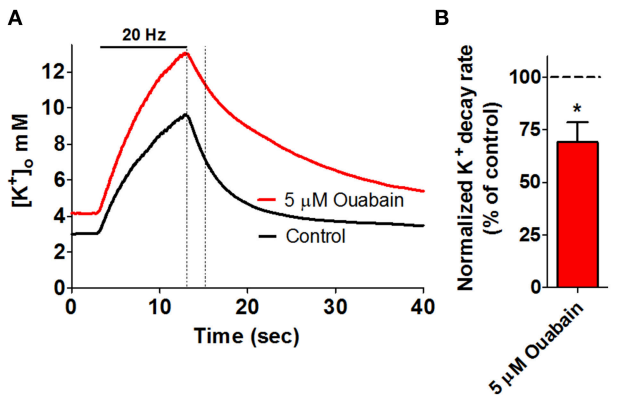

FIGURE 2 | Key role for the Na^+/K^+-ATPase in management of stimulus-evoked K^+ transients. High frequency electrical stimulation of rat hippocampal brain slices gives rise to an extracellular K^+ transient, as measured via ion-sensitive microelectrodes. **(A)** Addition of 5 μM ouabain (red trace), inhibiting α2 and α3 Na^+/K^+-ATPase, results in compromised K^+ clearance when compared to a control recording (black trace) obtained prior to addition of ouabain in the same slice. The dashed lines mark a 2-s linear segment used for quantification of the rate of K^+-clearance and is summarized in **(B)**, $^*P < 0.05$. Figure modified from Larsen et al. (2014) with permission.

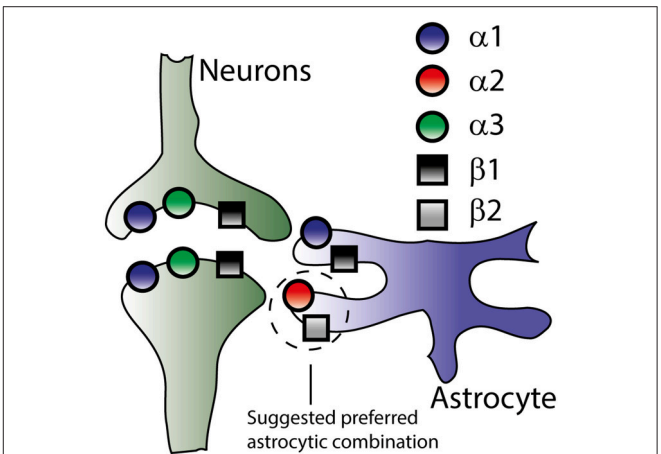

FIGURE 3 | Na^+/K^+-ATPase subunit isoform distribution in neurons and astrocytes. Though the literature offers no exact distribution of the Na^+/K^+-ATPase subunit isoforms (see text), this figure illustrates the overall consensus on isoform-specific expression of the Na^+/K^+-ATPase in neurons and astrocytes based on both mRNA and protein expression.

of neurons with altered isoform preference, altered expression in cultured neurons/astrocytes, and/or non-specific antibodies. Examples include detection of neuronal expression of the α2 isoform in late gestation mouse embryos (Moseley et al., 2003) and in cultures and sections of rat hippocampus (McGrail et al., 1991; Cameron et al., 1994) as well as neuronal expression of β2 in cerebellar sections and co-cultures (Peng et al., 1997).

As opposed to the uniform plasma membrane distribution of α1 in primary cultures of rat hippocampal astrocytes and neurons, α2 and α3 appeared to be organized in a reticular pattern paralleling the underlying endoplasmic reticulum (Juhaszova and Blaustein, 1997). In addition, α2 displayed co-localization with the Na^+/Ca^{2+} exchanger in the plasma membrane of rat astrocytes (Juhaszova and Blaustein, 1997) and localized to the glial leaflets surrounding dendritic spines (Cholet et al., 2002). The distinct cellular (α1, α3, β1 in neurons and α1, α2, β1, β2 in astrocytes) and subcellular localization pattern underscores the notion of the different Na^+/K^+-ATPase isoforms serving distinct physiological roles in the CNS.

Na^+ AND K^+ AFFINITIES OF THE NEURONAL AND ASTROCYTIC Na^+/K^+-ATPase ISOFORM COMBINATIONS

The obvious manner in which the Na^+/K^+-ATPase may be exerting isoform-specific roles in neurophysiology may be by the distinct apparent ion affinities (K'_m) of the different α and β isoform combinations. Of the isoforms expressed in the CNS, all subunit isoform combinations are able to assemble and be catalytically competent in heterologous expression systems (Crambert et al., 2000; Blanco, 2005; Larsen et al., 2014). The apparent ion affinities of the individual isoform combinations have been investigated in a number of cellular and membranous systems with each their advantages and disadvantages. Most commonly employed are leaky membrane preparations (non-sided preparations) obtained from membrane extracts of diverse tissue, mammalian cell lines (i.e., HeLa, COS-1), Sf-9 insect cells, or yeast cells all expressing select Na^+/K^+-ATPase isoforms (Sweadner, 1985; Jewell and Lingrel, 1991; Blanco et al., 1995; Therien et al., 1996; Müller-Ehmsen et al., 2001; Toustrup-Jensen et al., 2001), although intact cellular systems (sided preparations), i.e., Na^+/K^+-ATPase-expressing HeLa cells or *Xenopus* oocytes have also been employed (Munzer et al., 1994; Zahler et al., 1997; Hasler et al., 1998; Crambert et al., 2000; Horisberger and Kharoubi-Hess, 2002; Larsen et al., 2014). The advantage of the leaky membrane approach is the complete control of all ion concentrations, the experimental ability to address select steps of the Na^+/K^+-ATPase transport cycle, and the high degree of accuracy. An inherent disadvantage is the non-physiological ion concentrations facing the two sides of the Na^+/K^+-ATPase, which, due to the reciprocal influence of Na^+ and K^+ on the other ion's ability to bind, affect the apparent ion affinities of the Na^+/K^+-ATPase (Toustrup-Jensen et al., 2001). The transmembrane potential (or lack thereof in isolated membranes) also affects Na^+/K^+-ATPase activity and ion affinities in an isoform-specific manner (Larsen et al., 2014, also see **Figure 4A**). While the apparent K^+ affinity is uncomplicated to approach in intact cellular systems with physiological ion concentrations and membrane potential, determination of the apparent Na^+ affinity at the intracellular face of the Na^+/K^+-ATPase presents an experimental challenge. For both approaches, expression of the different Na^+/K^+-ATPase α isoforms in mammalian cells endogenously expressing β1 (Orlowski and Lingrel, 1988; Therien et al., 1996; Toustrup-Jensen et al., 2001) has prevented detailed analysis of the kinetic impact of β2 and β3 on the different α isoforms. Sf9 insect cells and *Xenopus laevis* oocytes express low levels of endogenous Na^+/K^+-ATPase and kinetic

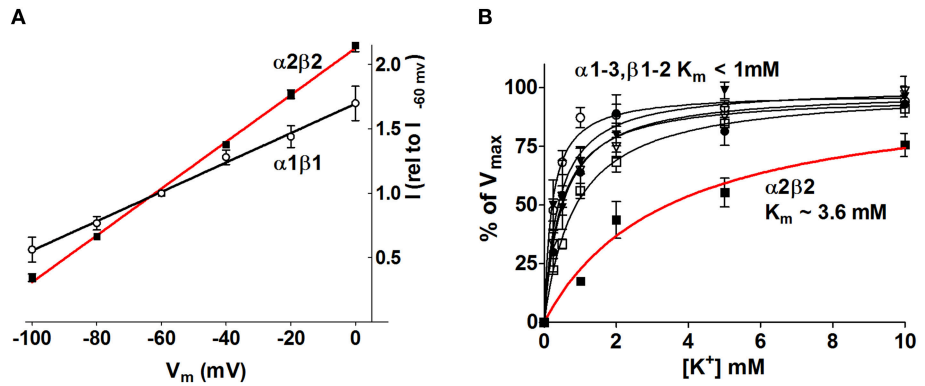

FIGURE 4 | The Na^+/K^+-ATPase activity increases in an isoform-specific manner as a function of $[K^+]_o$ and V_m. The Na^+/K^+-ATPase-mediated current was determined with two-electrode voltage clamp in *Xenopus* oocytes heterologously expressing the various astrocytic and neuronal subunit isoform combinations. **(A)** The α2β2 Na^+/K^+-ATPase displayed the most prominent voltage-sensitivity with increased Na^+/K^+-ATPase activity as a function of membrane depolarization. For clarity only α2β2 and α1β1 are illustrated, the other combinations all had intermediate I/V relationships. **(B)** the Na^+/K^+-ATPase-mediated current was determined as a function of $[K^+]$ at $V_m = -80$ mV. The α2β2 combination displayed a low apparent affinity for K^+, K'_m ~3.6 mM, whereas all other tested isoform combinations saturated at lower K^+ concentrations, $K'_m < 1.0$ mM. Figure modified from Larsen et al. (2014) with permission.

analysis of Na^+/K^+-ATPase expressed in these cell types have thereby paved the road toward determination of the apparent ion affinities of all isoform combinations (Blanco et al., 1995; Crambert et al., 2000; Larsen et al., 2014). With few studies comparing all, or the majority of, isoform combinations in parallel experimental sessions, a certain diversity exists in the obtained apparent ion affinities of the Na^+/K^+-ATPase. The general consensus emerging for the neuronal and astrocytic isoform combinations is as follows: When paired with the ubiquitous β1 isoform, the three α isoforms display similar apparent K^+ affinities in the 1–2 mM range, irrespective of the sidedness of the membrane preparations (Urayama and Nakao, 1979; Sweadner, 1985; Jewell and Lingrel, 1991; Therien et al., 1996; Crambert et al., 2000; Horisberger and Kharoubi-Hess, 2002; Larsen et al., 2014), although a few studies reported a slightly reduced K^+ affinity of the α2 isoform (Blanco et al., 1995; Müller-Ehmsen et al., 2001). The apparent Na^+ affinities of α1 and α2, when in constellation with β1, have been reported at quite a range of values (with K'_ms centered around 10-16 mM), although roughly similar between the isoforms in each study, with no general trend regarding which of the two displayed a tendency toward higher affinity than the other (Jewell and Lingrel, 1991; Blanco et al., 1995; Zahler et al., 1997; Crambert et al., 2000; Müller-Ehmsen et al., 2001; Horisberger and Kharoubi-Hess, 2002). α3, on the other hand, displayed a much reduced Na^+ affinity with values centered around K'_m of 30 mM, when determined in sided systems with physiological intracellular K^+ concentrations (Lytton, 1985; Zahler et al., 1997; Crambert et al., 2000; Horisberger and Kharoubi-Hess, 2002; Blanco, 2005). In the leaky membrane preparations, the apparent affinity of α3 toward Na^+ was either similar or only slightly different from that of α1 (Sweadner, 1985; Jewell and Lingrel, 1991; Therien et al., 1996). Pairing with the astrocytic β2 isoform did not significantly alter the apparent K^+ affinity of α1 or α3 whereas the α2β2 combination displayed a reduced K^+ affinity with K'_ms in the 3-5 mM range (Blanco et al., 1995; Crambert et al., 2000; Larsen et al., 2014, also see **Figure 4B**) while the apparent Na^+ affinity of α2 increased to around 7-8 mM upon pairing with β2 (Blanco et al., 1995). The increased apparent Na^+ affinity may be induced by a β2-dependent shift of the conformational equilibrium toward the E1P state (Hilbers et al., 2016). The isoform-specific relative ion affinities, which are of physiological relevance for clearance of K^+ from the extracellular space of the central nervous system, are summarized as follows:

$$K'_{m(K+)} : \alpha1\beta1 \approx \alpha1\beta2 \approx \alpha2\beta1 \approx \alpha3\beta1 < \alpha2\beta2$$
$$K'_{m(Na+)} : \alpha1\beta2 \approx \alpha2\beta2 < \alpha1\beta1 \approx \alpha2\beta1 << \alpha3\beta1$$

THE PHYSIOLOGICAL ROLE OF THE Na^+/K^+-ATPase ISOFORM COMBINATIONS IN K^+ CLEARANCE

The diverse roles of the Na^+/K^+-ATPase in management of $[K^+]_o$ in the central nervous system suggest a scenario in which explicit subunit isoform combinations perform distinct temporal and spatial roles. During the neuronal stimulus, the astrocytic Na^+/K^+-ATPase α2β2 combination appears specifically geared to respond to the resultant increase in extracellular $[K^+]$ due to its low apparent K^+ affinity: With saturation of all other isoform combinations at basal $[K^+]_o$, an affinity constant of α2β2 in the 3-4 mM range allows this isoform combination to increase its transport activity when faced with a K^+ load and thus increase the rate of K^+ clearance from the extracellular space and into the nearby astrocytes (Larsen et al., 2014, **Figure 4B**). In addition, the membrane depolarization brought about by the increased extracellular $[K^+]$ promoted enhanced transport activity of all isoform combinations although the voltage-sensitivity was most pronounced for the α2β2 combination (Larsen et al., 2014, **Figure 4A**). The select glial expression of the Na^+/K^+-ATPase α2β2 isoform combination (McGrail et al., 1991; Cameron et al., 1994; Zhang et al., 2014) and localization of α2 to the glial processes surrounding neuronal dendrites (Cholet et al., 2002) taken together with the voltage- and K^+-sensitivity of the α2β2 isoform combination, renders it an efficient responder to stimulus-evoked K^+ transients in the extracellular space and thus an important contributor to management of the extracellular K^+ during neuronal activity.

Given the nature of the Na^+/K^+-ATPase, the transport activity can, in addition to its K^+-dependent activation, also be governed by activity-dependent fluctuations in the intracellular Na^+ concentration. As neurons propagate action potentials with subsequent synaptic activity, neuronal Na^+ accumulation occurs gradually via ligand- and voltage-gated Na^+ channels in parallel to the release of K^+ to the extracellular space (Langer and Rose, 2009). This $[Na^+]_i$ increase prompts enhanced activation of the neuronal Na^+/K^+-ATPase of the isoform combination α3β1, with its characteristically low apparent Na^+ affinity (Lytton, 1985; Zahler et al., 1997; Crambert et al., 2000; Horisberger and Kharoubi-Hess, 2002; Blanco, 2005). The α3β1-mediated extrusion of Na^+ and uptake of K^+ permits the neurons to reestablish the concentration gradients of the two ions following the neuronal activity and the subsequent gradual release of the astrocytic K^+ stores via Kir4.1 (Chever et al., 2010). In this way the neuron is ready to propagate a succeeding volley of action potentials. Since the α3β1-mediated activity is governed primarily by the intracellular Na^+ concentration, its enhanced activity continues until normalization of $[Na^+]_i$ has occurred. As this process occurs toward the termination of the neuronal activity (Grafe and Ballanyi, 1987) and is independent of the $[K^+]_o$, it may lead to an undershoot of $[K^+]_o$ in which the concentration drops below baseline in the later phase of the stimulus-evoked K^+ transient prior to its stabilization (Ransom et al., 2000; D'Ambrosio et al., 2002).

Glutamatergic signaling represents the majority of the excitatory stimulus in the central nervous system (Danbolt, 2001). Upon vesicular release of glutamate into the synaptic cleft, glutamate is swiftly removed from the extracellular space by Na^+-coupled glutamate transporters primarily located in adjacent astrocytes (Bergles and Jahr, 1997; Danbolt, 2001). Of the five cloned isoforms of the glutamate transporter (Excitatory

Amino Acid Transporters, EAAT1-5), astrocytes express EAAT1 and EAAT2, with the latter being the dominantly expressed isoform in hippocampal tissue (Bar-Peled et al., 1997; Furuta et al., 1997; Lehre and Danbolt, 1998) and estimated to account for around 90% of the glutamate uptake in the mammalian brain (Danbolt et al., 1992). During one transport cycle, inward translocation of one glutamate is driven by cotransport of three Na^+ and one H^+ while one K^+ is extruded (Levy et al., 1998). Astrocytic Na^+ accumulation thus occurs upon glutamate transporter activity in cultured astrocytes (Chatton et al., 2000; Illarionava et al., 2014) and acute brain slices (Langer and Rose, 2009; Langer et al., 2012). The astrocytic glutamate transporters display some degree of co-localization with the Na^+/K^+-ATPase α2 in glial leaflets surrounding glutamatergic synapses (Cholet et al., 2002; Rose et al., 2009; Rose and Karus, 2013), suggesting functional interplay between these two transport proteins, as illustrated in **Figure 5**. Indeed, in cultured astrocytes, inhibition of the Na^+/K^+-ATPase instantly compromised glutamate uptake (Rose et al., 2009; Bauer et al., 2012; Illarionava et al., 2014) while Na^+ accumulation via glutamate transporter activity (or mimicking thereof via a Na^+ ionophor) stimulated Na^+/K^+-ATPase-mediated K^+ uptake driven by the α2 isoform (Pellerin and Magistretti, 1997; Bender et al., 1998; Munhoz et al., 2005; Sheean et al., 2013). However, this glutamate transporter-mediated activation of Na^+/K^+-ATPase activity in cultured cells was observed exclusively upon prolonged (up to 20 min) exposure to glutamate (Pellerin and Magistretti, 1997; Bender et al., 1998; Munhoz et al., 2005; Sheean et al., 2013) which could suggest an indirect activation of the Na^+/K^+-ATPase (Munhoz et al., 2005) rather than a direct effect of a Na^+ transient. It therefore remains to be resolved if the glial glutamate transporter-mediated Na^+ transients occurring during brief neuronal activity (Langer and Rose, 2009) directly activates the glial Na^+/K^+-ATPase from the cytosolic face of the membrane. For the Na^+/K^+-ATPase to respond to stimulus-evoked intracellular Na^+ transients, it follows that the intracellular face of the transport protein is not already saturated at the basal glial Na^+ concentration of ∼12 mM (Langer et al., 2012). With the above-mentioned challenges of recording the intracellular Na^+ affinity of various isoform combinations of the Na^+/K^+-ATPase in sided preparations of intact cells, especially in regard to β2, it remains to be revealed which glial isoform combination(s) may display Na^+ affinities in a range which would allow for increased activity when faced with stimulus-induced intracellular Na^+ transients.

It follows that the kinetic behavior and therefore physiological specialization of the different α isoforms may depend on the interacting β subunit and the physiological contribution of the different isoforms hinges on their quantitative expression in the different cellular structures of the CNS. Little is known about the quantitative distribution of the different isoforms at the protein level (due to challenges with comparison between different antibodies with different antigen specificity) as is their favored accessory subunit combinations, although α2 has been shown to favor interaction with the β2 subunit in mouse brain extracts (Tokhtaeva et al., 2012).

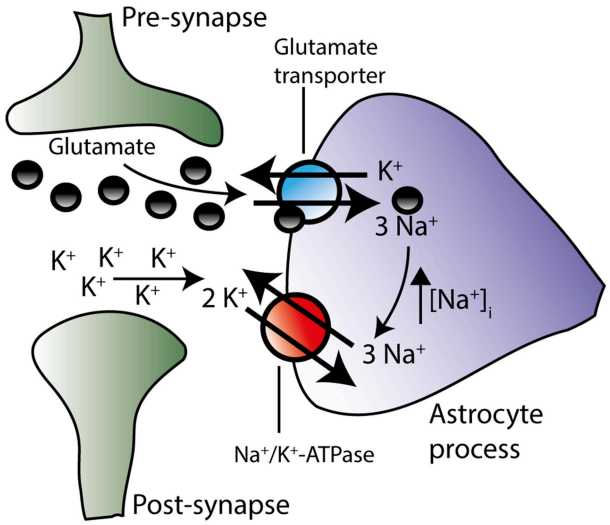

FIGURE 5 | The Na^+/K^+-ATPase is suggested to work in concert with the glutamate transporter. During action potential propagation, glutamate and K^+ are both released from the pre-synaptic neuron. Glutamate-induced depolarization of the post-synaptic neuron instigates further K^+ release. By diffusion glutamate and K^+ reach the peri-synaptic astrocyte processes located in close proximity to the synapse. Glutamate is transported into the astrocyte along with Na^+ and in exchange for K^+ (see text for stoichiometry, H^+ transport has been omitted on figure for clarity). The resultant rise in intracellular Na^+ may trigger the activation of the Na^+/K^+-ATPase isoform combinations, which are not already saturated at their Na^+ binding site. Increased activity of the Na^+/K^+-ATPase would result in enhanced extrusion of the accumulated Na^+ and, concomitantly, in amplified removal of extracellular K^+ in the synaptic area. Although this proposal (based on co-localization patterns and prolonged glutamate exposure of astrocytic cultures) remains to be verified in brain slices during neuronal activity, these two transporters may work in concert in order to efficiently clear both glutamate and K^+ from the extracellular space.

INVOLVEMENT OF Na^+/K^+-ATPase α2 AND α3 IN CENTRAL NERVOUS SYSTEM DISEASE

Alterations in expression of the Na^+/K^+-ATPase isoforms and/or dysfunctional mutant forms of the Na^+/K^+-ATPase may cause disturbances in the K^+ homeostasis and therefore affect a range of cellular parameters, i.e., membrane potential, synaptic signaling, rate of activity-evoked K^+ clearance, glutamate re-absorption etc. Underscoring the importance of these transport proteins for normal physiology, mice with homozygous deletion of the genes *ATP1A2* or *ATP1A3* (encoding α2 and α3) die immediately after birth (Ikeda et al., 2004, 2013). Severe neurological diseases thus arise following various mutations in *ATP1A2* and *ATP1A3* (de Vries et al., 2009; Benarroch, 2011; Bøttger et al., 2012): Familial hemiplegic migraine type 2 (FHM2) arises upon mutations in *ATP1A2* (De Fusco et al., 2003) and is a severe autosomal form of migraine with aura associated with hemiparesis and sometimes accompanied by manifestations such as epilepsy, seizures, ataxia and developmental disabilities (Pietrobon, 2007; Bøttger et al., 2012). A variety of different mutations in α2 has been detected in patients suffering from

FHM2 and knock-in mice expressing FHM2-derived mutant forms of α2 have been established and found to mimic FHM2-relevant disease traits (Leo et al., 2011; Bøttger et al., 2016 and reviewed elsewhere in this special issue by Lykke-Hartmann and colleagues): The heterozygous α2(+/W887R) knock-in mouse model displayed a lower induction threshold for cortical spreading depression (a phenomenon observed in association with migraine with aura) and an increased rate of propagation (Leo et al., 2011) whereas the α2(+/G301R) mouse model displayed mood depression, obsessive compulsive disorder, reduced regeneration after cortical spreading depression in males, as well as reduced glutamate uptake in astrocytic cultures obtained from embryonic homozygotes (Bøttger et al., 2016). A selection of the mutations found in human FHM2 patients have been generated *in vitro* to determine the functional impact on the Na^+/K^+-ATPase activity. Diverse kinetic implications arise as a consequence of the mutations, e.g., loss-of-function, reduced catalytic turnover, shifted E1↔E2 steady-state conformational equilibrium, increased/decreased apparent K^+ affinity and increased/decreased apparent Na^+ affinity (the latter associated with altered basal $[Na^+]_i$) (De Fusco et al., 2003; Segall et al., 2004, 2005; Schack et al., 2012; Toustrup-Jensen et al., 2014). The diverse functional outcome of these mutations, all giving rise to the same disease, underscores the complexity of the role of α2 in Na^+ and K^+ management in the central nervous system. However, it remains puzzling why such diverse alterations, e.g., oppositely directed shifts in K^+ affinity, disrupt neuronal signaling in a manner that gives rise to similar phenotypic outcome. Alterations in Na^+/K^+-ATPase α2 isoform expression have been indicated in several neuropathological situations: (i) migraine (with aura) patients displayed altered Na^+/K^+-ATPase isoform expression toward versions with higher ouabain sensitivity (Scarrone et al., 2007), (ii) reduced K^+ affinity of partially purified Na^+/K^+-ATPase from neocortices of human epileptic patients (Guillaume et al., 1991), (iii) reduced K^+ clearance rates observed in cortex of epileptic monkeys (Lewis et al., 1977), and (iv) glial fractions obtained from cats following freezing lesion-induced epilepsy lost their characteristic K^+-activation, suggesting that α2 and/or β2 are down regulated during the time between the freezing lesion and the occurrence of the epileptogenic states (Grisar et al., 1983). Oppositely, ciliary neurotrophic factor up regulated glial α2 mRNA with subsequent enhanced K^+ uptake and increased threshold for spreading depolarization (Seidel et al., 2015).

Mutations in the gene *ATP1A3* encoding the α3 isoform have been discovered in patients with rapid-onset dystonia parkinsonism (RDP), alternating hemiplegia of childhood (AHC), and early life epilepsy (de Carvalho Aguiar et al., 2004; Bøttger et al., 2012; Heinzen et al., 2014; Sweney et al., 2015). Expressional analysis of α3 demonstrated the presence of this subunit in anatomical regions correlating with RDP symptoms (Bøttger et al., 2011). Mice with heterozygous deletion of *ATP1A3* displayed movement abnormalities partly in line with the human RDP phenotype (Ikeda et al., 2013; Sugimoto et al., 2014) and were more susceptible to stress-induced depression-like phenotypes (Kirshenbaum et al., 2011). Mice expressing mutant forms of α3 exhibit a phenotype comparable to that of patients with AHC and display hyperexcitability, K^+-induced spreading depolarization of longer duration, and enhanced seizure susceptibility (Clapcote et al., 2009; Hunanyan et al., 2015; Kirshenbaum et al., 2015, reviewed in detail elsewhere in this special issue by Lykke-Hartmann and colleagues). Select human mutations in *ATP1A3* have been established *in vitro* and the kinetic alterations of the mutated α3 isoforms ranged from loss of function to reduced activity and modified cation binding (Einholm et al., 2010; Toustrup-Jensen et al., 2014; Weigand et al., 2014).

CONCLUSION

The Na^+/K^+-ATPase exhibits an isoform-specific expression pattern in the mammalian CNS. The distinct kinetic properties of the different catalytic α isoforms hinge, in part, on the accessory β subunit with which they pair and determine their temporal and spatial quantitative contribution to management of the extracellular K^+ transients occurring in the wake of neuronal activity. While the astrocytic α2 isoform appears specifically geared to respond to the activity-evoked K^+ transients upon pairing with β2, the excessively low apparent Na^+ affinity of the α3 ensures neuronal re-uptake of K^+ once the neuronal activity is terminated. Mutations in the genes encoding the neuronal α3 or the astrocytic α2 give rise to a range of severe neuronal pathologies, although the exact mechanism coupling each point mutation to a distinct disease pattern remains unresolved.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Flavonolignans As a Novel Class of Sodium Pump Inhibitors

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We examined the inhibitory effects of three flavonolignans and their dehydro- derivatives, taxifolin and quercetin on the activity of the Na^+/K^+-ATPase (NKA). The flavonolignans silychristin, dehydrosilychristin and dehydrosilydianin inhibited NKA with IC_{50} of 110 ± 40 μM, 38 ± 8 μM, and 36 ± 14 μM, respectively. Using the methods of molecular modeling, we identified several possible binding sites for these species on NKA and proposed the possible mechanisms of inhibition. The binding to the extracellular- or cytoplasmic C-terminal sites can block the transport of cations through the plasma membrane, while the binding on the interface of cytoplasmic domains can inhibit the enzyme allosterically. Fluorescence spectroscopy experiments confirmed the interaction of these three species with the large cytoplasmic segment connecting transmembrane helices 4 and 5 (C45). The flavonolignans are distinct from the cardiac glycosides that are currently used in NKA treatment. Because their binding sites are different, the mechanism of inhibition is different as well as the range of active concentrations, one can expect that these new NKA inhibitors would exhibit also a different biomedical actions than cardiac glycosides.

Keywords: sodium pump, Na^+/K^+-ATPase, flavonolignans, inhibition, binding sites

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INTRODUCTION

Sodium pump (Na^+/K^+-ATPase, E.C. 3.6.3.9, NKA) is an enzyme of crucial importance for all animal cells. It is the major determinant of cytoplasmic Na^+ and K^+ concentrations and the resting plasma membrane potential. The steep Na^+ gradient on plasma membrane is essential for variety of secondary active transporters, e.g., Na^+/Ca^{2+}- and Na^+/H^+- exchanger or Na^+-dependent glucose transporter, and hence, NKA indirectly regulates also concentrations of other physiologically important solutes.

It is not surprising that an uncontrolled inhibition of NKA can result in severe diseases, e.g., renal failure, hypertension or diabetic neuropathies (Kaplan, 2002) or even death, and that the most specific NKA inhibitor cardiac glycoside ouabain was originally used as an arrow poison (Newman et al., 2008). Despite these risks, extracts containing cardiac glycosides were used to control heart tonics already in ancient medicine, and the extracts were prepared either from plants in Arabic medicine (Brewer, 2004) or secretions of frog *Bufo bufo* in Chinese medicine (Watabe et al., 1996). Compounds like digitalis or digoxin are still prescribed for control of congestive heart failure (Gheorghiade et al., 2004). However, the use of cardiac glycosides is limited by their very

narrow useful concentration range (Newman et al., 2008), which stimulates further search for other NKA inhibiting compounds.

Silymarin is an extract from the seeds of milk thistle (*Silybum marianum)*. It contains numerous polyphenolic compounds and it was shown to possess antioxidant (Vacek et al., 2013; Pyszková et al., 2015), hepatoprotective (Loguercio and Festi, 2011) or anticancer effects (Agarwal et al., 2006). In this study, we have tested effects on NKA activity for a flavonoid taxifolin (TAX) and three flavonolignans, namely silybin (SB), silychristin (SCH), and silydianin (SD), which are major silymarin compounds (Biedermann et al., 2014); their structures are shown in **Figure 1**. The corresponding 2,3-dehydro derivatives (DHSB, DHSCH, and DHSD, the 2,3-dehydrotaxifolin is termed quercetin, QUE) were also tested.

The NKA catalytic cycle is usually described by the Albers-Post cycle (Jorgensen et al., 2003). It postulates that during the catalytic cycle, the enzyme adopts two major conformations, denoted as E1 and E2. In E1, the enzyme has high affinity to sodium and ATP and the binding sites are open to the cytoplasm, while in E2, the enzyme has high affinity to potassium, low affinity to ATP and the cation-binding sites are open to the extracellular space. High-resolution structures of NKA were obtained in several conformational states thanks to recent progresses in X-ray crystallography of membrane proteins (Morth et al., 2007; Ogawa et al., 2009; Nyblom et al., 2013). They revealed the binding sites for transported cations within the transmembrane domain, or binding site for some ligands, including ouabain. Notably, in the crystals assigned to the enzyme in E1 conformation, the cytoplasmic domains are assembled together (further referred to as a closed conformation), while in the E2 conformation, the cytoplasmic headpiece is widely opened (opened conformation).

These different structures provided a solid basis to figure out molecular processes responsible for the mechanisms of enzyme inhibition. Moreover, based on these high-resolution structures, molecular modeling allows further identification of the binding sites of flavonolignans that inhibit NKA, and strongly support rationalization of mechanisms of inhibition.

MATERIALS AND METHODS

Chemicals

Unless stated otherwise, all used chemicals were from Sigma-Aldrich Chemie (Steinheim, Germany).

Species Tested for the Effect on NKA Activity

Silymarin was purchased from Liaoning Senrong Pharmaceutical Co., Ltd. (China), SB, SCH, and SD were isolated from the

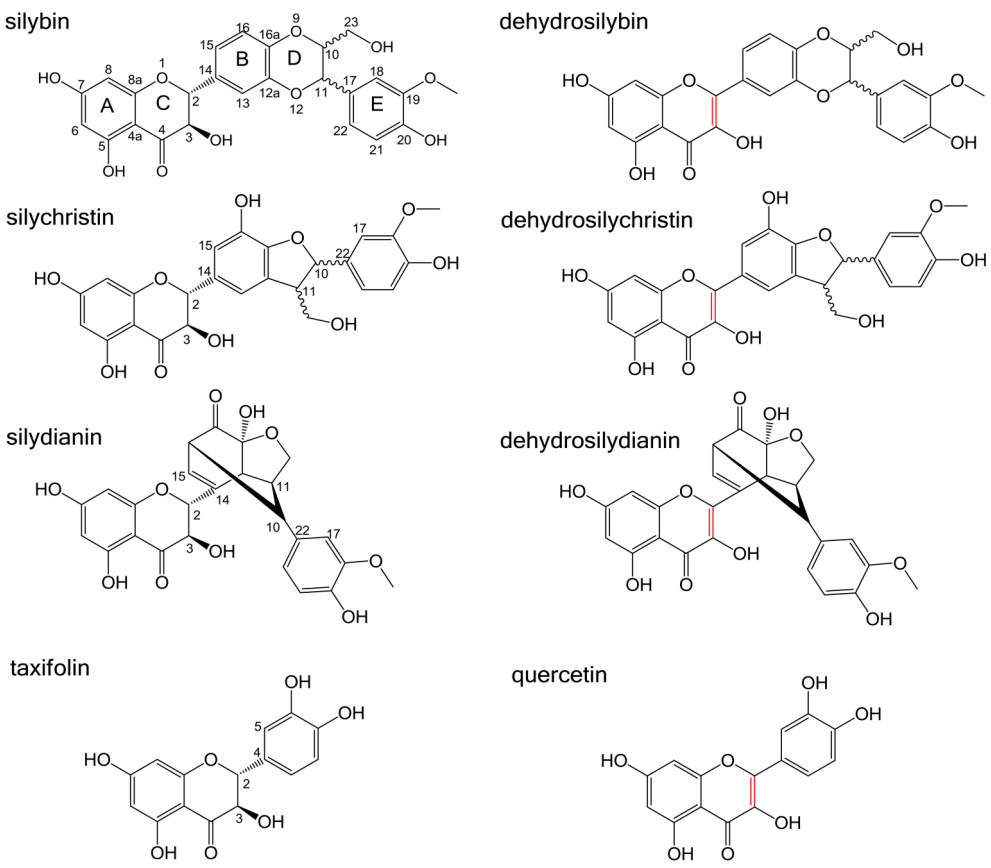

FIGURE 1 | Structures of silybin (SB), silychristin (SCH), silydianin (SD) and their dehydro-derivatives (DHSB, DHSCH, DHSD), taxifolin (TAX), and quercetin (QUE). The 2,3-double bond in dehydroderivatives is highlighted in red.

silymarin, and the dehydro- derivatives DHSB, DHSCH and DHSD were prepared as described previously (Džubák et al., 2006; Křenek et al., 2014; Pyszková et al., 2015). Silybin and silychristin were used as the natural diastereomeric mixture (ca. 1/1 and 95/5 respectively), silydianin is a single isomer. Dehydroderivatives were prepared from parent mixtures and are therefore enantiomeric mixtures (besides DHSD) (Pyszková et al., 2015). Taxifolin was purchased from Amagro (CZ). Quercetin was prepared by acidic hydrolysis of rutin with HCl/EtOH as described previously (Wang et al., 2011). Purity of all used compounds was over 95% (HPLC, PDA).

Isolation of Na^+/K^+-ATPase

The NKA was prepared from porcine kidney outer medulla using the method of Jorgensen and Klodos with some modifications (Jørgensen, 1988; Klodos et al., 2002; Kubala et al., 2014). An isolated enzyme was pipetted into small aliquots and stored at −20°C in ISE buffer (25 mM imidazole, 250 mM sucrose, 1 mM EDTA, pH 7.4) containing SDS detergent. The molar concentration of isolated NKA was estimated using the Bradford method with consideration of MW($\alpha + \beta$) = 165,000 Da. The protein purity >90% was estimated from SDS-PAGE (**Figure 2**).

Measurement of ATPase Activity

The measurements of NKA activity were performed using the Baginsky assay (Kubala et al., 2014). The assay was performed in microwell plates in 4 replicates for each point and automated in the pipetting station Freedom EVO (Tecan, Switzerland).

