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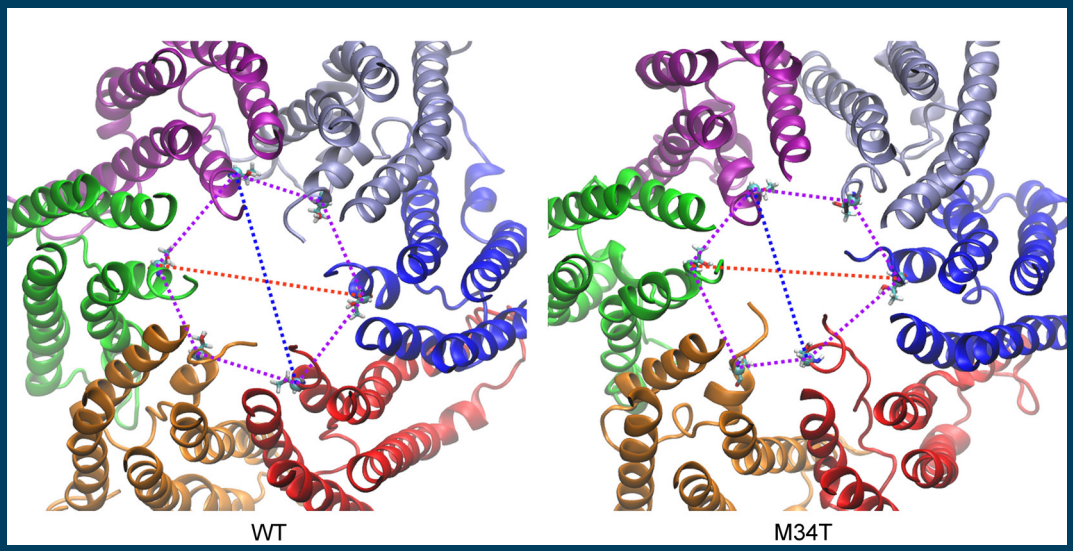

HEMICHANNELS; FROM THE MOLECULE TO THE FUNCTION

Topic Editors
Mauricio A. Retamal and Juan C. Sáez

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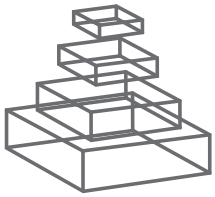

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HEMICHANNELS; FROM THE MOLECULE TO THE FUNCTION

Topic Editors:
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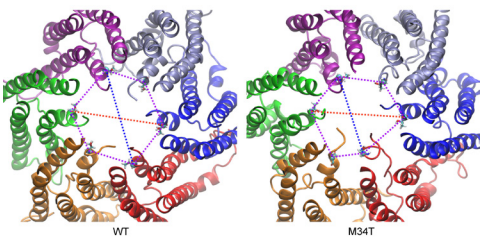

Molecular dynamics simulations highlight structural and functional alterations in deafness-related M34T mutation of connexin 26. Zonta F, Buratto D, Cassini C, Bortolozzi M, Mammano F. Front Physiol. 2014 Mar 4;5:85. doi: 10.3389/fphys.2014.00085. e Collection 2014.

Coordinated cell interactions are required to accomplish several complex and dynamic tasks observed in several tissues. Cell function may be coordinated by cell-to-cell communication through gap junctions channels (GJCs). These channels are formed by the serial docking of two hemichannels, which in turn are formed by six protein subunits called connexins (Cxs). It is well known that GJCs are involved in several functions, such as intercellular propagation of calcium waves, spread of electrotonic potentialsand spatial buffering of ions and metabolites. On the other hand, undocked hemichannels, which are not forming GJCs, can also serve other functions as "free hemichannels". Currently, it is recognized that undocked hemichannels may have functional relevance in cell physiology allowing diffusional exchange of ions and small molecules between intra- and extra-cellular compartments. Additionally, another family of proteins calls pannexins (Panx) also forms functional hemichannels at the plasma membrane. Recently, Panxhemichannels have been involved in both pathological and physiological processes. Controlled hemichannel opening allows the release of small signaling molecules including ATP, glutamate, NAD^+, adenosine, cyclic nucleotides, PGE_2. They also allow uptake of relevant signaling molecules (e.g., cADPR) and metabolites (e.g., glucose). Additionally, a growing body of evidence shows that hemichannels are involved in important processes, such glucose detection in tanicytes, activation of the inflammasome, memory consolidation in the basolateral amygdala, potentiation of muscle contraction and release of nitric oxide from endothelial cells, among others. However, hemichannels can also play an important role in the homeostatic imbalance observed in diverse chronic diseases. In fact, massive and/or uncontrolled hemichannel opening induces or accelerates cell death in several pathological conditions including Charcot-Marie-Tooth

disease, ischemia, oculodentodigital dysplasia, hydrotic ectodermic dysplasia, inflammatory responses, and deafness. Hemichannel-mediated cell death is due mainly to an entry of Ca^{+2}. The latter activates proteases, nucleases and lipases, causing irreversible cell damage. An increasing amount of evidence demonstrates that blockade of uncontrolled hemichannel opening greatly reduces the cellular damage observed in several chronic diseases models. Therefore, Cx and Panx-hemichannels appear as promising drug targets for clinical treatment of human chronic diseases. Therefore, pharmacological tools are urgently needed to further elucidate hemichannels functions and to validate them as drug targets for the development of novel therapies for connexin-based diseases. Thus, understanding the role of Cx and Panx-hemichannels under physiological conditions and recognizing the molecular mechanisms controlling them, may provide us with a better picture of the hemichannels participation in some diseases and of the signals underlying their malfunctioning.

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Hemichannels; from the molecule to the function

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Keywords: hemichannels, connexins, gap junction channels, pannexins, redox regulation, posttranslational modifications

INTRODUCTION

Coordinated cell interactions are required to accomplish diverse complex and dynamic tasks of several tissues in vertebrates and invertebrates. Cell functions, such as intercellular propagation of calcium waves and spread of electrotonic potentials, are coordinated by cell-to-cell communication through gap junction channels (GJCs). These channels are formed by the serial docking of two hemichannels (HCs), which in vertebrates are formed by six protein subunits called connexins (Cxs). In humans, a gene family encodes 21 different proteins with a highly variable C-terminal where most posttranslational modifications occur. Among them protein phosphorylation and/or oxidation (e.g., nitrosylation) induces functional changes.

Currently, it is believed that undocked HCs may have functional relevance in cell physiology allowing diffusional exchange of ions and small molecules between intra- and extra-cellular compartments. In support to this new concept, it has been shown that controlled HC opening allows the release of small signaling molecules (e.g., ATP, glutamate, NAD^+, adenosine, and cyclic nucleotides) and uptake of metabolically relevant molecules (e.g., glucose). Additionally, a growing body of evidences shows that HCs are involved in important and diverse processes, such PGE_2 release from osteocytes, glucose detection in tanicytes, T cell infection with AIDS virus, memory consolidation in the basolateral amygdala and release of nitric oxide from endothelial cells, among others. However, HCs can also play an important role in the homeostatic imbalance observed in diverse diseases. In fact, enhanced HCs opening induces or accelerates cell death in several pathological conditions. Hemichannel-mediated cell death is due mainly to Ca^{+2} influx and cellular overload. The latter activates proteases, nucleases and lipases, causing irreversible cell damage. Accordingly, blockade of HCs reduces the cellular damage observed in several animal models of human diseases. Additionally, another family of proteins called pannexins (Panxs) also forms channels at the plasma membrane and some of their functional and pharmacological sensitivities overlap with those of Cx HCs. Recently, Panx channels have been involved in both pathological and physiological processes. Therefore, Cx HCs and Panx channels appear as promising drug targets for clinical treatment of several inherited and acquired human diseases.

This research topic gathers 11 articles that give a broad view about the role of Cx- and Panx–based channels from purified molecules reconstituted in a lipid environment and posttranslational regulation, to physiological and pathological implications. In addition, it proposes a putative molecular explanation of HC malfunctioning in specific diseases.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Functional analysis and regulation of purified connexin hemichannels

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Gap-junction channels (GJCs) are aqueous channels that communicate adjacent cells. They are formed by head-to-head association of two hemichannels (HCs), one from each of the adjacent cells. Functional HCs are connexin hexamers composed of one or more connexin isoforms. Deafness is the most frequent sensineural disorder, and mutations of Cx26 are the most common cause of genetic deafness. Cx43 is the most ubiquitous connexin, expressed in many organs, tissues, and cell types, including heart, brain, and kidney. Alterations in its expression and function play important roles in the pathophysiology of very frequent medical problems such as those related to cardiac and brain ischemia. There is extensive information on the relationship between phosphorylation and Cx43 targeting, location, and function from experiments in cells and organs in normal and pathological conditions. However, the molecular mechanisms of Cx43 regulation by phosphorylation are hard to tackle in complex systems. Here, we present the use of purified HCs as a model for functional and structural studies. Cx26 and Cx43 are the only isoforms that have been purified, reconstituted, and subjected to functional and structural analysis. Purified Cx26 and Cx43 HCs have properties compatible with those demonstrated in cells, and present methodologies for the functional analysis of purified HCs reconstituted in liposomes. We show that phosphorylation of serine 368 by PKC produces a partial closure of the Cx43 HCs, changing solute selectivity. We also present evidence that the effect of phosphorylation is highly cooperative, requiring modification of several connexin subunits, and that phosphorylation of serine 368 elicits conformational changes in the purified HCs. The use of purified HCs is starting to provide critical data to understand the regulation of HCs at the molecular level.

Keywords: purification, method, gap junction, phosphorylation, PKC, calcium, ATP, permeability

INTRODUCTION

Gap-junction channels (GJCs) formed by connexins are responsible for cell-to-cell communication in eukaryotic cells (Mese et al., 2007; Nielsen et al., 2012; Abascal and Zardoya, 2013). GJCs are formed by head-to-head docking of two connexin hexamers referred to as hemichannels (HCs) or connexons, one from each of the neighboring cells (**Figure 1A**). GJCs and HCs are permeable to large hydrophilic solutes, depending on their isoform composition (Harris, 2001; Nielsen et al., 2012). There are 21 human connexin isoforms, varying in length from 226 to 543 amino acids, which display diverse properties in terms of solute permeabilities, regulation and associations with other proteins (Hua et al., 2003; Mese et al., 2007; Nielsen et al., 2012; Abascal and Zardoya, 2013). **Figure 1A** shows a representation of a connexin, a HC and a GJC. Connexins have four transmembrane α helices (M1-M4) that extend a significant distance outside the plasma membrane. The N- and C-terminal ends and the intracellular loop are intracellular. The available crystal structure of a Cx26 HC in **Figure 1B** shows M1 and M2 as the primary and secondary pore-lining helices, respectively, with the narrowest region of the pore near the extracellular surface of the membrane (Maeda et al., 2009; Nielsen et al., 2012). The extracellular loops, which contain six conserved cysteines that form intramolecular disulfide bonds, have an essential role in HC docking (Foote et al., 1998; Bao et al., 2004b). The primary sequence of the intracellular loop is not well conserved (Hua et al., 2003; Abascal and Zardoya, 2013); this region seems to interact with the C-terminal domain (CTD) during the regulation of HCs by intracellular acidification (Delmar et al., 2004; Hirst-Jensen et al., 2007). The N-terminal region has a role in voltage gating (Purnick et al., 2000; Gonzalez et al., 2007; Bargiello et al., 2012; Kronengold et al., 2012), whereas the CTD displays large variations in length among connexin isoforms and is involved in regulation and protein-protein interactions (Francis et al., 1999; Hua et al., 2003; Agullo-Pascual and Delmar, 2012; Herve et al., 2012; Abascal and Zardoya, 2013). Cx26 and Cx43 are two of the most different connexins regarding their primary sequence and length of the C-terminal sequence, which comprises ∼20 amino acids in Cx26 and ∼150 in Cx43.

GJCs are aqueous channels that communicate two compartments that normally have a very similar composition (the cytoplasms of adjacent cells), and are formed by head-to-head association of two HCs, one from each of the adjacent cells. HCs that are "free" at the plasma membrane (not forming GJCs) communicate two compartments of very different composition (intracellular and extracellular fluids). Whereas GJCs are mostly open and mediate electrical and chemical coupling between cells,

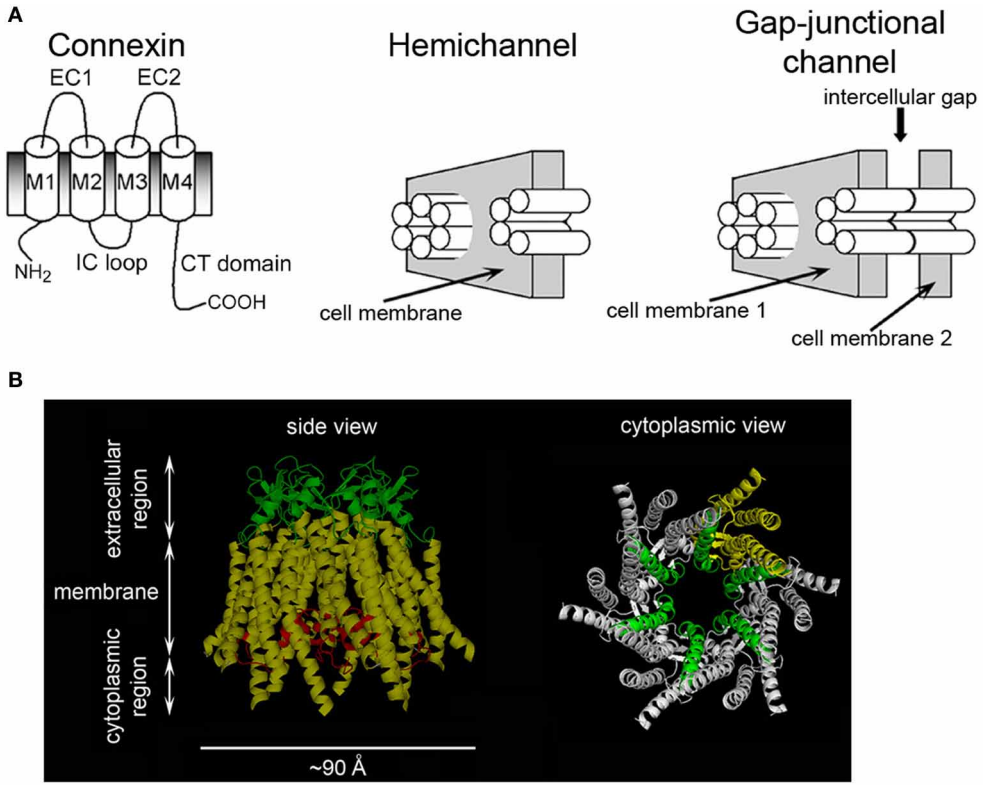

FIGURE 1 | Connexins, hemichannels, and gap-junctional channels. **(A)** Representation of a connexin, a HC and a GJC. M1-M4: trans-membrane helices; EC1 and EC2: extracellular loops; IC loop: intracellular loop. Modified from Bao et al. (2005). **(B)** Cx26 HC structure. Left: transmembrane helices (yellow), EC loops (green), and N-terminal region (red). Part of the latter forms a short α helix that inserts into the pore and has a role in voltage gating. Right: HC view showing M1 (green) lining the pore throughout. M2, M3, and M4 from one subunit are shown in yellow. The N-terminal region was removed to facilitate observation of M1.

HCs at the plasma membrane have a low open probability (Po) due, at least in part, to the combination of the high, cell-negative, membrane voltage, and the millimolar concentration of extracellular Ca^{2+} (Li et al., 1996; Contreras et al., 2003; Gonzalez et al., 2007; Fasciani et al., 2013). However, they still play roles in physiologic processes by mediating the transmembrane fluxes of hydrophilic molecules such as ATP, NAD^+, glutamate, glutathione, PGE_2 and glucose (Bruzzone et al., 2001; Ye et al., 2003; Cherian et al., 2005; Rana and Dringen, 2007; Retamal et al., 2007a; Kang et al., 2008; Orellana et al., 2011; Wang et al., 2013). Sustained opening of HCs under pathological conditions may result in water and solute fluxes that cannot be compensated by the cells. These fluxes include metabolite loss, Ca^{2+} influx, equilibration of ionic gradients and cell swelling and lead to cell damage. Normally, the combination of the cell-membrane voltage and millimolar concentrations of extracellular divalent cations keep HC Po low (Contreras et al., 2003; Bukauskas and Verselis, 2004; Chen et al., 2005; Sanchez et al., 2010; Fasciani et al., 2013). However, Cx43 HCs can be activated in the presence of extracellular divalent cations under a number of conditions including ischemia/hypoxia (John et al., 1999; Li and Nagy, 2000; Li et al., 2001; Contreras et al., 2002; Vergara et al., 2003b; Saez et al., 2005; Shintani-Ishida et al., 2007). Studies in cardiomyocytes, astrocytes, and renal proximal tubule cells strongly suggest that Cx43 HCs are activated by ATP depletion and that the mechanism may involve dephosphorylation and oxidation (John et al., 1999; Li and Nagy, 2000; Li et al., 2001; Contreras et al., 2002; Vergara et al., 2003b; Saez et al., 2005; Retamal et al., 2006; Shintani-Ishida et al., 2007).

CONNEXIN 26

Cx26 is expressed in a number of tissues, and its mutations are frequently associated with deafness and skin diseases (Forge and Wright, 2002; Forge et al., 2003; Gerido and White, 2004; Zhao et al., 2006; Nickel and Forge, 2008; Lee and White, 2009; Liu et al., 2009). Here we will focus on the role of Cx26 in the inner ear. In the inner ear, the cochlea houses the organ of Corti, a narrow spiral of epithelial sensory cells (hair cells) that transduces sound waves into electrical impulses. The cochlear gap-junctional communication network includes several different cell types and is essential for hearing (Steel, 1999; Forge and Wright, 2002; Forge et al., 2003; Wangemann, 2006; Nickel and Forge, 2008). Profound hearing loss of genetic origin is common (∼1 in 2000 children) and mutations of Cx26, the main connexin in the inner ear, are its major cause (Steel, 2000; Ravecca et al., 2005; Sabag et al., 2005; Apps et al., 2007; Nickel and Forge, 2008; Lee and White, 2009; Martinez et al., 2009; Laird, 2010; Terrinoni et al., 2010; Zoidl and Dermietzel, 2010).

The receptor function of the hair cells requires high [K^+] in the endolymph and a positive endolymphatic electrical potential (Steel, 1999; Forge and Wright, 2002; Forge et al., 2003; Teubner et al., 2003; Wangemann, 2006; Zhao et al., 2006; Nickel and Forge, 2008). Both are generated by K^+ transport by the *stria vascularis* and depend on an intact gap-junctional system (Steel, 1999; Forge and Wright, 2002; Forge et al., 2003; Wangemann, 2006; Nickel and Forge, 2008). Activation of the hair cells by sound waves opens non-selective cation channels near the tips of the stereocilia, eliciting influxes of Ca^{2+} and K^+ and membrane depolarization (Kikuchi et al., 2000; Steel and Kros, 2001; Forge and Wright, 2002; Forge et al., 2003; Nickel and Forge, 2008). It has been proposed that the K^+ that enters the hair cells moves across their basolateral membrane into the perilymphatic space between hair cells and supporting cells. This K^+ is then taken up by the supporting cells and recycled back into the endolymph *via* the cochlear gap-junctional network, moving from the supporting cells through the root cells, spiral ligament fibroblasts and the *stria vascularis*, from where it is secreted back to the *scala media* endolymph (Spicer and Schulte, 1998; Steel, 1999; Kikuchi et al., 2000; Forge and Wright, 2002; Forge et al., 2003; Zhao et al., 2006; Nickel and Forge, 2008; Liu et al., 2009). Absence of Cx26 results in death of the hair cells, but the mechanism is unknown. It has been speculated that deafness is a consequence of decreased K^+ recycling in the cochlea (Johnstone et al., 1989; Kudo et al., 2003; Wangemann, 2006). However, the notion of cochlear K^+ recycling has been questioned (Patuzzi, 2011), and there are deafness-associated Cx26 mutants that form K^+ permeable GJCs, but show more subtle permeability changes, such as decrease in pore size or changes in charge-selective permeability (Goldberg et al., 1999, 2002; Bruzzone et al., 2003; Beltramello et al., 2005; Chen et al., 2005; Zhao, 2005; Deng et al., 2006; Anselmi et al., 2008; Gossman and Zhao, 2008; Mese et al., 2008; Majumder et al., 2010). In this case (e.g., Cx26 V84L), the mutations may affect second messenger transport between cells.

It has also been proposed that "leaky" mutant HCs can lead to cell damage and deafness (Stong et al., 2006; Gerido et al., 2007; Lee et al., 2009). Water and solute fluxes through mutant "leaky" HCs (metabolite loss, Ca^{2+} influx, equilibration of ionic gradients, cell swelling) can lead to cell damage and deafness. It has also been speculated that ATP in the endolymph is essential to maintain healthy hair cells, and deafness could result from Cx26 mutations that decrease HC-mediated ATP secretion (Anselmi et al., 2008; Majumder et al., 2010). Recent observations suggest that ATP released through Cx26 and Cx30 HCs on the endolymphatic side of supporting cells activates purinergic receptors in inner hair cells, evoking bursts of action potentials in the developing organ of Corti (Gale et al., 2004; Anselmi et al., 2008; Majumder et al., 2010). Ca^{2+} signaling seems to be essential to maintain healthy hair cells, and therefore alterations in signaling may have an important role in genetic deafness. A role of cochlear Ca^{2+} signaling in the response to damaging stimuli has also been proposed, and an increase in Ca^{2+} permeability through HCs formed by a Cx26 mutant associated with keratitis ichthyosis deafness syndrome (Cx26 G45E) has been identified (Sanchez et al., 2010).

CONNEXIN 43

Cx43 is the most ubiquitous connexin isoform. Its CTD contains a number of domains involved in interactions with a variety of proteins such as tubulin, tyrosine kinases, ubiquitin ligase, zonula occludens 1 (ZO-1) and Na^+ channels (Warn-Cramer and Lau, 2004; Solan and Lampe, 2009; Agullo-Pascual and Delmar, 2012; Herve et al., 2012; Agullo-Pascual et al., 2013). Here, we will focus on the role of Cx43 in the cardiovascular system.

Mutations of Cx43 are associated with arrhythmias, oculodentodigital dysplasia, and other genetic disorders, and alterations in Cx43 expression and function play important roles in the pathophysiology of frequent medical problems such as those related to cardiac and brain ischemia as well as wound healing in diabetes (Solan and Lampe, 2009; Eugenin et al., 2012; Marquez-Rosado et al., 2012; Orellana et al., 2012; Churko and Laird, 2013; Giaume et al., 2013). Cx43 is by far the most abundant heart connexin, expressed at high levels in atrial and ventricular myocytes, and to a lower extent in parts of the ventricular conduction system (Fontes et al., 2012). Normally, Cx43 is located at the intercalated discs and its density is low at the lateral membranes (Miura et al., 2010; Duffy, 2012; Fontes et al., 2012; Jeyaraman et al., 2012; Remo et al., 2012). In combination with other factors, this distribution results in a faster longitudinal conduction velocity that is the basis for anisotropic conduction. The preferential location of Cx43 at the intercalated discs depends on a number of factors, including its association with ZO-1 (Remo et al., 2012). Cardiac remodeling occurs in response to a variety of cardiac disorders, and is characterized by structural and electrical alterations that decrease heart electrical stability (Miura et al., 2010; Duffy, 2012; Fontes et al., 2012). The changes in impulse conduction are associated with the phenomenon of lateralization, defined by decreased Cx43 expression, with a relative increase in lateral *vs.* intercalated-disc Cx43 expression, which is often associated with abnormal conduction and arrhythmias (Miura et al., 2010; Duffy, 2012; Fontes et al., 2012; Jeyaraman et al., 2012; Remo et al., 2012). Lateralization is observed in a variety of acquired and inherited arrhythmic syndromes, including ischemic heart disease, hypertrophic cardiomyopathy and arrhythmogenic right ventricular cardiomyopathy (Miura et al., 2010; Duffy, 2012; Fontes et al., 2012; Remo et al., 2012). Although the events leading to lateralization are not fully understood, it seems that under ischemia or conditions of cell stress, the tyrosine kinase Src is activated and binds to ZO-1, competing with the Cx43 binding site, which releases Cx43 from ZO-1. As a result, ZO-1 remains at the intercalated discs, while the plaques move to the lateral membrane (Duffy, 2012). Details on the regulation of Cx43 GJCs and HCs are presented later.

WHY STUDY PURIFIED HEMICHANNELS?

Extensive information has been obtained on GJC and HC expression and function from cell-biology and functional studies in cells and organs in normal and pathological conditions. For example, there is evidence for changes in Cx43 phosphorylation state under ischemia in the heart (Solan et al., 2007; Solan and Lampe, 2009; Miura et al., 2010; Jeyaraman et al., 2012; Marquez-Rosado et al., 2012), and opening of HCs seems to have a role in cardiomyocyte damage in ischemia (John et al., 1999; Li and Nagy, 2000; Li et al.,

2001; Contreras et al., 2002; Vergara et al., 2003b; Shintani-Ishida et al., 2007; Hawat et al., 2010). It is also known that HCs open in response to PKC inhibitors (Bao et al., 2004a). However, molecular mechanisms are hard to tackle in these systems because of their inherent complexities. Experiments *in vivo* are fundamental to understand biological processes, but *in vitro* studies using isolated systems under well-controlled conditions are also an essential component for a complete understanding of normal function and the molecular mechanisms of diseases. In this context, studies of purified HCs provide direct structural and conformational information that serves as an essential complement to the studies in more complex system.

HCs have physiological and pathophysiological significance (John et al., 1999; Bruzzone et al., 2001, 2003; Contreras et al., 2002; Vergara et al., 2003a; Ye et al., 2003; Beltramello et al., 2005; Cherian et al., 2005; Stong et al., 2006; Gerido et al., 2007; Rana and Dringen, 2007; Retamal et al., 2007a; Shintani-Ishida et al., 2007; Anselmi et al., 2008; Gossman and Zhao, 2008; Kang et al., 2008; Lee et al., 2009; Hawat et al., 2010), but the information obtained with HCs also contributes to our understanding of GJCs. Cx26 GJCs behave like two HCs in series (Chen et al., 2005; Sanchez et al., 2010), whereas Cx43 HCs expressed in HeLa cells display single-channel events with conductance and kinetics consistent with Cx43 GJCs, even though Cx43 HCs are activated only at very positive intracellular voltages, a feature different from the voltage sensitivity of Cx43 GJCs (Saez et al., 2005). In addition, the mechanisms of GJC and HC regulation overlap very well (Moreno et al., 1992, 1994; Takens-Kwak and Jongsma, 1992; Kwak et al., 1995; Lampe et al., 2000; Bao et al., 2004a,c, 2007; Delmar et al., 2004; Saez et al., 2005; Ek-Vitorin et al., 2006), and therefore studies on the mechanisms of regulation of HCs also contribute to our understanding of GJC regulation.

EXPRESSION/PURIFICATION AND CHARACTERIZATION OF PURIFIED CONNEXINS

Recombinant connexins have been expressed in a variety of systems for cell-biology, biochemical and functional studies, notably mammalian cell lines and frog oocytes. For detailed biochemical studies that require purified connexins, the proteins have been expressed in mammalian and insect cells. Although expression in mammalian cells has been used successfully (Koreen et al., 2004), the insect cell/baculovirus expression system is the only one available that yields milligram amounts of purified connexins. This system has been useful to express Cx26 and Cx43, as well as a variety of mutants (Stauffer, 1995; Bao et al., 2004c, 2007; Oshima et al., 2007, 2008; Gassmann et al., 2009; Maeda et al., 2009; Ambrosi et al., 2010; Fiori et al., 2012).

In most cases, we express connexins modified by insertion of a protease cleavage site and a poly-His tag at the C-terminal end (Bao et al., 2004c; Fiori et al., 2012). The protease cleavage sites are selective for either thrombin or TEV proteases. These connexin DNA sequences are cloned into baculovirus transfer vectors and used to generate recombinant baculoviruses. We have used the Invitrogen Bac-to-Bac system successfully. The viruses produced in Sf9 insect cells are used to infect insect cells for protein production, in either Sf9 or High-Five cells working well for connexin expression (Bao et al., 2004c, 2007; Fiori et al., 2012). We generally use Sf9 cells in suspension, grown in serum-free HyClone CCM3 medium supplemented with gentamycin. The cells grown at 26°C are infected (generally 2 viral particles/cell) and the cells are harvested ∼2 days after infection, when viability is ∼40%. For purification, the cell pellets are resuspended in a 1-mM bicarbonate solution containing 1 mM protease inhibitors, and lysed. The membranes are alkali-extracted by addition of NaOH, then solubilized with 2.5% *n*-dodecyl-β-D-maltoside in the presence of a high salt concentration (2 M NaCl), a chelator of divalent cations, a reducing agent and 10% glycerol. The solubilized material is then purified based on the affinity of the His tag for Co^{2+}, followed by size-exclusion chromatography. If needed, removal of the His tag is accomplished by site-specific proteolysis, which is followed by isolation of the untagged connexin by size-exclusion chromatography. The insect-cell/baculovirus expression system yields approximately 0.5 mg/l culture of highly-pure connexins suitable for biochemical, structural and functional studies (Bao et al., 2004c; Fiori et al., 2012).

Connexins expressed in insect cells have been successfully employed for structural studies using X-ray crystallography and cryo-electron microscopy (Hoh et al., 1993; Oshima et al., 2003, 2007, 2008, 2011; Gassmann et al., 2009; Maeda et al., 2009; Ambrosi et al., 2010). Electron-microscopy data point to Cx26 purified as HCs (hexamers), as opposed to GJCs (dodecamers), and our studies agree with that notion (Bao et al., 2004c; Fiori et al., 2012). We have recently performed a detailed biochemical and biophysical characterization of purified Cx26 (Fiori et al., 2012). A single absorbance peak was observed in size-exclusion chromatograms, coincident with the high degree of purity estimated from Coomassie blue-stained gels. The apparent molecular weight of the protein-detergent complex was estimated at 235 kDa, whereas its average hydrodynamic radius determined by dynamic light scattering was 5.4 nm. These values are consistent with a Cx26 hexamer-detergent complex. The purified Cx26 hexamers were highly-structured, with a calculated α-helix content of 59%, in reasonable agreement with the recent crystal structure of Cx26 that shows an approximate α helical content of 54%.

It is interesting to note that the properties of purified Cx26 and Cx43 differ. While Cx26 is purified as HCs that are highly stable (Fiori et al., 2012), purified Cx43 HCs are not stable in detergent, but are stable in lipids (Bao et al., 2007). In detergent and at low concentrations Cx43 is mostly present as monomers, whereas at higher concentrations it forms hexamers that allow for subunit exchange (Bao et al., 2007). The lower stability of Cx43 HCs in solution is very useful because it allows the generation of Cx43 HCs with controlled subunit composition (see below).

FUNCTIONAL ANALYSIS OF PURIFIED AND RECONSTITUTED CONNEXIN HEMICHANNELS

Recombinant purified GJCs and HCs expressed in Sf9 insect cells have been used extensively for structural studies. In contrast, functional studies are few, and have been performed without control of a number of variables known to affect HC gating, such as transmembrane voltage and redox state (Retamal et al., 2006, 2007b; Gonzalez et al., 2007; Bargiello et al., 2012). Functional assays for purified connexin HCs can be generally divided into

qualitative assays used to determine whether HCs are permeable to a solute or not, and quantitative/semi-quantitative assays that are more suitable to determine changes in HC permeability properties. The former include the transport-specific fractionation technique developed by Harris and collaborators (Harris et al., 1989; Harris and Bevans, 2001; Bao et al., 2004c, 2007; Fiori et al., 2012), and probe-permeation assays that use labeled solutes to determine whether they can enter into liposomes containing HCs or be released from liposomes pre-loaded with the probe (Bevans et al., 1998; Bao et al., 2004a; Fiori et al., 2012). The latter includes single-channel electrophysiological studies and assays of solute influx into liposomes containing HCs (Buehler et al., 1995; Rhee et al., 1996; Gassmann et al., 2009; Fiori et al., 2012).

The transport-specific fractionation technique is used to determine whether HCs are permeable or not to sucrose and other hydrophilic solutes (Harris et al., 1989; Harris and Bevans, 2001; Bao et al., 2004c, 2007; Fiori et al., 2012). Although qualitative, it is a very powerful technique because it depends directly on solute transport and rules out solute binding to the liposomes. The method is based on the migration of liposomes upon centrifugation in a linear isoosmolar sucrose gradient where the concentration of sucrose increases from top to bottom, and the concentration of urea decreases from top to bottom, in such a way that the osmolality remains constant. The liposomes or proteoliposomes containing HCs impermeable to sucrose remain in the upper part of the tube, buoyed up by the entrapped urea solution of lower density. The heavier sucrose-loaded liposomes containing sucrose-permeable HCs migrate as a narrow band to a lower position in the tube. A schematic representation of the technique is shown in **Figure 2A**. The experiments are simplified by the incorporation into the liposomes of traces of a fluorescent lipid, which allows for easy following of the liposome position in the gradient. An example is shown in **Figure 2B**. Also, transport-specific fractionation can be used in combination with permeability assays for other solutes. For these studies, the liposomes are pre-loaded with the probes (e.g., radiolabeled or fluorescent permeability probes), and permeability is determined from the retention of the probes in the liposomes (impermeable) or their loss (permeable). The transport-specific fractionation of liposomes is also very useful to determine the fraction of purified connexins that form functional HCs (Rhee et al., 1996; Bao et al., 2004c, 2007), a very important parameter to assess the quality of the preparation. For these studies, HCs reconstitution is done in such a way that the average yield is less than one HC/liposome. Under these conditions, the experimental data (percentage of sucrose-permeable liposomes with one or more functional HCs)

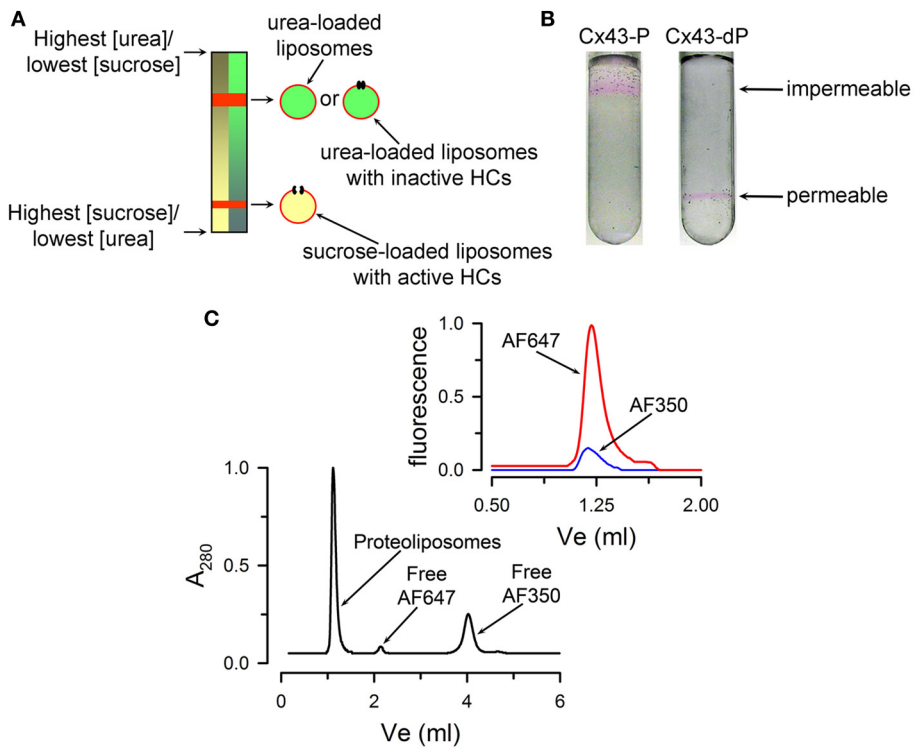

FIGURE 2 | Qualitative transport assays. (A) Schematic representation of the transport-specific assay. **(B)** Typical migration of liposomes containing purified Cx43 HCs on an isoosmolar linear sucrose density gradient. Images showing the position of the rhodamine B-labeled proteoliposomes after centrifugation. The HCs were formed by either fully dephosphorylated Cx43 (Cx43-dP, sucrose permeable) or Cx43 fully phosphorylated by PKC (all six Ser-368 residues phosphorylated, Cx43-P, impermeable to sucrose). Reconstitution was at an average HC/liposome ratio of 2.3. **(C)** Permeability of reconstituted HCs to Alexa Fluor probes. Liposomes pre-loaded with Alexa Fluor 350 (AF350) or 647 (AF647) were run on the size-exclusion column to separate free extraliposomal dyes from the dyes inside the liposomes. A_{280} is the absorbance at 280 nm. Top right: AF350 and AF647 fluorescence associated with the liposomes containing Cx26. The data were normalized to the peak emission of parallel experiments with liposomes without Cx26. Panels **(B)** and **(C)** were modified from Bao et al., 2004c and Fiori et al., 2012, respectively.

can be compared with the prediction based on the protein/lipid ratio and the size of the liposomes. Sucrose-impermeable liposomes will be those without HCs and those with non-functional HCs. Our studies showed that essentially all purified Cx26 and Cx43 HCs purified from insect cells form functional HCs (Bao et al., 2007; Fiori et al., 2012).

A more standard assay for solute permeability is based on probe uptake or efflux. For example, liposomes containing purified Cx26 HCs pre-loaded with Alexa Fluor 350 (349 Da) and Alexa Fluor 647 (1300 Da) can be separated from free probes by size-exclusion chromatography (Fiori et al., 2012). **Figure 2C** shows a typical size-exclusion chromatogram where liposomes are separated from the extraliposomal free dyes. Alexa Fluor 350 and 647 were retained by the liposomes without HCs, whereas only the latter was retained inside the proteoliposomes with Cx26 HCs. Similar experiments can be performed with physiologically-relevant solutes such as ATP, inositol phosphates and cyclic nucleotides (Ayad et al., 2006; Harris, 2007).

Single-channel analysis is the assay of choice to evaluate the permeability properties of connexin HCs to small inorganic ions. Unfortunately, the experience with purified HCs has been mixed at best, with HCs often not showing the single-channel conductance and kinetics expected from the studies in cells (Buehler et al., 1995; Rhee et al., 1996; Gassmann et al., 2009). This is clearly an area that needs additional experimental work. Therefore, for the more quantitative studies, we will focus on assays that we developed recently to study permeation of Ca^{2+}, Na^+, and H^+. In principle, intercellular Ca^{2+} signaling can be the result of movements through GJCs of second messengers such as IP3 and/or Ca^{2+}, but signaling is also possible through a paracrine pathway, by the release of ATP through HCs (Piazza et al., 2007; Anselmi et al., 2008; Kang et al., 2008; Mammano, 2013). In this case, activation of purinergic receptors in neighboring cells elicits Ca^{2+} influx in those cells. Although extracellular [Ca^{2+}] at millimolar concentrations decreases HC activity (Li et al., 1996; Contreras et al., 2003; Chen et al., 2005; Fasciani et al., 2013; Lopez et al., 2013), HCs can still be activated at high extracellular [Ca^{2+}] under a number of conditions, including ischemia, inflammation, connexin dephosphorylation, and extracellular alkalinization (John et al., 1999; Li and Nagy, 2000; Contreras et al., 2002; Bao et al., 2004a; Retamal et al., 2007b; Shintani-Ishida et al., 2007; Hawat et al., 2010; Schalper et al., 2010; Orellana et al., 2011; Eugenin et al., 2012). In spite of indirect evidence from studies in cells, the possibility of Ca^{2+} movement through HCs and GJCs had not been addressed directly until recently. Reasons include the minimal number of functional studies of purified HCs, and the absence of an assay to follow Ca^{2+} permeation in purified HCs. In order to address whether HCs are permeable to Ca^{2+}, we developed an assay to follow the time course of changes in intraliposome [Ca^{2+}] that result from influx through HCs, and tested the assay on purified Cx26 HCs (Fiori et al., 2012). Basically, we loaded liposomes with the low-affinity Ca^{2+}-sensitive fluorescent probe Fluo-5N, and then removed the extraliposomal probe by gel filtration. Fluo-5N is retained inside the liposomes in the presence or absence of Cx26 HCs because its size and charge (958 Da, -5 net charge). Typical experiments are shown in **Figure 3A**, where Fluo-5N emission

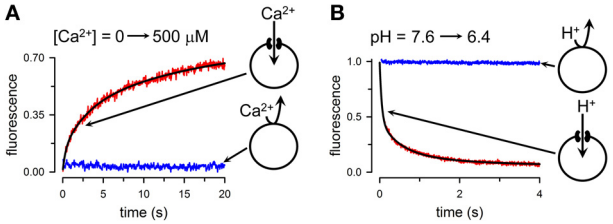

FIGURE 3 | Quantitative transport assays. (A) Rate of Ca^{2+} influx into liposomes containing purified Cx26 HCs. Extraliposomal free-[Ca^{2+}] was rapidly increased by mixing in a stop-flow cell from a few nM to 500 μM, and the rate of increase in emission from Fluo-5N trapped into the liposomes was followed. Red record: liposomes containing Ca^{2+}-permeable Cx26 HCs; blue record: liposomes without HCs. The black line is a multi-exponential fit to the data used to calculate the rate of Ca^{2+} influx. **(B)** Changes in intraliposomal pH followed by the pH-sensitive probe fluorescein attached to a phospholipid head group. Extraliposomal pH was rapidly reduced from 7.6 to 6.4 by mixing in a stop-flow cell, and the rate of decrease in pH was followed by the quenching of the fluorescein bound to the inner leaflet of the liposomes bilayer. The black line is a multi-exponential fit to the data used to calculate the rate of liposome acidification. Modified from Fiori et al., 2012.

increased following the elevation of free-[Ca^{2+}] from <10 nM to 500 μM in the liposomes containing HCs (red trace), but not in liposomes without HCs (blue trace). In these experiments, the increase in extraliposomal [Ca^{2+}] was achieved in <0.5 ms in a stop-flow cell. Analysis of the changes in fluorescence allows for estimations of Ca^{2+} influx and permeability. With a similar approach, but using the Na^+-sensitive fluorescent probe SBFI, we determined that the permeabilities of Cx26 HCs to Na^+ and Ca^{2+} are similar, pointing to a high Ca^{2+} permeability. In spite of the high Ca^{2+} permeability, since cytosolic [Ca^{2+}] and [Ca^{2+}] gradients are low, significant cell-to-cell Ca^{2+} fluxes through GJCs will depend critically on the number of permeable channels. In contrast, the much larger electrochemical driving force for Ca^{2+} influx into cells suggests a significant role of HCs in Ca^{2+} influx, at least under certain circumstances (e.g., ischemia), or in disease-causing mutants that display higher Ca^{2+} permeability (G45E mutant associated with keratitis ichthyosis deafness syndrome) (Sanchez et al., 2010).

We have also developed a variation of the methodology above to assess the permeation of H^+ equivalents (H^+/OH^-/buffer transport) (Fiori et al., 2012). For these studies, the liposomes contain traces of a phospholipid labeled with fluorescein at the head group. When extraliposomal pH is reduced from 7.6 to 6.4 in a stop-flow cell, fluorescein emission decreased in the Cx26-HC liposomes exposed to the pH gradient (**Figure 3B**, red trace), but not in the liposomes without HCs (**Figure 3B**, blue trace). Upon lowering extraliposomal pH, there is quenching of the fluorescein emission from the outer leaflet of the liposome bilayer. This quenching is independent of the presence of HCs, and is too fast for detection in the stop-flow setup. Only the slower fluorescence quenching that results from the effects of intraliposomal acidification on the inner-leaflet fluorescence is then recorded (**Figure 3B**). From the pH changes, buffer composition and other parameters, we estimated an H^+-equivalent permeability ~10-fold higher than that for Na^+. Since sucrose permeability

is low, and relative K^+/cAMP and K^+/Lucifer yellow permeability ratios are high (Kanaporis et al., 2008), Cx26 HC permeability to organic buffers such as HEPES is lower than that to Na^+, and therefore the data on H^+ equivalents/Na^+ permeability ratio is an underestimate of the true H^+/Na^+ ratio.

Other simple assays that we have employed are "traditional" rapid-filtration assays (Bao et al., 2004c). These are possible because the permeabilities of HCs to "large" hydrophilic solutes, including second messengers (e.g., ATP, cAMP, IP3) is not very high and equilibration between intra- and extraliposomal spaces in HCs containing ∼1 HC occurs in >10 s (Kanaporis et al., 2008; Fiori et al., 2012).

REGULATION OF CX43 HEMICHANNELS BY PHOSPHORYLATION

Phosphorylation of Cx43 plays a critical role in gap-junction remodeling, and plasma-membrane HC opening in response to ischemic damage in the brain, heart and kidney is likely linked to Cx43 dephosphorylation (Li and Nagy, 2000; Contreras et al., 2002; Hawat et al., 2010; Duffy, 2012). Following phosphorylation of Ser368 by PKC the electrical cell-to-cell coupling (mediated fluxes of small inorganic univalent ions though GJCs) is maintained, whereas the selectivity of chemical coupling (larger hydrophilic solutes) generally decreases, but with an altered selectivity; permeability to negatively-charged solutes decreases, but that to positively-charged ones increases (Kwak et al., 1995; Lampe et al., 2000; Bao et al., 2004a,c; Ek-Vitorin et al., 2006). Single-channel studies have shown that stimulation of PKC decreases the frequency of the dominant of Cx43 GJCs conductance state (∼100 pS), favoring a lower conductance state (∼50 pS) (Moreno et al., 1992, 1994; Lampe et al., 2000; Ek-Vitorin et al., 2006).

A consequence of Ser368 HC dephosphorylation is an increase in the permeability to large hydrophilic solutes that may cause cell damage due to losses of essential metabolites and second messengers (e.g., glutathione, cAMP, IP3) and/or perhaps influx of Ca^{2+}, whereas phosphorylation of GJCs at Ser368 decreases cell-to-cell chemical coupling, which could minimize spreading of the damage to healthy neighboring cells. Uncoupled HCs have been shown to exist in several cell types, and activation of large non-selective Cx43 HCs during ischemia may overwhelm the normal membrane-transport mechanisms and alter intracellular composition, contributing to cell injury. This notion is supported by data on cardiomyocytes, astrocytes, and renal proximal tubule cells that show Cx43 HC activation by ATP depletion (John et al., 1999; Li and Nagy, 2000; Li et al., 2001; Contreras et al., 2002; Vergara et al., 2003b; Hawat et al., 2010). One possibility is that ATP depletion activates the Cx43 HCs by decreasing their phosphorylation state, although other possibilities have been proposed (Kwak et al., 1995; Lampe and Lau, 2000; Lampe et al., 2000; Li and Nagy, 2000; Contreras et al., 2002; Bao et al., 2004a; Retamal et al., 2007b,c; Hawat et al., 2010).

There is significant information available on the role of changes in Cx43 phosphorylation in the heart during cardiac ischemia/hypoxia. The overall effect is a decrease in dephosphorylation that can be a consequence of ATP depletion and/or increased activity of protein phosphatase 1A (Solan and Lampe, 2009; Miura et al., 2010; Duffy, 2012). The changes in Ser368 phosphorylation state are complex. Several constitutively phosphorylated serines are dephosphorylated, including Ser365 (Solan et al., 2007; Solan and Lampe, 2009). Dephosphorylation of Ser365 could be potentially important because its phosphorylation prevents the Ser-368 phosphorylation by PKC (Solan et al., 2007). The combination of Ser365 dephosphorylation and he increase in heart PKC activity in response to ischemia/hypoxia can account for the increase in Ser368 phosphorylation in spite of the overall decrease in Cx43 phosphorylation (Ek-Vitorin et al., 2006; Solan et al., 2007; Solan and Lampe, 2009; Marquez-Rosado et al., 2012). The increase in S368 phosphorylation occurs at the intercalated discs, but Cx43 phosphorylated at S368 has not been found outside the intercalated discs (Ek-Vitorin et al., 2006; Solan et al., 2007; Solan and Lampe, 2009; Marquez-Rosado et al., 2012). Therefore, it seems that in cardiac ischemia total Cx43 expression decreases, but S368-phosphorylated Cx43 increases at the intercalated discs, and Cx43 dephosphorylated at S368 is present in the lateral membranes, probably including HCs. The resulting effects would be a reduction in cell-to-cell coupling at the intercalated discs (minimizing the spread of the damage), and an increase in HC activity that can contribute the ischemic damage.

REGIONS OF THE Cx43 C-TERMINAL DOMAIN THAT ARE ESSENTIAL FOR THE HEMICHANNEL REGULATION BY PKC-MEDIATED PHOSPORYLATION

The Cx43 CTD can be phosphorylated by several kinases, including PKA, PKC, p34cdc2/cyclin B kinase (p34cdc2), casein kinase 1 (CK1) and pp60src kinase (src), with effects on GJC and HC permeabilities, as well as trafficking, assembly and degradation (Solan and Lampe, 2009). The effects of phosphorylation by these kinases are varied, but here we will concentrate on PKC-mediated phosphorylation of Cx43.

It has been established that the effect of PKC-mediated phosphorylation of Cx43 depends on the presence of the CTD (Solan and Lampe, 2009). A number of low-resolution electron microscopy structures of Cx43 GJCs are available (Unger et al., 1997, 1999a,b; Yeager, 1998). However, these structures do not provide information on the CTD because it had to be removed to improve crystal resolution (Unger et al., 1997, 1999a,b; Yeager, 1998; Cheng et al., 2003). There are NMR structures of the isolated Cx43 CTD (Sorgen et al., 2002, 2004; Hirst-Jensen et al., 2007; Solan et al., 2007; Grosely et al., 2010), but it is presently unclear how closely they resemble the native CTD conformation in the full-length Cx43. One approach to start addressing the specifics of the mechanism is to determine which regions of the CTD are essential for the effect of PKC. To address this question, we performed functional studies in frog oocytes expressing HCs formed by mutants of Cx43 where regions of the CTD were deleted (Bao et al., 2004a). The functional assay consisted of measuring the increase in the uptake of the HC-permeable carboxyfluorescein in response to PKC inhibitors. In Cx43, the CTD extends from approximately E227, and consists of ∼155 amino acids. In HCs formed by a Cx43 with deletion of ∼60% of the CTD PKC inhibitors still increased carboxyfluorescein uptake. The absence of influence of this large

CTD deletion on the response to PKC was directly confirmed in equivalent experiments on purified HCs reconstituted in liposomes (Bao et al., 2004c). The CTD deletion included sequences before and after the Pro-rich region that is essential for the inhibitory effect of lowering intracellular pH and phosphorylation by tyrosine kinases (Warn-Cramer et al., 1996, 1998; Calero et al., 1998; Warn-Cramer and Lau, 2004). Further deletion of the Pro-rich region in our deletion mutant abolished the effect of PKC inhibitors, suggesting a critical role of the Pro-rich region, and therefore a similarity between the effects of acidification and PKC (Bao et al., 2004a). However, replacing of two prolines essential for the pH effect with alanines had no influence on the effect of PKC inhibitors (Bao et al., 2004a). Moreover, replacing 11 amino acids of the Pro-rich region with a 10-Gly linker also had no effect on the response to PKC inhibition (Bao et al., 2004a). Therefore, the mechanisms of changes in HC permeability by Tyr kinases and pH changes, on one side, or by PKC-mediated phosphorylation, on the other, are different. It is still unknown whether the absence of effect of PKC inhibition in the large CTD deletion that included the Pro-rich region is due to the inability of the CTD end (where Ser368 is located) to approach a target region of Cx43. In this context, a ball-and-chain mechanism has been proposed to explain the closure of the Cx43 HC pore in response to intracellular acidification and activation of insulin receptor tyrosine kinase; in this mechanism, the CTD would act as the "ball" that interacts with the C-terminal half of the intracellular loop and occludes the pore (Homma et al., 1998; Delmar et al., 2004; Warn-Cramer and Lau, 2004).

IDENTIFICATION OF THE PKC TARGET RESIDUE

The observations described above indicate that the CTD is essential for the regulation of HC permeability by PKC-mediated phosphorylation and that the majority of the amino acids in the CTD are not involved in the effect of PKC. It has been established that PKC phosphorylates Cx43 CTD serines, and that the phosphorylated residues are Ser368 and Ser372, neighboring residues near the end of the CTD (Bao et al., 2004a,c; Solan and Lampe, 2009). Replacement of these two residues with alanine individually or in combination indicates that Ser368 is the essential residue for the effect of PKC inhibitors in frog oocytes (Bao et al., 2004a). This is consistent with previous mutagenesis studies in mammalian cells (Lampe et al., 2000) and was directly corroborated by us using purified Cx43 HCs reconstituted in liposomes (Bao et al., 2004c). With this preparation, we first showed that purified Cx43 dephosphorylated by alkaline phosphatase is permeable to organic hydrophilic probes, including sucrose, and that phosphorylation by PKC *in vitro* produces sucrose-impermeable HCs. Then, we showed that this effect of PKC on purified HCs is absent in a Cx43 mutant where S368 was replaced with Ala. The HCs formed by the Cx43-Ser368A mutant are constitutively permeable to sucrose and carboxyfluorescein and do not respond to PKC (Bao et al., 2004a,c). These studies indicate that the effect of PKC on "large" solutes is direct (does not require regulatory intermediate steps), and that the effect is due to phosphorylation of Ser368.

CONFORMATIONAL CHANGES ASSOCIATED WITH THE REGULATION BY PKC

Since phosphorylation of Ser368 is responsible for the decrease in permeability of Cx43 HCs, phosphorylation of this target residue should elicit a conformational change in the HCs. The nature of this change is still unresolved. As mentioned earlier, a ball-and-chain (or particle-receptor) mechanism has been proposed to explain the closure of the HC pore in response to intracellular acidification and activation of insulin receptor tyrosine kinase (Homma et al., 1998; Delmar et al., 2004; Warn-Cramer and Lau, 2004). Although phosphorylation by PKC does not close the Cx43 HC pore completely (Moreno et al., 1992, 1994; Lampe et al., 2000; Ek-Vitorin et al., 2006), as v-Src and MAPK phosphorylation do (Kim et al., 1999; Cottrell et al., 2003; Warn-Cramer and Lau, 2004), a similar mechanism, but with partial pore closure, is still a possibility. Unfortunately, experimental data addressing the molecular mechanism of regulation of Cx43 GJCs and HCs is largely missing. In a recent study it was shown that mutation of Ser368 and Ser372 to Asp elicits conformational changes in a Cx43 fragment consisting of M4 and the CTD solubilized in detergent (Grosely et al., 2013). The percentage of α-helical structure, of the wild-type fragment, as determined from circular dichroism spectra, increased by lowering the pH from 7.5 (∼30%) to 5.8 (∼45%), whereas the PKC-phosphorylation-mimicking mutant had an intermediate α-helical content (∼40%) that was pH independent. Significant changes in chemical shifts in the PKC mutant fragment were detected by NMR spectroscopy for 14 residues. This is currently the only study where conformational changes were evaluated at the amino-acid level. Assuming that 100% of M4 in the M4-CTD fragment is α helical, it seems that 10–30% of the wild-type CTD is structured (depending on the pH), i.e., the majority is unstructured. In fact, it has been proposed that the CTD is an intrinsically disordered domain, where the phosphate can inhibit binding of the CTD to molecular partners directly, or indirectly, by altering the conformational preference of the disordered region. The latter is a potential mechanism for the change in Cx43 HC permeability.

Although interesting, it is presently unclear whether the structure of the CTD fragments is representative of that in the full-length Cx43. It is possible that interactions of the CTD with other regions of Cx43 result in a structure different from that of the isolated fragments. In this context, although not definitive, our studies suggest a more structured PKC-phosphorylated CTD of purified Cx43 reconstituted in liposomes (Bao et al., 2004c). We performed limited trypsin-proteolysis, and identified digestion products by immunoblotting using an antibody against the Cx43 CTD. We found trypsin-resistant CTD fragments containing the antibody epitope in digests of proteoliposomes containing PKC-phosphorylated Cx43, but not in those of dephosphorylated Cx43 or the Cx43-S368A phosphorylated by PKC. Based on their mobility, the trypsin-resistant fragments correspond to the complete CTD and the CTD plus the last two transmembrane segments. The protection of the entire CTD following phosphorylation of Ser368 would be an unlikely effect in an intrinsically disordered protein, however, it cannot be ruled out since exposure to cleavage sites is not the only factor determining sensitivity to trypsin digestion, but conformational kinetics also plays a role.

Independently on the structure of the CTD in full-length Cx43, we also observed a conformational change in response to phosphorylation of Ser368 in purified Cx43 solubilized in detergent. We found that phosphorylation decreases tryptophan fluorescence and produces a blue-shift of the emission peak, suggesting that one or more tryptophans are in a more hydrophobic environment when the protein is dephosphorylated (Bao et al., 2004c). In summary, it has been established that phosphorylation of Ser368 elicits a conformational change of Cx43, but the exact nature of the change and how it relates to the alteration in permeability are unknown.

PHOSPHORYLATION OF SER368 PRODUCES A PARTIAL CLOSURE OF THE Cx43 HEMICHANNEL

In spite of the minimal knowledge on the conformational changes that result from phosphorylation of Ser368, significant progress has been made on mechanistic aspects of the effect of phosphorylation on the purified HCs. One key finding is that contrary to the case of cytoplasmic acidification and MAPK-mediated phosphorylation (Burt and Spray, 1988; Kim et al., 1999; Delmar et al., 2004; Warn-Cramer and Lau, 2004), the closure of the HC pore in response to phosphorylation of Ser368 is partial (Bao et al., 2007). We obtained direct evidence for this in studies on purified HCs reconstituted in unilamellar liposomes (Bao et al., 2007). Phosphorylation of Ser368 by PKC abolishes sucrose permeability of reconstituted Cx43 HCs. However, phosphorylated HCs were still permeable to ethyleneglycol (*Mr* 62 vs. 342 for sucrose) (**Figure 4**). This observation indicates that phosphorylation by PKC decreases the cross-sectional area of the Cx43 HC pore, but a significant opening remains to allow for ethyleneglycol transport. Since the hydrodynamic radius of ethyleneglycol of 4.4 Å is larger than that of hydrated K^+ and Cl^- (~3.3 Å), it seems likely that PKC-phosphorylated HCs are still permeable to these ions. These results can explain why activation of PKC reduces dye transfer, but has no major effect on cell-to-cell gap-junction currents (Moreno et al., 1992; Takens-Kwak and Jongsma, 1992; Moreno et al., 1994; Kwak et al., 1995; Lampe et al., 2000; Ek-Vitorin et al., 2006).

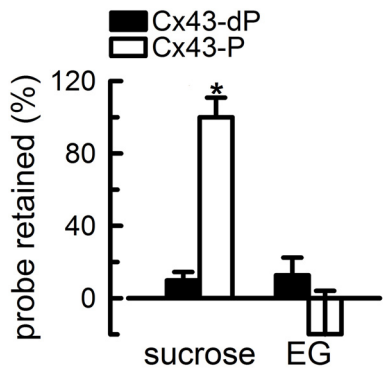

FIGURE 4 | Partial closure of Cx43 hemichannels by PKC-mediated phosphorylation. The proteoliposomes were loaded with radiolabeled probes, and the % retention was measured after gel filtration. The proteoliposomes contained HCs formed by either Cx43-dP or Cx43-P. Reconstitution was at an average of 2.3 HCs/liposome. Data are means ± s.e.m. of 4–7 experiments. The asterisk denotes $P < 0.05$ compared with proteoliposomes containing Cx43-dP HCs. Modified from Bao et al., 2007.

As mentioned under "regulation of Cx43 HCs by phosphorylation," stimulation of PKC decreases the frequency of the dominant conductance state of Cx43 GJCs of ~100 pS, favoring a lower conductance state of ~50 pS (Moreno et al., 1992, 1994; Lampe et al., 2000; Ek-Vitorin et al., 2006). It is possible that these lower conductance channels are formed by the phosphorylated HCs permeable to ethyleneglycol, but not sucrose. However, the level of Cx43 phosphorylation in cells' studies is undefined, and there is no simple correlation between single-HC conductance and permeability to large hydrophilic solutes by different connexin isoforms (Harris, 2001; Nielsen et al., 2012). Independently of these uncertainties, PKC-mediated phosphorylation of Cx43 could reduce fluxes of organic hydrophilic solutes such as ATP, cAMP, and IP3 (*Mr* 300–700) without major effects on small-inorganic ion fluxes and cell-to-cell electrical coupling.

PHOSPHORYLATION OF ALL SUBUNITS IS NECESSARY TO ABOLISH Cx43 HEMICHANNEL PERMEABILITY TO "LARGE" HYDROPHILIC SOLUTES

The observations that HCs formed by dephosporylated Cx43 (Cx43-dP) are permeable to sucrose, but those formed by Cx43 phosphorylated by PKC (Cx43-P) are not (Bao et al., 2004c), provided us with a tool to determine the number of subunits that need to be phosphorylated at Ser368 to abolish HC sucrose permeability. It is relatively simple to study the function of GJCs and HCs that are fully dephosphorylated at Ser368 by expressing the Cx43-S368A mutant in cell lines devoid of endogenous connexins. However, it is hard to express HCs with a known number of subunits phosphorylated at Ser368. Fortunately, Cx43 HC of controlled subunit composition can be generated *in vitro* from purified Cx43. As mentioned under "Charaterization of purified connexin hemichannels," purified Cx43 (but not Cx26) solubilized in detergent forms HCs where the connexins (subunits) exchange (Bao et al., 2007). This allowed us to generate and reconstitute Cx43 HCs of controlled average subunit composition.

We demonstrated exchange of subunits and determined subunit composition in semi-quantitative and quantitative manners, using size-exclusion chromatography and luminescence resonance energy transfer (LRET), respectively (Bao et al., 2007). For the former experiments, we mixed Cx43 and Cx43-EGFP (Cx43 with an enhanced green fluorescent protein fused to the C-terminus of the CTD). Each Cx43 fused to EGFP adds ~26 kDa, increasing the hydrodynamic size of the HCs significantly, and in proportion to the average number of Cx43-EGFP subunits in the HCs. Although this method is sensitive, it lacks accuracy to discriminate between, for example, HCs containing 3 or 5 Cx43-EGFP subunits. This was solved using LRET. With this spectroscopic technique, we measured energy transfer between donor and acceptor LRET probes reacted to Cx43 cysteines.

LRET measures energy transfer from the lanthanides Tb^{3+} or Eu^{3+} to fluorescent acceptor molecules (Selvin, 2002). It has atomic-resolution and high sensitivity, and can also provide dynamic information on conformational changes in the millisecond to minute time frame (Posson et al., 2005; Posson and Selvin, 2008; Rambhadran et al., 2011; Zoghbi et al., 2012; Cooper

and Altenberg, 2013; Zoghbi and Altenberg, 2013). Therefore, it will be useful for future studies of the conformational changes elicited by phosphorylation of Ser368. LRET has many advantages over traditional FRET (Selvin, 2002). Its basic principles and advantages have been described in detail, and are presented in **Figure 5**. The main advantages derive from the long lifetime of the Tb^{3+} and Eu^{3+} excited states, which allows for acquisition of long-lifetime donor emission in a gated mode (i.e., delaying acquisition, generally for 60–200 μs). Gated acquisition minimizes the light scattering effects of structures such as detergent micelles and liposomes, and results in minimal background with high signal-to-noise ratio. In addition, LRET-based distances can be measured in the 25–120 Å range and are more accurate than those based on FRET because donor and sensitized acceptor emission are unpolarized, and therefore uncertainty about κ (geometric factor related to the relative orientation of the donor and acceptor transition dipoles) is not an issue (Selvin, 2002; Zoghbi et al., 2012). Another major advantage is that the sensitized emission lifetime is independent of the labeling stoichiometry because long-lifetime acceptor emission can arise only from LRET (Selvin, 2002; Bao et al., 2007), i.e., labeling stoichiometry affects the intensity of the signal, but not its lifetime. Finally, the "atomic-like" lanthanide emission (sharp peaks with dark regions between them) allows for measurements of acceptor emission without contamination from the donor emission (Selvin, 2002; Zoghbi et al., 2012; Cooper and Altenberg, 2013).

To test LRET on Cx43 HCs, we labeled Cx43 with either fluorescein maleimide as acceptor or Tb^{3+}-DTPAcs124-EMCH as donor, by incubation with a 10-fold molar excess of the thiol reagents for 2 h at 4°C. Cx43 has 9 cysteines (Bao et al., 2007). Six are located in the extracellular loop, and are likely to form intramolecular disulfide bonds (Foote et al., 1998; Maeda et al., 2009). These may not be accessible for labeling. The remaining 3 cysteines are located in the CTD, and may be available for labeling. The labeling stoichiometry was 3 probe molecules/HC, suggesting that all CTD cysteines are solvent accessible. After labeling, we mixed fluorescein-labeled, Tb^{3+}-labeled and unlabeled Cx43 in different proportions, but always using a low proportion of Tb^{3+}-labeled Cx43 (0.5 mol/HC). Under these conditions, most HCs have either one Tb^{3+}-labeled subunit or none, with only ∼8% containing more than one donor-labeled subunit (calculated from the binomial distribution). Under these conditions, considering that essentially all subunits assemble as functional HCs, the fluorescein emission intensity due to energy transfer from Tb^{3+} (long lifetime sensitized fluorescein emission) depends on the number of fluorescein-labeled subunits/HC, and can be used to determine the HC subunit composition (**Figure 6**). Details and validation of the methodology have been published (Bao et al., 2007). The LRET-based method is very accurate; it can discriminate between HCs containing ± 1 acceptor-labeled subunits (Bao et al., 2007). In addition, measurements can be obtained from HCs in liposomes, which allows for parallel determinations of HC subunit composition and function.

After demonstrating that the average subunit composition of reconstituted HCs is that of the subunit mixes in detergent, we went back to the original question of the number of subunits that have to be phosphorylated by PKC to abolish HC sucrose permeability. To answer the question, we performed experiments with 0.8 HC/liposomes on average. In this way, very few liposomes contain more than one HC, and therefore the % of sucrose-permeable liposomes depends on whether the HC in that liposome is permeable or not. Also, if subunits form HCs randomly, the subunit composition of the HCs will follow the binomial distribution, e.g., for a 3/3 mixture of Cx43-dP/Cx43-P the most frequent HCs will contain 3 Cx43-dP and 3 Cx43-P subunits (∼31%), with lower frequencies of 2/4 and 4/2 (∼23% each), 1/5 and 5/1 (∼9% each), and 0/6 and 6/0 (∼2% each). Proteoliposomes with HCs formed only by Cx43-P are impermeable to sucrose, whereas those formed by Cx43-dP alone or Cx43-dP/Cx43-P mixtures of 5/1, 4/2 or 3/3 were permeable to sucrose (Bao et al., 2007). Therefore, 3 Cx43-P subunits/HC are insufficient to abolish sucrose permeability. With more than

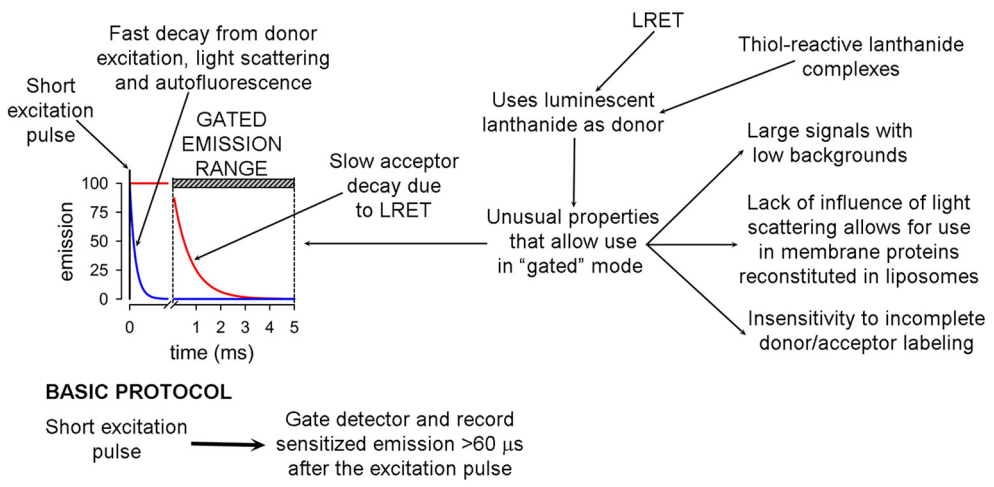

FIGURE 5 | Basic principles and advantages of luminescence resonance energy transfer. The basic gated-mode LRET protocol that takes advantages of the long lifetime of the donor compared to the fluorophore acceptor is summarized at the bottom.

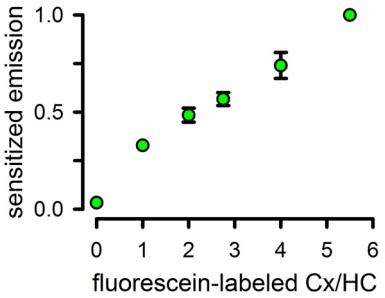

FIGURE 6 | Use of luminescence resonance energy transfer to assay hemichannels subunit composition. Sensitized fluorescence emission as a function of the average number of fluorescein-labeled subunits/HC. Data were normalized to the peak value of 1 Tb/5.5 fluorescein-labeled preparation and are means ± s.e.m. of 7–9 experiments. Reconstitution was at an average of 0.8 HCs/liposome. All values are statistically different from the previous one ($P < 0.001$), except for that at the 3/3 ratio. Modified from Bao et al., 2007.

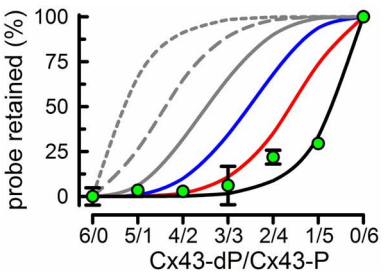

FIGURE 7 | All subunits have to be phosphorylated by PKC to abolish Cx43-HC sucrose permeability. Effects of varying Cx34-dP/Cx43-P average ratios on the % sucrose-permeable proteoliposomes preloaded with radiolabeled sucrose. Data are means ± s.e.m. of 4–7 experiments. Lines represent the expected % (calculated from the binomial distribution) if the number of Cx43-P subunits necessary to render the HC impermeable to sucrose are ≥1 (gray, short dash), ≥2 (gray, long dash), ≥3 (gray, solid), ≥4 (blue), ≥5 (red), or 6 (black). Modified from Bao et al., 2007.

3 Cx43-P subunits/HC, the percentage of sucrose-impermeable liposomes becomes significant and proportional to the number of Cx43-P subunits (Bao et al., 2007). However, even for an average of 5 Cx43-P/HC, the % of sucrose-impermeable liposomes was only ∼30%, even though ∼83% of the Cx43 is Cx43-P. If 5 Cx43-P/HC were sufficient to produce sucrose-impermeable HCs, the expected % of sucrose-impermeable liposomes in the 1/5 Cx43-dP/Cx43-P HCs would be ∼74%. This is the sum of the proteoliposomes containing 5 (∼40%) and 6 (∼34%) Cx43-P subunits according to the binomial distribution. Since this value is more than twice the value measured (∼30%), but very close to the percentage of proteoliposomes containing 6 Cx43-P subunits (∼34%), it appears that all 6 HC subunits must be phosphorylated to abolish sucrose permeability (**Figure 7**) (Bao et al., 2007). It is important that the transport-specific sedimentation assay used to determine whether the liposomes were permeable or impermeable to sucrose, provides steady-state information on the permeability cut-off, but not information on the rates of permeation. Therefore, our results cannot rule out that partial phosphorylation of Cx43 HCs decreases sucrose permeability, but show that complete phosphorylation is needed to abolish sucrose permeability. Studies of the kinetics of transport such as those that we have developed recently (see "Functional analysis of purified and reconstituted connexin hemichannels") can be used to address this issue.

SUMMARY AND PERSPECTIVES

Purified HCs constitute an excellent preparation for functional and structural studies of Cx26 and Cx43 HCs. Although still under development, there are assays to analyze the function and structure of these HCs under "basal" conditions, as well as to evaluate functional and structural changes in response to regulatory factors. Focusing on the regulation of Cx43 by phosphorylation, a number of significant findings have been made using purified HCs. These include: (a) the effect of PKC is direct on the HCs (no intermediate regulatory proteins), (b) phosphorylation of S368 is the only event needed for the alterations in permeability produced by PKC-mediated phosphorylation, (c) phosphorylation of S368 results in conformational changes of Cx43 HCs demonstrated by a blue shift of the peak emission of the native tryptophans and a protection of the CTD from trypsin digestion, (d) phosphorylation of S368 produces a partial closure of the HC pore that alters its selectivity: it abolishes sucrose permeability, but not that to the smaller hydrophilic solute ethyleneglycol, (e) to abolish sucrose permeability all six HC subunits have to be phosphorylated; 5 of the 6 is not sufficient. All indications are that important advances will be made in the near future on the molecular mechanism of HC regulation by correlating functional effects and structural changes determined by combining X-ray crystallography and spectroscopic techniques such as LRET.

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Hemichannels: new roles in astroglial function

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The role of astrocytes in brain function has evolved over the last decade, from support cells to active participants in the neuronal synapse through the release of "gliotransmitters." Astrocytes express receptors for most neurotransmitters and respond to them through Ca^{2+} intracellular oscillations and propagation of intercellular Ca^{2+} waves. While such waves are able to propagate among neighboring astrocytes through gap junctions, thereby activating several astrocytes simultaneously, they can also trigger the release of gliotransmitters, including glutamate, d-serine, glycine, ATP, adenosine, or GABA. There are several mechanisms by which gliotransmitter release occurs, including functional hemichannels. These gliotransmitters can activate neighboring astrocytes and participate in the propagation of intercellular Ca^{2+} waves, or activate pre- and post-synaptic receptors, including NMDA, AMPA, and purinergic receptors. In consequence, hemichannels could play a pivotal role in astrocyte-to-astrocyte communication and astrocyte-to-neuron cross-talk. Recent evidence suggests that astroglial hemichannels are involved in higher brain functions including memory and glucose sensing. The present review will focus on the role of hemichannels in astrocyte-to-astrocyte and astrocyte-to neuron communication and in brain physiology.

Keywords: astrocytes, hemichannel, calcium waves, tripartite synapse, connexins, brain functions

ASTROCYTES: GENERAL BACKGROUND

Astrocytes are spongiform-shaped glial cells (Bushong et al., 2002 and Ogata and Kosaka, 2002) that, contrary to common belief, are the most abundant cell type in the brain. They are divided into two major types based on their morphology, biochemistry, development, and location within the central nervous system (CNS): protoplasmic and fibrous (Miller and Raff, 1984). Given their numerous functions, several studies have tried to differentiate subpopulations of astrocytes (Lerea and McCarthy, 1989). However, such attempts have been unsuccessful due to the extraordinary capacity of astrocytes to adapt to their surrounding environment by changing the expression of a vast number of proteins. This is particularly evident in primary cultures, where they show fast changes in the expression of several receptors for neuro- and gliotransmitters (Shao and McCarthy, 1993; Shao et al., 1994). There is a remarkable heterogeneity in astrocyte populations, between different species, brain regions and within a brain region, in terms of their receptor expression, gap junction coupling, membrane currents, and their morphology (Matyash and Kettenmann, 2010; Zhang and Barres, 2010; Theis and Giaume, 2012).

Astrocytes have pivotal roles in brain function, including the maintenance of osmotic balance and optimal ionic conditions for neurons (Kimelberg, 2005), K^+ clearance from the extracellular space (Wallraff et al., 2006; Sibille et al., 2013), glucose and lactate metabolism (Allaman et al., 2011), neurotransmitter recycling of the two most abundant neurotransmitters in the brain, glutamate and GABA (Simard and Nedergaard, 2004), and immune responses (Dong and Benveniste, 2001; Farina et al., 2007). Moreover, astrocytes have end-feet that cover blood vessels and release vasoactive substances to regulate cerebral microcirculation (Anderson and Nedergaard, 2003; Zonta et al., 2003; Takano et al., 2006) and blood brain barrier (BBB) permeability (Alvarez et al., 2013). In fact, their end-feet physically constitute part of the BBB. Finally, astrocytes communicate with neurons through transmitters which are released into neighboring synapses, now called "gliotransmitters." It is not within the scope of the present review to comment on the above functions, for which we have cited very comprehensive reviews, which are highly recommended. The present review will focus on the possible role of hemichannels in astroglial function and brain physiology.

ASTROCYTES RESPOND TO SYNAPTIC NEUROTRANSMITTERS

Astrocytes express membrane receptors for almost all major neurotransmitters and neuromodulators, and possess ion channels and intracellular signaling cascades that allow them to respond within milliseconds to neuronal activity and neurotransmitters released at synapses. These fast responses occur mainly as changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (MacVicar and Tse, 1988; Marrero et al., 1989; Usowic et al., 1989; Barres et al., 1990; Salm and McCarthy, 1990; McCarthy and Salm, 1991). The mechanism by which astroglial activation occurs is believed to start when neurotransmitters released from neurons at the synapse activate receptors at the astroglial membrane, inducing activation of phospholipase C (PLC) and the concomitant production of IP_3. The latter then triggers the release of intracellular Ca^{2+} stored at the endoplasmatic reticulum (Sheppard

et al., 1997; Golovina and Blaustein, 2000; Scemes, 2000), which opens hemichannels (De Vuyst et al., 2009) and activates other Ca^{2+} dependent gliotransmitter release mechanisms including exocytosis. Hemichannels are hexameric plasma membrane channels formed by two different families of membrane proteins: connexins (Cx) and pannexins (Panx). Although these proteins do not share a relevant homologous primary structure, they have similar secondary and tertiary structures with four α-helical transmembrane domains, connected by one cytoplasmic and two extracellular loops, and intracellular N- and C-termini. Importantly, hemichannel opening allows the release of glutamate (Ye et al., 2003; Takeuchi et al., 2006; Kang et al., 2008; Jiang et al., 2011; Orellana et al., 2011a,b), ATP (Stout et al., 2002; Iglesias et al., 2009; Orellana et al., 2011a,b; Torres et al., 2012) and other gliotransmitters into the extracellular space. Given that astrocytes express NMDA receptors insensitive to blocking by extracellular Mg^{2+}, and are activated following physiological synaptic transmission (Verkhratsky and Kirchhoff, 2007) and through purinergic receptor channels (Idestrup and Salter, 1998; Zhu and Kimelberg, 2004; Lalo et al., 2008; Illes et al., 2012), ATP and glutamate released via hemichannels onto the extracellular space can activate purinergic or NMDA receptor channels located in the same astrocyte or in neighboring astrocytes, inducing changes in $[Ca^{2+}]_i$ (Zanotti and Charles, 1997; Guthrie et al., 1999). Moreover, because astrocytes envelope synapses, the release of glutamate, ATP, and other gliotransmitters also activates neighboring pre- and post-synaptic neurons, modulating synaptic activity (Dani et al., 1992; Nedergaard, 1994; Parpura et al., 1994; Kang et al., 1998; Parri et al., 2001). In fact, astrocytes stimulated by amyloid β-peptide (Aβ) release ATP and glutamate via connexin 43 (Cx43) hemichannels (Orellana et al., 2011a). Importantly, both of these gliotransmitters released by astrocytes have been shown to increase Panx1 hemichannel activity in neurons by activating P2X7 and NMDA receptors, resulting in further neuronal death. Given that high $[Ca^{2+}]_i$ enhances Panx1 hemichannel activity (Locovei et al., 2006), it is likely that purinergic and glutamatergic receptor activation leads to Panx1 hemichannel opening by inducing Ca^{2+} influx or by releasing Ca^{2+} from intracellular stores via activation of IP_3 receptors (Zanotti and Charles, 1997; Guthrie et al., 1999; Stout et al., 2002; Suadicani et al., 2006).

As reported by Cornell-Bell et al. (1990), both the initial increase, and sustained oscillation of $[Ca^{2+}]_i$ induced by glutamate in astrocytes, depend on the concentration of the latter. Indeed, under low glutamate concentrations (>1 μM), $[Ca^{2+}]_i$oscillations in single astrocytes appear locally, asynchronously and are short-lasting, whereas concentrations above 100 μM generate astrocyte-to-astrocyte propagating Ca^{2+} waves which last up to 30 min (Cornell-Bell et al., 1990). These intercellular Ca^{2+} waves can be propagated among adjacent astrocytes through Cx43 and Cx30 gap junction channels (GJCs) (Cornell-Bell et al., 1990; Charles et al., 1991; Enkvist and McCarthy, 1992; Finkbeiner, 1992; Venance et al., 1995; Leybaert et al., 1998; Scemes et al., 1998; Blomstrand et al., 1999; Suadicani et al., 2006) or by the Ca^{2+}-dependent release of ATP and glutamate and further activation of purinergic or glutamate receptors in neighboring astrocytes (Zanotti and Charles, 1997; Guthrie et al., 1999; reviewed in Bennett et al., 2003; Leybaert and Sanderson, 2012). GJCs are intercellular channels formed by docking of two hemichannels, one provided by each adjacent cell (Sáez et al., 2003). These channels connect the cytoplasmic compartments of adjacent cells, favoring the intercellular exchange of metabolites (e.g., ADP, ATP, glucose and glutathione), second messengers (e.g., cAMP and IP_3) and ions (e.g., Ca^{2+}, K^+ and Na^+).

To obtain a Ca^{2+} wave, the released Ca^{2+} needs to be significantly amplified. This amplification is mediated at least in part by the capacity of Ca^{2+} itself to activate both IP_3 receptors (Finch and Turner, 1991; Bezprozvanny and Ehrlich, 1995) and phospholipase C (Berridge, 1993; Venance et al., 1997) as well as through other mechanisms reviewed in Leybaert and Sanderson, 2012. It has been reported that such Ca^{2+} waves originate from a localized area of the cell (Shao et al., 1994) and spread throughout the cell and into other cells in a non-decremented manner (Shao et al., 1994). Moreover, it has been suggested that astroglial Ca^{2+} responses occur once a "threshold" is reached and in an "all-or-none" manner reminiscent of neuronal action potentials (Shao and McCarthy, 1993; Shao et al., 1994). In consequence, the formation of Ca^{2+} waves is intriguing, as it must include a mechanism that will set this threshold, which may depend on the isoform of the IP_3 receptor and on the concentration of IP_3 and Ca^{2+}. In neurons, during the generation of an action potential this threshold is defined by the membrane potential required to activate voltage-dependent Na^+ channels, which are densely located at the axon hillock and along the axon. The mechanism by which Ca^{2+} oscillations or fluctuations are integrated into a threshold that determines the triggering of a Ca^{2+} wave remains unclear, although some hypotheses have been postulated (see Leybaert and Sanderson, 2012). Nonetheless, the idea of a Ca^{2+} wave being a distinct phenomenon rather than just the consequence of a larger increase in $[Ca^{2+}]_i$ is also supported by other studies. In a study by McCarthy and Salm (1991), primary astrocytes were exposed to different neurotransmitter agonists and showed different Ca^{2+} responses to different neurotransmitters in distinct subpopulations. Interestingly, they found that astrocytes respond to neurotransmitter agonists by either a Ca^{2+} wave or $[Ca^{2+}]_i$ oscillations (McCarthy and Salm, 1991), that is, if a cell population responded to a given agonist with a Ca^{2+} wave, it may respond to another agonist with $[Ca^{2+}]_i$ oscillations and vice versa (McCarthy and Salm, 1991). This suggests, that in a manner similar to neuronal summation of post-synaptic evoked potentials, fluctuations in $[Ca^{2+}]_i$ may be integrated additively to generate propagating Ca^{2+} waves that can activate entire astroglial networks.

In cultured astrocytes, $[Ca^{2+}]_i$ oscillations can occur spontaneously in the absence of neuronal activation (Aguado et al., 2002; Perea and Araque, 2005), but can be regulated by neuronal activation and transmitter release. Ca^{2+} waves, on the other hand, appear in response to neurotransmitters, but given that astrocytes so far have been studied *in vitro*, it has been argued that Ca^{2+} waves may appear only in non-physiological conditions or in pathology (Scemes and Giaume, 2006). *In vivo* it is difficult to differentiate Ca^{2+} waves from $[Ca^{2+}]_i$ oscillations due to technical difficulties. However, $[Ca^{2+}]_i$ oscillations have been observed *in vivo* using imaging techniques under physiological

conditions. These $[Ca^{2+}]_i$ oscillations in astrocytes were found to be correlated to neuronal discharges (Hirase et al., 2004), and appear in response to sensory stimulation (Cirillo et al., 2012; Lind et al., 2013), electrical stimulation of afferent fibers (Johannssen and Helmchen, 2010) or ATP (Ding, 2012) and at speeds sufficiently fast to occur concomitantly with neuronal activity and hemodynamic changes (Lind et al., 2013). A very recent study has reported that whisker stimulation in awake, behaving mice induces very large Ca^{2+} astroglial responses spread over a large portion of cortex and which are modulated by subcortical noradrenergic input, but not by intracortical glutamate (Ding et al., 2013).

FUNCTIONAL HEMICHANNELS IN ASTROCYTES

Although the principal connexin in astrocytes is Cx43 (Dermietzel et al., 1989), they also express Cx30 GJCs (Nagy et al., 1999) and Pannexin 1 (Panx1; Iglesias et al., 2009) and Panx2 (Zappalá et al., 2007). Some studies, however, have also reported low levels of Cx26, Cx40, and Cx45 (Dermietzel et al., 1989, 2000; Nagy et al., 1997, 1999). Yet, despite the observations of these latter studies, astrocytes from Cx43/Cx30 double knockout mice fail to show gap junction-mediated communication (Wallraff et al., 2006; Rouach et al., 2008) indicating that Cx43 and Cx30 are the main functional connexins in astrocytes.

Cx43 hemichannels have mostly been studied *in vitro* using transfected and primary cells, as well as from acute slice experiments (Ye et al., 2003; Orellana et al., 2011a; Chen et al., 2012; Torres et al., 2012). The conditions found *in vitro* that favor Cx43 hemichannel opening seemed non-physiological at first, leading to a debate on its functionality under physiological conditions. This stems from an earlier belief that hemichannels opened at only highly depolarized membrane potentials (around 60 mV), making their opening virtually impossible in non-excitable cells like astrocytes, which show no large changes in membrane potential. However, recent studies have shown hemichannel opening also at negative membrane potentials (Retamal et al., 2007; Orellana et al., 2011a,b). Indeed, hemichannel-mediated uptake of several dyes (e.g., ethidium, propidium, TOPRO, YOPRO) occurs at resting membrane potentials (Contreras et al., 2003), suggesting that hemichannel opening may also be present at resting membrane conditions.

High levels of intracellular $[Ca^{2+}]_i$ and low extracellular Ca^{2+} ($[Ca^{2+}]_o$) increase opening probability of Cx43 hemichannels (Stout and Charles, 2003; Bao et al., 2004; Wang et al., 2012) whereas normal extracellular $[Ca^{2+}]_o$ closes them (Stout and Charles, 2003). Cx43 hemichannels have been reported to mediate the release of gliotransmitters (glutamate, ATP, glutathione) from astrocytes and glioma cells (Stout et al., 2002; Ye et al., 2003). Ye et al. (2003) demonstrated that low extracellular $[Ca^{2+}]_o$ induces glutamate release from astrocytes through Cx43 hemichannels in an exocytosis-independent manner and involves neither large pore anion channels, purinergic receptors, nor reversal of the glutamate transporter (Ye et al., 2003). This idea was further supported by reports showing ATP release from glioma cells overexpressing Cx43 and exposed to zero extracellular $[Ca^{2+}]_o$ (Ye et al., 2003; Contreras et al., 2004; Retamal et al., 2006). Other studies, however, have reported ATP release from astrocytes also mediated by the P2X7 receptor, Panx1 hemichannels, and exocytosis (Parpura et al., 1994; Coco et al., 2003; Bezzi et al., 2004; Mothet et al., 2005; Pascual et al., 2005; Garré et al., 2010). This suggests that ATP is released by astrocytes through different mechanisms. In a study by Garré et al. (2010), it was reported that pharmacological blockade of vesicles inhibited only early ATP release from astrocytes, while later release was reported to be mediated by P2X7 receptor activation as well by Panx1 and Cx43 hemichannel opening, suggesting that each release mechanism may occur at different periods.

ROLE OF ASTROGLIAL CONNEXIN AND PANNEXIN HEMICHANNELS IN GLIOTRANSMITTER RELEASE AT THE SYNAPSE

Astrocytes release gliotransmitters into neuronal synapses, giving rise to what is now known as the tripartite synapse (Araque et al., 1998), implying a synapse between a pre- and post-synaptic neuron and their bidirectional communication with one astrocyte. Glutamate is the most important and abundant excitatory neurotransmitter of the CNS and one the most ubiquitous gliotransmitters released by astrocytes (Navarrete et al., 2012). Multiple mechanisms have been proposed to explain the release of glutamate from astrocytes, including hemichannels (Ye et al., 2003), anion channels (Wang et al., 2013), and exocytosis (Parpura et al., 1994; Coco et al., 2003; Bezzi et al., 2004; Mothet et al., 2005; Pascual et al., 2005 but see Wang et al., 2013). The other major neurotransmitter is GABA, the principal inhibitory neurotransmitter of the CNS. Although GABA is abundantly released by interneurons, it is also released by astrocytes (Lee et al., 2011).

Perhaps some of the best known gliotransmitters are D-serine and glycine, which are required for NMDAR activation of postsynaptic neurons and necessary for glutamate-mediated synaptic plasticity (Panatier et al., 2006; Henneberger et al., 2010; Hogerton and Bowser, 2013; Kang et al., 2013). D-serine has been reported to be released from astrocytes via large vesicles (Kang et al., 2013) and exocytosis (Parpura et al., 1994; Coco et al., 2003; Bezzi et al., 2004; Mothet et al., 2005; Pascual et al., 2005). It must be noted that a recent report has suggested that neurons may also release D-serine and glycine (Balu et al., 2014 and Ehmsen et al., 2013) both involved in regulating synaptic plasticity (Rosenberg et al., 2013). Until now, there has been no evidence indicating that astroglial hemichannels can release either D-serine or glycine.

As stated earlier, ATP both activates astrocytes and is also released by them. Additionally, it appears to suppress glutamatergic synapses (Zhang et al., 2003; Cao et al., 2013a) and can be turned into adenosine, which decreases excitatory transmission (Dunwiddie and Diao, 1994; Dunwiddie et al., 1997, but see Fujita et al., 2012). ATP has been shown to be released through hemichannels (Stout et al., 2002; Kang et al., 2008), P2X7 channels (Suadicani et al., 2006) and exocytosis (Parpura et al., 1994; Coco et al., 2003; Bezzi et al., 2004; Mothet et al., 2005; Pascual et al., 2005). Another gliotransmitter, glutathione, is released in response to extracellular glutamate (Frade et al., 2008) through connexin hemichannels (Rana and Dringen, 2007). Other well-known gliotransmitters include BDNF (Parpura and Zorec, 2010) and taurine (Choe et al., 2012). Although, previous studies have shown that taurine could be released via astroglial hemichannels (Stridh 2006/2008), further studies are necessary to elucidate whether BDNF could be released by the same pathway.

ASTROCYTIC HEMICHANNELS IN BRAIN FUNCTION

Given that astrocytes participate in the tripartite synapse, their contribution to brain function is as wide as that of neurons; taking into account their other functions (microcirculation, BBB formation), perhaps even more so (for a schematic of main astrocytic signaling cascades see **Figure 1**). Hemichannels contribute to the release of glutamate which is necessary for NMDAR-dependent synaptic plasticity (Henneberger et al., 2010; Navarrete et al., 2012). In fact, recently it was shown that blockade of Cx43 hemichannels in the basolateral amygdala by microinjection of mimetic peptides impairs memory consolidation but not short-term memory (Stehberg et al., 2012). Given that this study was performed using rodent fear conditioning, which is the most accepted model of post-traumatic stress disorder in animals, it would be plausible to suggest that Cx43 hemichannels may have a role in the establishment of memories in general and traumatic memories in particular.

POTENTIAL ROLE OF ASTROGLIAL CONNEXIN AND PANNEXIN HEMICHANNELS IN PSYCHIATRIC DISEASES

There is to-date no direct evidence linking astrocytic hemichannels and psychiatric disorders, so one can only speculate what their role might be. Studies have shown abnormal expression of glial fibrillary acid protein (GFAP)—a marker for astrocytes—in the post-mortem brain of patients with major depression (Bowley et al., 2002; Altshuler et al., 2010; Rajkowska and Stockmeier, 2013), while other studies have shown reduced density of astrocytes from clinical (Ongur et al., 1998; Cotter et al., 2001; Bremner et al., 2002), post-mortem (Cotter et al., 2001), and preclinical (Banasr and Duman, 2008; Banasr et al., 2010) studies, suggesting that the density and reactivity of astrocytes are reduced in this mood disorder.

Moreover, accumulating evidence suggests that antidepressants act on astrocytes (for reviews see Czéh and Di Benedetto, 2013; Etiévant et al., 2013). These express a variety of receptors including monoaminergic transporters and receptors, leading to the possibility that antidepressants exert their effects at least in part through modifying astroglial function (Peng and Huang, 2012; Quesseveur et al., 2013a). In this sense, it's been demonstrated that application of antidepressants on rodent primary astrocyte cultures may elicit Ca^{2+} waves, Ca^{2+} oscillations, release of gliotransmitters, glucose metabolites, and neurotrophic factors (Hisaoka et al., 2011), whereas studies in post-mortem human brain tissue suggest that antidepressants may reverse major depression associated glial reductions in the amygdala (Bowley et al., 2002). Interestingly, many transmitters released by astrocytes have antidepressant or anxiolytic effects. To this effect, acute D-serine treatment (800–2700 mg/Kg) produces antidepressant-like effects in rodents (Malkesman et al., 2012), astroglial release of ATP has been shown to modulate depressive-like behaviors

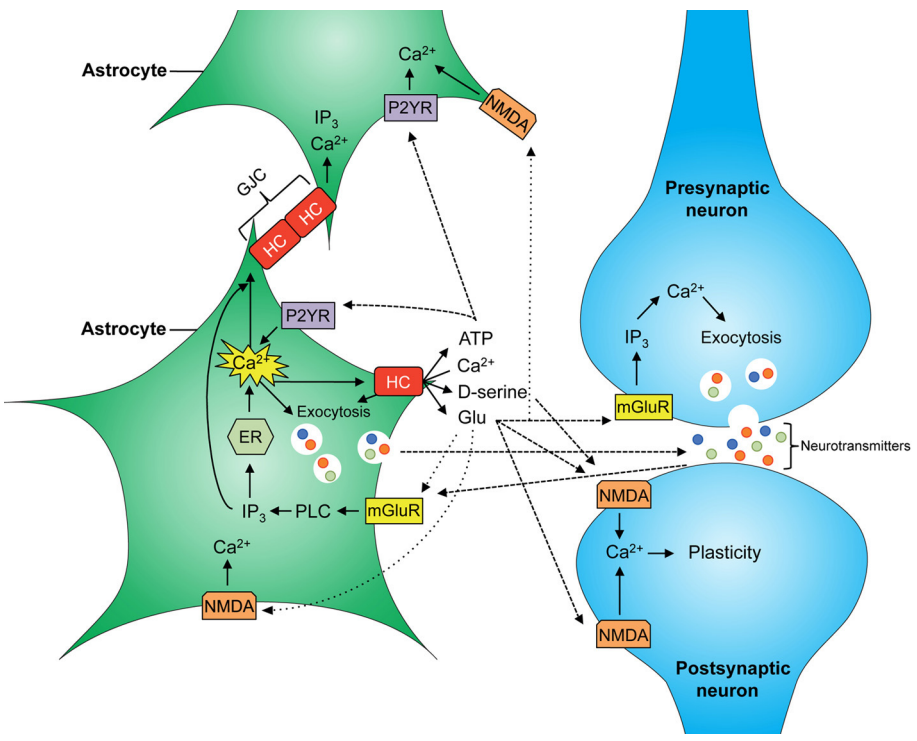

FIGURE 1 | Scheme of major astrocyte signaling associated to gliotransmitter release. Increased intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) allows the release of gliotransmitters into the synaptic cleft through vesicles and hemichannels (HCs). D-serine, glycine, and glutamate released by astrocytes can activate NMDA receptors at the post-synaptic neuron and modulate neuronal plasticity. Astroglial glutamate also binds to mGluR at the presynaptic neuron increasing neuronal release of glutamate into the synapse. In astrocytes, increasing $([Ca^{2+}]_i$ allows Ca^{2+} wave propagation between astrocytes, mediated by gap junction channels (GJCs) and by release of glutamate and ATP, resulting in further activation of NMDA and P2YR receptors at neighboring astrocytes, respectively.

(Cao et al., 2013b), GABA agonists are well known to have anxiolytic effects (the mechanism of action for benzodiazepines), while overexpression of astrocytic BDNF produces anxiolytic effects (Quesseveur et al., 2013b). Given that the release of all the above could be mediated at least in part by hemichannels, it is probable that mood depends on hemichannel activity.

Studies have also shown abnormal expression of GFAP in the post-mortem brain of patients with schizophrenia (Toro et al., 2006; Feresten et al., 2013). In fact, Khan and colleagues (Khan et al., 2001) found by electron microscopy that roughly 1/3 of D2 dopamine receptors in the cortex are expressed in astrocytes, and that D2 receptor agonist quinpirole increases astroglial intracellular $[Ca^{2+}]_i$, suggesting that astrocytes may be a target for antipsychotics.

Finally, current evidence also suggests that astrocytes could be involved in drug abuse (Miguel-Hidalgo, 2009). All in all, given the pivotal role astrocytes play in brain function, and their active release of gliotransmitters into synapses, it is highly probable that they will become a target in the treatment of psychiatric diseases. In this respect, hemichannels constitute an attractive candidate for such treatment as they mediate gliotransmitter release at the synapse of glutamate, activating NMDA, and non NMDA-dependent mechanisms critical for synaptic plasticity and the release of ATP and adenosine which may decrease neuronal network excitation. Moreover, antidepressants and antipsychotics may act, at least in part, through various astroglial monoamine receptors and transporters to modulate cytoplasmic Ca^{2+} that controls hemichannel activity.

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Connexin 43 hemichannels and intracellular signaling in bone cells

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Cell function and survival are controlled by intracellular signals, and modulated by surrounding cells and the extracellular environment. Connexin channels participate in these processes by mediating cell-to-cell communication. In bone cells, gap junction channels were detected in the early 1970s, and are present among bone resorbing osteoclasts, bone forming osteoblasts, and osteocytes - mature osteoblasts embedded in the mineralized matrix. These channels are composed mainly by Cx43, although the expression of other connexins (45, 46, and 37) has also been reported. It is now believed that undocked Cx43 hemichannels (connexons) formed in unopposed cell membranes facing the extracellular environment participate in the interaction of bone cells with the extracellular environment, and in their communication with neighboring cells. Thus, we and others demonstrated the presence of active hemichannels in osteoblastic and osteocytic cells. These hemichannels open in response to pharmacological and mechanical stimulation. In particular, preservation of the viability of osteoblasts and osteocytes by the anti-osteoporotic drugs bisphosphonates depends on Cx43 expression *in vitro* and *in vivo*, and is mediated by undocked hemichannels. Cx43 hemichannels are also required for the release of prostaglandins and ATP by osteocytes, and for cell survival induced by mechanical stimulation *in vitro*. Moreover, they are required for the anti-apoptotic effect of parathyroid hormone in osteoblastic cells. This review summarizes the current knowledge on the presence and function of undocked connexons, and the role of hemichannel regulation for the maintenance of bone cell viability and, potentially, bone health.

Keywords: connexin43 hemichannels, osteoblast, osteocyte, apoptosis, bone

INTRODUCTION

The amount of bone and its strength is maintained throughout life by the concerted actions of osteoblasts, the bone forming cells, and osteoclasts, the bone removing cells (**Figure 1A**). The action of these two cell types is coordinated by signals derived from osteocytes, the bone resident cells that derived from osteoblasts and are embedded in the bone matrix (**Figures 1A–D**). Osteocytes are highly communicated among themselves and with cells of the bone surface through cytoplasmic projections (**Figure 1C**).

The existence of gap junctions between osteocytes and osteoblasts on the bone surface was first suggested by electron microscopy observations of calvaria bones from newborn mice (Weinger and Holtrop, 1974). Although the level of resolution of the images did not allow for these authors to conclusive demonstrate the presence of gaps separating the cell surfaces of osteoblasts and osteocytes, they concluded that the structures seen were consistent with gap junctions. This was later confirmed at the ultramicroscopy level by the groups of Doty (1981) and Marotti (Palumbo et al., 1990). Further studies showed that Cx43 is the most abundant connexin expressed *in vitro* and *in vivo* in all type of bone cells: osteoblasts, osteocytes and osteoclasts (Schirrmacher et al., 1992; Civitelli et al., 1993; Jones et al., 1993; Donahue et al., 1995). This is exemplified on a murine bone section stained for Cx43 (**Figure 1D**).

The small molecules that are transferred through connexin channels, and might act as second messengers in bone cells have not been completely identified (see Stains et al., 2014 for a recent revision). Second messengers such as ATP and Ca^{2+} can move from one cell to another through gap junctions, or can be released to the extracellular media through hemichannels in osteoblastic cells (Jorgensen et al., 1997; Genetos et al., 2007). In addition, cAMP production induced by parathyroid hormone requires Cx43 expression in osteoblastic cells (Vander Molen et al., 1996; Bivi et al., 2011), and Cx43-mediated amplification of FGF2 effect on the osteoblast gene RUNX2 depends on the production of water-soluble inositol polyphosphates (Niger et al., 2013). Taken together, these pieces of evidence suggest that Cx43 not only can control the movement of second messengers, but also their synthesis.

The expression of Cx45, Cx46 and, more recently, Cx37 has also been demonstrated in bone cells (Kruger et al., 2000; Stains and Civitelli, 2005; Paic et al., 2009; Chaible et al., 2011; Pacheco-Costa et al., 2014). In particular, Cx37 is required for osteoclast differentiation and mice lacking Cx37 exhibit high bone mass due to defective bone resorption (Pacheco-Costa et al., 2014).

In addition to be part of gap junctions, connexin channels can be found in unopposed cell membranes forming undocked connexons or hemichannels. Although it was long known that bone cells express connexins, the presence of hemichannels in

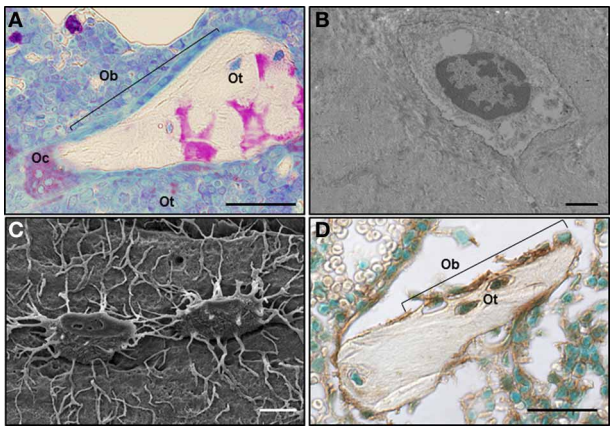

FIGURE 1 | Cell-to-cell interactions among osteoclasts, osteoblasts, and osteocytes in close proximity maintain bone homeostasis. (A) An osteoclast (Oc), a team of osteoblasts (Ob) and bone-embedded osteocytes (Ot) are shown in a rat bone section, in which osteoclasts were stained for the osteoclast-specific enzyme tartrate resistant acid phosphatase (TRAPase) (red) and counterstained using Toluidine blue. Scale bar indicates 50 μm. Picture contributed by Keith W. Condon (Indiana University School of Medicine). **(B)** Transmission electron microscope image of an osteocyte embedded in the bone matrix. Image was obtained at the Electron Microscopy Center of the Department of Anatomy and Cell Biology (Indiana University School of Medicine). Scale bar indicates 5 μm **(C)** Scanning electron microscope image of an acid-etched bone showing two osteocytes highly communicated through their cytoplasmic projections (Bellido et al., 2014). Scale bar indicates 1 μm. **(D)** Cx43 immunostaining of a section of cancellous bone showing osteoblasts (Ob) on the bone surface and osteocytes (Ot) embedded in the bone matrix stained for Cx43 (brown) and counterstained with Methyl green (Plotkin et al., 2008). Scale bar indicates 50 μm.

osteoblastic cells was not reported until 2001 (Romanello and D'Andrea, 2001). In the current review, the demonstration of the presence and function of connexin hemichannels in osteoblasts and osteocytes is discussed.

Cx43 AND BONE DEVELOPMENT: A ROLE FOR HEMICHANNELS?

The importance of Cx43 expression in osteoblasts and osteocytes for bone development, as well as for osteoblast and osteocyte differentiation, survival and function has been clearly established (for recent reviews see Civitelli, 2008; Loiselle et al., 2013; Plotkin and Bellido, 2013). Thus, global deletion of Cx43 results in delayed ossification and impaired osteoblast differentiation in the embryos (Lecanda et al., 2000). In addition, studies with tissue specific deletion of Cx43 have demonstrated that the adult skeleton is also affected by the absence of the connexin (Chung et al., 2006; Watkins et al., 2011; Zhang et al., 2011; Bivi et al., 2012a,b). Cx43 is also important for osteoclast differentiation, as demonstrated in mice in which the connexin was deleted from osteoclast precursors (Sternlieb et al., 2012). Because these studies were performed by deleting the whole Cx43 molecule, it is not possible to determine whether absence of cell-to-cell gap junction communication or the function of Cx43 in undocked hemichannels present in cell membranes (or even channel-independent functions of the connexin), or a combination of these functions, are responsible for the observed phenotypes. Nevertheless, recent developments discussed below support the presence and functionality of Cx43 hemichannels in bone cells *in vivo*.

Mutations of the Cx43 gene are associated with oculodentodigital dysplasia (ODDD), a condition with skeletal malformation that include craniofacial abnormalities and broad long bones (Paznekas et al., 2002). Interestingly, some of the Cx43 mutations leading to ODDD result not only in decreased gap junction communication, but also in enhanced hemichannel activity (Dobrowolski et al., 2007), suggesting that part of the phenotype of the patients might be due to exacerbated hemichannel function. Consistent with this, a study reported in the form of an abstract showed that osteocytic expression of the Cx43 mutant R76W, which does not form gap junctions but has the ability to form hemichannels, leads to decreased bone mineral density (Jiang et al., 2010). The phenotype of these mouse models might be due to the lack of intercellular gap junction communication or, alternatively, to excessive hemichannel activity in bone cells, resulting in skeletal defects.

Interestingly, stimuli that increase bone mass have been shown to increase Cx43 expression, its localization in the cell membrane, and the activation of gap junction and hemichannel activity. Thus, estrogen, increases the expression of Cx43 and gap junction communication in the osteocytic cell line MLO-Y4 (Ren et al., 2013); and the effect of sex steroid removal on the cortical bone is partially prevented in mice lacking Cx43 in osteoblastic cells (Watkins et al., 2012). However, we have shown that the anti-apoptotic effect of sex steroids on osteocytic cells does not require Cx43 function (Plotkin et al., 2002). On the other hand, as it will be discussed below, Cx43, and in particular, hemichannels, mediate at least in part the effect of the anti-osteoporotic drugs bisphosphonates (Plotkin et al., 2002, 2008), mechanical stimulation (Cherian et al., 2005; Zhang et al., 2011; Grimston et al., 2012; Bivi et al., 2013), and parathyroid hormone (Bivi et al., 2011).

THE BONE PROTECTING DRUGS BISPHOSPHONATES PRESERVE OSTEOBLAST AND OSTEOCYTE VIABILITY BY OPENING Cx43 HEMICHANNELS

Bisphosphonates, a family of drugs that include alendronate, have been used over the past 40 years to treat conditions with low bone mass such as osteoporosis, and to prevent bone fractures (Russell, 2011). Bisphosphonates block osteoclastic bone resorption, therefore preserving the amount of bone. However, stopping bone resorption cannot completely explain the ability of these drugs to prevent bone fractures. We therefore proposed that, in addition to inhibiting bone resorption, bisphosphonates have a positive effect on osteoblasts and osteocytes that can contribute to the anti-fracture properties of the drugs. Preservation of osteoblast life span should lead to prolonged matrix synthesizing activity, whereas maintenance of osteocyte viability should preserve their mechanosensory function, therefore improving bone strength. We found that bisphosphonates protect osteoblasts and osteocytes from apoptosis induced by several agents (**Figure 2A**) *in vitro* using osteoblastic cell isolated from neonatal calvaria bone and osteocytic MLO-Y4 cells, and by glucocorticoid excess *in vivo* using a mouse model of glucocorticoid-induced bone disease

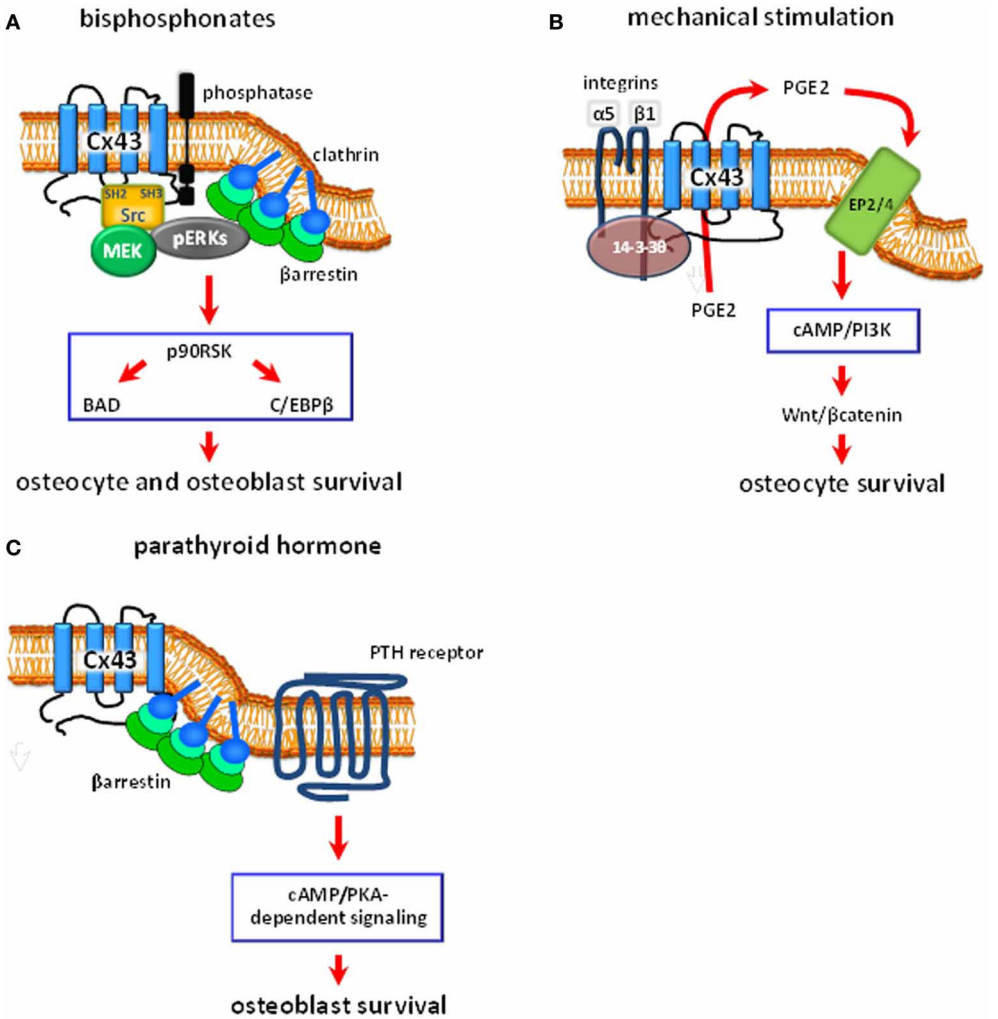

FIGURE 2 | Schematic representation of the proposed intracellular signaling pathways regulated by Cx43 hemichannels in bone cells. (A) Bisphosphonates bind to phosphatases present in the cell membrane. This induces Cx43 hemichannel opening, followed by activation of the kinases Src and MEK, and ERKs. ERKs activated downstream of Cx43 hemichannel opening are retained in the cytoplasm by a complex formed by βarrestin and clathrins. This leads to the phosphorylation of the cytoplasmic targets $p90^{RSK}$, BAD and C/EBPβ, which results in osteoblast and osteocyte survival. **(B)** Mechanical stimulation induces α5β1 integrin engagement and the association of the integrins with Cx43, by a mechanism that requires the protein 14-3-3θ. This results in hemichannel opening and the release of PGE2. PGE2, in turn, activates EP2/4 prostaglandin receptor by an autocrine/paracrine mechanism, leading to activation of the cAMP/PI3K signaling pathways, accumulation of βcatenin with the consequent activation of Wnt signaling, and inhibition of osteocyte apoptosis. **(C)** Parathyroid hormone (PTH), through binding to the PTH receptor, induces activation of the cAMP/PKA signaling pathway. Cx43, by sequestering β-arrestin away from the PTH receptor, facilitate cAMP/PKA-mediated downstream signaling and osteoblast survival.

(Plotkin et al., 1999). Although osteoblasts and osteocytes have distinct functions, they respond in similar fashion to bisphosphonates. Therefore, the studies described in this section were performed with both cell types.

The survival effect of bisphosphonates is mediated by the activation of the extracellular signal regulated kinases ERKs in cell cultures and *in vivo* (**Figure 2A**) (Plotkin et al., 1999, 2011). Thus, phosphorylation of ERKs is increased upon treatment of cells or mice with the bisphosphonate alendronate. Importantly, ERK activation is required for the protective effect of bisphosphonates since their effect was abolished by ERK pharmacological inhibitors and by transfection of a dominant negative form of MEK, the kinase that activates ERKs (Plotkin et al., 1999, 2002).

ERKs can be activated by an array of extracellular stimuli, including binding of growth factors and cytokines to their receptors. We then explored the possibility that bisphosphonates activate ERKs by acting on membrane channels. Because connexin channels are abundant in osteoblasts and osteocytes, and bisphosphonates are molecules of low molecular size (approximately 400 daltons), we examined whether inhibition of connexin channels affected the protective response to the bisphosphonate alendronate. We found that inhibiting connexin channels with 18 α-glycyrrhetinic acid (AGA) abolished the anti-apoptotic effect of alendronate, whereas the inactive analog of AGA, glycyrrizic acid (GA), did not modify this response (Plotkin et al., 2002). Similar to anti-apoptosis, AGA, but not GA, prevented ERK activation

induced by alendronate. These results suggested that the connexin channels are required for the ERK-mediated anti-apoptotic effect of bisphosphonates. However, prevention of apoptosis by alendronate does not require cell-to-cell contact, since the bisphosphonate inhibited apoptosis in cells plated at low density or maintained in suspension, suggesting that gap junctions do not mediate the effect of bisphosphonates and that hemichannels are involved. Moreover, treatment with alendronate did not affect gap junction communication. Instead, alendronate induced opening of hemichannels in MLO-Y4 cells measured as uptake of the fluorescent small dye Lucifer Yellow. Because Cx43 is the main connexin protein expressed in osteocytic MLO-Y4 cells, we next determined whether responsiveness to alendronate depends on its expression using several cell models (Plotkin et al., 2002). We found that the drug was able to protect from apoptosis embryonic fibroblasts derived from wild type mice, but it was unable to do so in cells derived from Cx43 deficient mice, and that transfecting Cx43 to deficient cells rescued responsiveness to alendronate. Similarly, ROS17/2.8 osteoblastic cells that express Cx43 were protected from apoptosis by alendronate, but UMR 106 cells which do not express Cx43 were not. And Cx43 transfection to UMR106 cells rescued responsiveness to alendronate. Moreover, authentic osteoblasts derived from Cx43 deficient mice did not respond to alendronate, whereas apoptosis was prevented by this agent in osteoblasts derived from wild type mice. Cx43, but not other connexins tested as representative of the three connexin subfamilies (26, 31, 32, 37, 40, and 45), was able to confer responsiveness to alendronate in HeLa cells, which do not express connexins (Plotkin et al., 2002). These results established the requirement of Cx43 for the anti-apoptotic effect of bisphosphonates *in vitro*.

The cytoplasmic C-terminal domains of connexins differ considerably, suggesting that the specific requirement of Cx43 for the effect of alendronate could be due to this region. Indeed, a Cx43 truncated mutant that lacks the cytoplasmic tail was unable to confer responsiveness to alendronate, response that was recovered by co-transfecting the truncated mutant and the cytoplasmic tail (Plotkin et al., 2002). However, the C-terminus tail alone was not able to mediate anti-apoptosis. These results suggest that the C-terminus of Cx43 contains domains required for anti-apoptosis by bisphosphonates and that the pore-forming region also contributes to the effect.