The reaction buffer was composed of 325 mM NaCl, 50 mM KCl, 7.5 mM $MgCl_2$, and 75 mM imidazole, pH 7.2. In the experiments where the K^+-dependence was estimated, the KCl concentration is ranging from 0 to 100 mM. The NKA (0.1 mg/mL) isolated from porcine kidney was mixed with reaction buffer without ATP. All inhibitors were solubilized in methanol immediately before the measurement and then premixed with the reaction buffer to the required concentration. Subsequently, 10 μL of inhibitor solution was added into 20 μL of enzyme solution and incubated for 2 min. In the control sample, only 10 μL of reaction buffer without inhibitor was added including corresponding amount of methanol. The reaction was started by the addition of ATP solution (20 μL, 7.5 mM in the stock, the final concentration in the reaction was 3 mM). The reaction proceeded for 6 min at room temperature and then was stopped by addition of 75 μL of staining solution, which was composed of 160 mM ascorbic acid, 3.7% (v/v) acetic acid, 3% (w/v) SDS, and 0.5% ammonium molybdate. The staining reaction was stopped after another 8 min by adding 125 μL of solution composed of 0.9% (w/v) bismuth citrate, 0.9% (w/v) sodium citrate and 3.7% HCl.

The Baginsky method detects a product of ATP hydrolysis, inorganic phosphate, which interacts with ammonium molybdate. The reaction results in a color change, which can be monitored as a change of absorbance at 710 nm, and was measured using microplate reader Synergy Mx (BioTek, USA). The calibration line was determined using KH_2PO_4 solutions, in 0–37.5 nM concentration range.

The specific NKA activity is standardly estimated using the treatment by ouabain, which serves as a specific inhibitor of NKA. The ATPase activity decreases to less than 10% in the presence of 10 mM ouabain. This residual activity in the presence of ouabain was subtracted from the total estimated ATPase activity in ouabain-untreated samples, and all data are presented as the ouabain-sensitive ATPase activity. The IC_{50} values were obtained from fitting the data to the logistic function.

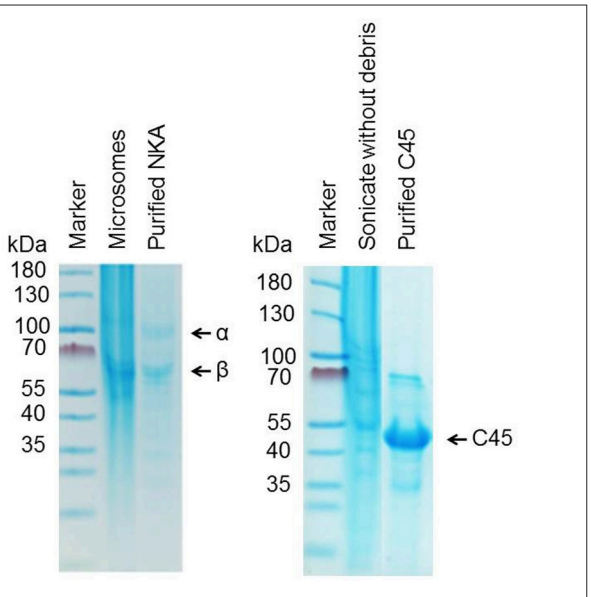

FIGURE 2 | Purity of isolated NKA (left) and C45 (right) as revealed by SDS-PAGE.

Expression and Purification of the Isolated Large Cytoplasmic Segment C45

The large cytoplasmic segment connecting the transmembrane helices 4 and 5 (C45 loop, residues L354-I777 of the mouse brain sequence) with a $(His)_6$-tag at the N-terminus was expressed in *E. coli* BL21 (Promega, USA) and purified using a Co^{2+}-based affinity resin (Clontech, USA) as described previously (Grycova et al., 2009). Immediately after elution, the protein was dialyzed into 20 mM Tris, 140 mM NaCl, pH 7.4 buffer and stored at −20°C.

Protein were analyzed by Coomassie blue stained SDS PAGE and concentration was determined using the Bradford assay (Bradford, 1976) using BSA as a standard.

Absorption and Fluorescence Spectroscopy

Spectroscopic experiments were performed in 20 mM Tris, 140 mM NaCl, pH 7.4 buffer. SCH, DHSCH and DHSD as well as protein C45 were diluted to 5 μM concentration.

Absorption spectra were measured on spectrometer Specord 250 Plus (Analytic Jena, Germany) in the range 300–600 nm, with the bandpass 2 nm, a step 1 nm and a scan-speed 2 nm/s. The reference spectrum was acquired using the cuvette with a pure buffer.

Fluorescence emission spectra were measured using Fluorolog-3 (Horiba Scientific, USA). The spectra were scanned with a step of 1 nm, both excitation- and emission bandpass 5 nm and integration time 1 s. For SCH, the excitation was 325 nm and emission was recorded in the 340–600 nm interval, for DHSCH and DHSD, the excitation wavelength was 380 nm and spectra were scanned in the 400–600 nm interval. Signal from pure buffer was subtracted as a background.

Computation of Structures for Molecular Docking

Geometry optimizations of all structures (**Figure 1**) was performed using the density functional theory (DFT) formalism with the software package Gaussian09 (Frisch et al., 2009). The hybrid functional B3P86 has been used because it has repeatedly succeeded in describing most of polyphenol properties (Trouillas et al., 2006, 2008). Gibbs energies (G) were computed at B3P86/6-31+G(d,p) level at 298 K, 1 atm. After a vibrational frequency analysis, ground-state geometries were confirmed by the absence of any imaginary frequency. Quantum calculations were performed in the gas phase.

The SB (stereoisomer A) and DHSB (stereoisomer A) initial structures were taken from Trouillas et al. (2008) and further re-optimized. Structure of the most stable isomers of SCH (stereoisomer A), DHSCH (stereoisomer A), SD and DHSD were already presented in Pyszková et al. (2015).

Molecular Docking

The compounds SCH, DHSCH, and DHSD were docked to the opened and closed structures of NKA (PDB, ID, 3KDP, and 4HQJ) using Autodock Tools (Morris et al., 2009) and Autodock Vina (Trott and Olson, 2010) with the grid covering the whole protein. The values of parameters exhaustiveness was set to 100 and num_modes to 9999 in order to reveal all possible docking modes. In the default setting, the bonds creating different conformers were freely rotable, to find optimal geometry of molecules interacting with the pump.

RESULTS

Conformational Analysis

Conformational analysis of both flavonoids TAX and its dehydro-analog QUE revealed two stable rotamers. In case of QUE, as reported previously (Trouillas et al., 2006), the planarity is observed, allowing π electron delocalization along the A, C and B rings, while for TAX, the planarity is lost due to the loss of the 2,3-double bond.

A conformation analysis of SB, DHSB, SCH, DHSCH, SD, and DHSD revealed 4 most stable representative conformers for all molecules within less than 1.6 kcal/mol difference in Gibbs energy (**Figure 3**). These conformers can be obtained by modification of the torsion angles Phi = C3–C2–C14–C15 and Psi = C11–C10–C22–C17 (Psi = C10–C11–C17–C22 for SB and DHSB) (**Table 1**), and the loss of the flavonoid moiety planarity is observed also for these molecules, when the 2,3-bond is hydrogenated (DHSB, DHSCH, and DHSD).

Effects on NKA Activity

Ouabain-sensitive ATPase activity was measured for increasing concentrations of all species (**Figure 4**). In the case of SB, DHSB, SD, and DHSD, the examined concentration range was

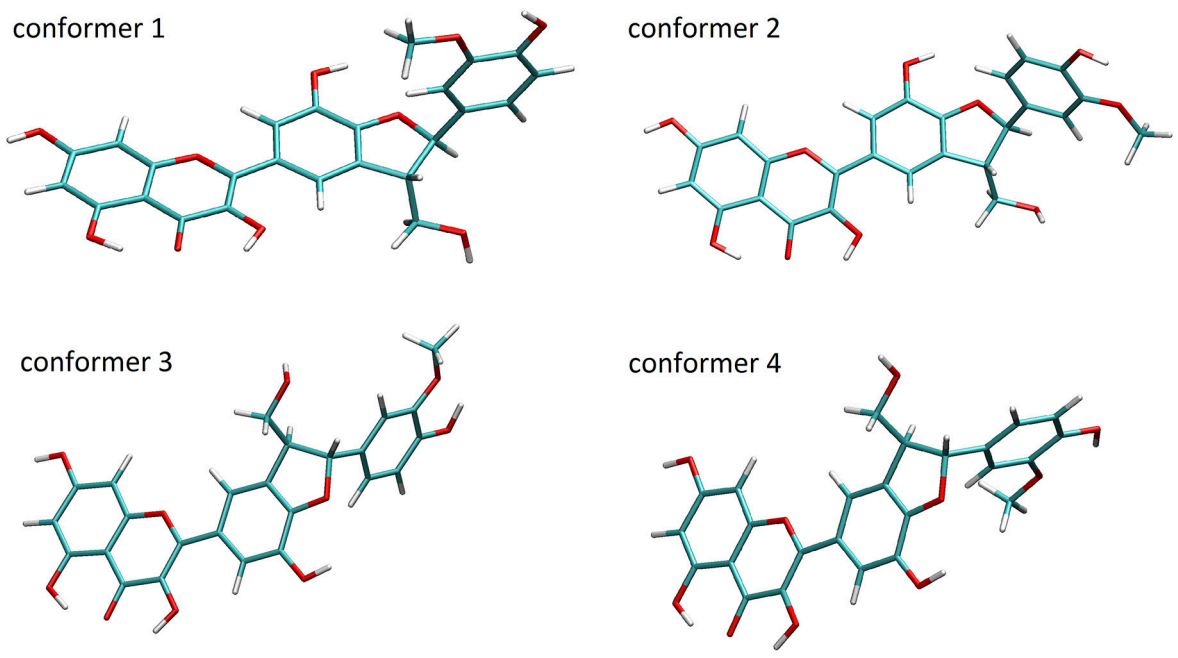

FIGURE 3 | Structures of stable conformers of DHSCH.

TABLE 1 | Structural parameters of the stable conformers.

Species	Conformer	Phi (°)	Psi (°)	Relative Gibbs energy (kcal/mol)
SB	1	77	−98	0
	2	83	81	0.4
	3	−96	−97	0.2
	4	−98	81	0.2
DHSB	1	3	−98	0
	2	−1	80	0.3
	3	−178	−98	0.1
	4	−176	83	0.5
SCH	1	81	−71	0
	2	82	98	0.9
	3	−105	52	0.1
	4	−97	−141	0.7
DHSCH	1	174	−52	0
	2	175	87	0.2
	3	9	97	0.1
	4	7	−55	1.6
SD	1	−3	−37	0
	2	−5	147	0.2
	3	−125	−42	0.5
	4	−123	142	0.4
DHSD	1	1	−45	0
	2	1	142	0.3
	3	166	−40	0.2
	4	166	145	0.8
TAX	1	−83		0
	2	80		0.1
QUE	1	180		0
	2	0		0.4

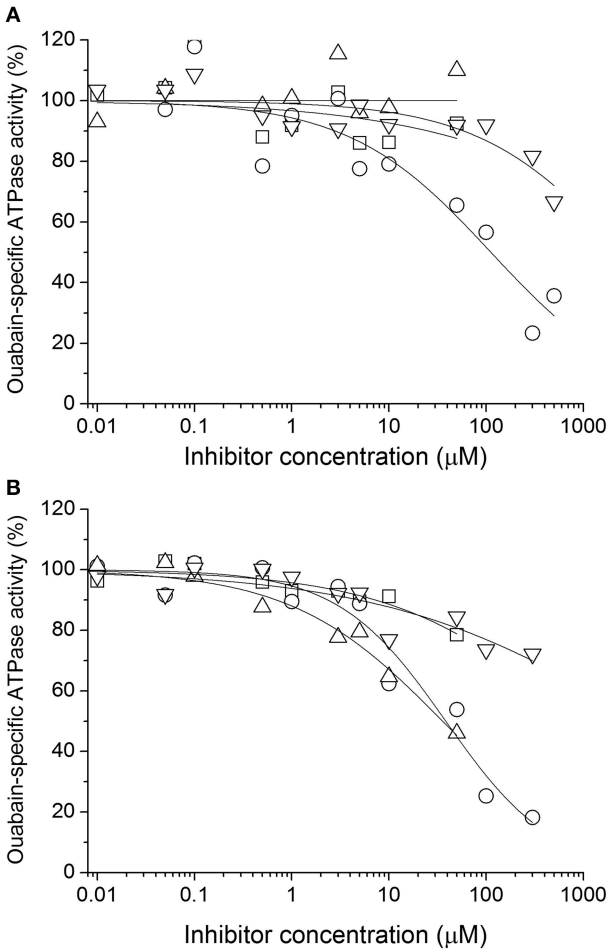

FIGURE 4 | Inhibition of NKA by (A) SB (squares), SCH (circles), SD (up triangles), TAX (down triangles) and (B) by the corresponding dehydro-derivatives DHSB, DHSCH, DHSD and QUE. The reference value (100%) represents ouabain-specific activity of untreated NKA.

limited due to lower solubility of these species. Substantial inhibition was observed only for SCH, DHSCH and DHSD with IC_{50} 110 ± 40 μM, 38 ± 8 μM, and 36 ± 14 μM, respectively. In all these cases, we observed a Hill coefficient < 1, indicating the presence of multiple binding sites and negative cooperativity. The data are summarized in **Table 2**, and SCH, DHSCH, and DHSD were subject to further analyses.

We have tested influence of these three species on the ouabain inhibition and K^+-dependence of NKA activation. None of the species substantially influenced the IC_{50} for ouabain or the K^+/ouabain antagonism (**Figure 5**). The NKA activity is K^+-dependent and it increased with increasing concentration of potassium with $K_{0.5}(K^+) = 4.2 \pm 0.6$ mM. In contrast to ouabain, which raised the $K_{0.5}(K^+)$ to 14.8 ± 0.1 mM, none of the flavonolignans significantly altered the K^+-dependence of NKA activity (**Figure 6**).

Molecular Docking

Molecular docking enables prediction of the binding sites for small ligands on large biomolecules. Binding to NKA was examined for both major conformations of the enzyme. All SCH, DHSCH, and DHSD bound with a similar affinities (−11 to −9 kcal/mol) to all sites in both opened and closed conformations. We identified five major binding sites, three of them were common to both the opened and closed conformations (**Figure 7**), one binding pose was exclusively observed only for the opened conformation, and another one in turn only for closed conformation. All conformers bound to at least one binding site, nevertheless, their relative preferences for individual binding sites differed (**Table 3**).

In the closed conformation, by far the most favored site was near the phosphorylation site. All the conformers can occupy this site, except for conformer 4 of DHSCH. All compounds bound also to another pose between the A and N domains on the other side near R248 (**Figure 8**). However, this site only exists in the closed conformation. It is much more selective and

TABLE 2 | Values of IC_{50} for inhibition of the NKA activity, n, Hill coefficient, $K_{0.5}$(ouabain) indicates the value obtained in the presence of 5 mM KCl (in parentheses in 40 mM KCl) and 40 μM concentration of flavonolignan, $K_{0.5}(K^+)$ denotes the values for K^+-dependent activation of NKA obtained in the presence of 40 μM flavonolignan, n.d., not determined.

Species	IC_{50} (μM)	n	$K_{0.5}$(ouabain) (μM)	$K_{0.5}(K^+)$ (mM)
None			2.6 ± 1.3 (23 ± 3)	4.2 ± 0.6
SB	>1000	n.d.	n.d.	n.d.
DHSB	900 ± 800	0.5 ± 0.1	n.d.	n.d.
SCH	110 ± 40	0.6 ± 0.1	4.0 ± 1.7 (18 ± 2)	8 ± 5
DHSCH	38 ± 8	0.8 ± 0.1	1.5 ± 0.5 (24 ± 6)	4 ± 1
SD	>1000	n.d.	n.d.	n.d.
DHSD	36 ± 14	0.6 ± 0.1	3.3 ± 1.4 (19 ± 7)	3 ± 2
TAX	>1000	n.d.	n.d.	n.d.
QUE	>1000	n.d.	n.d.	n.d.

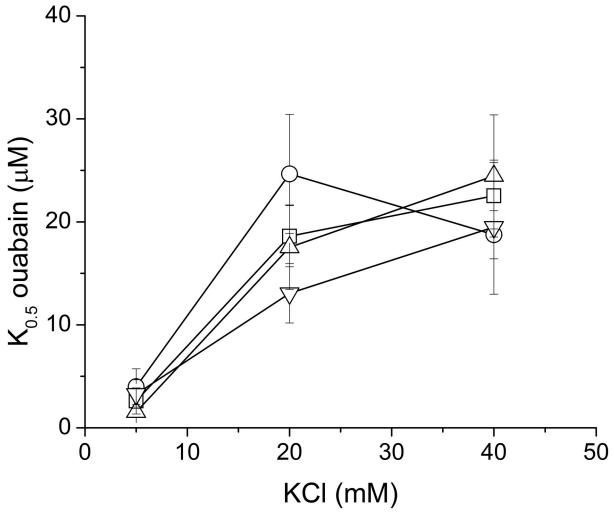

FIGURE 5 | The K^+/ouabain antagonism in the absence (squares) or in the presence of 40 μM SCH (circles), DHSCH (up triangles) or DHSD (down triangles).

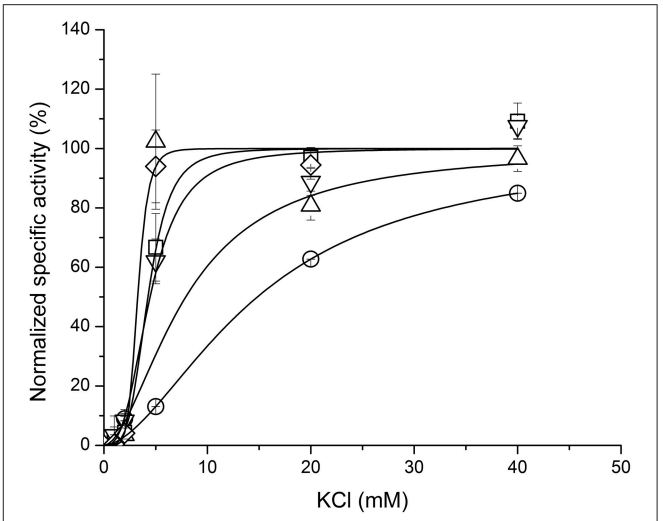

FIGURE 6 | The K^+-dependence of NKA activity in the absence of any ligand (squares) or in the presence of ouabain (circles), SCH (up triangles), DHSCH (down triangles) or DHSD (diamonds).

can be occupied only by conformer 3 of SCH, conformers 2 and 4 of DHSCH and conformer 1 of DHSD. Another possible binding location in the closed structure is on the extracellular side between E312 on TM4 and R886 (extracellular loop connecting TM7 and TM8), and it is the most favored site for conformer 4 of DHSCH. This site can serve as the entry for potassium ions during transport. Conformer 1 of SCH and conformers 1 and 4 of DHSCH are the only molecules that bind at the intracellular C-terminal site. Also this site was proposed to play a role in transport of cations through the plasma membrane (Toustrup-Jensen et al., 2009).

Also in the opened conformation, the binding to the groove between P and N cytoplasmic domains near the phosphorylation site was observed for all conformers, except for conformer 3 of DHSCH. It was the most preferred site for all SCH conformers and for conformers 1 of DHSCH and DHSD. The binding site specific only for the opened conformation is located under A domain, near the residues N353 and D740, where a regulatory potassium ion is bound (Schack et al., 2008). It is the most preferred site for conformers 2 and 4 of DHSCH and for conformer 2 of DHSD, but numerous other conformers can bind in this site as well. The site at the extracellular potassium entrance is the most preferred for the conformer 4 of DHSD. There is also a binding pose at the C-terminal of the protein, but it can be occupied only by DHSCH or DHSD, and for conformer 3 of both species it is the most preferred site.

Interaction with the Isolated Large Cytoplasmic Segment C45

Based on the prediction from molecular docking, we examined whether SCH, DHSCH, and DHSD could interact with the isolated large cytoplasmic segment C45 using absorption and fluorescence spectroscopy. Absorption spectra of SCH, DHSCH, and DHSD exhibited maxima at ca. 325, 385, and 375 nm, respectively, and the presence of C45 did not substantially alter the spectra (not shown), thus, providing no further clue about the interaction.

On the other hand, the interaction with C45 was clearly manifested in the fluorescence spectra for all three species. We observed no detectable fluorescence above background for the free SCH. The interaction with C45 protein turned out to be fluorogenic, and in the presence of C45, a bright SCH fluorescence appeared (quantum yield increased more than 1000 times compared to the free SCH) with a maximum at 394 nm (**Figure 9**). For the free DHSCH, we observed only very weak fluorescence with a maximum at 537 nm and an apparent shoulder at ca. 475 nm. In the presence of C45, the fluorescence intensity increased ca. 3-times, and two peaks at 466 and 527 nm could be resolved. Similarly for the free DHSD, there is only a weak fluorescence with a maximum at 472 nm. In the presence of C45, the three-fold intensity increase is accompanied by a shift of

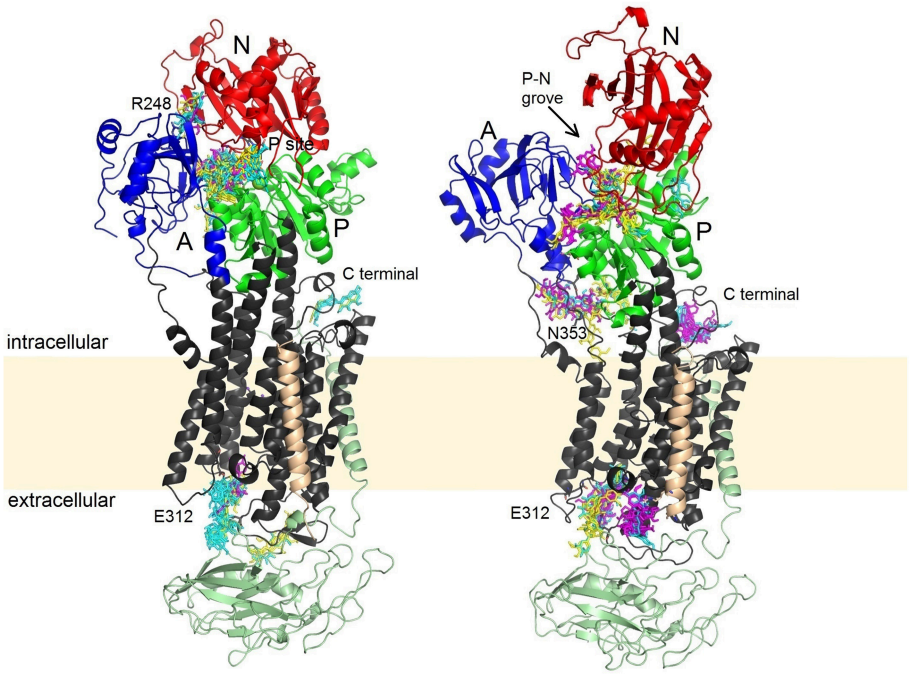

FIGURE 7 | Binding sites of SCH (yellow), DHSCH (cyan) and DHSD (magenta) on the opened and closed structure. Beta subunit is in light green, the FXYD protein light orange, A, domain blue; N, domain red; P, domain green.

the maximum to 459 nm, and there is also an apparent shoulder at 510 nm.

DISCUSSION

Recent pharmacology greatly benefits from the species isolated from the plants used in traditional medicine. In addition, these species also serve as precursors or inspiration for synthesis of new active derivatives, which can turn out to be even more effective in biochemical interactions. This study was focused on interactions of a series of phenolic compounds from silymarin (being or not hydrogenated on 2,3-bond of the flavonoid moiety) with one of the most important enzymes in the animal metabolism, the NKA.

Bioavailability of most polyphenolic compounds naturally found in silymarin is limited by their lower solubility in water, only SCH and SD exhibit substantially higher solubility in aqueous environment. In our experiments, we observed that only SCH inhibited the NKA with IC_{50} of 110 ± 40 μM, and similar concentrations can hardly be reached within the living organism. On the other hand, two of the 2,3-dehydroderivatives, DHSCH and DHSD, inhibited NKA substantially more efficiently with IC_{50} of 38 ± 8 μM and 36 ± 14 μM, respectively. Interestingly, none of the flavonolignans exhibited influence on the known K^+/ouabain antagonism (Müller-Ehmsen et al., 2001). Moreover, in contrast to ouabain, they did not alter the K^+-dependence of NKA activity, suggesting that the flavonolignans have different mode of inhibition than ouabain.

Interaction of small molecules with large biomolecules is one of the key issues in structural biology, and recently, it has gained benefits from the development in computational techniques. The classical key-and-lock concept assumes that small molecules can specifically interact with enzymes when their geometries fit each other. The induced-fit concept that was proposed later, has not that strict requirements on the geometries of the interaction partners, and it assumes that after the first contact, the enzyme can rearrange its conformation to fit the shape of the small ligand (Koshland, 1995).

Our calculations revealed that all flavonolignans (including the dehydro- derivatives) can adopt several stable conformations. Their stabilizing energies calculated in the gas phase appeared very similar, suggesting that they are all present in solution in roughly equimolar mixtures. Although differences between flavonolignans conformers may play no important role in, e.g., their redox properties, the precise geometry is crucial in the interaction with enzymes, and some conformers can be tightly bound to the enzyme, while the others can be inactive.

However, the situation is probably more complex in our case. The fitting of the inhibition curves revealed the Hill coefficient different from 1 for all SCH, DHSCH, and DHSD, indicating the presence of multiple binding sites. Indeed, molecular docking revealed one extracellular binding site and four other sites in the cytoplasmic segment of the protein, indicating multiple possibilities of how the flavonolignans could inhibit the enzyme. The main enzyme function is translocation of cations through the plasma membrane. The cations are transiently bound to their binding sites formed by residues TM4, TM5, TM6, and TM8 in the transmembrane domain (Morth et al., 2007), while the extracellular- and cytoplasmic pathways to these binding sites alternatively open and close. Binding of flavonolignans to the extracellular binding site or to the C-terminal binding site can

efficiently block these pathways, and thus, can stop the cation transport. The opening and closing of the extracellular- and cytoplasmic gates is accompanied by the change in the position of cytoplasmic domains, as a consequence of ATP-binding and hydrolysis (Kubala, 2006). Localization of the flavonolignan on the interfaces between N- and P- or N- and A-domains can hinder the interactions between cytoplasmic domains, and thus, inhibit the enzyme allosterically. All conformers of all SCH, DHSCH, and DHSD were able to bind to at least to one binding pose, but by far the most occupied site was the one at the interface of N- and P-domains near the phosphorylation site. It can be occupied by almost all conformers, it is present in both opened and closed enzyme conformations, and we experimentally verified binding of all SCH, DHSCH, and DHSD to the large cytoplasmic segment connecting TM4 and TM5 (C45) using fluorometry.

The C45 constitutes approx. 40% of the enzyme mass and forms the cytoplasmic domains N and P. It can be overexpressed without the rest of the enzyme in high quantities in *E. coli* (Grycova et al., 2009). So far, all the experiments indicate that it retains its structure, ability to bind ATP and TNP-ATP (Kubala et al., 2003a,b, 2004), and dynamic properties (Grycova et al., 2009; Kubala et al., 2009) as when it is a part of the entire enzyme. Its solubility greatly facilitates all subsequent

TABLE 3 | The fractions of conformers bound to the selected binding sites.

CLOSED	P site	R248	Extracellular	C terminal	Other
DHSD1	**0.23**	0.05	0.03	ND	ND
DHSD2	**0.15**	ND	ND	ND	ND
DHSD3	**0.25**	ND	0.03	ND	ND
DHSD4	**0.28**	ND	ND	ND	ND
DHSCH1	**0.28**	ND	ND	0.03	0.03
DHSCH2	**0.23**	0.03	0.08	ND	0.05
DHSCH3	**0.13**	ND	0.03	ND	ND
DHSCH4	ND	0.03	**0.05**	0.03	0.05
SCH1	**0.15**	ND	0.03	0.03	0.05
SCH2	**0.33**	ND	ND	ND	ND
SCH3	**0.18**	0.03	0.03	ND	0.03
SCH4	**0.15**	ND	0.03	ND	ND
OPENED	**P site**	**N353**	**Extracellular**	**C terminal**	**Other**
DHSD1	**0.05**	0.03	0.03	0.03	0.05
DHSD2	0.03	**0.05**	ND	0.03	ND
DHSD3	0.05	ND	0.03	**0.10**	0.05
DHSD4	0.08	0.08	**0.15**	ND	0.20
DHSCH1	**0.10**	0.03	0.05	0.03	0.10
DHSCH2	0.05	**0.13**	ND	0.03	0.20
DHSCH3	ND	ND	ND	**0.03**	0.05
DHSCH4	0.08	**0.08**	0.03	ND	0.05
SCH1	**0.10**	0.05	0.05	ND	0.15
SCH2	**0.10**	ND	ND	ND	0.10
SCH3	**0.08**	0.05	0.05	ND	0.03
SCH4	**0.13**	0.05	ND	ND	0.08

The most preferred binding site of each conformer in bold, "P site" stands for phosphorylation site. ND, binding to this site was not detected.

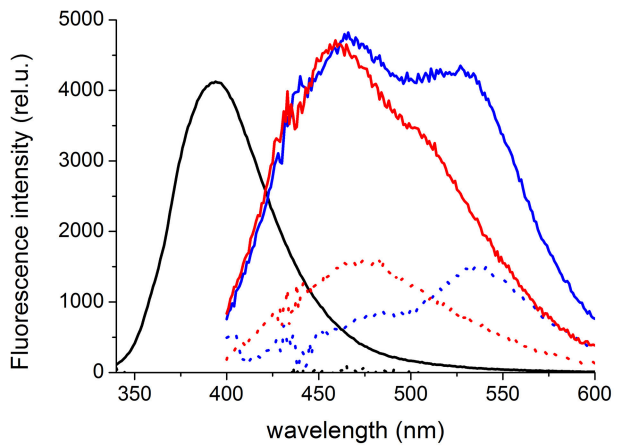

FIGURE 9 | Fluorescence emission spectra of 5 μM SCH (black), DHSCH (blue) and DHSD (red) in the presence (solid line) or absence (dotted line) of 5 μM C45. The fluorescence intensity in the spectrum of SCH in the C45 presence was divided by a factor of 20 to fit the graph.

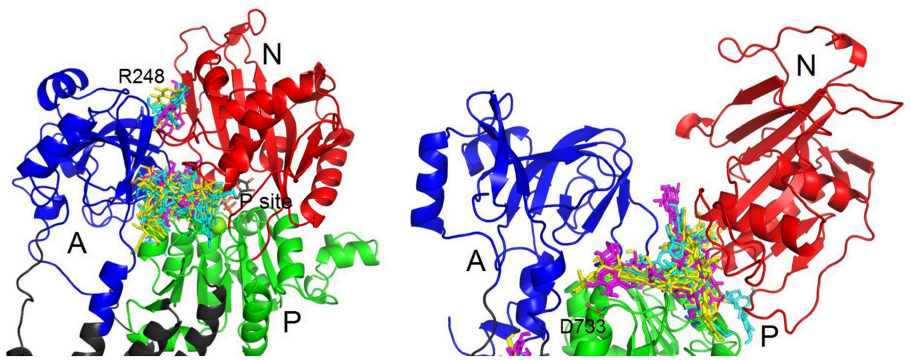

FIGURE 8 | Zoom on the binding sites of SCH (yellow), DHSCH (cyan) and DHSD (magenta) on cytoplasmic loops in the opened and closed structure. A, domain is blue; N, domain red; P, domain green.

experiments and this model system was successfully used for closer localization of binding sites of numerous other small molecules on the cytoplasmic part of NKA (Huličiak et al., 2012; Havlíková et al., 2013). Also in the case of SCH, DHSCH and DHSD, the spectroscopic experiments unambiguously confirmed that these molecules interact with C45. Moreover, in the case of DHSCH and DHSD, we could observe two peaks in the emission spectra of the protein-bound forms. It reveals that there are two binding modes of these species to the C45 and it is in line with predictions from molecular docking.

In conclusion, flavonolignans are proposed to be a novel class of NKA inhibitors, and particularly the 2,3-dehydroderivatives DHSCH and DHSD seem to be very promising agents. The flavonolignans are distinct from the cardiac glycosides that are currently used in NKA treatment. Because their binding sites are different, the mechanism of inhibition is different as well as the range of active concentrations, one can expect that these new NKA inhibitors would exhibit also a different biomedical actions than cardiac glycosides. Currently, the major problem seems to be the low specificity in interaction with biomolecules, which is probably partially related to significant number of stable flavonolignan conformers. Syntheses of more than 100 flavonolignan derivatives have been described (Biedermann et al., 2014), and further screening could identify a derivative that would be useful at lower concentrations and with higher specificity.

AUTHOR CONTRIBUTIONS

MK designed the study, prepared the manuscript, performed the absorption- and fluorescence spectroscopy experiments. PČ performed the molecular docking computations, contributed to the interpretation of data. JG isolated NKA and performed the ATPase activity measurements. MB performed the conformational analysis and prepared the flavonolignan molecules for docking. TŠ expressed and purified the C45 protein for spectroscopic analyses. PT participated to the conformational analysis and manuscript preparation. DB isolated, prepared, purified and supplied the compounds studied.

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frontiers in Cell and Developmental Biology

REVIEW
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FXYD5: Na^+/K^+-ATPase Regulator in Health and Disease

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FXYD5 (Dysadherin, RIC) is a single span type I membrane protein that plays multiple roles in regulation of cellular functions. It is expressed in a variety of epithelial tissues and acts as an auxiliary subunit of the Na^+/K^+-ATPase. During the past decade, a correlation between enhanced expression of FXYD5 and tumor progression has been established for various tumor types. In this review, current knowledge on FXYD5 is discussed, including experimental data on the functional effects of FXYD5 on the Na^+/K^+-ATPase. FXYD5 modulates cellular junctions, influences chemokine production, and affects cell adhesion. The accumulated data may provide a basis for understanding the molecular mechanisms underlying FXYD5 mediated phenotypes.

Keywords: FXYD5, dysadherin, Na^+/K^+-ATPase, polarity, cell adhesion, cell junctions, glycosylation

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FXYD5 (Dysadherin, RIC) is a type I membrane protein, that belongs to FXYD family. In mammalian cells this family of proteins consists of seven members (FXYD1-7) that share the conserved F-X-Y-D motif in the trans-membrane domain. All family members are known to interact with Na^+/K^+-ATPase and affect its kinetic properties in a tissue-specific manner (for a review see Garty and Karlish, 2006). FXYD5 was identified as a cell surface molecule by a monoclonal antibody that was developed to selectively recognize cancerous but not normal cells. Due to its effect of reducing cell–cell adhesion in transfected PLC/PRF/5 liver cancer cells the protein was termed dysadherin (Ino et al., 2002). Dysadherin and FXYD5 are synonyms for the same protein, consisting of 178 amino acid residues.

FXYD5 is expressed in a variety of cell types. Its role has been well characterized in epithelial tissues, probably due to the high abundance of FXYD5 in these cells and its up-regulation in several carcinomas, which originated from epithelial cells. FXYD5 was particularly abundant in intestine, spleen, lung, and kidney, and to much less extent in muscle tissues (Lubarski et al., 2005, 2007). Additional studies also recognized FXYD5 in endothelial cells and lymphocytes (Ino et al., 2002). The physiological significance of FXYD5 expression in the lymphatic system remains to be determined. The muscle tissues are a composite of a variety of cell types, and there is no indication yet, if FXYD5 expression in this tissue is myocyte specific. For instance, FXYD5 expression could be compartmentalized to endothelial cells, which are a part of the vascular supply to muscle bundle.