Further studies showed that bisphosphonates induce the transient opening of undocked Cx43 hemichannels resulting in activation of Src kinase, which is associated with Cx43, leading to ERK activation and cell survival (**Figure 2A**) (Plotkin et al., 2002). Since receptors for bisphosphonates have not been described, the requirement of Cx43 for bisphosphonate actions raised the possibility that Cx43 would be such receptor. However, we found that bisphosphonates not only bind to cells that express Cx43 but also to Cx43-deficient cells (Lezcano et al., 2012). Moreover, binding of labeled bisphosphonate to osteoblastic cells is displaced by unlabeled bisphosphonates, as expected, but also by protein phosphatase substrates. Furthermore, these substrates inhibit the anti-apoptotic effect of bisphosphonates (Morelli et al., 2011) Changes in the phosphorylation status of Cx43 C-terminus tail are associated with channel activity (reviewed in Herve and Sarrouilhe, 2006), suggesting that bisphosphonates might induce opening of Cx43 hemichannels thought their interaction with membrane bound phosphatases. However, the identity of the protein phosphatase that binds to Cx43, and whether it can dephosphorylate Cx43 and induce hemichannel opening remain to be determined. Taken together, these pieces of evidence indicate that although Cx43 is required for survival signaling, it is dispensable for bisphosphonate binding; and raises the possibility that bisphosphonates bind to a protein phosphatase that interact with Cx43 in the cell membrane.

An interesting feature of ERKs activated by bisphosphonates and the Cx43/Src pathway is that instead of undergoing nuclear translocation like ERKs activated by most stimuli, they are retained in the cytoplasm where ERKs modify cytoplasmic substrates (Plotkin et al., 2005a). We found that this phenomenon is due to the ability of Cx43 of interacting with β-arrestins (Plotkin et al., 2006b). Consistent with a role of β-arrestin in the retention of bisphosphonate-activated ERKs in the cytoplasm, ERKs stay in the cytoplasm in cells expressing endogenous β-arrestin or transfected with wild type β-arrestin. However, in the presence of a dominant negative from of β-arrestin, ERKs activated by alendronate translocate to the nucleus. Because cytoplasmic localization of ERKs is required for survival induced by bisphosphonates (Plotkin et al., 2005a), the dominant negative β-arrestin reversed anti-apoptosis induced by alendronate. Thus, Cx43 regulates the ERK signaling pathway due to its ability of function as a scaffold that foster interaction between β-arrestin and Src/ERKs.

Further support for the role of Cx43 on the survival effect of bisphosphonates was obtained *in vivo*, by the demonstration that administration of alendronate does not prevents glucocorticoid-induced osteoblast and osteocyte apoptosis in mice lacking Cx43 in these cells (Plotkin et al., 2008). However, the bisphosphonate was still able to prevent bone loss induced by glucocorticoids due to its potent inhibition of bone resorption. Similarly, bisphosphonates prevented bone loss induced by sex steroid removal in mice lacking Cx43 in osteoblasts and osteocytes, by reducing bone resorption (Watkins et al., 2012). On the other hand, IG9402, a bisphosphonate that does not affect osteoclast function by prevents osteoblast and osteocyte apoptosis, prevents bone loss induced by glucocorticoids in mice (Plotkin et al., 2006a, 2011). This suggest that in the absence of anti-resorptive effects, the protective effect of preventing osteocyte and osteocyte apoptosis through opening Cx43 hemichannels can be revealed. Thus, preservation of osteoblast and osteocyte apoptosis mediated by hemichannel opening might contribute to the anti-fracture efficacy of bisphosphonates.

MECHANICAL STIMULATION OPENS Cx43 HEMICHANNELS *IN VITRO*, A MECHANISM THAT MEDIATES CELL SURVIVAL INDUCED BY MECHANICAL FORCES

Adequate mechanical stimulation is required for maintaining bone mass and strength throughout life. Indeed, reduced or absent loading leads to decrease bone mass and elevated risk of fractures in the elderly, in immobilized patients, and in astronauts (Bikle et al., 1997; Marcus, 2002; Dolbow et al., 2011). Osteocytes are ideally positioned for detecting and responding to changes in mechanical strains imposed on bone. Since their description

at the microscopy level, connexin channels were thought to be the means by which the cells buried in the bone communicate with the cells on the bone surface and transmit the need for bone formation or removal.

Consistent with a role of connexin channels on mechanotransduction in bone, Cx43 expression and intercellular communication is increased by loading *in vitro* and *in vivo* (Ziambaras et al., 1998; Cheng et al., 2001b; Robinson et al., 2006; Tu et al., 2012). Early work by Donahue's group show that mechanical stimulation of osteoblastic cells induced by oscillatory fluid flow leads to release of prostaglandin (PG) E2 by a mechanism that requires intact Cx43 channels (**Figure 2B**) (Saunders et al., 2001). Thus, the release of PGE2 was inhibited in cells expressing a dominant negative form of Cx43 with reduced channel permeability. Moreover, PGE2 release induced by mechanical stimulation further increased gap junction communication (Cheng et al., 2001a) by a mechanism mediated by the prostaglandin EP2 receptor (Cherian et al., 2003) suggesting the existence of a positive feedback loop. This effect of mechanical loading was originally ascribed to a role of Cx43 in intercellular gap junction communication. However, based on previous studies showing that mechanical stimulation increases hemichannel opening in osteoblastic cells *in vitro* (Romanello et al., 2003), work from Jiang's group demonstrated that PGE2 released induced by mechanical stimulation requires opening of Cx43 hemichannels in osteocytic cells (**Figure 2B**) (Cherian et al., 2005). Interestingly, hemichannels present in the osteocytic cell body, and not in the dendritic cytoplasmic projections, are opened by mechanical stimulation (Burra et al., 2010). The release of PGE2 induced by fluid flow in osteocytic MLO-Y4 cells was prevented by using pharmacologic inhibitors of connexin channels and by an anti-sense oligonucleotide for Cx43 (Cherian et al., 2005). Moreover, prostaglandin release was enhanced when the cells were seeded at low density, which reduces intercellular gap junction communication. Intracellular levels of PGE2 production were not affected by inhibition of connexin channels, suggesting that PGE2 release but not its synthesis requires Cx43 hemichannel activity. Other authors have postulated, however, that P2X7 receptors (Li et al., 2005) and/or pannexin1 (Thi et al., 2012) are involved in the release of PGE2 induced by mechanical stimulation in bone cells. This suggests that the combined actions of these channels are required for prostaglandin release induced by mechanical stimulation in osteoblastic and osteocytic cells.

Further studies by Jiang's group demonstrated that engagement of α5β1 integrin in required for opening of Cx43 hemichannels in osteocytic cells (**Figure 2B**) (Batra et al., 2012). Thus, Cx43 interacts with α5β1 integrin, an interaction enhanced by mechanical stimulation (Batra et al., 2012, 2014). Cx43/α5β1 association is required for Cx43 hemichannel opening induced by loading and is mediated by activation of phosphatidylinositol-3 kinase (PI3K) and AKT. Consistent with a role of the integrins on mechanotransduction, we have shown that mechanical stimulation leads to the engagement of integrins α5 and β1, which in turn activate FAK/Src and the ERK pathway promoting osteocyte survival (Plotkin et al., 2005b).

More recently, Jiang's group showed that the scaffolding molecule 14-3-3θ is required for the interaction between integrin α5 and Cx43 and for plasma membrane delivery and function of Cx43 hemichannels (**Figure 2B**) (Batra et al., 2013). In particular, silencing of 14-3-3θ prevents the accumulation of Cx43 on the cell membrane and opening of hemichannels induced by fluid flow. Taken together, these studies support a role of Cx43 hemichannels on PGE2 release.

PGE2 release by osteocytes through Cx43 hemichannels subjected to mechanical forces is required for maintaining cell viability (**Figure 2B**). Indeed, prostaglandins prevent osteoblastic apoptosis via activation of EP4 receptors (Machwate et al., 2001); and mechanical stimulation prevents osteocyte apoptosis *in vitro* (Plotkin et al., 2005b; Aguirre et al., 2007; Kitase et al., 2010). Moreover, inhibition of glucocorticoid-induced apoptosis by mechanical stimulation is abolished by inhibiting prostaglandin synthesis using indomethacin (Kitase et al., 2010). The pro-survival effect of PGE2 release by osteocytes subjected to mechanical stimulation in mediated by activation of the EP2 and EP4 receptors, via cAMP/PKA signaling pathway. In addition, the study by Kitase and colleagues showed that activation of Wnt/βcatenin signaling downstream of PI3K/Akt contributes to PGE2/EP2/4-induced osteocyte survival (**Figure 2B**).

The participation of the Cx43/PGE2 survival pathway in skeletal homeostasis *in vivo* is not known. However, this potential role of Cx43 is supported by our work (Plotkin et al., 2008; Bivi et al., 2012a), later confirmed by others (Lloyd et al., 2013) showing that deletion of Cx43 from osteoblastic cells results in increased osteocyte apoptosis. Interestingly, recent work from Jiang's group (Xu et al., 2013) showed that transgenic mice expressing in osteocytes a dominant negative Cx43 mutant with impaired permeability (Cx43Δ130-136), which lacks both hemichannel and gap junction functions (Krutovskikh et al., 1998), also exhibit increased osteocyte apoptosis (Jean X. Jiang, personal communication). On the other hand, mice with osteocytic expression of the R76W Cx43 mutant, in which the ability to form gap junction channels is impaired, but that maintains hemichannel activity (Xu et al., 2013) did not exhibit increased osteocyte apoptosis compared to wild type controls (Jean X. Jiang, personal communication). This evidence supports the role of Cx43 hemichannels for maintain osteocyte survival *in vivo*; and suggests that in the absence of Cx43 hemichannels in osteocytes, the mechanical loading that occurs during normal ambulatory conditions cannot protect the cells from undergoing apoptosis. Indeed, one of the earliest effects of skeletal unloading is the accumulation of apoptotic osteocytes (Aguirre et al., 2006).

However, Cx43 is not required for the increased bone mass induced by mechanical stimulation in murine models. On the contrary, removal of Cx43 from osteoblasts and/or osteocytes results in increased anabolic response to mechanical stimulation of osteoblast on the periosteal bone surface (Zhang et al., 2011; Grimston et al., 2012; Bivi et al., 2013). The cause of this exacerbated response is not known. We have found that Cx43-deficient osteocytes exhibit elevated Wnt/βcatenin signaling (Bivi et al., 2013), a known mediator of mechanical signals in osteocytes (Robinson et al., 2006). This higher basal activation of Wnt signaling could explain the exacerbated response to loading in Cx43-deficient mice. Nevertheless, mounting evidence indicates that Cx43 hemichannel opening and the release of PGE2 mediate the survival effect of mechanical stimulation on osteocytes, suggesting that the increase in bone formation induce by

mechanical stimulation depends on signaling pathway different from those involved in loading-induced osteocyte survival.

Cx43 EXPRESSION AND CHANNEL PERMEABILITY BUT NOT GAP JUNCTION CHANNELS ARE REQUIRED FOR cAMP-MEDIATED ANTI-APOPTOTIC EFFECT OF PARATHYROID HORMONE ON OSTEOBLASTS

Intermittent administration of parathyroid hormone (PTH) is the only FDA-approved treatment to increase bone mass. Cx43 expression appears to be required to obtain a full anabolic response to intermittent PTH administration in mice (Castro et al., 2003; Chung et al., 2006). Thus, PTH does not increase bone mass, bone formation and osteoblast number when administered to heterozygous Cx43 deficient mice ($Cx43^{+/-}$) (Castro et al., 2003). Moreover, PTH-induced mineral appositional rate, a measure of the work of osteoblast teams, is reduced in mice lacking Cx43 in osteoblastic cells (in $Cx43^{fl/-}$; Col1a1-2.3kb-Cre mice) (Chung et al., 2006). Studies in mice have shown that the anabolic effect of intermittent PTH administration in cancellous bone is due, at least in part, to inhibition of osteoblast apoptosis (**Figure 2C**) (Jilka et al., 1999). Similarly, PTH related protein (PTHrP), the other ligand of the PTH receptor, as well as constitutive activation of this receptor in transgenic mice, also increases osteoblast number and decreases the prevalence of osteoblast apoptosis (Calvi et al., 2001; Martin, 2005; Miao et al., 2005; O'Brien et al., 2008). Taken together, these pieces of evidence suggest that intermittent PTH administration does not result in a full anabolic response in mice lacking Cx43 due to the inability of the hormone to prevent apoptosis of osteoblastic cells in the absence of Cx43.

In vitro mechanistic studies showed that, indeed, PTH does not prevent apoptosis in osteoblastic cells lacking Cx43, whereas overexpression of wild type Cx43 rescues the survival effect of the hormone (**Figure 2C**) (Bivi et al., 2011). A similar result is obtained by transfecting a Cx43 mutant in which cysteine residues of the extracellular domain have been mutated, rendering it unable to dock with other connexin molecules to form gap junction channels (Bao et al., 2004). On the other hand, a Cx43 mutant with impaired permeability was not able to confer responsiveness to PTH, suggesting that active Cx43 hemichannels are required for the survival effect of the hormone.

In addition, the interaction of Cx43 with β-arrestin modulates the response of osteoblasts to PTH (**Figure 2C**) (Bivi et al., 2011). Thus, PTH-induced anti-apoptosis is rescued by transfecting a dominant negative form of β-arrestin to cells lacking Cx43; and overexpression of β-arrestin induces the same inhibition on PTH-induced osteoblast survival as removing Cx43. Moreover, transfection of Cx43 decreases the association between the PTH receptor and β-arrestin, suggesting that Cx43 binds to β-arrestin, thus competing with the PTH receptor. Interestingly, Cx43 mutants lacking the C-terminus domain or lacking the phosphorylation site in serine 368 decrease the interaction between PTHR and β-arrestin, suggesting that Cx43 binds β-arrestin at the phosphorylated serine 368.

We found that PTH does not increase the expression of cAMP-target genes in osteoblastic cells lacking Cx43 (Bivi et al., 2011). Consistent with this, it was previously shown that the response to PTH on cAMP production is blunted in osteoblastic cells in which Cx43 expression has been reduced using anti-sense cDNA (Vander Molen et al., 1996). These findings, together with evidence that β-arrestin reduces cAMP responses of the PTH receptor (Premont and Gainetdinov, 2007), support the hypothesis that Cx43 interacts with β-arrestin decreasing β-arrestin binding to the PTH receptor, then facilitating cAMP dependent signaling induced by PTH. Thus, Cx43 hemichannel activity and the ability of the connexin to interact with intracellular signaling molecules, through specific phosphorylation sites in its cytoplasmic tail, regulates survival signaling induced by PTH in osteoblastic cells.

CONCLUSIONS

Since the first description of gap junction channels at the structural level in bone cells, substantial advances on our understanding of the role of connexins on bone cell physiology have been made (Civitelli, 2008; Loiselle et al., 2013; Plotkin and Bellido, 2013). The requirement of connexins, and in particular Cx43, for bone cell differentiation and function, as well as for the response of the cells to bone-acting stimuli has been clearly established *in vitro* and *in vivo*. Moreover, the role of the Cx43 hemichannels *in vivo* has begun to be unveiled. Indeed, while studies with genetically modified mice appear to agree that sustained opening of Cx43 hemichannels is deleterious for bone (Dobrowolski et al., 2007; Jiang et al., 2010), transient opening of hemichannels is beneficial for osteoblast and osteocyte survival (Plotkin et al., 2002, 2008, 2011; Kitase et al., 2010; Bivi et al., 2011). Studies using genetically modified mice currently underway will allow to conclusively demonstrate the role of Cx43 hemichannels on bone development and cell function.

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Pannexin 1 channels in skeletal muscles

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Normal myotubes and adult innervated skeletal myofibers express the glycoprotein pannexin1 (Panx1). Six of them form a "gap junction hemichannel-like" structure that connects the cytoplasm with the extracellular space; here they will be called Panx1 channels. These are poorly selective channels permeable to ions, small metabolic substrate, and signaling molecules. So far little is known about the role of Panx1 channels in muscles but skeletal muscles of $Panx1^{-/-}$ mice do not show an evident phenotype. Innervated adult fast and slow skeletal myofibers show Panx1 reactivity in close proximity to dihydropyridine receptors in the sarcolemma of T-tubules. These Panx1 channels are activated by electrical stimulation and extracellular ATP. Panx1 channels play a relevant role in potentiation of muscle contraction because they allow release of ATP and uptake of glucose, two molecules required for this response. In support of this notion, the absence of Panx1 abrogates the potentiation of muscle contraction elicited by repetitive electrical stimulation, which is reversed by exogenously applied ATP. Phosphorylation of Panx1 Thr and Ser residues might be involved in Panx1 channel activation since it is enhanced during potentiation of muscle contraction. Under denervation, Panx1 levels are upregulated and this partially explains the reduction in electrochemical gradient, however its absence does not prevent denervation-induced atrophy but prevents the higher oxidative state. Panx1 also forms functional channels at the cell surface of myotubes and their functional state has been associated with intracellular Ca^{2+} signals and regulation of myotube plasticity evoked by electrical stimulation. We proposed that Panx1 channels participate as ATP channels and help to keep a normal oxidative state in skeletal muscles.

Keywords: potentiation, sarcolemma leakage, muscular plasticity, phosphorylation, ATP

PANNEXIN 1 BASED CHANNELS: STRUCTURE AND EXPRESSION

Myotubes (Buvinic et al., 2009) and innervated myofibers (Cea et al., 2013; Riquelme et al., 2013) express pannexin1 (Panx1). Panx genes (PANX) are a small family; three of them have been cloned from mammals and are called PANX1, PANX2, and PANX3. They are orthologs of invertebrate innexins (Inxs) (Ambrosi et al., 2010), which form gap junction channels allowing the direct cytoplasm communication between adjacent cells (Sáez et al., 2003). Presently, it has been proposed that gap junction channels of chordates have to be formed exclusively by connexins (Cxs) (Dahl and Keane, 2012). Gap junction channel formation requires plasma membrane proximity of two cells and the docking of two hemichannels (half of a gap junction channel), each one provided by one of two adjacent cells (Sáez et al., 2003). However, mammalian Panxs and Inxs do not share significant gene and protein sequence homology with chordate Cxs. Nevertheless, Panx channels share some pharmacological properties with Cx-based channels and present some differences in sensitivity to different compounds and conditions including Probenecid, carbenoxolone, flufenamic acid, La^{3+} and cytoplasmic acidification (D'Hondt et al., 2009). Panx1 channels are also inhibited by brilliant blue G, a P2X receptor blocker, and by the food dye FD&C blue N° 1 [Brilliant Blue FCF (BB FCF) or Blue 1] (Wang et al., 2013), present in Blue Gatorade (see in nutritional facts on official web site www.gatorade.com) and Blue M&M (see in ingredients on official web site www.mms.com). Also, they share some permeability properties with Cx hemichannels, since they are permeable to small positively [i.e., ethidium (Etd^+), DAPI] and negatively (i.e., gluconate, glutamate, ATP, and aspartate) charged organic molecules and inorganic ions (Na^+, K^+, Ca^{2+}, and Cl^-). In addition, Panx1 forms gap junction channels in exogenous expression systems including Xenopus *laevis* oocytes and some mammalian cells lines (Vanden Abeele et al., 2006). Nevertheless, up to the present there is no *in vivo* evidence that Panxs form gap junction channels, as compared to the evidence for Cxs or Inxs (Dahl and Keane, 2012). Therefore, these channels seem to work exclusively as channels in the nonjunctional membrane, allowing the exchange of molecules between the cytoplasm and the extracellular space. Panx1 channels present in the reticular system and the plasma membrane are activated by positive membrane potentials, high extracellular K^+, extracellular ATP via P2 receptors, stretch-induced membrane deformation and posttranslational modifications (D'Hondt et al., 2013).

Panx1 is ubiquitously expressed in several tissues. Different levels of mRNA are detected in northern blots, being strongly expressed in heart, skeletal muscle, testis, and ovary. Medium levels are present in brain (Bruzzone et al., 2003), placenta, thymus,

kidney, prostate, and small intestine, and low to almost undetectable levels are found in lung, liver, pancreas, spleen, colon, and peripheral blood (Baranova et al., 2004). The pattern of expression of the other two Panxs is completely different; Panx2 is preferentially expressed in brain (Baranova et al., 2004) and Panx3, mostly expressed in bone and skin (Sandilos and Bayliss, 2012). Panx1 channels are oligomeric structures formed by 6 subunits of Panx1 protein (Boassa et al., 2007). Panx1 shows similar membrane topology than Cxs and is characterized by four transmembrane (TM) segments, two extracellular loops, and cytoplasmic localization of both amino and C-termini. Panxs 1 and 3 are glycoproteins, unlike Cxs that are not glycosylated. It has been proposed that glycosylation of Panxs prevents formation of gap junction channels (Boassa et al., 2008). Panx1 and Panx3 suffer N-linked glycosylation at Asp254 present in the second extracellular loop and Asp71 found at the first extracellular loop, respectively. However, a fraction of Panx1 mutant that is not glycosylated still traffics to the cell surface, suggesting that Panx1 glycosylated protein could interact and form channels with unglycosylated forms and/or that the unglycosylated Panx1 fraction forms gap junction channel-like structures (Penuela et al., 2009).

Structural analyses of the lining residues of the Panx1 pore using the substituted cysteine accessibility method (SCAM) have revealed that the TM1 region and domains of the first extracellular loop (E1) are exposed to the channel lumen. Also, C-terminal amino acids substitution and reagent perturbation suggest the contribution of this segment to the permeation pathway (Wang and Dahl, 2010). Panx1 channels activated by positive membrane potentials show several substates. The full open state presents a characteristically high conductance in different cell types including Xenopus oocytes, insulinoma cells and cardio myocytes (Bao et al., 2004; Iglesias et al., 2009; Kienitz et al., 2011). However, recent studies using different Panx1 transfectants revealed a characteristic unitary conductance of ∼60 pS (Ma et al., 2009; Romanov et al., 2012). Moreover, the permeability of Panx1 channels has been recently shown to be negligible to anions exceeding 250 Da, which would exclude ATP (Romanov et al., 2012). This apparent controversy might be explained by Panx1 channel variations due to pore properties, such as diameter and length changes caused by different interactions with other cellular proteins or due to different post-translational modifications in different cell types. Thus further studies maybe required to clarify this issue.

ACTIVATION OF Panx1 CHANNELS

Several stimuli increase the activity of Panx1 channels. Among them are: increase in extracellular K^+ concentration, positive membrane voltage over +40 mv, extracellular ligands, such as ATP [which activates Panx1 channels in micromolar concentrations (Locovei et al., 2006) but inhibit them in milimolar concentrations (Qiu and Dahl, 2009)], that enhance the intracellular free Ca^{2+} levels including like $P2Y_{1-2}$ receptors coupled to Gq proteins or $P2X_7$ receptors that are non-selective cationic channels with a slow kinetic of inactivation (Locovei et al., 2006, 2007; Iglesias et al., 2008). Panx1 channel opening has been induced by glutamate through NMDA receptor activation in neurons (Thompson et al., 2008; Orellana et al., 2011), neuronal stress induced by oxygen glucose deprivation (Thompson et al., 2008), hypertonic stress in lymphocytes (Woehrle et al., 2010) and increase in intracellular free Ca^{2+} levels induced by Ca^{2+} ionophore (Locovei et al., 2006). Although Panx1 does not have putative Ca^{2+} biding sites, it possesses multiple phosphorylation consensus sites in the C-terminal tail to several serine and threonine kinases (Penuela et al., 2007; Riquelme et al., 2013). A possible mechanism of activation of Panx1 channels that involves phosphorylation has been recently suggested. During repetitive skeletal muscle contraction, the Panx1 channel activity increases and the state of phosphorylation of Panx1 Ser and Thr residues is also increased (Riquelme et al., 2013). In contrast, phosphorylation of Tyr residues in Panx1 has not been detected yet (Iglesias et al., 2008; Riquelme et al., 2013). A protein phosphorylation-dependent activation could be followed by inactivation via a phosphoprotein phosphatase or by phosphorylation of a different amino acid residue by another protein kinase with less Ca^{2+} affinity, followed by complete dephosphorylation via a protein phosphatase.

An alternative mechanism of Panx1 channel activation in skeletal muscle could be a direct protein-protein dependent mechanism (Panx1 channel/dihydropyridine receptor) mediated by conformational changes of voltage activated dihydropyridine receptors induced by depolarizing membrane potentials. In support of this view it is possible to say that electrical stimulation induces ATP release and uptake of Etd^+ and fluorescent glucose derivatives in myofibers, indicating opening of Panx1 channels (Riquelme et al., 2013). Also, myotubes lacking the Cav1.1-α1 subunit released almost no ATP upon electrical stimulation, suggesting that Cav1.1 plays a critical role in this process (Jorquera et al., 2013).

ROLE OF Panx1 BASED CHANNELS IN NORMAL SKELETAL MUSCLES

POSSIBLE ROLE OF Panx1 CHANNELS IN MUSCULAR ONTOGENY

Skeletal muscles develop through a process partially coordinated by extracellular signaling. The coordinate response of cell groups includes the myogenic commitment of mesodermal pluripotent cells, myoblast alignment and fusion. In mice, this process requires the presence of Cx43 expression and functional gap junction channels (Kalderon et al., 1977; Proulx et al., 1997; Araya et al., 2003). In rats, these channels disappear at about 1 week postnatal age when skeletal muscles become innervated (Cea et al., 2012).

The acquisition of myogenic commitment requires increase of $[Ca^{2+}]_i$, and activation of calcineurin, a Ca^{2+}-dependent protein phosphatase that induces expression of the transcription factor myf-5 (Friday and Pavlath, 2001). Increases in $[Ca^{2+}]_i$ could be induced by activation of P2 receptors with extracellular ATP/ADP. In addition, activation of P2X receptors 2, 4 or 7 increases the cell membrane permeability to small molecules, including Lucifer yellow, Etd^+ and YO-PRO-1, in diverse cell types such as myoblasts and macrophages (North, 2002; Araya et al., 2005; Pelegrin and Surprenant, 2006). However, Panx1 has been proposed to mediate the plasma membrane permeabilization to dyes after activation of P2X/Y receptors (Pelegrin and Surprenant, 2006; Locovei et al., 2006, 2007).

Treatment with a concentration of β-glycyrrhetinic acid that blocks connnexin-based channels (gap junction channels and hemichannels) and Panx1 channels (Bruzzone et al., 2005) prevents the expression of myogenin and MRF4, two transcription factors that promote myogenesis and myotubes formation (Proulx et al., 1997). However, treatment with octanol, blocker of Cx-based channels but not Panx1 channels (Bruzzone et al., 2005; Pelegrin and Surprenant, 2006), does not block myogenesis as evaluated by the expression of the pro-myogenic transcription factor Myf-5 (Proulx et al., 1997). Thus, the presence of functional Panx1 channels might be enough to promote commitment and myogenesis *in vitro*, and probably Cx hemichannels and/or other Ca^{2+} channels have a redundant role that overcomes the lack of Panx1, since Panx1$^{-/-}$ mice do not show evident muscular phenotype changes. In agreement with the role of Cx-based channels coordinating the commitment of myoblast, Cx43 deficient muscles show a delay regeneration response after $BaCl_2$-induced damage (Araya et al., 2005).

POTENTIATION OF MUSCULAR CONTRACTION

The force generated in muscular contraction increases after repetitive twitches. This response is called potentiation and depends on an increase of intracellular free Ca^{2+} concentration (Sandonà et al., 2005; Zhi et al., 2005) due to Ca^{2+} release from intracellular stores and Ca^{2+} inflow from the extracellular space in fast and slow twitch muscles, respectively. In the latter, Ca^{2+} uptake depends on the activation of purinergic ionotropic $P2X_4$ receptors (Sandonà et al., 2005), which are highly expressed in this type of muscle. For fast twitch muscles, the potentiation response is independent of the extracellular Ca^{2+} concentration (Louboutin et al., 1996), but requires extracellular ATP. Accordingly, extracellular ATPases inhibit the potentiation response (Sandonà et al., 2005) and, together with the absence of P2X receptors, have led to suggest that the potentiation response in these muscles is mediated by activation of metabotropic P2 receptors as $P2Y_1$ receptors (Riquelme et al., 2013). Recently, Panx1 channels were proposed as a possible pathway for ATP release in skeletal muscles (Riquelme et al., 2013). In normal adult skeletal muscle, Panx1 was localized in the sarcolemma of the T-tubules system (Cea et al., 2012; Riquelme et al., 2013; Jorquera et al., 2013). Moreover, Panx1 channels were activated by electrical stimulation allowing the uptake of glucose (a fluorescent glucose analog, 2-NBDG) and release of ATP, which are necessary for potentiation of muscular contraction (Sandonà et al., 2005; Riquelme et al., 2013). In addition, the absence of Panx1 in myofibers of Panx1 deficient mice (Panx1$^{-/-}$ mice) does not exhibit potentiation of muscle contraction induced by electrical stimulation. Therefore, it was suggested that this response is due to the absence of ATP release (Riquelme et al., 2013). In agreement with this notion, the lost potentiation response of skeletal muscles from Panx1$^{-/-}$ mice can be reversed by addition of exogenous ATP to the bath, shown for the first time here (**Figure 1**). This finding confirms that Panx1 channels are necessary for the potentiation response, and is consistent with the hypothesis that potentiation *in vivo* could be due to permeability of Panx1 channels to ATP, at least in activated skeletal muscles. It further suggests that the presumptive Panx1 channel-dependent mechanism for ATP release is not compensated by other pathways of ATP release, or other possible mechanisms of potentiation.

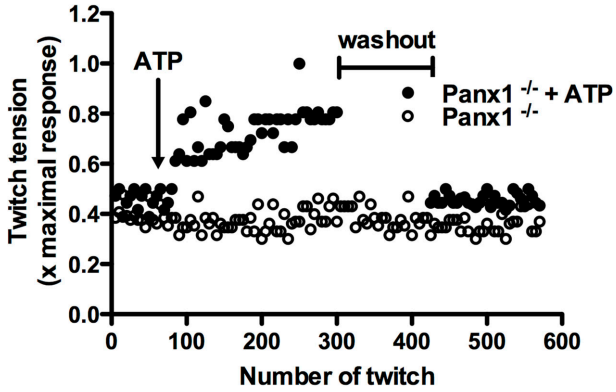

FIGURE 1 | Exogenous ATP reverses the absence of potentiation of muscular contraction in Panx1 deficient mice. Potentiation of muscular contraction was induced by electrical transmural stimulation (0.03 Hz, 45 V, 100 ms stimuli duration) in isolated Soleus muscle from Pannexin 1 deficient mice (Panx1$^{-/-}$), and exogenous ATP was applied (200 μM, final concentration, closed circles) after 65 twitches. Then, the muscle was rinsed during 2 min and the potentiation response was re-evaluated. In addition, the potentiation response with the same number of twitches was evaluated in absence of ATP and washout (open circles).

ROLE OF Panx1 CHANNELS IN PATHOLOGICAL CONDITIONS OF SKELETAL MUSCLES

Recently, denervation was shown to induce *de novo* expression of Cx-based hemichannels that mediate a drastic increase in sarcolemma permeability and leads to muscular atrophy (Cea et al., 2013). In addition, it was found that Panx1 channels do not play a crucial role on this phenomenon, since muscles of Panx1$^{-/-}$ mice showed similar increase in membrane permeability and atrophy to those observed in denervated muscles of wild type animals (Cea et al., 2013). Until now, this is the only work published in which a possible pathological role of Panx1-based channels in skeletal muscles has been evaluated. In addition, it was analyzed the production of thiobarbituric reactive substances (TBARS), including malondialdehyde (MDA), as a measure of oxidative stress in denervated skeletal muscles of Panx1$^{-/-}$ mice because denervation is known to increase the skeletal muscle levels of reactive oxygen species (ROS) (Abruzzo et al., 2010). We found, in this work, that the absence of Panx1 does not affect the basal levels of TBARS but prevented the increase in TBARS levels present in 7-day denervated wild type muscles (**Figure 2**), suggesting that Panx1 channels might allow loss of reducing agents and thus their absence would protect against ROS generation. Alternatively, constitutive Panx1$^{-/-}$ muscles might have developed more antioxidant phenotype and thus could be more resistant to the denervation-induced ROS generation.

SKELETAL MUSCLE PLASTICITY

The skeletal muscle activity induces remodeling of structure and functional performance of myofibers, changing the muscular force output, endurance and contractile velocity with respect to a functional demand (Tavi and Westerblad, 2011). All these

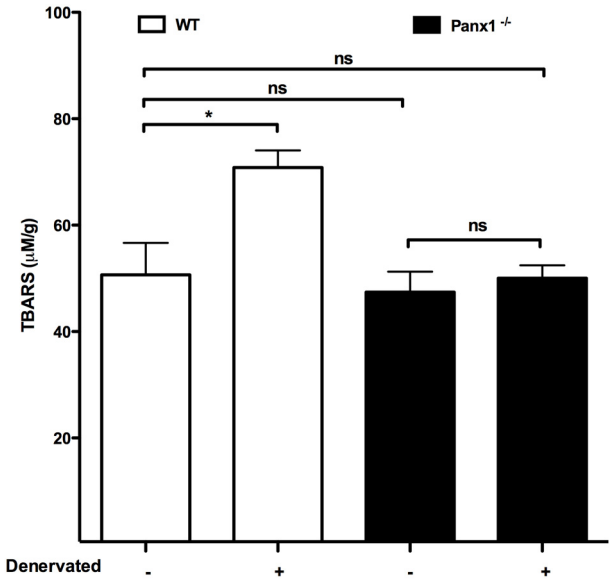

FIGURE 2 | The absence of Panx1 prevents the increase in levels of thiobarbituric reactive substances in denervated skeletal muscles. Seven days after denervation gastrocnemius muscles were isolated from wild type (wt, white bar), or Panx1 deficient mice (Panx1$^{-/-}$, black bar), and levels of thiobarbituric acid reactive substances (TBARS) were evaluated in denervated vs. not denervated muscles. *$P < 0.05$; ns, not significant.

changes are called skeletal muscle plasticity. This adaptive process depends on the frequency of repetitive fiber contraction, activation of intracellular signal pathways and gene expression that promotes the establishment of new myofiber characteristics (Tavi and Westerblad, 2011). The physiological importance of skeletal muscle plasticity is evident; however, the molecular mechanisms involved in muscle plasticity remain elusive. Electrical stimuli at frequencies that activate Panx1 channels (20 Hz) induce the expression of a molecular marker that reflects the transition between a fast to slow myofiber (Jorquera et al., 2013). Panx1 channels present in the T-tubule membrane are regulated for the dihydropyridine receptors; with high frequencies (90 Hz) the activity of Panx1 channels is low and the expression of plasticity marker in myofibers does not change (Jorquera et al., 2013). The electrical stimulation induces ATP release through Panx1 channels, eliciting an IP_3-dependent intracellular Ca^{2+} signal (Eltit et al., 2006) which is directly associated with gene expression changes (Semsarian et al., 1999; Jaimovich and Carrasco, 2002; Carrasco et al., 2003; Buvinic et al., 2009). These responses depend on the activation of P2 receptors by extracellular ATP because apyrase, an ATP hydrolase, or suramin, a general P2 receptors inhibitor, blocks both signals. The metabotropic Ca^{2+} signal induced by extracellular ATP was prevented by Panx1 channel inhibitors (^{10}Panx1 and oleamide) that reduced the calcium transients and the ATP release (Buvinic et al., 2009). Consequently, it was proposed that a train of action potentials with a defined frequency induces Ca^{2+} release events that differentially activate Ca^{2+}-dependent signaling pathways, which determine the expression of genes responsible for the slow or fast muscle phenotype (Tavi and Westerblad, 2011). These signaling pathways include calcineurin–NFAT-, Ca^{2+}/calmodulin-dependent kinases II and IV- and protein kinase C-dependent pathways (Tavi and Westerblad, 2011). These Ca^{2+} signals lead to muscular plasticity by modulating the expression of several genes including IL-6 and c-*fos*, and the switch between troponin isoform from fast to slow fiber (Buvinic et al., 2009; Jorquera et al., 2013).

CONCLUDING REMARKS

Panx1 channels are involved in several relevant physiological skeletal muscle processes, such as potentiation of skeletal muscle contraction since they do not occur in the absence of these channels. Currently, Panx1 channels have been clearly involved in potentiation of contraction of skeletal muscles. In addition, Panx1 channels appear to mediate the release of ATP involved in muscle remodeling. People who are active in sports should be cautious with food and drink additives that could reduce the Panx1 channel activity and consequently the potentiation response of muscle contraction. Moreover, to further understand the possible role of Panx1 channels in pathologic responses it may be needed to develop highly selective Panx1 channels inhibitors and/or acute down regulation of Panx1 expression using inducible KO animals or transfections with siRNA or morpholines. Finally, Panx1 channels act as ATP channels and the absence prevent the induction of reactive oxygen species; measured as levels of TBARS in this work (**Figure 2**), suggesting that Panx1 channels are relevant to keep healthy skeletal muscles.

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Connexin and Pannexin hemichannels are regulated by redox potential

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Connexins (Cxs) and Pannexins (Panxs) are two non-related protein families, having both the property to form hemichannels at the plasma membrane. There are 21 genes coding for different Cx based proteins and only 3 for Panx. Under physiological conditions, these hemichannels (Cxs and Panxs) present a low open probability, but when open, they allow the release of signaling molecules to the extracellular space. However, under pathological conditions, these hemichannels increase their open probability, inducing important lysis of metabolites, and ionic imbalance, which in turn induce the massive entry of Ca^{+2} to the cell. Actually, it is well recognized that Cxs and Panxs based channels play an important role in several diseases and -in many cases- this is associated with an aberrant hemichannel opening. Hemichannel opening and closing are controlled by a plethora of signaling including changes of the voltage plasma membrane, protein-protein interactions, and several posttranslational modifications, including protein cleavage, phosphorylation, glycosylation, hydroxylation and S-nitrosylation, among others. In particular, it has been recently shown that the cellular redox status modulates the opening/closing and permeability of at least Cx43, Cx46, and Panx1 hemichannels. Thus, for example, the gaseous transmitter nitric oxide (NO) can induce the S-nitrosylation of these proteins modulating in turn several of their properties. The reason is that the redox status of a cell is fundamental to set their response to the environment and also plays an important role in several pathologies. In this review, I will discuss how NO and other molecules associated with redox signaling modulate Cxs and Panx hemichannels properties.

Keywords: carbon monoxide, nitric oxide, connexin, S-Nitrosylation, redox signaling

CONNEXIN AND PANNEXIN HEMICHANNELS: GENERAL PROPERTIES

Connexins (Cxs) are a family of proteins constituted by 21 members, while Pannexins (Panxs) have only 3 members. These two protein families are formed by fourth transmembrane domains, two extracellular loops, one intracellular loop and both the N and C-terminus located at the cytoplasm (Milks et al., 1988; Yeager and Gilula, 1992; Wang and Dahl, 2010). In spite of their topological similarities, Cxs and Panx share a very low homology in terms of their amino acidic sequence. However, in both cases the oligomerization of six subunits forms a channel frequently call "hemichannel." But, it has been recently reported that Panx2 seems to form an octamer (Ambrosi et al., 2010), which would be the only exception to the hexameric hemichannels rule. Originally, the term "hemichannel" comes from the idea that the serial docking of two Cx -hemichannels form a gap junction channel (GJC). Therefore, a half GJC should be a hemi-channel. Even though the term hemichannel is widely used, some authors also used the word connexon to refer to these channels. As mentioned above, in the case of Panx, the term hemichannel is also used, but—as they seem not to form GJCs (Scemes et al., 2007) some authors have suggested to call them just channels (Sosinsky et al., 2011).

Cx- based GJC and hemichannels are formed by the same protein, but they have marked differences in terms of their cellular localization, opening and closing regulation and their roles in cellular processes. Thus, Cx-hemichannels are located at the plasma membrane zone that is not contacting with other cells, whereas GJC are located at the contacting zone. In the case of Panx- hemichannels, the history is more complex. There are a number of studies that support the idea that Panxs form only hemichannels *in vivo* (Scemes et al., 2007), probably because Panxs are glycoproteins and its posttranslational modification could interfere with the GJC formation (Penuela et al., 2007). However, Panx1 has been observed to form GJC in *Xenopus Laevis* oocytes heterologous expression system (Bruzzone et al., 2003), which indicates that at least Panx1, under certain conditions, can form intercellular channels. Due to their cellular localization, when hemichannels open the flow of molecules and ions between the intracellular compartment and the extracellular space is allowed. In particular, Cx- hemichannels have been associated with cell-cell autocrine/paracrine communication through ATP (Romanello and D'Andrea, 2001; Stout et al., 2002), glutamate (Ye et al., 2003), cyclic ADP-ribose [cADPR] (Bruzzone et al., 2001), cAMP (Valiunas, 2013) and PGE2 (release) and glucose uptake (Retamal et al., 2007a). Additionally, hemichannels

Abbreviations: CO, Carbon Monoxide; NO, Nitric oxide; Cx, Connexin; GJC, Gap junction channel; Panx, Pannexin; Cys, Cysteine.

are relevant players in calcium waves propagation (Cotrina et al., 1998; Stout et al., 2002), memory consolidation in the amygdala (Stehberg et al., 2012), cell proliferation (Song et al., 2010), cell migration (Cotrina et al., 2008), light processing by the retina (Kamermans et al., 2001; Vroman et al., 2013), among others. On the other hand, GJC allow the cells to share ions and metabolites directly (Sáez et al., 1989; Kam et al., 1998; Goldberg et al., 1999; Niessen et al., 2000). So far, Panx1 hemichannels have been shown to be permeable to ATP (Bao et al., 2004; Penuela et al., 2013) and, interestingly, it is probable to be the largest pore associated with the activation of the P2X7 receptor by extracellular ATP (Pelegrin and Surprenant, 2006; Iglesias et al., 2008). Thus, both Cx- and Panx- hemichannels are permeable to signaling molecules and, therefore, are associated with a great number of biological processes.

Taken together above evidence, it is now increasingly accepted that under physiological conditions Cxs- hemichannels can open, but with a low open probability (Contreras et al., 2003), which would be enough to participate in several cellular processes (Sáez et al., 2010; Rackauskas et al., 2010; Kar et al., 2012). However, under pathological conditions, Cx- hemichannels increase their overall activity most likely due to increasing the open probability and thus forming "leaky hemichannels" (Liang et al., 2005; Stong et al., 2006; Sánchez et al., 2010) and/or increasing their number at the plasma membrane (Retamal et al., 2006). This augmented hemichannel activity has been associated with an accelerated cell death in heterologous systems (Essenfelder et al., 2004; Gerido et al., 2007; Tong et al., 2011; Levit et al., 2012), supporting the idea that a low hemichannel activity can be related to several cell functions, but a high and/or uncontrolled hemichannel activity diminishes cell viability. Similarly, Panx- hemichannels also increase their activity under pathological conditions, thus Panx1 hemichannels increase their opening probability in cells metabolically inhibited (Domercq et al., 2010; Bargiotas et al., 2011), as well as under inflammatory conditions (Riteau et al., 2010; Orellana et al., 2011).

As presented before, maintaining a controlled opening/closing hemichannel is very important to preserve a normal cell function. Cx hemichannels are constantly under the control of several factors, including those acting intracellularly, as membrane potential (Ebihara, 2003; Bukauskas and Verselis, 2004; Kronengold et al., 2012), intramolecular interactions (Ponsaerts et al., 2010), pH (Peracchia, 2004) and posttranslational modifications, such as phosphorylation (Sáez et al., 1998; Lampe and Lau, 2000; Moreno, 2005), ubiquitination, SUMOylation, palmitoylation, caspasecleavage, S-Nitrosylation, hydroxylation and deamidation (reviewed by Johnstone et al., 2012; D'Hondt et al., 2013), as well as those acting extracellularly, such as Ca^{2+} and Mg^{2+} (Verselis and Srinivas, 2008; Bader et al., 2012). Similarly, Panx- hemichannels are also modulated by intracellular signaling molecules and posttranslational modifications, such as N-glycosylation in their extracellular loops [Panx1, Panx2, and Panx3, asparagine 254, 86, and 71, respectively] (Penuela et al., 2013). Notwithstanding, there is no confirmation yet that Panx are phosphoproteins; current evidence indicates that they can be so. Thus, a connection was observed between activation of kinases, such as Src (Weilinger et al., 2012) and Rho (Seminario-Vidal et al., 2009) and the Panx1 hemichannel opening. Finally, rises in the intracellular calcium concentration do increase the Panx1 hemichannel opening (Locovei et al., 2006). In conclusion, under physiological conditions, these molecular mechanisms act in combination to keep the hemichannels mostly closed. This mini review will focus on the effect of reactive oxygen species [ROS, i.e., nitric oxide (NO)] over the permeability and gating of Cxs and Panxs hemichannels.

CONNEXIN'S HEMICHANNELS AND THEIR CONTROL BY REDOX SIGNALING MOLECULES

Probably the first study suggesting that Cxs- hemichannels can be modulated by redox signaling molecules was the work performed by Contreras and his co-workers (2002). This work showed that rat's astrocytes in culture become permeable to fluorescent tracers Ethidium and Lucifer yellow, after being metabolically inhibited by 75 min with iodoacetic acid and antimycin A. This membrane permeabilization was blocked by Octanol, La^{3+} and 18α-glycyrrhetinic acid (AGA), three well known hemichannel blockers, and was not observed in astrocytes from mouse knockout for Cx43. These results indicate that metabolic inhibition induces the opening of Cx43 hemichannels. Additionally, it was observed that Trolox -a free radical scavenger- was very efficient in preventing the opening of hemichannels induced by metabolic inhibition (Contreras et al., 2002). This indicates that metabolic stress increases free radicals concentration by an unknown mechanism (at least in that date) inducing the Cx43 hemichannel opening. Interestingly, a progressive dephosphorylation of this protein was also observed in parallel with the metabolic inhibition progression. But even when Cx43 is dephosphorylated, which is a signaling to induce hemichannel opening (Bao et al., 2007), hemichannels were closed by Trolox indicating that the redox status of Cx43 is a control mechanism more important than dephosphorylation, at least in metabolic stress conditions. Then, Retamal et al. (2006) studied the molecular mechanism that control Cx43 hemichannel opening under metabolic inhibition. Here, it was observed that astrocytes under control conditions were permeabilized to Ethidium after being exposed to a nitric oxide (NO) donor (GSNO). This permeabilization was reverted by DTT, indicating that NO induces the opening of Cx43 hemichannels by a mechanism dependent of S-nitrosylation (Retamal et al., 2006). Because of the above, the effect of DTT was tested in astrocytes under metabolic inhibition, and it was observed that DTT was able to block the entry of Ethidium into the cells. A similar result was observed when a reduced gluthation that is able to cross the plasma membrane (GSH-EE) was added to the bath solution, but this reduction was not observed when GSH (which does not cross the membrane) was added (Retamal et al., 2006). This indicates that the hemichannel blocking induced by reducing agents is due to reduction of the oxidized intracellular cysteines (Cys). At this point, it was not yet determined which Cx43 Cys were modified by NO, but it was demonstrated that Cx43 is S-nitrosylated by GSNO and also by metabolic inhibition, thus changing some properties of these channels.

Then, we studied the effect of redox molecules in Cx43 expressed in HeLa cells (Retamal et al., 2007b). First, we studied the effect of GSNO on these hemichannels, and unexpectedly

no changes were observed in the activity of Cx43 hemichannels (data not published). However, it was evident that a reducing agent such as DTT presents a robust activation of these hemichannels, which was observed as an increase of dye uptake and by electrophysiology experiments. This indicates that HeLa cells somehow keep Cx43 hemichannel's Cys groups oxidized and, therefore, susceptible to be reduced by reducing agents such as dithiothreitol (DTT). Now, in astrocytes, oxidative stress open hemichannels and in HeLa cells reducing agents induce the opening of hemichannels. How can this be possible? To answer this question, Retamal et al. (2007b) added DTT to cells under metabolic inhibition at different times and it was observed that, when DTT was added before 20 min of metabolic inhibition, it induced an increase in Ethidium uptake. Moreover, when DTT was added around 30 min of metabolic inhibition no clear effect was observed, but when added after 40 min, it induced closure of Cx43 hemichannels. These data suggest that even when Cx43 hemichannels are affected by the cellular redox potential, the net effect is probably going to depend upon some other factors, such as the phosphorylation/dephosphorylation balance. Thus, it is necessary to elucidate the possible cross-talk between phosphorylation, pH, and membrane potential within the final effect induced by oxidation. Experiments based on this line of thought have been published (De Vuyst et al., 2009), but more research is still needed.

As mentioned before, Cx43 hemichannels are sensitive to redox potential, but until now there is no evidence showing which is or are the Cys groups that can be modulated by NO or other free radicals. It has been suggested that Cys271 could be a good candidate, because this Cys has been shown to be S-nitrosylated in GJC formed by Cx43 in endothelial cells (Straub et al., 2011). Interestingly, this posttranslational modification seems to decrease the permeability to IP_3 through Cx43 GJC, and it is constantly modulated by the S-nitrosogluthathion reductase (Straub et al., 2011).

In another experiment performed in astrocytes in culture, the effect of proinflammatory cytokines on GJC and hemichannels were analyzed. Under these conditions, GJC between astrocytes were found to be closed, measured as a decrease in the intercellular transference of Lucifer yellow, but in parallel it was observed an increase in Ethidium uptake, which would suggest the opening of Cx43 hemichannels (Retamal et al., 2007a). The increase of hemichannel activity was prevented by L-name (an inhibitor of the nitric oxide syntase) and also by DTT. But, the decrease of cell coupling was affected neither by DTT nor by L-name (Retamal et al., 2007a). Above findings suggest that Cx43 hemichannels are more sensitive to changes in the redox potential than GJC and/or the modifications in different Cys groups show differences in terms of sensitivity to reducing agents. Similar results have been observed in ryanodine receptors, where different Cys groups are differentially oxidized (i.e., by *S*-nitrosylation, *S*-glutathionylation, and disulfide oxidation) and induced different modification to channel properties (Aracena-Parks et al., 2006). On the other hand, the increase of Cx43 hemichannels activity in astrocytes induced by NO can lead to a massive neuronal death due to considerable efflux of glutamate from astrocytes (Froger et al., 2010) and the inhibition of Cx43 hemichannels by synthetic cannabinoids was neuroprotective (Froger et al., 2010). Therefore, more studies are necessary to find out the effect of other oxidizing agents that affect Cys groups, such as oxidized glutathione, which will help to understand the role of redox potential as controller of hemichannels in pathological and also physiological conditions.

It has been recently reported that Cx46 is also modulated by the NO donor GSNO (Retamal et al., 2009). In this work, GSNO was shown to induce changes in Cx46 hemichannel opening and closing kinetics, current—voltage relationship and also induces the appearance of a current relaxation at voltages over +40 mV. All of them are reverted by DTT, suggesting that GSNO can induce the oxidation of Cx46 in Cys groups. These modifications were not observed in a Cx46 without intracellular Cys (Cx46C3A), thus suggesting that some of the two intracellular Cys are S-nitrosylated. Additionally, it was observed that GSNO induces a slight (but statistically significant) decrease in the hemichannel permeability to large molecules (i.e., Lucifer yellow) (Retamal et al., 2009). To date, it has not been possible to elucidate which is/are the Cys involved in this phenomenon. However, these data seem to be relevant to understand the role of free radicals as initiators and/or enhancers of lens opacity (disease commonly known as cataracts) (Berthoud and Beyer, 2009; Retamal et al., 2011).

A recent study has shown that Cx32, Cx37, and Cx40 are also affected by NO (Figueroa et al., 2013). In this work, it was determined that NO induced the opening of Cx37 and Cx40, whereas it induced the closure of Cx32. In all cases (including Cx43), hemichannels were permeable to NO (Figueroa et al., 2013). This work suggests that hemichannels formed by Cx32, 37, 40, and 43 could be good pathways for the diffusion of NO between endothelial cells and smooth muscle cells.

Finally, hemichannel opening has been observed in other models of oxidative stress, such as cadmium-induced

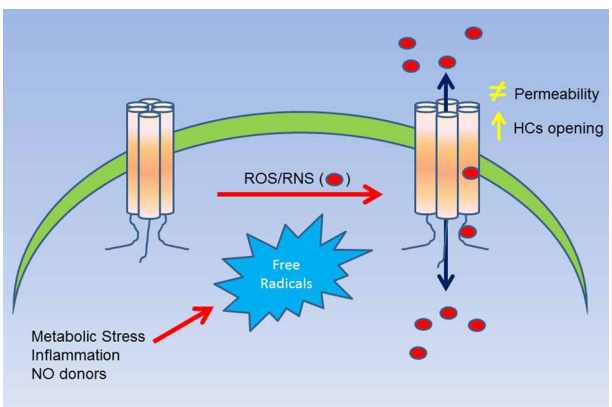

FIGURE 1 | Summary of the effect of ROS/RNS over Cxs hemichannels. A cellular stress (i.e., metabolic inhibition) induces an increase of ROS/RNS production, which in turn, can affect directly Cx hemichannels (as observed in Cys271 of Cx43). This molecular modification can induce and increase in the open probability of hemichannels and/or changes in their permeability to large molecules. Additionally, the posttranslational modification induced by ROS/RNS can also lead to an increase of the permeability of nitro oxide and possibly of other free radicals as well.

Table 1 | List of all Cxs and Panx hemichannels that are known to be affected by changes of cellular redox potential.

Connexin/ Pannexin	Molecule/ Condition	Experimental model	Amino acid modified	Effect	Ref
Cx32	NO donor	Fleta		Decrease hemichannel opening	Figueroa et al., 2013
Cx37	NO donor	Hela		Increase hemichannel opening	Figueroa et al., 2013
Cx40	NO donor	Hela		Increase hemichannel opening	Figueroa et al., 2013
Cx43	Metabolic Inhibition! NO donor	Astrocytes		Increase hemichannel opening	Contreras et al., 2002
	NO donor	Astrocytes		Increase hemichannel opening	Retamal et al., 2006
	DTT	HeLa		Increase hemichannel opening	Retamal et al., 2007b
	Inflammation like condition	Astrocytes		Increase hemichannel opening	Retamal et al., 2007b
	NO donor	Endothelial Cells	C271 -S-nitrosylation	Decrease IP3 permeability of GJCs	Straub et al., 2011
	smoking-induced cell Injury	N2A		Increase hemichannel opening	Ramachandran et al., 2007
	Cadmium-induced oxidative str.e.	Fibroblast		Increase hemichannel opening	Fang et al., 2011
Cx46	NO donor	Xenopus Oocyte		Modify Voltaje sensitivity and opening-closing kinetics	Retamal et al., 2009
Panxl	Ischemic-like conditions	Neurons		Increase hemichannel opening	Thompson et al., 2006
	Ischemic-like conditions	Neurons		Increase hemichannel opening	Zhang et al., 2008
	TCEP	N2A		Decrease hemichannel opening	Bunse et al., 2009
	NO donor	HEK293T	Cys 40 and 346—S-nuosyla:lon	Decrease hemichannel opening	Lohman et al., 2012

This table specifies the model in which the experiments were performed, the observed effects on hemichannel activity and which Cys group was modified.

oxidative stress (Fang et al., 2011) and smoking-induced cell injury (Ramachandran et al., 2007). In the work of ramachandran, they observed that CSE (cigarette smoke extract) and h_{2o2} were able to cross the plasma membrane through the open hemichannels. This is consistent with the work of Figueroa et al. (2013), suggesting that hemichannels are not only affected by free radicals but also are permeable to them (**Figure 1**). Interestingly, in 2012 a mutant of Cx31 (Cx31R42P) was reported to produce hemichannels with a gain in activity, which induced cell death (Chi et al., 2012). This cell death was prevented by hemichannel blockers, increasing the extracellular concentration of Ca^{2+}, and by the ROS scavenger butylated hydroxyanisole (BHA) (Chi et al., 2012). In this work, authors suggested that this mutation somehow would induce an increase in the ROS production by the cells. Moreover, as Cx31 has several cys groups in their c-terminus, it is possible that these ROS are oxidizing Cx31 hemichannels, which in turn would induce the hemichannel opening, similarly to previous observation made in Cx43 hemichannels expressing cells (Contreras et al., 2002; Retamal et al., 2006).

To date, no direct evidence is available to support the possibility that gsgg, h_{2o2}, carbon monoxide (co), hydrogen sulphide (h_{2s}) or any other oxidant molecules may affect the properties of cx hemichannels. Therefore, it is absolutely essential to investigate the effect of these molecules in hemichannels formed by cxs *in vitro* and *in vivo* in order to better understand the role of ROS in hemichannel function.

PANNEXIN'S HEMICHANNELS AND THEIR CONTROL BY REDOX SIGNALING MOLECULES

Panx1 is expressed in several types of cells (Bruzzone et al., 2003). As observed for Cx43 hemichanels in astrocytes, neurons under ischemic-like conditions open their Panx1 hemichannels (Thompson et al., 2006), phenomenon that was inhibited by DTT and L-NAME, and suggesting that NO is involved in neuronal Panx1 hemichannel opening (Zhang et al., 2008). In the same line of evidence, Panx1 hemichannels are closed by reducing agents (Bunse et al., 2009). Accordingly, mutation of Cys 40 and 346 prevent the GSNO induced S-nitrosylation of this protein and also prevent Panx1 hemichannels opening (Lohman et al., 2012). The substitution of Cys40 or Cys346 by Serine induced the appearance of hemichannels constitutively open (Bunse et al., 2009, 2010). In contrast, mutation of Zebra fish Cys282 to tryptophan (C282W)

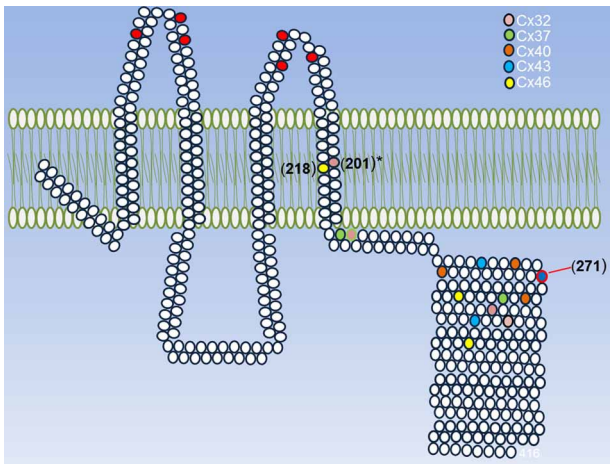

FIGURE 2 | Summary of Cys that can be modified by ROS/RNS. A representative Cx is shown and its conserved extracellular Cys is highlighted (red circles) and each cysteine present in Cxs 32, 37, 40, 43, and 46 are shown in different colors. These Cxs were chosen because they are sensitive to redox potential. The exact position for each Cys was taken according to their last aa in the TM4. The asterisk in Cys201 of Cx32 indicates that their mutations induce the appearance of a disease and the Cys 271 of Cx43 is the only one that has proved to be S-nitrosylated.

reduced the hemichannel activity (Prochnow et al., 2009). Above results suggest that these Cys groups are probably the redox potential sensor in this protein, and they can vary depending on the species. Interestingly, it has been recently proposed that Panx1 hemichannels can help NO to cross the plasma membrane (Campanucci et al., 2012), phenomenon recently shown for Cx- hemichannels (Figueroa et al., 2013). Taking into account all the evidence, it is possible to suggest that under physiological conditions, intracellular reduced glutathione (GSH) keeps Panx1 hemichannel closed, but when the free radical concentration increases, due to (for example) metabolic stress, Panx1 hemichannels become open. In summary, Panx1 is affected by ROS, but the exact molecular mechanism is not completely understood yet. At present, there are no data showing the effects of ROS upon the activity/properties of hemichannels formed by Panx2 and Panx3.

REDOX SIGNALING MEDIATES HEMICHANNELS FUNCTION IN PATHOLOGICAL CONDITIONS

The role of Cxs and Panx hemichannels on pathological conditions has been extensively reviewed (Bennett et al., 2012; Orellana et al., 2013). Therefore, this section will be focused on the role of redox signaling as intermediary of cellular response mediated by Cxs and Panx hemichannels in unhealthy cells. It is well accepted that under pathological conditions there is an increase in ROS/RNS production, which can affect different proteins through posttranslational modifications (Kolluru et al., 2013; Nakamura et al., 2013), thus, affecting diverse cellular functions (Gaston et al., 2003). In this context, an enhanced production of for example NO will lead to an increased hemichannel opening, which in turn will have several consequences in the cellular function as discussed before. Now, it is important to mention that the net effect of NO on hemichannels is going to depend on the level of NO produced. Thus, moderated levels of NO will induce a moderate hemichannel opening, which will have a profound effect in the autocrine/paracrine communication. This is because it is known that Cx43 hemichannel opening allows the release of signaling molecules to the extracellular space such as ATP (Stout et al., 2002), which is a well-recognized molecule involved in inflammatory processes (Eltzschig et al., 2012), mainly through P2X7 activation (Arulkumaran et al., 2011). Thus, hemichannel opening induced by NO could be a key point in several inflammatory processes. On the other hand, a large NO production may induce a massive hemichannel opening that is a signal that enhances and/or accelerates cell death (Retamal et al., 2006; Sáez et al., 2010). Therefore, depending on the NO production, the hemichannel activity may lead to a wide range of responses, from tissue inflammation to massive cell death. **Table 1** summarizes the effect of ROS/RNS over Cx and Panx hemichannels.

Another important issue about redox control of hemichannel activity in physiological and pathological conditions is that at least Cxs that seem to be sensitive to NO. As connexins, ryanodine receptors show several Cys groups and have been shown that they can be differentially modify by NO (S-nitrosylation) or GSSG (S-gluthationylation) (Aracena-Parks et al., 2006). These differences in Cys modifications depend on their microenvironment in which a given Cys is located (Lam et al., 2010). Similarly, it can be postulated that different Cys in different regions on a given Cxs or Panx, could be affected by NO or GSSG in a different way and that may induce different changes in the hemichannels properties. Therefore, it is very important to study the effect of other oxidant molecules, such as H_2S, CO, GSSG on hemichannel activity and test which are the Cys that are modified by these molecules. Also, it would be interesting to study if these modifications are present in proteins expressed in cells under pathological conditions. Following this line of evidence, our research group found that in rat's lenses that present cataract, Cx46 is S-nitrosylated, which suggests that at least this Cxs is modified by NO in pathological conditions, hence validating the data obtained *in vitro*. In **Figure 2**, it is observed all Cys groups present in Cxs sensitive to redox potential that are located in the TM4 and mostly in a region between 20 and 70 aa far from the beginning of the C-terminus. Although there are no conserved Cys, it is possible to postulate a zone sensitive to ROS/RNS. Actually, a Cys271 of Cx43 (which is already S-nitrosylated) is found in this putative zone. Cys in TM4 could be important redox sensors because rCx46 truncated C-terminus (Cx46ΔCT, which lacks Cys group in their C-terminus) and hCx46WT (which has only a Cys in TM4) are sensitive to nitric oxide (personal observation). Cys in a similar position—Cys201 (Morlé et al., 2000) and their homologous in Cx26—Cys202 (Sillén et al., 1998) -when mutated- induce the appearance of X-linked Charcot-Marie-Tooth disease and deafness, respectively. It is important to emphasize that there are Cxs with differences between their expression of Cys in their C-terminus (**Table 2**, Cx40 and Cx46), but there are others Cxs presenting important homologies (**Table 2**, Cx32 and Cx37) (**Table 2**).

NO is a highly reactive gas that participates in several physiological and pathological processes (Wang et al., 2010; Anand and Stamler, 2012; Nakamura et al., 2013). Recently, this

Table 2 | Alignment of C-terminus of human, rat and mouse Cxs and Panx modified by nitric oxide.

Cx32
H-evvyliiracarraqrrsnppsrkgsgfghrlspeykqneinkllseqdgslkdilrrspgtgaglaeksdrcsac
R-evvyliiracarraqrrsnppsrkgsgfghrlspeykqneinkllseqdgslkdilrrspgtgaglaeksdrcsac
M-evvyliiracarraqrrsnppsrkgsgfghrlspeykqneinkllseqdgslkdilrrspgtgaglaeksdrcsac

Cx37
H-vhllcrclsrgmrarqgqdapptqgtssdpytdqvffylpvgqgpssppcptynglssseqnwanltteerlassrpplfldpppqngqkppsrpsssaskkqyv
R-vhllcrcvsreikarrdhdtrpaqgsasdpypeqvffylpmgegpssppcptynglssteqnwanlteerltstrpppfvnaapqgggqkssrpnssaskkqyv
M-vhllcrcvsreikarrdhdarpaqgsasdpypeqvffylpmgegpssppcptynglssteqnwanlteerltssrpppfvntapqggrkspsrpnssaskkqyv

Cx40
H-yhlgwkkirqrfvkprqhmakcqlsgpsvgivqsctpppdfnqclengpggkffnpfsnnmasqqntdnlvteqvrgqeqtpgegfiqvrygqkpevpngvspghrlphgyhsdkrrlskasskarsddlsc
R-yhlgwkkirqrlaksrqg-dkhqllgpstslvqgltpppdfnqclknspdekffsdfsnnmgsrknpdplateevpnqeqipeegfihtqyggqkpeqpsgasaghrfpqgyhsdkrrlskasskarsddlsc
M-yhlgwkkirqrfgksrqgvdkhqlpgpptslvqsltpppdfnqclknssgekffsdfsnnmgsrknpdalatgevpnqeqipgegfihmhysqkpeyasgasaghrlpqgyhsdkrrlskasskarsddlsv

Cx43
H-elfyvffkgvkdrvkgksdpyhatsgalspakdcgsqkyayfngcssptaplspmsppgyklvtgdrnnsscrnynkqaseqnwanysaeqnrmgqagstisnshaqpfdfpddnqnskklaaghelqplaiv
R-elfyvffkgvkdrvkgrsdpyhattgplspskdcgspkyayfngcssptaplspmsppgyklvtgdrnnsscrnynkqaseqnwanysaeqnrmgqagstisnshaqpfdfpddnqnakkvaaghelqplaiv
M-elfyvffkgvkdrvkgrsdpyhattgplspskdcgspkyayfngcssptaplspmsppgyklvtgdrnnsscrnynkqaseqnwanysaeqnrmgqagstisnshaqpfdfpddsqnakkvaaghelqplaiv

dqrpssrassrassrprpddlei
dqrpssrassrassrprpddlei
dqrpssrassrassrprpddlei

Cx46
H-eiyhlgwkklkqgvtsrlgpdaseaplgtadppplppssrppavaigfppyyahtaaplgqaravgypgapppaadfkllalteargkgqsaklynghhhllmteqnwanqaaerqppalkaypaastpaaps
R-eiyhlgwkklkqgvtnhfnpdasevrhkpldplseaansgppsvsiglppyythpacptvqgkatgfpgapllpadftvvtlndaqgrghpvkhcnghh--ltteqnwaslgaepqtpaskpssaassp----
M--iyhlgwkklkqgvtnhfnpdasearhkpldplptatssgppsvsigfppyythpacptvqakaigfpgaplspadftvvtlndaqgrnhpvkhcnghh--ltteqnwtrqvaeqqtpaskpssaassp----

pvgssspplaheaeagaaplllldgsgsslegsalagtpeeeeqavttaaqmhqpplplgdpgraskasrassgrarpedlai
----------hgrkg-----ltdssgssleesalvvtp-egeqalattvemhspplvlldperssk---sssgrarpgdlai
----------dgrkg----lidssgsslqesalvvtpeegeqalattvemhspplvlldpgrssk---ssngrarpgdlai

Panx1
H-tlfvpfrqktdvlkvyeilptfdvlhfksegyndlslynlfleenisevksykclkvleniksgqgidpmllltnlgmikmdvvdgktpmsaemr-eeqgnqtaelqgmnidsetkanngeknarqrllds
R-tlfvpfrqktdvlkvyeilptfdvlhfksegyndlslynlfleeniselksykclkvleniksnqgidpmllltnlgmikmdvidgkvpmslqtkgedqgsqrmdfkdldlssetaanngeknsrqrllns
M-tffipfrqktdilkvyeilptfdvlhfksegyndlslynlfleeniselksykclkvleniksnqgidpmllltnlgmikmdiidgkiptslqtkgedqgsqrvefkdldlsseaaanngeknsrqrllnp

SC
SC

Cys in each C-terminus protein is shown in yellow. Note that all Cys are conserved in Cx32 in spite of large differences in Cx46. Human Cx46 has no Cys in C-terminus, but a Cys is present in position 218, in transmembrane 4. It has been reported that Cys located in the membrane can be S-nitrosylated (Leclerc et al., 2006). Therefore, the possibility that hCx46 can be redox sensitive cannot be ruled out.

gaseous transmitter has been shown to be permeable through hemichannels formed by Cxs (Figueroa et al., 2013). Considering that Cxs and Panx hemichannels are modulated by NO (Retamal et al., 2006, 2009; Lohman et al., 2012), it is possible to suggest that under pathological conditions hemichannels are playing an amplifier role of signaling pathways activated by NO, and –additionally- allowing NO to diffuse easily and widely in a tissue. On the other hand, it is known that there is a good correlation between intracellular Ca^{2+} levels and NO production. Recently, it has been demonstrated that Cx26 and Cx43 hemichannels are permeable to this cationic divalent ion (Sánchez et al., 2010; Schalper et al., 2010; Fiori et al., 2012). Thus, under pathological conditions where hemichannels increase their open probability, hemichannels can induce intracellular Ca^{2+}increase, which in turn can activate intracellular pathways, such as caspases (Ishiura, 1981) and NO production (Schmidt et al., 1992). In this context, it has been recently shown that hemichannels participate in the NO production in injured endothelial cells of aorta (Berra-Romani et al., 2013). Therefore, several cellular responses induced by redox molecules (oxidant or reducing ones), such as; increased intercellular communication mediated by ATP and activation of Ca^{2+} dependent intracellular signaling pathways, can be mediated, at least in part, by inducing hemichannel opening. Finally, since the intracellular Ca^{2+} concentration is a good indicator of cellular health (Rasmussen et al., 1990), hemichannel opening under pathological conditions can set not only the cell response to an injury, but also determine the final destination of a cell.

CONCLUSIONS AND PERSPECTIVES

For many years, much research has been performed to elucidate the molecular mechanism involved in the control of opening and closing of Cx and Panx- hemichannels, while the exact molecular mechanism has not yet been resolved. The most studied are probably: changes of membrane potential, phosphorylation, pH and extracellular divalent cations. Recently, another control mechanism has been proposed and it seems to affect several different types of Cxs and- at least- Panx1 hemichannel function. This new control mechanism is linked to changes of redox potential. Presently, the effect of NO upon Cx- and Panx hemichannels has been the most studied. However, there are other important free radicals that act in both pathological and physiological conditions. I would like to point out that carbon monoxide (CO) and hydrogen sulphide (H_2S) are emerging as important gaseous transmitters with relevant roles in cell physiology (Wilkinson and Kemp, 2011; Ju et al., 2013). Thus, future lines of research will be probably focused on: (1) the effect of CO and H_2S upon Cxs and Panx- hemichannels and GJCs; (2) Which is or are—at molecular level- the redox sensor(s) in different Cxs and Panxs, (3) Which are (if any) the hypothetical interactions between the redox sensor and the well-recognized slow and fast gating of Cxs hemichannels, and (4)

which are the modifications on the structure of the Cxs in terms of folding, protein-protein bindings and intramolecular interaction.

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Connexin hemichannels in the lens

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The normal function and survival of cells in the avascular lens is facilitated by intercellular communication through an extensive network of gap junctions formed predominantly by three connexins (Cx43, Cx46, and Cx50). In expression systems, these connexins can all induce hemichannel currents, but other lens proteins (e.g., pannexin1) can also induce similar currents. Hemichannel currents have been detected in isolated lens fiber cells. These hemichannels may make significant contributions to normal lens physiology and pathophysiology. Studies of some connexin mutants linked to congenital cataracts have implicated hemichannels with aberrant voltage-dependent gating or modulation by divalent cations in disease pathogenesis. Hemichannels may also contribute to age- and disease-related cataracts.

Keywords: connexin46, connexin50, cataract, lens, gap junction

THE LENS AND CATARACTS

The lens is a transparent organ whose main function is to transmit light and focus it on the retina. It sits suspended between two clear fluids (the aqueous humor and the vitreous) and has no direct blood supply. The lens is comprised of two cell types: epithelial cells that form a single layer along the anterior surface and fiber cells that form the bulk of the organ (**Figure 1**). At the lens equator, epithelial cells differentiate into fiber cells, a process that involves cell elongation, loss of nuclei and organelles, and synthesis of very high concentrations of small soluble proteins called crystallins. These proteins act as chaperones and increase the refractive index of the lens without interfering with its transparency. This differentiation process, which occurs throughout the lifespan of the organism, leads to generation of two cell types which differ in their metabolic capacities: the surface epithelial cells, which are nucleated and contain most of the metabolic, synthetic, and active transport machinery of the lens and mature fiber cells which have limited metabolic activities, are non-dividing, and must survive for the lifespan of the organism (Mathias and Rae, 2004).

A cataract is an opacity or cloudiness in the lens that may cause a decrease in vision and could eventually lead to blindness. The specific biochemical and structural changes associated with cataract formation are diverse, but a common biochemical change is the generation of high molecular weight insoluble protein aggregates (Moreau and King, 2012).

Because the lens does not have a direct blood supply, the nutrients for the organ all derive from the fluids in which it is suspended. Specifically, the aqueous humor (which is dynamically produced from the plasma) provides the main source for inorganic and organic ions, carbohydrates, glutathione, amino acids, and oxygen. The aqueous humor is also the repository for metabolites and carbon dioxide produced by lens cells. Ions and nutrients reach cells in the interior through an internal "circulation" in which flow of ions and water drives the movement of solutes throughout the organ. A model of this circulation has been developed based on surface currents recorded from lenses (Robinson and Patterson, 1982; Parmelee, 1986; Mathias et al., 1997) and measurements of hydrostatic pressures at different depths within the lens (Gao et al., 2011). In this model, current carried by ions (and associated water and solutes) enters the lens along the extracellular spaces at the anterior and posterior poles, it crosses fiber cell membranes in the lens interior, and it flows back to the surface at the equator (via a cell-to-cell pathway) (Mathias et al., 2007, 2010). The hydrostatic pressure gradient also drives water flow toward the exterior (Gao et al., 2011). The lens circulatory system provides a pathway for internal fiber cells to obtain essential nutrients, remove potentially toxic metabolites, and maintain resting potentials (Goodenough, 1979; Piatigorsky, 1980).

LENS GAP JUNCTIONS AND CONNEXINS

Intercellular communication among the cells of the lens is facilitated by an extensive network of gap junctions. Gap junctions are membrane specializations that contain clusters of intercellular channels that are permeable to ions and small solutes ($\leq$1 kDa). Epithelial and fiber cells contain morphologically and physiologically distinct gap junctions (Rae and Kuszak, 1983; Miller and Goodenough, 1986). Epithelial cells are functionally coupled through gap junction channels. Lens fiber cells also share ions and small metabolites through gap junction channels, and consequently behave as a functional syncytium (Goodenough et al., 1980; Mathias and Rae, 1989). The extent of epithelial-to-fiber cell coupling is somewhat controversial.

Gap junction channels are oligomeric assemblies of members of a family of related proteins called connexins (Cx) (Beyer and Berthoud, 2009). Six connexins oligomerize to form a connexon (hemichannel), and two connexons dock to form a complete (dodecameric) intercellular channel. Channels formed by diverse connexins differ in physiological properties including unitary conductance, permeability, gating, and regulation by different protein kinase-dependent pathways (reviewed in Harris, 2001, 2007; Sáez et al., 2003). Thus, the regulation of intercellular communication and the permeation of different molecules in different

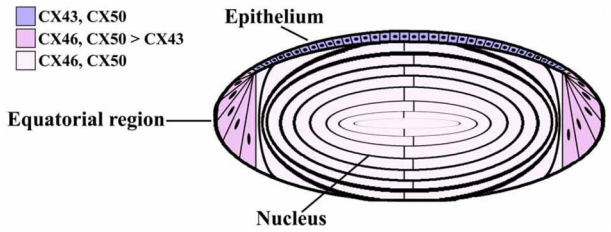

FIGURE 1 | Diagram of the lens showing the distribution of connexin isoforms. Cells from the anterior epithelial cell layer express Cx43 and Cx50, differentiating fiber cells express Cx43, Cx46, and Cx50, and fiber cells contain Cx46 and Cx50.

regions of the lens are determined by the repertoire of connexins expressed.

Three connexins have been identified in the lens with somewhat overlapping expression patterns (**Figure 1**). Cx43 is expressed in lens epithelial cells (Musil et al., 1990), but its expression is turned off as epithelial cells in the equatorial region differentiate into fiber cells. Cx50 is also expressed in epithelial cells (TenBroek et al., 1994; Dahm et al., 1999; Rong et al., 2002). Cx46 and Cx50 become abundantly expressed in the differentiating cells and are the two most abundant connexins in lens fiber cells (Paul et al., 1991; White et al., 1992). These two connexins co-localize at gap junction plaques and can form mixed hexamers (Paul et al., 1991; Jiang and Goodenough, 1996). Transcripts for a fourth connexin, Cx23, have been detected in the zebrafish embryo lens (Iovine et al., 2008) and in the mouse lens (Puk et al., 2008). This connexin has been implicated in fiber cell differentiation, because fiber cells do not elongate properly in mice expressing a missense mutation of Cx23 (Puk et al., 2008). While lens cells from other mammalian species may also express Cx23, Cx23 transcript was not detected in RNA isolated from human lenses (Sonntag et al., 2009). Moreover, while Cx23 protein has been detected in proteomic studies of mouse lens membrane proteins (Bassnett et al., 2009), it was not detected in human samples (Wang et al., 2013). Even in the mouse, the cellular distribution of the Cx23 protein is unknown, because there are no good antibodies for its detection. Therefore, Cx23 is not included in **Figure 1**.

STUDIES OF Cx46- OR Cx50-NULL MICE IMPLICATE CONNEXINS IN CATARACT FORMATION

The importance of gap junction-mediated lens intercellular communication for the maintenance of lens transparency has been substantiated by a number of genetic studies in mice. Targeted deletion of either Cx46 or Cx50 results in the development of cataracts in homozygous (but not heterozygous) null mice (Gong et al., 1997; White et al., 1998). The Cx50-null mice have a milder cataract than the Cx46-null mice (Gerido et al., 2003). Cx50-null mice have microphthalmia and small lenses, while eye and lens sizes are similar in Cx46-null and wild type mice (Gong et al., 1997; White et al., 1998; Rong et al., 2002). Double knock-out mice lacking both Cx46 and Cx50 have small lenses with dense opacities that are far more extensive than those observed in either Cx46 or Cx50 single null mice (Xia et al., 2006). Cx43 may not have a critical importance in normal lens function, because the lenses of animals with a conditional deletion of Cx43 are transparent and develop normally through at least 6 months of age, even though intercellular transfer of neurobiotin and Lucifer yellow among epithelial cells is decreased (DeRosa et al., 2009).

LENS CONNEXIN MUTATIONS ARE LINKED TO CONGENITAL CATARACTS

Mutations in lens connexins have been linked to disease in people and rodents. Missense and frame-shift mutations of the genes encoding Cx46 and Cx50 (*GJA3* and *GJA8*) have been identified in members of human families with inherited cataracts of various different phenotypes. Nearly all of the cataracts are inherited as autosomal dominant traits. These mutants and their associated cataract phenotypes have been reviewed recently (Beyer et al., 2013). In several mutant mouse strains, the cataract trait has also been mapped to mutations of Cx46 and Cx50.

The functional and cellular abnormalities associated with cataract-linked connexin mutants have been thoroughly studied for some of the mutants. The most frequently observed phenotype is induction of no or insignificant levels of intercellular conductance and formation of no or very few gap junction plaques. Examples include Cx50R23T, Cx50D47N, Cx50P88S, Cx50P88Q, and Cx46fs380 (Berthoud et al., 2003; Minogue et al., 2005; Arora et al., 2006, 2008; Thomas et al., 2008). All of the mutants with this general phenotype (loss of function and a severe reduction in the number or complete absence of gap junctions) should reduce intercellular communication between lens fiber cells, regardless of the differences in the mechanisms for their trafficking impairment, retention, or accumulation.

Other mutants (e.g., Cx50W45S, Cx46D3Y, and Cx46L11S) make abundant gap junction plaques, but have no gap junction channel activity when expressed by themselves implying that they form non-functional channels (Tong et al., 2011, 2013). They have an open probability of zero (or no unitary conductance).

A connexin mutant may also contribute to cataractogenesis through interactions with the co-expressed wild type connexins. In the lens, expression of a mutant connexin may affect the abundance/stability of the wild type connexins. For instance, in both heterozygous and homozygous lenses of *No2* mice, expression of Cx50D47A leads to severe reductions in the levels of both Cx50 and Cx46 (likely by increasing degradation of the wild type and mutant connexins) (Berthoud et al., 2013). Intercellular communication between fiber cells of *No2* mouse lenses is likely severely reduced. A mutant connexin may also affect the function of the wild type connexins. Exogenous expression experiments show that some mutants (e.g., Cx50P88S, Cx50P88Q, Cx50W45S, Cx50E48K, Cx46D3Y, Cx46L11S) decrease the junctional conductance supported by their wild type counterparts, likely through co-oligomerization (Pal et al., 1999; Arora et al., 2006; Banks et al., 2009; Tong et al., 2011, 2013).

Several different mechanisms have been invoked to explain how changes in intercellular communication lead to cataract formation. Reductions of intercellular communication should decrease the circulation of gap junction permeant molecules (including water, ions, and metabolites) between lens cells.

Consistent with that prediction, Cx46- and Cx50-null mice have reduced coupling conductances between lens fibers (reviewed in Mathias et al., 2010) and a decrease in the gradient of intracellular hydrostatic pressure that normally runs from the center to the periphery of the lens (Gao et al., 2011). Data from Cx46-null mice suggest that impairment of lens intercellular communication leads to increased levels of intracellular calcium within the lens which contribute to cataract formation by stimulating calpain-dependent proteolysis of crystallins (Baruch et al., 2001; Gao et al., 2004).

In the lens, mutant connexins may also contribute to cataracts by altering the trafficking or function of other (non-connexin) lens fiber cell proteins. The mutant connexins may also interfere with lens development and differentiation and, consequently, alter the expression of these other lens proteins.

CONNEXIN HEMICHANNELS

In addition to forming intercellular channels, connexins can form functional hemichannels that induce large conductances in single plasma membranes. These conductances are caused by permeation of ions through "undocked" single connexons. This phenomenon has been best demonstrated in expression systems and in cultures of various cells that endogenously express connexins.

Cx46 was the first connexin demonstrated to form hemichannels in *Xenopus* oocytes and has been one of the most studied. After cloning rat Cx46 cDNA, Paul et al. (1991) injected the *in vitro* transcribed cRNA into oocytes, and they were surprised to observe depolarization and osmotic lysis. Voltage-clamp experiments revealed that Cx46 cRNA-injected oocytes developed a large, non-selective cation current that was activated on depolarization and was inhibited by external divalent cations (Ebihara and Steiner, 1993). Subsequent studies showed that while Cx46 hemichannels allow permeation of both cations and anions, they are more permeable to cations (Trexler et al., 1996). Examples of the hemichannel currents induced by expression of the bovine Cx46 ortholog (Cx44) are shown in **Figure 2A**. By recording both non-junctional and junctional currents from the time of pairing, Gupta et al. (1994) showed that hemichannels could be recruited into intercellular channels (reproduced in **Figure 2B**). Cx46 hemichannels are gated closed by low intracellular pH; this closure is voltage dependent (Trexler et al., 1999). The single channel conductance of Cx46 hemichannels exhibits significant rectification; it is 300 pS at −50 mV, but only 135 pS at +50 mV (Trexler et al., 1996).

Like Cx46, all of the other lens connexins (including Cx43, Cx50, and Cx23) can form functional hemichannels (Beahm and Hall, 2002; Contreras et al., 2003; Srinivas et al., 2005; Sonntag et al., 2009). However, detection of comparable levels of hemichannel current may require injection of larger amounts of RNA for Cx43 and Cx50 than for Cx46 (Tong and Ebihara, 2006). The hemichannels formed by each of these connexins are regulated by voltage and extracellular divalent cations; hyperpolarization and elevated divalent cation concentrations promote closure. Synergistic action of these two mechanisms may prevent the opening of hemichannels under physiological conditions. Among the lens connexins, only Cx46 has a weak enough Ca^{2+}-sensitivity that it may exhibit significant opening at physiological concentrations of extracellular Ca^{2+}. Quantification of the differences in divalent cation sensitivity has shown that hemichannel currents induced by chicken Cx45.6 (the ortholog of mammalian Cx50) can only be detected when the external calcium concentration is reduced to zero nominal concentration, whereas Cx46 hemichannel currents (and those of its chicken ortholog, Cx56) are detectable at much higher external calcium concentrations (0.7 mM Ca^{2+}) (Ebihara et al., 1995). It is not completely clear what determines the difference in Ca^{2+} sensitivity between these connexins, nor the mechanism by which Ca^{2+} promotes hemichannel closure. In the case of Cx46 hemichannels, Verselis and Srinivas (2008) have shown that calcium only closes Cx46 hemichannels in excised patches when added from the extracellular side. In the case of Cx50, Zhang et al. have proposed that Ca^{2+}regulates Cx50 hemichannels by influencing calmodulin binding to the cytoplasmic side (Zhang et al., 2006), similar to the Ca^{2+}/calmodulin regulation of other connexin intercellular channels (Peracchia et al., 2000; Zhou et al., 2009; Xu et al., 2012). Observations following application of a thiol reactive compound to rat Cx46 containing a cysteine substitution for leucine35 imply that Ca^{2+} gates the channel closed at a position that is extracellular to leucine35 (Pfahnl and Dahl, 1999).

Expression studies have shown that the lens connexins form hemichannels that also differ in some other properties. Their hemichannel currents exhibit differences in activation and deactivation kinetics (Ebihara et al., 1995). They have unique single channel conductances. They differ in their permeabilities to some small molecules.

When expressed in HeLa cells or in *Xenopus* oocytes, Cx50 forms high conductance (352 or 470 pS) single hemichannels (Valiunas and Weingart, 2000; Srinivas et al., 2005). Hemichannels formed of Cx50 are sensitive to extracellular monovalent cations. Replacement of extracellular Na^+ with K^+ may reduce the ability of Ca^{2+} (or other divalent cations) to close Cx50 hemichannels (Srinivas et al., 2006).

Cx43 hemichannels have largely been described and studied in non-lens cell types such as astrocytes. They have unitary conductances of ∼220 pS (about twice the conductance of a single Cx43 intercellular channel) (Contreras et al., 2003). In addition to opening provoked by low concentrations of extracellular divalent cations, Cx43 hemichannels open in response to various cellular insults like metabolic inhibition, ischemia, and lowering of the intracellular redox potential (Contreras et al., 2002, 2004; Retamal et al., 2006, 2007). Some of these opening events are associated with S-nitrosylation of Cx43 (Retamal et al., 2006). Cx43 hemichannels are permeable to a variety of common dye tracers (like Lucifer yellow, ethidium, and propidium) and can allow the release of cytoplasmic small molecules (including ATP and glutamate) (Orellana et al., 2011). Cx23 is a unique member of the connexin family. Unlike other connexins, it only contains four (instead of six) cysteines in its extracellular loops. When studied in stably transfected HeLa cells, mouse Cx23 supported release of ATP (even in calcium containing medium), but did not induce detectable intercellular currents nor it allow passage of microinjected tracers (Sonntag et al., 2009). These observations suggested that mouse Cx23 formed hemichannels, but not

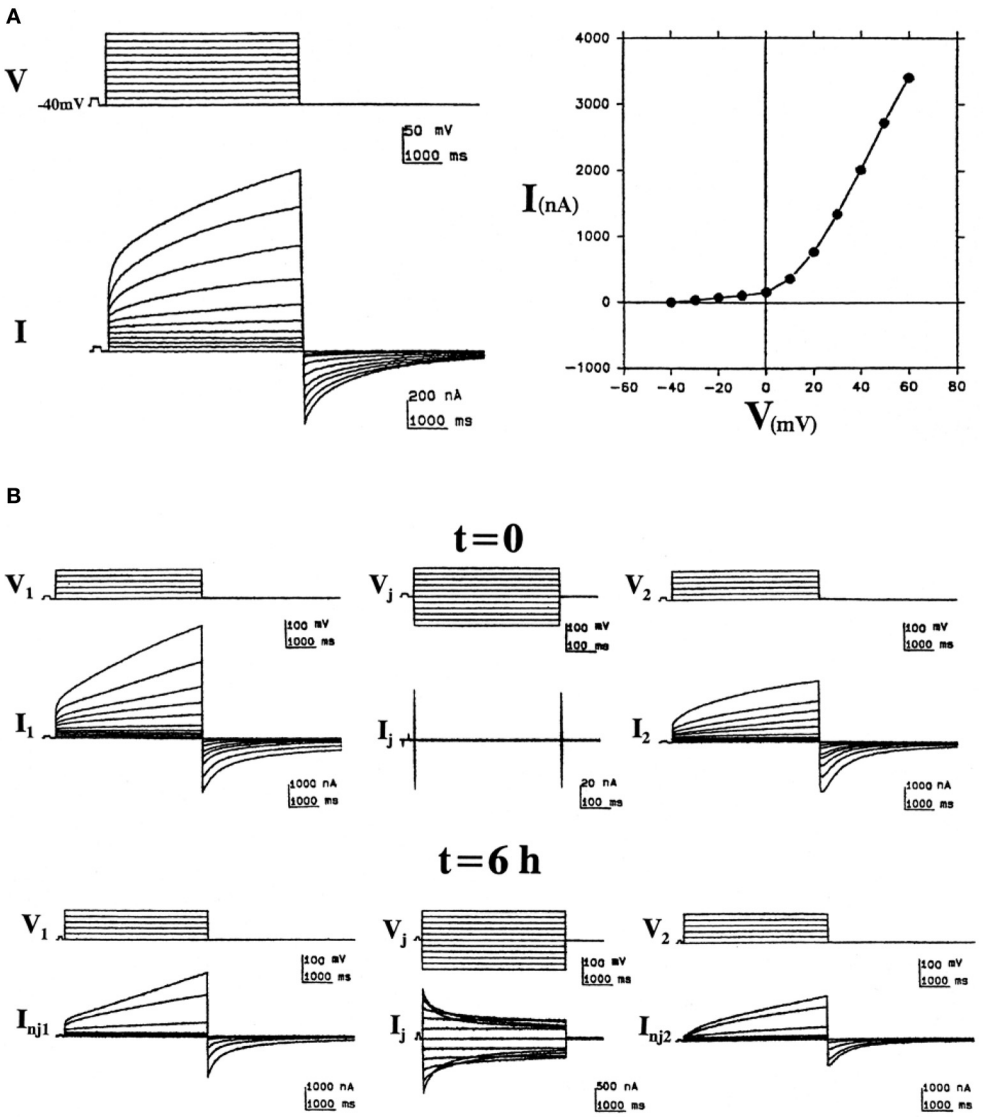

FIGURE 2 | "Hemichannel" currents induced by expression of the bovine Cx46 ortholog (Cx44) in *Xenopus* oocytes. (A) Bovine Cx44-induced non-junctional currents in isolated oocytes. Left: Sample recordings illustrate the induction of an outward time- and voltage-dependent current (*I*) activated by depolarizing voltage pulses (*V*) of 5 s from a holding potential of −40 mV to +60 mV in increments of 10 mV. At pulse-off, the currents deactivated returning to baseline. Right: The corresponding *I*/*V* curve, plotting the current values at the end of the pulses vs. voltage. **(B)** Formation of junctional channels from bovine Cx44 hemichannels. Left and right panels show non-junctional currents of bovine Cx44-injected oocytes (1 and 2) at the time of pairing ($t = 0$) and 6 h after pairing ($t = 6$ h). The middle panels show the junctional currents at these times (reproduced from Gupta et al., 1994).

gap junction channels. In contrast, in another study, the Cx23 orthologs from zebrafish formed both gap junction channels and hemichannels (Iovine et al., 2008). Likely because its expression cannot be detected in primates (including humans) (Sonntag et al., 2009), there have been very few studies of Cx23 and its hemichannels.

There have been some studies of the pharmacology of hemichannels formed by the lens connexins. These hemichannels are generally inhibited by similar concentrations of the non-selective blockers that inhibit most gap junctional channels (including carbenoxolone, α-glycyrrhetinic acid, flufenamic acid, heptanol, and octanol). Cx46 hemichannels are modulated by PKC-dependent phosphorylation. Treatment with the PKC activators, phorbol-12-myristate-13-acetate (TPA) or 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), reduces the amplitude of hemichannel currents and leads to their inactivation after prolonged incubation, an effect that can be reverted by PKC inhibitors (Ngezahayo et al., 1998; Jedamzik et al., 2000). Treatment with a casein kinase II inhibitor (2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole) during the expression period also reduced the amplitude of the currents evoked by a 60 mV depolarizing pulse (Walter et al., 2008). Cx46 hemichannels are also sensitive to nitric oxide donors (e.g., *S*-nitrosoglutathione); Cx46 hemichannel currents show a faster activation rate, increased voltage sensitivity,

and increased tail currents with altered kinetics in single *Xenopus* oocytes incubated in the presence of *S*-nitrosoglutathione. These effects have been ascribed to nitrosylation of cytoplasmic cysteines, because they were not observed when these cysteines were mutated to alanines (Retamal et al., 2009). Unsaturated fatty acids can also modulate Cx46 hemichannel function. Linoleic acid has a biphasic effect on Cx46, increasing hemichannel currents at 0.1 μM and decreasing them at concentrations of 100 μM or higher (without affecting gap junction channels) (Retamal et al., 2011).

Like Cx50 gap junction channels, Cx50 hemichannels are inhibited by mefloquine and other quinine derivatives (like N-benzylquininium) (Cruikshank et al., 2004; Rubinos et al., 2012). Cx43 channels and hemichannels are also inhibited by this drug, but much higher concentrations are required. Cx46 is virtually insensitive.

In expression systems, some of the lens connexins can form functional heteromeric hemichannels with characteristics that differ from those of either connexin alone. Co-expression of chicken Cx56 (orthologous to mammalian Cx46) with Cx50 (or its chicken ortholog, Cx45.6) produced hemichannels with intermediate properties in several parameters including the threshold for activation, rate of deactivation, unitary conductance, steady state open probability, and mean open times at negative potentials (Ebihara et al., 1999).

Because Cx46 and Cx50 form hemichannels in expression systems, they have been extensively analyzed after mutagenesis in structure-function studies. Sequential replacement of individual amino acid residues with cysteines in Cx46 and determination of their accessibility to modifying reagents has implicated residues in the first transmembrane and first extracellular domains in formation of the channel pore (Zhou et al., 1997; Kronengold et al., 2003). Other studies have implicated the first extracellular domain of Cx46 in determining charge selectivity (Trexler et al., 2000). Reciprocal substitution experiments have implicated N-terminal amino acids as critical determinants of the differences in voltage gating properties between Cx46 and Cx50 (Tong and Ebihara, 2006). Truncations that remove portions of the C-terminus of Cx46 have implicated this domain in hemichannel function (Zeilinger et al., 2005; Walter et al., 2008). In some studies, these truncation mutants induce hemichannel currents of smaller magnitude than those of the full length wild type protein, which may result from decreased trafficking to/from the plasma membrane (Schlingmann et al., 2013).

CONNEXIN HEMICHANNELS IN THE LENS

Several lines of evidence demonstrate the presence of "hemichannel-like" activities in the lens. Their sensitivities to calcium and to some pharmacologic inhibitors suggest that they may be formed by connexins. When Rae et al. (1992) removed extracellular divalent cations from whole lenses, they observed a decrease in resting membrane potential and a large increase in input conductance, which they ascribed to activation of a stretch activated, non-selective cation channel. Eckert et al. (1998) identified a large, slowly-activating, non-selective current in lens fiber cells isolated under calcium-free conditions. Hyposmotic stress induced ATP release and uptake of propidium in intact lenses that were blocked by 18α-glycyrrhetinic acid and probenecid (Shahidullah et al., 2011, 2012).

Ebihara et al. (2010) further examined hemichannel currents in isolated lens fiber cells by whole cell patch clamping. Upon removal of divalent cations from the external solution, they detected large, non-selective currents that activated on depolarization. A single channel conductance of 241–243 pS was measured in a few cells. Uptake of propidium iodide and 4′-6-diamidino-2-phenylindole (DAPI) was observed in divalent cation-free medium, and the dye uptake was inhibited by gap junction blockers including Gd^{3+}, flufenamic acid, and octanol. The calcium-sensitive currents and dye uptake were detected in fiber cells isolated from Cx50-null mice, but they were absent in cells isolated from Cx46/Cx50 double-null mice. These data suggest that Cx46 hemichannels are responsible for the calcium-sensitive currents and dye uptake, and that Cx46 hemichannels may be functional in the lens *in vivo*. Opening of hemichannels in the lens may have significant physiological roles (**Figure 3A**). Because Cx46 hemichannels are mechanosensitive, it has been proposed that their openings allow rapid fluid equilibration during lens accommodation (Bao et al., 2004b). Ebihara et al. (2010) have suggested that connexin hemichannels may provide a normal pathway for influx of calcium and sodium in fiber cells. Although Cx46 hemichannels have a low probability of opening under normal conditions (i.e., at membrane resting potentials and 1 mM $[Ca^{2+}]_o$), they might be sufficient to account for the rather low sodium influx that occurs under physiological conditions in fiber cells.

OTHER LENS CHANNELS THAT RESEMBLE CONNEXIN HEMICHANNELS

There are also other proteins that form channels with properties similar to connexin hemichannels and may contribute to lens physiology and pathophysiology.

The pannexins are a family of proteins with three members in mouse and man (Panx1, Panx2, and Panx3). They were originally discovered based on their sequence similarity to the innexins which form gap junctions in invertebrates (Panchin et al., 2000). Although most expression studies indicate that pannexins do not form intercellular channels, there is agreement that they form large pore, non-selective transmembrane channels with some similarities and differences as compared with connexin hemichannels. The properties of pannexin channels, their roles in various different cell types and comparisons to connexin hemichannels have been extensively reviewed (MacVicar and Thompson, 2010; Sosinsky et al., 2011).

Among the pannexins, Panx1 has been most extensively studied. Panx1 channels can open in the presence of physiological extracellular calcium concentrations. They are mechanosensitive, and they can be activated by high extracellular K^+ (Bao et al., 2004a; Pelegrin and Surprenant, 2006; Silverman et al., 2009). Panx1 channels allow uptake of various dye tracers (Bao et al., 2004a; Pelegrin and Surprenant, 2006; Silverman et al., 2009) and release of ATP from erythrocytes, taste receptors, and other cells (Locovei et al., 2006; Huang et al., 2007). Panx1 associates

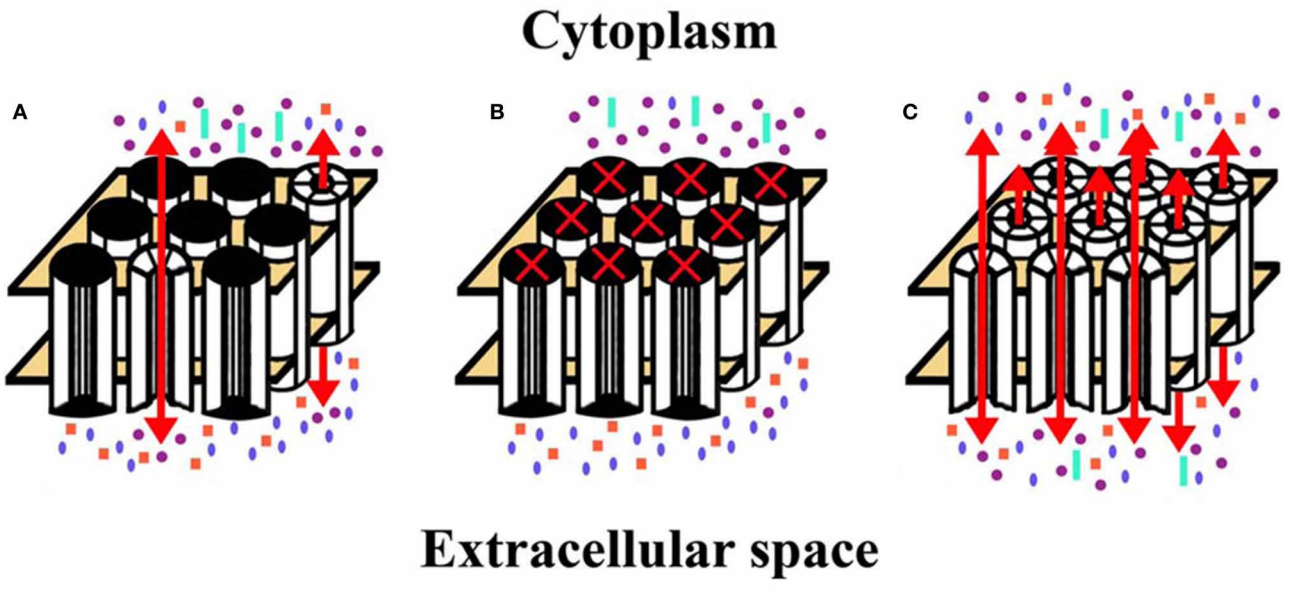

FIGURE 3 | Diagrams represent hemichannel function in the lens in normal physiology and in pathology. (A) Under normal conditions, few connexin hemichannels are open, permitting only a small flux of ions according to their concentration gradients. This would mostly include influx of Na^+ and Ca^{2+} and efflux of K^+. **(B)** Reduction (or complete blockade) of hemichannel opening would reduce the normal, physiologic transmembrane passage of permeant ions and solutes leading to alterations of the normal ionic concentrations across the plasma membrane. **(C)** If hemichannel opening was pathologically increased, ions and other small molecules would flow across the membrane according to their concentration gradients. Movement of electrolytes like Na^+ and K^+ would lead to loss of transmembrane potentials. Entry of Ca^{2+} might lead to opening of additional hemichannels, and activation of several signaling cascades and calcium-dependent proteases. A significant increase in the intracellular Ca^{2+} concentration may lead to cell death by different mechanisms (Orrenius et al., 2003). Increased hemichannel opening would also lead to loss of ATP, NAD^+, glutathione, and other permeant cytoplasmic small molecules. All of these changes would contribute to loss of homeostasis and cytotoxicity. Na^+, blue ellipses; K^+, purple circles; Ca^{2+}, orange squares; ATP (or NAD^+, glutathione, etc.), aquamarine rectangles.

with the $P2X_7$ purinergic receptor and contributes to formation of the inflammasome (Pelegrin and Surprenant, 2006; Silverman et al., 2009). Panx1 channels are blocked by some of the same agents as connexins (like carbenoxolone, flufenamic acid, and mefloquine) but also by some others (like probenecid) that do not block connexin channels (Iglesias et al., 2008; Ma et al., 2009).

There is rather limited information regarding pannexins in the lens. Both Panx1 and Panx2 mRNAs are expressed in the lens, and immunoreactive Panx1 is detected in lens epithelial and fiber cells (Dvoriantchikova et al., 2006). It has been hypothesized that Panx1 forms the probenecid-inhibitable channels that release ATP from lens epithelial cells after exposure to hyposmotic stress (Shahidullah et al., 2011, 2012). Gunning et al. (2012) have found that lens fiber cells contain a non-specific cation conductance that may be distinct from connexin hemichannels. It is stimulated by hypertonic stress or isosmotic cell shrinkage and may be involved in volume regulation. Members of the transient receptor potential (TRP) family of cation channels are also expressed in the lens, and activation of TRPV4 channels may participate in the osmotic stress-stimulated release of ATP from the lens (Shahidullah et al., 2011, 2012). However, connexin hemichannels could also contribute to this process, since Cx43 hemichannels have been implicated in regulation of cell volume in other cells (Quist et al., 2000).

CONNEXIN HEMICHANNELS AND CATARACTS

Studies of cataract-linked connexin mutants suggest several ways that connexin hemichannels contribute to the pathogenesis of cataracts (**Figures 3B,C**).

The ability of some mutant lens connexins to form functional hemichannels has been assessed. Unlike wild type Cx46, many of the cataract-associated Cx46 mutants do not form functional hemichannels (e.g., Cx46L11S, Cx46fs380). Others exhibit a reduced ability to form them (e.g., Cx46D3Y, Cx46N63S) (Pal et al., 2000; Tong et al., 2013). These mutants would lead to less than normal connexin hemichannel activity in the lens (**Figure 3B**).

Cataract-associated mutants may also form hemichannels with altered properties (e.g., gating or charge selectivity) as compared with the wild type connexin. For example, the cataract-associated mutant Cx46N63S (which does not form functional gap junction channels) is impaired in its ability to induce hemichannel currents under standard recording conditions (i.e., 0.7 mM $[Ca^{2+}]_o$ and 0.8 mM $[Mg^{2+}]_o$), but Cx46N63S-induced currents increase in magnitude when the concentration of the divalent cations is decreased (Ebihara et al., 2003). This mutant forms hemichannels with increased sensitivity to the extracellular concentration of magnesium ions (Ebihara et al., 2003). Cx46D3Y forms hemichannels that have altered charge selectivity and voltage-dependent gating (Tong et al., 2013).

A very striking alteration of hemichannel properties is exemplified by Cx50G46V, a mutant found in a patient with total cataract (Minogue et al., 2009). This mutant forms gap junction plaques and supports intercellular communication normally. However, unlike wild type Cx50, Cx50G46V has a greatly increased ability to form functional hemichannels (Minogue et al., 2009; Tong et al., 2011). Expression of this mutant increases the proportion of apoptotic cells and causes cell death (Minogue et al., 2009) (**Figure 4**), suggesting that opening of the hemichannels would also cause severe cell damage *in vivo*. This cytotoxicity appears dominant, since co-expression of Cx50G46V with wild type Cx46 or Cx50 also decreases cell (oocyte) viability (Tong et al., 2011). Recently, a cataract-linked human Cx46 mutant (Cx46G143R) was identified that had increased hemichannel function, but no gap junction channel function (Ren et al., 2013). Connexin mutants with enhanced hemichannel activity (**Figure 3C**) may cause fiber cell death through a complex sequence of events including disruption of transmembrane ion gradients leading to loss of membrane potential, and entry of calcium ions, leading to activation of intracellular proteases and decreased metabolic activity.

POSSIBLE CONTRIBUTIONS OF CONNEXIN HEMICHANNELS TO ACQUIRED CATARACTS

The roles of connexin hemichannels in age- or disease-related cataracts are unknown and largely unexplored. However, there are a variety of reasons to hypothesize that alterations of the lens connexins and their hemichannel activities might contribute to the development of these cataracts.

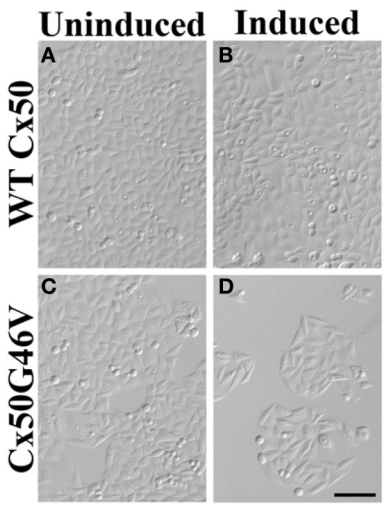

FIGURE 4 | Expression of Cx50G46V (but not wild type Cx50) decreased the number of cells. HeLa cells were stably transfected with constructs that allowed the inducible expression of wild type (WT) Cx50 or Cx50G46V. Phase-contrast photomicrographs obtained from cultures of cells transfected with WT Cx50 **(A,B)** or with Cx50G46V **(C,D)** that were left untreated **(A,C)** or that were induced by treatment with 1 μM ponasterone A **(B,D)** for 96 h. While a dramatically reduced number of cells remained in the culture 96 h after induction of Cx50G46V, the cell density was not affected by induction of wild type Cx50. This reduction is consistent with the cytotoxicity anticipated for this mutant due to its increased hemichannel function. Bar, 111 μm (reproduced from Minogue et al., 2009 with minor modifications).

Connexin hemichannels may facilitate the disturbances of calcium homeostasis that contribute to the pathogenesis of various different kinds of cataracts. The hemichannels can be activated by depolarization, which is frequently associated with the onset of cataractogenesis and allow entry of Ca^{2+} from the extracellular space. Studies in isolated lens fiber cells have shown that influx of calcium ions can provoke a process termed "disintegrative globulization" that may mimic cataractogenesis (Bhatnagar et al., 1995). This process can also be induced by osmotic changes or hyperglycemia as may occur in association with diabetes (Wang et al., 1997; Chandra et al., 2002). Although chloride channels and L-type calcium channels have been implicated in this process (Wang et al., 1996, 1997), it is reasonable to hypothesize that lens connexin hemichannels might also participate as conduits for entry of calcium ions (Ebihara et al., 2010).

Aberrant hemichannel opening may also be involved in cataracts that are associated with various stress factors. Age-related cataracts are thought to result from the cumulative effects of oxidative stress on lens components (i.e., DNA, proteins, and lipids) and the decreased efficiency of repair mechanisms. In astrocytes, the metabolic inhibition-induced opening of Cx43 hemichannels was associated with S-nitrosylation of the protein and blocked by high intracellular concentrations of reduced glutathione (Retamal et al., 2006). In the normal lens, the high concentrations of glutathione that are present may similarly keep connexin hemichannels closed and protect the lens from their potential deleterious consequences. However, in cataractous lenses where levels of glutathione are decreased (Truscott and Augusteyn, 1977), increased hemichannel opening might lead to further deterioration of the organ. However, the regulation of connexin hemichannel opening by reducing agents may depend on the cell type and the initial state of the cell. Studies of connexin constructs expressed by retroviral infection of chicken embryo fibroblasts have suggested that ultraviolet radiation stimulates caspase-dependent cleavage of Cx50 which leads to closure of hemichannels and reduction of intercellular communication (Wang et al., 2012). These authors speculate that this might be a mechanism for the lens to protect itself against this cataract-causing radiation.

On the other hand, studies have also suggested that some of the cellular components that contribute to lens pathology might reduce the normal physiologic opening of hemichannels. Such alterations would disrupt normal Cx46 hemichannel functions including serving as conduits for the entry of sodium and calcium ions into lens cells. Indeed, unsaturated fatty acids like linoleic acid block Cx46 hemichannels at the concentrations found in the lens and thus might contribute to cataract formation (Retamal et al., 2011).

CONCLUSIONS AND PERSPECTIVES

It is established that the lens connexins can make functional hemichannels in exogenous expression systems and in lens cells and that a connexin mutant that causes aberrant opening of hemichannels causes cataracts. Future studies should expand and

clarify our understanding of the roles of connexin hemichannels in the physiology of the normal lens and in the pathogenesis of cataracts due to many different causes.

A low level of hemichannel function may contribute to the normal physiology of the lens (as illustrated in **Figure 3A**). The best evidence implicates Cx46 in the connexin hemichannel openings detected in isolated lens cells (Ebihara et al., 2010). However, it is likely that Cx43 and Cx50 also contribute to the hemichannels present in the lens and that heteromeric hemichannels are formed among the three connexins.

It will be important to answer several questions regarding the roles of connexin hemichannels in normal lens physiology. How is the opening of these hemichannels regulated in the lens? Are connexin hemichannels responsible for the chronic Na^+ current in the normal lens? Do connexin hemichannels participate in the volume regulation accompanying accommodation? Do they participate in the lens circulation of water and solutes?

Reduced opening of connexin hemichannels (**Figure 3B**) should disrupt all of these normal processes. It might affect accommodation or even lead to cataracts.

Conversely, increased hemichannel opening (**Figure 3C**) might also lead to lens pathology. In the rare connexin mutant exhibiting gain of hemichannel function, the mechanism of cataract formation needs to be clarified. It can be anticipated that it involves disruption of transmembrane ion gradients and loss of cytoplasmic components (like ATP). In addition, it is possible that abnormal opening of connexin hemichannels contributes to the pathogenesis of cataracts that have a high incidence due to non-genetic etiologies. The disease mechanisms for such cataracts include activation of kinases, imbalances of redox potentials, and accumulation of calcium which might increase hemichannel opening following pathophysiologic modification of the connexins by phosphorylation, oxidative damage, etc.

Direct testing of these hypotheses would be facilitated by development of selective pharmacologic inhibitors of the connexin hemichannels or by genetic approaches like generation of mutant mice. For example, a mouse could be generated by replacing the wild type gene encoding Cx46 or Cx50 with a mutant that only makes functional intercellular channels (but not hemichannels). In these mice, experiments could evaluate whether the mice develop cataracts (either normally or in response to stresses like ultraviolet radiation or diabetes) and whether the fiber cells have normal resting potentials and ionic currents. A positive outcome of such studies might imply the potential therapeutic value of a hemichannel-inhibiting drug as a treatment to prevent development of cataracts.

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Connexin and pannexin (hemi)channels in the liver

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The liver was among the first organs in which connexin proteins have been identified. Hepatocytes harbor connexin32 and connexin26, while non-parenchymal liver cells typically express connexin43. Connexins give rise to hemichannels, which dock with counterparts on adjacent cells to form gap junctions. Both hemichannels and gap junctions provide pathways for communication, via paracrine signaling or direct intercellular coupling, respectively. Over the years, hepatocellular gap junctions have been shown to regulate a number of liver-specific functions and to drive liver cell growth. In the last few years, it has become clear that connexin hemichannels are involved in liver cell death, particularly in hepatocyte apoptosis. This also holds true for hemichannels composed of pannexin1, a connexin-like protein recently identified in the liver. Moreover, pannexin1 hemichannels are key players in the regulation of hepatic inflammatory processes. The current paper provides a concise overview of the features of connexins, pannexins and their channels in the liver.

Keywords: connexin, pannexin, hemichannel, gap junction, hepatocyte, cell death, inflammation

INTRODUCTION

The liver is a unique organ endowed with a plethora of specialized functions and a strong regenerative capacity. The establishment of communicative networks between the different liver cell types is therefore indispensable. Non-parenchymal liver cells preferentially have paracrine or juxtacrine contacts amongst themselves and with other hepatic cell types (Kmieć, 2001). Hepatocytes, the most prominent liver cell population, directly communicate with each other through gap junctions. The latter are formed by head-to-head docking of 2 hemichannels of neighboring cells, which in turn are composed of 6 connexin (Cx) proteins (**Figure 1**) (Vinken et al., 2006, 2008, 2009, 2010a,b, 2011; Decrock et al., 2009, 2011). Historically, these hemichannels have been considered as merely structural precursors of gap junctions. In the last decade, an accumulating body of evidence points to independent roles for hemichannels in cellular signaling by connecting the intracellular compartment with the extracellular environment (Vinken et al., 2006, 2010b; Decrock et al., 2009, 2011). More recently, a novel class of connexin-like proteins, the pannexin (Panx) proteins, has been identified. They assemble in a hemichannel configuration and are also named "single membrane channels" instead of hemichannels, as they do not appear to form gap junctions (D'Hondt et al., 2011; Bennett et al., 2012; Dahl and Keane, 2012; Wang et al., 2013a). In contrast to gap junctions, connexin and pannexin hemichannels seem to be mainly involved in pathological processes, including cell death and inflammation (Decrock et al., 2009; D'Hondt et al., 2009; Chekeni et al., 2010; Ganz et al., 2011; Bennett et al., 2012). These emerging roles for connexin and pannexin hemichannels are discussed in the present paper with focus on their relevance to liver (dys)functionality. Furthermore, an updated overview of the currently available knowledge regarding hepatic connexin and pannexin expression as well as liver gap junctions is provided.

Abbreviations: ATP, adenosine 5′ triphosphate; Cx, connexin; GJIC, gap junctional intercellular communication; IP3, inositol triphosphate; P_2X_7R, P_2X_7 receptors; Panx, pannexin; UTP, uridine 5′ triphosphate.