FXYD5: STRUCTURE, MOLECULAR WEIGHT VARIABILITY, AND POST TRANSLATIONAL MODIFICATIONS

Unlike other family members, FXYD5 has an atypically long extracellular domain >140 amino acids, including a cleavable signal peptide. FXYD5 has a short intracellular C-terminal segment of only 15 amino acids (Lubarski et al., 2005). The calculated molecular mass of FXYD5 is ~20 kDa, and without signal peptide the predicted molecular mass is ~17 kDa. In a variety of normal mouse epithelial tissue, a ~24 kDa polypeptide was reported to bind to two different antibodies raised

independently against the synthetic C-terminal peptide and the truncated N-terminal domain (Lubarski et al., 2005, 2007). A similar molecular mass was also found for HA-tagged recombinant protein translated from the mouse FXYD5 cDNA in several cell lines (Lubarski et al., 2011, 2014). In agreement, Miller and Davis demonstrated that mature human Flag-tagged FXYD5 cDNA transfected into HEK 293 or LA4 cells produced protein that migrated as an indistinct band of ∼35 kDa, and in mouse as a 25 kDa band (Miller and Davis, 2008a). However, in several cancerous cell lines antibodies recognized natively expressed FXYD5 of different molecular sizes, ranging between 50 and 55 kDa (Ino et al., 2002; Shimamura et al., 2004). The N-terminal domain, has a high abundance of Ser, Thr, and Pro residues, that were reported to be extensively *O*-glycosylated, an observation that could explain the variability in the apparent molecular weight of the protein (Ino et al., 2002; Tsuiji et al., 2003). Nevertheless, other explanations cannot be excluded. For example, detergent-resistant multimers in membrane preparations of cells expressing a FXYD5-Flag construct have been observed (Miller and Davis, 2008b). A similar phenomenon has also been reported for other FXYD proteins (Garty and Karlish, 2006; Wypijewski et al., 2013). Thus, the general conclusion is that some *O*-glycosylation is always present, since the migration size of native and expressed FXYD5 in all of the reported expression systems is consistently higher than the predicted molecular weight, and the presence of sugar moieties was demonstrated by lectin binding to the 24 kDa protein (Lubarski et al., 2011). Thus, the 50–55 kDa molecular species could be either a dimeric form of FXYD5 or a highly glycosylated species that is prevalent mainly in cancerous cells. In either case this issue deserves further investigation.

As discussed below, whereas the FXYD5 trans-membrane segment acts as a kinetic modulator of Na^+/K^+-ATPase activity, neither the N nor C extra-membrane segments of FXYD5 have been characterized in terms of possible functional effects (Lubarski et al., 2007). The intracellular region of FXYD5 is considerably shorter than that of the other FXYD family members and consists of only 15 amino acids. Interestingly, our observations have shown that any alterations to the C-terminus, such as deletion or tagging, results in failure of the protein to express at the surface, suggesting that the C-terminus is essential for correct folding or plasma membrane trafficking or both functions. In spite of its short length the C-terminus is known to undergo various post-translational modifications. For example, Ser^{163}, a conserved residue in all FXYD proteins, has been suggested as a target for kinase-mediated regulation of cell motility in an *in vitro* model of airway epithelia. The authors concluded that phosphorylation at Ser^{163} regulates the FXYD5/Na^+/K^+-ATPase interaction and that this interaction modulates cell migration across a wound in airway epithelial cells (Miller and Davis, 2008b).

FXYD5 has two cysteines in its intracellular region, Cys^{168} and Cys^{170}. Neither residue is exclusive for FXYD5 and both are located in equivalent positions throughout the FXYD family. Although the equivalent cysteines in all FXYD proteins are predicted to undergo palmitoylation, this modification has been demonstrated experimentally only for FXYD1 and FXYD5 (Tulloch et al., 2011; Martin et al., 2012). Palmitoylation of FXYD1 was reported to be promoted by PKC phosphorylation and to be required for inhibition of the Na^+/K^+-ATPase activity (Tulloch et al., 2011). The functional role of FXYD5 palmitoylation, if any, has not yet been characterized. Nevertheless, since all FXYD members have at least one conserved cysteine residue, palmitoylation may be a general means of regulating the pump.

At least one splice variant has been identified for FXYD5 with a non-canonical GT/CC splice site (Lubarski et al., 2007). Due to elimination of the original stop codon this splice variant creates a longer transcript encoding 10 additional residues at the C-terminus. Splice variants have also been reported for FXYD2 and FXYD3 (Kuster et al., 2000; Bibert et al., 2006). In both cases, differential expression of the two variants was observed, and in the case of FXYD3 different functional effects were also noted (Bibert et al., 2006). The functional effect of FXYD5 splice variant has not yet been analyzed. **Table 1** summarizes the published data of all FXYD5 modifications and mutations.

FUNCTIONAL INTERACTION OF FXYD5 WITH NA^+/K^+-ATPase

The specific interaction of FXYD5 with Na^+/K^+-ATPase was demonstrated by co-immunoprecipitation in several expression systems (Lubarski et al., 2005, 2007, 2011; Miller and Davis, 2008b). Similar to FXYD1 (Crambert et al., 2002), but unlike FXYD4 and FXYD2 (Garty et al., 2002), association between FXYD5 and the Na^+/K^+-ATPase was found efficient also in the absence of Rb^+/ouabain that preserve the native pump structure. The stability of the αβ/FXYD5 complex in detergent solubilized membranes and its high efficiency of co-immunoprecipitation are determined by its trans-membrane domain, as demonstrated by structure-function studies of FXYD5/FXYD4 chimeras expressed in *Xenopus* oocytes (Lubarski et al., 2007). A series of experiments, using point mutations, identified three trans-membrane residues as particularly important for the FXYD5/Na^+/K^+-ATPase interaction (Lubarski et al., 2007). All three residues, Ala^{150}, Ile^{160}, and Leu^{161}, are common to all FXYD proteins, except FXYD4, and their significance for association with the pump were described in earlier studies (Lindzen et al., 2003; Li et al., 2004, 2005). A residue unique to FXYD5, Arg^{145}, is located at the membrane- extracellular interface. Mutation of Arg^{145} to Gly (Arg^{145}Gly), a residue located at the equivalent position in most of the FXYD family members, significantly increases the stability of the FXYD5/Na^+/K^+-ATPase complex, as demonstrated by co-immunoprecipitation experiments in HEK293 cells (Lubarski et al., 2014). Due to the location of Arg^{145} at the membrane-extracellular interface, the charge may have a major effect on the position of FXYD5 within the plasma membrane.

One of the well characterized functional effects of FXYD5 is to increase the pump activity (V_{max}), measured either as ouabain-blockable and K^+-induced outward current, or as ouabain-inhibitable $^{86}Rb^+$ uptake. Co-expression of FXYD5 together with the Na^+/K^+-ATPase in *Xenopus laevis* oocytes produces a more

TABLE 1 | Summary of all published FXYD5 modifications and mutations.

Residues	Location	Modification	Function	Expression system	References
1–21	N-terminus	Signal peptide	Plasma membrane targeting	*Xenopus* oocytes	Lubarski et al., 2005
22–145	N-terminus	*O*-Glycosylation	Up-regulated in cancer?	PLC/PRF/5	Tsuiji et al., 2003
S^{163}	C-terminus	Phosphorylation	Reduce association with Na^+/K^+-ATPase	MDCK	Miller and Davis, 2008b
C^{168}, C^{170}	C-terminus	Palmitoylation	Not tested	T-cells	Martin et al., 2012
^{179}AYRVINMKES^{188}	C-terminus	Splice variant	Not tested	Dendritic cells	Lubarski et al., 2007
A^{150}, I^{160},L^{161}	Transmembrane	Mutation to G,M,A	Reduce association with Na^+/K^+-ATPase	*Xenopus* oocytes, HEK293	Lubarski et al., 2007
R^{145}	N-terminus, membrane interphase	Mutation to G	Increase association with Na^+/K^+-ATPase	HEK293	Lubarski et al., 2014

than two-fold increase in the V_{max}, without affecting the $K_{0.5}$ for external K^+ (Lubarski et al., 2005, 2007). In a separate study using Madin-Darby canine kidney cells (MDCK), transfected with FXYD5, the effect on V_{max} was also demonstrated. In addition, FXYD5 was shown to elevate the apparent affinity for Na^+ about two-fold, and decrease the apparent affinity for K^+ by 60% (Miller and Davis, 2008a).

FXYD5-FXYD4 chimeras were used to study the structure-function relations of different domains, measured by Rb^{86} uptake in *Xenopus* oocytes. These indicated that the FXYD5 trans-membrane segment is involved in the effect to increase the pumping rate. Other parameters were not tested in these experiments (Lubarski et al., 2007). Since the plasma membrane expression of Na^+/K^+-ATPase, as quantified by surface biotinylation, was not altered by FXYD5 expression, it was concluded that FXYD5 elevates the turnover rate of the pump (Lubarski et al., 2011).

The effects of FXYD5 on kinetic parameters of the Na^+/K^+-ATPase are small, about two-fold, similar to those reported for other FXYD proteins (Lubarski et al., 2005; Garty and Karlish, 2006). However, they are likely to be physiologically significant. The physiological role of the kinetic effect of FXYD5 can be proposed on the basis of its observed localization in normal tissue and on the basis of phenotypic analysis of differential FXYD5 expression under pathophysiological conditions. Interestingly, in kidney the FXYD5 cellular expression pattern correlates with low abundance of Na^+/K^+-ATPase. FXYD5 is expressed in cortical collecting duct intercalated cells, which have almost undetectable levels of the pump (Lubarski et al., 2005). Therefore, in principle, a FXYD5-mediated increase in V_{max} could serve as a homeostatic mechanism that acts to moderate or restore increased intracellular Na^+ or decreased intracellular K^+ ion concentrations, without altering the number of pumps in the cell. FXYD5 was found to be up-regulated during several pathological events. Patients with spinal cord injury (SCI) exhibited down-regulation of Na^+/K^+-ATPase, while FXYD5 expression was significantly increased (Boon et al., 2012). In alveolar epithelial cells, during hypoxia, up-regulation of FXYD5 was demonstrated, while several studies reported down regulation in Na^+/K^+-ATPase, at the transcriptional level and a decrease in the number of pumps at the basolateral membrane (Planès et al., 1996; Clerici and Matthay, 2000; Wodopia et al., 2000; Dada et al., 2003; Igwe et al., 2009). Up-regulation in FXYD5 under these conditions may serve as a compensatory mechanism to overcome the decrease in Na^+/K^+-ATPase unit numbers, by increasing the pumping rate. A significant increase in FXYD5 was found also in the lungs and nasal epithelium of cystic fibrosis (CF) mice, at the level of both protein and mRNA, and this up-regulation was directly correlated with loss of Cystic fibrosis transmembrane conductance regulator (CFTR) function (Miller and Davis, 2008a). Increased FXYD5 expression observed in CF airway epithelia may contribute to the increased Na^+/K^+-ATPase activity, which in turn may add to the greater sodium reabsorption seen in CF. The regulatory mechanism of FXYD5 increase under these pathological conditions is unknown yet. Nevertheless, FXYD5 up-regulation was reported following addition of pro-inflammatory cytokines TNFα or IL-1β (Miller and Davis, 2008a). Since inflammation is a common outcome of the above disorders it is a logical starting point in the evaluation of pathways regulating FXYD5 expression.

While structural interaction between FXYD proteins and the pump have been partially resolved by the crystal structures (Kanai et al., 2013), the mechanistic details of FXYD5 functional effects are still to be determined. It is certain, however, that the kinetic effects detected *in vitro* are not the only outcome of the FXYD5/Na^+/K^+-ATPase association as discussed below.

FXYD5 EXPRESSION IS UP-REGULATED IN VARIOUS HUMAN TUMORS

FXYD5 has been identified as a cancer-associated protein. In a number of clinical studies it was shown that there is a statistically significant correlation between the FXYD5 abundance and the progression of malignancies, accompanied by poor outcome of patients with various cancers (for a review see Nam et al., 2007). Initial studies showed that FXYD5 overexpression was correlated with reduced cell–cell adhesiveness, giving rise to the name "dysadherin" (Ino et al., 2002). In support of this concept, it was found that the mouse FXYD5 gene was induced in NIH 3T3 fibroblasts, transformed by a variety of oncogenes, including *E2a-Pbx1, v-Ras, Neu,* and *v-Src* (Fu and Kamps, 1997). Hence, clues to the basis of this FXYD5 mechanism of action is of high interest.

Some evidence has suggested the link of FXYD5 to known cancer promoting signaling pathways. In some cancer cell lines and tumors FXYD5 overexpression has been correlated with down-regulation of E-cadherin (Nakanishi et al., 2004; Batistatou et al., 2005; Kyzas et al., 2006; Sato et al., 2013). It has long been recognized that E-cadherin, acting as the cell–cell adhesion receptor, has an important role in suppression of tumor progression (Schipper et al., 1991; Oka et al., 1993; Umbas et al., 1994). Therefore, it was proposed that FXYD5 might promote metastasis by down-regulating E-cadherin. However, FXYD5 expression was correlated with changes in cell morphology *in vitro,* and with promotion of metastasis *in vivo,* in cell lines and tumors that did not express any E-cadherin. These findings suggest that E-cadherin dependence is not a general phenomenon for all FXYD5 expressing tumor types (Shimamura, 2003; Shimada, 2004; Nishizawa et al., 2005; Tamura et al., 2005; Lubarski et al., 2011).

A study, using a global gene expression analysis approach, identified chemokine (C-C motif) ligand 2 (CCL2) as the transcript most effected by silencing FXYD5 in MDA-MB-231 breast cancer cells (Nam et al., 2006). Investigations in animal model systems have recognized CCL2 as a mediator of inflammation with pro-malignant activities. CCL2 was shown to promote angiogenesis and act directly on the tumor cells to increase their migratory and invasion-related properties (Soria and Ben-Baruch, 2008). Correlation of FXYD5 expression with CCL2 secretion was demonstrated in different cell lines (Nam et al., 2006; Schüler et al., 2012). Furthermore, the ability of FXYD5 to promote invasion of MDA MB231 cells *in vivo* was inhibited by suppression of CCL2 (Nam et al., 2006).

In addition, more global, molecular changes were found to be associated with FXYD5 overexpression in breast cancer model. It was reported that FXYD5 expression results in enhancement of NF-κB transcriptional activity (Nam et al., 2006). The presence of FXYD5 was also accompanied by increased activation of AKT, demonstrated by elevated abundance of phospho-AKT in breast cancer tumors. Inhibition of AKT activity suppressed the ability of FXYD5 to promote the activation of the NF-κB pathway. Therefore, it was suggested that NF-κB activation might be a downstream signaling of FXYD5-mediated AKT activation (Lee et al., 2012).

Due to its ability to control cell proliferation and to suppress apoptosis, the transcription factor NF-κB is broadly related to carcinogenesis. Furthermore, the NF-κB pathway has been associated with control of metastasis and angiogenesis (Bassères and Baldwin, 2006; Karin, 2006). AKT is a serine/threonine kinase that functions through its ability to phosphorylate a number of key pro-oncogenic targets that promote cell growth or inhibit apoptotic pathways (Hay, 2005; Manning and Cantley, 2007). The NF-κB transcription factor has been identified as a target of the AKT signaling pathway (LoPiccolo et al., 2007).

In MDA-MB-231 cells, several hundred genes were reported to be differentially expressed associated with FXYD5 silencing, as determined by global gene expression analysis (Nam et al., 2006). Since NF-κB is a transcription factor that is responsible for a broad spectrum of cellular activities, it is possible that some of the pathways described above are initiated as a consequence of FXYD5-mediated NF-κB activation. However, it is not clear how FXYD5 alters NF-κB or any of the affected proteins described above. Since all the reported signaling events are modified at the transcriptional level, it is also unclear whether the proposed mechanisms are primary or secondary consequences of FXYD5 expression. Furthermore, direct interaction with FXYD5 has not been established with any protein other than Na^+/K^+-ATPase.

FXYD5 OVER EXPRESSION ALTERS EPITHELIAL MORPHOLOGY

Since FXYD5 normally resides in kidney epithelia, it was logical to study the effects of its up-regulation in these cells. FXYD5 over expression was first examined in the rodent M1 cell, on account of their ability to create and maintain tight epithelial monolayer *in vitro*. In these cells, FXYD5 was shown to induce dilation of tight and adherent junctions (TJ, AJ) and promote expansion of interstitial spaces between the neighboring cells. These morphological changes were observed by electron microscopy and were further confirmed by re-distribution of TJ and AJ markers (ZO-1, occludin, and β-catenin) in FXYD5 expressing monolayers (**Figure 1**). As a consequence, FXYD5 transfected M1 cells lost their tight epithelial integrity, measured by reduced paracellular electrical resistance and increased trans-cellular permeability to macro molecules (Lubarski et al., 2011).

The effects of FXYD5 were also studied at a single cell level. It was found that the presence of FXYD5 results in impaired adhesion of cells to the matrix, as observed by the reduced rate of cell transformation from spherical to a flatter elongated shape, with inhibited anterior-posterior polarity. This morphological effect was seen consistently with several cell lines, and was demonstrated both by silencing FXYD5 in cells that normally express it (H1299, Panc-1), or transfecting FXYD5 into cells that lack this protein (M1, HEK293). Cells expressing FXYD5 exhibit a less flattened elongated shape, demonstrated also by a reduced number of focal adhesion points, as established by differential immuno-staining of paxillin, the focal adhesion-associated adaptor protein. Changes in focal adhesions were also correlated with less organized actin fibers structure (Lubarski et al., 2014).

Interestingly, there are some conflicting results concerning effects of FXYD5 on motility parameters in different cell lines. In some cell lines, the presence of FXYD5 evoked increased cell migration, in agreement with previously published data (Ino et al., 2002; Shimamura et al., 2004), whereas in others (M1 and H1299) the opposite results were obtained. This discrepancy was apparent for both single and collective cell movement. However, changes in FXYD5-mediated adhesion were constant and more robust finding that is not dependent on cell type (Lubarski et al., 2014).

Looser cell–cell contacts and impaired cell-matrix association could explain morphological changes associated with the role of FXYD5 in the metastatic processes. However, since FXYD5 is expressed in a variety of normal epithelia the effects described above might also be a component of its inherent physiological role. Distribution of FXYD5 in "leaky" epithelia of

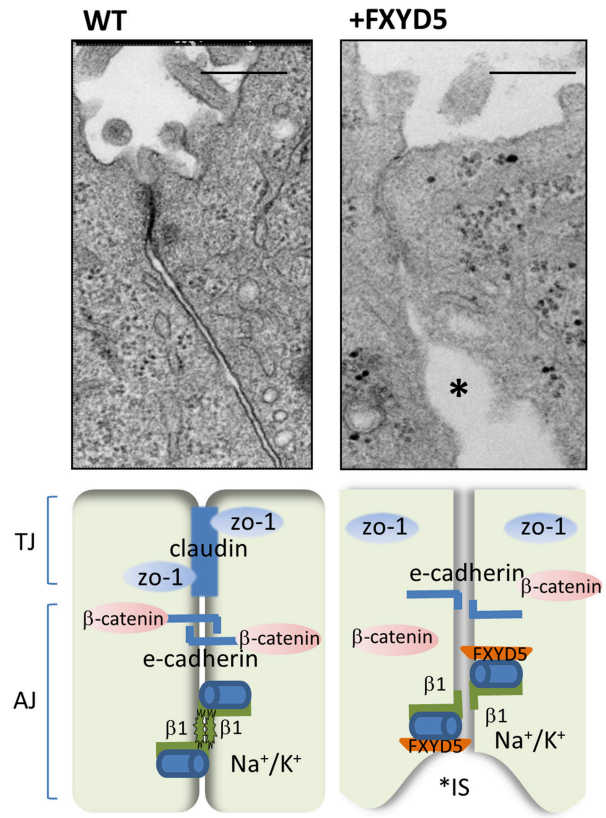

FIGURE 1 | FXYD5 impairs cell–cell junction formation. (**Upper**) High magnification of electron microscopy images of normal and FXYD5 transfected M1 cell monolayer. Large expansions of interstitial spaces (IS) located just under the TJ evoked by FXYD5 expression (asterisks). Bar = 200 nm. The EM images were originally published in (Lubarski et al., 2011). (**Lower**) Experimental mode of FXYD5 function. FXYD5 expression in M1 polarized monolayer results in down regulation in membrane expression of TJ markers ZO-1 and occludin. E-cadherin membrane localization remained intact, while β-catenin, has been redistributed and appeared perpendicular to the membrane plane. A reduced form of β glycosylation has been observed, indicating an additional condition for promotion of "leaky" epithelia.

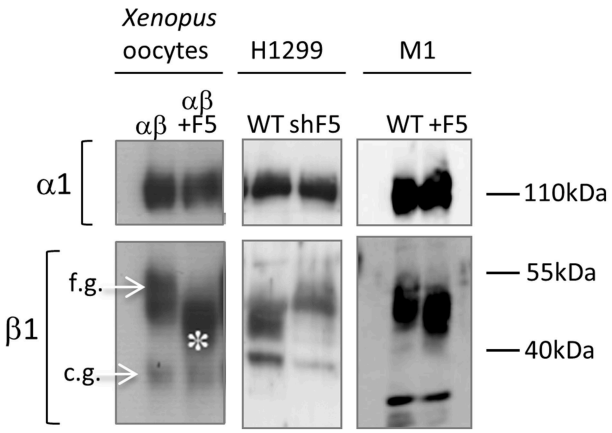

FIGURE 2 | Expression of FXYD5 effects β1-Na^+/K^+-ATPase glycosylation. Western blot of microsomes: left panel- *Xenopus* oocytes, injected with αβ or αβ/FXYD5, middle panel- H1299 WT cells and shFXYD5 cells (shF5), right panel- M1 WT cell and FXYD5 transfected cells (+F5). β1-Na^+/K^+-ATPase is represented by two bands, one for fully glycosylated (f.g) and the other for core glycosylated (c.g.) form (marked with arrows). FXYD5 expression reduces glycosylation level of fully glycosylated form (asterisk). The figure was originally published in (Lubarski et al., 2007, 2011).

the gastro-intestinal tract could support this claim. An additional possibility is that FXYD5 is transiently up-regulated under conditions that require enhancement of paracellular permeability as, for example, during nutrient absorbtion or for infiltration of leukocytes during infection (Capaldo and Nusrat, 2009; Turner, 2009). The mechanism underlying additional functions of FXYD5 might also be explained as secondary effects following the increased pump activity. However, the phenotypes observed *in vitro* were apparent at very different pumping rates, arguing against this notion (Lubarski et al., 2011).

FXYD5 ADDITIONAL EFFECTS MIGHT BE MEDIATED BY ITS ASSOCIATION WITH NA^+/K^+-ATPase

Another interesting effect of FXYD5 is the modification of the glycosylation state of the β1-subunit of Na^+/K^+-ATPase. The Na^+/K^+-ATPase pump consists of the catalytic α and structural β subunits. The classical role of β is to control assembly and stability of alpha-beta heterodimers and plasma membrane transport of sodium pumps (McDonough et al., 1990). The effect of FXYD5 on β1 glycosylation was noticed initially in *Xenopus* oocytes, and has been observed consistently with various mammalian expression systems that either normally express FXYD5 or have been transfected with FXYD5 cDNA (**Figure 2**; Lubarski et al., 2005, 2007, 2011). The phenomenon is manifested as increased migration of the highly glycosylated β1 form in SDS-PAGE gels, when co-expressed with FXYD5, with no change in its core unglycosylated protein. In general, this phenomenon may imply ER retention of an immature protein form. However, experiments determining the glycosylation state of surface biotinylated β1 seem to argue against such a possibility. Also, deglycosylation of β1 has no effect on the stability of the αβ complex, as reflected by surface expression of the α subunit in mammalian cells, and intact catalytic activity measured in *Xenopus* oocytes (Lubarski et al., 2005, 2011).

Various FXYD5/FXYD4 chimeras, constructed for structure-function studies, revealed that the presence of both trans-membrane and extracellular domains of FXYD5 are required for the reduced glycosylation of β1. Surprisingly, β deglycosylation is the only experimentally observed effect attributable to the long glycosylated extracellular portion of FXYD5, reported until now. Interestingly, a similar effect was demonstrated for FXYD3, but in this case the reduced glycosylation depended on the presence of uncleaved signal peptide (Crambert et al., 2005).

FXYD5-dependent modification of β1 glycosylation might be explained by a direct FXYD5-β interaction that may obstruct addition of sialic acid residues at all sites, or interfere with glycosylation at one or more positions. Although, according to

the crystal structure (Toyoshima et al., 2011; Kanai et al., 2013) the transmembrane helices of the β and FXYD subunits are located at opposite sides of the α subunit, interactions between their extracellular domains cannot be ruled out. Nevertheless, if FXYD5-β interaction is possible, there is no certainty that it has an effect on β processing. At least in the case of FXYD3, it was demonstrated that the effect on glycosylation is not exclusive to β and furthermore, is not dependent on direct interaction with the Na^+/K^+-ATPase (Crambert et al., 2005). This evidence could indicate that FXYD5- mediated modification of glycosylation is not a direct effect, and might involve more global changes in protein sorting or in glycosylation machinery, or alternatively, that FXYD5 interacts with membrane proteins other than the Na^+/K^+-ATPase.

The functional significance of the above behavior may explain additional functions attributed to the β1 subunit. For example, several studies have provided evidence that the β1 subunit of Na^+/K^+-ATPase has a role as an adhesion molecule. Transcellular β1–β1 interactions have been shown to be involved in preservation and regulation of epithelial junctions, and the sugars moieties of the β extracellular domain were found to be important for maintaining β mediated cell–cell contacts (for a review see Vagin et al., 2012). Hence, FXYD5-mediated interference in β glycan structure, either direct or indirect, may result in "looser" junctions, characteristic of FXYD5 expressing cells.

The effect of FXYD5 on cell-matrix adhesion was also found to be mediated through its interaction with Na^+/K^+-ATPase. Specifically, two point mutations in the transmembrane segment demonstrated that association of FXYD5 with the pump is directly related with changes in cell morphology (Lubarski et al., 2014). Similar conclusions were drawn from $Ser^{163}Asp$ mutation. The negative charge at the mutated $Ser^{163}Asp$ residue, that mimics phosphorylation, has been proposed to regulate FXYD5/Na^+/K^+-ATPase association and this interaction has been correlated with modulation of collective cell movement in airway epithelial cells (Miller and Davis, 2008b). However, in contrast to the transmembrane mutations, the $Ser^{163}Asp$ mutation also interfered with plasma membrane localization of FXYD5.

KEY CONCEPTS AND FUTURE RESEARCH PERSPECTIVES

FXYD5 is an established modulatory subunit of Na^+/K^+-ATPase, expressed in a variety of epithelial cells. Nevertheless, the function of its unique extracellular structural domain still remains elusive (Lubarski et al., 2005, 2007; Miller and Davis, 2008a). FXYD5 is up-regulated under several pathological conditions and its increased expression and differentially-modified molecular form in various malignancies pointed to possible functions in addition to kinetic modulation of Na^+/K^+-ATPase activity (Ino et al., 2002; Nam et al., 2007; Igwe et al., 2009; Boon et al., 2012). FXYD5-mediated alterations in cell morphology *in vitro,* and substantial transcriptional modification cannot be explained only by changes in Na^+/K^+-ATPase activity. The proposed molecular mechanisms underlying its effects in adhesion, motility, paracellular permeability, and metastatic progression vary between different cell types. The only consistently observed interaction of FXYD5 is with Na^+/K^+-ATPase. In addition to increasing the Na^+/K^+-ATPase pumping rate, FXYD5 has been shown to modify the glycosylation state of the β1 subunit (Lubarski et al., 2005, 2007, 2011). In parallel, a role of Na^+/K^+-ATPase as an adhesion molecule and the contribution of the β1 subunit to adherent junction formation have been established (Vagin et al., 2012). Therefore, a correlation between these processes has been proposed.

For the future a major focus should be on the physiological relevance underlying the effect of FXYD5 on Na^+/K^+-ATPase activity. The nature of FXYD5-mediated β1 carbohydrate modifications is a major question. This could call for a search for more global changes, since it appears obvious, that glycosylation of more than one protein is affected by FXYD5. In support of this notion, a role of another FXYD protein that shares this feature, FXYD3, has also been suspected in carcinogenesis (Maxwell et al., 2003). Altered glycosylation is a universal feature of cancer cells and it may explain several of FXYD5-induced morphological changes (Varki et al., 2009).

Finally, a most intriguing line of research could be to search for the trigger that activates transformation or modification of FXYD5 from its role in normal tissue to that in the malignant state. The reported up-regulation of FXYD5 in pathological conditions that require stabilization of the pump activity may shed light on the initial event that elicits malignant transformation.

AUTHOR CONTRIBUTIONS

IL approved final version of manuscript, prepared figures, drafted manuscript, edited, and revised manuscript.

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Ouabain Enhances ADPKD Cell Apoptosis via the Intrinsic Pathway

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Progression of autosomal dominant polycystic kidney disease (ADPKD) is highly influenced by factors circulating in blood. We have shown that the hormone ouabain enhances several characteristics of the ADPKD cystic phenotype, including the rate of cell proliferation, fluid secretion and the capacity of the cells to form cysts. In this work, we found that physiological levels of ouabain (3 nM) also promote programmed cell death of renal epithelial cells obtained from kidney cysts of patients with ADPKD (ADPKD cells). This was determined by Alexa Fluor 488 labeled-Annexin-V staining and TUNEL assay, both biochemical markers of apoptosis. Ouabain-induced apoptosis also takes place when ADPKD cell growth is blocked; suggesting that the effect is not secondary to the stimulatory actions of ouabain on cell proliferation. Ouabain alters the expression of BCL family of proteins, reducing BCL-2 and increasing BAX expression levels, anti- and pro-apoptotic mediators respectively. In addition, ouabain caused the release of cytochrome c from mitochondria. Moreover, ouabain activates caspase-3, a key "executioner" caspase in the cell apoptotic pathway, but did not affect caspase-8. This suggests that ouabain triggers ADPKD cell apoptosis by stimulating the intrinsic, but not the extrinsic pathway of programmed cell death. The apoptotic effects of ouabain are specific for ADPKD cells and do not occur in normal human kidney cells (NHK cells). Taken together with our previous observations, these results show that ouabain causes an imbalance in cell growth/death, to favor growth of the cystic cells. This event, characteristic of ADPKD, further suggests the importance of ouabain as a circulating factor that promotes ADPKD progression.

Keywords: Na, K-ATPase signaling, polycystic kidney disease, programmed cell death, BCL-2 proteins, cytochrome c, caspase

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INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is the most common genetic disorder of the kidney, characterized by the formation and progressive enlargement of numerous fluid filled cysts, which severely distort renal morphology and function, reviewed in Grantham (2008) and Paul and Vanden Heuvel (2014). ADPKD cysts are formed *in utero* and continue progressing after birth at a relatively slow, but relentless rate throughout the life of the affected individual (Grantham et al., 2010). Patients with ADPKD eventually develop renal insufficiency and end-stage renal disease (ESRD), requiring dialysis or kidney replacement therapy (Alam and Perrone, 2010; Grantham et al., 2011; Kanaan et al., 2014).

ADPKD is caused by mutations in the genes that encode for polycystin-1 and polycystin-2 (*Pkd1* and *Pkd2* respectively); however, progression of the disease is highly influenced by factors circulating in the bloodstream (Pei, 2011; Fedeles et al., 2014; Ong and Harris, 2015). We have shown that the hormone ouabain, in concentrations similar to those present in plasma, stimulate the proliferation of renal epithelial cells obtained from kidney cysts of patients with ADPKD (ADPKD cells), the growth of microcysts generated by ADPKD cells, and cyst-like tubule dilations in embryonic kidneys from a mouse model of ADPKD (Nguyen et al., 2007; Jansson et al., 2012). In contrast, ouabain does not significantly influence cell proliferation and cyst formation in normal kidney cells (NHK cells) and metanephric organs from wild type mice (Blanco and Wallace, 2013).

The slow progression of ADPKD is difficult to explain in a condition that is primarily characterized by continuous cell proliferation. Cell growth is maintained by a balance between cell proliferation and apoptosis, a process of programmed cell death (Green and Llambi, 2015; Savitskaya and Onishchenko, 2015). Interestingly, an imbalance between increased rates of cell apoptosis have been reported in kidneys from animal models of ADPKD and in humans carrying the disease, a phenomenon that may contribute to the uncontrolled, but slow progression of the disease (Lanoix et al., 1996; Zhou and Kukes, 1998; Murcia et al., 1999; Torres, 1999; Edelstein, 2005; Ibrahim, 2007; Goilav et al., 2008; Ibraghimov-Beskrovnaya and Bukanov, 2008).

Apoptosis is an essential process during normal tissue development and aging and is also found in several pathological situations (Elmore, 2007; Tezil and Basaga, 2014; Arya and White, 2015; Labi and Erlacher, 2015). Apoptosis involves an intricate cascade of molecular events, with the B-cell lymphoma 2 (BCL-2) protein family and a series of cysteine proteases, the caspases, being essential mediators of the process. The BCL-2 family include several members that are pro-survival and pro-apoptotic factors, such as BCL-2 and BAX respectively. The proteolytic caspases include the "initiator" caspases-8, -9, and -10, and the "executioner" caspases 3 and 7 (Elmore, 2007; Green and Llambi, 2015; Zheng et al., 2015). Two main caspase-mediated pathways control programmed cell death. The extrinsic pathway, a ligand triggered and transmembrane receptor mediated cascade (Ashkenazi, 2015), and the intrinsic pathway, which comprises mitochondrial changes and the release of cytochrome c from the mitochondrial intermembrane space to the cell cytosol (Brenner and Mak, 2009). Both intrinsic and extrinsic pathways converge to stimulate the activity of caspases-3 and -7, which are responsible for the events that are characteristic of apoptosis, including DNA fragmentation, protein cross-linking and degradation, and cell disintegration into apoptotic bodies (Salvesen and Riedl, 2008).

While apoptosis has been described as a feature of ADPKD, the factors and mechanisms that influence programmed cell death in ADPKD cells are poorly understood. Ouabain has been shown to influence programmed cell death in a cell type specific manner. For instance, ouabain has pro-apoptotic effects in normal neuronal cells, neuro- and glioblastoma cells, hepatic cells, blood peripheral lymphocytes, lymphoma cells, and prostate cancer cells (Olej et al., 1998; Xiao et al., 2002; Huang et al., 2004; Esteves et al., 2005; Kulikov et al., 2007; Panayiotidis et al., 2010; Xu et al., 2010; Fu et al., 2013; Yan et al., 2015). In contrast, ouabain protects endothelial cells, cerebellar granule cells, renal proximal tubule, and COS-7 cells against apoptosis (Isaev et al., 2000; Orlov et al., 2004; Trevisi et al., 2004, 2006; Li et al., 2010; Dvela et al., 2012; Burlaka et al., 2013); and has a dual pro- and anti-apoptotic effect in smooth muscle, umbilical vein endothelial cells, and fibroblast (Chueh et al., 2001; Winnicka et al., 2010; Ren et al., 2014). At present, the role of ouabain in ADPKD apoptosis is unknown. In this work, we show that ouabain enhances apoptosis in ADPKD, but not in NHK cells, by activating the intrinsic pathways of programmed cell death.

MATERIALS AND METHODS

Cell Culture

Primary cell cultures of ADPKD cells, derived from surface cysts of ADPKD kidneys and NHK cells were generated by the PKD Biomaterial Core at University of Kansas Medical Center (KUMC). A protocol for the use of discarded human kidney tissues was approved by the Institutional Review Board at KUMC. Primary cultures were prepared as described (Wallace et al., 1996). Cells were seeded and grown in DMEM/F12 supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin G and 0.1 mg/ml streptomycin, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite (ITS). Twenty-four hours before, cells were subjected to various experimental manipulations, serum was reduced to 0.002% and ITS removed. As previously shown, these cells are epithelial in nature and they stain positive for specific lectin markers for the collecting duct and distal nephron, indicating that they are derived from the distal nephron (Yamaguchi et al., 2003).

Annexin-V and Propidium Iodide Staining

Alexa Fluor 488 labeled-Annexin-V and propidium iodide (PI) staining were utilized as biomarkers for the detection of apoptosis and necrosis in ADPKD and NHK cells by flow cytometric analysis, following the manufacturer's protocols (Invitrogen, Carlsbad, CA, USA). Briefly, cells were trypsinized, washed in PBS, and resuspended in binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4). After addition of 5 μL Annexin-V and 1 μL PI, cells were incubated at 37°C and protected from light for 15 min. Samples were diluted with binding buffer and were analyzed using a LSRII flow cytometer (Beckton Dickinson, Franklin Lakes, NJ). Alexa Fluor 488 labeled-Annexin-V, which detects changes in distribution of phosphatidylserine and phosphatidylethanolamine at the cell plasma membrane; and propidium iodide (PI) staining, which reveals loss of cell plasma membrane integrity, allowed distinguishing cells undergoing early and late stages of apoptosis, and necrosis (Ravichandran, 2010).