CONNEXINS AND PANNEXINS IN THE LIVER

At present, more than 20 connexin proteins have been identified in human beings and rodents. They all share a similar structure consisting of 4 membrane-spanning domains, 2 extracellular loops, a cytoplasmic loop, and cytosolic N-terminal and C-terminal regions (**Figure 1**). The different connexin family members are typically named after their molecular weight expressed in kilodaltons (Decrock et al., 2009; Vinken et al., 2009). Hepatocytes express Cx32 and to a lesser extent Cx26, which represents about 90 and 5%, respectively, of the total connexin amount in rat and human livers. In contrast, most non-parenchymal liver cells, including stellate cells and Kupffer cells, mainly harbor Cx43, while liver vascular cells predominantly express Cx37 and Cx40 (**Figure 2**) (Kumar and Gilula, 1986; Paul, 1986; Nicholson et al., 1987; Zhang and Nicholson, 1989; Chaytor et al., 2001; Bode et al., 2002; Fischer et al., 2005; Shiojiri et al., 2006). A typical hallmark of the liver includes zonation, which is also manifested at the connexin level. Indeed, Cx32 is uniformly distributed throughout the liver, whereas Cx26 is preferentially expressed in the periportal acinar area (Berthoud et al., 1992; Kojima et al., 1995; Iwai et al., 2000). Liver connexin

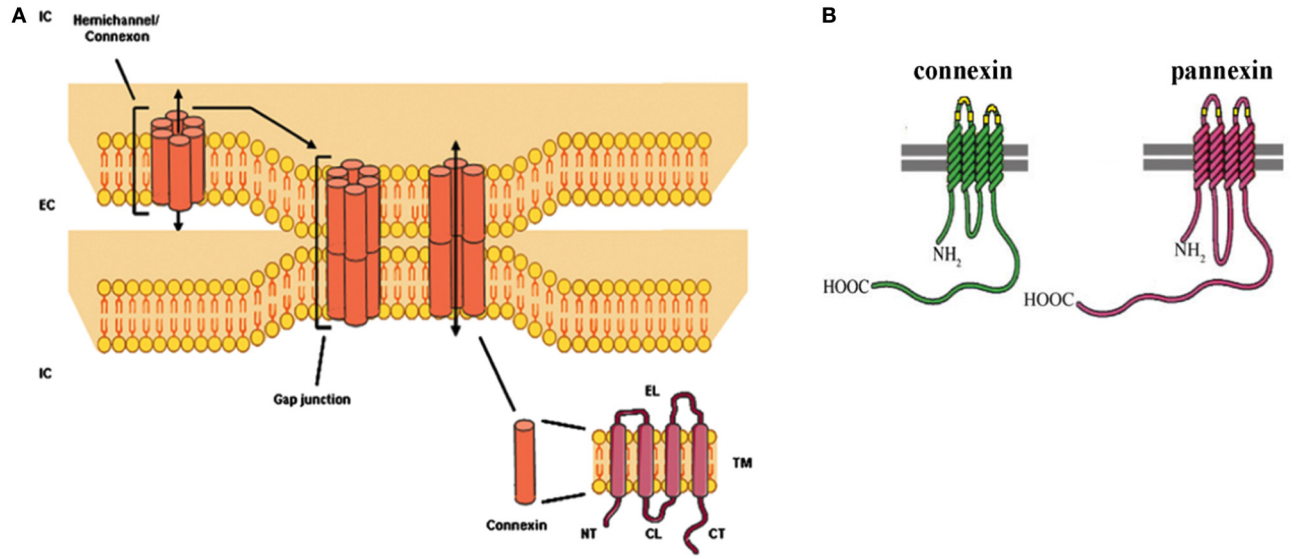

FIGURE 1 | (A) *Molecular architecture of gap junctions.* Gap junctions are grouped in plaques at the cell plasma membrane surface of 2 adjacent cells and are composed of 12 connexin proteins organized as 2 hexameric hemichannels. The connexin protein consists of 4 membrane-spanning domains (TM), 2 extracellular loops (EL), 1 cytoplasmic loop (CL), 1 cytoplasmic aminotail (NT) and 1 cytoplasmic carboxytail (CT) (EC, extracellular; IC, intracellular) (Vinken et al., 2008; Decrock et al., 2009). **(B)** *Structural comparison of connexins and pannexins* (Chekeni et al., 2010).

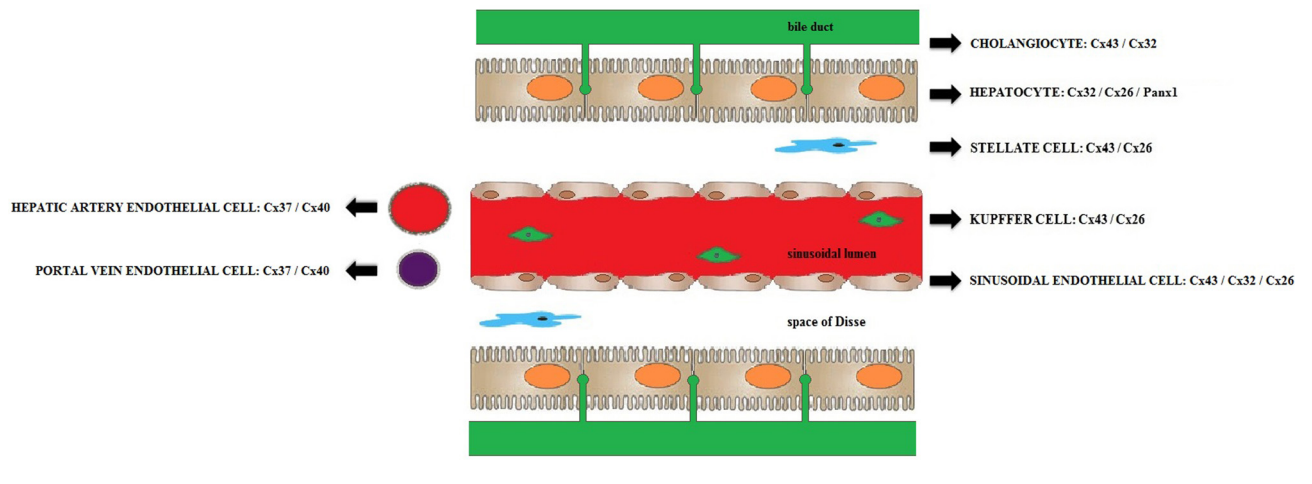

FIGURE 2 | Connexins and pannexins expressed in the liver. Schematic overview of the most relevant connexin and pannexin species expressed in rodent and human livers (Kumar and Gilula, 1986; Paul, 1986; Nicholson et al., 1987; Zhang and Nicholson, 1989; Chaytor et al., 2001; Bode et al., 2002; Fischer et al., 2005; Shiojiri et al., 2006; Xiao et al., 2012).

expression patterns undergo drastic changes upon both differentiation (i.e., liver organogenesis) and dedifferentiation (i.e., liver disease) (Vinken, 2012; Vinken et al., 2008, 2012a). Specifically, early hepatic progenitor cells switch from Cx43 to Cx26, but especially to Cx32 during differentiation into hepatocytes (Zhang and Thorgeirsson, 1994; Neveu et al., 1995; Naves et al., 2001; Paku et al., 2004). An inverse process is observed when chronic liver disease, such as liver fibrosis and cirrhosis, progressively turns into liver cancer (Vinken, 2012). Moreover, connexin expression and subcellular localization are significantly altered during liver cell growth (Traub et al., 1983; Kren et al., 1993; Temme et al., 2000). Proliferation is a rare event in the adult liver, but upon partial hepatectomy, the remaining liver lobes start to grow and the original size becomes restored within a week (Taub, 2004). Cx32 expression was found to increase during early hepatocyte cell cycling, followed by a sharp decline (Traub et al., 1983; Dermietzel et al., 1987; Sugiyama and Ohta, 1990; Miyashita et al., 1991; Kren et al., 1993; Temme et al., 2000; Kojima et al., 2003). Less consistent changes have been reported for Cx26 (Kren et al., 1993; Temme et al., 2000). Furthermore, Cx37 and Cx40 are clearly upregulated, while Cx43 tends to stay unaffected during liver regeneration (Kren et al., 1993).

Only 3 pannexin proteins have yet been characterized. They show structural similarity with connexin proteins, though pannexins typically have longer C-terminal regions and extracellular loops (D'Hondt et al., 2011; Wang et al., 2013a). Unlike connexins, very little is known about the occurrence of pannexins in the liver, although a number of studies have demonstrated the presence of Panx1 (Bruzzone et al., 2003; Csak et al., 2011; Ganz et al., 2011). In a more recent study, Panx1 mRNA was detected in cultured rat primary hepatocytes as well as in rat and human cancerous hepatocytes (Xiao et al., 2012). A single study also showed expression of Panx2 in the lateral plasma membrane fraction of primary rat hepatocytes (Li et al., 2008).

GAP JUNCTIONS IN THE LIVER

In the liver, the vast majority of gap junctions are formed between hepatocytes. They occupy as much as 3% of the hepatocellular membrane surface and form gap junctional plaques ranging from 0.2 to 1 μm in diameter and containing from 10 to more than 10,000 gap junction channels. Hepatocellular gap junctions in the pericentral acinar area mainly consist of homomeric hemichannels (i.e., containing Cx32), whereas their periportal counterparts can also be built up by heteromeric hemichannels (i.e., composed of both Cx26 and Cx32) (Vinken et al., 2008). Cx32 in the liver interacts with several other junctional proteins, such as occludin and claudin1 (Kojima et al., 2001), but also with mitochondrial proteins, including sideroflexin1 (Fowler et al., 2013). Gap junctions provide a generic pathway for communication between adjacent cells, called gap junctional intercellular communication (GJIC). GJIC includes the passive diffusion of small and hydrophilic molecules, such as cyclic adenosine monophosphate, adenosine 5' triphosphate (ATP), inositol triphosphate (IP3) and ions (Alexander and Goldberg, 2003; Dbouk et al., 2009; Decrock et al., 2009; Vinken et al., 2011). Homotypic Cx26 gap junctions favor cation transfer, whereas homotypic Cx32 gap junctions promote anion passage (Bukauskas et al., 1995). Likewise, ATP passes significantly better through gap junctions formed by Cx43 compared to Cx32-based channels (Goldberg et al., 2002).

Hepatocellular gap junctions are indispensable for maintaining the metabolic competence of the liver. In particular, Cx32-based GJIC underlies a number of liver-specific functions, including glycogenolysis (Stumpel et al., 1998), albumin secretion (Yang et al., 2003), bile secretion (Temme et al., 2001), ammonia detoxification (Yang et al., 2003) and xenobiotic biotransformation (Neveu et al., 1994; Shoda et al., 1999, 2000). Curiously, genetic ablation of Cx26 and Cx32 in mice does not drastically alter basal liver functionality (Ott et al., 2006). In line with this finding, Cx26 gene mutations in humans have been associated with skin diseases and deafness, but not with abnormalities in the liver (Lee and White, 2009), yet these patients display a gain of Cx26 hemichannel function (Mhaske et al., 2013). Similarly, human Cx32 gene mutations typically lead to neurological disorders, while leaving the liver largely unaffected (Abrams et al., 2000). However, Cx32-deficient mice, unlike their Cx26-lacking counterparts (Marx-Stoelting et al., 2008), have been found more susceptible to spontaneously occurring and chemically induced hepatocarcinogenesis (Temme et al., 1997).

The biochemical identity of the messengers that travel through hepatocyte gap junctions and that affect liver functionality remains largely elusive, though an exception exists for glycogenolysis. Breakdown of glycogen to glucose is triggered by hormonal and neuronal stimuli and predominantly occurs at the periportal acinar pole. Pericentral hepatocytes also show glycogenolytic activity, albeit to a lesser extent (Stumpel et al., 1998; Saez et al., 2003). Cx32-based gap junctions therefore drive the propagation of the glycogenolytic response from the periportal to the pericentral area. In fact, they facilitate the intercellular exchange of IP3, which activates calcium release from endoplasmic reticulum stores, in turn evoking calcium waves throughout the acinar tract (Clair et al., 2001; Saez et al., 2003; Gaspers and Thomas, 2005). Indeed, Cx32 knock-out mice display lowered blood glucose levels upon glycogenolytic stimulation (Nelles et al., 1996; Stumpel et al., 1998). Similarly, the spread of calcium waves through Cx43-based gap junctions controls ductular secretion from cholangiocytes and thus bile formation (Nathanson et al., 1999; Bode et al., 2002).

GJIC is clearly involved in liver cell growth. However, the exact role and overall relevance of gap junctions in hepatocyte cell cycling is matter of debate. In the regenerating liver of rats treated with an inhibitor of the mitogen-activated protein kinase pathway, the reduction of Cx32 production is counteracted, with no effects on hepatocyte proliferative activity (Kojima et al., 2003). This indicates that downregulation of GJIC may occur independently of hepatocyte proliferation. In the regenerating liver of Cx32 knock-out mice, hepatocellular proliferative activity is not affected, but the extent of synchronous initiation and termination of DNA synthesis is decreased (Kojima et al., 1997; Temme et al., 2000). Based on this observation, reduction of GJIC does not provide a direct signal for hepatocytes to divide, but rather permits cell cycle progression upon mitogenic stimulation. On the other hand, a plethora of studies have shown determinate functions for gap junctions in liver cell proliferation control, rather than merely an assisting role in growth progression (Koffler et al., 2000; Ruch, 2000; Yano et al., 2001). It should be stressed, however, that such studies typically rely on genetic ablation of connexin expression, thus impeding discrimination between GJIC and connexin hemichannel communication.

CONNEXIN AND PANNEXIN HEMICHANNELS IN THE LIVER

Although still surrounded by a lot of controversy, it is now accepted that connexin hemichannels autonomously establish a pathway for cellular signaling between the cytosol of individual cells and their extracellular environment (Decrock et al., 2009; Chandrasekhar and Bera, 2012; Kar et al., 2012). The messengers that diffuse through connexin hemichannels are very similar to those that can permeate gap junctions and typically include ATP, glutamate and glutathione. However, in contrast to gap junctions, connexin hemichannels have a low probability to be open. They can be opened by a number of stimuli that are of pathological origin, such as changes in extracellular or intracellular calcium concentration, oxidative stress, induced metabolic inhibition, ischemia/reperfusion insults and inflammatory conditions. Hence, connexin hemichannels are frequently referred to as pathological pores (Decrock et al., 2009; Chandrasekhar

and Bera, 2012; Kar et al., 2012). This has been well exemplified in the context of cell death. Although a limited set of reports described cytoprotective functions for connexin hemichannels (Plotkin et al., 2002; Okuda et al., 2013), most scientific evidence available points to pro-active roles for connexin hemichannels in the cell death process, involving the formation of a toxic pore or contributing to a paracrine cell death pathway (Kalvelyte et al., 2003; Takeuchi et al., 2006; Ramachandran et al., 2007; Decrock et al., 2009). Connexin hemichannels not only occur at the plasma membrane surface, but also reside at other subcellular locations, such as the mitochondria, where they have been linked to cell survival (Goubaeva et al., 2007; Lu et al., 2010; Azarashvili et al., 2011; Trudeau et al., 2012; Fowler et al., 2013). In this context, Cx43 translocates to the mitochondria where it interacts with Bax to initiate the mitochondrial apoptotic pathway in pancreatic cancer cells (Sun et al., 2012). Furthermore, mitochondrial Cx43 plays a role in myocardial ischemia-reperfusion injury by interfering with reactive oxygen species signaling (Ruiz-Meana et al., 2008) and facilitates ATP production (Boengler et al., 2012). More recently, mitochondrial Cx43-based hemichannels were found to assist in mitochondrial potassium uptake (Boengler et al., 2013).

Our group was the first to show the functional presence of connexin hemichannels in hepatocytes. Upon induction of Fas-mediated apoptosis in cultures of primary hepatocytes, GJIC rapidly declines, which is associated with a decay of the gap junctional Cx32 protein pool. At the same time, levels of newly synthesized Cx32 protein increase and gather in a hemichannel configuration. This becomes particularly evident toward the end stages of the cell death process (Vinken et al., 2010c). Subsequent experiments showed that Cx32-based hemichannels facilitate the apoptotic-to-necrotic transition in hepatocytes. Primary hepatocytes in culture are known to progressively lose their differentiated status, whereby Cx43 becomes *de novo* produced. Work with specific channel inhibitors demonstrated that Cx43 signaling, also involving hemichannels, underlies the onset of spontaneous apoptosis, which accompanies the dedifferentiation process in cultures of primary hepatocytes (Vinken et al., 2012b). In a more recent study, Cx43 production in cultured hepatocytes was epigenetically silenced followed by global protein and metabolite profiling. Among the proteins altered were several mitochondrial proteins. These data thus could further substantiate the existence of a mitochondrial connexin pool, and can be reconciled with a role for Cx43 signaling in spontaneously occurring apoptosis in primary hepatocyte cultures (Vinken et al., 2013).

Pannexin hemichannels have also been identified as mediators of apoptotic processes (Chekeni et al., 2010; Qu et al., 2011; Sandilos et al., 2012). Pannexin hemichannels can be opened by various pathological stimuli such as oxygen glucose deprivation, metabolic inhibition and S-nitrosylation (Thompson et al., 2006; Zhang et al., 2008). Similar to connexins (Yin et al., 2001; Theiss et al., 2007), Panx1 is an established target for caspases, which results in the formation of a constitutively open channel and the release of the so-called "find-me" signals, such as ATP and uridine 5′ triphosphate (UTP), at the earliest stages of cell death in order to recruit phagocytes (Chekeni et al., 2010; Sandilos et al., 2012). Both nucleotides act as "damage-associated molecular patterns," which are released during immunogenic cell death and serve as a signal to initiate and amplify cell death as well as to induce inflammation (Elliott et al., 2009; Chekeni et al., 2010). In fact, Panx1 plays a major role in the regulation of inflammatory processes and thus in innate immunity. Panx1 is instrumental for activating the inflammasome, a multiprotein complex involved in innate immunity and caspase 1 activation, and subsequent processing and release of the pro-inflammatory cytokines interleukin 1 beta and interleukin 18. Activation of the inflammasome has been seen in lipopolysaccharide-stimulated macrophages (Pelegrin and Surprenant, 2006, 2007) as well as in astrocytes and neurons (Silverman et al., 2009). Further investigation demonstrated that Panx1 hemichannel opening, induced by ATP stimulation of P_2X_7 receptors (P_2X_7R), facilitates the entry of bacterial inflammatory signals into the cytosol (Kanneganti et al., 2007). Panx1 is known to co-localize with the P_2X_7R and to form a "death receptor" complex (Locovei et al., 2007). Here, prolonged stimulation of the P_2X_7R results in the opening of a non-selective pore that may correspond to the Panx1 hemichannel. Extracellular ATP acts on the P_2X_7R, leading to Panx1 hemichannel opening (Pelegrin and Surprenant, 2006, 2007; Locovei et al., 2007; Iglesias et al., 2008). This mechanism may also apply for connexins, since Cx43 and P_2X_7R were seen to co-localize and co-immunoprecipitate in mouse macrophages and J774 cells (Fortes et al., 2004). However, a more recent study based on the use of $Panx1^{-/-}$ mice and $P_2X_7R^{-/-}$ mice demonstrated that Panx1 and the P_2X_7R function in distinct signaling pathways. Whereas only the P_2X_7R was necessary for inflammatory responses in lipopolysaccharide-primed macrophages, Panx1 was dispensable in the migrating phagocyte but was essential for the release of the "find me" signals from apoptotic cells to recruit macrophages (Qu et al., 2011).

Treatment of mice with lipopolysaccharide, results in elevated levels of Panx1 in the liver. Also, an increased production of active interleukin 1 beta and interleukin 18 is observed under these circumstances (Ganz et al., 2011). In addition to driving the inflammasome, Panx1 contributes to pathophysiological ATP release in lipoapoptosis induced by saturated free fatty acids, a key morphologic and pathological feature of human non-alcoholic steatohepatitis. Using a rat liver cell line, it has been shown that saturated free fatty acids increase extracellular ATP concentrations. Extracellular ATP release as well as cytosolic uptake of an indicator dye were partly inhibited by suppressing Panx1 expression (Xiao et al., 2012). Thus, Panx1-based hemichannels may play an important role in hepatic inflammation by mediating an increase in extracellular ATP levels in lipotoxic liver injury.

CONCLUSIONS AND PERSPECTIVES

Although still being in its infancy, it has become clear in the last few years that connexin and pannexin hemichannels fulfill critical functions in the regulation of cell death and inflammation. Only a handful of published reports have addressed these features in the liver and all of those rely on the use of *in vitro* settings (Decrock et al., 2009; D'Hondt et al., 2009; Chekeni et al., 2010; Ganz et al., 2011). Although some physiological roles have been attributed to hemichannels (Penuela et al., 2013), this remains to be demonstrated in the liver. It will be challenging in the upcoming years to evaluate the *in vivo* relevance of hepatic connexin and pannexin hemichannels. Several liver diseases, such as hepatitis, fibrosis and

cholestasis, are associated not only with the onset of cell death and inflammation, but also with modifications in connexin expression patterns and activity (Vinken, 2012). In fact, as discussed in the current paper, it is conceivable to assume that connexin, but especially, pannexin hemichannels drive the process of hepatocyte cell demise by releasing "find me" signals to initiate their clearance (Chekeni et al., 2010). Furthermore, recent data suggest the critical involvement of Panx1 hemichannels in liver inflammation (Ganz et al., 2011; Xiao et al., 2012). The knowledge that will be gained in this respect in the upcoming years could open new perspectives for clinical therapy. Thus far, 2 studies have pinpointed the potential of interfering with hepatic connexin signaling in the clinical management of acute (Patel et al., 2012) and acute-on-chronic liver failure (Balasubramaniyan et al., 2013). However, these studies did not distinguish between GJIC and connexin hemichannel communication. Given the opposite roles of gap junctions and connexin hemichannels in the (dys)regulation of the homeostatic balance in the liver, such discrimination is key for a targeted and efficient clinical outcome. Specific and *in vivo*-applicable connexin hemichannel inhibitors have become available only very recently (Iyyathurai et al., 2013; Wang et al., 2013b). In this respect, a peptide called Gap19 was found to specifically block Cx43-based hemichannel signaling and to reduce cell death in a mouse model of cardiac ischemia/reperfusion insult (Wang et al., 2013c). Future efforts should be focused on the further development of such tools as well as on the testing of their clinical applicability, stressing the importance of the field of hepatology.

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Cx43-hemichannel function and regulation in physiology and pathophysiology: insights from the bovine corneal endothelial cell system and beyond

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Intercellular communication in primary bovine corneal endothelial cells (BCECs) is mainly driven by the release of extracellular ATP through Cx43 hemichannels. Studying the characteristics of Ca^{2+}-wave propagation in BCECs, an important form of intercellular communication, in response to physiological signaling events has led to the discovery of important insights in the functional properties and regulation of native Cx43 hemichannels. Together with ectopic expression models for Cx43 hemichannels and truncated/mutated Cx43 versions, it became very clear that loop/tail interactions play a key role in controlling the activity of Cx43 hemichannels. Interestingly, the negative regulation of Cx43 hemichannels by enhanced actin/myosin contractility seems to impinge upon loss of these loop/tail interactions essential for opening Cx43 hemichannels. Finally, these molecular insights have spurred the development of novel peptide tools that can selectively inhibit Cx43 hemichannels, but neither Cx43 gap junctions nor hemichannels formed by other Cx isoforms. These tools now set the stage to hunt for novel physiological functions for Cx43 hemichannels in primary cells and tissues and to tackle disease conditions associated with excessive, pathological Cx43-hemichannel openings.

Keywords: intercellular communication, hemichannels, loop/tail interactions, actin/myosin contractility, selective inhibition of Cx43 hemichannels, corneal endothelial cells

PROPERTIES OF THE BOVINE CORNEA WITH FOCUS ON THE ENDOTHELIAL CELL LAYER

MORPHOLOGICAL PROPERTIES

The cornea is a transparent, convex, avascular continuation of the sclera covering the front part of the eye. Light enters the eye through the cornea, which provides about 2/3 of the eye's refractive power and forms together with the lens the focusing power of the eye (Maurice, 1957). At the posterior side of the cornea lies the anterior segment, which contains aqueous humor. The delicate balance between the production and absorption of aqueous humor keeps the anterior chamber pressurized (∼20 mm Hg) contributing to the maintenance of the curvature of the cornea. When this balance is disturbed glaucoma can occur (Tandon and Autar, 1989). Unlike most tissues in the body, the cornea is avascular; it contains no blood vessels to nourish it or to protect it against infection. Instead, tiny vessels at the outermost edge of the cornea along with the tears and aqueous humor take care of cell nourishment. The adult human cornea is ∼0.5 millimeter thick and is arranged in five basic layers: the stratified epithelium, Bowman's membrane, stroma, Descemet's membrane and the endothelium (**Figure 1A**). The bovine cornea is thicker than the human cornea due to a thicker stroma and more epithelial cell layers (**Figure 1B**). The endothelium is the innermost layer of the cornea, located just underneath Descemet's membrane, and forms an interface between the stroma and anterior chamber. The corneal endothelium is a 4–6 μm thick, non-regenerative monolayer of polygonal-shaped, mostly hexagonal cells (**Figures 1C,D**) with a diameter of about 20 μm (Joyce, 2003). The human corneal endothelium consists of about 400,000 cells. On the apical cell surface facing the anterior chamber, numerous small microvilli are present, and extensive interdigitations appear on lateral and basal plasma membranes. A circumferential band of actin filaments, located toward the apical aspect of the cells, helps to maintain cell shape and mediates cellular migration. The corneal endothelial monolayer is able to persist thanks to the complex interplay between the extracellular matrix, integrins, interendothelial junction proteins and the actin cytoskeleton. As in vascular endothelium, corneal endothelial cells are interconnected by a complex set of interendothelial junction proteins that comprise tight junctions, adherens junctions and gap junctions. Whereas gap junctions form plaques of transmembrane channels between adjacent cells, tight junctions form a belt around the apical pole of a polar cell making a tight connection with the belt around the neighboring cells and adherens junctions form pericellular zipperlike structures along the cell border through their transmembrane homophilic adhesion (for review see Mehta and Malik, 2006).

PHYSIOLOGICAL PROPERTIES

The cornea with its smooth, transparent, strong and durable characteristics has several functions in the eye. The transparent convex surface of the cornea acts as the eye's outermost optical element of

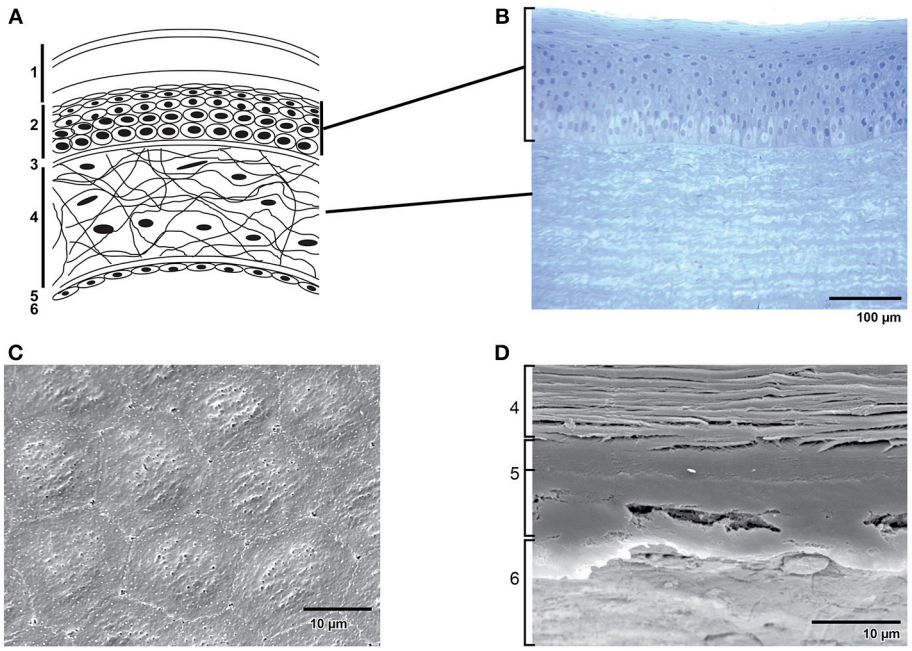

FIGURE 1 | Structure of the cornea. (A) Schematic representation of the structure of the human cornea: 1. Tear film with (starting from the outermost surface) a lipid layer, aqueous layer and a mucous layer. 2. Epithelial cells. 3. Bowman's membrane. 4. Stroma with keratocytes in an extracellular matrix of collagen fibrils and glycoaminoglycans. 5. Descemet's membrane. 6. Endothelial monolayer. **(B)** Transverse section of the epithelium and part of the stroma in bovine cornea. **(C)** Scanning electron microscopy of bovine corneal endothelium. Note the hexagonal shape of the cells. **(D)** Scanning electron microscopy of a transverse section of bovine cornea showing (from bottom to top) endothelium (6), the two distinct layers of Descemet's membrane (5) and a part of the stroma (4).

fixed focal length and enables focusing light onto the retina of the eye, hereby making the cornea act as an important lens. The avascular nature of the cornea and the regular arrangement of the collagen fibrils of the stroma ensure the transparency of the cornea (Davson, 1949; Dikstein and Maurice, 1972; Maurice, 1972; Hull et al., 1977; Riley, 1985; Bonanno and Giasson, 1992a,b; Bonanno, 2003), which is crucial for good vision. The cornea also serves as a filter, screening out some of the most damaging ultraviolet (UV) wavelengths in sunlight. Without this protection, the lens and the retina would be highly susceptible to injury from UV radiation. The cornea also functions as a barrier against influx of solutes, dust, germs, pathogens, and other injurious matter, into the eye. This protective task is primarily carried out by the epithelium but is shared with the eyelids, the eye socket, tears, and the sclera. Bowman's membrane and Descemet's membrane serve as a protective barrier against infection and injuries (Beuerman and Pedroza, 1996). The corneal epithelium, with its microvilli and microplicae, acts as a mucous surface that absorbs oxygen and nutrients from tears. The endothelium absorbs nutrients from the aqueous humor in order to provide the rest of the cornea of these nutrients.

The main physiological role of the corneal endothelium is maintenance of corneal transparency by controlling stromal hydration (Davson, 1949; Dikstein and Maurice, 1972; Maurice, 1972; Hull et al., 1977; Riley, 1985; Bonanno and Giasson, 1992a,b; Bonanno, 2003). Since the paracellular space between the corneal endothelial cells is partially occluded by the discontinuous focal adhesion tight junctions and the sinuous interdigitations of lateral membranes of adjacent cells, the endothelial barrier is leaky and permits paracellular passage of fluid and nutrients from the aqueous humor into the avascular cornea. The endothelium counteracts the tendency of the corneal stroma to swell by removing excess of stromal fluid and by regulating paracellular permeability through a complex interplay of cellular adhesive forces balanced against counter-adhesive forces generated by actomyosin molecular motors and through an active fluid transport from the stroma into the anterior chamber of the eye (reviewed in Bonanno, 2003). Both the "pump" and barrier functions of the endothelium are essential for maintaining the relatively dehydrated state of the stroma required for transparency. This continuous maintenance of equilibrium is referred to as the pump-leak mechanism (Davson, 1949; Maurice, 1972).

CORNEAL DYSFUNCTION

Regulation of barrier integrity

In order to maintain corneal function, regulation of the barrier integrity is crucial. The barrier function of the endothelial monolayer is controlled by the activation of different signaling mechanisms that affect paracellular and transcellular pathways. Although much is known about the identity of ion transport mechanisms in the corneal endothelium (Bonanno, 2003), the mechanisms of cell signaling that regulate barrier integrity are just beginning to be understood (Riley et al., 1996, 1998; Bonanno, 2003). An increase in contractility of the cortical perijunctional

actomyosin ring (PAMR) induces a centripetal force that opposes the tethering forces at tight junctions and adherens junctions and results in a breakdown of the endothelial barrier integrity (i.e., an increase in paracellular permeability) (Garcia and Schaphorst, 1995; Turner et al., 1997; Stevenson, 1999; Turner, 2000). The contractility of the actin cytoskeleton, including that of PAMR, is regulated through actomyosin interaction that is induced by the phosphorylation of the regulatory light chain of myosin II, also called myosin light chain (MLC) (Somlyo and Somlyo, 2000; Kamm and Stull, 2001). Phosphorylation of MLC, influenced by MLCK, PKC, or Rho kinase, induces an altered contractility of the actin cytoskeleton (Dudek and Garcia, 2001; Bogatcheva et al., 2002; Satpathy et al., 2004), which results in a significant breakdown of barrier integrity and the formation of interendothelial gaps (Garcia et al., 1995; Zhao and Davis, 1999; Van Nieuw Amerongen et al., 2000; Vouret-Craviari et al., 2002). Restoration of barrier integrity mainly occurs through relaxation of the actin cytoskeleton as a result of a decreased MLC phosphorylation via PKA-mediated inactivation of MLCK (Garcia et al., 1997; Somlyo and Somlyo, 2000; Kamm and Stull, 2001). In BCEC, barrier integrity is controlled via MLCK-, PKC- and Rho kinase-mediated pathways that regulate the MLC phosphorylation status (Satpathy et al., 2004, 2005; Srinivas et al., 2004).

Cell loss

In humans, corneal endothelial cell count averages around 3000 (Waring et al., 1982) to 6000 (Bourne, 2003) cells/mm^2 at birth and then slowly declines with age (Bourne and Brubaker, 1982, 1983; Bourne et al., 1997). With advancing age corneal endothelial cells also display greater morphological heterogeneity, a smaller percentage of hexagonal cells (Bourne and Brubaker, 1983; Bourne, 2003), and a decreased endothelial cell density and corneal thickness (Armitage et al., 2003; Zhu and Joyce, 2004; Mimura and Joyce, 2006) (for review see Moller-Pedersen, 1997; Bourne, 2001; Joyce, 2003). This cell loss can be accelerated by damage (Bourne and Brubaker, 1982, 1983; Bourne et al., 1997), pathological factors, such as primary corneal endotheliopathies (Schultz et al., 1984; Gagnon et al., 1997) (e.g., Fuchs's dystrophy), inflammation, glaucoma, prolonged UV exposure, a number of drugs and mechanical injury during intraocular surgery (Rao et al., 1978; Brooks and Gillies, 1991; Armitage et al., 2003) or laser procedures (Bergmann et al., 1991).

In the healthy adult cornea, endothelial cell count is between 2000 and 2500 cells/mm^2. When this cell count falls below a critical level (500–1000 cells/mm^2) endothelial dysfunction occurs (Edelhauser, 2000; Bonanno, 2003; Bourne, 2003; Bourne and McLaren, 2004; Bourne et al., 2004). Loss of an intact endothelial monolayer induces corneal edema, which causes the cornea to become cloudy, resulting in a loss of visual acuity (Riley, 1985; Landshman et al., 1988; Tuft and Coster, 1990; Riley et al., 1998; George and Larkin, 2004). In this situation, corneal transplantation is required to restore a functional endothelium (George and Larkin, 2004) as there are no pharmacological approaches to overcome endothelial dysfunction.

Wound repair

In many cell types, including corneal epithelium, cell division contributes to wound repair. However, evidence strongly suggests that cell division, if it occurs, plays only a minor role as a repair mechanism in mature corneal endothelium *in vivo*. Numerous studies indicate that wound healing in mature corneal endothelium *in vivo* mainly occurs by cell enlargement, in which flattening of the cells occurs, and cell migration, in which individual cells move from the wound edge to repopulate the wound (for review see Joyce, 2003). As a consequence of this form of repair, there is not only an age-related increase in cell size (polymegathism) of corneal endothelial cells, but also a gradual alteration from the typical hexagonal shape to a more polygonal, and finally pleomorphic shape (pleomorphism) (Laing et al., 1976; Matsubara and Tanishima, 1983; Murphy et al., 1984; Hoppenreijs et al., 1992). Stimulation of the cAMP pathway enhances individual cell migration, and prostaglandin E2 (PGE2) may be an endogenous stimulator of this response during corneal endothelial wound repair (Joyce and Meklir, 1994).

When only a small number of cells have been injured, healing occurs solely by enlargement of cells immediately adjacent to the wound. Repair appears to be initiated by membrane ruffling into the wound area. Once cells have made contact with each other, they stop ruffling and establish mature cell-cell contacts (for review see Joyce, 2003). In large wounds, repair occurs mainly as the result of coordinated enlargement and migration of cells adjacent to the wound and a few rows behind the wound edge. Cells initially enlarge and elongate into the wound area, causing movement of the monolayer as a sheet to cover the wound. Without losing contact with neighboring cells, the enlarged cells subsequently contract and pull surrounding cells into the wound area (Ichijima et al., 1993a,b). This form of repair has been designated as monolayer "spreading" (Joyce et al., 1989). Both monolayer spreading and cell migration result, at least in part, from alterations in the expression and organization of cytoskeletal elements, such as actin filaments and microtubules.

INTERCELLULAR COMMUNICATION IN BOVINE CORNEAL ENDOTHELIAL CELL MONOLAYERS

CONNEXIN AND PANNEXIN EXPRESSION PROFILE

Intercellular communication between bovine corneal endothelial cells typically has been studied using a local mechanically induced stimulus applied to a single cell. Communication between cells can be easily monitored by measuring intercellular Ca^{2+}-wave propagation within the BCEC monolayer that is loaded with a fluorescent Ca^{2+} dye, Fluo-4. A detailed description and visualized representation of the method can be found elsewhere (D'hondt et al., 2013a). The focus on Ca^{2+} signaling in this type of experiments is due to the fact that mechanical stimulation elicits an increase in cytosolic [Ca^{2+}], involving several Ca^{2+}-flux mechanisms, including the inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R)-mediated release of Ca^{2+} from the endoplasmic reticulum due to the activation of phospholipase C (PLC) (Leybaert and Sanderson, 2012; D'hondt et al., 2013a) (**Figure 2A**). A detailed analysis of the spatio-temporal activation of PLC upon mechanical stimulation has been described by others in MDCK cells (Tsukamoto et al., 2010), although the

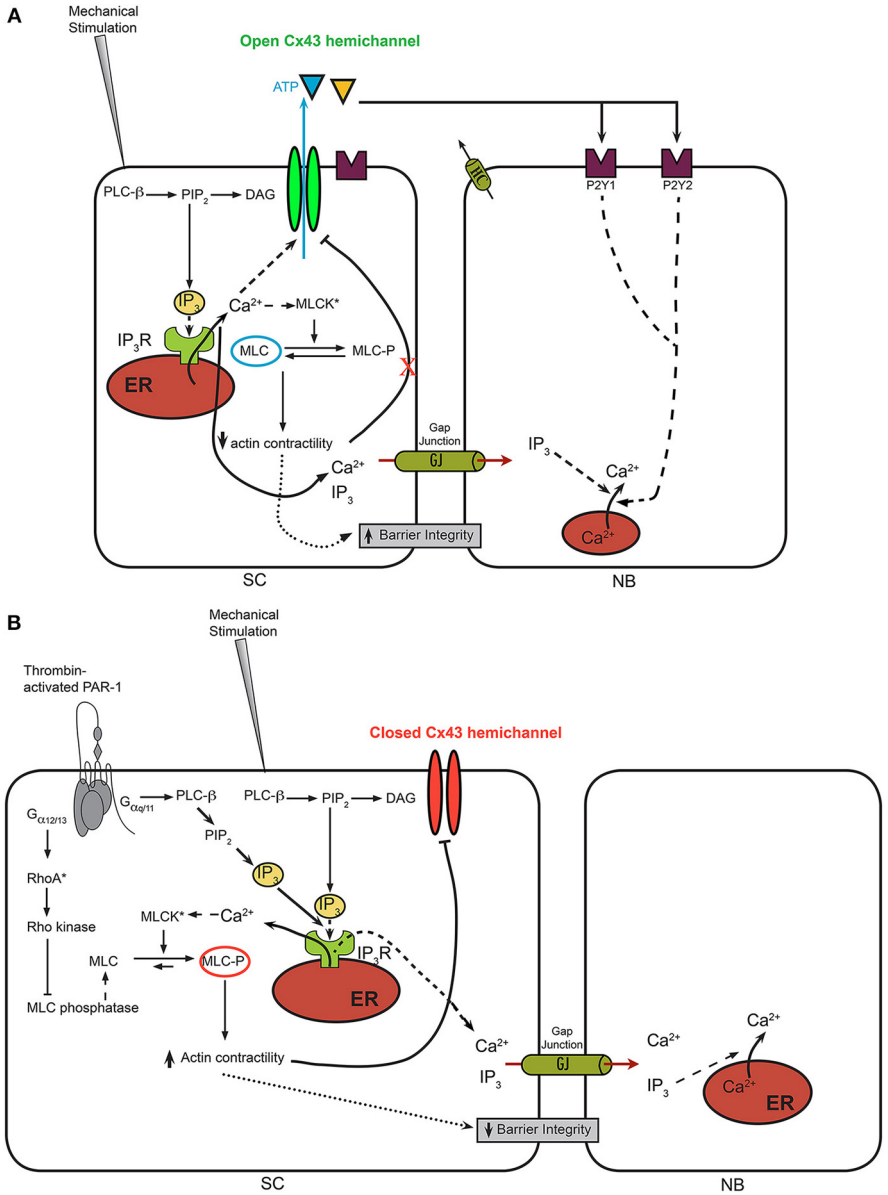

FIGURE 2 | A model for Ca^{2+}-wave propagation in BCECs in normal conditions vs. thrombin-treated conditions. (A) In normal BCECs, it is hypothesized that mechanical stimulation leads to a moderate rise in cytosolic [Ca^{2+}] via IP_3-dependent signaling mechanisms, which leads to the opening of Cx43 hemichannels and the flux of ATP from the cytosol into the extracellular environment. This allows the propagation of the Ca^{2+} from the "stimulated cell" (SC) to neighboring (NB) cells via activation of purinergic receptor and downstream IP_3-induced Ca^{2+} signaling. Under these conditions, it is anticipated that MLC remains in a dephosphorylated state, because of excess MLC phosphatase activity despite the potential activation of MLC kinases through Ca^{2+}/CaM. **(B)** In thrombin-pre-treated BCECs, the concerted activation of IP_3/Ca^{2+} signaling, leading to the activation MLC kinase combined with the activation RhoA/Rho kinase, leading to the inhibition of MLC phosphatase, leads to an enhanced contractility of the actin/myosin cytoskeleton. The latter seems linked to the Cx43 hemichannels, likely through its C-terminal tail. Increased actin/myosin contractility is proposed to displace the C-terminal tail from the cytoplasmic loop, thereby annihilating loop/tail interactions essential for Cx43-hemichannel opening. As such, Ca^{2+} signaling triggered by mechanical stimulation will not be able to lead to opening of these "locked"/'closed" Cx43 hemichannels, preventing extracellular ATP release and suppressing ATP-driven Ca^{2+}-wave propagation to neighboring cells. In addition to the effects of the actin/myosin contractility on Cx43 hemichannels, actin/myosin contractility may alter tight junctions and the ability of "head-to-head" docked hemichannels to form gap junction channels.

exact mechanisms underlying this PLC activation remain elusive. Importantly, the properties of the intercellular Ca^{2+} wave, including the speed of propagation and the extent of propagation (i.e., active area, corresponding to the area of cells responding with a cytosolic [Ca^{2+}] rise) provide an invaluable tool to analyze the properties of communicating channels, like connexins and pannexins (Leybaert and Sanderson, 2012). Furthermore, the occurrence of intercellular Ca^{2+} waves has been described

in a variety of cell systems and tissues, including the retina, cochlea, blood vessels, brain, liver with important physiological functions and pathophysiological consequences (Leybaert and Sanderson, 2012). Furthermore, the method impinges on the endogenously expressed channels and receptors and physiological signaling molecules present within the primary cell system. In particular, the presence of different connexin isoforms, including Cx26, Cx30.3, Cx31, Cx32, Cx36, Cx43, Cx45, Cx46, Cx46.6, and Cx50 and pannexin isoforms, including Panx1, Panx2, and Panx3, has been confirmed at the mRNA level (Ponsaerts et al., 2010a). At the protein level, the presence of Cx43 in BCEC lysates could be demonstrated via immunoblotting (Ponsaerts et al., 2010b), while immunocytochemistry showed the presence of Cx26 and Cx43 between corneal endothelial cells (Laux-Fenton et al., 2003). While these observations do not allow making conclusions about the relative expression of Cx/Panx isoforms or about the predominant Cx/Pan isoforms, the presence of a plethora of Cx/Panx isoforms do underpin that BCECs are very well suited for studying intercellular communication. Furthermore, these aspects are not limited to BCECs, since human corneal endothelial cells also express connexin isoforms, including Cx43, and display intercellular communication (Williams and Watsky, 2002). The upregulation of Cx43 at the protein level seems an important marker for the assessment of novel strategies to improve the preservation and maintenance of functional human donor cornea. In particular, the treatment with vasoactive intestinal peptide and ciliary neurotrophic factor, endogenous autocrine molecules from corneal endothelial cells, seem to be very promising agents to promote the differentiation and survival of corneal endothelial cells (Koh et al., 2011; Koh, 2012). Finally, the presence of connexins in corneal endothelial cells also seems to prevent their proliferation. As such, Cx43 knockdown has been implicated as a novel therapeutic strategy to accelerate wound healing in the corneal endothelium (Nakano et al., 2008) and to promote re-epithelialization by suppressing stromal oedema and inflammatory responses (Grupcheva et al., 2012).

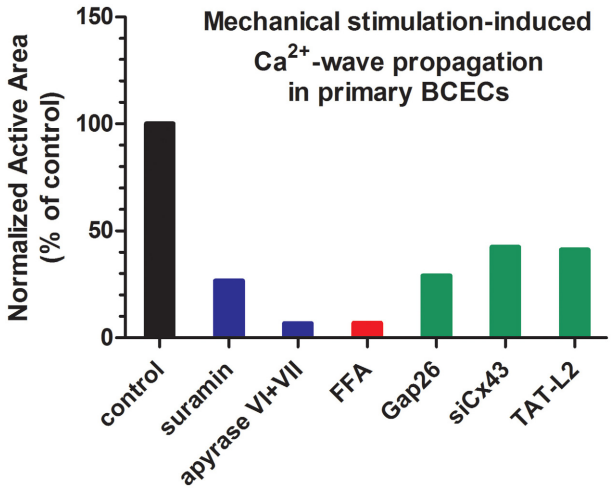

FIGURE 3 | Characteristics of intercellular communication in BCECs. A graph depicting the characteristics of intercellular communication in BCECs, based on mechanical stimulation-induced Ca^{2+}-wave propagation data (active area) previously published in (Gomes et al., 2005a,b; Ponsaerts et al., 2010b). Data were further normalized to their respective controls (set at 100%). The graph is intended to indicate the relevance of ATP release (blue bars), hemichannels (red bar) and Cx43-based hemichannels (green bars). In general, the data indicate that mechanical stimulation-induced Ca^{2+} wave propagation is almost completely driven by release of ATP into the extracellular environment (~90% inhibition by ATP-degrading enzymes) and that Cx43 hemichannels are a major release pathway for this ATP (~60% inhibition upon Cx43 knockdown or inhibition), although other Cx and/or Panx isoforms likely contribute to ATP release. Since this graph is intended to provide a general view, readers should access the original research paper for obtaining information about the mean data and their respective SEM values.

PARACRINE SIGNALING

Intercellular communication, including intercellular Ca^{2+}-wave propagation, can occur via two main pathways: (i) direct coupling via gap junction channel, allowing the passage of signaling molecules that can mobilize intracellular Ca^{2+}, like IP_3, and (ii) indirect coupling via hemichannels, allowing the release of signaling molecules, like ATP, that can trigger intracellular Ca^{2+}signaling by acting on ionotropic and/or metabotropic receptors, like the P2X and P2Y purinergic receptors (Leybaert and Sanderson, 2012) (**Figure 2A**). In BCECs, a prominent role for paracrine signaling via extracellular ATP was found as the main driving mechanism for intercellular Ca^{2+}-wave propagation in response to mechanical stimulation (Gomes et al., 2005b), although gap junctions are present and are operative in BCECs (Gomes et al., 2006). Indeed, exogenous application of the apyrase VI + VII cocktail dramatically diminished the active area of the intercellular Ca^{2+}-wave (**Figure 3**), while inhibiting endogenously expressed ectonucleotidases using exogenously applied inhibitors, like ARL-67156, dramatically increased the intercellular communication (Gomes et al., 2005b). These observations were supported by direct luciferin/luciferase bioluminescent measurements of the extracellular ATP levels, showing that mechanical stimulation induced ATP release in the extracellular environment. Extracellular ATP-driven intercellular Ca^{2+}-wave propagation requires the presence of ATP receptors that initiate Ca^{2+} signaling upon activation. At the mRNA level, different ionotropic P2X receptors, including P2X3, P2X4, P2X5, and P2X7 and different metabotropic P2Y receptors, including P2Y1, P2Y2, P2Y4, P2Y6, P2Y10, and P2Y12 are expressed (Gomes et al., 2005b; Ponsaerts et al., 2010a), correlating with previous functional evidence (Srinivas et al., 1998). Also mRNA of the ectonucleotidases, CD39 and CD73, was detected, confirming the presence of ATP-degrading enzymes. The activity of ectonucleotidases seems to critically control this ATP-driven communication, since aged BCECs display increased ectonucleotidase activity and concomittant reduced ATP-dependent intercellular communication (D'hondt et al., 2009). Consistent with the presence of the purinergic receptors, exogenously added ATP using concentrations in the (sub)μM range was able to induce intracellular Ca^{2+} release with an EC50 of ~1.5 μM. Particularly, P2Y receptors might be responsible for the ATP-induced Ca^{2+} rises and ATP-driven Ca^{2+}-wave propagations, since suramin, a non-selective P2X and P2Y antagonist, dramatically reduced intercellular Ca^{2+}-wave propagation (Gomes et al., 2005b) (**Figure 3**).

However, the latter result should be interpreted with caution, since a recent report indicated that suramin could also suppress Cx43-hemichannel activity and its associated membrane permeability (Chi et al., 2014).

Cx43 HEMICHANNELS

An important mechanism responsible for the release of ATP seems to be mediated via hemichannels (Gomes et al., 2005a). Similar to the application of exogenous apyrases, the presence of flufenamic acid (FFA), a known hemichannel inhibitor, strongly reduced mechanical stimulation-induced Ca^{2+}-wave propagation in BCECs and almost completely blunted mechanical stimulation-induced ATP release without affecting gap junction coupling (Gomes et al., 2005a) (**Figure 3**). Yet, data obtained using FFA have to be interpreted with caution, since FFA is a broad spectrum ion channel modulator, impacting a variety of non-selective cation channels and K^+, Ca^{2+}, Na^+ and Cl^--permeable channels (see Guinamard et al., 2013 for a detailed discussion). Consistent with the prominent expression of Cx43 in BCECs and the relevance of Cx43 as one of the major Cx isoforms responsible for intercellular communication and intercellular Ca^{2+} waves, Gap26, a Cx43-mimetic peptide, suppressed intercellular communication and ATP release (Gomes et al., 2005a) (**Figure 3**). It is important to note that Gap26 can also inhibit gap junction-mediated intercellular communication. However, as shown in a recent electrophysiological study, the kinetics for inhibition of hemichannels vs. gap junctions by Gap26 seems to be very different. Indeed, short-term incubations (∼5 min) were sufficient to readily inhibit Cx43-hemichannel currents, while long-term incubations (∼30 min) were required to inhibit Cx43 gap junction-mediated electrical coupling in cell pairs (Desplantez et al., 2012). The inhibition of Cx43 hemichannels by Gap26 has been further characterized, displaying a right-ward shift of about 20–30 mV for the activation of Cx43 hemichannel currents, suppressing Cx43-hemichannel currents potentiated by physiological elevations in intracellular $[Ca^{2+}]$, and inhibiting Cx43-hemichannel currents with an IC50 of about 80 μM (Wang et al., 2012, 2013a). Not only ectopically expressed Cx43 hemichannels could be inhibited, but Gap26 also suppressed native Cx43 hemichannels from pig ventricular cardiomyocytes (Wang et al., 2012). In BCECs, Gap26 applications that are able to inhibit Cx43 hemichannels did not impair gap junctional coupling in fluorescence recovery after photobleaching (FRAP) using carboxyfluorescein (Gomes et al., 2005a). More recently, siRNA-based knock down approaches were applied to directly assess the contribution of Cx43 in this process (Ponsaerts et al., 2010b) (**Figure 3**). Reducing the endogenous Cx43-protein levels by about 90% resulted in a more than 70% reduction in the active area of the intercellular Ca^{2+} wave in response to mechanical stimulation. Also using Ca^{2+}-free solution (EGTA) as a trigger for the opening of hemichannels, the knockdown of Cx43 resulted in a more than 60% reduction in EGTA-induced ATP release. Collectively, these data indicate Cx43 as the main connexin isoform responsible for forming hemichannels and for mediating hemichannel-mediated ATP release in BCECs, thereby driving intercellular Ca^{2+}-wave propagation.

BOVINE CORNEAL ENDOTHELIAL CELL MONOLAYERS AS A PRIMARY CELL MODEL FOR THE STUDY OF Cx43-HEMICHANNEL PROPERTIES AND REGULATION

Cx43 HEMICHANNELS AS INTEGRATORS OF SIGNALING PROCESSES

Cx43 hemichannels can be activated via membrane depolarizations, decreases in extracellular $[Ca^{2+}]$ and moderate increases in intracellular $[Ca^{2+}]$ (Wang et al., 2013a,c). While strong membrane depolarization (above +50 mV) are needed for the opening of Cx43 hemichannels, increases in cytosolic $[Ca^{2+}]$ in the physiological range of 100–500 nM provoke a left-ward shift in the voltage-dependent opening of Cx43 hemichannels (Wang et al., 2012). In this study, moderate intracellular $[Ca^{2+}]$ elevations could induce Cx43-hemichannel opening in response to a membrane depolarization in the order of +30 mV, thus shifting the threshold for voltage activation toward the physiological range (Wang et al., 2012). Furthermore, increasing the cytosolic $[Ca^{2+}]$ to about 500 nM seems sufficient to open Cx43 hemichannels, allowing the flux of ions and of signaling molecules like ATP (De Vuyst et al., 2009). In that sense, it is not surprising that mechanical stimulation, which triggers cytosolic Ca^{2+} rises, leads to Cx43 hemichannel opening. Furthermore, this Cx43-hemichannel opening seems to be strongly influenced by other physiological signaling cascades in response to extracellular factors. For instance, activation of the plasmalemmal thrombin-sensitive PAR-1 receptors using thrombin or the thrombin receptor activating peptide 6 (TRAP6; SFLLRN) inhibited Cx43-hemichannel activity in BCECs in response to mechanical stimulation (D'hondt et al., 2007a) (**Figure 2B**). The underlying signaling cascades triggered by thrombin exposure involved the concerted activation of myosin light chain kinase (MLCK), Rho kinase and protein kinase C (PKC) (Garcia et al., 1996; D'hondt et al., 2007a) (**Figure 2B**). Separate inhibition of one of the signaling cascades nearly completely restored Cx43-hemichannel opening in thrombin-treated BCECs. A common denominator of the different signaling pathways initiated by thrombin is that they all contribute to increased myosin light chain (MLC) phosphorylation (Satpathy et al., 2004), an event that results in increased contractility of the actin cytoskeleton in non-muscle cells (Burridge and Chrzanowska-Wodnicka, 1996). The critical role of the MLC phosphorylation status for controlling Cx43-hemichannel activity has been supported by experiments applying adenosine (D'hondt et al., 2007b), which has previously been demonstrated to induce MLC dephosphorylation in BCEC (Srinivas et al., 2004). Extracellularly added adenosine counteracts RhoA activation via a cAMP-dependent mechanism, prevents the inhibition of MLC phosphatase and opposes thrombin-induced MLC phosphorylation (D'hondt et al., 2007b). Interestingly, pre-incubation of the BCECs with adenosine completely alleviated the thrombin-induced inhibition of Cx43 hemichannels. The critical role of RhoA in the downstream signaling cascade initiated by thrombin and cross-talk to Cx43 hemichannels was supported by the use of C3 toxin, a cell-permeable RhoA inhibitor (Ponsaerts et al., 2012a). This compound completely abolished the thrombin-induced inhibition of Cx43 hemichannels in BCECs. Consistent with this, RhoA was rapidly activated in BCECs exposed to thrombin and C3 toxin pre-treatment completely prevented this RhoA activity.

Cx43-HEMICHANNEL OPENING IS NEGATIVELY REGULATED BY THE ACTIN CYTOSKELETON

Given the prominent role of MLC phosphorylation in controlling the contractility of the actin/myosin cytoskeleton and the functional link between MLC phosphorylation and decreased Cx43 hemichannel activity, the impact of the actin/myosin contractility on Cx43-hemichannel opening was assessed using a myosin II ATPase inhibitor, (-)-blebbistatin (Straight et al., 2003; Ponsaerts et al., 2008). This compound prevents the contraction of the actin/myosin cytoskeleton (Straight et al., 2003; Lucas-Lopez et al., 2008). Importantly, (-)-blebbistatin does not prevent the thrombin-induced increase in the MLC phosphorylation status, indicating that it does not affect the upstream signaling pathways controlling the activity of kinases or phosphatases that impact the MLC phosphorylation status (Ponsaerts et al., 2008). Strikingly, (-)-blebbistatin completely prevented the thrombin-induced inhibition of Cx43 hemichannel activity in BCECs brought about either mechanical stimulation or by Ca^{2+}-free solution. These data indicated that activation of the actomyosin contractile system serves as an endogenous brake counteracting the opening of Cx43 hemichannels (Ponsaerts et al., 2012b). The negative regulation of Cx43 hemichannels seems not restricted to BCECs, but seems to be a generally present and physiologically relevant mechanism (Ponsaerts et al., 2010b). Indeed, follow-up work in HeLa cells and C6 glioma cells ectopically expressing Cx43 revealed a bell-shaped dependent regulation of Cx43-hemichannel activity in response to cytosolic $[Ca^{2+}]$. Moderate rises in cytosolic $[Ca^{2+}]$ in the range of 100–500 nM triggered Cx43-hemichannel opening, while excessive rises in cytosolic $[Ca^{2+}]$ above 500 nM and the μM range suppressed Cx43-hemichannel opening (De Vuyst et al., 2009; Ponsaerts et al., 2010b). Interestingly, the inhibition of Cx43-hemichannel opening brought about by high cytosolic $[Ca^{2+}]$ rises (triggered by 10 μM A23187, a Ca^{2+} ionophore) could be completely prevented by (-)-blebbistatin (Ponsaerts et al., 2010b). This mechanism may not only occur during steady-state rises in cytosolic $[Ca^{2+}]$, but may also occur during physiological agonist-induced Ca^{2+} signaling (De Bock et al., 2012b). In recent work, it was shown that bradykinin-triggered Ca^{2+} oscillations in the MDCK epithelial cell line led to the Ca^{2+}-dependent opening of Cx43 hemichannels (De Bock et al., 2012b). Interestingly, the opening of Cx43 hemichannels provided a non-selective Ca^{2+}-entry pathway and led to a further rise in cytosolic $[Ca^{2+}]$, causing inhibition of Cx43-hemichannel opening. Thus, it may be anticipated that activation of the actomyosin cytoskeleton may have dual function: (i) by acting as a "safety" mechanism to prevent excessive Cx43-hemichannel opening, which would be detrimental for the cell due to loss of ionic, metabolic and energetic gradients, and (ii) by serving as a "Ca^{2+}-dependent" shutdown mechanism of Cx43 hemichannels, allowing a bimodal regulation of Cx43-hemichannel opening by cytosolic $[Ca^{2+}]$. The Ca^{2+}-dependent inhibition of Cx43 hemichannels promotes the occurrence of sustained Ca^{2+} oscillations, leading to increased survival [e.g., by increasing mitochondrial bioenergetics (Hajnoczky et al., 1995)] or controlling physiological functions like endothelial cell membrane permeability (De Bock et al., 2012a, 2013). In the absence of Ca^{2+}-dependent inhibition of Cx43 hemichannels, the Ca^{2+}-dependent opening of Cx43 hemichannels could lead to a sustained Ca^{2+} influx, causing intracellular Ca^{2+} overload and cell death.