Measurement of Fragmented DNA by TUNEL Assay

Cells cultured on glass coverslips were treated with 3 nM ouabain for 24 h and analyzed for apoptosis using the DeadEnd fluorometric TUNEL system (Promega, Madison, WI). This

method determines nuclear fragmentation, an important biochemical marker for cell apoptosis, by using terminal deoxynucleotidyl transferase to transfer fluorescein (FITC)-12-dUTP to the free 3′-OH of cleaved DNA. Cells were counter-stained with DAPI to label the nuclei. The percentage of cells undergoing apoptosis was determined using fluorescence microscopy. Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoro-methylketone (z-VAD-fmk), at a concentration of 20 μM and camptothecin, at 5 μM (Promega Corporation, Madison, WI), were applied as an inhibitor and inducer of apoptosis, respectively.

Immunoblot Analysis

Cells treated with and without 3 nM ouabain for 24 h were washed once with ice-cold phosphate buffered saline (PBS) and lysed in a solution containing 10 mM Tris-Cl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, and 0.1% NP-40. Samples were centrifuged at 10,000 × *g* for 10 min. Protein amounts were determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Fifty μg of the cleared lysates were subjected to SDS-PAGE (15% gel) and blotted on to nitrocellulose membranes. Immunoblots were probed with different primary antibodies that recognize PARP-1, BCL-2, BAX, caspase-3 or caspase-8 (Cell Signaling Technology, Boston, MA). Species-specific secondary antibodies conjugated to horse-radish peroxidase and enhanced chemiluminescence was used for protein detection (Santa Cruz Biotechnology, Dallas, TX). Protein expression levels were determined by densitometry and were expressed as a ratio of the corresponding untreated controls.

Cytochrome C Analysis

Cytochrome c release from mitochondria was studied in NHK and ADPKD cells after treatment with or without 3 nM ouabain for 24 h by immunoblot and immunocytochemistry. For the immunoblot analysis, cells were harvested and cytosolic or mitochondrial fractions were prepared using the Cell Fractionation Kit ab109719, according to the manufacturer's instructions (AbCam, Cambridge, UK). Samples were subjected to SDS/PAGE and proteins transferred to nitrocellulose membranes. Cytochrome c was determined using a monoclonal antibody from BD Biosciences (San Diego, CA) and horse-radish peroxidase conjugated secondary antibodies; and its levels were estimated by densitometric analysis of the bands obtained from the cytoplasmic fractions. For the immunocytochemical analysis, cells were plated on coverslips and treated with or without ouabain for 23.5 h. Then, 100 nM MitoTracker Red CMXRos (Thermo Fisher Scientific, Waltham, MA) was added to the cells and they were incubated for an additional 30 min at 37°C, protected from light. Cells were fixed with 3.7% paraformaldehyde in serum-free media for 15 min at 37°C. Samples were washed in PBS and permeabilized with acetone for 5 min. Anti-cytochrome c antibody (BD Biosciences, San Diego, CA) (1:75) was applied to the cells overnight at 4°C. Coverslips were washed once with PBS, and then slides were incubated with secondary Alexa 488-conjugated antibodies for 1 h at room temperature. Samples were washed in PBS and mounted onto microscope slides with DAPI Slowfade Gold solution (Thermo Fisher Scientific, Grand Island, NY). Slides were viewed using a Eclipse 80i Upright microscope (Nikon Instruments, Inc., Melville, NY). Analysis of cytochrome c release from the mitochondria was quantified as the ratio of cytochrome c release was determined from the obtained images, by quantifying the number of pixels in the cell cytosol divided by the number of pixels in mitochondria. Values were expressed as the ratio of cytosolic/mitochondrial cytochrome c levels, as previously described (Gao et al., 2001). This allowed characterizing the mitochondrial to cytosolic distribution of cytochrome c.

Caspase-3/7 Activity Determination

Caspase-3/7 activity was determined using the Caspase-Glo 3/7 Assay according to manufacturer's instructions (Promega, Madison, WI). Briefly, NHK and ADPKD cells were plated into black-walled, clear-bottomed 96-well plates (Corning Inc., Corning, NY) at a density of 4000 cells per well and treated with or without ouabain for 24 h. The luminescent caspase-3/7 substrate was added following the manufacturers specifications. The luminescent signal resulting from cleavage of the substrate specific to caspase-3 or -7 is proportional to the amount of caspase activity present. Data were expressed as a percentage of untreated controls.

Data Analysis

Statistical significance of the differences between ouabain treated and untreated controls was determined by Student's *T*-test. Also, Bonferroni's test was applied as another way to confirm statistically significant differences. Statistical significance was defined as $P < 0.05$.

RESULTS

Ouabain Stimulates Apoptosis in ADPKD but Not in NHK Cells

Ouabain has been reported to stimulate apoptosis in a cell type specific manner (Silva and Soares-da-Silva, 2012). We explored weather ouabain affected programmed cell death in NHK and ADPKD cells. For this, we treated NHK and ADPKD cells with 3 nM ouabain, a concentration of this hormone that is within the levels commonly found to be circulating in blood. Twenty-four hours later, we determined cell apoptosis and necrosis using Alexa Fluor 488 labeled-Annexin-V and PI labeling and flow cytometry. Sorting of the cells based on these markers showed that ouabain treatment caused a modest but significant increase in the number of ADPKD cells undergoing apoptosis compared to untreated controls (**Figure 1A**, top panel). This increase corresponded to cells showing signs of late apoptosis, while the number of ADPKD cells showing early manifestations of apoptosis, or undergoing necrosis did not significantly change with ouabain administration (**Figure 1A**, bottom panel). Different from ADPKD cells, ouabain did not induce programmed cell death (either early or late apoptosis), or necrosis in NHK cells (**Figure 1A**, top and bottom panels).

To further estimate apoptosis, we determined DNA nuclear fragmentation by TUNEL assay in NHK and ADPKD cells. In agreement with the Alexa Fluor 488 labeled-Annexin-V

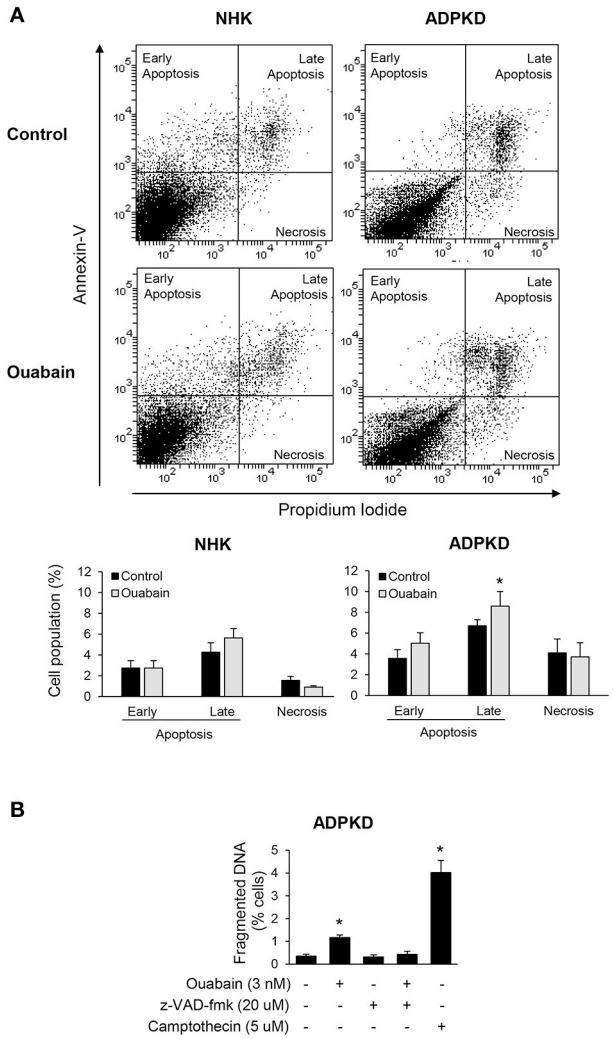

FIGURE 1 | Ouabain induces apoptosis in ADPKD, but not in NHK cells. **(A)** Alexa Fluor 488 labeled-Annexin V and PI staining. After treatment with ouabain for 24 h, cells were labeled and sorted using flow cytometry. The top panel shows representative plots for the sorted NHK and ADPKD cells, without and with of ouabain. Different cell populations undergoing necrosis, early and late apoptosis, identified by differential annexin-V/PI labeling, were quantified and expressed as percent of total cells (bottom panel). **(B)** DNA fragmentation assays. Cells seeded onto glass coverslips were incubated with 3 nM ouabain and the indicated experimental conditions for 24 h, fixed and analyzed for apoptosis using the Dead-End Fluormetric TUNEL System. Fragmented DNA was labeled by incorporation of fluorescein-12-dUTP(a) at 3′-OH DNA ends. The nuclei were counterstained with DAPI and visualized by immunofluorescence microscopy. At least 10 random fields were analyzed from each of three different ADPKD kidneys. In all graphs, bars represent the mean ± SEM of three different experiments. The asterisks indicate values that are statistically different compared to the corresponding untreated control, with $P < 0.05$.

and PI studies, ouabain slightly, but statistically significantly increased TUNEL staining in ADPKD cells (**Figure 1B**). This ouabain-induced increase in DNA fragmentation could be rescued by the pan-caspase inhibitor, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoro-methylketone (z-VAD-fmk), further suggesting that ouabain is inducing apoptosis in ADPKD cells. The topoisomerase I inhibitor, camptothecin, was used as a positive control for apoptosis (**Figure 1B**). Altogether, these experiments show that ouabain stimulates apoptosis in ADPKD, but not in NHK cells.

Ouabain Modulates Expression of BCL-2 Protein Family Members in ADPKD Cells

Whether a cell undergoes apoptosis is in part determined by the ratio of pro- to anti-apoptotic members of the BCL-2 protein family (Green and Llambi, 2015). Within the BCL-2 members, BAX and BAK function as pro-apoptotic agents, while BCL-2 behaves as an anti-apoptotic mediator (Zheng et al., 2015). To further characterize the mechanisms by which ouabain induces cell death in renal normal and cystic cells, we examined the role of these apoptotic regulators in NHK and ADPKD cells. Ouabain treatment for 24 h did not alter the expression levels of either BAX or BCL-2 proteins in NHK cells (**Figure 2A**). However, in ADPKD cells, ouabain caused a significant decrease in the anti-apoptotic BCL-2 protein, with a concomitant increase in the pro-apoptotic BAX protein levels (**Figure 2B**). This change toward a pro-apoptotic protein ratio supports the role of ouabain as an inducer of apoptosis in ADPKD cells, and suggests that its effects are mediated via the intrinsic pathway of programmed cell death.

Ouabain Enhances Cytochrome C Release from ADPKD Cell Mitochondria

A pro-apoptotic change in BCL-2/BAX protein ratio is commonly followed by the release of cytochrome c from mitochondria, another event in the activation cascade of programmed cell death (Li and Dewson, 2015; Um, 2015). Therefore, we determined the release of cytochrome c from mitochondria, by measuring the levels of cytochrome c in cytoplasmic fractions from NHK and ADPKD cells by immunoblot. As expected, and as shown in **Figure 3A**, cytochrome c levels were much higher in the mitochondrial than in the cytosolic fractions of both NHK and ADPKD cells. Importantly, cytochrome c was significantly augmented by ouabain in cytosolic fractions from ADPKD, but it was slightly decreased in NHK cells (**Figure 3A**). Due to the disparity between the high saturating levels of cytochrome c, typical of mitochondria, and the normal low levels of cytochrome c found in cytoplasm, we used higher exposure times for the development of the immunoblots corresponding to the cytosolic than the mitochondrial cell fractions from the same blot. This allowed us to perform a better quantification of the cytochrome c bands in the cell cytosol; which, along with the sole presence of VDAC in mitochondria, support the lack of any major cross contamination between mitochondrial and cytoplasmic samples. The increase in cytochrome c in the cytoplasm suggests that the change in BCL-2/BAX ratio in ADPKD cells did result in release of cytochrome c from the cell mitochondria.

In addition, changes in cytochrome c localization were studied by immunocytochemistry. For this, cells were labeled with MitoTracker Red, a dye which allows the visualization of mitochondria, and an anti-cytochrome c. Then, the ratio of cytoplasmic to mitochondrial localization of cytochrome c

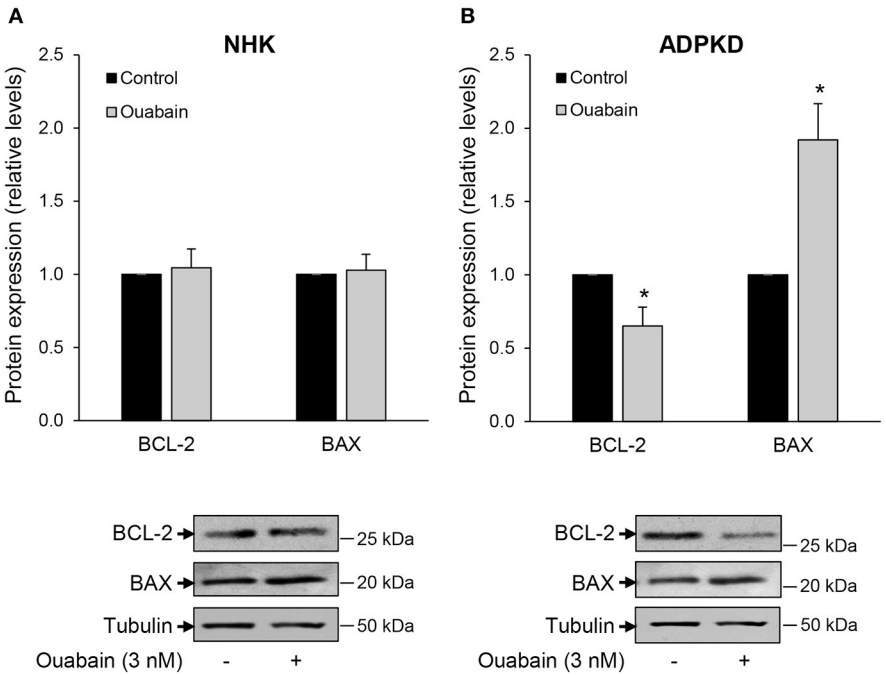

FIGURE 2 | Ouabain modifies the expression of BCL-2 and BAX protein levels in ADPKD, but not NHK cells. NHK **(A)** and ADPKD **(B)** cells were treated in the absence and presence of 3 nM ouabain and 24 h later, expression levels of BCL-2 and BAX were determined by immunoblot. Tubulin was used as a loading control. Top panels show the densitometric analysis of the protein bands, while bottom panels show representative blots. Bars are the mean ± SEM of three experiments. Asterisks indicate statistically different values, with $P < 0.05$ vs. untreated control.

was determined by quantification of pixel density as described (Gao et al., 2001). As shown in **Figure 3B**, the ratio of cytoplasmic/mitochondrial cytochrome c did not change with ouabain treatment in NHK cells. In contrast, this ratio was significantly increased by ouabain in ADPKD cells (**Figure 3B**). Altogether, these results show that ouabain promotes the release of cytochrome c from the mitochondria of ADPKD cells and agree with the notion that ouabain induces apoptosis in these cells.

Ouabain Activates Caspase-3/7 in ADPKD Cells

Proteases of the caspase family play an essential role in the cleavage of specific substrates that mediate cell apoptosis (Zhivotovsky, 2003; Poreba et al., 2013). To assess the involvement of caspases in ouabain-induced apoptosis of ADPKD cells, we treated NHK and ADPKD cells in the presence and absence of 3 nM ouabain for 24 h and determined activation of the "executioner" caspase-3 via its cleavage status. Caspase-3 cleavage was determined by immunoblot. As shown in **Figure 4A**, caspase-3 cleavage was not modified by ouabain in NHK cells (left panels). In contrast, in ADPKD cells, ouabain significantly increased the cleavage of pro-caspase-3 into its active large (p17) and small (p12) fragments (right panels).

In addition, we directly measured the levels of caspase-3 activity, in ADPKD cells treated with and without 3 nM ouabain. Once activated, caspase-3 cleaves the same peptide sequences than caspase-7 and their activities cannot be distinguished with the assay that we used. In any case, both caspase-3 and -7 are "executioner" caspases, involved in downstream cleavage of substrates that mediate many of the typical biochemical and morphological events of apoptosis (Zhivotovsky, 2003; Poreba et al., 2013). As shown in **Figure 4B**, ouabain significantly stimulated caspase-3/7 activity of ADPKD cells.

Ouabain dependent caspase-3 activation by ouabain was also estimated by immunoblot analysis of poly (ADP-ribose) polymerase-1 (PARP-1), a known target of caspase-3 action (Kauppinen and Swanson, 2007). Consistent with activation of caspase-3, ouabain increased the cleavage of PARP-1 in ADPKD cells, but not in NHK cells (**Figure 4C**).

Ouabain Does Not Activate the Extrinsic Pathway of ADPKD Cell Apoptosis

The factors involved in triggering cell apoptosis can act through two main mechanisms, the intrinsic and extrinsic pathways (Green and Llambi, 2015). Our findings that ouabain stimulates the release of cytochrome c from mitochondria suggest a role for the intrinsic pathway in the mechanisms leading to ouabain-mediated apoptosis in ADPKD cells. To determine the involvement of the extrinsic pathway in ouabain-induced ADPKD apoptosis, we measured the activation of caspase-8. This protease is involved in the extrinsic pathway of programmed cell death and its cleavage is a marker for its activation (Salvesen, 1999). We treated NHK and ADPKD cells in the presence and absence of 3 nM ouabain for 24 h and determined the total and cleaved forms of caspase-8 by immunoblot. Compared to

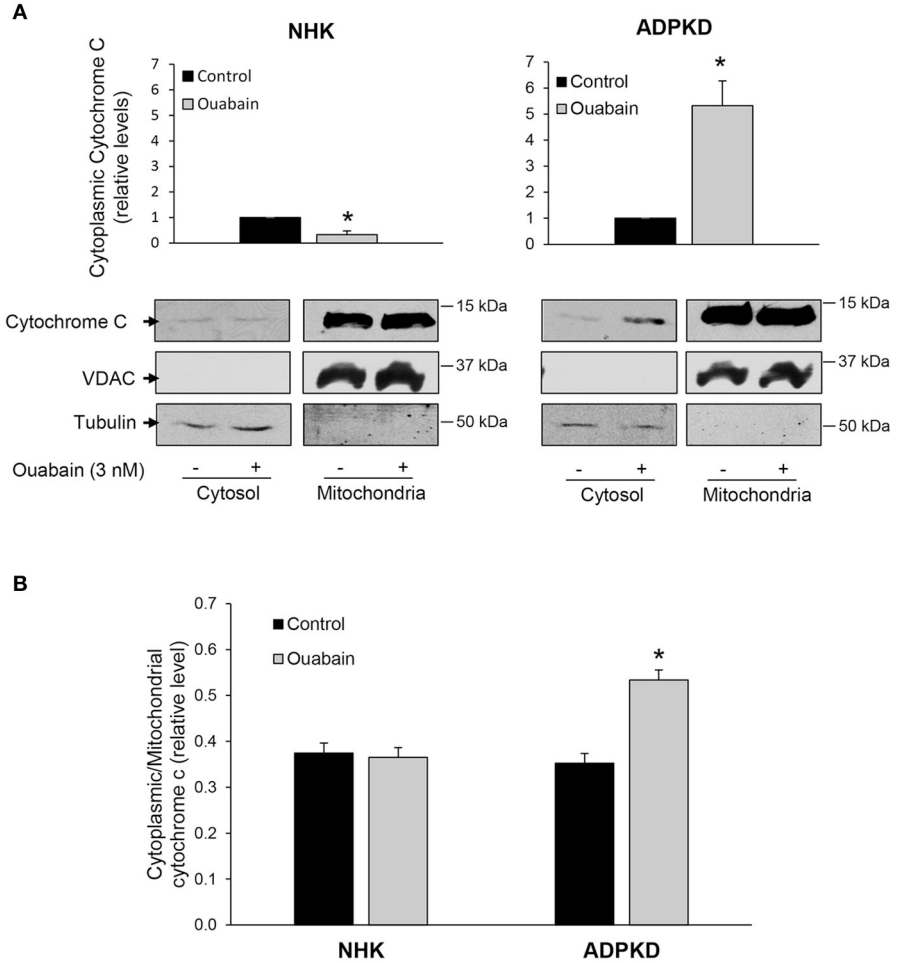

FIGURE 3 | Ouabain stimulates cytochrome C release from mitochondria in ADPKD, but not NHK cells. (A) Immunoblot analysis. After treatment with or without 3 nM ouabain for 24 h, cells were processed to obtain cell cytoplasmic and mitochondrial fractions, and samples were subjected to immunoblot analysis to determine cytochrome c levels. Tubulin was used as a loading control, while VDAC was used as a mitochondrial marker. The bottom panels show representative immunoblots. Relative densitometric levels for cytochrome c in the cytoplasm are shown in the upper panels and they represent data compiled from four different experiments. Due to the difference between mitochondrial and cytoplasmic cytochrome c levels, and to better quantify the cytoplasmic bands, different exposure times for the cytochrome c cytosolic and mitochondrial samples from the same immunoblots were used. **(B)** Immunocytochemical analysis. After treatment with 3 nM ouabain for 24 h, NHK and ADPKD cells were labeled for cytochrome c and MitoTracker, to visualize mitochondria. Cytochrome c release was quantified and expressed as the cytosol to mitochondrial ratio. Bars represent the compiled data from 3 different experiments. In A and B, bars represent the mean ± SEM. Asterisks indicate statistically different values, with $P < 0.05$ vs. untreated control.

untreated controls, ouabain unexpectedly, decreased caspase-8 cleavage in NHK cells (**Figure 5A**), but it did not affect caspase-8 cleavage in ADPKD cells (**Figure 5B**). These results suggest that the apoptotic effects of ouabain in ADPKD cells are not mediated by caspase-8 and the extrinsic pathway of programmed cell death. In addition, the reduction in caspase-8 in NHK cells suggests a protective effect of ouabain toward apoptosis in normal cells.

The Effect of Ouabain on ADPKD Apoptosis is Independent from Cell Proliferation

We have previously shown that ouabain enhances the growth of ADPKD cells (Nguyen et al., 2007). The ouabain-induced activation of apoptosis that we observe in ADPKD cells could just be secondary to the high proliferative effects that ouabain has in these cells. To investigate this possibility, we inhibited cell proliferation in NHK and ADPKD cells with thymidine, and treated the cells with 3 nM ouabain for 24 h. Finally, we determined apoptosis levels by cell sorting, after labeling the cells with Alexa Fluor 488 labeled-Annexin-V. As shown in **Figure 6A**, ouabain had no effect on NHK cell early or late apoptosis, either in the presence of absence of thymidine. In ADPKD cells, ouabain activated programmed cell death, even when cell proliferation was blocked with thymidine (**Figure 6B**). This shows that the effects of ouabain on ADPKD cell apoptosis directly target programmed cell death and they are not an indirect consequence

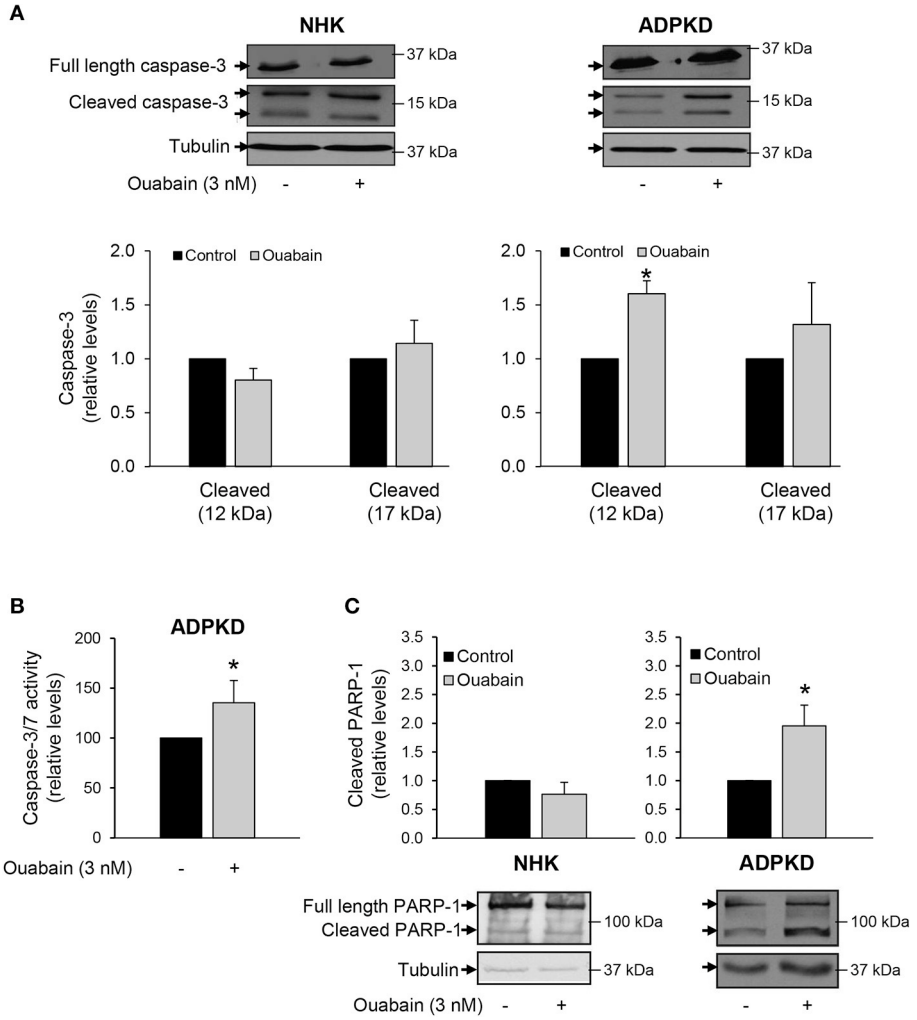

FIGURE 4 | Ouabain stimulates caspase-3 cleavage and activity in ADPKD, but not NHK cells. (A) Caspase-3 cleavage. After treatment with 3 nM ouabain for 24 h, the total (35 kDa) and cleaved products (17 and 12 kDa) of caspase-3 were determined in NHK and ADPKD cells by immunoblot and densitometric analysis. Upper panels show representative blots and bottom panels the densitometric analysis from three different experiments. Bars are the mean ± SEM of three experiments. **(B)** Relative caspase-3 and -7 activity levels. ADPKD cells were treated in the absence and presence of 3 nM ouabain and caspase activity was measured using the Caspase-3/7 Glo Assay. Data are the mean ± SEM of sextuplicate experiments. **(C)** PARP-1 cleavage. After ouabain treatment for 24 h, NHK and ADPKD cell lysates were subjected to immunoblot to determine fragmentation of the caspase-3 substrate, PARP-1. The top panels show the densitometric analysis of the total and cleaved (89 kDa) PARP-1 bands. Cleaved PARP-1 is expressed relative to the corresponding untreated controls. The bottom panels show representative immunoblots. Bars represent the mean ± SEM of 5 different experiments. Asterisks show statistically different values, compared to untreated controls and with $P < 0.05$.

of the exacerbated growth that the hormone causes in the cells.

DISCUSSION

In this work we have shown that ouabain stimulates apoptosis in human ADPKD cells. Thus, ouabain causes changes in plasma membrane phospholipids, induces DNA fragmentation, alters the balance of BCL-2 protein expression, favors release of cytochrome c from mitochondria, and activates caspase-3/7; all of which are typical events of programmed cell death. We found that the levels of apoptosis that ouabain promotes are modest; however, this agrees with the apoptosis seen in ADPKD cells, which cannot compensate for the proliferative nature of the disease (Woo, 1995; Lanoix et al., 1996). Also, we found that apoptosis levels varied depending on the method used for cell death determination, being slightly higher when Alexa Fluor 488 labeled-Annexin-V/PI was used, as compared to tunel assay. This may reflect differences in the end points measured in each case (plama membrane vs. DNA changes respectively), or differences in the sensitivity of each assay. Ouabain-induced ADPKD apoptosis occurs even when cell proliferation in the cells is blocked. This suggests that the enhancement of ADPKD cell apoptosis by ouabain is due to a direct action of ouabain

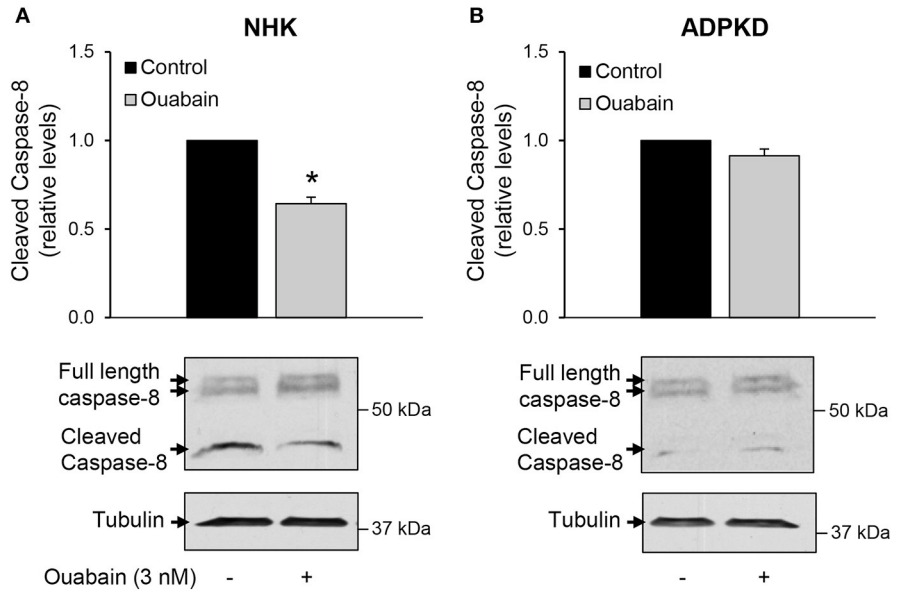

FIGURE 5 | Ouabain does not affect the extrinsic pathway for apoptosis in ADPKD cells. NHK **(A)** and ADPKD **(B)** cells were treated with 3 nM ouabain for 24 h and the total (41/43 kDa) and cleaved (37 kDa) forms of caspase-8 were determined by immunoblot analysis and quantified by densitometry. The top panels show the densitometric analysis of the bands from 4 different experiments. The bottom panels show representative blots. Values are the mean ± SEM. Asterisks indicate statistically different values, with $P < 0.05$ vs. untreated control.

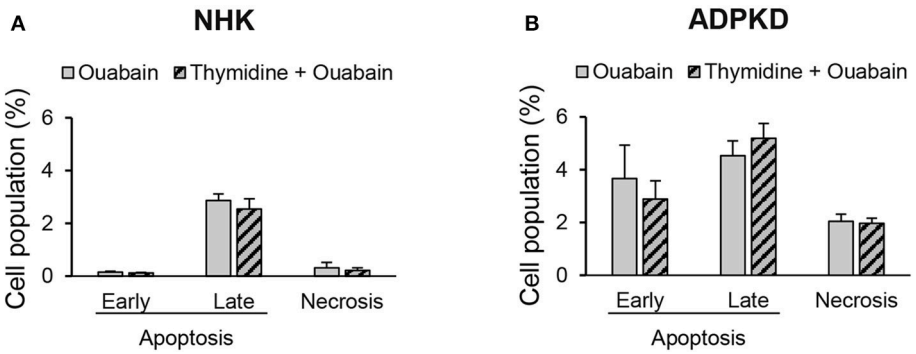

FIGURE 6 | Ouabain induces ADPKD cell apoptosis independent from its proliferative effects. NHK **(A)** and ADPKD **(B)** cells were treated with or without 3 nM ouabain, in the presence and absence of 2.5 M thymidine to arrest cell growth. Alexa fluor-labeled-annexin-V and PI staining and cell sorting were utilized to detect apoptosis/necrosis. Bars represent the mean ± SEM of 3 different experiments. While the values for ouabain induced apoptosis were statistically significant between NHK and ADPKD cells ($P < 0.05$), no statistical differences were found between cells treated with ouabain in the presence and absence of thymidine, both for NHK or ADPKD samples.

on programmed cell death, and not a secondary consequence of increased cell growth, which is another effect induced by ouabain in these cells (Blanco and Wallace, 2013). The role of ouabain on apoptosis has been shown to be cell type dependent (Silva and Soares-da-Silva, 2012). Similar to ADPKD cells, various cancer cell types either undergo apoptosis when treated with ouabain, or are sensitized for apoptosis triggered by other compounds (Huang et al., 2004; Winnicka et al., 2007; Qiu et al., 2008; Bloise et al., 2009; Xu et al., 2010; Platonova et al., 2011; Alonso et al., 2013; Chen et al., 2014; Wang et al., 2014). Therefore, ADPKD are not the only diseased cells that react to ouabain with an apoptotic response.

We show that, different from ADPKD cells, ouabain does not induce apoptosis in NHK cells. In agreement with these observations, ouabain does not trigger programmed cell death in renal proximal tubule cells and explanted embryonic kidneys from normal rats. Moreover, in those studies, ouabain was shown to have a protective effect against apoptosis induced by serum-starvation (Li et al., 2010) and Shiga toxin-2 infection (Burlaka et al., 2013). While the goal of our experiments was not to explore the effects of ouabain under stimuli that challenge the cells to undergo apoptosis, we find a decrease in cytochrome c release from mitochondria, a reduction of ouabain-induced caspase-8 cleavage, and a trend for lower caspase-3 cleavage in NHK

cells after ouabain treatment. This suggests that ouabain may be shifting NHK cells toward a pro-survival phenotype. In any case, it appears that, despite the differences in species and renal origin, ouabain acts as a pro-survival agent in normal kidney cells.

Interestingly, increased apoptosis is a feature of early and late stages of human ADPKD (Woo, 1995; Lanoix et al., 1996), and it is linked to cystogenesis in various animal models of polycystic kidney disease (Moser et al., 1997; Trudel et al., 1998; Lin et al., 1999; Lager et al., 2001). ADPKD apoptosis has been shown to take place in the epithelial cells lining the kidney cysts, and in cysts formed by Madin-Darby canine kidney cells (MDCK) grown in collagen matrix, a model of kidney cystic disease (Woo, 1995; Lin et al., 1999; Ecder et al., 2002; Ibrahim, 2007). Moreover, polycystin 1, the primary gene altered in ADPKD, has been shown to be a regulator of programmed cell death in renal cells, and its over-expression confers the host cells resistance to apoptosis (Boletta et al., 2000). Apoptosis has also been detected in non-cystic tissue in ADPKD kidneys, which suggests that programmed cell death of the normal remaining kidney contributes to the progressive deterioration of ADPKD renal function (Woo, 1995; Ibrahim, 2007; Goilav et al., 2008). Therefore, it appears that apoptosis is an essential concomitant event that helps kidney cyst development, and along with cell proliferation contributes to the pathophysiology of ADPKD. Our results show that in human cells, the target for ouabain-induced apoptosis is the cystic, ADPKD cells, and not the NHK cells. The apoptotic effect of ouabain in ADPKD cells, along with the aberrant increase that ouabain promotes in ADPKD cell growth (Nguyen et al., 2007), places ouabain as a modulator of two essential mechanisms involved in the pathophysiology of ADPKD. It is clear that that the increase in apoptosis caused by ouabain is not sufficient to overcome the ouabain-induced ADPKD cell growth. Therefore, ouabain functions as a factor that creates a dysregulation of ADPKD cell growth/death, in favor of cell proliferation.

Our studies used amounts of ouabain that are within those circulating in plasma (Bagrov et al., 2009). This highlights the relevance of ouabain as a factor that contributes to the pathophysiology of ADPKD. At present, the molecular basis for the different responses of ADPKD and NHK cell to ouabain is unclear; however, we have previously found that, different from NHK cells, ADPKD cells contain a fraction of the total Na,K-ATPase with an abnormal higher affinity for ouabain (Nguyen et al., 2007). Due to their increased ouabain affinity, ADPKD cells may be just more susceptible to the endogenous circulating levels of ouabain, to which NHK cells do respond to the same extent. In addition to their differential sensitivity to ouabain, ADPKD cells also differ from NHK cells in the activity of several intracellular signaling pathways. For example, it is known that the kinase B-Raf and the ERK pathway have an abnormal reactivity to different circulating factors in ADPKD cells (Yamaguchi et al., 2003; Rajagopal and Wallace, 2015). It is therefore possible that, in ADPKD cells, ouabain impinges on pathways which respond in an exacerbated manner on apoptotic effectors to cause programmed cell death. Undoubtedly, more studies are necessary before we can fully understand the molecular mechanisms by which ADPKD cells respond differently to the variety of stimuli that circulate in blood, including ouabain.