Different mechanisms responsible for the negative regulation of Cx43 hemichannels by the actin/myosin contractile systems may be considered, including a direct or indirect link between Cx43 hemichannels and the actin/myosin cytoskeleton (Ponsaerts et al., 2012b). Unfortunately, while many cytoskeletal proteins have been shown to interact with Cx43, most, if not all, evidence has been obtained for Cx43 gap junctions (Herve et al., 2012). In that sense, it is interesting to note that increased contractility also negatively impacted Cx43-mediated gap junctional coupling in BCECs (D'hondt et al., 2007a). One of the underlying mechanisms proposed was the loss of tethering forces essential for stabilizing the interactions of the transmembrane proteins of tight junctions and the subsequent disrupted barrier integrity (Garcia et al., 1996; Satpathy et al., 2004). Hence, sufficient level of tethering forces may facilitate the docking of hemichannels from adjacent cells to form and maintain gap junction channels. Another aspect proposed was that increased actin/myosin contractility could interfere with the interaction of Zona Occludens-1 (ZO-1) with Cx43, thereby affecting Cx43 gap junction assembly (Hunter et al., 2005; Rhett et al., 2011). Loss of Cx43/ZO-1 interaction has been shown to increase gap junction size (Hunter et al., 2005). This promoted the trafficking of and assembly of the "undocked" hemichannels from the perinexus into gap junctional plaques, thereby increasing gap junctional communication while decreasing hemichannel-mediated signaling (Rhett et al., 2011). In BCECs, the binding of ZO-1 to Cx43 hemichannels was not detectable. In addition, it seemed that the inhibition of hemichannel opening by thrombin could be overcome by both TAT-CT10 and TAT-CT10ΔI, which lacks the last Ile residue critical for binding the PDZ2 domain of ZO-1 (Giepmans and Moolenaar, 1998; Ponsaerts et al., 2010b). Furthermore, the loss of tethering forces and barrier integrity could definitely not account for an inhibition of Cx43-hemichannel activity, since such an effect may rather lead to an increase in the ratio of "undocked" Cx43 hemichannels over docked Cx43 gap junctions. While it is clear that actin contractility negatively impacts Cx43-hemichannel activity, proper cytoskeletal organization is definitely required for ATP release and subsequent intercellular Ca^{2+} signaling, since these phenomena are impaired upon cytoskeletal destabilization using cytochalasin D exposure (Cotrina et al., 1998).

Cx43 HEMICHANNEL OPENING IS CONTROLLED BY INTRAMOLECULAR LOOP/TAIL INTERACTIONS

Since Cx43 gap junctions are critically and dynamically controlled by intramolecular interactions between the C-terminal tail and the second part of the cytoplasmic loop (L2 region) (Duffy et al., 2002; Delmar et al., 2004; Seki et al., 2004; Hirst-Jensen et al., 2007) and since Cx43 hemichannels consists of the same protein building blocks as Cx43 gap junctions, it was plausible that Cx43-hemichannel opening too was influenced by loop/tail interactions and that the actin/myosin cytoskeleton could impact Cx43-hemichannel activity by interfering with loop/tail interactions. An important insight was provided by the application of cell-permeable peptides covering the last 10 amino acids of the

C-terminal tail of Cx43 (TAT-CT10) (Ponsaerts et al., 2010b). These residues harbor two important functional domains: (i) the C-terminal Ile^{382}, which serves as the binding site for the PDZ2 domain of ZO-1 (Giepmans and Moolenaar, 1998), and (ii) the Asp^{378} and Asp^{379} residues, which serve as residues involved in the interaction with the L2 domain (Hirst-Jensen et al., 2007). Strikingly, pre-incubation of BCECs with TAT-CT10 peptide completely prevented the thrombin-induced inhibition of Cx43 hemichannels (Ponsaerts et al., 2010b). On the one hand, a TAT-CT10 peptide but lacking the C-terminal Ile residue (TAT-CT10ΔI) remained capable of alleviating the thrombin-induced inhibition of Cx43-hemichannel activity, indicating that altered Cx43/ZO-1-complex formation was not involved in this process (Ponsaerts et al., 2010b). On the other hand, a TAT-CT10 version in which Asp^{378} and Asp^{379} have been mutated to Ala residues completely lost its ability to alleviate the thrombin-induced inhibition of Cx43-hemichannel activity (D'hondt et al., 2013b). Consistent with this, while biotinylated CT10 peptides immobilized to a streptavidin-coated sensor chip could bind the L2 and cytoplasmic loop region in surface plasmon resonance experiments, biotinylated CT10 peptides in which Asp^{378} and Asp^{379} were changed into Ala residues, completely lost this property (Ponsaerts et al., 2010b; D'hondt et al., 2013b). Similar findings were observed for Cx43 hemichannels ectopically expressed in HeLa and C6 glioma cells. Indeed, the inhibition of ectopically expressed Cx43 hemichannels by high cytosolic $[Ca^{2+}]$ could be alleviated by treatment of the cells with either TAT-CT10 or TAT-CT10ΔI but not with TAT-$CT10^{DD/AA}$ (Ponsaerts et al., 2010b). Furthermore, the electroporation of a peptide covering the last 9 amino acids of the C-terminal tail of Cx43 (CT9) in MDCK cells completely abolished the occurrence of bradykinin-induced Ca^{2+} oscillations (De Bock et al., 2012b). This indicates that the loss of endogenous loop/tail interactions can occur during physiological Ca^{2+} signaling and is the underlying mechanism responsible for the Ca^{2+}-dependent inhibition of Cx43 hemichannels.

Since the L2 region was identified as the target for the CT10 peptide, the effect of a cell-permeable L2 peptide on Cx43 hemichannel opening was also examined (Ponsaerts et al., 2010b). It was found that TAT-L2 prevented Cx43-hemichannel opening in BCECs induced by mechanical stimulation or by Ca^{2+}-free solution (**Figure 3**). TAT-L2 also inhibited Cx43-hemichannel opening in Cx43-expressing HeLa/C6-glioma cells induced by moderate increases in cytosolic $[Ca^{2+}]$. Interestingly, a mutant version of TAT-L2 (i.e., TAT-$L2^{H126K/I130N}$) previously shown to be impaired for its ability to interact with the C-terminal tail (Seki et al., 2004), failed to inhibit the activity of endogenously and ectopically expressed Cx43 hemichannels (Ponsaerts et al., 2010b). In contrast, gap junctional coupling did not seem to be inhibited by TAT-L2. Hence, it is proposed that TAT-L2 by targeting the CT10 region of Cx43 hemichannels interferes with the occurrence of endogenous loop/tail interactions essential for Cx43-hemichannel activity. As such, the inhibitory effect of TAT-L2 could be prevented by co-application of TAT-CT10 (Ponsaerts et al., 2010b). Moreover, from these measurements and previous studies of the Delmar group (Seki et al., 2004), it also became clear that L2 oppositely affects Cx43 gap junctions, namely favoring their open state, while preventing/inhibiting Cx43 hemichannel opening. These findings were supported by the fact that $Cx43^{M239}$-based hemichannels, which lack the complete C-terminal tail, fail to open. In contrast, $Cx43^{M239}$-based gap junctions are active and remain open, even during conditions of acidification (Moreno et al., 2002). Therefore, it was anticipated that loop/tail interactions present in Cx43 gap junctions also exist in Cx43 hemichannels but with a functional outcome. To scrutinize this concept, it was shown that TAT-CT10 and TAT-CT10ΔI, but not TAT-$CT10^{DD/AA}$, could restore $Cx43^{M239}$-hemichannel activity (Ponsaerts et al., 2010b). It is important to note that the addition of TAT-CT10 is not sufficient to open Cx43 hemichannels, but rather facilitates their opening in response to triggers like voltage steps to positive membrane potentials or increased cytosolic $[Ca^{2+}]$. Hence, our model proposes that loop/tail interactions are required to bring Cx43 hemichannel in a "ready to open" state (Wang et al., 2013a).

Further studies revealed a stretch of 9 amino acids within the L2 region of the cytoplasmic loop as the target of CT10 (Wang et al., 2013b). This Lys-rich stretch of amino acids is mainly positively charged, thereby allowing electrostatic interactions with the two negatively charged Asp residues within the CT10 region. Strikingly, a nonapeptide, called Gap19, displayed similar properties as TAT-L2, thereby causing inhibition of Cx43 hemichannels but not Cx43 gap junctions (Wang et al., 2013b). Gap19 naturally displayed strong cell-permeable properties, likely due to the Lys-rich stretch, thereby resembling the positively charged residues in the TAT cell-penetrating sequence. Consistent with this, Gap19 completely mimicked the actions of TAT-L2, thereby inhibiting the opening of Cx43 hemichannels in response to low extracellular $[Ca^{2+}]$ or moderate rises in cytosolic $[Ca^{2+}]$ (Wang et al., 2013b). The inhibitory effect of Gap19 was also analyzed at the electrophysiological level using whole cell patch clamp experiments, allowing the characterization of its effect on Cx43 hemichannel unitary currents (Wang et al., 2013a,b). Gap19 decreased the frequency of single Cx43-hemichannel openings, thereby reducing the open probability of the Cx43 hemichannels. Interestingly, Gap19 was also found to inhibit native Cx43 hemichannels present in ventricular cardiomyocytes, acutely isolated from pig hearts. The inhibition seems to be due to a right-shift of about 30 mV in the voltage-dependent opening of Cx43 hemichannels.

Finally, it is anticipated that the loss of loop/tail interactions essential for Cx43-hemichannel activity may underlie the inhibition of Cx43-hemichannel activity in response to increased actin/myosin contractility. Indeed, $Cx43^{M239}$-based hemichannels fail to open, even in response of the myosin II ATPase inhibitor, (-)-blebbistatin (Ponsaerts et al., 2010b).

TARGETING LOOP/TAIL INTERACTIONS IN Cx43: SELECTIVE Cx43-HEMICHANNEL INHIBITORS AS NOVEL TOOLS IN CELL BIOLOGY, PHYSIOLOGY AND PATHOPHYSIOLOGY

The concept of loop/tail interactions differentially controlling Cx43 gap junctions vs. hemichannels has spurred the development of peptide tools, including TAT-L2 and Gap19, to selectively inhibit Cx43 hemichannels while unaffecting Cx43 gap junctions in a variety of primary cell systems and tissues (Evans et al., 2012; Iyyathurai et al., 2013; Wang et al., 2013a). As such, these

tools supplement knockdown/knockout approaches, which do not allow discriminating between gap junctions and hemichannels. Furthermore, these tools are not only selective for Cx43 hemichannels vs. gap junctions, but are also unlikely to target other Cx or Panx isoforms. In particular, the L2 region of Cx43 is very divergent among different Cx isoforms and the region that is targeted by L2 or Gap19, i.e., the last 10 amino acids of Cx43, is not present in other Cx isoforms (see Table 2 in Iyyathurai et al., 2013 for the BLAST results obtained by using the L2 and CT10 sequences of human Cx43 as sources). This is supported by experimental evidence, showing that Gap19 did neither inhibit Cx40 hemichannels nor Panx1 channels (Wang et al., 2013b).

These tools now set the stage for the discovery of novel cell biological and physiological roles for Cx43 hemichannels and the inhibition of pathological, excessive Cx43 hemichannel openings in disease conditions, including ischemia/reperfusion in the heart (Iyyathurai et al., 2013; Saez and Leybaert, 2014). Furthermore, these tools not only target plasmalemmal Cx43 hemichannels, but may also target Cx43 hemichannels present in the inner mitochondrial membrane (Boengler et al., 2013). For instance, Cx43 has been found in the inner mitochondrial membrane of subsarcolemmal mitochondria from ventricular cardiomyocytes (Boengler et al., 2009). Cx43 has been implicated in mitochondrial K^+ uptake (Miro-Casas et al., 2009). Genetic ablation of Cx43 as well as Gap19 decreased the rate of mitochondrial K^+ uptake in subsarcolemmal mitochondria (Boengler et al., 2013). Hence, the acute inhibition of Cx43 hemichannels using Gap19 elucidated a novel cell biological role for Cx43 hemichannels at the level of the mitochondria modulating mitochondrial K^+ uptake.

TAT-L2 has also been applied in the context of the brain. In particular, micro-injection of the TAT-L2 peptide in the basolateral amygdala, a region of the brain involved in memory consolidation, of auditory fear-conditioned rats resulted in ablation of memory consolidation without impacting short-term memory, locomotion, or shock reactivity or without affecting synaptic transmission or interastrocyte gap junctional communication (Stehberg et al., 2012). The amnesic effect of TAT-L2 was related to an impaired gliotransmitter release from astrocytes, since a cocktail of gliotransmitters including glutamate, glutamine, lactate, D-serine, glycine, and ATP could restore the learning capacity of the rats. As such, TAT-L2 as a selective Cx43-hemichannel inhibitor led to the discovery of a novel physiological role for Cx43 hemichannels as release pathways for gliotransmitters essential for memory consolidation in the basolateral amygdala.

TAT-CT10 has been applied in the context of satellite glial cells, the main glia in sensory ganglia. They are proposed to prevent the formation of electrical and chemical synapses between neighboring neurons. However, paracrine signaling between glial cells and sensory neurons may occur. A role for hemichannels has been implicated in increased vagal nerve activity after nodose neuron exposure to Ca^{2+}-free solution. Interestingly, TAT-CT10 displayed a similar effect as Ca^{2+}-free solution on these sensory neurons, thereby facilitating paracrine signaling between satellite glial cells and neurons (Retamal et al., 2014).

Gap19 has been applied in the context of the heart exposed to ischemia/reperfusion (Wang et al., 2013b). Importantly, Gap19 not only inhibited the "physiological" opening of native Cx43 hemichannels present in ventricular cardiomyocytes obtained from healthy hearts, but also suppressed the excessive, "pathophysiological" opening of Cx43 hemichannels from ventricular cardiomyocytes exposed to metabolic inhibition triggered by a mitochondrial uncoupler and a glycolysis inhibitor (Wang et al., 2013b). Consistent with this, Gap19 also suppressed the deleterious effects of ischemia/reperfusion on cardiomyocyte viability *in vitro*, thereby maintaining the cell volume of cardiomyocytes and limiting the occurrence of cell death. Finally, *in vivo* ischemia/reperfusion experiments in mice showed that pre-incubation with Gap19 significantly decreased the infarct size.

REMAINING QUESTIONS AND FUTURE DIRECTIONS

THE LINK BETWEEN THE ACTIN/MYOSIN CYTOSKELETON AND Cx43 HEMICHANNELS?

Two models for the regulation of Cx43 hemichannels by actin/myosin contractility, including a "direct" linker protein that bridges the C-terminal tail of Cx43 with the actin/myosin cytoskeleton and an "indirect" membrane-embedded "sensor" of the contractile state of the actin/myosin cytoskeleton that can also bind to the C-terminal tail of Cx43 hemichannels via an adaptor protein (Ponsaerts et al., 2012b). Different proteins associated with the actin/myosin cytoskeleton have been implicated in interactions with Cx43 proteins, but also actin may directly bind Cx43 proteins (Butkevich et al., 2004; Li et al., 2005; Wall et al., 2007; Sin et al., 2009; Vitale et al., 2009; Herve et al., 2012). In addition, we may not exclude that these interactions could be modulated by Ca^{2+}-dependent changes in the phosphorylation state of Cx43 hemichannels due to the activation of Ca^{2+}-dependent kinases or phosphatases (Solan and Lampe, 2009; O'quinn et al., 2011; Palatinus et al., 2011; Marquez-Rosado et al., 2012).

OTHER DOMAINS WITHIN THE C-TERMINAL TAIL CONTRIBUTING TO THE CONTROL OF Cx43-HEMICHANNEL ACTIVITY?

The current model for loop/tail interactions controlling Cx43-hemichannel activity involves the Gap19 region in the cytosplasmic loop and the CT10 region in the C-terminal tail. Although these domains are sufficient to either inhibit Cx43-hemichannel opening or to restore Cx43-hemichannel activity, it cannot be excluded that other domains in the C-terminal tail of Cx43 can modulate or contribute to the interactions with the Gap19/L2 region of the cytoplasmic loop and thus the activity of Cx43 hemichannels. In particular, previous studies in Cx43 gap junctions have implicated other domains acting as the gating particle controlling Cx43 gap junction activity. For instance, a 17-mer peptide corresponding to the region 271 to 287 of Cx43 is able to inhibit Cx43 gap junctions (Calero et al., 1998). However, the relevance of this region and/or other regions within the C-terminal tail for controlling Cx43-hemichannel activity remains to be elucidated.

LOOP/TAIL INTERACTIONS AS A GENERAL CONCEPT IN CONTROLLING THE ACTIVITY OF OTHER Cx ISOFORMS?

It also remains to be elucidated whether the concept of loop/tail interactions being critical for the activity of Cx hemichannels is

limited to Cx43 or can be a general mechanism operative in other Cx isoforms. In any case, although the "hot spots" for loop/tail interactions in Cx43 seem not highly conserved among other Cx isoforms (see Iyyathurai et al., 2013 for a detailed discussion), it could be possible that other amino acid stretches or motifs within the loop and tail of other Cx isoforms may establish such interactions. For instance, a recent report from Jiang and co-workers indicates that loop/tail interactions may exist in Cx46 hemichannels (Ren et al., 2013). Indeed, the $Cx46^{G143R}$ mutation decreases gap junctional coupling while increasing hemichannel activity. Consistent with this, a GST-fusion protein containing the Cx46 loop region displayed enhanced Cx46-binding properties when containing the cataract-causing G143R mutation (Ren et al., 2013). These data seem to suggest that at least for Cx isoforms belonging to the gap junction family, loop/tail interaction may be operative and control the opening of hemichannels. Interestingly, the introduction of an additional positive charge in the loop enhancing Cx46-hemichannel activity and promoting Cx46 binding seems to suggest a prominent role for charge-based interactions, similar to Cx43. Until now, there is no evidence whether other Cx isoforms belonging to other gap junction families can be controlled by loop/tail interactions. Of note, Cx32 also displays a bell-shaped dependence toward cytosolic $[Ca^{2+}]$ rises (De Vuyst et al., 2006), but it remains to be established whether this is due to the occurrence of loop/tail interactions and their modulation by contractility.

CONCLUSIONS

We hereby show that primary BCECs are a good model system for studying the properties of native Cx43 hemichannels and their regulation by physiological signaling events. These studies ought to be complemented with knockdown experiments and ectopically expressed Cx43 hemichannels, allowing the expression of mutated and truncated Cx43 versions. The integrated approach has led to important discoveries, including the negative regulation of Cx43-hemichannel opening by the actin/myosin contractile system, the essential role of loop/tail interactions for Cx43-hemichannel activity and to the development of novel peptide tools that allow selective Cx43-hemichannel inhibition without affecting Cx43 gap junctions or hemichannels formed by other Cx isoforms.

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Role of Pannexin-1 hemichannels and purinergic receptors in the pathogenesis of human diseases

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In the last decade several groups have determined the key role of hemichannels formed by pannexins or connexins, extracellular ATP and purinergic receptors in physiological and pathological conditions. Our work and the work of others, indicate that the opening of Pannexin-1 hemichannels and activation of purinergic receptors by extracellular ATP is essential for HIV infection, cellular migration, inflammation, atherosclerosis, stroke, and apoptosis. Thus, this review discusses the importance of purinergic receptors, Panx-1 hemichannels and extracellular ATP in the pathogenesis of several human diseases and their potential use to design novel therapeutic approaches.

Keywords: HIV, inflammation, connexins, atherosclerosis, ATP

INTRODUCTION

In recent years it has become evident that pannexin (Panx)-1 hemichannels in concert with extracellular adenosine triphosphate (ATP) and purinergic receptors are involved in several physiological and pathological conditions. Recent evidence suggests the participation of Panx-1 hemichannels, extracellular ATP and purinergic receptors in the coordination of events such as cellular activation, apoptosis, stress signals, secretion of inflammatory cytokines, and HIV replication have been explored (Pelegrin, 2008; Schenk et al., 2008; Chekeni et al., 2010; Woehrle et al., 2010; Qu et al., 2011; Seror et al., 2011; Orellana et al., 2013). This review will describe these interactions in the context of several human diseases.

The Panx family consists of three members, Panx-1, -2, and -3 (Baranova et al., 2004). Panx-1 is ubiquitously expressed (Scemes et al., 2009). Panx-2 is mainly expressed in the central nervous system (CNS), while Panx-3 is localized in osteoblasts, synovial fibroblasts and chondrocytes (Barbe et al., 2006; Ray et al., 2006). Originally, it was speculated that Panx hemichannels could form gap junction channels between adjacent cells (Bruzzone et al., 2003, 2005; Bruzzone and Dermietzel, 2006). However, current evidence suggests that Panxs cannot form intercellular channels (Boassa et al., 2007). Asparagine residues found on the extracellular domains of Panx are glycosylated and therefore make docking between two Panxs highly unlikely (Boassa et al., 2007; Penuela et al., 2007).

Panxs are structurally similar to connexins (Cxs), although they share no sequence homology. Panx consist of a cytosolic N-terminal domain, four transmembrane domains with two extracellular loops and a cytosolic C-terminal domain (Boassa et al., 2007; Penuela et al., 2007). Panxs form large pore channels located on the plasma membrane, which are known to open during membrane depolarization, by changes in intracellular Ca^{2+} signaling, vasodilation, vasoconstriction, taste sensation, airway defense, learning/memory, cellular differentiation, cell death and during innate, and adaptive immune responses (Chekeni et al., 2010; MacVicar and Thompson, 2010; Prochnow et al., 2012). Upon opening of these hemichannels small signaling molecules such as ATP are released to the extracellular space, which then signal through surface receptors including purinergic receptors.

Purinergic receptors are divided into two groups, Adenosine receptors (ARs) for adenosine and P2 receptors for ATP/ADP receptors (Fredholm et al., 1994; Ralevic and Burnstock, 1998). P2 receptors are subdivided into ionotropic P2X and metabotropic P2Y receptors. P2X receptors are ligand gated ion channels that form trimeric structures using individual subunits (P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, and P2X7) (Fredholm et al., 1994; Ralevic and Burnstock, 1998). P2Y receptors are G-protein coupled receptors with eight subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14). Purinergic receptor signaling is fundamental in many cellular processes such as platelet aggregation, exocrine and endocrine secretion, endothelial-mediated vasodilation, nociceptive mechanosensory transduction, neuromodulation, neuroprotection, cell proliferation, differentiation, migration, embryological development, wound healing, inflammation, and cytokine secretion (Abbracchio and Burnstock, 1998; Burnstock and Knight, 2004; Burnstock and Verkhratsky, 2010; Burnstock, 2012). Upon release of ATP into the extracellular space, several enzymes degrade ATP into ADP, AMP and

adenosine, which also signals through purinergic receptors and adenosine receptors.

ARs (A1, A2A, A2B, and A3) were initially classified as P1 receptors until it was discovered that their agonist was adenosine (Fredholm et al., 1994; Junger, 2011). As indicated above, the production of extracellular adenosine is achieved by hydrolyzing ATP in a stepwise manner by ectonucleotidases (Yegutkin, 2008). These enzymes include the Ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family comprised of three enzyme subtypes, which hydrolyses ATP to AMP, and Ecto-nucleoside triphosphate diphosphydrolase (E-NTDPase) family comprised of eight enzyme subtypes, which can hydrolyze ATP to ADP or AMP. Finally, Ecto-5'-nucleotidase/ CD73 in tandem with CD38 can hydrolyze AMP to adenosine, which then activates the ARs (Yegutkin, 2008; Junger, 2011). In **Figure 1** we summarized the interaction between Panx-1 hemichannels, purinergic receptors, adenosine receptor, as well as the extracellular metabolism of ATP (**Figure 1**). In the next sections we will discuss the involvement of this complex in several human diseases.

PURINERGIC RECEPTORS, PANX-1 HEMICHANNELS AND THEIR INVOLVEMENT IN ISCHEMIC STROKE

According to the World Health Organization (WHO), 15 million people suffer stroke worldwide each year, resulting in 5 million deaths and another 5 million survivors that are permanently disabled (www.WHO.int). Ischemic stroke results from a permanent or transient decrease in cerebral blood flow. This decrease in blood flow is usually caused by the obstruction of a cerebral artery by an embolus or local thrombosis (Katsura et al., 1994; Martin et al., 1994; Dirnagl et al., 1999). Brain tissue requires a high intake of glucose and oxygenation for proper cerebral function. The restriction of cerebral blood flow impairs the delivery of glucose and oxygen and consequently leads to tissue damage by mechanisms dependent on excitotoxicity, peri-infarct depolarizations, inflammation and programmed cell death (Katsura et al., 1994; Martin et al., 1994; Dirnagl et al., 1999).

Thompson et al. demonstrated a connection between Panx-1 hemichannels and ischemia using acutely isolated hippocampal neurons in which oxygen and glucose deprivation (OGD) resulted in opening of Panx-1 hemichannels (Thompson et al., 2006). Blocking NMDA (N-methyl-D-aspartate), AMPA (2-amino-3-[5-methyl-3-oxo-1,2-oxazol-4-yl] propanoic acid) and P2X7 receptors failed to modify the large anoxic depolarization activated by OGD, which corresponded to opening of Panx-1 hemichannels. Therefore, the mechanism by which Panx-1 hemichannels are opened during OGD was thought to be independent from ligand-gated receptors (Thompson et al., 2006). Recent evidence suggests that anoxia induces NMDA receptor

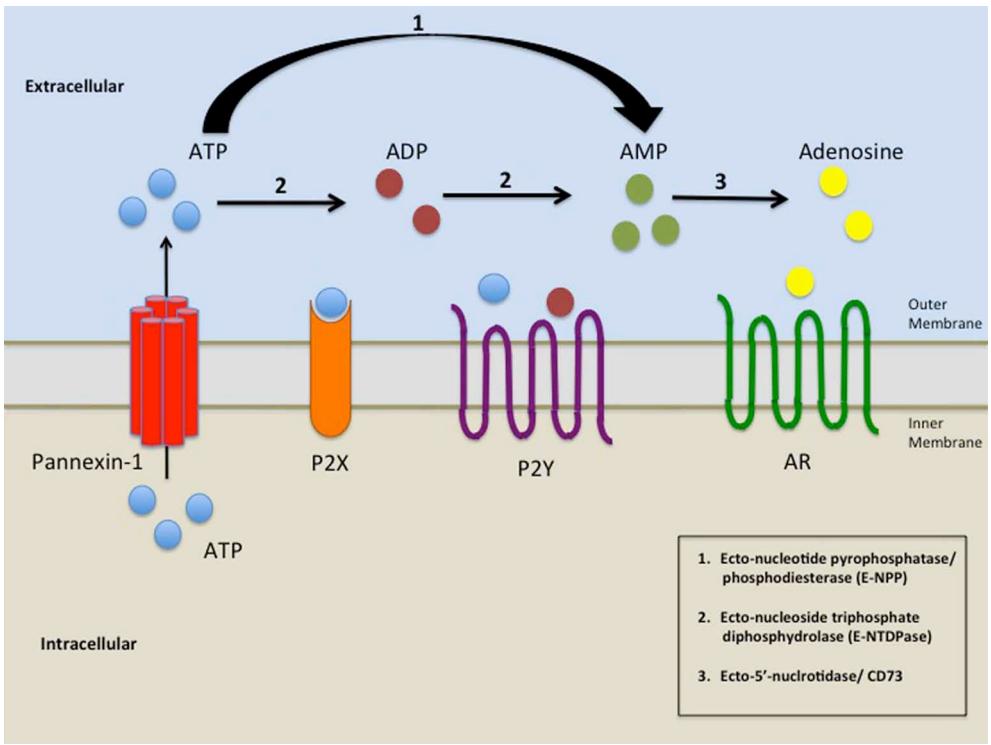

FIGURE 1 | A schematic representation of the elements involved in the release of ATP by opening of Panx-1 hemichannels and subsequent activation of purinergic signaling. Pathological or physiological stimuli result in the opening of Panx-1 hemichannels promoting the release of ATP from the cell. ATP/ADP/AMP could then bind to P2X and P2Y receptors. Ecto-nucleoside triphosphate diphosphydrolase (E-NTDPase) including ecto-ATPase and ATP-diphosphohydrolase promotes the hydrolysis of ATP to ADP or from ADP to AMP (2). Ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) hydrolysis ATP to AMP (1). AMP is further hydrolyzed by Ecto-5'-nucleotidase/ CD73 (3) which promotes the formation of adenosine. Adenosine then activates adenosine receptors (AR).

activation, which activates Src kinases, that participates in the opening of Panx-1 hemichannels (Weilinger et al., 2012). This suggests a signaling pathway involving the coupling of NMDA receptors using Src Kinases to Panx-1 hemichannels. Furthermore overstimulation of NMDA receptors activates the opening of Panx-1 hemichannels in neurons (Thompson et al., 2008). However, another study demonstrated that inhibiting glutamate receptors of hippocampal pyramidal slices prevented anoxic depolarization and that Panx1 hemichannels did not generate a large inward current associated with anoxic depolarization (Madry et al., 2010). Therefore, future studies are required to clarify the participation of Panx-1 hemichannels in response to anoxia/ischemia.

ATP is a neurotransmitter that mediates communication between CNS cells, including astrocytes and neurons. Initially, it was believed that the mechanism by which ATP was released from astrocytes involved Connexin43 (Cx43) hemichannels. However, experiments conducted using wild type, Cx43 null, and Panx-1 knocked down astrocytes provided evidence indicating that downregulation of Panx-1 prevented the release of ATP from astrocytes (Iglesias et al., 2009). Downregulation of Cx43 has no affect on the release of ATP from astrocytes. In contrast, experiments using conditional Cx43 knockout demonstrated that ATP release and recruitment of microglia/macrophages following injury was reduced and the recovery of the animals was improved, suggesting a role for Cx43 hemichannels in inflammation but also in recovery (Huang et al., 2012). Orellana et al. demonstrated that under hypoxic conditions astrocytes release ATP and glutamate activating neuronal Panx-1 hemichannels via P2X and NMDA receptors resulting in neuronal death (Orellana et al., 2011). This study demonstrates that neurons could be protected from ischemia-associated damage by blocking NMDA/P2X receptors as well as Panx-1 hemichannels (Orellana et al., 2011).

Furthermore experiments conducted in the double Panx-1 and Panx-2 knockout mice subjected to permanent right middle cerebral artery occlusion (MCAO) demonstrated that Panx channels contributed to ischemic brain injury *in vivo* (Bargiotas et al., 2011). The double knockout mice subjected to MCAO demonstrated improved neurological deficit, reduced movement latency and infarct size as compared to the wild type (Bargiotas et al., 2011). Single knockouts of either Panx-1 or Panx-2 did not differ in ischemic brain injury from wild type; however, Panx-2 knockout mice were partially protected from ischemic injury. These data suggests that Panx-1 and Panx-2 work together to regulate response to injury.

Ischemia induces astrocytes to release ATP, which rapidly activates microglia resulting in the formation of a barrier between the healthy and injured tissue in order to promote repair (Davalos et al., 2005; Nimmerjahn et al., 2005). Moreover, an excess release of nucleotides can result in accelerated neurodegeneration (Di Virgilio et al., 2009). An excessive level of extracellular ATP in oligodendrocytes induces a rise in cytosolic Ca^{2+} by activating P2 receptors and P2Y7 receptors (Kirischuk et al., 1995; James and Butt, 2001). Using primary cultures of oligodendrocytes it was demonstrated that OGD induced the release of ATP and blocking of P2X7 receptors using periodate oxidized ATP (oATP) or Brilliant Blue G (BBG) reduces the ischemic-induced ionic imbalance. In addition, reduction in opening of Panx hemichannels using blockers such as mefloquine and flufenamic acid reduced extracellular ATP levels after OGD attenuating ischemic damage. These data indicates that OGD opens Panx hemichannels inducing the release of ATP which then activates P2X7 receptors causing oligodendrocytes failure, myelin damage and axon dysfunction (Domercq et al., 2010).

Furthermore, elevations in the expression of several P2 receptors (P2X1, P2X2, P2X4, P2X7, and P2Y4) during ischemia have been demonstrated suggesting increased sensitivity of neurons to extracellular concentration of ATP (Cavaliere et al., 2002, 2003, 2007). Using spontaneously hypertensive rats (SHR) subjected to MCAO Lammer et al. demonstrated that inhibition of P2 receptors by pyridoxalphosphate-6-azophenyl-2', 4'-disulfonate (PPADS) improved the recovery of the cortical electrophysiological and motor functions (Lammer et al., 2006, 2011). PPADS does not pass through the blood brain barrier; therefore, the rats were infused by intracerebroventricular administration for 7 days after MCAO. Furthermore, analysis of motor coordination demonstrated that blockade of P2 receptors by PPADS resulted in improved motor recovery when compared to non-PPADS treated rats subjected to MCAO (Lammer et al., 2011). Thus, opening of Panx-1 hemichannels and activation of P2 receptors play a major role in the pathogenesis of ischemia and blocking or knocking down these hemichannels/receptors could provide additional therapeutic interventions to reduce damage and to improve recovery in response to ischemic events.

PANX HEMICHANNELS, PURINERGIC RECEPTORS AND INFLAMMATION

Tissue damage causes the release of ATP from injured cells, resulting in P2 receptor mediated purinergic signaling and the initiation of inflammation (Bours et al., 2006; Kanneganti et al., 2006; Mariathasan et al., 2006). During this process in both immune and parenchymal cells, hemichannels are open in concert with activation of purinergic receptors to control cellular migration, inflammation, and damage.

As indicated above an essential aspect of inflammation is the migration of inflammatory cells into areas of injury. Cellular migration requires mechanisms to allow orientated movement including sensing changes in the chemoattractant gradient, activation of G-protein coupled receptors, and downstream signaling resulting in cytoskeletal rearrangement leading to movement toward the chemotactic signal. Recent evidence suggests that Panx-1 hemichannels and P2X7 could initiate an intracellular signaling cascade which, results in rearrangement of the F-actin microfilament network in C6 glioma cells causing the assembly of large tumor cell aggregates (Bao et al., 2012). A similar actin microfilament rearrangement as mentioned above is a critical step in cellular migration. Intracellular ATP is released through Panx-1 hemichannels and then binds to the P2X7 receptor (see **Figure 1**), which causes an increase in intracellular calcium resulting in actin microfilament organization (Cotrina et al., 1998; Suadicani et al., 2006). Current evidence suggests that the release of ATP by a Panx-1 hemichannel mediated mechanism from apoptotic cells function as "Find me signals" in order to recruit monocytes to

areas of damage (Chekeni et al., 2010). Our laboratory demonstrated that chemokines that bind to CCR5 or CXCR4 transiently open Panx-1 hemichannels in T lymphocytes, suggesting that these channels also play a key role in surveillance and inflammation. Thus our work and the work of others propose that Panx-1 hemichannels, extracellular ATP and purinergic receptors are essential in immune surveillance and inflammation.

Migration in a chemotactic gradient requires excitatory signals at the front of the cell and inhibitory signals at the back of the cell (Berzat and Hall, 2010). In this context ATP released by Panx-1 hemichannels stimulates the P2Y2 receptors, which provides the excitatory signal at the front of the cell (Chen et al., 2006, 2010). Bao et al. demonstrated that Panx-1 hemichannels provides the ligand for the adenosine A2A receptors that plays a role in the inhibitory signal at the back of the cell (Bao et al., 2013). Resting neutrophils had a uniform distribution across the cell of A2A receptors and polarized cells had the A2A receptors redistributed to the back of the cell where they provided the inhibitory signal. Inhibition of Panx-1 hemichannels blocked A2A receptor stimulation preventing the accumulation of cAMP, impairing the polarization and migration of neutrophils in a chemotactic gradient (Bao et al., 2013). These results suggest that chemoattractant receptors require opening of Panx-1 hemichannels in order to provide excitatory and inhibitory signals for efficient chemotaxis of neutrophils.

Inflammasomes are large multiprotein complexes leading to caspases-1-activated maturation of IL-1β and IL-18. The NLRP3 inflammasome is the most studied inflammasome containing NLRP3 as a scaffold protein (Schroder and Tschopp, 2010; Davis et al., 2011). NLRP3 inflammasomes are activated via danger-associated molecular patterns (DAMPs) such as extracellular ATP, which act through P2X7 receptors (Lich et al., 2006; Meylan et al., 2006; Said-Sadier and Ojcius, 2012). There are several proposed mechanisms, which induce NLRP3 inflammasome activation such as, reactive oxygen species (ROS) production and apoptosis (Said-Sadier and Ojcius, 2012). Cell induced ROS production and immune activation, have been shown to induce caspase-1 activation (Cruz et al., 2007; Said-Sadier and Ojcius, 2012). Hung et al. demonstrated that activation of P2X4 and P2X7 in response to ATP released by Panx-1 hemichannels contributed to ATP induced ROS production and inflammasome activation in gingival epithelial cells (Hung et al., 2013). Inhibitors of P2X4, P2X7, and Panx-1 significantly reduced ATP dependent production of ROS. Reduced expression of P2X4, P2X7, and Panx-1 using siRNA demonstrated that both purinergic receptors and Panx-1 hemichannel were required for ATP-induced ROS production in primary and immortalized gingival epithelial cells (Hung et al., 2013). Furthermore, recent evidence identifies that the NLRP3 inflammasome is activated during the phagocytosis of dying autophagic cells. This mechanism involves the release of ATP through Panx-1 hemichannels of the dying autophagic cell, P2X7 receptor activation and potassium efflux (Ayna et al., 2012).

In agreement, patients who have chronic lung inflammation such as allergic asthma or chronic obstructive pulmonary disease have enhanced extracellular ATP levels in the bronchoalveolar space, as well as in the bronchoalveolar lavage fluids (BALF) suggesting that the enhanced lung inflammation observed in these individuals may be associated with ATP dysregulation and purinergic receptor activation (Idzko et al., 2007; Lommatzsch et al., 2010). Furthermore, P2X7 receptor deficient mice have been shown to have less neutrophil airway influx and Panx-1 hemichannel inhibitors partially prevent further neutrophil airway influx and cytokine production (Riteau et al., 2010). Extracellular ATP serves as a danger signal to the immune system by binding to P2X7 receptor and activating NALP3 and caspase-1 which then leads to the maturation and release of IL-1β, eventually forming the NALP3 inflammasome (Ferrari et al., 2006; Kanneganti et al., 2006; Mariathasan et al., 2006; Sutterwala et al., 2006; Di Virgilio, 2007). Extracellular ATP induced caspase-1 activation and IL-1β maturation requires P2X7 receptor and Panx-1 hemichannel (Pelegrin and Surprenant, 2006; Locovei et al., 2007). These data further suggests the involvement of Panx-1 hemichannels, purinergic receptors and extracellular ATP in inflammasome activation.

Another human disease involving Panx-1 hemichannels is inflammatory bowel diseases (IBD) including ulcerative colitis, and Crohn's disease. These diseases are chronic conditions associated with gut dysfunction resulting from alterations in the enteric nervous system leading to severe symptoms (Mawe et al., 2009). Currently not much is known about the expression of Panx-1 in the intestines. However, one study showed the expression of innexins in the gut of nematodes, which were needed for gut motility (Peters et al., 2007). Recently Gulbransen et al. showed using a mouse model of colitis that Panx-1 hemichannels are required for P2X7 receptor mediated enteric neuron cell death in intestinal inflammation (Gulbransen et al., 2012). They identified that Panx-1 hemichannels play a key role in enteric neuronal damage, leading to organ dysfunction. Inhibition of Panx-1 hemichannels protects neurons and maintains proper control of the colonic muscles preserving motility (Gulbransen et al., 2012). Diezmos et al. described the expression of Panx-1 in the human colon; they also described alterations in the expression of Panx-1 in IBD patients (Diezmos et al., 2013). Panx-1 mRNA and protein was present in all layers of the human colon. There was also dense expression of Panx-1 on the submucosal and myenteric ganglia further confirming the involvement of Panx-1 in neural control of colonic motility (Diezmos et al., 2013). These findings suggest a critical role of Panx-1 hemichannels in the pathophysiology of enteric plexus damage during IBD.

As described above ATP release by opening of Panx-1 hemichannels results not only in activation of ATP receptors but also in degradation of ATP to ADP, AMP, and adenosine. CD39 and CD73 are ectonucleotidases, which degrade ATP, ADP, and AMP to adenosine (see **Figure 1**). These ectonucleotidases play an essential role in maintaining immune homeostasis. Regulatory T cells (Tregs) are mediators of inflammatory response. High levels of CD39 and CD73 are expressed on the surface of $Foxp3^+$ Treg cells (Mandapathil et al., 2009; Schuler et al., 2011). Murine Treg cells increase CD39 activity after activation of the T cell receptor and non-activated cells had inactive CD39 (Borsellino et al., 2007). Adenosine derived from the enzymatic breakdown of ATP by CD39 and CD73 mediates a considerable portion of the anti-inflammatory activities of Treg cells (Deaglio et al., 2007). Romio et al. showed that adenosine produced by Treg cells in concert

with A2A receptors downregulated nuclear factor- κB (NF- κB) activation in T effector cells, which in turn reduced the release of proinflammatory cytokines and chemokines (Romio et al., 2011). Activation of the A2A receptor on Treg cells promotes the expansion of these cells, thereby increasing immune regulation (Ohta et al., 2012). In humans 90% of Foxp3$^+$ Treg cells also express CD39 however CD73 expression is minimal (Mandapathil et al., 2009; Dwyer et al., 2010; Mandapathil et al., 2010). Antonioli et al. speculated that CD73 may be secreted from human Treg cells and is responsible for the production of adenosine (Antonioli et al., 2013). Qiu et al. demonstrated that cells co-expressing Panx-1 hemichannel and P2Y or P2X7 receptors exposed to high levels of ATP, only transiently activates Panx-1 hemichannel (Qiu and Dahl, 2009). ATP instead of causing a positive feedback loop is actually causing a negative feedback loop and inactivating Panx-1 hemichannel. This mechanism could provide another mode of immuneregulation suppressing the immune response in order to prevent damage caused by prolonged inflammation.

PURINERGIC/ADENOSINE RECEPTORS AND THEIR ROLE IN ATHEROSCLEROSIS

Atherosclerosis is a chronic inflammatory disease affecting the vessel wall and a major health issue worldwide (Koupenova et al., 2012a,b). One of the major components of atherosclerosis is the formation of arterial plaques. The progression of atherosclerosis begins with the recruitment of inflammatory monocytes to the area of lipid deposition or arterial injury (Glass and Witztum, 2001; Reiss and Glass, 2006). As discussed in the previous section migration is a Panx-1 hemichannel, purinergic receptor and extracellular ATP dependent process. Infiltrating macrophages in the arterial wall take up large amounts of oxidized low-density lipoprotein (ox-LDL) becoming foam cells loaded with cholesterol (Stary et al., 1994). The accumulation of foam cells leads to the formation of fatty streaks, increase in arterial wall thickness, reduction of oxygen diffusion into the tissue and development of advanced atherosclerosis (Gessi et al., 2010; Hansson and Hermansson, 2011; Moore and Tabas, 2011; Koupenova et al., 2012a,b).

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor comprised of α and β subunits (Wang and Semenza, 1995). HIF-1 adapts cells to low oxygen partial pressure and induces target genes that influence energy metabolism, cell proliferation, hematopoiesis, vascular development, and vasotone (Semenza et al., 1994; Liu et al., 1995; Carmeliet et al., 1998; Kourembanas et al., 1998; Lacombe and Mayeux, 1998; Rose et al., 2002). Zones of hypoxia occur in the atherosclerotic plaque, a result of impaired oxygen diffusion due to the thickness of the lesion, as well as high oxygen consumption by the foam cell. Furthermore, it was demonstrated that ox-LDL induce HIF-1α accumulation in human Mono-Mac-6 (MM6) macrophages (Shatrov et al., 2003). Jiang et al. investigated the gene expression profiles of cultured human U937 cells transfected by HIF-1α-siRNA in response to 24 h of exposure to ox-LDL. Their results indicated that HIF-1α-siRNA inhibits the development of macrophage derived foam cells with ox-LDL by inhibiting the expression of HIF-1α (Jiang et al., 2007). A key function of HIF-1α is the expression of vascular endothelial growth factor (VEGF). VEGF is a regulator of angiogenesis during embryogenesis, skeletal growth and reproductive functions (Ferrara et al., 2003). Together HIF-1α and VEGF are involved in angiogenesis and atherogenesis. As described above the expression of HIF-1α in macrophages under atherogenic conditions promotes the formation of foam cells. Foam cells, macrophages and the U937 myelomonocytic cell line were individually cultured and treated with adenosine under hypoxic conditions which resulted in the accumulation of HIF-1α in all the cells (Gessi et al., 2010). When A1, A2A, A2B, and A3 receptors were knocked down using siRNA there was a reduction in the accumulation of HIF-1α in the cells. In addition, the production of VEGF in foam cells was increased when adenosine was added and strongly reduced with the addition of A2B, and A3 antagonist respectively (Gessi et al., 2010). Hypoxia stabilizes HIF-1α, resulting in the accumulation of adenosine (Gessi et al., 2010). Notably, it can be speculated that in this adenosine-mediated atherosclerosis mechanism, Panx-1 hemichannels may play a role in the release of intracellular ATP into the extracellular environment leading to the formation of adenosine (**Figure 1**).

Conversely Koupenova et al. determined that the absence of A2B adenosine receptor expression in the liver resulted in a worse atherosclerotic outcome in the double knockout mouse model of apolipoproteinE (ApoE) and A2B adenosine receptor which were fed a high fat/high cholesterol diet (Koupenova et al., 2012a,b). Lack of A2B adenosine receptor led to an elevation of plasma lipids and plaque formation. In this model the liver is responsible for contributing to the anti-atherosclerotic phenotype. Under normal conditions the liver expresses low levels of A2B adenosine receptor. However, with a high fat diet, levels of A2B receptors in the liver increase. Activation of A2B receptor in hepatocytes *in vivo* and *in vitro* causes a decrease in the transcription factor sterol regulatory element binding protein-1 (SREBP-1), which regulates lipid synthesis. Moreover, eliminating A2B adenosine receptor in the liver of the mouse model increased the levels of SREBP-1 and its downstream targets acetyl coenzyme-A carboxylase- α (ACC) and fatty acid synthase (FAS) resulting in upregulation of lipid synthesis. Resulting in the formation of foam cells and the development of atherosclerotic plaques. (Koupenova et al., 2012a,b).

As mentioned above the formation of an atherosclerotic plaque begins with the uptake and accumulation of cholesterol by macrophages and is also influenced by endothelial dysregulation. These atherosclerotic plaques are composed of smooth muscle cells (SMCs), which under normal physiological conditions are found in the medial layer of the artery wall. However, under atherosclerotic conditions SMCs lose their contractile element and gain the ability to replicate and migrate into the intima of the arterial wall (Gorski and Walsh, 1995). Once in the intima SMCs proliferate and begin depositing fibrotic connective tissue (Watson et al., 1998). All of these deregulated cells act as a cover for the fibrous cap that stabilizes the plaque by covering the lipid rich regions. Adenosine and ATP mediate endothelial cell growth, migration, proliferation and death (Burnstock, 2006). ATP binding of P2Y2 and/or P2Y4 stimulates SMC cell proliferation via a mitogen-activated protein kinase (MAPK) cascade contributing to the development of atherosclerosis (Hou et al., 2000). However, adenosine derived from the enzymatic breakdown of ATP by ecto-5′-nucleotidase (see

Figure 1) acts as an endogenous modulator protecting against vascular inflammation and immune cell recruitment, therefore, preventing the progression of atherosclerosis (Buchheiser et al., 2011). Adenosine in concert with A2A and A2B receptors has also been shown to stimulate endothelial cell proliferation and regulate the release of platelet-derived growth factor (PDGF) a smooth muscle mitogen from platelets (Jonzon et al., 1985; Adair, 2005).

Moreover, inflammation stimulated by accumulation of ox-LDL in the atherosclerotic plaque activates the release of cytokines and metalloproteinases resulting in degradation of the fibrous cap (Erlinge and Burnstock, 2008). These events result in a weak plaque, which can potentially rupture and release its content into the circulation. This content is highly thrombogenic and produces activation of platelets causing the formation of local thrombus occluding the artery or embolising and resulting in ischemic stroke or myocardial infarction (Erlinge and Burnstock, 2008) Pinheiro et al. demonstrated using human subcutaneous fibroblast that the release of histamine induces an increase in intracellular Ca^{2+} resulting in the release of ATP via Panx-1 hemichannels (Pinheiro et al., 2013). Furthermore, the release of ATP activates P2 receptors and results in fibroblast proliferation and collagen production. The principal cell type of vascular adventitia is fibroblast therefore increase proliferation of this cell type could contribute to atherosclerotic lesion progression and eventual rupture. This evidence suggests a complex mechanism, which results in plaque destabilization, and involves mast cells, histamine, P2 receptors, ATP, Panx-1 hemichannels and fibroblasts.

ROLE OF PANX-1 HEMICHANNELS IN APOPTOSIS

There are two main types of cell deaths, apoptosis and necrosis. Morphological features such as cell rounding, DNA fragmentation, externalization of phosphatidyl serine, caspase activation and the lack of an inflammatory reaction characterize apoptosis. Necrosis is characterized by swelling of organelles and plasma membrane, followed by the collapse of the plasma membrane and ending in the uncontrolled release of intracellular contents after the membrane has ruptured which leads to an inflammatory response. Intact apoptotic cells have been shown to release ATP and UTP without extrusion of additional cellular contents, suggesting the opening of relatively large membrane pores such as Cxs or Panx hemichannels during the apoptotic process (Harris, 2007; Elliott et al., 2009; Ghiringhelli et al., 2009; Scemes et al., 2009; MacVicar and Thompson, 2010).

Chekeni et al. showed in Jurkat cells that the channels involved in the release of ATP and UTP in apoptotic cells were Panx hemichannels and not Cxs hemichannels (Chekeni et al., 2010). Inhibition of these channels using 18-alpha-glycyrrhetinic acid (18AGA) or flufenamic acid (FFA), which are efficient Cxs hemichannel blockers had no effect on, the release of ATP induced by intact apoptotic cells (Chekeni et al., 2010). However, when specific Panx hemichannel blockers were used such as probenecid, the release of ATP was blocked from intact apoptotic cells. ATP is a chemoattractant for immune cells thus blocking ATP release by inhibiting Panx-1 hemichannel opening results in a decrease in monocyte recruitment (Chekeni et al., 2010).

Overexpression of Panx-1 increases the release of nucleotides during apoptosis, subsequently increasing monocyte migration. Activation of caspases 3/7 results in opening of Panx-1 hemichannels by a mechanism that involves cleavage of the Panx-1 intracellular carboxy terminal region increasing the release of ATP and UTP which is vital for apoptosis. Using whole cell patch clamp it was determined that opening of Panx-1 hemichannels and subsequent release of ATP occurs in the early events of apoptosis, and no opening of Panx-1 hemichannels was observed in the later events of apoptosis (Chekeni et al., 2010). Sandilos et al. determined that the C-terminus functions as a dissociable channel blocker, capable of inhibiting C-terminally truncated Panx-1 hemichannels and relief of C-terminal inhibition followed by cleavage does not happen if the C terminus is covalently tethered to the channel pore (Sandilos et al., 2012). This evidence suggests a role for Panx-1 hemichannels in the early events of apoptosis.

Divergent from the idea that Panx-1 hemichannels do not form gap junctions Vanden Abeele et al. demonstrated that overexpression of Panx-1 induces formation of Ca^{2+}permeable gap junction channels between cells allowing cellular Ca^{2+} diffusion and facilitating intercellular Ca^{2+} wave propagation (Vanden Abeele et al., 2006). Panx-1 overexpression also increased the Ca^{2+} permeability of the endoplasmic reticulum (ER) membrane and affected intraluminal ER Ca^{2+} concentration. Using human prostate cancer epithelial cells (LNCaP) and human embryonic kidney cells (HEK-293) they demonstrated that while overexpression drastically reduced intraluminal Ca^{2+}, endogenous Panx-1 depletion using siRNA increased the content of Ca^{2+} in the ER. This data suggests that Panx-1 hemichannels are not only found on the plasma membrane but also in the ER membrane, and it participates in ER Ca^{2+} leak and intracellular Ca^{2+}movement. Vanden Abeele et al speculated that the reduced concentration of Ca^{2+} associated with Panx-1 overexpression could be caused by increase of the BCL-2 family of proteins which plays an important role in the regulation of calcium leak from the ER and is an anti-apoptotic protein (Pinton et al., 2000; Vanden Abeele et al., 2002, 2006; Bassik et al., 2004). It may also be due to a deficiency of two pro-apoptotic proteins Bax and Bak (Scorrano et al., 2003; Oakes et al., 2005). This data suggests that Panx-1 could be involved in apoptotic events taking place in endomembranous compartments such as the ER.

PURINERGIC RECEPTORS AND PANX-1 HEMICHANNEL IMPORTANCE IN HIV-1 INFECTION

The first clinical observations of acquired immune deficiency syndrome (AIDS) were reported in 1981. Since the identification of HIV as the virus responsible for AIDS the countries affected and the numbers of those infected rose to overwhelming numbers. As of 2011, there are 34 million people worldwide living with HIV according to the World Health Organization. A total of 2.5 million new infections were reported in 2011 with 1.7 million deaths related to AIDS.

The established model for HIV entry into cells is mediated by the binding of HIV glycoprotein (gp) 120 to the cellular CD4 receptor. This interaction induces a conformational change to allow the glycoprotein to bind to the co-receptors CXCR4 and/or CCR5. The interaction of gp120 with these two host receptors creates a stable attachment between the virus and the cell membrane facilitating successful viral entry into the cell. In the past

two decades studies have documented that binding of HIV or gp120 to the cell rapidly increases the intracellular free calcium concentration (Weissman et al., 1997; Arthos et al., 2000; Liu et al., 2000; Balabanian et al., 2004; Melar et al., 2007). This rapid increase of intracellular free calcium suggests the potential involvement of other membrane receptors or channels in the early stages of HIV infection.

Our laboratory demonstrated that HIV infection of peripheral blood mononuclear cells (PBMCs) and CD_4^+ T lymphocytes causes opening of Panx-1 hemichannels in a biphasic manner (Orellana et al., 2013). Binding of the virus to its receptor (CD4) and co-receptors (CXCR4 and/or CCR5) induces opening of Panx-1 hemichannels. Opening of Panx-1 hemichannels in response to the virus resulted in ATP release and subsequent purinergic receptor activation. We also showed that opening of Panx-1 hemichannels was required for HIV entry and replication in CD_4^+ T lymphocytes. We propose that opening of Panx-1 hemichannels results in an increase of intracellular calcium and subsequent actin rearrangement, which is a necessary step that allows the virus to fuse with the host cell membrane. The details of these mechanisms are currently under investigation.

Our laboratory recently described a novel role for purinergic receptors in HIV replication in macrophages (**Figure 2**). We identified that P2X1, P2X7, and P2Y1 participate in HIV replication. Demonstrating that P2X1 is key in controlling viral entry into human macrophages. Although P2X7 and P2Y1 did not inhibit entry, it is highly likely that these receptors participate in later stages of the viral life cycles (Hazleton et al., 2012). We also identified the gp120's binding to primary human macrophages induces the release of ATP which facilitates autocrine activation of purinergic receptors. Panx-1 hemichannels, purinergic receptors and extracellular ATP play a key role in HIV infection and replication of HIV in immune cells by contributing to entry and possibly in other steps of the viral life cycle. In agreement another study using cell lines and PBMCs indicates that extracellular ATP activates P2Y2 receptors resulting in Pyk2 Kinase activation (Seror et al., 2011). It has been reported that Panx-1 hemichannels, P2Y2 and Pyk2 are physically recruited to the infection synapse (the contact site between the viral and cellular membrane) in order to facilitate infection (Seror et al., 2011). We propose that this process causes membrane depolarization and assists in membrane-to-membrane fusion allowing viral entry.

POTENTIAL HEMICHANNEL AND PURINERGIC RECEPTOR THERAPIES

Purinergic receptor, Panx-1 hemichannel, and extracellular ATP, ADP, AMP, and adenosine blockers and their potential use as therapeutic agents are under current investigation. Commercially

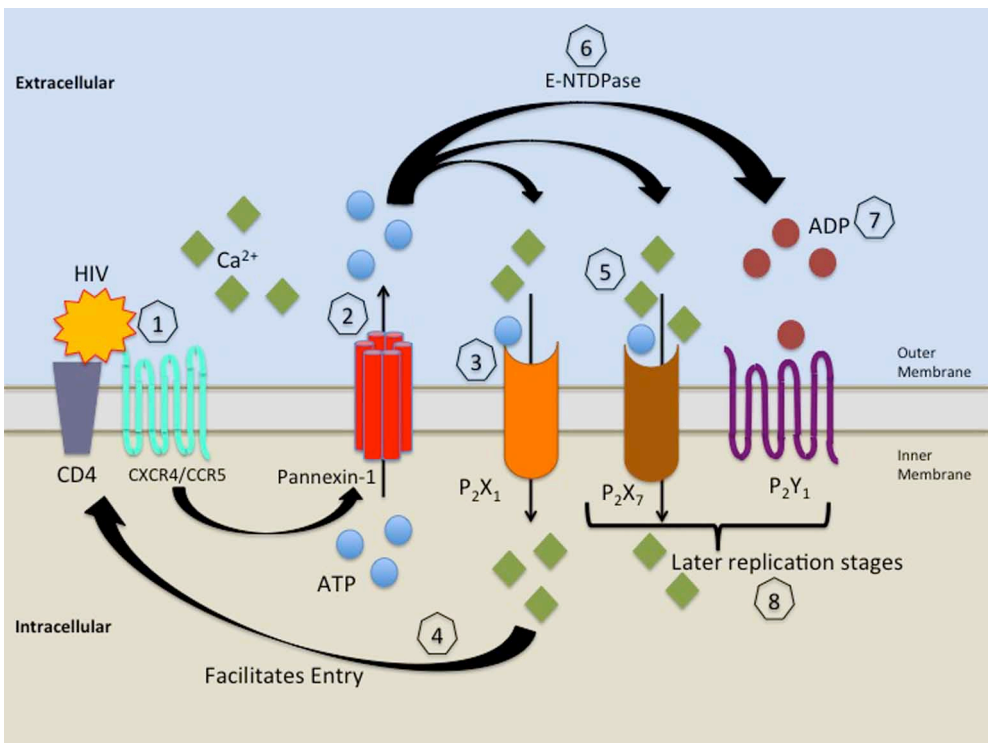

FIGURE 2 | Proposed model for the role of Panx-1 hemichannels and purinergic receptors in HIV infection. HIV's binding to CD4 and CXCR4/CCR5 (1) induces signaling which leads to opening of Panx-1 hemichannels and release of ATP (2). Extracellular ATP released through open Panx-1 hemichannels binds to and activates P2X1 receptors causing calcium influx (3), which facilitates HIV entry (4). The release of ATP continues and activates P2X7 and P2Y1 receptors (5), causing further calcium influx inducing downstream signaling, which facilitates later stages of the HIV life cycle (8). Ecto-nucleoside triphosphate diphosphydrolase (E-NTDPase) converts ATP to ADP (6), which activates P2Y1 receptors (7), which increases intracellular calcium, which causes signaling that facilitates later stages of the HIV life cycle (8).

available pharmaceutical adenosine, such as adenocard and adenoscan, are currently used to treat supraventricular tachycardia, is an example of an ion channel targeted treatment (Delacrétaz, 2006; Jacobson and Gao, 2006). Other clinically used drugs include dipyridamole and methotrexate, which are used to alter the extracellular adenosine concentration as well as signaling. Currently the US Food and Drug Administration (FDA) approved the A2A receptor agonist regadenoson (Lexiscan; Astellas Pharma) for myocardial perfusion imaging in patients with suspected coronary artery disease (Ghimire et al., 2013).

Among purinergic receptor blockers, which are, consumed daily are food dyes such as Brilliant Blue G (BBG) and Brilliant Blue FCF (BB FCF) which are found in most soft drinks. These dyes are shown to block at least P2X7 receptors, and Panx-1 hemichannels respectively (Jiang et al., 2000; Wang et al., 2013). In addition millions of people worldwide consume caffeine, which antagonize adenosine receptors and is used to treat premature apnoea. Actually, nothing is known of the effect that these dyes and caffeine have in physiological and pathological conditions. For example daily ingestion of these compounds in HIV positive individuals could cause the virus to adapt to these blockers changing the course of the disease. As mentioned above these channels/receptors play a role in inflammation and immune response, therefore individuals who consume these dyes in large quantities could also suffer from a suppressed immune response leading to numerous pathologies and susceptibility to pathogens. Further studies are required to investigate the effect that these dyes have among the human population.

The P2Y12 platelet receptor plays an important role in the genesis of platelet aggregation (Power et al., 2012; Tam et al., 2012). Current treatments blocking adenosine diphosphate (ADP) binding to the P2Y12 receptor, which inhibits platelet aggregation, are commercially available as well as in clinical trials. The first generation thienopyridine drugs which are used for their anti-platelet activity was ticlopidine, which bound irreversibly to P2Y12 platelet receptor (Cattaneo, 2010; Mohelmani and Jackson, 2012). However, its toxicity led to the development of second-generation thienopyridine clopidogrel (Ji and Hou, 2011). Clopidogrel also has its limitations, such as a delay in platelet block because the prodrug requires activation in the liver, and clopidogrel therapy is irreversible which can lead to increase bleeding and transfusion risk in cardiothoracic surgery (Power et al., 2012; Tam et al., 2012). Third generation thienopyridine prasugrel addressed the issue of delayed platelet blocking by being relatively independent of hepatic activation, however, it still remained irreversible and patients were still at risk for increase bleeding (Ferraris et al., 2012). Ticagrelor is an orally administered direct acting platelet blocker, which binds reversibly to the P2Y12receptor. This drug does not require metabolic conversion. It also belongs to a new class of drugs called cyclopyrimidines, which bind, non-competitively to the P2Y12receptor independently of the ADP binding site (van Giezen and Humphries, 2005). Ticagrelor when compared to prasugrel has demonstrated a more promising outcome with fewer side effects.

Numerous clinical trials are on going using various adenosine receptor agonist and antagonist. The expectation are high and could provide treatments for many physiological and pathological conditions such as lipolysis, renal blood flow, immune function, sleep regulation, angiogenesis, inflammatory diseases, ischemia-reperfusion, and neurodegenerative disorders (Sun et al., 2001; Huang et al., 2005; Fredholm, 2007; Johansson et al., 2007; Haskó et al., 2008; Rosenberger et al., 2009; Liu et al., 2010; Eltzschig and Carmeliet, 2011; Eltzschig and Eckle, 2011; Lazarus et al., 2011; Grenz et al., 2012). However, developing adenosine receptor targets is challenging because adenosine signaling is widespread. Therefore it is necessary to use ligands, which could be successfully administered to affect the area of interest, while being safe to use in a clinical setting.

Probenecid is a Panx-1 inhibitor, and has been on the market for decades as a treatment for gouty arthritis. High levels of extracellular potassium ion induce inflammasome activation and caspase 1 cleavage in neurons and astrocytes. In addition probenecid has been shown to attenuate the caspase 1 cleavage in cultured neurons induced by extracellular potassium ions (Peng et al., 2009). Recent evidence using a mouse model has shown that administering probenecid prior to and after stroke induced reduced infarct size, decreased cerebral water content, inhibited neuronal death, and reduced inflammation in the brain (Xiong et al., 2014). These results suggest that probenecid could be used as a treatment for stroke. Another Panx-1 inhibitor is carbenoxolone prescribed to treat oesophageal ulceration and inflammation. Probenecid and carbenoxolone could be ideal candidates as a treatment for pathological as well as physiological conditions were the inhibition of Panx-1 hemichannels could be useful. Other possible treatments could involve the use of mimetic peptides, which are designed with sequences found in the two extracellular loops of the Panx protein. These peptides mimic the loop-to-loop interaction between two hemichannels and activate a docking gate keeping the hemichannel closed. The designs of better and more specific blockers are required in the treatment of diseases involving Panx-1 hemichannels, purinergic receptors and ATP/adenosine.

CONCLUSION

In this review we have discussed the role which purinergic receptors and Panx-1 hemichannels play in the pathogenesis of several human diseases. It is crucial to understand the contribution of these receptors and channels in physiological and pathological conditions, in order to design new and improved therapeutic approaches. The contribution that purinergic receptors and Panx-1 hemichannels play in the HIV viral life cycle has only been recently described and unlocking this relationship could hold the key to the development of new preventative therapies and treatments. Purinergic receptor, Panx-1 hemichannel, and extracellular ATP, ADP, AMP, and adenosine are important modulators of many cellular events and hold great potential in understanding and treating many pathological and physiological conditions. The pathologies discussed in this review contribute to a large number of fatalities worldwide. Although much progress has been made in the advancement of treatments for these pathologies, there are still many avenues, which have not been explored. As more information regarding Panx-1 hemichannels and purinergic receptors emerge, the possibility of new therapeutic opportunities for these pathologies emerges as well.

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Molecular dynamics simulations highlight structural and functional alterations in deafness–related M34T mutation of connexin 26

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Mutations of the GJB2 gene encoding the connexin 26 (Cx26) gap junction protein, which is widely expressed in the inner ear, are the primary cause of hereditary non-syndromic hearing loss in several populations. The deafness–associated single amino acid substitution of methionine 34 (M34) in the first transmembrane helix (TM1) with a threonine (T) ensues in the production of mutant Cx26M34T channels that are correctly synthesized and assembled in the plasma membrane. However, mutant channels overexpressed in HeLa cells retain only 11% of the wild type unitary conductance. Here we extend and rationalize those findings by comparing wild type Cx26 (Cx26WT) and Cx26M34T mutant channels *in silico*, using molecular dynamics simulations. Our results indicate that the quaternary structure of the Cx26M34T hemichannel is altered at the level of the pore funnel due to the disruption of the hydrophobic interaction between M34 and tryptophan 3 (W3) in the N–terminal helix (NTH). Our simulations also show that external force stimuli applied to the NTHs can detach them from the inner wall of the pore more readily in the mutant than in the wild type hemichannel. These structural alterations significantly increase the free energy barrier encountered by permeating ions, correspondingly decreasing the unitary conductance of the Cx26M34T hemichannel. Our results accord with the proposal that the mutant resides most of the time in a low conductance state. However, the small displacement of the NTHs in our Cx26M34T hemichannel model is not compatible with the formation of a pore plug as in the related Cx26M34A mutant.

Keywords: genetic deafness, conductance, gating, gap junction channels, potential of mean force, mean first passage time

INTRODUCTION

Connexins are integral transmembrane proteins that form intercellular channels in vertebrates. Six connexins form a hexamerical assembly, known as connexon or hemichannel, which delineates an aqueous pore with a minimum diameter of ∼1.2 nm. When two hemichannels from adjacent cells dock and join, leaving a gap of ∼2–3 nm, they may form an intercellular gap junction channel which spans the two plasma membranes and allows the exchange of cytoplasmic molecules with size up to ∼1 kDa (Goodenough and Paul, 2009). The importance of electrical and molecular signaling through gap junction channels is widely recognized (Evans et al., 2006; Harris, 2007). It is also well established that connexin hemichannels open in response to various types of stimuli and conditions, including mechanical, shear, ionic and ischemic stress and provide a pathway for the release of intracellular ATP, glutamate, NAD^+ and prostaglandin E2, which act as paracrine messengers (Evans et al., 2006).

Virtually all cells in solid tissues are coupled by gap junctions (Goodenough and Paul, 2009), thus it is not surprising that mutations in connexin genes have been linked to a variety of human diseases, including cardiovascular anomalies, peripheral neuropathy, skin disorders, cataracts, and deafness (Wei et al., 2004; Laird, 2006; Dobrowolski and Willecke, 2009). Of notice, about half of all cases of human deafness in countries surrounding the Mediterranean have been linked to mutations in the *GJB2* gene, which encodes Cx26 (Zelante et al., 1997; Petit et al., 2001). In this paper, we focus on hemichannels formed by the deafness–associated Cx26M34T mutant. According to the published X–ray model of the human Cx26WT gap junction channel (Maeda et al., 2009), M34 interacts with W3 of the NTH belonging to an adjacent connexin. The six NTHs fold inside the pore and the M34–W3 hydrophobic interactions stabilize their position at the cytoplasmic mouth (see Figure 5 of Maeda et al., 2009).

The Cx26M34T mutant, which encodes full–length products, was originally described by Kelsell et al. (1997) who associated it with a dominant form of non-syndromic deafness (DFNA3) and also noted that M34 is conserved across several species both in Cx26 and in the closely related connexin 32 (Cx32) protein. Cx26M34T was also linked to a recessive form of hearing loss by Houseman et al. (2001). However, subsequent studies on the family first described by Kelsell et al. (1997) uncovered the association of dermatological signs in deaf patients and

identified another dominant mutation in *GJB2* segregating with the disease, casting doubts on the significance of the Cx26M34T variant. Other authors reported normal hearing in heterozygous carriers of Cx26M34T associated with either Cx26G35del or other Cx26 recessive mutations (Denoyelle et al., 1997; Kelley et al., 1998; Scott et al., 1998; Feldmann et al., 2004) and classified it as a benign polymorphism. An attempt to rationalize these results noted that, even though Cx26M34T is significantly overrepresented among patients, its relative penetrance is about 1/10 of that of undisputedly pathogenic mutations (Pollak et al., 2007).

The functional expression of the Cx26M34T connexin in *Xenopus* oocytes showed that the mutant exerts a dominant negative effect on Cx26WT (White et al., 1998). Later on Thonnissen et al. (2002) observed low levels of dye transfer between HeLa cells overexpressing Cx26M34T, providing the first evidence that this mutant could traffic to the cell membrane and form intercellular channels in a mammalian expression system, albeit with reduced efficiency. In contrast, Oshima et al. (2003) reported that assembly of Cx26M34T in HeLa and Sf9 cells resembles that of Cx26WT and that dye transfer in these cells is close to normal. Other electrophysiological studies performed in paired *Xenopus* oocytes concluded that Cx26M34T was capable of forming functional heterotypic channels with Cx32, albeit with abnormal gating properties (Skerrett et al., 2004). Based on these results, it was suggested that Cx26M34T/Cx32 heterotypic channels are not fully open at rest but are activated when positive transjunctional voltages are applied to the Cx26M34T side (Skerrett et al., 2004).

The pathogenetic role of M34T was confirmed in a study by Bicego et al. (2006) showing that, at a cellular level, Cx26M34T is correctly synthesized and targeted to the plasma membrane in HeLa cells, but inefficiently forms intercellular channels that display an abnormal electrical behavior and retain only 11% of the unitary conductance of Cx26WT. Moreover, Cx26M34T channels failed to support the intercellular diffusion of fluorescent tracers and the spreading of mechanically induced intercellular Ca^{2+} waves.

In the strictly homologous Cx32 protein, several mutations of Met34 have been associated with X linked Charcot–Marie–Tooth disease (M34T, M34I Tan et al., 1996, M34V Latour et al., 1997, M34K Yum et al., 2002). Single–channel recordings performed in transfected N2A cells showed that Cx32M34T mutants reside in a low–conductance (15 pS) substate 98% of the time at −80 mV (Oh et al., 1997).

Here, starting from previously described molecular models (Zonta et al., 2012, 2013) based on the 3.5 Å X–ray data (Maeda et al., 2009), we constructed a model of the Cx26M34T hemichannel and analyzed it by use of molecular dynamics simulations. The results we present provide an interpretative framework, at atomic scale, of the reduced opening probability and conductance observed in the residual open state of the mutant hemichannel. This work advances our understanding of the molecular mechanisms that underline ion permeation and gating of connexin hemichannels and provides a mechanistic link between connexin mutations and hereditary deafness.

METHODS

EQUILIBRIUM MOLECULAR DYNAMICS OF Cx26WT AND Cx26M34T CONNEXONS

We generated the Cx26M34T hemichannel model starting from an equilibrium configuration of the Cx26WT model published in Refs. (Zonta et al., 2012, 2013) and mutating the 34th amino acid of each connexin protomers with the *mutate* tool of the Swiss PDB–Viewer (Guex and Peitsch, 1997). As done previously for Cx26WT, we embedded the Cx26M34T hemichannel in a plasma membrane represented by 494 Palmytoyl Oleoyl Posphatidly Choline molecules (POPC), following the same methodology described in Pantano et al. (2008). In order to achieve a faster convergence of molecular dynamics trajectories, coordinates for the original phospholipid bilayer were obtained from an equilibrium configuration of the membrane model described in Pantano and Carafoli (2007). The system then was solvated with full atom TIP3P water containing Cl^- and K^+ ions at a concentration of ∼0.15 M to neutralize the positive net charge of the connexon and to mimic a physiological ionic strength. The whole system comprised 204664 and 205825 atoms respectively for Cx26M34T and Cx26WT simulations.

We initially performed a short energy minimization run, followed by equilibrium molecular dynamics under periodic boundary condition using unitary cells of 12 × 12 × 11 nm for both systems, consistent with the channel density measured in a Cx26 gap–junction plaque by atomic force microscopy (Muller et al., 2002). Equilibrium molecular dynamics simulations, performed with GROMACS 4.6 software (Hess et al., 2008) using the Amber03 force field (Duan et al., 2003) under constant *nPT* conditions, lasted 40 ns, the last 18 ns of which were retained for data analysis. Temperature T and pressure P were kept constant, at 300 K and 1 atm respectively, using the Berendsen thermostat and barostat (Berendsen et al., 1984). Fast smooth Particle–Mesh Ewald summation (Darden et al., 1993) was used for long–range electrostatic interactions, with a cut off of 1.0 nm for the direct interactions.

Root mean squared deviation (RMSD) of the transmembrane domain stabilized after 15 ns, whereas short range interaction between membrane and protein (short range Lennard Jones plus short range Coulomb potential) stabilized after 20 ns. Moreover, root mean squared fluctuations (RMSF) were well equilibrated in the last 20 ns time window of the simulation (data not shown).

ESTIMATE OF THE POTENTIAL OF MEAN FORCE (PMF) FROM STEERED MOLECULAR DYNAMICS

Simulations were performed under constant volume conditions on the previously equilibrated systems. To force the passage of a K^+ ion through the channel pore (**Figure 1**), we connected it to one end of a linear spring with elastic constant k of 2000 kJ $mol^{-1}nm^{-2}$ and zero resting length. The other end of the spring shifted along pore axis (z direction) from the cytoplasmic to the extracellular side of the hemichannel at a constant velocity of 0.5 nm ns^{-1}. The shifting spring was stiff enough to keep the K^+ in the proximity of the z axis, with a standard deviation of 0.034 nm. The simulations spanned a total of 8.6 nm in 17.2 ns for each system. The mean force $F(x, y, z)$ exerted on the ion by

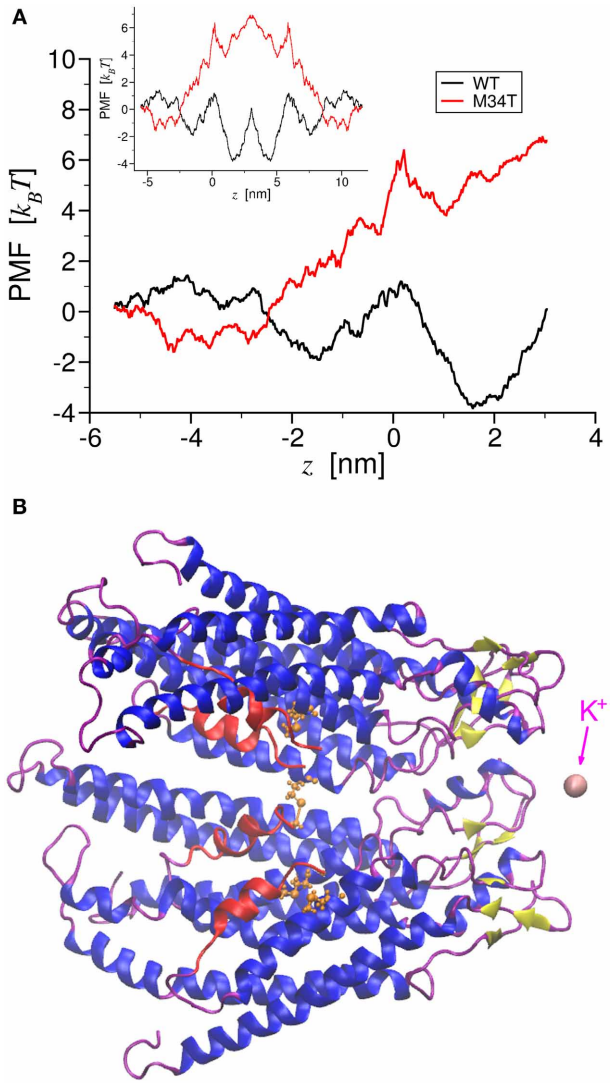

FIGURE 1 | Potential of Mean Force of potassium ion permeation through Cx26WT and Cx26M34T. Panel **(A)** shows the total PMF for a single K^+ ion permeating through the two different hemichannels as a function of the pore axial coordinate. The black trace corresponds to the wild type, while the red trace to the mutant. The inset shows the corresponding PMFs for the entire gap junction channel. In panel **(B)**, a cartoon representation of a section made of four connexins protomers of the Cx26M34T hemichannel is drawn in register with the axis coordinate in A. The NTHs are colored in red, while in orange we show a ball and stick representation of Thr34. This also frame shows the K^+ ion (in pink) at the final position, just outside the extracellular mouth of the hemichannel. Note that the two PMFs diverge largely in the region around the NTHs.

the hemichannel amino acids was gauged by the instantaneous spring extension averaged over 40 ps time intervals.

$F(x, y, z)$ balanced effectively all other forces acting on the ion at each point along the K^+ trajectory, therefore the work profile

$$W_{HC}(z) = \int_0^z F_z\,(0, 0, \zeta)\; d\zeta \tag{1}$$

which is known as the PMF, has the meaning of a free energy profile for the permeation of a single K^+ ion (Kirkwood, 1935; Roux and Karplus, 1991a,b; Park and Schulten, 2004) through the hemichannel (HC) pore (**Figure 1A**). We derived the PMF for the full gap junction channel $W_{GJ}(z)$ (**Figure 1A**, inset) by reflecting $W_{HC}(z)$ about a vertical axis passing through the z coordinate corresponding to extracellular end of the hemichannel (Zonta et al., 2013).

COMPUTATION OF IONIC CONDUCTANCE

To link quantitatively PMF and ionic conductance, let us consider the first time an unforced K^+ ion reaches one end the full gap junction channel (labeled r) given that it started at point (0, 0, z_0). Such *first passage time* of the permeation process has mean value τ given by:

$$\tau = \frac{1}{D}\int_{z_0}^{r} e^{\beta W_{GJ}(y)}dy \int_{l}^{y} e^{-\beta W_{GJ}(z)}dz \tag{2}$$

which can be computed provided the PMF $W_{GJ}(z)$ and the bulk diffusion coefficient D are known (Szabo et al., 1980; Zwanzig, 1988). In Equation 2 the factor $\beta = (k_B\,T)^{-1}$, where T is absolute temperature and k_B is the Boltzmann constant. The numerical value we used for $D = 1.957 \cdot 10^{-9}\; m^2/\,\mathrm{s}$ was experimentally determined in Samson et al. (2003) and accords with an independent estimate we obtained from molecular dynamics simulations of K^+ in the bulk.

If a single ion were to occupy the pore at any time, the transition rate would be

$$\kappa = \frac{1}{\tau} \tag{3}$$

and net unitary current could be estimated as the difference between forward ($\kappa_{l\to r}$) and reverse ($\kappa_{r\to l}$) transition rates multiplied by the charge q of the ion:

$$I = q(\kappa_{l\to r} - \kappa_{r\to l}) \tag{4}$$

In equilibrium conditions the current is obviously null. Therefore, to compute the unitary conductance

$$\gamma_0 = \frac{I}{\Delta V} \tag{5}$$

an electrical potential difference ΔV must be applied between the two ends of the channel. Assuming that the electrical potential is a linear function of the pore axial coordinate z (Tao et al., 2012), we can take its presence into account by adding a suitable term to the PMF, yielding a new function

$$U(z) = W_{GJ}(z) + \frac{q\Delta V}{L}(L - x) \tag{6}$$

After replacing $W_{GJ}(z)$ with $U(z)$ in Equation 2, we numerically computed the forward and reverse transition rates as:

$$\kappa_{l\to r} = \left[\frac{1}{D}\int_{z_0=l}^{r} e^{\beta U(y)}dy \int_{l}^{y} e^{-\beta U(z)}dz\right]^{-1} \tag{7a}$$

$$\kappa_{r\to l} = \left[\frac{1}{D}\int_{z_0=r}^{l} e^{\beta U(y)}dy \int_{r}^{y} e^{-\beta U(z)}dz\right]^{-1} \tag{7b}$$

for ten different values of the imposed ΔV. For each value, we estimated the net current I using Equation 4. We finally plotted I vs. ΔV and derived γ_0 as the slope of the interpolating line.

STATISTICAL ANALYSIS

Means are quoted ± standard error of the mean (s.e.m.) and p-values are indicated by letter p. Statistical comparisons were made using the Mann–Whitney U-test (Mann and Whitney, 1947); $p < 0.05$ was selected as the criterion for statistical significance.

RESULTS

MOLECULAR DYNAMICS PREDICTS CONDUCTANCE VALUES THAT ACCORD WITH EXPERIMENTAL RESULTS FOR BOTH Cx26WT AND Cx26M34T

To gain insight into the role played by the M34T mutation, we used steered molecular dynamics to derive the PMFs for K^+ ion permeation (**Figure 1**; see Methods). The PMF profiles for Cx26WT and Cx26M34T differ primarily in the narrowing region of the pore (**Figure 1A**), where the six NTHs fold inside the cytoplasmic mouth of the hemichannel (**Figures 1B**, **Supplementary Movie S1**). We used these PMFs to compute the full channel unitary conductance γ_0 as detailed in the Methods and obtained the values reported in **Table 1**, first column. Although those figures are one order of magnitude smaller than their respective measured counterparts (**Table 1**, third column), their ratio $R_{MD} = \gamma_{0,\,M34T}/\gamma_{0,\,WT} = 9.5\%$ is in good agreement with the experimental result ($R_{EXP} = 11.4\%$) (Bicego et al., 2006).

As detailed in the Methods section, the conductance values we computed were derived from transition rates of ion permeation through the channel assuming that a single ion can occupy the channel at a given time. This assumption is probably unrealistic. Therefore, to obtain a better estimate for the ionic conductance, we contemplated the possibility that N_I K^+ ions occupy the channel simultaneously. The maximum conductance is achieved when the ions permeate the channel with minimal reciprocal interaction. Indeed, ionic conductance saturates with increasing salt concentration, and, when many ions are allowed to occupy the channel simultaneously, deviation from independent ion transition is observed (Hille and Schwarz, 1978).

To estimate N_I, we assumed that electrostatic repulsion is negligible if the ions are found at the relative distance $2\lambda_D$, where

$$\lambda_D = \sqrt{\frac{\varepsilon_0 \varepsilon_r k_B T}{2e^2 N_A c}} \approx 0.79 \text{ nm} \tag{8}$$

is the Debye length (a Debye sphere is a volume whose radius is the Debye length, outside of which charges are electrically screened; in the formula above, e is the proton's charge, N_A is Avogadro's number and c the concentration). We then computed

$$N_I = \frac{L}{2\lambda_D} = \frac{17.2 \text{ nm}}{1.58 \text{ nm}} \approx 10.89 \tag{9}$$

Table 1 | Comparison between conductance predicted by molecular dynamics simulations and experimental values.

	Single ion [pS]	Multi–ion correction [pS]	Experimental value [pS]
WT	9.64	105	114
M34T	0.92	10	13
Ratio M34T/WT	9.6%	9.6%	11.4%

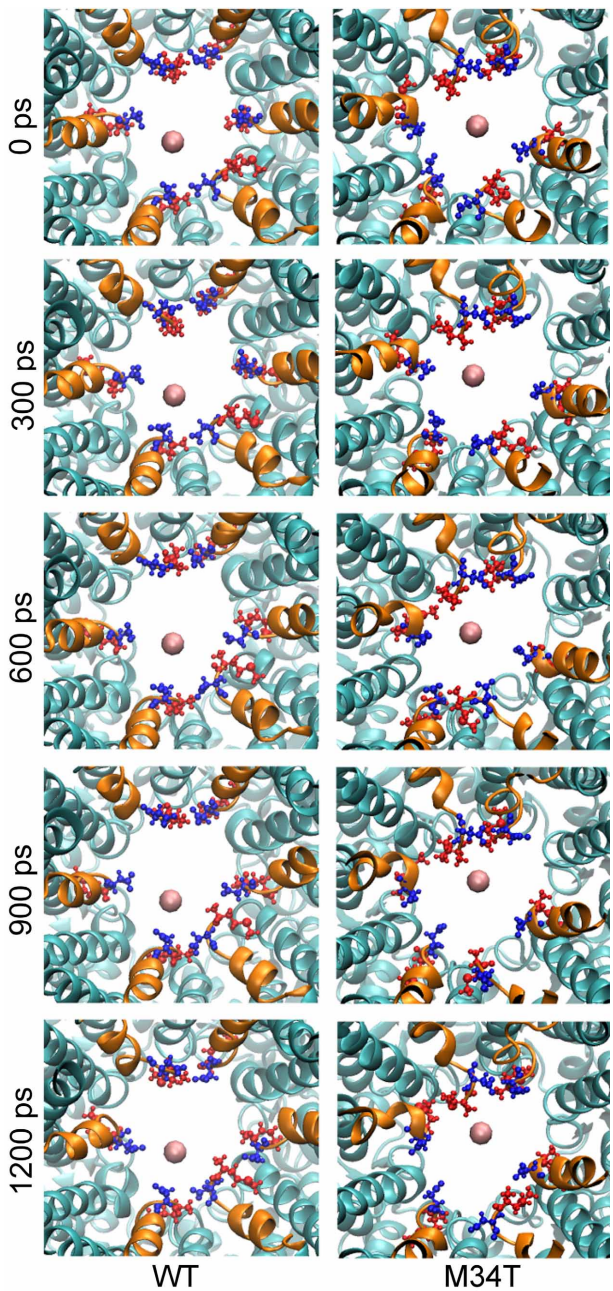

FIGURE 2 | Snapshots of potassium steered molecular dynamics. The pictures show, from a cytoplasmic point of view, five different snapshots in a region proximal to the NTHs. The K^+ ion is shown as a pink sphere and the NTHs are highlighted in orange. M1 (blue) and D2 (red) residues are drawn in ball and stick representation. In the mutant, these residues protrude more toward the center of the pore and consequently the energy of interaction with K^+ is higher and results in an increased total PMF.

where L is the length of the full gap junction channel. We then re-estimated the unitary conductance as $\gamma = N_I\gamma_0$ for both Cx26WT and Cx26M34T. The corrected results (**Table 1**, second column) agree far better with the experimental data (**Table 1**, third column).

The reduced conductance of Cx26M34T correlates with the observation that the first residues (M1, D2) of the NTHs in the Cx26M34T hemichannel protrude more toward the center of the pore than their WT counterparts and thus interact more efficiently with the drifting K^+ ions (**Figure 2**, **Supplementary Movie S2**).

ANALYSIS OF EQUILIBRIUM MOLECULAR DYNAMICS TRAJECTORIES REVEALS AN ASYMMETRIC CONFIGURATION OF NTHs IN Cx26M34T HEMICHANNELS

As shown in **Figure 3**, replacing a hydrophobic M with a polar T in position 34 disrupts the hydrophobic interaction between M34 and W3 in the NTH of the adjacent connexin. Consequently, the six NTHs of the Cx26M34T hemichannel rearranged in a more asymmetric configuration in the course of the 40 ns equilibration process. To measure these changes and to compare Cx26M34T to Cx26WT quantitatively, let us introduce an *eccentricity coefficient E* that gauges departure from a perfect hexagonal symmetry. Specifically, we define E as the ratio between the maximum (D) and the minimum (d) diameter of a hexagon built on the alpha carbons of corresponding amino acids in the six protomers (**Figure 4**). We computed E for amino acids number 2 to 14 of the NTHs, averaged the results over 100 configurations spanning the last 18 ns of equilibrium molecular dynamics, yielding $E_{WT} = 1.10 \pm 0.05$ (mean $\pm$ s.e.m.) for the Cx26WT hemichannel and $E_{M34T} = 1.16 \pm 0.11$ (mean $\pm$ s.e.m.) for the Cx26M34T hemichannel. Since the distributions of E-values are not normal, we analyzed the significance level of the observed difference using the Mann Whitney U-test (Mann and Whitney, 1947). The p-value of 0.03 returned by the test indicates that, compared to Cx26WT, the distribution of Cx26M34T data is significantly shifted toward larger E values (i.e., it has a higher degree of asymmetry).

We further analyzed the dynamical behavior of the NTHs by tracking the angles of the hexagon built on the alpha carbon of the six T5 residues, which are located roughly half way along the NTHs. In **Figure 5** we plot angular values during the last 18 ns of equilibrium dynamics for both Cx26WT (**Figure 5A**) and Cx26M34T (**Figure 5B**) vs. time. Note that angles in the Cx26M34T hemichannel display a higher degree of instability compared to Cx26WT and, correspondingly, the distribution of their values differ significantly (**Figure 5C**, $p = 0.01$, Mann–Whitney U-test).

THE M34T MUTATION REDUCES THE INTERACTION BETWEEN THE NTHs AND THE INNER WALL OF THE HEMICHANNEL

It has been proposed that, for a gap junction channel to reside in the fully open state, the six NTHs must be attached to the inner wall of its cytoplasmic mouth via hydrophobic interactions between W3 and M34 (Maeda and Tsukihara, 2011; Fasciani et al., 2013). To test this hypothesis, we performed a series of steered molecular dynamics simulations by connecting the center of mass of one NTH (residues 1 to 12) to one end of a linear spring with zero resting length and elastic constant of 100 kJ $mol^{-1}nm^{-2}$ (**Figure 6**). **Figure 6B** shows six different pull force traces (one per NTH) for Cx26WT (black) and Cx26M34T (red). At the beginning of each run, the two spring ends coincided and the pull

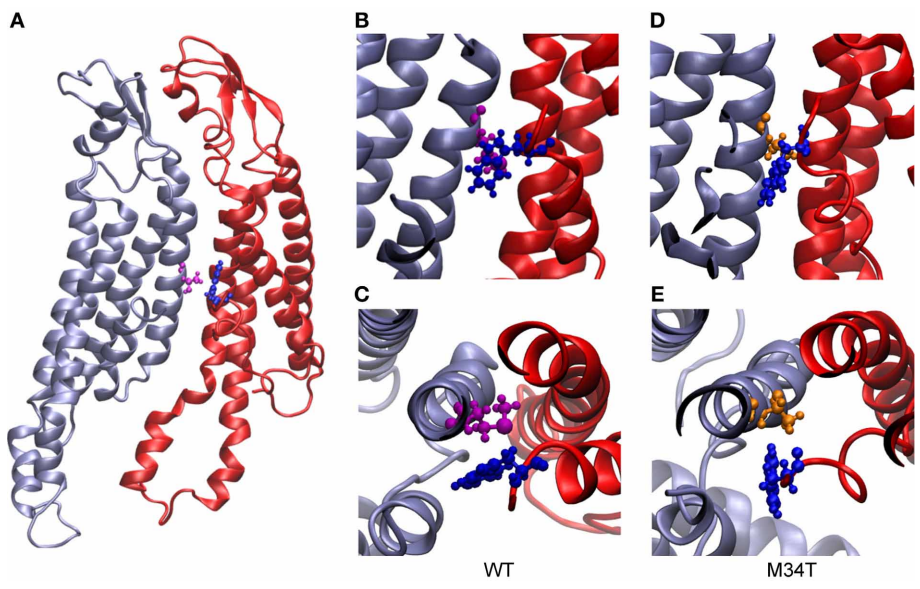

FIGURE 3 | Structural comparison between Cx26WT and Cx26M34T. In panel **(A)**, two adjacent wild type connexins are shown in ribbon representation. The two residues highlighted in ball and stick representation are M34 (purple) and W3 (blue). Panels **(B)** (top view) and **(C)** (side view) show details of the hydrophobic interaction between these two residues. **(D)** and **(E)**, same as **(B)** and **(C)** for Cx26M34T; W3 is again represented in blue, while T34 in orange. Note that the interaction present in the wild type is disrupted in the mutant.

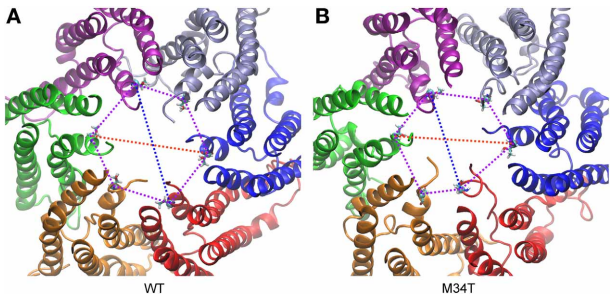

FIGURE 4 | Analysis of symmetry index. (A) Cx26WT connexon model. **(B)** Cx26M34T connexon model. Shown are the major (red) and minor (blue) diameter and the angles (purple) of the hexagon built on T5 alpha carbons for a snapshot of the equilibrium dynamics. The six connexins are rendered with different colors and represented in ribbons. Each T5 alpha carbon is represented with its Van der Waals radius, while the rest of the amino acid is represented in licorice.

force was null. Setting the free end of the spring into motion with constant velocity of 1 nm ns^{-1} toward the center of the pore generated a centripetal force on the NTH. In the typical scenario, the pulled NTH did not follow immediately because the pull force was insufficient to overcome the interaction that kept the NTH attached to the inner wall of the hemichannel. Consequently the spring extended and the pull force increased linearly, until it reached a value sufficient to break the NTH–wall interaction. At this point the NTH started to move, the spring relaxed and the pull force dropped abruptly. In the Cx26M34T hemichannel the pulled NTH started to move after about 1 ns, whereas in the Cx26WT hemichannel the movement occurred at the end of the simulation period (after ∼3 ns). To highlight the differences between the two data sets, we averaged the six different traces and computed a running average over these mean traces to reduce the effect of thermal noise (**Figure 6C**). Note the clear departure of the average traces around 105 (kJ/mol)/nm (**Figure 6D**). We interpret this value as the detaching force for the Cx26M34T mutant. The corresponding value for Cx26M34T was in excess of 250 (kJ/mol)/nm, almost three–fold larger.

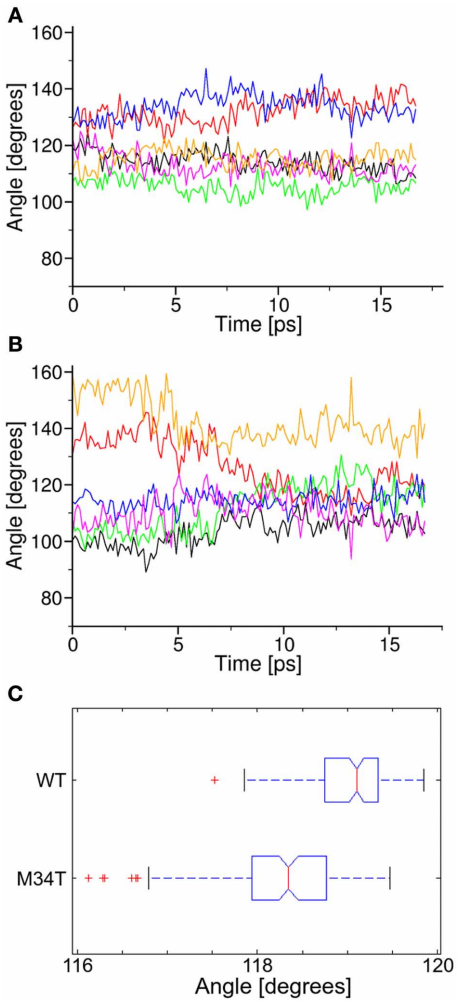

FIGURE 5 | Analysis of angular parameters. Panels **(A,B)** show the time course of angular values during equilibrium dynamics for Cx26WT and Cx26M34T, respectively (see also **Figure 3**). **(C)** Box plots of the two data distribution, in which we interpreted each angle as a representation of the corresponding observable. The difference between the two distribution is significant (see text).

DISCUSSION

At the cellular level, the deafness–associated Cx26M34T mutant is correctly synthesized and targeted to the plasma membrane in HeLa cells, but inefficiently forms intercellular channels that display an abnormal electrical behavior and retain only 11% of the unitary conductance of Cx26WT (Bicego et al., 2006). Moreover, Cx26M34T channels fail to support the intercellular diffusion of fluorescent tracers and the spreading of mechanically induced intercellular Ca^{2+} waves (Bicego et al., 2006). It has also been suggested that Cx26M34T/Cx32 heterotypic channels are not fully open at rest but are activated when positive transjunctional voltages are applied to the Cx26M34T side (Skerrett et al., 2004).

Our molecular dynamics simulations of Cx26WT and Cx26M34T hemichannels indicate that the quaternary structure of the mutant is altered at the level of the NTHs due to the disruption of the hydrophobic interaction between M34 (in the first transmembrane helix) and W3 (in the NTH) (**Figure 3**). The mutation destabilizes the NTH binding to the cytoplasmic mouth of the channel altering its shape, which is significantly more asymmetric in the mutant hemichannel model compared to the wild type model (**Figures 4**, **5**).

The NTHs are thought to participate in voltage gating, which is rather complex in channels formed by human Cx26 that exhibit a bipolar behavior (Gonzalez et al., 2006). Single channel recordings indicate that the opening of Cx26 hemichannels upon depolarization at negative potentials involves a transition from the fully closed state to a main open state. As depolarization progresses, hemichannels remain stable in this high conductance state until polarization reaches larger positive potentials, whereupon they inactivate by closing partially to a subconductance or residual open state (Gonzalez et al., 2006).

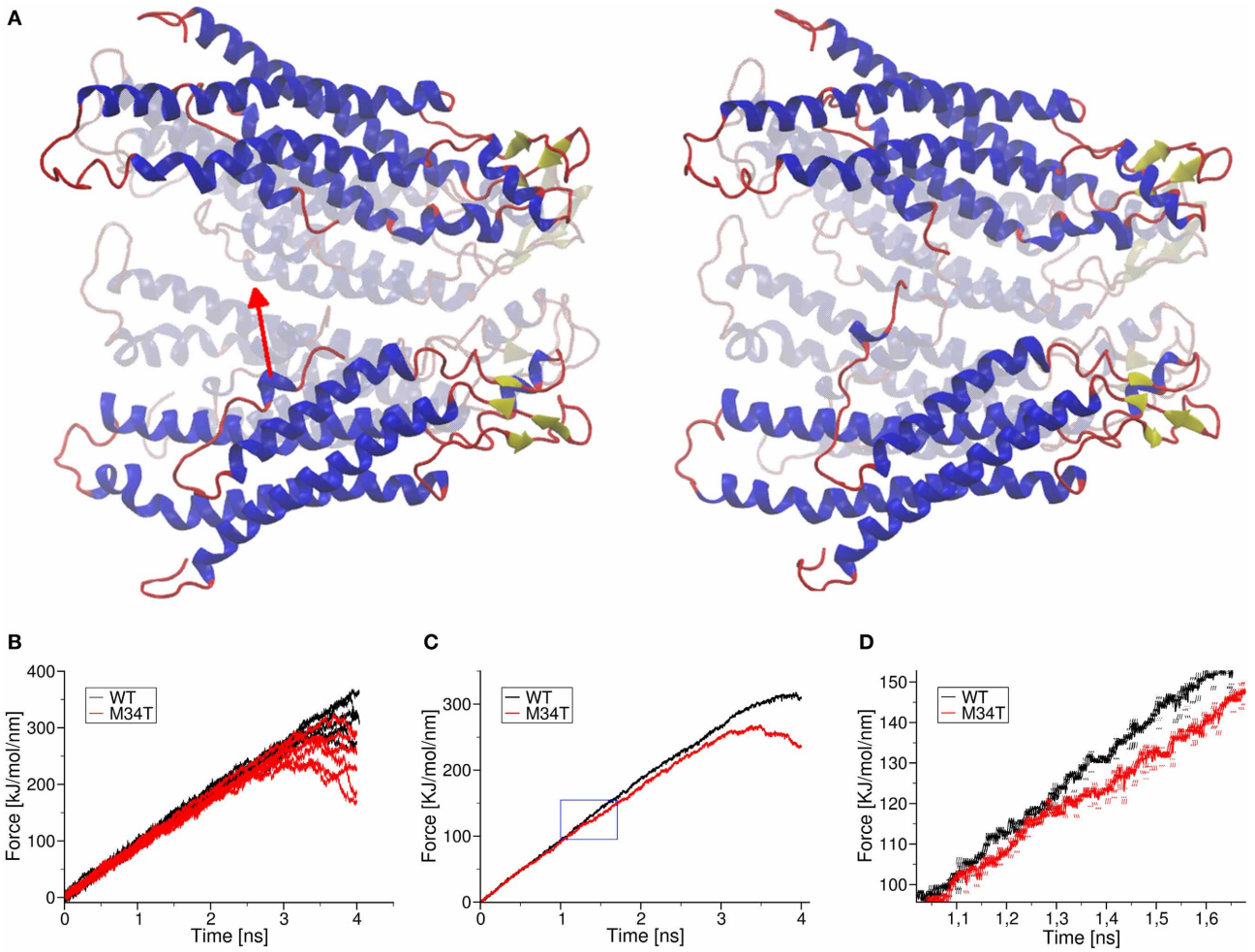

FIGURE 6 | Effect of a pull force applied to the NTHs. Panel **(A)** shows schematically the effect of pulling one NTH: shown are the initial and final frames of a pulling simulation. In panel **(B)**, we plot raw pull force data for each trajectory corresponding to the six different helices of Cx26WT (black traces) and Cx26M34T (red traces). Panel **(C)** shows the mean traces obtained from the corresponding raw traces, after application of a further running average over 200 fs in order to reduce thermal noise. The blue box is magnified in panel **(D)** to show more clearly the point where the two mean traces separate. Error bars are standard deviation obtained from the running average. Visual inspection of the molecular dynamics trajectories revealed that, in the mutant, the detached helix interacts with a neighboring NTH, due to the more asymmetric shape of the pore mouth. This interaction obstacles the motion of the helix toward the center, until the pull force is large enough to break it. This effect was not observed in wild the type Cx26WT (**Supplementary Movie S3**).

To explain these observations, it has been proposed that connexin hemichannels possess two gating mechanisms. One mechanism has the same polarity for all the hemichannels independently of their connexin isoform composition and depends critically on the extracellular Ca^{2+} concentration (Ebihara and Steiner, 1993; Pfahnl and Dahl, 1999; Muller et al., 2002). A study performed on Cx32 hemichannels concluded that (i) Ca^{2+} can block both voltage gated opening to the higher conductance open state and ion conduction through the partially open hemichannels and (ii) the effect depends on Ca^{2+} binding with millimolar affinity within the extracellular vestibule of the pore (Gomez-Hernandez et al., 2003). In our prior work using molecular dynamics simulations, we provided an interpretative model showing that Ca^{2+} ions linger within the negatively charged extracellular mouth at a membrane potential of −80 mV. Upon depolarization to 0 mV the interactions weaken and the position of the Ca^{2+} ions shifts significantly toward the extracellular space (Zonta et al., 2012). This scheme is supported by the presence of negatively charged amino acids facing the pore in the extracellular mouth, in particular D46 and E47, that are highly conserved across connexin isoforms. E47 is also believed to undergo post-translational gamma carboxylation (Locke et al., 2009), which increases it affinity for Ca^{2+} ions. This crucial point is analyzed in Zonta et al. (2014).

The second gating mechanism shows different polarity among different connexin isoforms, and depends on the total charge of the NTHs (Verselis et al., 1994). It has been proposed that the NTHs move in response to changes in electrical potential and close the channel (Maeda and Tsukihara, 2011; Fasciani et al., 2013). Our simulations indicate that the NTHs in the

Cx26M34T mutant are less bound to the channel wall (**Figure 6**) and we speculate that, for this reason, the gating mechanism is compromised, as proposed in Skerrett et al. (2004). The slight but significant modifications highlighted by our relatively brief (40 ns) equilibrium molecular dynamics simulations, reflect in a sizeable (90%) reduction of the unitary conductance (**Table 1**), in quantitative accord with the experimental results (Bicego et al., 2006). Our simulation work also agree with the proposal that the M34T mutant channels resides most of the time in a low conductance state (13 pS for Cx26M34T, 15–20 pS for Cx32M34T). However, the displacement of the NTHs in our equilibrium dynamics, is not marked enough to be compatible with the formation of the pore plug as described in Oshima et al. (2007) for the closely related Cx26M34A mutant. Further simulation work is required to test whether such a plug can be formed by Cx26M34T mutant connexins.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIALS

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fphys.2014.00085/abstract

Supplementary Movie S1 | Steered molecular dynamics of K^+ permeation. In this simulation, we force a K^+ ion to permeate through the pore of Cx26WT and Cx26M34T hemichannels. The total PMFs for the two cases (black trace for wild type and red trace for the mutant) is shown in register with the permeating ion position (represented with its Van der Waals radius, in pink). The NTHs are highlighted in red, and two key residues are represented in ball and stick; T34 is color in green and K41 (corresponding to the maximum of the total PMF) in orange.

Supplementary Movie S2 | Steered molecular dynamics of K^+ permeation seen from the cytoplasmic point of view. The movie compares wild type (on the left) and mutant hemichannel (on the right). See also the caption of **Figure 2**.

Supplementary Movie S3 | Effect of pulling one NTH toward the center of the pore. The pulled helix is colored in red, while the other NTHs in blue. The initial position is shown as a reference in white. The pulling of a helix in the wild type (on the left) and in the mutant (on the right) reveals different qualitative behaviors, in addition to the quantitative differences described in **Figure 6**. While in the wild type hemichannel the pulled helix moves abruptly at the end of the simulation, for the mutant this motion occurs earlier, but is then hampered by the interaction of the pulled helix with a neighboring NTH which are more mobile and do not keep the symmetric configuration of the wild type.

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Possible role of hemichannels in cancer

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In humans, connexins (Cxs) and pannexins (Panxs) are the building blocks of hemichannels. These proteins are frequently altered in neoplastic cells and have traditionally been considered as tumor suppressors. Alteration of Cxs and Panxs in cancer cells can be due to genetic, epigenetic and post-transcriptional/post-translational events. Activated hemichannels mediate the diffusional membrane transport of ions and small signaling molecules. In the last decade hemichannels have been shown to participate in diverse cell processes including the modulation of cell proliferation and survival. However, their possible role in tumor growth and expansion remains largely unexplored. Herein, we hypothesize about the possible role of hemichannels in carcinogenesis and tumor progression. To support this theory, we summarize the evidence regarding the involvement of hemichannels in cell proliferation and migration, as well as their possible role in the anti-tumor immune responses. In addition, we discuss the evidence linking hemichannels with cancer in diverse models and comment on the current technical limitations for their study.

Keywords: hemichannels, connexins, pannexins, cancer

OVERVIEW OF MAMMALIAN HEMICHANNELS AND METHODOLOGICAL CONSIDERATIONS

Multicellular organisms have evolved with sophisticated routes for cell-to-cell communication. Such interactions are multidimensional (e.g., they occur between different types of cells, in diverse spatial orientation/direction, environmental conditions and time frames) and create signaling circuits within tissues, ultimately allowing for a more efficient use of resources and coordination of responses. In addition to a myriad of membrane receptors, channels and transporters, mammalian cells are equipped with relatively large, low-resistance proteinaceous conduits to transmit signaling molecules such as ions (e.g., Na^+ and K^+), second messengers (e.g., Ca^{2+} and phosphatidylinositol 1,4,5-trisphosphate [IP_3]), nucleotides (e.g., cAMP, ADP, and ATP) and metabolites (e.g., glucose, adenosine, glutamate and glutathione) across electrochemical gradients. Such structures correspond to aqueous pores formed by hexamerization of subunits of two topologically related vertebrate protein families commonly referred to as "gap junction-type proteins": connexins (Cxs) and pannexins (Panxs). In humans, there are 21 different Cxs genes with a wide genomic distribution and 3 genes encoding Panxs located in chromosomes 11 (Panx1 and 3) and 22 (Panx2) (Baranova et al., 2004; Söhl and Willecke, 2004). Cxs and Panxs show no sequence homology and there is no evidence supporting formation of mixed Cxs/Panxs based channels. Alternative splicing variants and pseudogenes for some of these transcripts have been described in humans, but their biological relevance is uncertain. Paradoxically (and for historical reasons), when such channels are recognized as single structures they are termed "hemichannels" or "connexons," although they really are whole, functional cell membrane channels allowing the passage of relatively small solutes up to ∼1.2 kDa. When two of such channels/connexons, each from one adjacent cell, converge to areas of intercellular membrane contact and serially dock allowing for continuous communication of cytosols, they are termed intercellular gap junction channels (Sáez et al., 2003). In a conservative view, hemichannels have been considered to remain preferentially closed in resting conditions and to serve mainly as structural precursors of gap junction channels through lateral diffusion and clustering after membrane insertion (Gaietta et al., 2002). However, independent site-specific and cytoskeleton-dependent membrane targeting routes for hemichannels, either to areas of cell-cell junctions or to unopposed membrane clusters, have been described (Shaw et al., 2007). The relevance of Cx-based hemichannels and intercellular channels in physiology has become evident by the demonstration of disease phenotypes associated with Cxs mutations affecting protein conformation, turnover and channels function (reviewed in Schalper et al., 2009; Pfenniger et al., 2011).

Diverse structural and functional factors limit the study Cx and Panx hemichannels and intercellular gap junction channels in biological systems. The first major challenge is clear discrimination between hemichannels and intercellular channels. Hemichannels and intercellular channels formed by the same Cx or Panx usually coexist in cells and show overlapping

permeability to ions/molecules and a similar (not highly specific) pharmacological sensitivity (Retamal et al., 2007a; Giaume and Theis, 2010; Fiori et al., 2012). In addition, hemichannels and gap junction channels formed by most studied Cxs and Panxs share a common amino acid sequence, secondary structure and transcriptional/post-transcriptional regulation (Sáez et al., 2003; Riquelme et al., 2013a). Therefore, molecular methods or conventional antibody-based detection are usually limited to study the levels, location, stoichiometry and participation of each channel type in any given response. In addition, the difficulties to recapitulate *in vitro* the complex multi-cellular/multidimensional tissue conditions have limited a clear dissection of the relative contribution of each channel type to various physiological and pathological processes. To overcome some of the aforementioned limitations, mimetic peptides and antibodies targeting specific regions at the extracellular (docking) domains have been used to allow structure-specific recognition/blockade of hemichannels (recently reviewed in Riquelme et al., 2013a).

The second major problem is discriminating between the contribution of Cx and Panx-based channels to any given response. Channels and hemichannels formed by Cxs or Panxs have functional, pharmacological similarities and overlapping expression patterns. In particular, Panxs have been shown to have glycosylation sites on the extracellular loop and a high glycosylation level could preclude the serial docking of Panx hemichannels (Boassa et al., 2008; Peñuela et al., 2013). This led to the notion that Panxs form exclusively hemichannels and not intercellular gap junction channels (Sosinsky et al., 2011). However, recent studies confirmed the early findings by Bruzzone et al. (2003) showing that at least Panx1 and 3 can form functional intercellular gap junction channels with independent properties (Sahu et al., 2014). Future studies exploring diverse cell/tissues and various experimental conditions will be required to support and extend this concept. Further details on the transcriptional regulation of Cx and Panx genes, structural and functional characteristics of Cx- and Panx-based channels, post-translational modifications, pharmacological properties and methodological considerations are discussed in comprehensive reviews published elsewhere by our group and by others (Goodenough and Paul, 2003; Sáez et al., 2003, 2010; Baranova et al., 2004; Söhl and Willecke, 2004; Panchin, 2005; Schalper et al., 2008b; Giaume and Theis, 2010; Kar et al., 2012; D'Hondt et al., 2013; Peñuela et al., 2013).

The aforementioned methodological limitations for the study of hemichannels both *in vitro* and *in vivo* and the possible "contamination" of results by additional yet anonymous transmembrane routes have pointed out possible flaws in the interpretation of correlative dyes/molecules uptake or release and electrophysiological studies demonstrating hemichannel existence and functions (Spray et al., 2006). However, the evidence on intercellular gap junction channels also largely relies on comparable correlative expression/function studies using dye transfer and electrophysiological experiments combined with pharmacological blockade. Direct intercellular communication pathways different from gap junction channels termed intercellular nanotubes have recently been described (reviewed in Sherer, 2013) and should be considered in the interpretation of gap junction studies. In addition, the intercellular transfer of regulatory molecules in specialized small bi-layered membranous vesicles termed exosomes (or ectosomes) could also contribute to some of the responses attributed exclusively to gap junction channels, particularly in the central nervous system (Kalani et al., 2014), immune system (Hwang, 2013) and cancer cells (Azmi et al., 2013). Channel-independent functions of Cxs and Panxs have also been well described and add difficulty to the interpretation of results (Vinken et al., 2012). Most studies evaluating the functions and properties of intercellular channels in various conditions have not simultaneously addressed possible changes in hemichannel functions. Thus, a comparable degree of skepticism should exist on the notion of the exclusive involvement of intercellular channels in many studies correlating Cx and Panx expression with certain responses or phenotypes. Finally, visual localization of hemichannels and gap junction channels has been performed largely using antibodies, some of which have not been thoroughly validated regarding their specificity, optimal titration/dynamic range, reproducibility and stability over time. The lack of specificity and reproducibility of commercial and in-house established antibodies represents a common flaw in biomedical research (Bordeaux et al., 2010). In this regard, personal experience working in the gap junction field, as well as reports by other authors have highlighted possible limitations and conflicting results using presumably specific antibodies (Yahalom et al., 1991; Coppen et al., 2002; Shurman et al., 2005; Brisset et al., 2009; Cone et al., 2013). Moreover, the relative contribution of technical error and biological variations in antibody-based results (e.g., diverse protein folding, sample fixation, antigen retrieval, blocking solutions, alternative transcripts, and posttranslational modifications) are not easy to discern. Careful validation of reagents and, more important, systematic reporting of antibodies lacking specificity/reproducibility could help to overcome this complex scenario.

GAP JUNCTION PROTEINS AND CANCER

After the seminal work by Drs. Werner Loewenstein and Yoshinobu Kanno showing impaired intercellular electrical coupling in chemically-induced and xenografted rat hepatocarcinomas (Loewenstein and Kanno, 1966, 1967), the role of gap junctions in cancer cells has been a subject of interest for the last five decades. A considerable number of articles on this subject have been published and the literature has been periodically reviewed (see Loewenstein, 1980; Trosko et al., 1983; Kanno, 1985; Yamasaki, 1990; Mesnil and Yamasaki, 1993; Mesnil et al., 1995; Trosko and Ruch, 1998; Mesnil, 2002; Naus, 2002; Mesnil et al., 2005; Cronier et al., 2009; Kandouz and Batist, 2010; Naus and Laird, 2010). In general, cancer cells from various types and after diverse experimental challenges have been shown to have lower expression of their native gap junction proteins than non-tumor samples. Also, highly proliferative tumor cells show atypical (e.g., predominantly cytoplasmic) expression of these proteins and impaired gap junctional intercellular communication. Restoration of the gap junction-type proteins and/or intercellular communication is frequently associated with reduced cell proliferation, which led to the broad concept that Cxs and Panxs were tumor suppressors. However, emerging data on the role of these proteins in tumor cell migration and metastasis have challenged this paradigm and pointed to situations in which

these proteins could actually favor cancer progression (Cronier et al., 2009; Kandouz and Batist, 2010; Naus and Laird, 2010). Unfortunately, little of the knowledge on this subject has been translated to cancer medicine and the possible role of hemichannels in carcinogenesis and tumor progression remains largely unexplored. Previous studies on hemichannels have used transformed cell models. However, key experiments addressing the functions and impact of hemichannels in cancer cells have not yet been communicated and include: (i) detailed characterization of the presence and relative abundance of hemichannels in cancer cells; (ii) evaluation of hemichannel-mediated molecule uptake/release in tumor cells as compared to non-tumor cells; (iii) functional consequences of hemichannels activation and blockade in tumor cells and neoplasms and (iv) prognostic and predictive value of hemichannels expression/activation in human malignancies.