As shown from our labeling experiments with PI, ouabain does not cause cell necrosis, which suggests that ouabain effects are non-toxic for the ADPKD cells. This agrees with the notion that, in ADPKD cells, ouabain is not completely inhibiting Na,K-ATPase ion transport, but that at relatively low, physiologic amounts, it activates downstream effectors, as we have shown before (Nguyen et al., 2011). In further support of this, we here found that ouabain activates several mediators in the signaling pathway that leads to apoptosis. Thus, in ADPKD, but not NHK cells, ouabain causes an inbalance in the expression of the BCL-2 and BAX proteins, involved in anti- and pro-apoptotic effects respectively. Ouabain slightly inhibited BCL-2 expression and augmented BAX levels, agreeing with induction of apoptosis in the cells. Interestingly, BCL-2 deficient mice show increased kidney apoptosis and the development of renal cyst disease (Veis et al., 1993). The downregulation of BCL-2 and the pro-apoptotic effects of ouabain may represent mechanisms, which together with those on cell growth and fluid secretion, contribute to the enhancement of the cystic phenotype of ADPKD cells. The decrease in BCL-2 protein concentration in response to ouabain is not unique to ADPKD cells and it has been found in other cell types (Kulikov et al., 2007; Sapia et al., 2010; Trenti et al., 2014). Moreover, overexpression of either BCL-2 or BCL-XL has been reported to abrogate the pro-apoptotic effects of ouabain in a lymphoma cell line (Gilbert and Knox, 1997). Interestingly, the involvement of the BCL-2 family of proteins in ouabain-mediated effects in cells is also supported by the finding that the Na,K-ATPase α subunit contains BH1- and BH3-like motifs, similar to those involved in the pairing of BCL-2 family proteins among each other (Zha et al., 1996; Lauf et al., 2015), and that the BCL-2 family member proteins, BCL-XL and BAK1 co-immunoprecipitate with Na,K-ATPase in A549 lung cancer cells and in fetal human epithelial lens cells (Lauf et al., 2015). While it is unknown whether Na,K-ATPase acts as a scaffolding protein to mediate pro-apoptotic effects via its BH1- and BH3-motifs in ADPKD cells, it is clear that BCL family proteins are involved in ouabain-induced apoptotic effects in the renal epithelial cystic cells.

Besides stimulating the expression of BCL-2 protein, ouabain also impacts on other important mediator of apoptosis in ADPKD cells, such as the mitochondria, through the release of cytochrome c. Moreover, ouabain activates the executioner caspases 3 and 7. Instead, ouabain does not activate the cleavage of caspase-8, an essential effector of the extrinsic apoptotic pathway. Therefore, ouabain induces ADPKD cell apoptosis via specific activation of the intrinsic pathway of programmed cell death. Ouabain has been shown to activate the intrinsic apoptotic pathway in other cell types (Xiao et al., 2002; Kulikov et al., 2007; Alonso et al., 2013). Importantly, our results concur with studies in the Han:SPRD rat, a rodent model of ADPKD, in which the increase in apoptosis is dependent on activation of the intrinsic pathway of programmed cell death (Edelstein, 2005; Tao et al., 2005). In this manner, ouabain enhances an apoptotic route, which commonly participates in ADPKD programmed cell death.

In summary, we have further advanced our understanding of the effects of ouabain in ADPKD cells and found that physiological amounts of ouabain stimulate apoptosis in these cells through activation of the intrinsic pathway of programmed cell death. Activation of this characteristic event of ADPKD, together with other enhancing actions of ouabain on the ADPKD phenotype (Blanco and Wallace, 2013), further supports the role of ouabain as a non-genetic factor that can modulate renal cystogenesis and the progression of ADPKD.

AUTHOR CONTRIBUTIONS

Conception and design: GB; completion of experiments: JV; analysis and interpretation of data: JV and GB; drafting the article: JV; article revision and approval of the final version of the manuscript: JV and GB.

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MINI REVIEW
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Beneficial Renal and Pancreatic Phenotypes in a Mouse Deficient in FXYD2 Regulatory Subunit of Na,K-ATPase

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The fundamental role of Na,K-ATPase in eukaryotic cells calls for complex and efficient regulation of its activity. Besides alterations in gene expression and trafficking, kinetic properties of the pump are modulated by reversible association with single span membrane proteins, the FXYDs. Seven members of the family are expressed in a tissue-specific manner, affecting pump kinetics in all possible permutations. This mini-review focuses on functional properties of FXYD2 studied in transfected cells, and on noteworthy and unexpected phenotypes discovered in a *Fxyd2*$^{-/-}$ mouse. FXYD2, the gamma subunit, reduces activity of Na,K-ATPase either by decreasing affinity for Na^+, or reducing V_{max}. FXYD2 mRNA splicing and editing provide another layer for regulation of Na,K-ATPase. In kidney of knockouts, there was elevated activity for Na,K-ATPase and for NCC and NKCC2 apical sodium transporters. That should lead to sodium retention and hypertension, however, the mice were in sodium balance and normotensive. Adult *Fxyd2*$^{-/-}$ mice also exhibited a mild pancreatic phenotype with enhanced glucose tolerance, elevation of circulating insulin, but no insulin resistance. There was an increase in beta cell proliferation and beta cell mass that correlated with activation of the PI3K-Akt pathway. The *Fxyd2*$^{-/-}$ mice are thus in a highly desirable state: the animals are resistant to Na^+ retention, and showed improved glucose control, i.e., they display favorable metabolic adaptations to protect against development of salt-sensitive hypertension and diabetes. Investigation of the mechanisms of these adaptations in the mouse has the potential to unveil a novel therapeutic FXYD2-dependent strategy.

Keywords: gamma subunit, knockout mouse, renal sodium transporters, hypertension, adaptation, pancreatic islets, beta cells

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REGULATORY SUBUNITS OF NA,K-ATPASE

FXYDs are established regulators of Na,K-ATPase (Garty and Karlish, 2005; Geering, 2006). This is a family of seven small single span membrane proteins (FXYD1-FXYD7) with differential and actively regulated expression in tissues and cells (Sweadner and Rael, 2000; Geering, 2006; **Figure 1A**). Association of each with Na,K-ATPase leads to modulation of activity of the enzyme in all possible ways: inhibition or activation of V_{max}, and increase or decrease of affinity for Na^+, K^+, or ATP. A remarkable feature of FXYD proteins is their interchangeability, which allows fine-tuning of kinetic properties of the Na,K-ATPase in order to adjust to any given physiological or

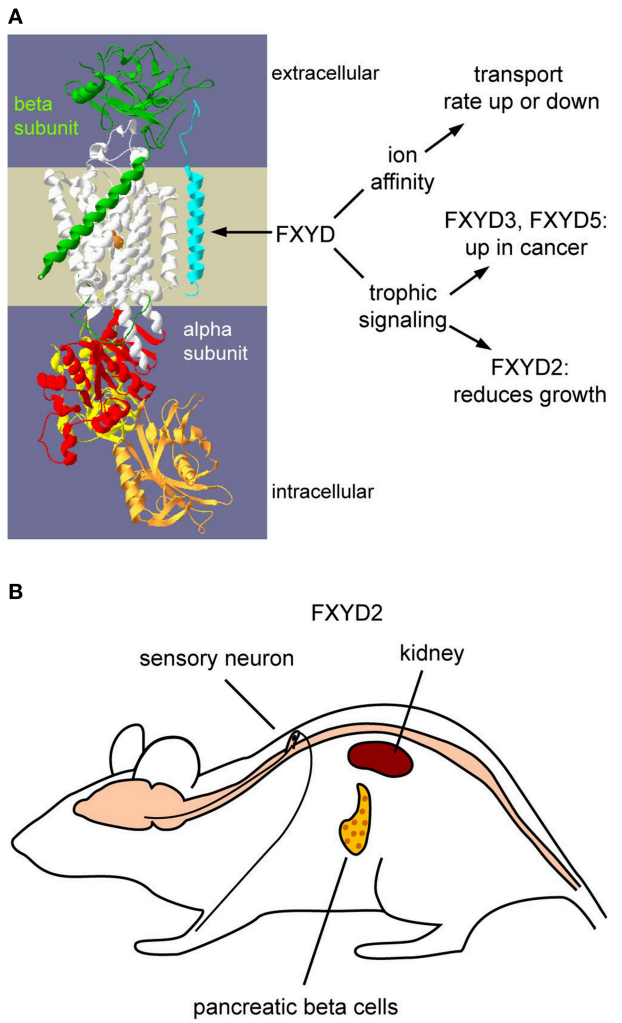

FIGURE 1 | Functions of FXYDs and the tissue distribution of FXYD2. **(A)** Schematic representation of how FXYD proteins alter functions of Na,K-ATPase. Effects on affinities for substrates are well-characterized. How FXYD proteins function at the molecular level for the demonstrated alterations of signaling pathways has yet to be determined. **(B)** Expression sites of FXYD2 in rodents. The principal sites are renal tubules, pancreatic beta cells, and sensory neurons. Mammary glands may also express FXYD2, but this remains to be systematically investigated.

pathological situation (Arystarkhova et al., 2007). Based on crystal structures of Na,K-ATPase, the FXYD subunit is positioned on the periphery of the complex (Toyoshima et al., 2011), which may be a structural basis for "easy" exchange of regulatory subunits. Association of FXYD proteins with alpha/beta complex occurs post-translationally either in Golgi or even at the plasma membrane (Crambert et al., 2002; Pihakaski-Maunsbach et al., 2008; Moshitzky et al., 2012). This may facilitate exchange of regulatory subunits within an already-functioning complex.

FXYD2 protein was the first member of the family associated with Na,K-ATPase: it was identified as a proteolipid labeled with a photosensitive-derivative of ouabain, specific inhibitor of Na,K-ATPase, along with the alpha and beta subunits (Forbush et al., 1978). Further analysis of the "gamma subunit" (FXYD2) confirmed its intimate relation to Na,K-ATPase by co-localization and co-immunoprecipitation with the alpha subunit from kidney membranes (Mercer et al., 1993). Distribution of FXYD2 (at least in rodent) is quite limited: it is highly expressed in kidney with lower representation in pancreas and even lower in mammary gland (Sweadner et al., 2000) and dorsal root ganglia (Venteo et al., 2012; Wang et al., 2015; **Figure 1B**). FXYD2 stands out from the family by being present in two splice variants: FXYD2a and FXYD2b, that differ only at the small N-terminal segment exposed on the extracellular side (Küster et al., 2000; Sweadner et al., 2000; Arystarkhova et al., 2002b). The distribution patterns of the FXYD2 splice variants in kidney are not identical (Pu et al., 2001; Arystarkhova et al., 2002b). Co-expression of FXYD2a and FXYD2b is seen in medullary thick ascending limb. Proximal convoluted tubules however, express only FXYD2a, while only FXYD2b is found in distal convoluted tubules and connecting tubules. The data suggest engagement of the distinct segments of FXYD2a and FXYD2b in extracellular association with tubule-specific partners that are not identified yet. It should be noted that association with multiple partners has been described for other FXYD proteins, such as FXYD1 with Na^+/Ca^{2+} exchanger (Wang et al., 2006) or L-type Ca^{2+} channel (Guo et al., 2010), and FXYD4 with KCNQ1 K^+ channel (Jespersen et al., 2006).

FXYDs can also influence cell growth through downstream signaling pathways. FXYD3 and FXYD5 (dysadherin) are expressed in some normal tissues but are elevated in some types of cancer (Arimochi et al., 2007; Nam et al., 2007). We have demonstrated that FXYD2 decreases cell growth rates in culture (Arystarkhova et al., 1999, 2002a, 2007; Wetzel et al., 2004), while FXYD3 has been demonstrated to increase growth rates (Kayed et al., 2006). This is yet another example of the multiplicity of ways that different FXYDs can act.

FUNCTIONAL PROPERTIES OF FXYD2 LEARNED FROM TRANSFECTED CELLS

Remarkably, no endogenously expressed FXYD2 protein is found in any renal mammalian cell line (Arystarkhova et al., 2007); this is also true of a pancreatic beta cell line (Arystarkhova et al., 2013). Functional assessment of FXYD2 has been explored in expression systems. Reduction of the affinity for Na^+ is the most widely-supported effect of FXYD2 (Béguin et al., 1997; Arystarkhova et al., 1999; Therien et al., 1999). Notably, both splice variants, FXYD2a and FXYD2b, similarly inhibited Na,K-ATPase by decreasing apparent affinity for Na^+ (Arystarkhova et al., 2002a). This rules out the N-terminal splice variants for modulation of this kinetic parameter. Further structure-function studies implicated the transmembrane segment of FXYD proteins in the control of Na^+ affinity (Lindzen et al., 2003).

Unexpectedly though, an unidentified post-translational modification relieved FXYD2a-mediated reduction of apparent Na^+ affinity in stably-transfected cells (Arystarkhova et al.,

2002a). Post-translational modification is a common theme in the FXYD family: phosphorylation (Silverman et al., 2005; Fuller et al., 2009), palmitoylation (Tulloch et al., 2011), *O*-glycosylation (Tsuiji et al., 2003; Crambert et al., 2004), and *S*-glutathionylation (Bibert et al., 2011) have all been reported for various FXYDs. The palmitoylation and *S*-glutathionylation entail modification of one or both cysteine residues immediately following the transmembrane span. Palmitoylation was required for Na,K-ATPase inhibition by FXYD1 (Tulloch et al., 2011), while *S*-glutathionylation of FXYD1 correlated with reduction of inhibition mediated by *S*-glutathionylation of the β1 subunit (Bibert et al., 2011). FXYD2 is not susceptible to *S*-glutathionylation (Bibert et al., 2011), but in silico analysis of FXYD2a structure predicts Cys52 (FXYD2a numbering) as a potential site for palmitoylation (Tulloch et al., 2011). However, whether FXYD2 is indeed palmitoylated and whether this kind of regulation takes place *in vivo* awaits further investigation.

In addition to mRNA splicing and post-translational modifications, we observed that mRNA for FXYD2b could be selectively edited, at least in transfected cells (Sweadner et al., 2011). Single base substitution C172U of the RNA results in introduction of a premature in-frame stop codon. The truncated protein was retained intracellularly due to exposure of an ER retrieval signal, -KKXX. The RNA editing was observed in several established cell lines of different origin (NRK-52E renal epithelial cells, C6 glioma, and L6 myotubes) thus generalizing the phenomenon. Functionally, expression of the truncated form of FXYD2b completely abrogated the reduction of apparent Na^+ affinity of Na,K-ATPase activity. Cells expressing truncated FXYD2b also exhibited no growth delay as opposed to transfectants with full length FXYD2b. Remarkably, no truncation was found for FXYD2a, suggesting selective post-transcriptional control of FXYD2b. This is a potential indirect mechanism for differential regulation of Na^+ affinity by the splice forms. Whether the modification of the transcript plays a protective role, ensuring that FXYD2b is inactive even if made in cells where it may be deleterious, or the truncated protein has another biological role, remains to be discovered.

FXYD2a was upregulated in cells by several types of cellular stress, including hyperosmotic and oxidative (Wetzel et al., 2004). In these conditions it reduced the V_{max} of the enzyme. The stress-related response was observed in cells of different origins, indicating a general cell response via a signaling process linked to the primary insult. In parallel to inhibition of Na,K-ATPase activity, expression of FXYD2 (either by transfection or induction) correlated with a reduction in rate of cell growth (Wetzel et al., 2004). An escape from cell growth delay was achieved by selective siRNA silencing of FXYD2 during ongoing stress, suggesting the adaptive value of FXYD2 as a general cellular mechanism of regulation of cell growth.

Taken together, FXYD2 can be considered an endogenous inhibitory subunit of Na,K-ATPase affecting the pump's activity via reduction of affinity for Na^+ and V_{max}. The control occurs at multiple levels, such as alternative splicing, post-translational modification, and mRNA editing. The FXYD proteins evidently also participate in fine-tuning the signaling role of Na,K-ATPase.

FUNCTIONAL PROPERTIES OF FXYD2 LEARNED FROM KNOCKOUT MICE

Generation of knockout mice was an essential step toward characterization of FXYD2. Global knockout of FXYD2 was performed via replacement of the transmembrane domain coding sequence with a LacZ cassette with a selectable marker (Jones et al., 2005). The higher Na^+ affinity in assays of kidney membranes from *Fxyd2*$^{-/-}$ knockout mice confirmed that FXYD2 normally reduces the Na^+ affinity of Na,K-ATPase, thus complementing studies from transfected cells. The observed changes were in a physiological range suitable for modulation of Na,K-ATPase by hormones. No significant variation of K^+ affinity or reduction in the affinity for ATP was observed between in *Fxyd2*$^{-/-}$ and WT, suggesting that alterations observed earlier in different expression systems (Therien et al., 1999; Arystarkhova et al., 2002a; Pu et al., 2002) might be cell specific.

FXYD2$^{-/-}$ MICE HAVE A PANCREATIC PHENOTYPE

Although viable and fertile, the knockout mice had obvious problems with reproduction: reduced apparent conception rate, smaller surviving litter size, and reduced percentage of successful litters in pairs with *Fxyd2*$^{-/-}$ or even *Fxyd2*$^{+/-}$ dams (Arystarkhova et al., 2013). Thus deletion or even dose reduction of the *Fxyd2* gene was unfavorable for female reproduction. However, if pups survived the perinatal period (0–3 days), knockout mice lived a normal life-span with growth parameters not much different from wild type mice.

A renal phenotype was expected since kidney has the highest level of expression of FXYD2 (Arystarkhova et al., 2002a; Wetzel et al., 2004). However, initial analysis did not reveal any significant impairment in renal function in *Fxyd2*$^{-/-}$ mice kept under optimal care. Instead, a reduced blood glucose level was seen in *Fxyd2*$^{-/-}$ mice either under fed or fasted conditions (Arystarkhova et al., 2013). Although this could indicate peripheral resistance to insulin, either young or mature *Fxyd2*$^{-/-}$ mice revealed insulin sensitivity indistinguishable from their wild type controls. Remarkably, there was a dramatic improvement in glucose tolerance in the knockout animals. All of the above imply a metabolic phenotype of low blood glucose in *Fxyd2*$^{-/-}$ mice. Reduction in blood glucose correlated with an elevation in circulating insulin (2–2.5 fold) either under basal or glucose-stimulated conditions (Arystarkhova et al., 2013). Elevated insulin was sustained in pregnant *Fxyd2*$^{-/-}$ and *Fxyd2*$^{+/-}$ dams close to delivery (E18–E20) consistent with maternal pathophysiology as the reason for the fragility and low survival rate of newborn pups, by affecting either milk production or nurturing behavior.

Based on GEO profiles, pancreas is the second tissue (after kidney) in abundance of FXYD2 expression. By RT-PCR and Western blots, both splice variants of FXYD2 were identified in pancreatic islets from rodent and human tissues (Flamez et al., 2010). Immunofluorescence, however, revealed unusual localization of the protein considering its role as a regulatory

subunit of Na,K-ATPase. While some of the FXYD2 was found at the plasma membrane (together with Na,K-ATPase), most FXYD2 was observed in the cytoplasm (Flamez et al., 2010; Arystarkhova et al., 2013). Whether the apparent separation of FXYD2 from Na,K-ATPase indicates intracellular trafficking of FXYD2 within beta cells in response to yet an unidentified physiological stimulus requires further investigation. We did not detect the edited mRNA product by RT-PCR. In pancreas FXYD2 may play a biological role distinct from regulation of Na,K-ATPase, and the molecular mechanism of intracellular retention may be interaction with another protein.

Functionally, there was no significant difference in ability to secret insulin from freshly isolated pancreatic islets from WT and *Fxyd2*$^{-/-}$ mice (Arystarkhova et al., 2013). However, morphometric analysis revealed a higher number of insulin-producing beta cells per islet cross-sectional area in the knockout mice compared to WT. This led to an increase in total beta cell mass/pancreas from the *Fxyd2*$^{-/-}$ knockouts (Arystarkhova et al., 2013). Thus depletion of FXYD2 from pancreatic islets apparently increases the rate of cell division, consistent with the effect of FXYD2 on growth rate in transfected cells in culture. Beta cell hyperplasia may underlie the elevation of insulin production that in turn may explain the observed phenotype of low blood glucose. No morphological changes were seen in acinar cells from *Fxyd2*$^{-/-}$ mice, implying preferential hyperplasia of the endocrine islet compartment. It should be noted that enhanced proliferation of beta cells in *Fxyd2*$^{-/-}$ mice stayed within the physiological range, i.e., unlike insulinomas. All together the data suggest that removal of FXYD2 is beneficial for pancreatic beta cells.

On the molecular level, freshly isolated islets from *Fxyd2*$^{-/-}$ mice exhibited significantly higher levels of phosphorylation of Akt (PKB; Arystarkhova et al., 2013), one of the central players in regulation of beta cell mass and function (Bernal-Mizrachi et al., 2001; Blandion-Rosano et al., 2012). This may underlie the islet hyperplasia observed in *Fxyd2*$^{-/-}$ mice, and reflect ongoing signaling in adults. Experimentally, inducible expression of FXYD2 in rat INS 832/13 cells was paralleled by a reduction in phosphorylation of Akt, whereas time-dependent degradation of FXYD2 resulted in its gradual elevation (Arystarkhova et al., 2013). PI3 kinase is the upstream activator of Akt kinase, and it had been shown to interact with Na,K-ATPase (Yudowski et al., 2000). A potential scenario is that depletion of FXYD2 leads to a conformational change in Na,K-ATPase and, as a result, sustained activation of PI3K, leading to enhanced activity of Akt. This would implicate FXYD2 as an upstream player in the Akt signaling pathway in pancreatic beta cells. Activation of Akt is one of the important links between growth signals and regulation of β -cell expansion (Bernal-Mizrachi et al., 2001; Elghazi et al., 2007). Downstream targets of Akt, tuberous sclerosis complex 1 and 2 (TSC1/2) and mechanistic target of rapamycin complex 1 (mTORC1), are prime candidates for enhancing cell cycle progression (Blandino-Rosano et al., 2012). The hope is that further investigation of FXYD2 will lead to novel regulators of beta cell mass to enhance insulin secretion for therapeutic purposes.

USEFUL ADAPTATIONS TO FXYD2 DEPLETION FROM KIDNEY

Na,K-ATPase is located in basolateral membranes in renal tubules where it generates the driving force for Na^+ entry across the apical membrane. Theoretically deletion of the inhibitory subunit FXYD2 should enhance renal Na^+ reabsorption by increasing both Na^+ affinity and V_{max} of Na,K-ATPase in the proximal tubule. Measured renal Na,K-ATPase activity in cortical membranes from the knockout mice was elevated as predicted (Arystarkhova et al., 2014), and so a renal phenotype was expected. Under basal conditions no significant differences between WT and knockout mouse were found in plasma concentration of Na^+ or in plasma osmolality, implying no increase in sodium retention (Jones et al., 2005). Urine electrolytes (mg/mg creatinine) remained within the normal range in FXYD2-deficient mice (Jones et al., 2005). In addition, FXYD2-deficient mice were normotensive and demonstrated sodium balance indistinguishable from their littermate controls (Arystarkhova et al., 2014). Taken together, the experimental data imply that NaCl uptake or efflux in appropriate renal segments is adaptively controlled in the *Fxyd2*$^{-/-}$ mice.

Regulation of inflow through apical Na^+ transporters is one possibility. Distal convoluted tubule (DCT) and thick ascending limb (TAL) are the segments with the highest level of expression of both Na,K-ATPase and FXYD2. The major apical Na^+ transporters there are sodium/chloride cotransporter (NCC; Subramanya and Ellison, 2014) and sodium/potassium/chloride cotransporter (NKCC2; Ares et al., 2011) in DCT and cortical TAL (cTAL), respectively. Both belong to a family of electroneutral cation-coupled chloride co-transporters. Functionally, both transporters face the lumen of the tubules and perform uptake of Na^+ from the tubular fluid driven by the sodium gradient created by Na,K-ATPase. Activity of both transporters is regulated by expression, trafficking and phosphorylation. In humans, reduced activity of NKCC2 leads to severe salt and volume loss and decreased blood pressure (Bartter syndrome), while reduction in NCC function is associated with salt wasting and low blood pressure (Gitelman syndrome). In contrast, gain of function of either transporter is associated with an increase of Na^+ reabsorption and development of salt-sensitive hypertension (Ares et al., 2011; Hoorn et al., 2011).

If either TAL or DCT were involved in compensation of Na^+ balance in the *Fxyd2*$^{-/-}$ mice, luminal sodium uptake should be reduced in those segments, i.e., activity of NKCC2 or NCC transporters should be diminished. Surprisingly, there was significant up-regulation of NCC protein expression (by 30%) and highly augmented phosphorylation of both cortical NKCC2 (at least two-fold) and NCC (4–6 fold at two phosphorylation sites, pT53 and pS71; Arystarkhova et al., 2014). The level of phosphorylation of both transporters is usually taken as a proxy of their activity. Thus high phosphorylation of NCC and NKCC2 in *Fxyd2*$^{-/-}$ mice would predict even higher Na^+ reabsorption in cTAL and DCT that should eventually lead to hypertension. In practice, *Fxyd2*$^{-/-}$ mice revealed no significant difference in mean arterial pressure (either males or females) under basal conditions when compared to their wild type littermates

(Arystarkhova et al., 2014). Thus the compensated physiology of the mouse and lack of Na^+ retention cannot be explained by downregulation of apical Na^+ entry in TAL and DCT segments.

Acute saline challenge of WT and *Fxyd2*$^{-/-}$ mice also did not reveal a difference in rate of Na^+ excretion between genotypes (Arystarkhova et al., 2014). Therefore activation of both cortical NKCC2 and NCC must be explained by compensation in either more distal or more proximal segments in the knockout mice. No difference was seen in the expression level or activity of the distally-expressed amiloride sensitive epithelial sodium channel, ENaC (Arystarkhova et al., 2014), ruling it out for compensation for an increase in Na,K-ATPase activity, and leaving proximal tubules as a likely site of Na^+ absorption adjustments in the *Fxyd2* knockout mouse.

A testable hypothesis is that increase in activity of Na,K-ATPase in proximal tubule, the principal site of Na^+ reabsorption from filtrate, would cause excess reabsorption of Na^+ into interstitial spaces and lead to activation of the intrarenal dopamine response (Aperia, 2000; Carey and Padia, 2013). This reduces the activity of apical NHE3 transporter, which is thought to be rate-limiting for Na^+ reabsorption (McDonough, 2010), by trafficking it toward the base of the apical villi (Chen et al., 2009; McDonough, 2010), and internalizes Na,K-ATPase as well (Pedemonte et al., 2005; Gildea et al., 2009). The Na^+ concentration in the lumen in proximal tubules would then stay high, and the observed hyperstimulation of the downstream NKCC2 and NCC transporters would be adaptive, bringing the FXYD2 knockout mouse into Na^+ balance.

CONCLUSIONS

To summarize, *Fxyd2*$^{-/-}$ mice are in a desirable state when considering the risk factors of metabolic syndrome. The animals are resistant to elevation of glucose in blood as well as to Na^+ retention, i.e., they display features unfavorable for development of diabetes or salt-sensitive hypertension. In addition, deletion of FXYD2 from dorsal root ganglion neurons correlated with a marked reduction in a nociceptive adaptation (allodynia) to inflammation (Wang et al., 2015). Is there a common trait in these beneficial phenotypes observed in different tissues? We showed that FXYD2 modulates both ion transport (Arystarkhova et al., 1999, 2002a) and signaling (Arystarkhova et al., 2013). Investigation of mechanism(s) underlying favorable metabolic adaptations in the knockout mouse will be a challenge for the coming years.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Na,K-ATPase Isozymes in Colorectal Cancer and Liver Metastases

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The goal of this study was to define Na,K-ATPase α and β subunit isoform expression and isozyme composition in colorectal cancer cells and liver metastases. The α1, α3, and β1 isoforms were the most highly expressed in tumor cells and metastases; in the plasma membrane of non-neoplastic cells and mainly in a cytoplasmic location in tumor cells. α1β1 and α3β1 isozymes found in tumor and metastatic cells exhibit the highest and lowest Na^+ affinity respectively and the highest K^+ affinity. Mesenchymal cell isozymes possess an intermediate Na^+ affinity and a low K^+ affinity. In cancer, these ions are likely to favor optimal conditions for the function of nuclear enzymes involved in mitosis, especially a high intra-nuclear K^+ concentration. A major and striking finding of this study was that in liver, metastasized CRC cells express the α3β1 isozyme. Thus, the α3β1 isozyme could potentially serve as a novel exploratory biomarker of CRC metastatic cells in liver.

Keywords: Na/K-ATPase isozymes, sodium pump isozymes, colorectal cancer, colorectal cancer liver metastases, Na/K-ATPase isoforms colorectal cancer immunohistochemistry

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INTRODUCTION

Colorectal cancer (CRC) is one of the major causes of neoplasia-related morbidity and mortality, representing the second major cause of disease incidence among females and the third among males (Jemal et al., 2011). In the western world, CRC is the 4th leading cause of death (Ferlay et al., 2010). Metastatic CRC cells can invade, populate and flourish in a new niche and ultimately cause organ dysfunction and death. CRC spawns metastases in liver, lungs, bone marrow and brain (Chiang and Massague, 2008). However, it is the liver where CRC cells metastasize most frequently (Hess et al., 2006). The first line treatment for CRC involves surgery and adjuvant oxaliplatin based chemotherapy. A common side effect of this treatment strategy is oxaliplatin-induced peripheral neuropathy (Pachman et al., 2014).

Previous research from our group has led to the identification of several genes, which were shown to be significantly up-, or down- regulated in peripheral white cells (PWCs) of CRC patients, due to oxaliplatin-based chemotherapy (Morales et al., 2014). Interestingly, one of the differentially expressed genes was the isoform α3 of the *Na,K-ATPase*; mRNA levels of *Na,K-ATPase* α3 subunit

were down-regulated 2.6-fold. Moreover, an alteration in the intracellular location of Na,K-ATPase α3 isoform has been reported in human CRC tumor cells vs. normal colon (Sakai et al., 2004). Additionally, other laboratories have shown differential expression in cells, altered subcellular localization and down regulation of the β subunit of the Na^+/K^+-ATPase in carcinoma cells (Rajasekaran et al., 1999, 2001a,b, 2010).

Na,K-ATPase is an integral protein in the plasma membrane of all animal cells that transports three sodium ions out and two potassium ions into the cell, against electrochemical gradient (Skou, 1957; Jorgensen et al., 2003). This activity is necessary for the regulation of the cellular ionic homeostasis and maintaining the electrochemical gradient required for ion channel function and secondary active transport (Mobasheri et al., 2000). Recently, additional functions for the Na,K-ATPase in the cell have been proposed, as a signal transducer and transcription activator (Aizman et al., 2001; Miyakawa-Naito et al., 2003; Harwood and Yaqoob, 2005; Yuan et al., 2005; Zhang et al., 2006) affecting cell proliferation (Abramowitz et al., 2003), cell motility (Barwe et al., 2005), and apoptosis (Wang and Yu, 2005). Besides this, the Na,K-ATPase is the receptor of cardiotonic glycosides. It is functionally composed of catalytic α (100–112 kDa) and regulatory β (45–55 kDa) subunit and an optional γ (6.5–10 kDa) subunit belonging to the FXYD family of proteins (Mercer et al., 1993).

Na,K-ATPase is expressed as several isozymes. Four different isoforms of the α subunit have been found in humans (Blanco, 2005). The α1 isoform (*ATP1A1* gene) is expressed almost in all tissues. Isoform α2 (*ATP1A2* gene) is the predominant isoform in skeletal muscle (Hundal et al., 1992), brain (astrocytes) (McGrail et al., 1991), heart (Zahler et al., 1992), and adipose tissue (Lytton et al., 1985). The α3 isoform (*ATP1A3* gene) is primarily found in the brain (neurons) (Hieber et al., 1991; McGrail et al., 1991) and isoform α4 (*ATP1A4* gene) is only expressed in testis (Woo et al., 2000). In reference to the β subunit, three different isoforms have been identified: β1 (*ATP1B1* gene), β2 (*ATP1B2* gene) and β3 (*ATP1B3* gene). While β1 has a generalized expression in almost all tissues and cells, the expression of the other β isoforms are more restricted to certain tissues and cells. The β2 isoform is found in skeletal muscle (Lavoie et al., 1997), pineal gland (Shyjan et al., 1990), and nervous tissues (Peng et al., 1997), whereas β3 is present in testis, retina, liver, and lung (Malik et al., 1996; Zahler et al., 1996; Arystarkhova and Sweadner, 1997; Martin-Vasallo et al., 2000). The expression pattern of the Na,K-ATPase subunit-isoforms is subjected to developmental and hormonal regulation and can be altered during disease (Book et al., 1994; Charlemagne et al., 1994; Charlemagne and Swynghedauw, 1995; Ewart and Klip, 1995; Zahler et al., 1996).

The purpose of this study was to determine the cellular and subcellular localization of the α and β subunit isoforms of Na,K-ATPase in CRC and its liver metastasis using a panel of well-characterized isoform-specific antibodies. The primary hypothesis of this study was that metastatic cancer cells possess a unique expression phenotype of Na,K-ATPase isozymes, similar to that of CRC cells.

MATERIALS AND METHODS

Tissue Samples

The Ethics Committee of the Universidad de La Laguna (ULL) and Ethical Committee of the Hospital Universitario Nuestra Señora de Candelaria (HUNSC) approved this study. All patients signed an informed-consent document for diagnosis and research on tissue specimen before being enrolled in the project. All the study subjects were treated with FOLFOX CT: day 1 oxaliplatin 100 mg/m^2 iv over 2 h; leucovorin calcium 400 mg/m^2 iv over 2 h; followed by 5-fluorouracil 400 mg/m^2 iv bolus and by 5-fluorouracil 2400 mg/m^2 iv over 46 h; every 14 days. Paraffin-embedded tissue samples and clinical data were obtained from 15 patients (7 males, 8 females) and 1 control male from the reference medical areas of HUNSC.

Antibodies

Table 1 shows antibodies and references used in this study. Secondary antibodies used were goat anti-rabbit IgG or goat anti-mouse IgG. Biotinylated secondary antibody was used for immunohistochemistry (IHC), whereas secondary antibodies targeted with specific fluorochromes were used for immunofluorescence (IF).

Immunohistochemistry

Five-micron thick paraffin embedded tissue sections were deparaffinized in xylene and hydrated in graded series of alcohol baths. Heat mediated antigen retrieval was performed in an autoclave at 120°C for 10 min in sodium citrate buffer pH 6.0 before commencing the IHC staining protocol. To remove endogenous peroxidase activity, sections were incubated with 3% H_2O_2 in methanol for 15 min at room temperature. Non-specific sites were blocked with 5% Fetal Bovine Serum (FBS), 0.3% Triton-X-100 in Tris-buffered saline (TBS) for 1 h at room temperature. Endogen biotin was blocked with the Avidin/Biotin Vector Blocking Kit (Vector Laboratories Inc., #SP-2001, Burlingame, CA 94010, USA) according to the manufacturer's instructions. Primary antibodies (see **Table 1**) were incubated O/N at 4°C. Slices were then incubated for 2 h at 37°C with biotin-conjugated secondary antibodies (see **Table 1**). Antibodies for IHC were diluted in TBS, 5% FBS, 0.1% Triton. To amplify the specific antibody staining, ABC complex (Pierce, Thermo Fisher Scientific Inc., #32020, Waltham, MA, USA) was applied to the sections, prepared according to manufacturer's instruction and incubated for 1 h at room temperature. 3,3′-diaminobenzidine (DAB) Substrate Concentrate (Bethyl Laboratories Inc., #.IHC-101F, Montgomery, Texas, USA) was used to visualize immunoperoxidase activity. Slides were counterstained with Harris Hematoxylin solution DC (Panreac, #256991.1610 Barcelona, Spain) to visualize cell nuclei. Samples were mounted with Eukitt (Panreac, #253681, Barcelona, Spain) and optical light microscope (Olympus BX50, Tokyo, Japan) was used to visualize IHC staining results. Images were acquired using the Olympus DP70 camera and the DP controller software 2.1.1.183 (Copyright 2001–2004 Olympus Corporation). Negative control experiments were carried out by

TABLE 1 | Antibodies used in this study. α1(620) (Sztul et al., 1987), α3 (Pietrini et al., 1992), α3 (XVIF9-G10) (Arystarkhova and Sweadner, 1996), SpETβ1 and SpETβ2 (Gonzalez-Martinez et al., 1994).

Antibody	Target	Host	Type	Dilution	Source
α1(620)	α1-isoform*	R	P	1:1000	M. J. Kashgarian
α3	α3-isoform*	R	P	1:600	M. Caplan
α3 (XVIF9-G10)	α3-isoform*	M	Mc	1:5	Arystarkhova and Sweadner
SpETβ1	β1-isoform*	R	P	1:600	P. Martin-Vasallo
SpETβ2	β2-isoform*	R	P	1:600	P. Martin-Vasallo
Anti-proliferating cell antigen (Anti-PCNA)	PCNA	M	Mc	1:100	Boehringer Mannheim
Anti-rabbit IgG (H+L), biotin conjugated (2°)	Rabbit-IgG	G	P	1:300	Pierce
Anti-rabbit IgG (whole molecule), FITC-conjugated (2°)	Rabbit-IgG	G	P	1:200	Sigma
Anti-mouse IgG, DyLight®650-conjugated (2°)	Mouse-IgG	G	P	1:100	Abcam

R, rabbit; G, goat; M, mouse; Mc, monoclonal; P, polyclonal.
**subunit-isoforms of the Na,K-ATPase, 2°: secondary antibody.*

following the procedure stated above but without incubating with primary antibody.

Double Immunofluorescence Simultaneous Staining

As with the IHC samples, tissue sections for IF staining were paraffin embedded. After deparaffinization, hydration and heat-induced epitope retrieval procedure (as described above for the IHC staining), slides were incubated with 5%BSA, 0.3%Triton-X-100 in TBS to block non-specific sites. Then tissue sections were incubated simultaneously with a mixture of two distinct primary antibodies (e.g., rabbit against human target 1 and mouse against human target 2) overnight at 4°C. Slices were then incubated for 1 h at room temperature in dark with a mixture of two secondary antibodies (see **Table 1**) conjugated to two different fluorochromes (i.e., FITC-conjugated against rabbit-Sigma and DyLight®650-conjugated against mouse-Abcam). Antibodies for IF were diluted in TBS, 1% bovine serum albumin (BSA), 0.1% Triton. Slides were mounted with ProLong®Diamond Anti-fade Mountant with DAPI (Molecular Probes by Life Technologies, #P36962, Eugene, Oregon, USA) to visualize cell nuclei. Slides were acquired and analyzed using Olympus confocal microscope (Olympus FV1000, Tokyo, Japan) and the software FV10-ASW1.3; Lasers: Diode 405 nm, Argon multiline 458/488/514, HeNe 633 nm. Images were acquired by sequential scan (first sequence Diode and HeNe, second sequence Argon) to avoid overlapping of channels. Image resolution 1024 × 1024. Objective lens: 60X/1.35 NA oil Plan-Apochromat. Negative control experiments were carried out by following the same immunohistochemical procedure but with the primary antibody omitted.

Image Analysis and Scoring

Samples were evaluated by two independent observers who were blinded to the clinical data. Scores were graded as absent (−), moderate (+) or strong (+ + +) for any specific kind of cell. These cut-offs were established by consensus of all investigators. For all tumors this grading was applied to three different patterns of Na,K-ATPase α and β subunit isoform staining in tumor cells: staining of the plasma membrane; staining of the nuclear envelope and staining of the cytoplasm.

Final results were computed as the product of staining intensities. In cases where scorings differed, the observers re-evaluated samples to consensus. All samples were analyzed and scored.

RESULTS

Na,K-ATPase α1 and α3 Isoform Expression in CRC

In healthy colon tissue (**Figures 1A,C**), the α1 isoform was detected at the basolateral side of the plasma membrane of epithelial cells lining the colonic *mucosae* of Lieberkühn Crypts and in discrete stromal cells in the connective tissue surrounding the crypts. In turn, α1 isoform was mainly detected in a peri-nuclear location in tumor cells (**Figures 1B,D**). Mesenchymal cells from the stromal tissue surrounding the tumor also exhibited positive immunostaining (**Figures 1B,D**).

The Na,K-ATPase α3 isoform was detected on epithelial cells lining the colonic crypts and on cells from the *lamina propria* in healthy colon (**Figure 1E**). The α3 isoform was mainly detected in or near the plasma membrane of epithelial cells and in the cytoplasm of positively stained cells in the stroma. In CRC tumor samples, the α3 isoform was mainly located in a peri-nuclear location in CRC tumor cells, while in the plasma membrane of these cells staining was negative (**Figure 1F**). Stromal cells surrounding the tumor were also α3-positive. Immunolabeling for the α3 isoform was also detected in microvascular endothelial cells (**Figure 1H**) and in cells from the inflammatory reaction associated with CRC within the stroma (**Figure 1G**), where the α3 isoform showed an intense and specific peri-nuclear labeling.

Na,K-ATPase β1 Isoform Expression in CRC

The β1 isoform of Na,K-ATPase was detected in epithelial cells from the normal colonic *mucosae* (**Figure 2A**). There was a high positive staining at the baso-lateral side of polarized epithelial cells that line the colonic Lieberkühn crypts (**Figure 2B**).

In CRC tumors, the β1 isoform presented a less defined expression pattern. This isoform was detected in some tumor cells, but the location was not well-defined, and was seen in

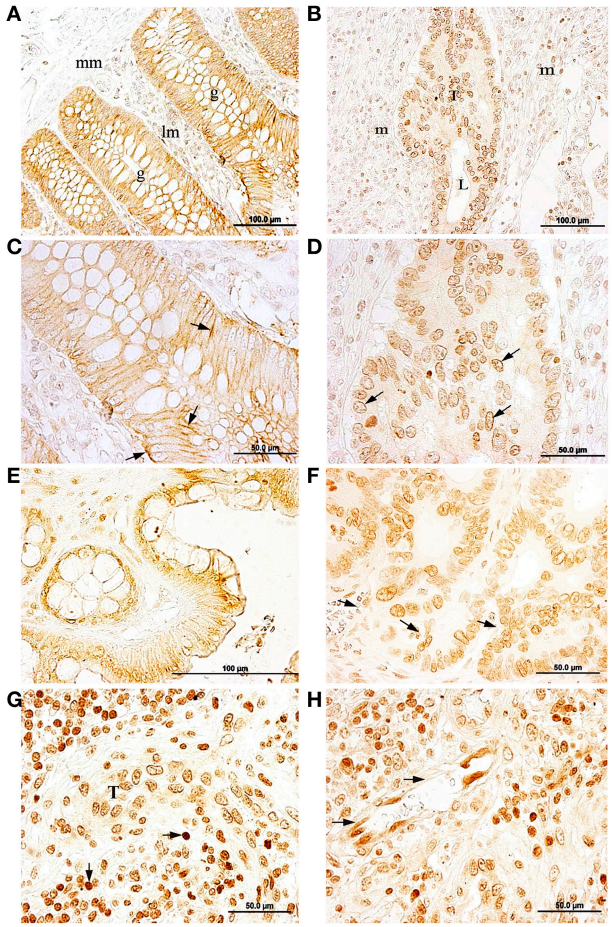

FIGURE 1 | Immunolocalization of the Na,K-ATPase α1 and α3 subunit isoforms in normal colon and colorectal cancer (CRC). (A,C) α1 immunostaining in baso-lateral side of polarized epithelial cells in healthy colonic *mucosae*, black arrow. g, colonic gland (Lieberkühn crypts); mm, *muscularis mucosae*; lm, *lamina propia mucosae*. **(B,D)** peri-nuclear labeling for the α1 isoform in tumor cells. T, tumor; L, lumina; m, mesenchymal tissue. **(E)** α3 immunostaining in plasma membrane of healthy colon epithelial tissue. **(F)** α3 expressed peri-nuclearly in CRC tumor (arrows). **(G)** α3 immunostaining in tumor cells (T) surrounded by immune cells (arrows). **(H)** Endothelial cells expressing the α3 isoform in a peri-nuclear location (arrows).

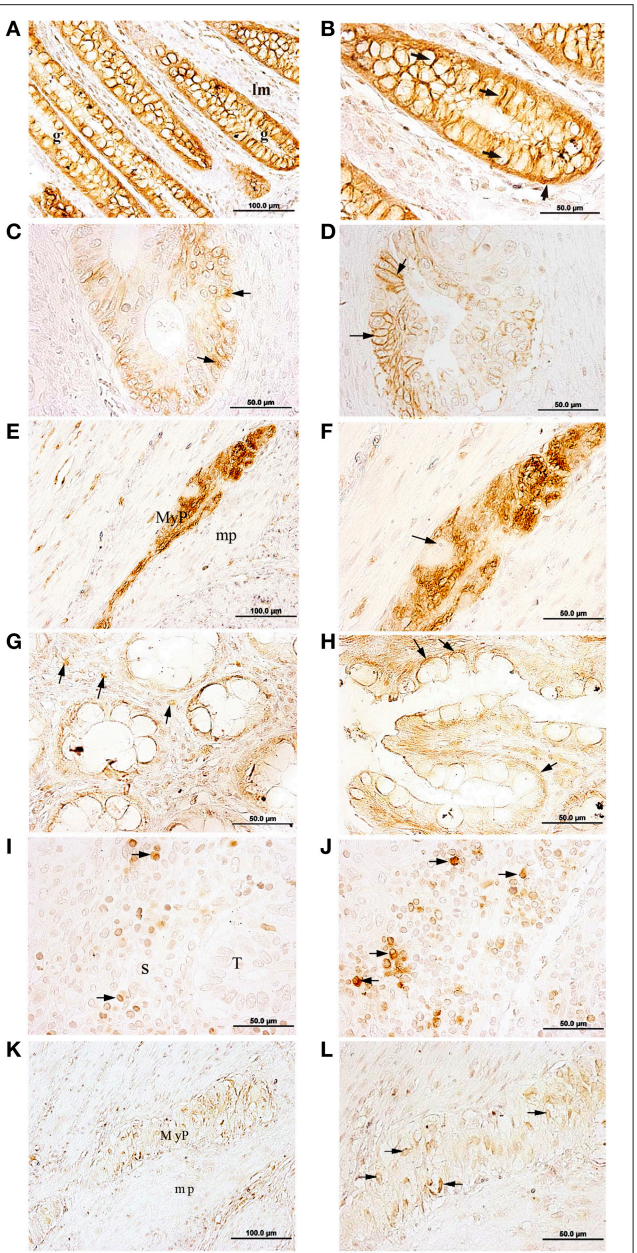

FIGURE 2 | Immunolocalization of the Na,K-ATPase β1 and β2 isoforms in normal colon and CRC. (A,B) β1 is mainly located at the baso-lateral side of polarized epithelial cells from the normal colonic *mucosae* (arrows in **B**). **(C)** β1 is located in the cytosol of tumor cells (arrows). **(D)** β1 is located at the cell membrane of tumor cells (arrows). **(E)** Cells from the myenteric plexus (MyP) at the *muscularis propia* (mp) express β1. **(F)** β1 isoform is mainly located in or near the cytoplasmic membrane of the neurons and/or glia; a neuron nucleus with visible nucleolus is indicated using an arrow. g, colonic gland (Lieberkühn crypts); lm: *lamina propia mucosae*. **(G)** Selected cells from the connective tissue of the *mucosae* express the β2 isoform (arrows). **(H)** β2 is mainly located at the baso-lateral side of polarized epithelial cells from the normal colon *mucosae* (arrows). **(I)** β2 was not detected in tumor cells from adenocarcinomatous glands (T); however, some stroma (S) cells express β2 (arrows). **(J)** Possible leukocytes of the stroma expressing β2 (arrows). **(K)** Cells from the myenteric plexus (MyP) at the *muscularis propia* (mp) express β2. **(L)** β2 isoform is mainly located at the soma of glia cells (arrows).

several subcellular locations within the tumor. In some tumor cells β1 staining was peri-nuclear (**Figure 2C**), while in others a peripheral location was observed (**Figure 2C**).

In addition, in healthy tissue, β1 was detected in cells of the myenteric plexus, also known as the Auerbach's plexus, within the muscular tissue of the *muscularis propia* (**Figure 2E**). In the cells from the plexus, Na,K-ATPase β1 isoform was detected in or near the cytoplasmic membrane of axons and dendrites of neurons and glial cells (**Figure 2F**).

Na,K-ATPase β2 Isoform Expression in CRC

The β2 isoform was detected at the baso-lateral side of polarized epithelial cells from the normal colonic *mucosae* and in

selected fibroblastic and immune cells from the *lamina propria* (**Figure 2G**) and (**Figure 2H**). In colon adenocarcinoma cells, the β2 isoform was not detected (**Figures 2I,J**). However, some immune cells located in the stromal tissue surrounding the tumor, were β2 positive while others remained negative (**Figures 2I,J**). In immunopositive cells, β2 staining was detected peri-nuclearly and also throughout the cytoplasm. Immunostaining intensity for the β2 isoform varied from strong to weak across cells in this region. In myenteric plexus (**Figure 2K**) the β2 isoform was detected in the soma of neural cells (**Figure 2L**).

Co-Expression of Na,K-ATpase α3 and β1 Isoforms and PCNA

In CRC, some cells from adenocarcinomatous glands showed positive staining to both PCNA (nuclei) and Na,K-ATPase α3 isoform (cytoplasm) (**Figure 3**), and PCNA and β1 isoform (plasma membrane) (**Figure 3**).

Na,K-ATPase α1 and α3 Isoform Expression in CRC Metastases in Liver

In healthy liver tissue, hepatocytes were immunopositive for α1, in the plasma membrane (**Figure 4A**). Bile ducts cells were also α1 positive (**Figure 4B**), and the staining was detected mainly at the baso-lateral side of the plasma membrane. In metastases, the Na,K-ATPase α1 isoform was detected in cytoplasm and in the cytoplasmic membrane of cells of metastatic tumor niches (**Figures 4C,D**).

In normal healthy liver, Na,K-ATPase α3 isoform was not detected in any cell types (**Figure 4E**). However, in metastatic tumor cells within the liver, the α3 isoform was detected (**Figure 4F**; Supplementary Figure S1D) in a peri-nuclear location and spread across the cytoplasm. Staining intensity varied among cells, from strong to weak labeling. In addition to tumor cells, this isoform was also detected in immune cells located at the outermost part of the liver (**Figure 4G**). In apparently healthy liver tissue surrounding metastases, Na,K-ATPase α3 isoform was detected peri-nuclearly and also throughout the cytoplasm of hepatocytes (**Figure 4H**).

Na,K-ATPase β1 and β2 Isoforms Expression in Metastasis

In metastasized liver, the β1 isoform was detected at the plasma membrane of hepatocytes (**Figure 5A**), bile ducts epithelial cells (**Figure 5B**) and peri-nuclearly and/or in the cytoplasm of cells in disorganized and necrotic tissue (**Figures 5C,D**; Supplementary Figure S1C).

In healthy liver tissue, the β2 isoform was detected in the cytoplasm of some cells and in peri-nuclear locations in others (**Figures 5E,F**), with variable staining intensities among cells ranging from strong to weak. A weak but specific signal for the β2 isoform was also detected in bile ducts cells at the portal triads (**Figure 5G**). However, the β2 isoform was not detected in metastatic tumor cell niches (**Figure 5H**).

We were unable to detect the α2 and β3 subunit isoforms as their expression levels were probably below the threshold of the ABC amplified immunohistochemical detection technique employed in this study.

Na,K-ATPase α3 and β1 Isoforms Coexpression in Metastasis

In order to further confirm the co-expression of the α3 and β1 subunits isoforms in the same metastatic cells in the liver, using a different and monoclonal antibody, we performed confocal microscopy co-localization experiments. Image analysis and scoring was done using same procedure as in all other cases

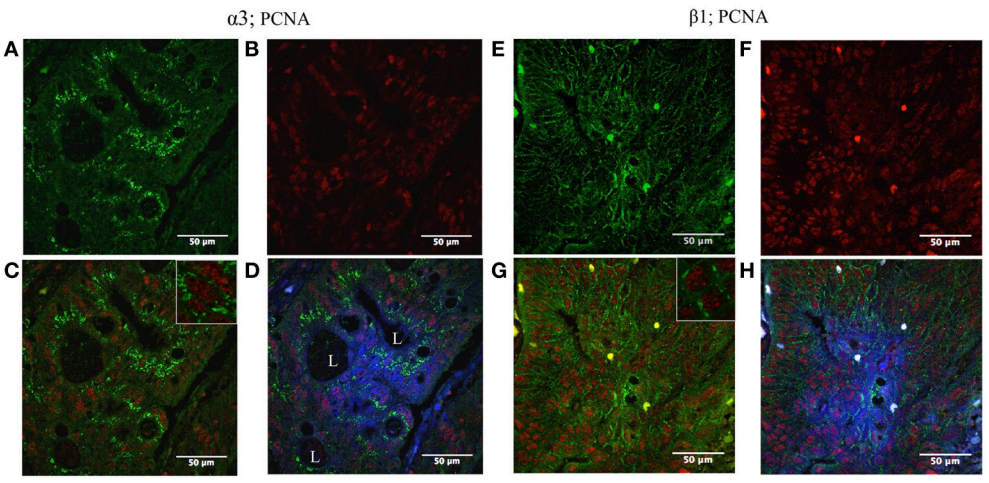

FIGURE 3 | Double immunofluorescence localization of Na,K-ATPase α3 and β1 isoform and proliferating cell nuclear antigen (PCNA) in CRC. Left panel: **(A)** α3 isoform is expressed in colon tumor cells (green). **(B)** High numbers of tumor cells express PCNA (red). **(C)** The tumor cells express both PCNA and the α3 isoform (blue and green merged). **(D)** α3 isoform is mainly located internally at the cytoplasm; blue (DAPI), red (PCNA), and green (α3 isoform) merged image. L, lumina. Right panel: **(E)** β1 isoform is expressed in colonic tumor cells (green). **(F)** High number of tumor cells expresses PCNA (red). **(G)** The tumor cells express both PCNA and β1 isoform (red and green merged). **(H)** Blue (DAPI), red (PCNA), and green (β1 isoform) merged image.

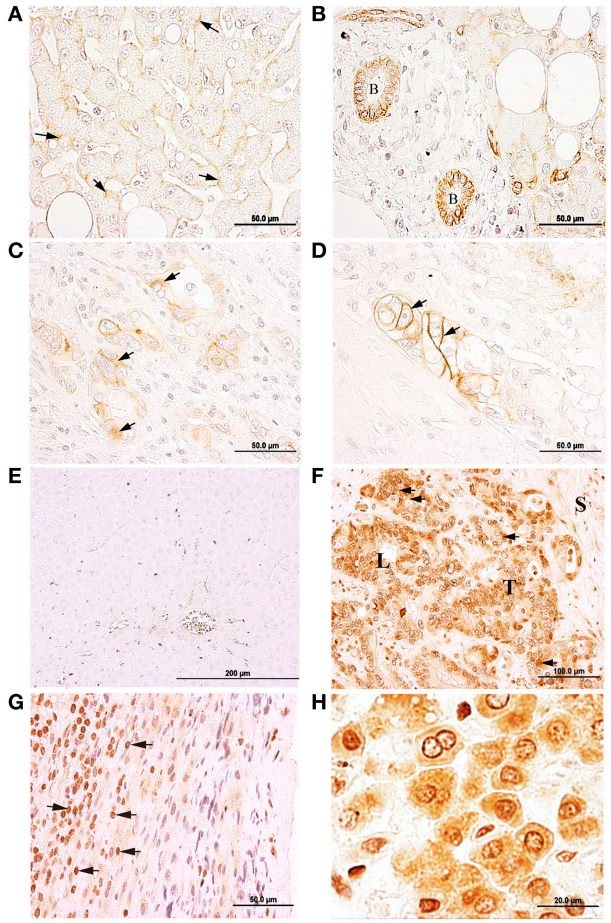

FIGURE 4 | Immunolocalization of the Na,K-ATPase α1 and α3 isoforms in normal liver and liver metastasis. (A) α1 isoform is located at the plasma membrane of the hepatocytes (arrows). **(B)** Bile duct epithelial cells (B) express α1 isoform, mainly at the baso-lateral side of cell membrane. **(C)** α1 isoform is located at the cytosol of metastatic tumor cells (arrows). **(D)** α1 is present at a peripheral position in metastatic tumor cells (arrows). **(E)** Negative immunolocalization of the α3 isoform in normal liver tissue. **(F)** α3 isoform is mainly located at a peri-nuclear position but also detected all-over the cytoplasm. **(G)** Inflammatory reaction established at the outermost part of the liver; α3 IHC-positive cells may correspond to cells from the immune system (arrows), such as leukocytes. **(H)** In normal liver tissue from a metastasized liver, hepatocytes express α3 in peri-nuclear and cytoplasmic locations.

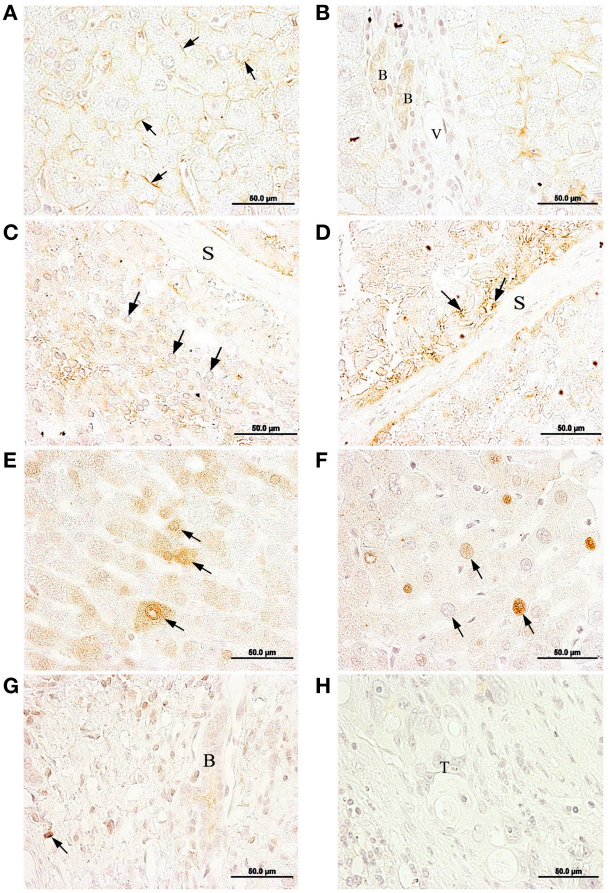

FIGURE 5 | Immunolocalization of the Na,K-ATPase β1 and β2 isoforms in normal liver tissue and liver metastasis. (A) β1 isoform is mainly located at the cytoplasmic membrane of hepatocytes (arrows) from normal liver. **(B)** Bile duct epithelial cells (B) express β1, V, venule. **(C)** Nuclei of metastatic tumor cells (arrows). **(D)** β1 IHC-positive staining at necrotic tissue is signaled with arrows; S, septum. **(E)** β2 is differentially expressed at the cytoplasm of neighboring hepatocytes (arrows) of normal liver. **(F)** Hepatocytes with different degrees of β2 immunostaining at a peri-nuclear position in neighboring hepatocytes (arrows) of normal liver. **(G)** In normal liver, a bile duct (B) and cells from the lining connective tissue (arrow) express β2. **(H)** Metastatic tumor cells (T) do not express β2.

and stated in the Materials and Methods section. As shown in **Figure 6** and in Supplementary Figure S1, both of them co-localize in a number of metastatic cells in percentages ranging from + to +++, depending on the sample and on the area within the same sample. Most of them showed further more than 2/3 of total metastatic cells.

DISCUSSION

In this study we explored the cellular and subcellular localization of the α and β subunit isoforms of Na,K-ATPase in CRC and its liver metastases. The aim of this work was to test the hypothesis that metastatic cancer cells possess a unique expression phenotype of Na,K-ATPase isozymes, which may be similar to that of CRC cells. **Table 2** summarizes the cell-specific Na,K-ATPase subunit-isoforms expression and **Table 3** highlights the possible cell-specific Na,K-ATPase isozymes present in healthy colon, colorectal cancer, healthy liver and metastasized liver.

Based on the results presented we propose that the predominating isozymes in tumor cells from the colon and metastases in the liver are α1β1 and α3β1. The α3β1 isozyme of Na,K-ATPase is only present in liver metastases but not in healthy liver, thus, the α3β1 isozyme could serve as a novel exploratory biomarker of CRC metastatic cells in liver. Further studies should be carried out to test the utility of this observation.

Subunits of Na,K-ATPase have the ability to form functional isoenzymes by a promiscuous association of α and β isoforms

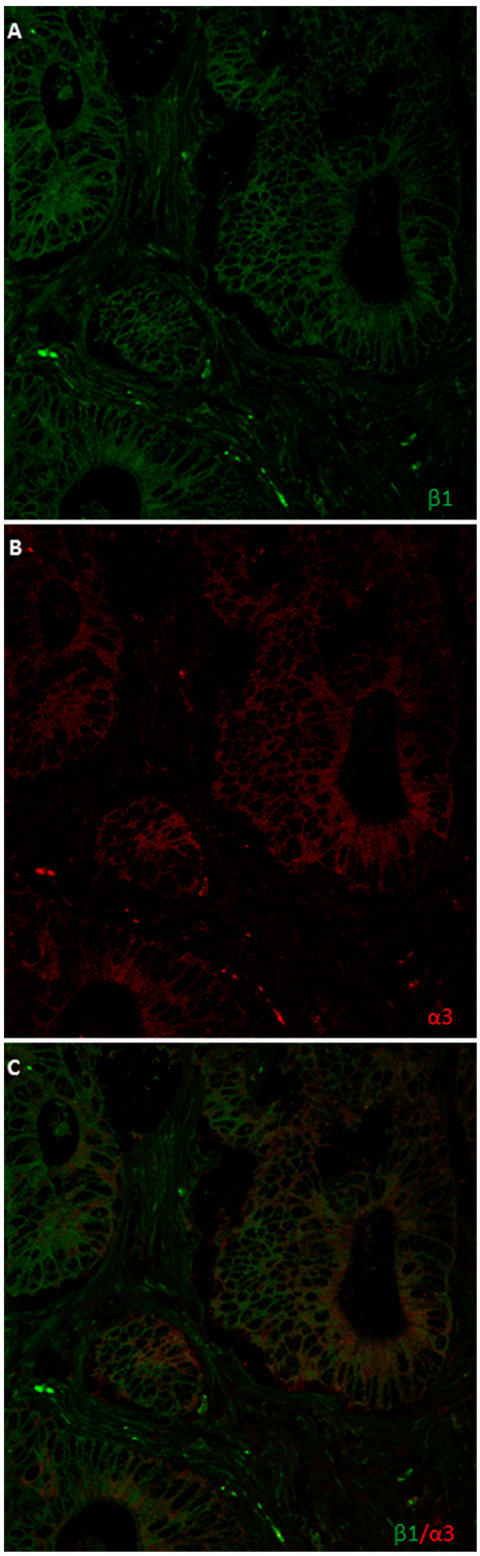

FIGURE 6 | Double immunofluorescence localization of Na,K-ATPase α3 and β1 isoform in liver metastasis. (A) α3 isoform is located at a peri-nuclear position and all-over the cytoplasm. **(B)** β1 isoform is mainly located at the plasma membrane of metastases cells and in nuclear envelope. **(C)** Merge.

TABLE 2 | Cell-specific Na,K-ATPase subunit-isoform expression in normal colon, colorectal cancer, normal liver, and metastasized liver.

	α1	α3	β1	β2
Normal colon				
Epithelial cells (*Mucosae*)	+++	+++	+++	+
Mesenchymal cells (*Submucosae*)	+	+	+	+
Smooth muscle cells (*Muscularis mucosae*)	?	?	?	?
Neurons (Myenteric plexus)	?	+	+++	?
Glia cells (Myenteric plexus)	?	?	+++	+++
Smooth muscle cells (*Muscularis propia*)	?	+	–	+
Colorectal cancer				
Tumor cells	+++	+++	+++	–
Mesenchymal cells (not immune system cells)	+	+++	–	+
Immune system cells	?	+++	?	?
Endothelial cells	?	+++	–	?
Epithelial cells (*Mucosae*)	+++*	+++	+++	–
Normal liver				
Hepatocytes	+++	–	+++	+
Ephitelial cells (bile duct)	+++	–	+	+
Endothelial cells	–	–	–	–
Mesenchymal cells (connective tissue)	–	–	–	+
Metastasized liver				
Tumor cells	+++	+++	+	–
Hepatocytes	?	+	?	–*
Epithelial cells (Bile duct)	+++	?	?	?
Mesenchymal cells (connective tissue)	–	+	–	–
Immune system cells	–	+++	?	?

*+ + +, indicates a high staining level; +, indicates low staining level; –, indicates no staining detected; and ?, indicates indeterminate staining, according to our observations. *Data not shown.*

to confer significantly different kinetic and biological properties. The apparent affinities for cations and ouabain have been determined by expressing recombinant enzymes in heterologous systems. Affinities of human isozymes expressed in *Xenopus laevis* oocytes are α1β1>α2β1>α3β1 for Na^+ and α3β1=α1β1>α1β3>α1β2>α2β1>α3β3>α3β2>α2β3>α2β2 for K^+ (Blanco and Mercer, 1998; Crambert et al., 2000). Tumor cells and metastatic cells have isozymes with highest and lowest Na^+ affinity and surrounding mesenchymal cells possess isozymes with medium range affinity. Regarding K^+ affinity, tumor and metastasis cells possess Na,K-ATPase isozymes of high K^+ affinity and mesenchymal cells low K^+ affinity. These are the isozyme combinations that permit an optimal performance of the enzymes involved in protein synthesis and transfer of phosphor groups (Glynn, 1985) processes both involved in carcinogenesis.

Another objective of this study was to correlate the mitotic index related to the expression of isoforms by co-localization of those along with PCNA, the clamp subunit of DNA polymerase δ marker of cell proliferation (Kubben et al., 1994; Bleau et al., 2014) and carried out further analysis by confocal microscopy. **Figure 3** shows cells expressing the α3 and β1 isoforms in CRC tumor cells, in which no correlation was seen between sodium pump isoforms and PCNA protein expression, that is, high

TABLE 3 | Possible cell-specific Na, K-ATPase isozymes present in normal colon, colorectal cancer, normal liver, and metastasized liver.

	α1β1	α1β2	α3β1	α3β2
NORMAL COLON				
Epithelial cells (*Mucosae*)	+++	+	+++	+
Mesenchymal cells (*Submucosae*)	+	+	+	+
Smooth muscle cells (*Muscularis mucosae*)	?	?	?	?
Neurons (Myenteric plexus)	?	?	+++	?
Glia cells (Myenteric plexus)	?	?	?	?
Smooth muscle cells (*Muscularis propia*)	–	?	–	+++
COLORECTAL CANCER				
Tumor cells	+++	–	+++	–
Mesenchymal cells (Not immune system cells)	–	+	–	+++
Immune system cells	?	?	?	?
Endothelial cells	–	?	–	?
Epithelial cells (*Mucosae*)	+++	–	+++	–
NORMAL LIVER				
Hepatocytes	+++	+	–	–
Ephitelial cells (Bile duct)	+++	+++	–	–
Endothelial cells	–	–	–	–
Mesenchymal cells (Connective tissue)	–	–	–	–
METASTIZED LIVER				
Tumor cells	+++	–	+++	–
Hepatocytes	?	–	?	–
Epithelial cells (Bile duct)	?	?	?	?
Mesenchymal cells (Connective tissue)	–	–	–	–
Immune system cells	–	–	?	?

+ + +, Indicates a possible high level of the isozyme; +, indicates a possible low presence of the isozyme; –, indicates no possibility; and ?, indicates indeterminate staining, according to our observations.

expression of PCNA can be found in cells with either, high or low, expression level of α3 or β1 and vice versa.

Hideki Sakai and co-workers (Sakai et al., 2004) used western blotting to demonstrate a decrease in α1 isoform expression in CRC and, inversely, an increase in the α3 isoform compared to the accompanying healthy *mucosae*. In addition they did not observe a significant expression level of Na,K-ATPase α2 isoform either in the CRC or in the accompanying healthy *mucosae*. Our study, confirms their observations. In addition, in recent studies of hepatocellular carcinoma, a significantly higher α3 level expression was shown in western blots compared to the accompanying non-tumor tissues, whereas no significant increases in expression of α1 and α2 proteins was observed (Shibuya et al., 2010).

Recently, it has been suggested that the cellular distribution and expression of Na,K-ATPase α3 isoform affects the anti-proliferative effects of oleandrin, a cardiac glycoside that inhibits the Na,K-ATPase (Yang et al., 2014). These authors demonstrated that healthy, as opposed to neoplastic colonic and lung tissues, exhibit different distributions of the α3 isoform. While the α3 isoform was predominantly located in the cytoplasmic membrane in healthy colon and lung, the distribution of this isoform was shifted to a predominantly peri-nuclear location in tumors. These observations have been corroborated by our laboratory. Furthermore, our results showed a subcellular location shift for the α1 isoform, which was mainly located at the basolateral side of the plasma membrane of healthy colonic epithelial cells, shifting to a peri-nuclear position in CRC tumor cells.

The α1 and α3 subunit isoforms were detected in all cells lining the colonic crypts. These isoforms were not only expressed in epithelial cells in healthy colon *mucosae*, but they were also detected in mesenchymal cells from the *lamina propria*. The α3 isoform presents a high expression level in neurons of the central nervous system (Hieber et al., 1991; McGrail et al., 1991). The present study shows specific staining for α3 in neurons from myenteric plexus.

Regarding the β subunit, it has been reported that expression of both the β1 and β2 mRNAs were decreased in renal, lung and hepatocellular carcinomas (Akopyanz et al., 1991), and that expression levels of the corresponding proteins was decreased in human clear cell renal cell carcinoma (Rajasekaran et al., 1999) and bladder carcinoma (Espineda et al., 2004). Previous work from our laboratory (Avila et al., 1997) reported, by western blot technique, that gastric and colon adenocarcinoma showed opposite patterns of β1 isoform expression. While gastric adenocarcinomas showed lower expression levels of β1 than the healthy tissue, colonic adenocarcinomas showed higher expression of this isoform compared to healthy surrounding tissue. In addition, the β2 isoform was neither detected in healthy colon, nor in stomach adenocarcinomas. In the present immunohistochemical study, we detected the β1 and β2 isoforms at the baso-lateral side of the plasma membrane in the healthy colon *mucosae*, but only β1 was found in CRC samples. Which, in a certain manner, resembles our previous findings in Na,K-ATPase in dog and rat prostate cancer where we found a downregulation and a reduced expression of sodium pump (Mobasheri et al., 2000, 2003a,b,c).

Na,K-ATPase β1 and β2 isoforms were detected in the myenteric plexus of healthy colon tissue. It is well-established that the β2 isoform of Na,K-ATPase, is an adhesion molecule on glia (AMOG) (Antonicek et al., 1987; Gloor et al., 1990).

In tumor samples, the β1 isoform presented a less defined pattern of expression. This isoform was detected in some tumor cells but not all, also the subcellular location differed among cells within a given adenocarcinomatous area, while some tumor cells where immunopositive for β1 at the cytoplasmic membrane location (**Figure 2D**) other cells presented immunostaining in a peri-nuclear position (**Figure 2C**).

Research by Rajasekaran and colleagues reported that Na,K-ATPase β subunit is required for epithelial polarization, suppression of invasion, and cell motility (Rajasekaran et al., 2001b), not only presence of Na,K-ATPase in the cell membrane but also Na,K-ATPase activity was important to form proper tight junctions, desmosomes, and induction of polarity in epithelial cells (Rajasekaran et al., 2001b). Further studies suggested that the transcription factor Snail might be repressing the β1 isoform and E-cadherin expression in carcinomas, associating these events to epithelial-mesenchymal transition (EMT) (Espineda et al., 2004).