In this article, we hypothesize that functional hemichannels can be present in tumor cells (at least of some tumor subtypes or tumor cell subpopulations) and they could alter tumor cell proliferation and disease progression through the transmembrane exchange of signaling solutes such as nucleotides and Ca^{2+}. To test this theory, we review the literature regarding the involvement of hemichannels in cancer-related processes such as cell proliferation/death and cell migration; and revisit some of the gap junction/cancer studies from a "hemichannel-centric" perspective. We also speculate on the possible role of hemichannels in cancer progression and as a clinically useful biomarker.

REGULATION OF Cx AND Panx EXPRESSION IN HUMAN CANCER

Most tumor cells harbor genetic and epigenetic defects that lead to altered protein expression and signaling. The presence of gene amplification, rearrangements and acquisition of the so-called "driver" activating mutations are hallmarks of many clinically actionable oncogenes including HER2, EGFR, KRAS, c-KIT, and ALK. In addition, malignant cells frequently downregulate or silence transcripts associated with anti-tumor characteristics (e.g., tumor suppressor genes) such as P53, RB, and PTEN. Silencing of such alleles can occur by gene deletion, inactivating mutations or by epigenetic alterations (e.g., promoter hypermethylation and histone modifications). Finally, tumor cells can also alter the expression of transcripts post-transcriptionally by microRNA-mediated silencing or using the more recently described long non-coding RNAs (lncRNAs) system (Hauptman and Glavac, 2013).

Cxs and Panxs have been frequently shown to display altered expression in human tumors and cancer cell lines. Diverse studies have addressed the underlying mechanisms and they are summarized in **Table 1**. Of note, recent efforts in exome/genome-wide sequencing using massive parallel sequencing strategies of diverse human malignancies such as the TCGA (The Cancer Genome Atlas) have been completed and to date, mutations in Cxs and Panxs have not emerged as frequent events. Moreover, naturally occurring mutations have been reported in Cx genes and are frequently associated with complex disease phenotypes (Schalper et al., 2009). To our knowledge, no mutations in human Panx genes have been communicated. Taken together, this suggests that regulation of gap junction-type proteins in cancer cells could be more frequently associated with epigenetic and post-transcriptional events, as suggested in pioneering experiments characterizing mRNA transcripts using substractive hybridization methods in cultured normal and tumor breast epithelial cells (Lee et al., 1991).

Cx AND Panx GENE MUTATIONS IN HUMAN CANCER

Early efforts looking for Cx mutations in cancer specimens found that 4 of 7 primary human hepatic angiosarcomas carried a proline-to-serine substitution in codon 319 of the Cx37 gene (Saito et al., 2000). The same genetic variant was found in DNA extracts from adjacent non-tumor tissue, suggesting that it corresponded to a germline mutation or a single nucleotide polymorphism. The amino acid 319 is located in the cytoplasmic tail of the protein that contains diverse regulatory motifs. Of note, Cx37 has been shown to form functional hemichannels (Wong et al., 2006) and its expression reduced the proliferation of cultured rat insulinoma cells (Burt et al., 2008). In follow-up studies it was shown that introduction of Cx37 mutations that abrogate both the intercellular channel and hemichannel functions (T154A and C61A-C65A) failed to suppress the proliferation (Good et al., 2011, 2012). The inablity of the mutant protein to mimic the effect of the wild type Cx37 in cell proliferation indicates that the passage of molecules through Cx37-based channels is required to influence cell growth/survival. In addition, association between the C1019T polymorphism of the Cx37 gene, gastric adenocarcinomas and H. pylori infection was identified in a retrospective Chinese cohort (Jing et al., 2012).

Another study looking for Cx mutations in sporadic colorectal cancers found 2 frameshift Cx43 mutations in 3 of 6 studied tumors (a single nucleotide deletion A311V in 2 samples and a single nucleotide insertion I358N in 1 tumor) (Dubina et al., 2002). No alterations in Cx32 gene were identified. The Cx43 variants were mapped to the carboxy-terminal tail of the peptide and were not detected in DNA extracts from peripheral blood samples, confirming their somatic nature. However, such mutations have not been mechanistically characterized or further reported in larger tumor series.

Using a high-resolution array-based CGH platform to screen human hepatocellular carcinomas, deletions in the region encoding the Cx40.1 gene were identified (Zender et al., 2008). In support of a tumorigenic effect of this variant, an *in vivo*, xenograft-based RNAi screen showed that Cx40.1 downregulation using shRNA elicited a prominent acceleration of tumor growth. Little is known about the properties of human Cx40.1. For instance, it is expressed in the human heart (Söhl and Willecke, 2004), but its channel forming capacity remains to be proven.

Deletions of an 817 kb area at 6q14-21 containing the Cx62 gene were found in DNA extracts from 21 of 55 human prostatic adenocarcinomas using a high-resolution single nucleotide polymorphism array (Liu et al., 2007). However, the levels of Cx62 mRNA from the ONCOMINE database were comparable between tumor samples and normal controls, suggesting that another transcript located in this deleted segment such as MAP3K7 might have tumor suppressive actions.

Table 1 | Possible mechanisms responsible for reduced expression of connexins in human cancers and cell lines.

Protein	Tumor	Alteration
Cx26	Squamous cell carcinoma oral cavity	Missense heterozygous single nucleotide mutation F142L (Rednam et al., 2011)
	Transitional carcinoma cell lines	Histone demethylation (Li et al., 2013)
	Small cell carcinoma (SCLC) and SCLC cell lines	Promoter hypermethylation (Chen et al., 2005)
	Invasive breast carcinoma (IBC) and IBC cell lines	Promoter hypermethylation (Tan et al., 2002)
Cx30	Colorectal carcinoma (CRC) and CRC cell lines	Promoter hypermethylation (Sirnes et al., 2011)
Cx32	Renal cell carcinoma cell lines	Promoter hypermethylation (Hirai et al., 2003)
	Gastric carcinoma	Promoter hypermethylation (Schalper et al., 2014, this report)
Cx36	Colorectal carcinoma (CRC) and CRC cell lines	Promoter hypermethylation (Sirnes et al., 2011)
Cx37	Colorectal carcinoma	Promoter hypermethylation (Sirnes et al., 2011)
	Hepatic angiosarcoma	High frequency of codon 319 polymorphism in vinyl chloride-related tumors (Saito et al., 2000)
Cx40.1	Hepatocellular carcinoma	Deletion (Zender et al., 2008)
Cx43	Colorectal carcinoma	Single nucleotide deletion A311V, Single nucleotide insertion I358N (Dubina et al., 2002)
	Non-small cell lung carcinoma	Promoter hypermethylation (Jinn and Inase, 2010)
	Prostate carcinoma cell lines	Histone deacetylation (Hernandez et al., 2006), miR-20a expression is inversely correlated with Cx43 expression (Li et al., 2012a,b)
	Glioblastoma multiforme cell line	miR-221/222 targets Cx43 mRNA, decreasing its expression (Hao et al., 2012)
	Nasopharyngeal carcinoma (NPC) and NPC cell line	miR-218 targets Cx43 mRNA and is downregulated in NPC (Alajez et al., 2011)
Cx45	Colorectal cancer cell lines	Promoter hypermethylation (Sirnes et al., 2011)
Cx62	Prostate carcinoma	6q14-21 deletion (Liu et al., 2007)

Aggressive oral squamous cell carcinomas are predominantly associated with alcohol and tobacco use and are extremely rare in children. Interestingly, the case of a 6-year old girl was reported with psoriasiform dermatitis and sensorineural hearing loss that presented with a high grade, 3.3 cm palatal and nasal squamous cell carcinoma (Rednam et al., 2011). Due to the clinical findings the patient was screened and the single nucleotide Cx26 mutation F142L was found. Although there was no demonstration of causal relationship between the tumor and the Cx26 variant, other Cx26 mutations associated with excessive keratinocyte proliferation and syndromic hearing loss have been shown to produce hemichannels with increased function and altered permeability (Sánchez et al., 2010; Mese et al., 2011).

EPIGENETIC REGULATION OF EXPRESSION OF Cxs AND Panxs IN CANCER

Epigenetic regulation of Cxs is a well-described process (reviewed in Vinken et al., 2009; Oyamada et al., 2013). Reduction in the expression of diverse Cxs with concomitant promoter hypermethylation has been shown in multiple human neoplasms and tumor cell lines. Downregulation of Cx32 gene expression by promoter hypermethylation was shown using bisulphite modified DNA-based PCR and a restriction enzyme-based assay in HK-2 and Caki-2 human renal cell carcinoma cells (Hirai et al., 2003). Treatment of cells with the demethylating agent 5-Aza-2′-Deoxycytidine restored Cx32 expression, confirming that Cx32 expression was suppressed. Similarly, downregulation of Cx43 in nasopharyngeal carcinoma CNE-1 cells was found to be associated with *GJA1* (Cx43) gene hypermethylation (Yi et al., 2007). A high frequency of Cx30, Cx36, and Cx37 promoter hypermethylation was also detected in colorectal carcinoma samples, as well as in Cx30, Cx36, and Cx45 in colon cancer cell lines (Sirnes et al., 2011). However, correlation between reduced transcript levels and the presence of promoter methylation was found only for Cx45. A more recent study identified Cx45 promoter hypermethylation in 33% of 485 colorectal carcinomas and a positive association with the actionable oncogenic BRAF exon 15 mutation (e.g., V600E) was noted (Ahmed et al., 2011).

Low Cx43 protein levels in human non-small cell carcinomas were associated with Cx43 CpG island promoter methylation and with heavy tobacco use (Jinn and Inase, 2010). Strikingly, the presence of Cx43 promoter hypermethylation in the non-tumor peritumoral lung tissue, but not in the tumor tissue proper, was significantly associated with lymph node positivity in non-small cell cancer patients (Chen et al., 2003). The biological determinants of this association are not clear. Reduced Cx26 protein and mRNA levels in a variety of lung carcinomas (including small-cell neuroendocrine carcinomas) and cell lines was also correlated with promoter hypermethylation and reverted by 5-Aza-2′-Deoxycytidine (Chen et al., 2005). Cx26 promoter

hypermethylation was also found in 11 of 20 human invasive breast carcinomas and in cell lines (Tan et al., 2002). However, the absence of Cx26 expression in human squamous esophageal carcinoma cells was not correlated with the presence of Cx26 promoter hypermethylation, suggesting the presence of a different silencing mechanism (Loncarek et al., 2003). Using methylation specific PCR, we found Cx32 promoter hypermethylation in 4 of 9 frozen samples from human gastric adenocarcinomas (Schalper et al., unpublished observation, **Figure 1**). The 246 bp methylation-specific band was detected exclusively in the tumor and not in non-tumor areas of the samples (**Figure 1A**). However, we found no clear relationship between the presence of Cx32 promoter hypermethylation and Cx32 protein levels as detected by immunohistochemistry (**Figures 1B,C**). Taken together, these findings suggest that although more frequent in cancer tissues, promoter hypermethylation in Cxs genes is not unequivocally related with reduced/absent protein.

To our knowledge, regulation of Panx genes by epigenetic control has not been reported. Using the NIH PROSCAN webtool (http://www-bimas.cit.nih.gov/molbio/proscan/), we evaluated a 1000 bp region upstream of the initiation codon of the human Panx1 gene. We identified a 434 bp region with diverse transcription factor binding motifs and a significant probability of being a promoter region (Promoter score = 88.86; Promoter cutoff = 53.00). In the same genomic region and using the EMBL-EBI Cpgplot tool (https://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/) we detected a 554 bp region with >50% C/G nucleotides, consistent with a CpG island. The latter suggests that human Panx1 has a CpG nucleotide-rich promoter region and could be modulated by hypermethylation.

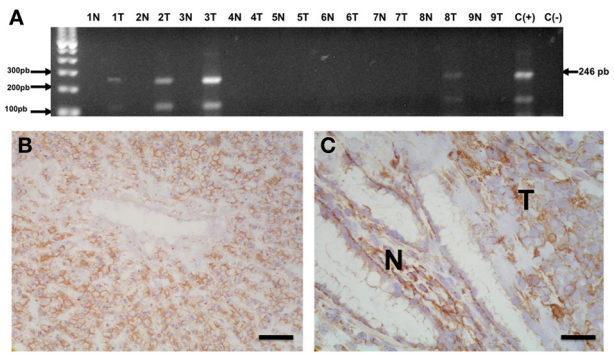

FIGURE 1 | Methylation of Cx32 promoter region in human gastric adenocarcinomas. (A) Agarose gel showing the results from methylation specific PCR of sodium bisulphite-modified DNA extracted from 9 frozen gastric adenocarcinomas (1T-9T) and non-tumor tissue from the same subjects (1N-9N). In 4 cases, a 246 bp band corresponding to the methylated sequence in the Cx32 promoter region was identified. C(+) = Positive control sample of methylated DNA; C(−) = Negative control lacking template DNA. **(B,C)** Microphotographs showing Cx32 immunostaining in morphologically normal human liver **(B)** and non-tumor (N)/tumor (T) interface from one gastric adenocarcinoma sample **(C)**. Liver was used as positive control and showed homogenous and intense membranous-like staining. Cx32 immunoreactivity was also detected in the normal foveolar gastric epithelium (N) and in malignant epithelial carcinoma cells (T). The previously reported (Sánchez et al., 2009) monoclonal anti-Cx32 antibody clone 72F (dilution 1:1500, overnight incubation) was used to stain frozen sections. Preparations were then counterstained with hematoxilin. Bar = 100 μm.

Few events associated with histone modifications have been shown to affect Cx26 and Cx43 genes in human neoplasms. Li and collaborators (Li et al., 2013) recently found an inverse relationship between Cx26 and JARID1B (also known as KDM5B) histone demethylase protein levels in transitional cancer cell lines and advanced human bladder tumors. Overexpression of JARID1B was associated with reduced Cx26 protein in HT1376 and T24 cells, indicating that this demethylase represses Cx26 expression.

In androgen dependent (LNCaP) and androgen-independent human prostate cancer cell lines (DU145 and PC3), treatment with the histone deacetylase inhibitor Trichostatin A prominently increased Cx43 mRNA and protein expression. The intercellular transfer of the fluorescent dye Lucifer yellow was also increased by Trichostatin A, indicating increased gap junction mediated intercellular communication (Hernandez et al., 2006). Similar findings were observed in cultured non-malignant human peritoneal mesothelial cells (Ogawa et al., 2005).

POST-TRANSCRIPTIONAL REGULATION OF EXPRESSION OF Cxs AND Panxs IN CANCER

Post-transcriptional modulation by small non-coding RNAs of Cx genes has also been reported in human tumors. Li et al. (2012a,b) found an inverse relationship between miR-20a and Cx43 protein and mRNA expression in human prostate tumors and cell lines. In this model, downregulation of miR-20a caused a 4-fold increase in Cx43 expression and reduced the cell proliferation. Using a luciferase reporter assay, the miR-20a antagomir increased the luciferase activity using the wild type Cx43 sequence, but was ineffective with Cx43 mutated at the miR-20a binding site at the 3′UTR region. The latter demonstrates that Cx43 is a direct target of miR-20a. Using a similar approach, miR-221/222 was shown to bind Cx43 mRNA, reduce Cx43 expression and promote cell growth and invasion in the human glioblastoma multiforme cell line U251 (Hao et al., 2012). Finally, transfection of miR-218 in nasopharyngeal carcinoma cells reduced Cx43 expression and was associated with increased apoptosis. Moreover, miR-218 transfection reduced the tumor growth after intramuscular implantation of C666-1 cells in SCID mice (Alajez et al., 2011). Although likely to occur, regulation of Cxs expression by lncRNAs has not been reported yet. In addition and to our knowledge, modulation of Panxs by non-coding RNAs has not been communicated.

HEMICHANNELS IN CELL PROLIFERATION AND TUMOR PROGRESSION

The cellular consequences of altered hemichannel functions are believed to be mediated mainly by defective transmembrane transport of signaling molecules passing through them, leading to altered activation of intracellular pathways and autocrine/paracrine signals. Key molecules associated with cell proliferation/survival and shown to permeate activated hemichannels include NAD^+ (nicotinamide adenine dinucleotide), cADPR (cyclic ADP-ribose), ATP, Ca^{2+},

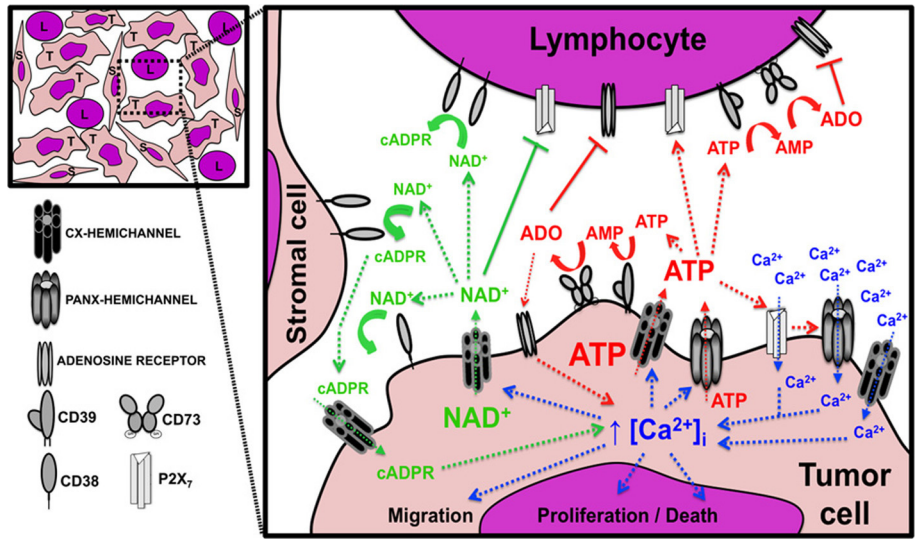

FIGURE 2 | Possible role of hemichannels in tumor growth and progression. Diagram showing the paracrine interactions mediated by the passage of nucleotides and Ca^{2+} through hemichannels between tumor cells (T), lymphocytes (L), and stromal cells (S). The image in the upper left corner depicts the tumor microenvironment components with intimate relationship between the tumor and non-tumor cells. The expanded inset on the right represents a magnification of one tumor area and indicates the signaling circuits associated with NAD^+ (green text and arrows), ATP (red text and arrows), and Ca^{2+} (blue text and arrows). The image also shows the presence and possible interactions between connexin (Cx) and pannexin (Panx) hemichannels, ectonucleotidases, purinergic receptors, and adenosine receptors. Arrows with dashed lines indicate a positive/stimulatory effect, arrows with continuous lines indicate a negative/inhibitory effect; and curved lines indicate (enzymatic) metabolic activity. ADO, adenosine; AMP, adenosine monophosphate; ATP, adenosine triphosphate; cADPR, cyclic adenosine diphosphate-ribose; $[Ca^{2+}]_i$, free intracellular Ca^{2+} concentration; NAD^+, nicotinamide adenine dinucleotide; $P2X_7$, P2X purinoreceptor 7. See text for detailed explanation of the biological consequences of each pathway.

IP_3, glutathione, and prostaglandin E_2 (Wang et al., 2013a,b). However, additional, yet unidentified, relatively small-sized solutes could also mediate autocrine/paracrine effect in cells. In particular, the nucleotides NAD^+ and ATP have been directly involved in hemichannel mediated cell proliferation (of non-tumor cells) by increasing the intracellular Ca^{2+} levels. Therefore, the exchange of these nucleotides and Ca^{2+} through hemichannels will be the main focus of this section. Also, the transmembrane passage of nucleotides constitutes a unique feeder system for extracellular nucleotidases that have a prominent role in the anti-tumor immune responses (see below). In addition, the increasingly recognized paracrine signaling between tumor and stromal cells that may occur through hemichannels could also support their involvement in tumor growth and cancer progression. A summary of the possible functions of hemichannels in cancer biology is presented in **Figure 2**.

HEMICHANNELS AND NAD^+ METABOLISM

The cellular NAD^+ homeostasis is tightly regulated in all organisms and contributes to the maintenance of the energetic balance, intracellular redox potential and signal transduction. Tumor cells undergo metabolic adaptations to support growth and survival, including a pronounced shift from oxidative phosphorylation toward more NAD^+ and lactate production through aerobic glycolysis, a phenomenon known as the Warburg effect (Chiarugi et al., 2012). The net cellular NAD^+ content is the result from its synthesis by various enzymatic cascades involving niacin (vitamin B3), tryptophan, aspartic acid and reutilization of intracellular nicotinamide-related compounds, as well as NAD^+ degradation through enzymatic hydrolysis, ribosylation and deacetylation. CD38 is the major mammalian NAD^+ glycohydrolase and ADP-ribosyl cyclase, thus directly participating in NAD^+ metabolism and production of second messengers (Chiarugi et al., 2012). However, the catalytic domain of CD38 is located in the extracellular domain, apart from its cognate intracellular substrate.

The first experimental evidence for the involvement of Cx43 hemichannels in transmembrane NAD^+ transport came from studies using cultured NIH3T3 fibroblasts, HeLa cells and proteoliposomes (Bruzzone et al., 2001). In these models, NAD^+ efflux required (and was paralleled by) Cx43 expression. Moreover, the NAD^+ release was increased by lowering the extracellular Ca^{2+} concentration and prominently blocked by beta-glycyrrhetinic acid, La^{3+} and a Cx43 monoclonal antibody, thus implicating Cx43 hemichannels (Bruzzone et al., 2001). Shortly after, the same group convincingly demonstrated the involvement of Cx43 hemichannels in the $[Ca^{2+}]_i$-dependent, cADPR-induced cell proliferation in co-cultured mouse NIH/373 fibroblasts with or without CD38 expression (Franco et al., 2001; De Flora et al., 2004). In this model, the efflux of NAD^+ through activated hemichannels allowed the interaction of this intracellularly produced dinucleotide with the extracellular catalytic segment of CD38, giving a mechanistic response to this topographical paradox. After its production by CD38-mediated NAD^+ ribosylation, the ubiquitous second messenger cADPR can re-enter the cells to induce IP_3-pathway independent-/ryanodine receptor induced Ca^{2+} transients and ultimately mediate cell cycling and

proliferation. Later studies by another research group extended these observations and showed that cADPR re-uptake is mediated by Cx43 hemichannels through bidirectional NAD^+ extrusion/cADPR import upon FCγ receptor stimulation in cultured murine J774A.1 macrophages (Song et al., 2011) (**Figure 2**, green colored arrows and text).

Although CD38 is expressed in various mammalian tissues including brain, prostate and muscle, it is most prominently found in lymphoid/blood cells (Deaglio et al., 2008). CD38 is frequently upregulated and has been indicated as a prominent oncogenic and adverse prognostic factor in hematologic malignancies including multiple myeloma and chronic lymphocytic leukemia. Moreover, humanized monoclonal antibodies targeting CD38 (e.g., daratumumab, MOR03087, and SAR650984) are under evaluation in early phase clinical trials to treat patients with advanced hematological B-cell malignancies (www.clinicaltrials.gov; trial identifiers NCT01615029, NCT00574288, NCT01749969, NCT01084252 and NCT01421186). As an integral component of the CD38-Cx43-cADPR axis and NAD^+ degradation pathway, it is tempting to speculate that expression of Cx43 in neoplastic cells could serve as a prognostic/predictive biomarker for such compounds and modulation of Cx43 hemichannels may itself represent a novel anti-tumor therapeutic target, especially in CD38 expressing B cell malignancies.

Cx43 was shown to be expressed in isolated human B cells from tonsil and peripheral blood and Cx-based channels participate in immunoglobulin production and B cell maturation (Oviedo-Orta et al., 2000, 2001). In addition, Cx43 defective knockout mice have lower IgM-positive B cells than their wild type littermates and the defect is more pronounced in the homozygous than in the heterozygous animal, supporting a gene-dosage effect (Montecino-Rodriguez and Dorshkind, 2001). Although some authors have ascribed the functional effects of lymphocyte Cx43 to their participation in the pool of intercellular channels located at the cell-cell contacts between activated immune cells (e.g., "the immunological synapse"), the involvement of hemichannels in these processes has not been excluded. This is particularly relevant in immune cells that are expected to spend a considerable part of their lifespan detached from other cells. In this regard, the reduction in immunoglobulin levels observed by Oviedo-Orta and collaborators in primary human lymphocytes was triggered by traditional Cx-channel blockers and by a Cx43 mimetic peptide corresponding to the second extracellular loop (e.g., Gap27) (Oviedo-Orta et al., 2001), known to inhibit Cx43 hemichannels (Evans et al., 2006; Wang et al., 2012). Notably, additional short peptides corresponding to the intracellular domains of Cx43 (e.g., Gap18 and Gap20) were ineffective in this model.

More recently, Cx43 was shown to be required for activation and migration of cultured murine B cells (Machtaler et al., 2011). In this study, B cell responses were independent of intercellular gap junction channels and immunofluorescence experiments of Cx43 in the murine B lymphoma cell lines WEHI 231 and A20, and in primary murine B splenocytes, showed a predominant homogenous membranous distribution. The latter indicates a lack of preferential clustering/enrichment of Cx43 in areas of cell-cell contact. A similar diffuse membranous Cx43/CD38 co-localization staining pattern was observed in cultured J774A.1 macrophages (Song et al., 2011).

Surprisingly few studies have explored the expression of Cxs in native human lymphoid tissues and human lymphoid neoplasms. For instance, Cx43 was detected in germinal centers of fresh samples from human tonsil and spleen (Krenacs et al., 1997). Of note, Cx43 protein and mRNA were predominantly located in cells with irregular shape and co-expressing CD21 and CD35, consistent with follicular dendritic cells. Only scarce, more rounded cells with lymphocyte appearance and of undefined lineage were found to carry the Cx43 transcript in this study.

Aiming to characterize Cx43 expression in native human lymphoid tissues and lymphoid malignancies, preliminary experiments from our group using chromogenic immunohistochemistry detected low levels of Cx43 protein in formalin fixed-paraffin embedded samples from 4 non-tumor lymph nodes and absence of signal in a set of 22 malignant B cell lymphomas (13 Diffuse large B cell lymphomas [DLBCL] and 9 classical Hodgkin lymphomas [CHL]) (**Figure 3**). While distinctive Cx43-positive plaques were evident toward the intercalated discs of human myocardiocytes used as positive control (**Figures 3A,B**), only focal labeling of Cx43 in occasional germinal centers from reactive-type lymph nodes was seen in cells with morphology consistent with endothelial cells, macrophages and follicular dendritic cells (**Figures 3C,D**). The latter finding is somewhat consistent with previous observations by Krenacs and collaborators (Krenacs et al., 1997) and points to the possibility that Cx43 expression in lymphocytes might be too low to be detected by immunohistochemistry in unstimulated lymph nodes (e.g., lower than in myocardium). Alternatively, other Cxs known to be present in lymphocytes such as Cx40 (Oviedo-Orta et al., 2001) or Panxs could represent the main hemichannel forming pool in native human lymphoid tissues and lymphomas. Of note, Panx1 hemichannels have been previously shown to be expressed in T lymphocytes and to allow the passage of ATP that is structurally related with NAD^+ and has a similar molecular mass (MW $NAD^+ = 663.42$ vs. ATP $= 551.14$) (Woehrle et al., 2010). In addition, we found membranous-like Panx1 expression in samples from high grade, DLBCL that showed prominent co-localization with the pan-B cell marker CD20 in confocal stacks (Shoji et al., unpublished observation). Future studies using more sensitive methods and functional assays will be required to clarify this.

The presence of hemichannels in tumor cells even without CD38 expression might confer on them growth/migration advantages through increased autocrine/paracrine NAD^+ signaling and the possible interaction with CD38 present in the membrane of adjacent non-tumor cells (e.g., stromal cells). In addition, relatively low increases in the extracellular NAD^+ concentration (in the micromolar range) through cell death/lysis or release through activated hemichannels can exert local immune suppressive effects through activation of ionotropic $P2X_7$ purinergic receptors in immune cells, leading to effector cytotoxic T cell death and expansion of immune suppressive regulatory CD4+/CD25+/FoxP3+ T cells (Tregs) (Scheuplein et al., 2009; Hubert et al., 2010). The latter effect could favor tumor progression by shielding cancer cells from immune-mediated cell killing

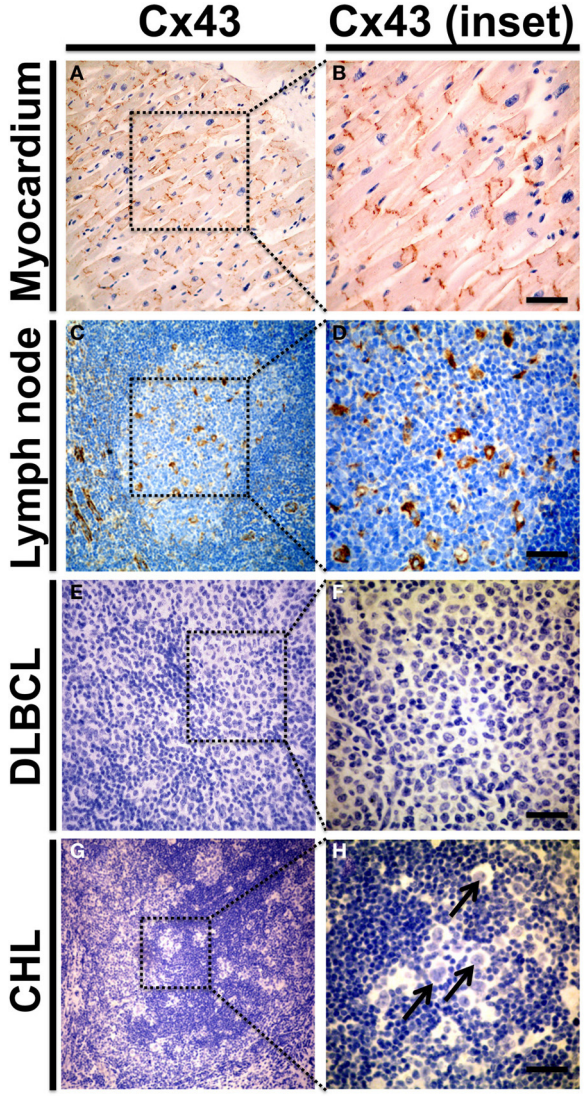

FIGURE 3 | Cx43 protein expression in human reactive lymph nodes and lymphomas. Representative microphotographs from formalin fixed paraffin embedded samples of morphologically normal human myocardium (*n* = 3, **A,B**), reactive lymph node with follicular and parafollicular hyperplasia (*n* = 4, **C,D**), diffuse large B cell lymphomas (*n* = 13 DLBCL, **E,F**) and classical Hodgkin's lymphoma (*n* = 9 CHL, **G,H**). Samples were included in a tissue microarray and stained with chromogenic immunohistochemical method for Cx43 using a mouse monoclonal antibody at 1:100 dilution (Zymed Laboratories). Preparations were counterstained with hematoxylin to visualize nuclei. Right panels **(B,D,F,H)** show magnified insets from low power images on the left **(A,C,E,G)**. **(A,B)** Depicts control Cx43 distribution in intercalated discs of human myocardiocytes. In non-tumor lymph nodes **(C,D)** Cx43 staining was predominantly detected in endothelial cells; and in cells with morphological features more consistent with macrophages and follicular dendritic cells. Cx43 was not detected in DLBCL and CHL samples **(E–H)**. Arrow in **(H)** indicates Hodgkin/Reed-Stemberg cells. Bar = 50 μm.

(**Figure 2**). Indeed, increasing studies have found elevated Cx43 and/or Cx26 to be associated with more aggressive (e.g., shorter survival) and disseminated disease in diverse human malignancies and *in vitro* cancer models (Kanczuga-Koda et al., 2006; Tate et al., 2006; Naoi et al., 2007; Kyo et al., 2008; Cronier et al., 2009; Bui et al., 2011; Lamiche et al., 2012; Stoletov et al., 2013; Tang et al., 2013; Ghosh et al., 2014). In addition, a recent study demonstrated that increased post-treatment Cx43 levels were significantly associated with response to neoadjuvant chemotherapy in human breast carcinomas (Teleki et al., 2013) and high tumor Cx43 expression by immunohistochemistry was predictive of response to platinum-based chemotherapy in non-small cell lung cancer (Du et al., 2013). Notably, many of these studies reported predominant cytoplasmic location of Cxs and failed to detect a corresponding increase in the intercellular gap junctional communication. Recently available antibodies capable of selectively detecting (and blocking) Cx hemichannels through recognition of extracellular loop sequences (Riquelme et al., 2013a) might shed light on the relative contribution of hemichannels and intercellular channels in these responses.

HEMICHANNELS IN ATP RELEASE AND PURINERGIC SIGNALING

ATP is responsible for energy storage and transfer in the form of renewable high-energy phosphoryl bonds. In addition to its role in cell metabolism, intracellular ATP participates in the maintenance of electrochemical gradients through the fueling of pumps, active vesicular transport and also serves as a coenzyme and mediator for signal transduction and DNA synthesis. The cellular levels of ATP are determined by its constant production occurring mostly through oxidation of a variety of carbon-based substrates (e.g., aerobic cellular respiration); and degradation by hydrolysis into the lower level nucleotide phosphoforms ADP and AMP.

In addition to the prominent intracellular function, extracellular ATP serves as a pleiotropic intercellular messenger through its interaction with ionotropic ($P2X_1$–$P2X_7$) and metabotropic ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) P2 purinergic membrane receptors (Falzoni et al., 2013). Examples of this include its function as neurotransmitter, proliferation stimulating agent, pro-inflammatory mediator and signaling factor between immune cells. In particular, extracellular ATP has been recognized to have a key role in the tumor-host interactions (reviewed in Di Virgilio, 2012). Despite its prominent extracellular role, the routes for cellular ATP release in both tumor and non-tumor cells are not well characterized. Diverse mechanisms have been proposed including passive transmembrane diffusion, vesicular release/exocytosis, cellular lysis and extrusion through specific chloride channels, ABC transporters, $P2X_7$ receptors and Cx/Panx hemichannels (Di Virgilio, 2012; Baroja-Mazo et al., 2013; Falzoni et al., 2013; Orellana et al., 2013). The first direct indication of ATP release occurring through Cx hemichannels came from experiments showing that the propagation of Ca^{2+} waves in cultured astrocytes required the presence of extracellular ATP and Cxs expression, but not of functional intercellular channels (Cotrina et al., 1998). In addition, cultured C6, HeLa and U373 cells showed UTP-induced ATP release only after transfection with Cx26, 32, or 43 and the responses were mimicked by removal of extracellular Ca^{2+} (Cotrina et al., 1998). Later on, numerous studies confirmed the permeability of Cx hemichannels to ATP in diverse cell types and using an extensive repertoire of experimental conditions including the removal of extracellular divalents (Arcuino et al., 2002; Stout et al., 2002; Stout and

Charles, 2003; Gomes et al., 2005; Pearson et al., 2005; Zhao et al., 2005; Bahima et al., 2006), mechanical stimulation (Arcuino et al., 2002; Stout et al., 2002; Gomes et al., 2005; Zhao et al., 2005; Richter et al., 2014), increased $[Ca^{2+}]_i$ (Braet et al., 2003a,b; De Vuyst et al., 2009), hypoxia/ischemic like conditions (Faigle et al., 2008; Clarke et al., 2009), membrane depolarization (Kang et al., 2008; Nualart-Marti et al., 2013), application of bacterial lypopolysaccharide (De Vuyst et al., 2007), treatment with amyloid beta peptide (Orellana et al., 2011), exposure to hypotonic stress (Lu et al., 2012), after spinal cord injury (Huang et al., 2012), in activated polymorphonuclear granulocytes (Eltzschig et al., 2006), induced by gamma irradiation (Ohshima et al., 2012) and after air-stimulation of cultured keratinocytes (Barr et al., 2013). Hemichannels formed by Panx1 have also been consistently shown to allow the passage of ATP in diverse cell types and experimental models (Bao et al., 2004; Locovei et al., 2006; Huang et al., 2007; Reigada et al., 2008; Schenk et al., 2008; Buvinic et al., 2009; Iglesias et al., 2009; Qiu and Dahl, 2009; Ransford et al., 2009; Chekeni et al., 2010; Garré et al., 2010; Murata et al., 2010; Sridharan et al., 2010; Woehrle et al., 2010; Seminario-Vidal et al., 2011; Dolmatova et al., 2012; Iglesias and Spray, 2012; Li et al., 2012a,b; Lohman et al., 2012; Sandilos et al., 2012; Xia et al., 2012; Xiao et al., 2012; Pinheiro et al., 2013; Riquelme et al., 2013b). Moreover, Panx1 hemichannels are functionally coupled to P2X and P2Y receptors activation (Locovei et al., 2006; Pelegrin and Surprenant, 2006; Wang et al., 2013a,b).

Hemichannels, ATP release and anti-tumor immune suppression

The levels of extracellular ATP correspond to the result between cellular release through diverse routes including hemichannels and degradation by ectonucleotidases (e.g., CD39 and CD73). Such enzymes mediate the extracellular conversion of ATP to AMP (CD39) and to adenosine (CD73) that interacts with predominantly immune inhibitory adenosine receptors (e.g., A2A), thus driving the shift from ATP driven pro-inflammatory environment to an adenosine-driven antiinflammatory/immune suppressive state (Antonioli et al., 2013) (**Figure 2**, red arrows and text). The latter mechanism might represent a negative regulatory switch to limit the inflammatory response and control tissue damage. However, relatively high concentrations of ATP (milimolar range) acting on P2X and P2Y receptors can induce marked suppressive effects in lymphocytes (Trabanelli et al., 2012; Burnstock and Di Virgilio, 2013). Consequently, increased extracellular ATP can potentially favor or suppress the local anti-tumor immune response, depending on its concentration, the presence and relative abundance of adenosine and P2 receptors in tumor and inflammatory cells, and the rate of conversion to adenosine by nucleotidases (Di Virgilio, 2012). In general, tumor microenvironments are considered to be purine-rich and CD39 and CD73 are commonly upregulated by hypoxia in diverse cancers leading to a more immune-suppressive tumor niche (Di Virgilio, 2012; Ghiringhelli et al., 2012; Antonioli et al., 2013). Consistent with this notion, expression of CD39 and CD73 are associated with more aggressive and metastatic tumors. The recent emergence of immunostimulatory drugs blocking immune inhibitory checkpoint pathways such as CTLA-4 and the PD-1/PD-L1 axis have shown unprecedented prominent and lasting clinical responses in patients with advanced solid tumors (Quezada and Peggs, 2013). Therefore, it is not surprising that strategies to inhibit CD39 and CD73 in cancer patients are being actively pursued. Unfortunately, most available *in vitro* and animal models to study the role of hemichannels in cancer cells are not helpful in evaluating their possible effect in the anti-tumor immune response (e.g., monocultures and immune defficient animals). Future studies using reconstituted cell culture systems including tumor cells and lymphocytes/APCs or immunocompetent animal models will help clarifying this issue.

Hemichannels, ATP release and cell proliferation

In addition to the inflammatory/immune effect, it has long been known that nucleotides have direct actions on tumor cells and most human neoplasms express a wide variety of purinergic receptors (Stagg and Smyth, 2010; Roger and Pelegrin, 2011; Burnstock and Di Virgilio, 2013). The effect of extracellular ATP in tumor cell proliferation/survival is complex and apparently depends on the ATP concentration, secretion pattern and the reportoir of purinergic receptors available in the target cell (Burnstock and Di Virgilio, 2013). Moreover, activation of the same receptor can induce apoptosis or even increase cell proliferation in different cell types. Extracellular nucleotides have been shown to induce anti-cancer effect in diverse tumor types and strategies to exploit this effect using ATP infusions or synthetic ATP analogs are ongoing. However, it is also clear that low doses of ATP as seen during spontaneous release from cells can have a pro-proliferative and growth promoting effect (Di Virgilio, 2012; Burnstock and Di Virgilio, 2013). In general, tumor cells have very high ATP content compared with non-tumor cells and strategies to reduce ATP have also been shown to enhance the activity of anti-cancer agents (Burnstock and Di Virgilio, 2013).

One of the most studied, yet still controversial P2 receptor in cancer cells is the so-called cytolytic $P2X_7$ receptor. Although relatively high (millimolar range) concentrations of extracellular ATP can induce cell death through $P2X_7$ receptor activation, more typically secreted (micromolar range) ATP concentrations are associated with promotion of cell survival/growth (Roger and Pelegrin, 2011; Di Virgilio, 2012). In support of this notion, expression of $P2X_7$ receptor in some tumor models is associated with increased growth rate and metastases (Stagg and Smyth, 2010; Roger and Pelegrin, 2011; Di Virgilio, 2012). Also, signaling through some P2Y receptors such as $P2Y_2$ has been associated with increased cell survival, proliferation and migration (White and Burnstock, 2006; Stagg and Smyth, 2010; Burnstock and Di Virgilio, 2013). In addition, it was recently shown that ATP released by non-tumor osteocytic cells treated with alendronate can either inhibit or stimulate the cell growth and migration of cultured MDA-MB231 breast cancer cell lines depending on the extracellular ATP concentration present (Zhou et al., 2014). While the inhibitory effect was seen with relatively low ATP concentrations and required $P2X_7$ expression/activation; higher extracellular ATP levels were associated with increased tumor cell migration that was mediated by adenosine signaling through the A2A receptor. Similar observations were made in MDA-MB231 mouse xenographs, confirming the importance of this mechanism *in vivo*. Taken together, this indicates that both the release of ATP by adjecent

non-tumor cells and the rate of conversion to adenosine participate in the ATP-mediated tumor cell proliferative responses. The biphasic effect of extracellular ATP could partially explain the dissimilar impact of ATP signaling in cell proliferation and survival seen in different models (**Figure 2**). Moreover, it suggests that treatment with non-hydrolizable forms of ATP could have prominent anti-cancer effects by limiting its conversion to pro-tumorigenic and immune supressive adenosine.

Notably, ATP release through activated Cx-hemichannels has been detected after stimulation with growth factors including nerve growth factor (NGF) (Belliveau et al., 2006); basic fibroblast growth factor (FGF-2) (De Vuyst et al., 2007) and acidic fibroblast growth factor (FGF-1) (Garré et al., 2010). NGF signaling has been implicated in diverse human malignancies (Molloy et al., 2011). In particular, NGF and its receptors are prominent oncogenic mediators in invasive human breast carcinomas and their expression in malignant cells is associated with adverse patient outcome (Dollé et al., 2004; Noh et al., 2013). Moreover, blockade of NGF signaling using monoclonal antibodies or reducing NGF expression with siRNA attenuates the proliferation and angiogenesis in MDA-MB-231 cells mouse xenografts (Adriaenssens et al., 2008). Aberrant FGFs signaling is also directly implicated in developmental tissue growth and in the progression of diverse human neoplasms and the genes encoding the four major FGF receptors are frequently amplified and/or mutated in human cancers (Dieci et al., 2013; Dienstmann et al., 2014). Research is ongoing to identify effective strategies to block FGF signaling using small molecule inhibitors and monoclonal antibodies to achieve anti-tumor effect with acceptable toxicity/safety profile. The aforementioned observations suggest that increased hemichannel activation possibly induced by excessive growth factors signaling could favor tumor cell proliferation and expansion. Supporting this possibility, Cx43 hemichannels were shown to mediate the proliferation of neural progenitor cells from the ventricular zone and the retinal epithelium through release of ATP, paracrine activation of purinergic P2Y receptors and subsequent $[Ca^{2+}]_i$ transients (Weissman et al., 2004; Pearson et al., 2005). In addition, two naturally occurring mutations in the human Cx30 gene (G11R and A88V) associated with a hyperproliferative keratinocytic genodermatosis termed hidrotic ectodermal dysplasia (or Clouston syndrome) have been demonstrated to produce defective Cx30 hemichannels with increased ATP release (Essenfelder et al., 2004). Although not directly measuring ATP, blockade of Cx43 hemichannels using the Gap26 mimetic peptide or carbenoxolone reduced the neointimal formation after vascular injury through decreased proliferation of smooth muscle cells (Song et al., 2009) and opening of Cx43 hemichannels mediated the survival signals induced by alendronate in osteocytic/osteoblastic cells through a Src-MAP kinases transduction dependent mechanism (Plotkin et al., 2002). The possible involvement of increased extracellular adenosine signaling in these responses has not been studied.

Similar to Cx hemichannels, ATP release through Panx1 hemichannels has been associated with increased proliferation of stem cells, neural progenitors (Wicki-Stordeur et al., 2012) and primary human cultured fibroblasts stimulated with histamine (Pinheiro et al., 2013). In the latter model, $P2Y_1$ receptor-dependent $[Ca^{2+}]_i$ transients were triggered by Panx1-mediated ATP release. The release of ATP through activated Panx1 hemichannels was also shown to accelerate the assembly of multicellular C6 tumor cells aggregates in a 3D culture system (Bao et al., 2012). The latter effect was mediated by $P2X_7$ receptors activation and remodeling of the F-actin cytoskeleton, and ultimately suggests that Panx1 could particiapte in tumor cell motility.

Consistent with a pro-tumorigenic effect, Panx1 (but not other Panxs) was found at relatively high levels in the melanoma cell lines B16 and was associated with membranous-like location and increased hemichannel function (Peñuela et al., 2012). In this model, down regulation of Panx1 using siRNAs significantly reduced the expression of the malignant melanoma markers vimentin, hsp70 and B-catenin, and diminished the cell density and motility, but increased melanin production suggesting acquisition of a more differentiated phenotype. Moreover, Panx1 silencing reduced the tumor size and the frequency of liver metastases in a chicken embryo tumor implantation model, suggesting that Panx1 hemichannels favor melanoma growth and progression (Peñuela et al., 2012). Similarly, silencing of Panx2 induced the acquisition of a more differentiated neuronal-like phenotype in N2A neuroblastoma cells (Swayne et al., 2010). Therefore, it would not be surprising to find specific tumor types that use hemichannels mediated mechanisms for their growth and dissemination. Also, the presence of subpopulations of cells with activated hemichannels, and clones with closed hemichannels or even lacking hemichannels in their membranes within a given tumor is possible. Dynamic adaptations of tumor cells to environmental pressure could also support the opportunistic transit between the functional states of hemichannels in tumor cells (e.g., proliferation vs. migration or invasion).

Anti-tumor effects of ATP release through hemichannels

Although a good deal of evidence points to the possible pro-tumorigenic and proliferative effect of both Cx and Panx1 hemichannels activation, it is relevant to consider that association with reduced cell proliferation/survival is also apparent. Moreover, sustained or de-regulated opening of hemichannels can be deleterious for cells. In fact, activation of Cx43 or Panx1 hemichannels by noxious stimuli such as ischemic-like conditions or oxidative stress can accelerate self and paracrine cell death through massive ATP-release and $[Ca^{2+}]_i$-related mechanisms (Contreras et al., 2004; Retamal et al., 2007b; Decrock et al., 2011; Carette et al., 2014) (**Figure 2**). Consistent with an anti-tumor effect, exogenous Panx1 expression was associated with decreased proliferation, motility and anchorage-independent growth in cultured C6 glioma cells (Lai et al., 2007). Similar findings were obtained after expression of Panx2 (Lai et al., 2009). In addition, Panx1 and Panx2 expression were associated with reduced tumor volume in murine C6 tumor implants, which led to propose them as tumor suppressor genes. In line with this observation, forced expression of Panx1 and Panx3 increased the dye uptake and reduced the proliferation of cultured rat epidermal keratinocytes (Celetti et al., 2010). Data from our group revealed that exogenous Panx1 expression significantly reduced the cell density of cultured HeLa cells (**Figure 4A**). In addition and using a previously reported antibody (Buvinic et al.,

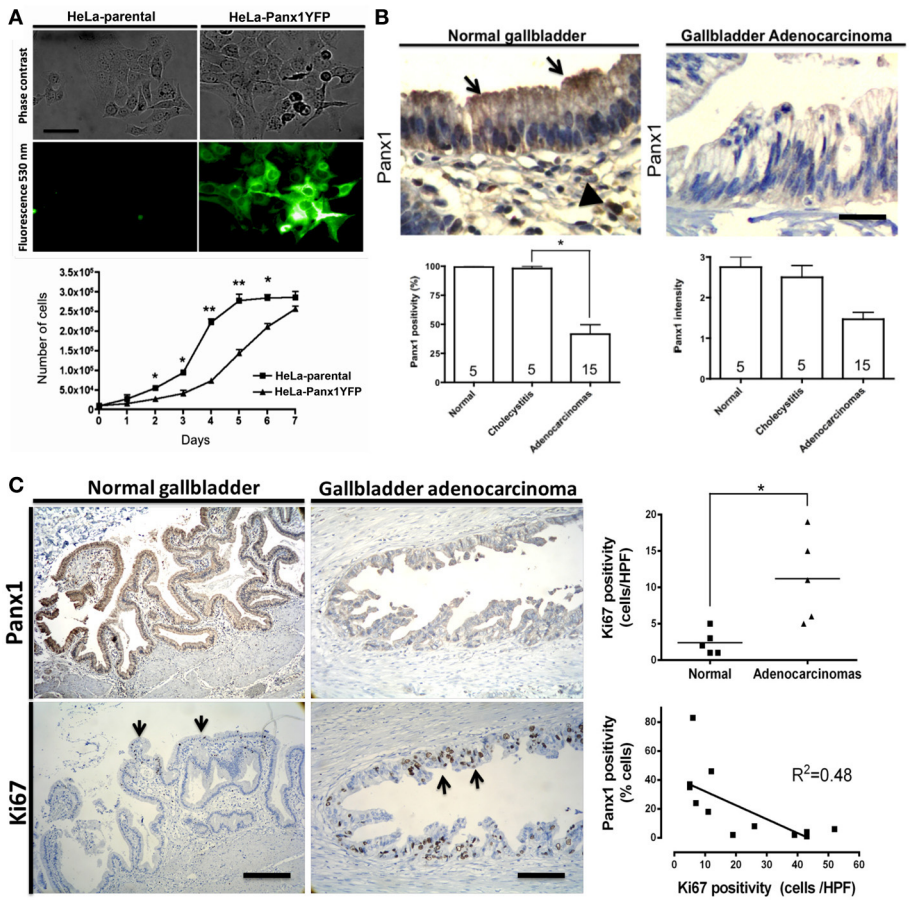

FIGURE 4 | (A) Exogenous Panx1 expression reduces cell density in cultured HeLa cells. Microphotograph showing phase contrast (upper panel) and fluorescence images (lower panel) of cultured parental HeLa cells (left) and HeLa cells stably expressing Panx1-YFP (right, green channel 530 nm). Graph showing the cell density of parental (boxes) and Panx1-expressing HeLa cells (triangles) at different days of culture. Cells were exposed to 10% fetal bovine serum and counted each 24 h for 7 days. Mean ± s.e.m. of 6 independent experiments is shown. Bar = 30 μM. **(B)** Panx1 expression is lower in gallbladder adenocarcinomas. Upper-left panel: Immunohistochemical Panx1 expression using a previously reported antibody (Buvinic et al., 2009) in morphologically normal gallbladder showing positivity in epithelial cells (arrows) and mononuclear inflammatory cells from the lamina propria (arrowheads). Upper-right panel: Panx1 expression in gallbladder adenocarcinoma showing lower Panx1 expression in tumor cells. Lower panels: Panx1 expression (left) and signal intensity (right) in normal gallbladders, chronic cholecystitis, and gallbladder adenocarcinomas. The amount of positive cells is expressed as percentage ± s.e.m. Intensity is expressed as arbitrary levels (1: weak; 2: moderate; 3: strong). Experimental conditions remained the same in all experiments. The number of cases in each category is indicated within the bars. Bar = 25 μm **(C)** Panx1 expression is inversely related with proliferation in gallbladder carcinomas. Representative microphotographs showing immunohistochemical Panx1 signal (upper panels) and Ki67 nuclear staining (lower panels, arrows) in normal gallbladder (left) and in gallbladder adenocarcinoma (right). Graph showing the Ki67 index in normal samples (black boxes) and in carcinomas (triangles). Linear regression analysis showing inverse relationship between Panx1 and Ki67 positivity in gallbladder carcinomas. $R^2 = 0.48$; $^*p < 0.05$ and $^{**}p < 0.01$. Bar = 400 μm.

2009), we found that Panx1 is present in morphologically normal human gallbladder epithelium and its levels are lower in gallbladder adenocarcinomas (**Figure 4B**). Also, in gallbladder tumors Panx1 signal is inversely related to the proliferative activity as determined by the nuclear Ki-67 positivity rate (**Figure 4C**). Of note, the rate of nuclear Ki-67 positive cells is frequently used as a metric of the tumor proliferation grade and has prognostic value in diverse human malignancies such as gliomas, breast carcinomas and melanomas (Weigel and Dowsett, 2010).

The apparent discordances between the effects of Panx1 expression in proliferation and motility of melanoma, glioma and epithelial tumor cells could be due to tumor cell-specific and/or cell lineage-specific functions of hemichannels. Distinct alterations in oncogenic pathways between melanocytic, glial and epithelial tumors are well known.

HEMICHANNELS IN INTRACELLULAR CA^{2+} BALANCE, CELL SURVIVAL AND MIGRATION

Intracellular Ca^{2+} is recognized as key second messenger and master regulator of cell fate. Altered cytosolic Ca^{2+} can be associated with cell death and cell proliferation, depending on the cell type, concentration and biological context. The intracellular Ca^{2+} balance is tightly regulated and active mechanisms have evolved to keep its levels in the low nanomolar range under resting conditions. The extracellular levels of Ca^{2+} are several orders of magnitude higher (e.g., milimolar range), creating a

prominent inward concentration gradient. A complex signaling system administrates dynamic intracellular Ca^{2+} reservoirs within organelles such as the endoplasmic reticulum, mainly through the function of endomembranous ion channels, Ca^{2+} ATPases and binding to Ca^{2+} rich proteins (Berridge et al., 2003). Also, this system allows the functional coupling between intracellular Ca^{2+} stores and extracellular signals through activation of plasma membrane receptors. Such surface receptors include the dimeric tyrosine kinase growth factor-type receptors (e.g., EGFR, FGFR, PDGFR, etc.) and a wide array of seven-transmembrane domain G-protein coupled receptors (e.g., P2Y purinergic receptors, peptide hormone receptors, chemokine receptors, neurotransmitters, etc.). In addition, the influx of Ca^{2+} through ligand-activated channels also participates in the intracellular Ca^{2+} homeostasis. Intracellular Ca^{2+} signaling and the generation of intercellular Ca^{2+} waves have been involved in key cancer-related process including escape from apoptosis, perpetuation of growth, cell migration, metastasis and promotion of angiogenesis (Monteith et al., 2007; Parkash and Asotra, 2010; Monteith et al., 2012). In particular, Ca^{2+} increases have been shown to favor cell proliferation through altered transcription and enhance cell motility through remodeling of actin cytoskeleton and focal adhesions (Lee et al., 2011; Monteith et al., 2012). Moreover, remodeling of Ca^{2+} signaling is a feature of diverse cancers and consists frequently in altered expression and function of Ca^{2+} channels and pumps (Roderick and Cook, 2008; Monteith et al., 2012). For instance, purinergic receptors, TRP channels, store operated Ca^{2+} channels (e.g., ORAI and STIM) and Ca^{2+} pumps are deregulated in diverse human tumors (Lee et al., 2011; Chen et al., 2013). Intracellular effectors commonly involved in the pro-oncogenic effects of Ca^{2+} include protein kinases (e.g., PKC), phosphatases (e.g., calcineurin), transcription factors (e.g., NFAT) and Ca^{2+}-sensitive signaling proteins (e.g., calmodulin).

As discussed, the activation of hemichannels is linked to the intracellular Ca^{2+} balance by mediating the autocrine/paracrine signaling through transmembrane exchange of nucleotides and Ca^{2+}-modifying second messengers (reviewed in Wang et al., 2013a,b) (**Figure 2**, blue arrows and text). In addition, early observations suggested that hemichannels activated by metabolic inhibition induced massive Na^{+} and Ca^{2+} entry in cultured rabbit myocardiocytes (Li et al., 2001). In support of this notion, recent data using reconstituted systems support that hemichannels formed by Cx26 (Fiori et al., 2012), Cx32 (Sánchez et al., 2009) and Cx43 (Schalper et al., 2010) allow Ca^{2+} influx (reviewed in Orellana et al., 2012). Notably, increasing the $[Ca^{2+}]_i$ with endogenous ligands (e.g., FGFs) or a Ca^{2+} ionophore enhances the activation of hemichannels formed by Cx32 and Cx43 (De Vuyst et al., 2006, 2009; Schalper et al., 2008a; Garré et al., 2010). Also, autocrine/paracrine signaling by additional (non-nucleotide) hemichannels permeants such as IP_3, glutamate and prostaglandins involve increased intracellular Ca^{2+} (Schwartz and Alford, 2000; Meves, 2006; Gossman and Zhao, 2008; Traynelis et al., 2010), suggesting the possibility of hemichannels-mediated Ca^{2+} influx/Ca^{2+}-increase/Ca^{2+} influx loops (see **Figure 2**). To our knowledge, Panx hemichannels have not yet been proven to be Ca^{2+} permeable. However, they can also be activated by increased $[Ca^{2+}]_i$ and the permeability properties of Panx1 hemichannels suggest that they are likely to allow the passage of ions. Taken together, these data suggest that under specific pro-tumorigenic circumstances (e.g., altered signal transduction and tissue microenvironment) activated hemichannels could favor cancer progression by directly increasing intracellular Ca^{2+}-dependent tumor cell proliferation and motility.

CONCLUSIONS AND FUTURE PERSPECTIVES

Cxs and Panxs are frequently altered in human tumors and cell lines. Emerging data has challenged the notion that these proteins are tumor suppressors and suggest that they could favor tumor growth and dissemination in some circumstances. Although diverse technical aspects limit the study of hemichannels, current data support their involvement in the modulation of cell proliferation and migration. The presence, regulation and characteristics of hemichannel functions in cancer cells remain largely unexplored, but their permeability to nucleotides and Ca^{2+} suggest a possible role in favoring tumor growth and disease progression. Future efforts to characterize the expression and specific functions of hemichannels in diverse human malignancies will support their use as prognostic/predictive markers and to design novel anti-cancer therapeutic strategies.

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