Taken together, these studies and our results indicate that the level of expression and the location of the β subunit in epithelial cells are important for maintaining their well-differentiated phenotype, which disappears during cancer progression. Research published by our group on sodium pump isoform expression levels in stem cells has confirmed that adipose-derived mesenchymal stem cells express all known Na,K-ATPase isoforms, but some of these genes are turned off along differentiation (Acosta et al., 2011). In CRC cells or its metastases in liver we have never seen expression of all isoforms, rather, we have detected the expression signatures specified in **Table 2**.

Regarding liver metastases, to our knowledge, there have not been any reported studies in reference to Na,K-ATPase isoforms in liver metastasis. In this study the α1 isoform was detected in metastatic tumor cell niches within the liver, exhibiting a cytoplasmic subcellular localization in some cells and a membrane localization in others. The α3 isoform, however, was mainly detected at a peri-nuclear location and was more diffusely expressed across the cytoplasm of tumor metastatic cells. The healthy hepatic tissue presented the α1 isoform at the cytoplasmic membrane where it may establish the functional heterodimer with a β1 isoform and/or β2 isoform. Our observations of the subcellular localization of the α1 isoform are consistent with the first reported immunolocalization of this subunit in hepatocytes of healthy liver tissue (Sztul et al., 1987). However, in healthy liver tissue, the α3 isoform was not detected.

The β2 isoform was detected both at a more peri-nuclear position in some hepatocytes and throughout the cytoplasm in others, but not at the cytoplasmic membrane. The reason for this is unclear at present. It is possible that β2 isoform performs other *moonlighting* protein functions in hepatocytes. In metastasized liver, we detected the β1 isoform in disordered and semi-necrotic tumor tissue. However, β2 isoform was not detected in liver metastases. This may be related to the fact that these metastatic cells arise from CRC tumor cells, which did neither express β2 isoform or at very insignificant levels. Interestingly, apparently in healthy hepatic tissue surrounding the metastatic zone, the hepatocytes expressed the α3 isoform, a phenotype not detected in healthy hepatocytes from non-CRC patient according to our results. It might be possible that the CRC and the FOLFOX-CT affecting this patient could be influencing these hepatocytes driving them to express other genes, *ATP1A3* in this case.

The high levels of peri-nuclear and cytoplasmic α3 isoform in liver metastatic cells is potentially indicative of other *moonlighting* functions of this isoform besides ion transport (Jeffery, 2014; Magpusao et al., 2015; Min et al., 2015). In addition, the α3β1 isozyme may have utility as a novel exploratory biomarker for metastases cells. However, further studies need to be performed in order to confirm both, the moonlighting and biomarker assessments.

AUTHOR CONTRIBUTIONS

Conceived and designed the study and experiments: MM and PV. Patients were selected by: MM. Performed the experiments: MB, DR, MdCM, MG, and JÁ. Analyzed and discussed the data and discussed the written manuscript: All authors. Wrote the manuscript: MB, DR, AM, and PV. Constructed the figures and tables: MB, DR, and JÁ.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2016.00009

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REVIEW
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Transcriptional regulators of Na,K-ATPase subunits

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The Na,K-ATPase classically serves as an ion pump creating an electrochemical gradient across the plasma membrane that is essential for transepithelial transport, nutrient uptake and membrane potential. In addition, Na,K-ATPase also functions as a receptor, a signal transducer and a cell adhesion molecule. With such diverse roles, it is understandable that the Na,K-ATPase subunits, the catalytic α-subunit, the β-subunit and the FXYD proteins, are controlled extensively during development and to accommodate physiological needs. The spatial and temporal expression of Na,K-ATPase is partially regulated at the transcriptional level. Numerous transcription factors, hormones, growth factors, lipids, and extracellular stimuli modulate the transcription of the Na,K-ATPase subunits. Moreover, epigenetic mechanisms also contribute to the regulation of Na,K-ATPase expression. With the ever growing knowledge about diseases associated with the malfunction of Na,K-ATPase, this review aims at summarizing the best-characterized transcription regulators that modulate Na,K-ATPase subunit levels. As abnormal expression of Na,K-ATPase subunits has been observed in many carcinoma, we will also discuss transcription factors that are associated with epithelial-mesenchymal transition, a crucial step in the progression of many tumors to malignant disease.

Keywords: Na,K-ATPase α-subunit, Na,K-ATPase β-subunit, transcription, promoter analysis, cancer, epigenetics

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INTRODUCTION

Na,K-ATPase is an integral membrane protein that mainly functions as an ion pump, hydrolyzing one molecule of ATP to pump three Na^+ out of the cell in exchange for two K^+ entering the cell per pump cycle. This function is crucial to maintaining the ion gradient across the membrane and is critical for the resting membrane potential, electrical activity of muscle and nerve, Na^+-coupled transport, and osmotic balance and cell volume regulation (Blanco et al., 1998; Kaplan, 2002). Besides being an ion pump, Na,K-ATPase acts as a signal transducer (Xie and Askari, 2002; Pierre and Xie, 2006; Rajasekaran and Rajasekaran, 2009; Reinhard et al., 2013). Both α- and β-subunit associate with various signaling molecules, including Src, phosphoinositide 3-kinase (PI3K), caveolin-1, protein phosphatase 2, and EGFR thereby activating a number of intracellular

Abbreviations: CRE, cAMP response element; EMT, epithelial-mesenchymal transition; FGFs, fibroblast growth factors; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HRE, hypoxia response element; MR, mineralocorticoid receptor; MRE, mineralocorticoid response element; Na,K-ATPase, sodium,potassium-adenosine triphosphatase; NRF1, nuclear respiratory factor 1; PGE1, prostaglandin E1; PGRE, prostaglandin response element; PR, progesterone receptor; PRE, progesterone response element; SBD, Smad binding domain; Sp, specificity protein; SRE, serum response element; TGF-β, transforming growth factor-β; TR, thyroid hormone receptor; TRE, thyroid hormone response element; ZEB1, zinc finger E-box binding homeobox.

signaling pathways, including MAPK and Akt signaling, to modulate cell polarity, cell growth, cell motility and gene expression (Haas et al., 2002; Barwe et al., 2005; Cai et al., 2008; Kimura et al., 2011). Na,K-ATPase also functions as a receptor for the cardiac glycoside ouabain, a specific inhibitor of the pump. Studies with ouabain at concentrations that do not inhibit the Na,K-ATPase pump function have been particularly useful in elucidating the pump-independent signaling pathways associated with Na,K-ATPase (Xie and Askari, 2002; Silva and Soares-da-Silva, 2012; Reinhard et al., 2013). Na,K-ATPase also regulates the formation and stabilization of intercellular junctions and the β_1 and β_2-subunits act as cell adhesion molecules (Gloor et al., 1990; Rajasekaran et al., 2001a,b; Vagin et al., 2006, 2012).

Na,K-ATPase is an abundantly expressed protein and can use up to about 2/3 of the energy expenditure of a cell (Howarth et al., 2012) thus requiring tight regulation. Aberrant expression and/or function of Na,K-ATPase has been associated with many disorders, with cancer being one of the more recent ones (Litan and Langhans, 2015). This review will focus on the transcriptional regulation of Na,K-ATPase by hormones, transcription factors, growth factors, extracellular stimuli, and epigenetic modification in general; followed by the role of transcription factors involved in epithelial-mesenchymal transition (EMT) and carcinoma.

THE EXPRESSION PROFILE OF Na,K-ATPase

Na,K-ATPase is a heteromeric transmembrane protein composed of an essential α- and β-subunit (Lingrel and Kuntzweiler, 1994), and an optional third subunit belonging to the FXYD proteins which are more tissue specific regulatory subunits of the enzyme (Geering, 2006). The α-subunit is the catalytic subunit responsible for transport activities of the enzyme. It contains 10 transmembrane segments; a large intracellular domain and a simple extracellular domain. An ATP-binding site and a phosphorylation site locate within the large cytoplasmic loops. The extracellular domain and the transmembrane regions harbor the binding sites for cardiac glycosides such as digoxin and ouabain (Kaplan, 2002; Sandtner et al., 2011; Laursen et al., 2013). The β-subunit is a highly glycosylated transmembrane protein, which increases the translation efficiency and stability of the α-subunit and targets the α-subunit to the plasma membrane (Blanco et al., 1998; Rajasekaran et al., 2004; Benarroch, 2011). It also modulates the affinity of Na^+ and K^+ to the enzyme (Blanco et al., 1998) and functions as a cell adhesion molecule (Gloor et al., 1990; Rajasekaran et al., 2001b; Barwe et al., 2005; Kitamura et al., 2005; Shoshani et al., 2005; Vagin et al., 2006, 2012). Unlike the α-subunit, the β-subunit spans the plasma membrane only once and has a very short intracellular N-terminus and a large extracellular C-terminal domain that contains several N-glycosylation sites (Blanco et al., 1998; Benarroch, 2011). In recent years, the FXYD family has been identified as another subunit of Na,K-ATPase. They are a group of small, single-span membrane proteins that are characterized by the FXYD motif in the extracellular domain, two conserved glycine residues within the membrane, and a serine residue at the membrane-cytoplasm interface (Garty and Karlish, 2006; Geering, 2008). FXYD proteins modify the affinity for Na^+, K^+, and ATP, pump kinetics and transport properties and stabilize Na,K-ATPase (Garty and Karlish, 2006; Geering, 2006, 2008; Mishra et al., 2011).

The Na,K-ATPase subunits are highly conserved across species and among isoforms. Four isoforms of α-subunit (α_1–α_4) and three isoforms of β-subunit (β_1–β_3) have been identified so far, which are encoded by different genes (Blanco, 2005). In human, there is high identity in amino acid sequence among the α-subunit isoforms with 87% identity between α_2 and α_1 and 88% between α_3 and α_1. α_4 is a little more divergent, but still sharing 79% identity with α_1. The β-subunit isoforms are more diverse. Compared to β_1, β_2 shows 39% identity and β_3 is 36%. Seven different FXYD proteins have been identified (FXYD1 to FXYD7), which show 43 $\sim$ 51% identity in their amino acid sequence.

The α- and β-subunits are expressed in a tissue specific manner (Herrera et al., 1987; Orlowski and Lingrel, 1988; Sweadner, 1989; Blanco et al., 1998; Benarroch, 2011). The α_1-subunit is present in most tissues and the highest expression is found in kidney, brain and heart. Compared to the broad tissue distribution of the α_1-subunit, the expression of other α-subunit isoforms is more restricted. The α_2-subunit predominates in skeletal muscle, brain and heart; the α_3-subunit is abundant in nervous tissue and heart. The α_4-subunit was discovered as a testis-specific subunit (Shamraj and Lingrel, 1994), but now has also been found in adult human and mouse skeletal muscle (Keryanov and Gardner, 2002). Within the nervous system, the α-subunit isoforms are expressed in a cell type specific manner. For example, the α_1-subunit is expressed in both neurons and glial cells, the α_2-subunit is primarily found in astrocytes and oligodendrocytes and the α_3-subunit is abundant in neurons (Benarroch, 2011). Like the α_1-subunit, the β_1-subunit is ubiquitously expressed in almost all tissues with the greatest levels in brain and kidney (Orlowski and Lingrel, 1988). The β_2-subunit is mainly found in nervous tissue, kidney and heart (Avila et al., 1998), whereas the β_3-subunit predominates in kidney and adrenal gland but is also presents in brain, lung and liver (Malik et al., 1998). The FXYD proteins show a tissue-specific distribution as well. FXYD1 (phospholemman) is expressed in heart and skeletal muscle; FXYD2 (γ-subunit) in kidney; FXYD3 (MAT-8) in stomach and colon; FXYD4 (CHIF) in kidney and colon; FXYD5 (dysadherin) in intestine, lung and kidney; FXYD6 and FXYD7 in brain (Garty and Karlish, 2006; Geering, 2006). In addition, the expression levels of α- and β-subunit change during development. For example, in brain, all three α-subunit isoforms increase in abundance from fetus to adult (Orlowski and Lingrel, 1988). However, the α_1-subunit is more abundant in fetal kidney and heart than in adult tissues (Herrera et al., 1987). In rat heart, the α_3-subunit decreases sharply after birth and is replaced by α_2-subunit in adults (Zahler et al., 1996). The β_1-subunit increases in the early stage of embryonic development, but decreases to the basal level during embryo implantation (Deng et al., 2013). With these diverse tissue distributions of Na,K-ATPase subunit isoforms, multiple

Na,K-ATPase isozymes can form, rendering cells able to respond more precisely to extracellular stimuli. For instance, while in kidney $\alpha_1\beta_1$ is the major $\alpha\beta$ complex, all combinations of $\alpha\beta$ complexes have been found in heart and brain (Mobasheri et al., 2000).

The spatial and temporal control of Na,K-ATPase expression occurs at the transcriptional, post-transcriptional, translational and post-translational level. Here, we will review findings on the transcriptional regulation of Na,K-ATPase over the past decades (**Table 1**). Extracellular stimuli, such as growth factors play a key role in the regulation of Na,K-ATPase by activating intracellular signaling pathways including protein kinase A (PKA), protein kinase C (PKC), and other Ca^{2+}-regulated signaling pathways. Numerous DNA-binding-site-specific transcription factors have been reported to be involved in the regulation of Na,K-ATPase expression, including hormone receptors, Snail1, specificity protein (Sp) and Zinc finger E-box-binding homeobox 1 (ZEB1). Recently, it has been shown that hypermethylation in promoter regions of human *ATP1B1* gene is associated with reduced expression of β_1-subunit (Selvakumar et al., 2014), indicating that epigenetic modification might be another mechanism of transcriptional regulation of Na,K-ATPase subunits.

PROMOTER ANALYSIS OF Na,K-ATPase α- AND β-SUBUNITS

Transcription factors activate or repress Na,K-ATPase expression by binding to specific DNA elements on the promoter regions. There are numerous sequence-specific elements on the promoter regions of the various Na,K-ATPase subunits, of which β_1 has been extensively studied (**Figure 1**).

α_1-subunit

The α_1-subunit is encoded by the gene *ATP1A1*. The *ATP1A1* promoter has very high GC content, which is a common characteristic of all housekeeping genes. It contains a potential TATA box (−27 bp; Shull et al., 1990), two mineralocorticoid/glucocorticoid response element (MRE/GRE) sites (−598 to −574 bp and −252 bp; Kolla et al., 1999); potential binding sites for AP-1(−507 and −453 bp), AP-2 (−568 and −380 bp), AP-3 (−286 bp), and several CTF/NF-1 and potential Sp1 binding sites (Shull et al., 1990). The nucleotide sequence from around −100 bp to the transcription initiation site is highly conserved among the human and rat *Atp1a1* genes (Kobayashi et al., 1997). An asymmetrical ATF/CRE site (−77 to −64 bp) is found in the rat *Atp1a1* promoter (Kobayashi and Kawakami, 1995). In addition, the rat *Atp1a1* promoter contains a Sp1 consensus sequence (−56 to −51 bp) and a Sp1 binding site (−120 to −106 bp; Suzuki-Yagawa et al., 1992).

α_2-subunit

The α_2-subunit is encoded by the gene *ATP1A2*. The promoter region of human *ATP1A2* contains a potential TATA box, two potential Sp1 recognition sites, numerous potential AP-1, AP-2, and NF-1 binding sites, and several sequences that are similar to the glucocorticoid receptor binding site (Shull et al., 1989; Malyshev et al., 1991). The major positive regulatory elements are located at the position from −175 to −108 bp on the rat *Atp1a2* gene, harboring two E boxes (−144 to −139 bp and −135 to −130 bp), a Spl binding consensus sequence (−123 to −118 bp) and a Sp1 binding site (−114 to −109 bp; Ikeda et al., 1993).

α_3-subunit

The α_3-subunit is encoded by the gene *ATP1A3*. The human *ATP1A3* promoter has high GC content, but no conventional TATA box (Malyshev et al., 1991). Sequence analysis reveals several potential Sp-1 binding sites and AP-1 sites, two AP-2 sites, two AP-4 sites, three GRE elements, two CACCC sequences, a NF-1 binding site, a couple of TRE elements (Pathak et al., 1990), and the binding sites for CREB/ATF and NF-Y/C-EBP (Benfante et al., 2005). These sites are well conserved between the human and rat *Atp1a3* gene (Pathak et al., 1990). It was subsequently shown that two Sp1 binding sites (−110 to −100 bp; −59 to −47 bp), a CCAAT box (−64 to −61 bp) and a half CRE-like site (−87 to −83 bp) are functional. Mutations in the Sp1 binding site (−110 to −100 bp) or the CCAAT box significantly reduced the *ATP1A3* promoter activity and these two sites worked synergistically in inducing *ATP1A3* promoter activity. The CRE-like element itself did not affect *ATP1A3* promoter activity, but cooperated with the upstream Sp1 site (Benfante et al., 2005). Three TRE elements (−636 to −457 bp; −218 to −106 bp; and −106 to −6 bp) showed strong and specific interaction with thyroid hormone receptor (TR) and modulated *ATP1A3* promoter activity (Bajpai et al., 2001). In addition, the region from −210 bp to the transcription initiation site was sufficient to direct brain-specific expression of the rat α_3-subunit (Pathak et al., 1994).

α_4-subunit

The α_4-subunit is encoded by the gene *ATP1A4*. In silicon sequence analysis of putative promoter regions of human and mouse *ATP1A4* showed that they do not contain a TATA-box sequence, but have potential binding sites for transcription factors AP-1, AP-4, GATA3, NF-Y, MYOD, NF-1, NFAT (Keryanov and Gardner, 2002) and two consensus CRE sites (Rodova et al., 2006). However, *ATP1A4* does not have any Sp1 binding site that is common on the promoter regions of other subunits (Keryanov and Gardner, 2002).

β_1-subunit

The β_1-subunit is encoded by the gene *ATP1B1*. It contains three potential MRE/GRE half sites (−1048 to −1027 bp; −650 to −630 bp; −276 to −271 bp; Derfoul et al., 1998), four E-boxes (−752 to −747 bp; −708 to −703 bp; −654 to −649 bp; −509 to −504 bp), a non-canonical E-box (−71 to −66 bp), a NF-1 binding site (−657 to −653 bp; Espineda et al., 2004), three prostaglandin response elements (PGRE: −445 to −438 bp; −226 to −216 bp; −100 to −92 bp; Matlhagela et al., 2005; Matlhagela and Taub, 2006), a putative hypoxia response element (HRE: −750 to −746 bp; Mony et al., 2013), a Smad binding domain (SBD: −728 and −721 bp; Mony et al., 2013), three thyroid hormone response elements (TRE; Feng et al., 1993), three progesterone element (PRE) half sites (Cochrane et al., 2012) and several Sp1 binding sites (Matlhagela et al., 2005; Matlhagela and

TABLE 1 | Examples of factors that transcriptionally regulate Na,K-ATPase.

Regulator	The roles in regulating Na, K-ATPase transcription	References
11-Dehydrocorticosterone	Increased α_1 and β_1 mRNA in vascular smooth muscle cells	Muto et al., 1998a
8-Bromo-cAMP	Increased α_1 and β_1 mRNA in a rat kidney epithelial cell line	Whorwood and Stewart, 1995
Aldosterone	Increased α_1 and β_1 mRNA in A6 kidney cells from Xenopus laevis	Verrey et al., 1987, 1988, 1989
	Increased α_1 and β_1 mRNA in adult and neonatal rat cardiocytes	Ikeda et al., 1991
	Increased α_1 and β_1 mRNA in vascular smooth muscle cells	Oguchi et al., 1993
	Increased α_3 mRNA in rat hippocampus	Farman et al., 1994
	Increased α_1 and β_1 mRNA in rat vascular smooth muscle cells	Muto et al., 1996
	Increased α_1 in rat cortical collecting duct	Tsuchiya et al., 1996
	Increased α_3 and β_1 in hippocampus, gyrus dentatus and periventricular gray substance	Grillo et al., 1997
	Increased α_1 mRNA in the renal cortex	Seok et al., 1999
	Increased β_1 mRNA in alveolar type 2 (AT2) cells	Olivera et al., 2000
	Increased α_1, but not β_1 mRNA in cortical collecting duct cells	Blot-Chabaud et al., 2001
	Increased $\alpha_2$and β_1 mRNA in human skeletal muscle	Phakdeekitcharoen et al., 2011
Ammonia	Increased α_2, but not α_1 mRNA, decreased α_3 mRNA	Xue et al., 2010
Angiotensin II	Increased α_1 and β_1 mRNA	Isenovic et al., 2004
Betamethasone	Increased α_1 and β mRNA level in 10-day-old rats, but not in adult rats	Celsi et al., 1991
	Increased α_1, α_2, β_1, but not α_3 mRNA in infant rat heart	Wang and Celsi, 1993
	Increased α_1 and β_1 mRNA in infant rat kidney	Wang et al., 1994
C peptide	Increased α_1 mRNA in human renal tubular cells	Galuska et al., 2011
Caffeine	Decreased α_1 and β_1 mRNA in rat kidney	Lee et al., 2002
Cholera toxin	Increased β_1, but not α_1 mRNA in a rat kidney epithelial cell line	Whorwood and Stewart, 1995
Corticosterone	Increased α_1 and β_1 mRNA in vascular smooth muscle cells	Muto et al., 1998a
Cyclic stretch	Increased $\alpha_1$and α_2 mRNA in aortic smooth muscle cells	Sevieux et al., 2001
db-cAMP	Increased α_1, but not β_1 mRNA	Dagenais et al., 2001
Dexamethasone	Increased α_2, but not α_1, α_3, and β mRNA in cultured neonatal rat cardiac myocytes	Orlowski and Lingrel, 1990
	Increased α_1 and β_1 mRNA in a rat liver cell line	Bhutada et al., 1991
	Increased α_1 and β_1 mRNA in fetal rat lung epithelial cell line	Chalaka et al., 1999
	Increased α_3 and β_1 mRNA in rat spinal cords	González et al., 1994, 1996
	Increased α_1 and β_1 mRNA in rat vascular smooth muscle cells	Muto et al., 1996
	Increase β_1, but not α_1 mRNA in alveolar epithelial type II cells	Barquin et al., 1997
	Increased β_1, but not α_1 mRNA in fetal lungs	Ingbar et al., 1997
	Increased α_1 and β_1 mRNA in a fetal rat lung epithelial cell line	Chalaka et al., 1999
	Increased β_1, but not α_1 mRNA in rat alveolar epithelial cells	Dagenais et al., 2001; Hao et al., 2003a,b
	Decreased α_1 mRNA in rat capsule-epithelium of lenses	Xie and Askari, 2002
DNA methylation	Decreased α_3, β_1 and *FXYD1* mRNA	Henriksen et al., 2013; Selvakumar et al., 2014
Dopamine	Increased β_1 mRNA in rat alveolar epithelial cells	Guerrero et al., 2001
EGF	Increased $\alpha_1$and β_1 mRNA in alveolar epithelial cells	Danto et al., 1998
	Increased α_2 but not α_1 mRNA in primary cultures of mouse astrocytes	Xue et al., 2010
Elevated Ca^{2+}	Increased α_1 and β_1 mRNA in rat kidney	Rayson, 1991
Elevated intracellular Na^{+}	Increased α_1 and β_1 mRNA in rat kidney epithelial cells	Muto et al., 2000
FGF	Increased α_1 and β_1 mRNA in vascular smooth muscle cells	Nemoto et al., 1997
Forskolin	Increased α_1 and β_1mRNA in a rat kidney epithelial cell line	Whorwood and Stewart, 1995
Glucose	Increases α_1 and β_1 mRNA	Muto et al., 1998a
Glycyrrhetinic acid	Decreased α_1 and β_1 mRNA in rat kidney epithelial cells	Whorwood and Stewart, 1995
High-fat diet	Increased α_1 mRNA in nuclear extracts from gastrocnemius muscle	Galuska et al., 2009
Hyperoxia	Selectively increased β_1 mRNA in MDCK cells	Wendt et al., 1998
Hypoxia	Down-regulated the expression of Na,K-ATPase in alveolar cells, renal proximal tubule cells and lung cancers	Planes et al., 1997; Adachi et al., 2004; Yu and Hales, 2011

(Continued)

TABLE 1 | Continued

Regulator	The roles in regulating Na, K-ATPase transcription	References
IL-2	Increased α_1 and β_1 mRNA in human blood lymphocytes	Karitskaya et al., 2010
Insulin	Increased α2, not α1 mRNA, decreased β1 mRNA in 3T3-L1 cells	Russo and Sweadner, 1993
	Increased α2, not α1 mRNA in VSMC cells	Tirupattur et al., 1993
Ischemia and reflow	Decreased α_1 and β mRNA level in rat kidney	Van Why et al., 1994
KCl	Increased α_1, α_3, and β_1 mRNA in neurons	Johar et al., 2012, 2014
KGF	Increased α_1, but not β_1 mRNA in alveolar type II cells	Borok et al., 1998
Low K^+	Increased α_1 and β_1 mRNA in cultured renal proximal tubule cells	Tang and McDonough, 1992
	Increased α_1 and β_1 mRNA in rat cardiac myocytes	Qin et al., 1994; Zhuang et al., 2000; Wang et al., 2007b
Mannitol	Increased $\alpha_1$and β_1 mRNA	Muto et al., 1998b
Manganese	Decreased α_3 mRNA in mice	Wang et al., 2013
Nitric oxide	Decreased α_1 mRNA in medullary thick ascending limb of Henle (MTAL) cell lines	Kone and Higham, 1999
NRF1	Increased β_1 but decreased α_1 mRNA	Johar et al., 2012
Ouabain	Increased α_1 and β_1 mRNAs in cultured rat astrocytes	Hosoi et al., 1997
	Increased α_1 and β_1 mRNA in rat kidney epithelial cells	Muto et al., 2000
	Regulated α_3 and β_1 mRNA in cultured neonatal rat cardiac myocytes	Kometiani et al., 2000
Pertussis toxin	Increased α_1 and β_1 mRNA in a rat kidney epithelial cell line	Whorwood and Stewart, 1995
PHA	Increased α_1 and β_1 mRNA in human blood lymphocytes	Karitskaya et al., 2010
Progesterone	Increased β_1 mRNA	Cochrane et al., 2012
	Increased β_1 mRNA in mouse uterus	Deng et al., 2013
Prostaglandin E1	Increased α and β mRNA in MDCK cells	Taub et al., 1992, 2004; Matlhagela and Taub, 2006
	Increased α and β mRNA in rabbit renal proximal tubule cells	Herman et al., 2010
Prostaglandin E2	Increased *ATP1B1* promoter activity in rabbit renal proximal tubule cells	Herman et al., 2010
Serum	Increased α_1 and β_1 mRNA in a rat liver cell line, Clone 9	Kirtane et al., 1994
	Increased α_1 and β_1 mRNA in vascular smooth muscle cells	Nemoto et al., 1997
Snail1	Selectively repressed β_1, but not α_1 mRNA in MCF7 and MDCK cells	Espineda et al., 2004
Sp (Sp1, Sp3, Sp4)	Increased α_1, α_3, and β_1 mRNA in murine neurons	Johar et al., 2014
T3	Increased α and β mRNA in rat kidney cortex	Gick and Ismail-Beigi, 1990
	Increased α, but not β mRNA in rat liver	Gick and Ismail-Beigi, 1990
	Increased α and β mRNA in rat kidney	McDonough et al., 1988
	Increased α_2, α_3, and β, but not α_1 mRNAs in neonatal rat cardiac myocytes	Orlowski and Lingrel, 1990
	Increased α_1 and β mRNA in a rat liver cell line Clone 9	Gick and Ismail-Beigi, 1990
	Increased α_1, α_3, and β_1 mRNA in neonatal rat myocardium	Melikian and Ismail-Beigi, 1991
	Increased α_1, α_2, and β_1 mRNA in cardiac myocytes	Hensley et al., 1992
	Increased α_1, α_2, α_3, and β_1 mRNA in cultured neonatal rat cardiocytes	Kamitani et al., 1992
	Increased α_2 and β_2 mRNA in skeletal muscle	Azuma et al., 1993
	Increased α_1 and β_1 mRNA in cultured rat mesangial cells	Ohara et al., 1993
	Increased α_1 and β_1 mRNA in in rat jejunum and Caco-2 cells	Giannella et al., 1993
	Increased α_2 mRNA in neonatal rat cardiac myocytes	Huang et al., 1994
	Increased α and β mRNA in rabbit renal proximal tubule cells	Lin and Tang, 1997
	Increased α_1, α_2, and α_3 mRNA in rat brain	Bajpai and Chaudhury, 1999
	Increased α_2 and β_1 mRNA in rat heart	Shao et al., 2000
	Increased α_2 and β_1 mRNA in human skeletal muscles	Phakdeekitcharoen et al., 2007
T4	Increased α_1, α_2, and β_1 mRNA in rat heart	Shao et al., 2000
Tetrodotoxin	Decreased α_1, α_3, *and* β_1 mRNA in murine neurons	Johar et al., 2012, 2014
TGF-β_1	Decreased β_1, α_1, α_2, and α_3 mRNA in young FRTL-5 cells	Pekary et al., 1997
TGF-β_2	Selectively decreased β_1 mRNA in ARPE-19 cell	Mony et al., 2013
Uremia	Decreased α_1, but increased α_2 in rat skeletal muscle	Bonilla et al., 1991
Vasopressin	Increased α_1, but not β_1 mRNA in cortical collecting duct cells	Blot-Chabaud et al., 2001
Veratridine (a Na^+ channel activator)	Increased α_1 and β_1 mRNA in rat vascular smooth muscle cells	Yamamoto et al., 1994

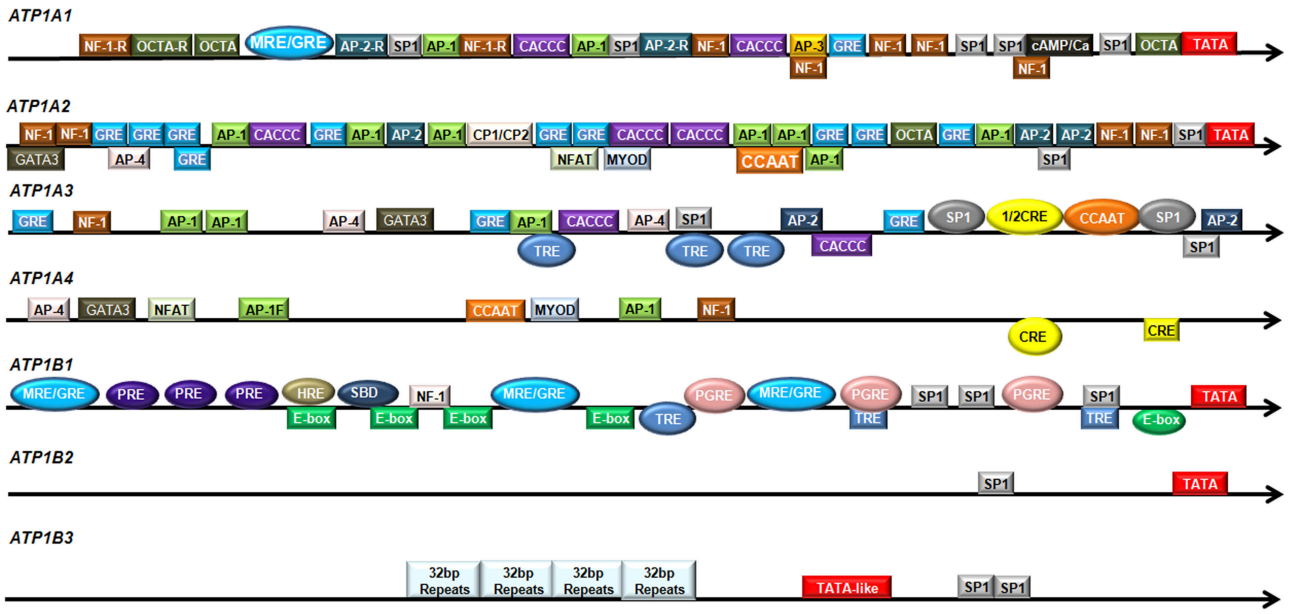

FIGURE 1 | Regulatory elements in the promoter regions of human Na,K-ATPase subunits. The promoter regions of human Na,K-ATPase are shown in the direction from 5′ to 3′. The colored boxes indicate potential transcription factor binding sites. The colored ovals represent experimentally verified transcription factor binding sites.

Taub, 2006). Furthermore, the mouse *Atp1b1* promoter region also contains a Cebpb binding site (Deng et al., 2013). The 5′ flanking region of rat *Atp1b1* shows 68% homology with human *ATP1B1*. It contains a consensus TATA box sequence (−31 bp), two CAAT boxes, four GC boxes, five half-site TREs, several GRE and calcium and serum response elements (Liu and Gick, 1992).

β_2-subunit

The β_2-subunit is encoded by the gene *ATP1B2*. The region from −280 bp to the transcriptional initiation site on the promoter of *ATP1B2* gene is highly conserved between rat, mouse and human. The human *ATP1B2* promoter contains a consensus TATA sequence at position −94 bp and a Sp1 binding site at position −120 bp (Avila et al., 1998). The mouse *Atp1b2* promoter has a TATA-like sequence (−23 to −28 bp), a putative CAAT box (−137 to −142 bp), and a GC box (−144 to −149 bp; Shyjan et al., 1991). Sequence analysis revealed a potential TATA box (−29 bp); two Sp1 binding sites (−55 and −147 bp) and a serum response element (SRE: −263 bp) on the rat *Atp1b2* promoter region (Kawakami et al., 1990). In addition, an AMOG regulatory element (AMRE: GAGGCGGGG at position −87 to −79 bp) and a functional Sp1 binding site (−147 to −142 bp) were found in rat *Atp1b2* promoter regions (Kawakami et al., 1992, 1993).

β_3-subunit

The β_3-subunit is encoded by the gene *ATP1B3*. The human *ATP1B3* promoter contains a TATA-like sequence (−413 to −408 bp) and two Sp1-like elements (−323 to −315 bp and −312 to −304 bp). Moreover, the human *ATP1B3* also has four identical 32-nucleotide-long repeats from position −760 to −633 bp. The sequence from −1 to −328 bp on the 5′ flanking region of human and mouse *Atp1b3* shows high homology. The mouse *Atp1b3* promoter has one Sp1-like sequence (−319 to −310 bp), but does not have any TATA-like sequence. No CAAT or GC boxes are present at the promoter region of human or mouse *Atp1b3* (Malik et al., 1998).

TRANSCRIPTIONAL REGULATION OF Na,K-ATPase BY HORMONES AND NUCLEAR RECEPTORS

Hormones are major regulators of Na,K-ATPase expression. Mineralocorticoids (Verrey et al., 1987, 1988, 1989; Ikeda et al., 1991; Oguchi et al., 1993; Farman et al., 1994; Muto et al., 1996; Tsuchiya et al., 1996; Grillo et al., 1997; Seok et al., 1999; Olivera et al., 2000; Blot-Chabaud et al., 2001; Phakdeekitcharoen et al., 2011), glucocorticoids (Orlowski and Lingrel, 1990; Bhutada et al., 1991; Celsi et al., 1991; González et al., 1994, 1996; Muto et al., 1996; Barquin et al., 1997; Ingbar et al., 1997; Chalaka et al., 1999; Dagenais et al., 2001; Hao et al., 2003a,b), thyroid hormone (McDonough et al., 1988; Gick and Ismail-Beigi, 1990; Orlowski and Lingrel, 1990; Melikian and Ismail-Beigi, 1991; Hensley et al., 1992; Kamitani et al., 1992; Azuma et al., 1993; Giannella et al., 1993; Ohara et al., 1993; Huang et al., 1994; Lin and Tang, 1997; Bajpai and Chaudhury, 1999; Shao et al., 2000; Phakdeekitcharoen et al., 2007), insulin (Russo and Sweadner, 1993; Tirupattur et al., 1993; Sweeney and Klip, 1998), progesterone (Cochrane et al., 2012; Deng et al., 2013), androgen (Blok et al., 1999), and vitamin D3 (Billecocq

et al., 1997) have been reported to activate or repress Na,K-ATPase transcription. These hormones bind to and activate their receptors, which belong to the family of nuclear receptors with C4 zinc-fingers that then translocate into the nucleus to bind to the promoter regions of the target genes to regulate gene expression.

Mineralocorticoid Receptor (MR) and Glucocorticoid Receptor (GR)

MR and GR are members of the nuclear receptor family. They have three major functional domains: a N-terminal regulatory domain (NTD); a DNA-binding domain (DBD) and a ligand-binding domain (LBD; Kumar and Thompson, 2005). The MR is highly homologous to the GR in the DBD and LBD but not in the NTD (Kolla et al., 1999) and while mineralocorticoids only activate the MR, glucocorticoids activate both GR and MR. The activated MR and GR translocate into the nucleus and bind to a specific palindromic DNA sequence known as glucocorticoid/mineralocorticoid response element (GRE/MRE) located in the promoter regions of target genes, thus modulating gene transcription. The GRE is composed of imperfect inverted hexanucleotide repeats separated by three nucleotides (GGTACA NNN TGTTCT; Nordeen et al., 1990).

Glucocorticoids regulate the transcription of Na,K-ATPase in a variety of tissues including lung, kidney, liver, heart, spinal cord, cardiac muscle and smooth muscle (Orlowski and Lingrel, 1990; Bhutada et al., 1991; Celsi et al., 1991; Wang and Celsi, 1993; González et al., 1994, 1996; Wang et al., 1994; Muto et al., 1996; Barquin et al., 1997; Ingbar et al., 1997; Chalaka et al., 1999; Dagenais et al., 2001; Hao et al., 2003b). This regulation is specific to the cell type and the developmental stage. For example, dexamethasone, a synthetic potent glucocorticoid, increased the mRNA levels of α_1- and β_1-subunit in a rat liver cell line and in a fetal rat lung epithelial cell line (Bhutada et al., 1991; Chalaka et al., 1999). However, in cultured neonatal rat cardiac myocytes, dexamethasone only induced α_2-subunit mRNA, and α_1-, α_3-, and β-subunit transcripts remained unchanged (Orlowski and Lingrel, 1990). Injection of betamethasone, another synthetic analog of glucocorticoid, increased α_1- and β-subunit mRNA abundance in 10-day-old rats, but not in adult rats (Celsi et al., 1991).

Mineralocorticoids, like aldosterone, also regulate the mRNA levels of Na,K-ATPase in diverse tissues such as kidney, heart, brain, vascular smooth muscle and skeletal muscle (Verrey et al., 1987, 1988, 1989; Ikeda et al., 1991; Oguchi et al., 1993; Farman et al., 1994; Tsuchiya et al., 1996; Grillo et al., 1997; Seok et al., 1999; Olivera et al., 2000; Blot-Chabaud et al., 2001; Phakdeekitcharoen et al., 2011). Aldosterone increased both α_1- and β_1-subunit transcripts in kidney, heart and vascular muscles and this up-regulation of β_1-subunit was much stronger than that of α_1-subunit (Verrey et al., 1987, 1988, 1989; Ikeda et al., 1991; Oguchi et al., 1993). Aldosterone also increased α_2- and β_1-subunit mRNA in human skeletal muscle (Phakdeekitcharoen et al., 2011). In addition, aldosterone selectively up-regulated α_3- and β_1-subunit mRNA in some brain regions, such as hippocampus, gyrus dentatus and periventricular gray substance (Farman et al., 1994; Grillo et al., 1997). The transcriptional regulation of α_1- and β_1-subunit by aldosterone was mediated mostly by MR (Muto et al., 1996; Kolla et al., 1999), whereas dexamethasone-mediated induction of β_1-subunit mRNA occurred through GR (Muto et al., 1996). Inhibiting GR and MR activities by their specific antagonists also reduced α_1- and β_1-subunit levels (Whorwood et al., 1994; Hao et al., 2003b). In monkey kidney CV-1 cells, which have no detectable MR and GR, activation of exogenous MR or GR alone by aldosterone or triamcinolone acetonide increased β_1-subunit gene promoter activity. However, activation of both receptors inhibited transcription from the β_1-subunit gene promoter (Derfoul et al., 1998) and the N-terminal region of MR mediated this inhibitory effect (Derfoul et al., 2000).

The promoter region of the *ATP1B1* gene contains three potential MRE/GREs at nucleotide position −1048 to −1027 bp; −650 to −630 bp and −276 to −271 bp. The first two contain two half-binding sites separated by 10 and 9 base pairs, respectively, whereas the last one consists of a single half-site. Although all three MRE/GREs were involved in the induction of *ATP1B1* promoter activity by corticoids, the one at position −650 to −630 bp was the strongest (Derfoul et al., 1998). Both MR and GR bound to the GRE/MRE (−650 to −630 bp) and activated the transcription from a heterologous neutral promoter in response to corticoids (Derfoul et al., 1998; Hao et al., 2003b). A similar element was found in the human *ATP1A1* promoter region at position −598 to −574 bp in which two half sites are separated by 12 base pairs. This MRE/GRE bound both MR and GR and activated a heterologous neutral promoter activity (Kolla et al., 1999). In addition, a GRE (+434 to +448 bp) in the coding region of the rat *Atp1b1* gene has been reported in a rat lung epithelial cell line. This element acted as an enhancer and increased the transcription from a heterologous promoter by dexamethasone (Hao et al., 2003a).

Progesterone Receptor

Progesterone receptor (PR) also belongs to the nuclear receptor family. PR's sequence and architecture is very similar to MR and GR (Leo et al., 2004). Like MR and GR, PR is activated by its ligand progesterone and then translocates into the nucleus, forms a homodimer and then binds to specific progesterone response elements (PREs) within the promoter of target genes to modulate transcription (Leonhardt et al., 2003). Progesterone increased the mRNA and protein levels of β_1-subunit (Cochrane et al., 2012; Deng et al., 2013), and this up-regulation was enhanced by estradiol-17β (Deng et al., 2013). RU486, a PR antagonist, completely blocked the increase in β_1-subunit mRNA by progesterone (Deng et al., 2013). In addition, human β_1-subunit was up-regulated by both PR-A and PR-B, two different PR isoforms (Richer et al., 2002). Moreover, PR bound to three PRE half sites on the *ATP1B1* promoter; Progestin, a synthetic progestogen with similar effect to progesterone, induced *ATP1B1* promoter activity, an induction that was suppressed by RU486 (Cochrane et al., 2012).

Thyroid Hormone Receptor

Thyroid hormone receptor (TR) is a member of the nuclear receptor superfamily and is activated by thyroid hormone, triiodothyronine (T3) and its prohormone, thyroxine (T4). Although TR alone can bind as monomer or homodimer to specific DNA sequences in the gene promoter regions known as thyroid hormone response elements (TREs), it preferentially forms heterodimers with retinoid X receptor (RXR) and then binds to TREs, thus enhancing or inhibiting the expression of genes that are involved in development, homeostasis, cellular proliferation and differentiation (Wu and Koenig, 2000). T3 has been shown to regulate the abundance of Na,K-ATPase mRNA and protein in heart, kidney, liver, intestines, brain, and skeletal muscle among others (McDonough et al., 1988; Gick and Ismail-Beigi, 1990; Orlowski and Lingrel, 1990; Melikian and Ismail-Beigi, 1991; Hensley et al., 1992; Kamitani et al., 1992; Azuma et al., 1993; Giannella et al., 1993; Ohara et al., 1993; Huang et al., 1994; Lin and Tang, 1997; Bajpai and Chaudhury, 1999; Shao et al., 2000; Phakdeekitcharoen et al., 2007). In general, T3 up-regulated the transcription of Na,K-ATPase subunits. For example, T3 increased the mRNA levels of α_1-, α_2-, α_3-, and β_1-subunit in cultured neonatal rat cardiocytes (Kamitani et al., 1992) and α_2-, β_1-, and β_2-subunit in skeletal muscle (Azuma et al., 1993; Phakdeekitcharoen et al., 2007). In hypothyroid rat brain, the mRNA level of α_1-, α_2-, and α_3-subunit was reduced during the first 3 weeks of brain development (Chaudhury et al., 1996). The transcription of these mRNAs was decreased in nuclei from hypothyroid cerebra and pretreatment with T3 increased the rates of transcription (Bajpai and Chaudhury, 1999). The promoter regions of *Atp1a1, Atp1a2, Atp1a3,* and *ATP1B1* genes have TRE sequences (Kamitani et al., 1992; Feng et al., 1993; Giannella et al., 1993). In addition, two negative thyroid receptor elements (−116 and −6 bp and −6 and +80 bp) were found in rat *Atp1a3* promoter. The TR/RXR complex bound to these elements and inhibited T3-mediated gene expression in neonatal rat cardiac myocytes, which only occured with prolonged incubation with T3 (He et al., 1996).

Insulin

Insulin is an important regulator of Na,K-ATPase. It binds to insulin receptor and activates various cellular signaling pathways, thus regulating cell functions. Insulin modulates Na,K-ATPase activity by increasing Na^+ influx; increasing the affinity of Na,K-ATPase for intracellular Na^+; altering phosphorylation status of Na,K-ATPase and facilitating the translocation of intracellular Na,K-ATPase to the plasma membrane. In addition, insulin also regulates the transcription, translation and stability of Na,K-ATPase (Ewart and Klip, 1995; Lavoie et al., 1996; Sweeney and Klip, 1998; Therien and Blostein, 2000; Clausen, 2003). The transcriptional regulation of Na,K-ATPase by insulin occurs in an isoform specific manner. For example, insulin selectively increased α_2, but not α_1 mRNA level in vascular smooth muscle cells (VSMC) and 3T3-L1 cells (Russo and Sweadner, 1993; Tirupattur et al., 1993; Sweeney and Klip, 1998). However, it reduced the β_1 mRNA level (Russo and Sweadner, 1993).

TRANSCRIPTION FACTORS REGULATING Na,K-ATPase

Specificity Protein

Specificity protein (Sp) is a small family of zinc-finger transcription factors with four members: Sp1, Sp2, Sp3, and Sp4. Sp1, Sp2, and Sp3 are ubiquitously expressed, while Sp4 is expressed mainly in neurons and testes. The Sp family members contain three zinc fingers close to the C-terminus, which is DBD, and preferentially bind to the GC box [(G/T)(G/A)GGC(G/T)(G/A)(G/A)(G/T)] on the promoter regions of target genes to enhance gene expression. Sp1, Sp3, and Sp4 have a highly conserved DBDs and they recognize the same consensus GC rich element with identical affinities. However, Sp2, in which the important histidine residue within the first zinc finger is replaced by a leucine residue, does not bind to the GC box but instead binds to a GT rich element (Suske, 1999). Potential Sp1 binding sites were found on the promoter regions of almost all Na,K-ATPase subunits, including *Atp1a1, Atp1a2, Atp1a3, Atp1b1,* and *Atp1b2* (Shull et al., 1989, 1990; Kawakami et al., 1990; Yagawa et al., 1990; Malyshev et al., 1991; Suzuki-Yagawa et al., 1992; Ikeda et al., 1993; Yu et al., 1996; Avila et al., 1998; Benfante et al., 2005). It has been shown that Sp1 and Sp3 bind to GC boxes on the promoter regions of rat *Atp1a1* (Kobayashi et al., 1997) and *Atp1b1* (Murakami et al., 1997). Mutations in the GC box sequences decreased basal *Atp1b1* transcription in neonatal rat cardiac myocytes (Zhuang et al., 2000). Sp1, Sp3 and Sp4 also bind to two Sp1 binding sites (−110 to −100 bp; −59 to −47 bp) on human *ATP1A3* promoter. Mutations in Sp1 binding site (−110 to −100 bp) significantly reduced the promoter activity (Benfante et al., 2005). Recently, Sp1, Sp3, and Sp4 had been shown to increase α_1-, α_3-, and β_1-subunit transcription in murine neurons by binding to their promoter regions (Johar et al., 2012). Mutating the Sp binding sites on *Atp1a1, Atp1a3, and Atp1b1* promoters led to a significant decrease in their promoter activity. Silencing Sp1, Sp3, or Sp4 significantly decreased the mRNA levels of α_1-, α_3-, and β_1-subunit, while overexpressing Sp1, Sp3, and Sp4 increased their transcription (Johar et al., 2012). Sp1 also binds to two Sp binding sites located near its TATA box on rat *Atp1b2* promoter and enhanced the expression of β_2-subunit. In addition, the functional Sp-binding site on *Atp1a1* promoter is well conserved in rat brain, kidney and liver (Suzuki-Yagawa et al., 1992; Kobayashi et al., 1997; Nemoto et al., 1997), indicating that it might contribute to the constitutional expression of α_1-subunit. The Sp binding sites are also required for the regulation of Na,K-ATPase transcription in response to extracellular stimuli. For example, hyperoxia selectively up-regulated β_1-subunit transcription in MDCK cells (Wendt et al., 1998). Hyperoxia increased the binding of Sp1 and Sp3 to the *Atp1b1* promoter and deletion of a GC rich element resulted in loss of both basal and hyperoxia-activated transcription (Wendt et al., 2000). Sp might also augment transcriptional activation of Na,K-ATPase through other transcription factors. For example, Sp1 significantly enhanced MR and GR expression (Kolla et al., 1999) and elevated MR and GR might synergize with Sp1 to upregulate Na,K-ATPase transcription.

Snail

Snail1 is the first identified member of the Snail family, which belongs to the zinc-finger transcription factors and has three members in vertebrates: Snail1, Snail2, and Snail3. The Snail family has a highly conserved C-terminal region, which is the DBD containing four to six zinc fingers; and a divergent N-terminal region, which contains a SNAG (Snail/Gfi) domain and recruits the transcription repressor complex (such as sin3A/HDAC). The Snail family functions as a transcriptional repressor by binding to the E-box (CAGGTG) on the promoter regions of target genes which are important for embryonic development as well as tumor progression (Nieto, 2002). Four E-boxes and a non-canonical E-box are present on the promoter region of the *ATP1B1* gene. Snail1 binds to the non-canonical E-box located at position −71 to −66 bp and selectively repressed the expression of β_1-subunit without affecting the α_1-subunit level (Espineda et al., 2004). Overexpression of Snail1 repressed *ATP1B1* promoter activity in a dose dependent manner and markedly reduced the level of β_1-subunit in MCF7 and MDCK cells. Knock-down of Snail by RNA interference increased the abundance of β_1-subunit mRNA (Espineda et al., 2004).

Activating Transcription Factor 1 (ATF-1)/cAMP Response Element Binding Protein (CREB) Family

ATF/CREB belongs to the basic leucine zipper factor (bZIP) family, which bind to DNA as dimers and function as activators. The ATF/CREB family is composed of different ATFs, CREB, CREM (cAMP response element modulator) and related proteins. This family contains an N-terminal DBD and a C-terminal bZIP domain that binds other bZIP transcription factors to form homo- and heterodimers. Both ATF and CREB bind to the cAMP response element (CRE: GTGACGT A/C A/G). Phosphorylation of ATF-1 and CREB by multiple kinases enhances the binding to CRE and activates the transcription of target genes which play an important role in cell survival and cell growth (Hai and Hartman, 2001). Dibutyryl cAMP (db-cAMP), a cell-permeable cAMP analog that activates cAMP dependent protein kinase (PKA) and phosphorylates the CREB family, induced the mRNA of α_1-subunit but not β_1-subunit (Dagenais et al., 2001). The rat *Atp1a1* promoter has an asymmetrical ATF/CRE site at position −70 to −66 bp. The ATF-1/CREB heterodimer bound to this site and induced constitutive expression of α_1-subunit. Blocking the binding of ATF-1/CREB to the CRE site reduced α_1-subunit transcription (Kobayashi and Kawakami, 1995). Phosphorylated ATF-1 increased rat α_1-subunit transcription and dephosphorylation of the ATF-1/CREB heterodimer by alkaline phosphatase reduced its binding to the CRE site on rat *Atp1a1* gene (Kobayashi et al., 1997). In addition, human *ATP1A4* promoter region has consensus CRE sites. db-cAMP and ectopic expression of CREMtau, a testis specific splice variant of CREM activated the *ATP1A4* promoter activity. This activation required the CRE site located at −263 bp relative to the transcription initiation site (Rodova et al., 2006).

Zinc Finger E-Box Binding Homeobox 1 (ZEB1)

The ZEB family has two members in vertebrates: ZEB1 (also known as Areb6) and ZEB2. They contain five major domains: two zinc-finger clusters separated by a central homeodomain, a Smad interacting domain (SID) and a CtBP interacting domain (CID). Each zinc finger cluster contains 3 or 4 zinc fingers and is responsible for DNA binding. ZEB binds to E-boxes (CACCT and CACCTG) on the promoter of target genes and recruits co-activators (PCAF or p300), thus activating gene transcription (Vandewalle et al., 2009). The rat *Atp1a1* promoter has a consensus Areb6 (ZEB1) binding sequence and Areb6 (ZEB1) had been shown to activate gene transcription in a cell-specific manner (Watanabe et al., 1993). High-fat diet also induced the binding of ZEB to rat *Atp1α1* promoter and increased the mRNA and protein level of α_1-subunit in nuclear extracts from gastrocnemius muscle (Galuska et al., 2009). Moreover, C-peptide increased the binding of ZEB to rat *Atp1α1* promoter region. Partial silencing of ZEB markedly reduced the effect of C-peptide on the increase in α_1-subunit expression (Galuska et al., 2011). In addition, ZEB binding sites were also found on *FXYD1* promoter at positions −270 to −295 bp and −329 to −354 bp (Galuska et al., 2009).

CCAAT Box Binding Transcription Factors (CBFs)

CCAAT box is one of the most common elements in eukaryotic promoters, which is often found between -60 to -100 bp relative to the transcription initiation site. Several transcription factors have been reported to bind to the CCAAT box, including NF-Y, NF-1, and CCAAT/enhancer-binding protein (C/EBP) isoforms (CEBP-α, CEBP-β, CEBP-γ, CEBP-δ, and CEBP-ε), which are essential for optimal transcriptional activation of target genes (Mantovani, 1998). CCAAT boxes have been found in *Atp1a2*, *Atp1a3,* and *Atp1b1* promoter regions (Pathak et al., 1990; Malyshev et al., 1991; Benfante et al., 2005; Deng et al., 2013). The CCAAT box (−61 to −64 bp) on human *ATP1A3* promoter is well conserved between rat and human and NF-YB had been shown to bind to this CCAAT box. The presence of the CCAAT box significantly increased *ATP1A3* promoter activity in both neuron and non-neuron cell lines, while mutations in the CCAAT box reduced the promoter activity (Benfante et al., 2005). In addition, the CCAAT/enhancer binding protein beta (Cebpb) might be involved in regulating β_1-subunit expression in human luminal epithelial cells. The active isoform of Cebpb significantly increased *Atp1b1* promoter activity, but the truncated isoform of Cebpb had no effects on *Atp1b1* promoter activity (Deng et al., 2013).

GROWTH FACTORS AND TRANSCRIPTIONAL REGULATION OF Na,K-ATPase

Transforming Growth Factor-β

Transforming growth factor-β (TGF-β) is a secreted protein that regulates numerous physiological processes, including cell

differentiation, proliferation, development and survival. TGF-β has three isoforms in mammals: TGF-β_1, TGF-β_2, and TGF-β_3, which are encoded by different genes. Aberrant TGF-β signaling has been linked to cancer initiation, invasion and metastasis (Lamouille et al., 2014). TGF-β treatment reduced mRNA and protein levels of Na,K-ATPase α- and β-subunits in primary cultures of renal proximal tubule cells (Tang et al., 1995). However, TGF-β_1 seems to regulate Na,K-ATPase subunit expression in a cell type specific manner. Some studies showed that TGF-β_1 reduced β_1-subunit as well as α_1-, α_2-, and α_3-subunit mRNA levels in young FRTL-5 rat thyroid cells (Pekary et al., 1997). Studies from our group found that although TGF-β_1 decreased the surface levels of β_1-subunit in kidney cells, this seemed to occur at the post-translational level since no significant changes in mRNA levels were observed between control and TGF-β_1-treated cells (Rajasekaran et al., 2010). TGF-β_1 also prevented the increase in α_1-subunit mRNA induced by steroid hormones (Husted et al., 2000). Nevertheless, TGF-β_2 selectively decreased the transcription of β_1-subunit in ARPE-19 cells, a human retinal pigmented epithelial cell line, involving two transcription factors: hypoxia inducible factor (HIF) and Smad3 (Mony et al., 2013). HIF belongs to the basic helix-loop-helix (bHLH) superfamily and is a heterodimer composed of a hypoxia induced HIF-α subunit and a constitutively expressed HIF-1β subunit. Three HIF-α subunits have been identified so far: HIF-1α, HIF-2α, and HIF-3α. HIF-1α contains three major domains: a N-terminal domain, which is a DBD containing a bHLH; an oxygen-dependent degradation domain (ODDD) that mediates oxygen-regulated protein stability and a C-terminal domain, which recruits transcriptional coactivators such as CBP/p300 to activate gene transcription. The HIF complex associates with hypoxia response elements (HREs) in the regulatory regions of target genes and binds the transcriptional coactivators to induce gene expression. Besides hypoxia, HIF-1 is also regulated in an oxygen-independent manner (Ke and Costa, 2006). TGF-β_2 increased HIF-1α expression in ARPE-19 cells and blocking the binding of HIF-1α to HREs with echinomycin prevented the TGF-β_2-induced decrease of the β_1-subunit (Mony et al., 2013). Since HIF-1α generally functions as a transcriptional activator, this observation raised the possibility that other transcription repressors may antagonize the function of HIF-1α in the regulation of Na,K-ATPase expression. The *ATP1B1* promoter region also contains a SBD in close proximity to a putative HRE. Smads are transcription modulators usually activated by TGF-β signaling that can inhibit gene expression by preventing the action of transcriptional activators (Massague et al., 2005). Studies in ARPE19 cells indeed support the idea that both Smad3 and HIF-1α cooperated in regulating the expression of β_1-subunit expression by TGF-β_2 (Mony et al., 2013).

Fibroblast Growth Factor

Fibroblast growth factors (FGFs) control a wide range of biological functions and regulate cellular proliferation, survival, migration, and differentiation. FGFs are a family of glyocoproteins that are generally sequestered by the extracellular matrix and cell surface heparan sulfate proteoglycans. Activation of FGF signaling occurs upon release of the factor and binding to one of the four highly conserved FGF receptors, FGR1, FGR2, FGR3, and FGR4 (Turner and Grose, 2010). Incubation of cells in serum rapidly increased α_1- and β_1-subunit mRNA levels in VSMC and rat liver cells and induced *Atp1a1* and *Atp1b1* promoter activity, suggesting that serum regulates Na,K-ATPase gene expression at the transcriptional level (Kirtane et al., 1994; Nemoto et al., 1997). Serum differentially regulated α_1- and β_1-subunit mRNA induction and activation of PKC and tyrosine kinase activity was required for up-regulation of α_1-subunit mRNA but not for β_1-subunit. Further studies revealed that FGF treatment stimulated *Atp1a1* and *Atp1b1* promoter activities, similar to the observation in serum treated VSMC (Nemoto et al., 1997). Furthermore, keratinocyte growth factor (KGF), a member of the FGF family and a polypeptide mitogen secreted by fibroblasts and endothelial cells that acts primarily on epithelial cells, increased the abundance of α_1-subunit, but not β_1-subunit mRNA in alveolar type II cells (Borok et al., 1998).

PROSTAGLANDIN AND Na,K-ATPase TRANSCRIPTION

Prostaglandin E1 (PGE1) is a member of the prostaglandin family, which are lipid mediators produced from arachidonic acid by cyclooxygenase and prostaglandin synthases. Prostaglandins exert their effects by activating G protein-coupled receptors and four PGE receptors have been reported: EP1 (E prostanoid receptor 1), EP2, EP3, and EP4 (Sugimoto and Narumiya, 2007). Prostaglandins are important regulators of ion transport in the kidney (Bonvalet et al., 1987) and PGE1 increased the transcription of α- and β-subunit of Na,K-ATPase in MDCK cells (Taub et al., 1992, 2004; Matlhagela and Taub, 2006). The increase in the transcription of β_1-subunit induced by PGE1 was mediated by EP1 and EP2 receptors and PGE receptor antagonists inhibited the promoter activity of human *ATP1B1* induced by PGE1 (Matlhagela and Taub, 2006). Three prostaglandin response elements (PGRE) have been identified within the human *ATP1B1* promoter, which are located at position −445 to −438 bp (PGRE1: TGACCTTC); −226 to −216 bp (PGRE2: GTCCCTCA); and −92 to −100 bp (PGRE3: AGTCCCTGC; Matlhagela et al., 2005; Matlhagela and Taub, 2006) and PGRE1 is well conserved among species (Matlhagela and Taub, 2006). The PGRE sequence is similar to the consensus CRE, and CREB had been shown to bind to these sequences indicating that PGREs might be cAMP response elements. The binding affinity of CREB to three PGREs was varied with PGRE3 > PGRE1 > PGRE2 (Matlhagela et al., 2005). Sp1, Sp3 as well as CREB all bound to PGRE (Matlhagela and Taub, 2006) and the increase in transcription of β_1-subunit was mediated through CREB binding to PGRE1 and PGRE3 as well as Sp1 binding to an adjacent Sp1 site (Matlhagela et al., 2005; Matlhagela and Taub, 2006). Mutations in PGRE3 or the GC box (−118 to −112 bp) immediately downstream of PGRE3 failed to mediate the increase in *ATP1B1* promoter activity induced by PGE1 (Matlhagela and Taub, 2006). Besides PGE1, PGE2 and PGF2α also have been shown to induce *ATP1B1* promoter activity in primary renal proximal tubule (RPT) cells (Herman et al., 2010).

Na,K-ATPase ACTIVITY AND TRANSCRIPTIONAL REGULATION

Chronic inhibition of Na,K-ATPase activity by ouabain leads to an increase in the abundance of Na,K-ATPase. For example, ouabain increased the mRNA levels of α_1- and β_1-subunit in cultured rat astrocytes (Hosoi et al., 1997; Muto et al., 2000), which was abolished by actinomycin D, a transcription inhibitor acting by interfering with mRNA synthesis (Muto et al., 2000). Ouabain also regulated the transcription of α_3- and β_1-subunit in cultured neonatal rat cardiac myocytes. Ouabain augmented the *Atp1b1* promoter activity and the effect of ouabain on the regulation of Na,K-ATPase subunits was dependent on extracellular Ca^{2+} and calmodulin (Kometiani et al., 2000). The inhibition of Na,K-ATPase activity by ouabain can be mimicked by incubating cells in a low K^+ environment and low extracellular K^+ increased α_1- and β_1-subunit mRNA levels in rat cardiac myocytes (Qin et al., 1994; Zhuang et al., 2000; Wang et al., 2007a). Sp and CREB family transcription factors were required for the up-regulation of Na,K-ATPase subunits induced by low K^+. Rat *Atp1a1* promoter region has a CRE/ATF site (at −70 to −63 bp) and a GC box motif (at −57 to −48 bp). Mutations in the CRE/ATF site or GC box substantially reduced low K^+-mediated *Atp1a1* promoter activity. Low K^+ increased the expression of Sp1, Sp3 and CREB-1 and enhanced the binding of these transcription factors to the GC box and, to a lesser extent, to the CRE/ATF site on rat *Atp1a1* promoter (Wang et al., 2007b). The sequences between -102 to +151 bp on rat *Atp1b1* were required for low K^+-induced trans-activation of reporter gene expression (Qin et al., 1994). Low K^+ enhanced the binding of Sp1 and Sp3 to GC box elements on the *Atp1b1* promoter (Zhuang et al., 2000). Mutations in potential GC box sequences decreased basal and low K^+-mediated up-regulation of β_1-subunit transcription in neonatal rat cardiac myocytes (Zhuang et al., 2000). Inhibiting the binding of Sp1 and Sp3 to GC boxes by mithramycin, which has high affinity for GC-rich DNA sequences (Matlhagela et al., 2005), blocked low K^+-mediated up-regulation of *Atp1a1* and *Atp1b1* promoter activity. Multiple cellular signaling pathways were involved in this low K^+-induced increase in the transcription of α_1- and β_1-subunits. Protein kinase A (PKA), ERK1/2, and histone deacetylase (HDAC) were required for up-regulation of α_1-subunit transcription, whereas the transcription of β_1-subunit was dependent on protein kinase C (PKC), c-Jun-N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK; Wang et al., 2007a). However, it is important to point out that inhibition of Na,K-ATPase and therefore an increase in intracellular sodium regulates Na,K-ATPase on multiple levels including transcription, transport, endocytosis and degradation (Vinciguerra et al., 2003, 2004; Wang et al., 2014).

NEURONAL ACTIVITY AND Na,K-ATPase TRANSCRIPTIONAL REGULATION

α_1-, α_3-, and β_1-subunits are abundantly expressed in most neurons in the central nervous system and neuronal activity modulates the transcription of Na,K-ATPase subunits. Depolarization by KCl increased the promoter activities as well as the mRNA levels of α_1-, α_3- and β_1-subunits; however, impulse blockade induced by tetrodotoxin (TTX), a voltage-dependent Na^+ channel blocker, significantly reduced α_1-, α_3-, and β_1-subunit transcripts in murine neurons (Johar et al., 2012, 2014). At least two transcription factors were involved in this activity-dependent transcriptional regulation in neurons, nuclear respiratory factor 1 (NRF1) and the Sp family (Johar et al., 2012, 2014).

NRF1 is a transcription factor which was first identified as an activator of cytochrome C (Evans and Scarpulla, 1989). NRF1 increases the expression of nuclear genes required for mitochondrial biogenesis and function, but also binds to the promoter region of target genes which control cell cycle, cell growth, cell adhesion, migration, and tumor invasion (Okoh et al., 2011). Increased NRF1 levels have been observed in hepatoma, thyroid oncocytoma and breast cancers (Dong et al., 2002; Savagner et al., 2003; Okoh et al., 2011). The consensus NRF1 binding site is (T/C)GCGCA(C/T)GCGC(A/G), which is a GC-rich element. In addition, NRF1 binds to sequences with an invariant GCA core flanked by GC-rich regions, which can be found in 5′ flanking regions of *Atp1a1*, *Atp1a3,* and *Atp1b1* genes. It has been reported that NRF1 binds to the promoter regions of *Atp1a1* and *Atp1b1* in murine neurons at binding sites that are conserved among mice, rats and humans. Mutations in the NRF1 binding sites significantly decreased *Atp1b1* promoter activity, but increased *Atp1a1* promoter activity, which indicates that NRF1 differentially regulates Na,K-ATPase subunits. Indeed, silencing of NRF1 significantly decreased β_1-subunit transcript but increased the mRNA level of α_1-subunit and overexpression of NRF1 increased the mRNA level of the β_1-subunit but decreased the α_1-subunit transcript. NRF1 itself is regulated by neuronal activity (Yang et al., 2006). Depolarization by KCl increased NRF1 expression, and TTX reduced the NRF1 expression (Johar et al., 2012) and knockdown of NRF1 with small interference RNA blocked the up-regulation of the β_1-subunit and down-regulation of the α_1-subunit induced by KCl, whereas overexpression of NRF1 rescued the down-regulation of the β_1-subunit and up-regulation of the α_1-subunit by TTX. The inverse regulation of Na,K-ATPase α_1- and β_1-subunit by NRF1 in response to KCl and TTX points to additional transcription factors and an intricate network regulating Na,K-ATPase expression by neuronal activity.

Sp transcription factors are regulated by neuronal activity and depolarization by KCl increased Sp1 and Sp4 expression, while TTX reduced the expression of Sp1 and Sp4 (Johar et al., 2014). Sp1, Sp3, and Sp4 bound to the promoter regions of *Atp1a1*, *Atp1a3,* and *Atp1b1* in murine neurons, and Sp4 showed the highest binding affinity. Mutations in the Sp binding sites of *Atp1a1*, *Atp1a3,* and *Atp1b1* promoters significantly decreased the promoter activity. Silencing Sp1, Sp3, and Sp4 decreased α_1-, α_3-, and β_1-subunit mRNA levels; while overexpression of Sp1, Sp3, and Sp4 increased α_1-, α_3-, and β_1-subunit transcripts. Silencing Sp1 or Sp4 also blocked the KCl-induced up-regulation of Na,K-ATPase subunits and overexpression of Sp1, Sp3, or Sp4

rescued the down-regulation of Na,K-ATPase subunits by TTX (Johar et al., 2014).

EPIGENETIC MECHANISMS IN THE REGULATION OF Na,K-ATPase TRANSCRIPTION

DNA methylation is an important epigenetic mechanism in the regulation of gene expression. Methylation generally occurs at the cytosine bases of CpG sequences throughout the entire genome, with the exception of CpG islands usually found in gene promoter regions (Cedar and Bergman, 2009). Methylation patterns often depend on tissue type and developmental stage, correlating with transcriptional activity. Aberrant DNA methylation patterns have been linked to altered gene expression in various genetic diseases and tumors (Bird, 2002). Higher methylated CpGs were found in the first exon of *Atp1a3*, which correlated with the lower expression of this gene in pig liver (Henriksen et al., 2013). Manganese exposure led to sustained *Atp1a3* promoter hypermethylation and downregulation of its transcript level in mice (Wang et al., 2013). CpG islands are also found in *ATP1B1* and *Atp1b2* promoter regions (Alvarez de la Rosa et al., 2002; Selvakumar et al., 2014), which are conserved between human and mice and remain unmethylated (Alvarez de la Rosa et al., 2002). However, *ATP1B1* promoter is hypermethylated in tumor samples of clear cell renal cell carcinoma, which showed reduced β_1-subunit levels (Selvakumar et al., 2014). Knock-down of the tumor suppressor gene von Hippel-Lindau (VHL) enhanced *ATP1B1* promoter methylation and decreased β_1-subunit expression, while inhibition of methyltransferase by 5-Aza-2′-deoxycytidine rescued β_1-subunit mRNA expression in VHL-knockdown cells (Selvakumar et al., 2014).

Methylation is also involved in transcriptional regulation and differential distribution of FXYD1. The *FXYD1* promoter contains methylated cytosines and a predicted CpG island. 5′-aza-cytidine markedly increased *FXYD1* mRNA levels. Mouse heart showed lower methylation in *fxyd1* promoter and higher fxyd1 expression; while brain had a higher methylation of the *fxyd1* promoter and lower fxyd1 expression (Deng et al., 2007). In brain, the fxyd1 mRNA level is lower in the frontal cortex than in cerebellum, which correlates with the more frequent promoter methylation found in frontal cortex (Banine et al., 2011). The *fxyd1* promoter is a target of the Methyl CpG binding protein 2 (MeCP2), a common transcriptional modulator. MeCP2 directly bound to methylated CpG in the *fxyd1* promoter and repressed *fxyd1* transcription (Deng et al., 2007). On the other hand, activating histones, such as histone 3 acetylated at lysines 9 and 14 (H3K9/14ac) and histone 3 methylated at lysine 4 (H3K4me3), disassociated with the *fxyd1* promoter to augment the inhibitory effect (Banine et al., 2011).

CONCLUSIONS

The expression of Na,K-ATPase is transcriptionally regulated by hormones, growth factors, lipid mediators and other extracellular stimuli through mediating transcription factor binding to promoter regions of Na,K-ATPase subunits. Many regulators of Na,K-ATPase are involved in the response to demands of cellular activity and ion homeostasis. Together with the spatial and temporal regulation of Na,K-ATPase subunits and their individual isoforms, this allows for a complex and controlled regulation of Na,K-ATPase in response to physiological demands in a tissue-specific manner. Interestingly, various recently identified transcriptional regulators of Na,K-ATPase are transcription factors that are also activated during development, in stem cells and during epithelial-mesenchymal transition (EMT). EMT is a switch in which epithelial cells undergo a shift from a well-differentiated polarized epithelial phenotype to a fibroblastic, mesenchymal phenotype and is a natural developmental process in tissue and organ formation. EMT can also be activated during wound healing, in tissue fibrosis, and is a major factor in cancer developing into a malignant disease (Kalluri and Weinberg, 2009). Vice versa, EMT activated transcription factors regulate the expression of Na,K-ATPase. For example, TGF-β and FGF up-regulate the expression of ZEB1, ZEB2, and Snail; steroid hormones, IGF-1 and PGE2 also induce ZEB1 and ZEB2. And ZEB1 and Snail1 regulate the transcription of Na,K-ATPase. Most recently, we identified the β_1-subunit as a target of the Sonic hedgehog (Shh) signaling pathway (Lee et al., 2015). Shh is a critical morphogen involved in patterning of the early embryo and organogenesis, including the neural tube and limb system. The correlation between these signaling pathways and the transcriptional regulation of Na,K-ATPase is intriguing as elucidating the cross-regulation network among these factors may help in our understanding of the role of Na,K-ATPase with its pump-dependent and pump-independent functions in normal development, stem cell biology, and disease.

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