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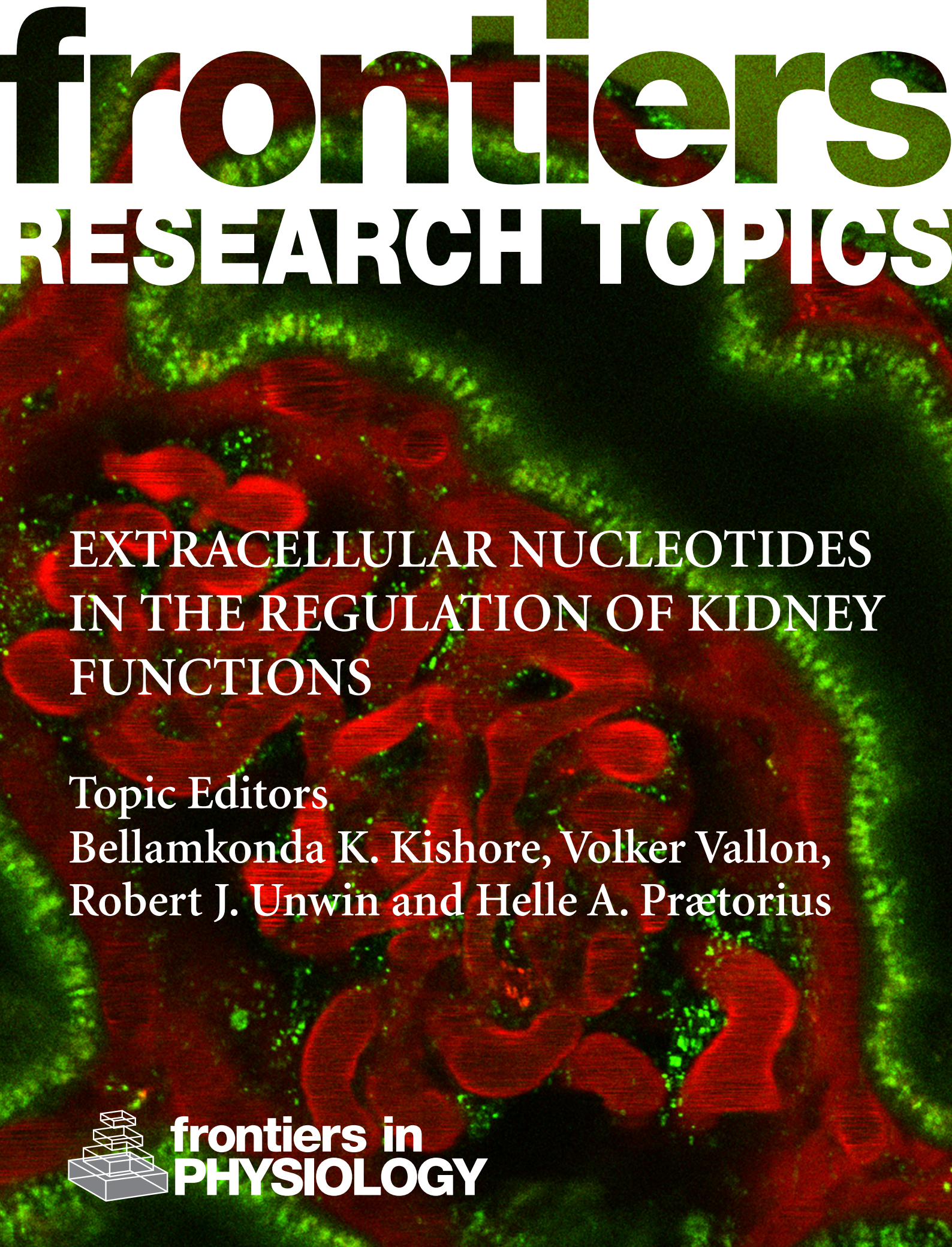

# EXTRACELLULAR NUCLEOTIDES IN THE REGULATION OF KIDNEY FUNCTIONS

Topic Editors
Bellamkonda K. Kishore, Volker Vallon,
Robert J. Unwin and Helle A. Prætorius

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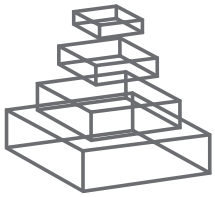

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# EXTRACELLULAR NUCLEOTIDES IN THE REGULATION OF KIDNEY FUNCTIONS

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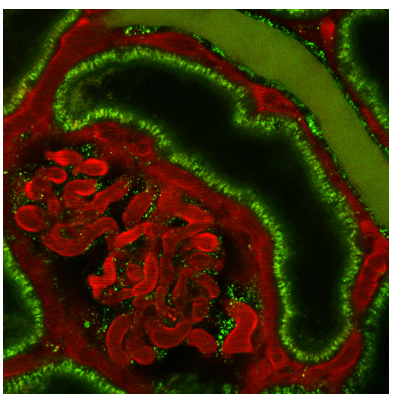

Intravital fluorescence image of the rat kidney showing a glomerulus and surrounding proximal and distal tubule segments, the key anatomical structures within the kidney where purinergic signaling is critically important. Plasma was labeled red using Alexa594-albumin, tubular fluid in the collecting duct was labeled green using Lucifer yellow. (Courtesy: Dr. Janos Peti-Peterdi).

ATP is normally regarded as the major source of fuel for the energy-demanding processes within cells; however, ATP and other nucleotides (such as ADP, UTP, UDP) can be released from cells, where they act as autocrine or paracrine signaling molecules to affect cellular and tissue functions. In response to various stimuli, ATP and other nucleotides are released from cells in a regulated fashion, either by exocytosis of nucleotide-containing vesicles, or through channels in the plasma membrane. This process occurs in virtually every organ or cell in the body. The cellular effects of these extracellular nucleotides are mediated through specific membrane receptors (P2X and P2Y). These nucleotide signals can be terminated by rapid degradation of the ligand molecules by ecto-nucleotidases (e.g., NTPDases and NPPs). Many of the molecular components essential to nucleotide signaling have been cloned and characterized in detail, and their crystal structures are beginning to emerge. The collected data on extracellular nucleotides suggest a vivid and dynamic signaling system that is modulated by the expression and sensitivity of specific receptors on cells, and by the regulated release and extracellular degradation of ATP and other nucleotides; thus creating a microenvironment of highly regulated paracrine or autocrine control mechanisms.

Within the kidney, extracellular nucleotides have emerged as potent modulators of glomerular, tubular, and microvascular functions. These functions include, but are not limited to, tubular transport of water and sodium, tubuloglomerular feedback and auto-regulation, regulation of blood pressure and the microcirculation, oxidative stress, and cell proliferation/ necrosis/apoptosis. Moreover, studies have also uncovered the interaction of nucleotide signaling with other mediators of renal function, such as vasopressin, aldosterone, nitric oxide, prostaglandins, angiotensin II, and the ATP-break down product adenosine. These insights have provided a more comprehensive and cohesive picture of the role of extracellular nucleotides in the regulation of renal function in health and disease. The availability of transgenic mouse models of the key proteins involved in nucleotide signaling has markedly enhanced our understanding of the physiological and pathophysiological roles of the different components of the system in the kidney. Although at a preliminary stage, the pathophysiological significance of this system in the kidney holds the key for the development of an entirely new class of drugs for the treatment of disease conditions, including disorders of water and/or sodium homeostasis, hypertension, acute kidney injury, etc.

Thus, the regulation of renal function by extracellular nucleotides is clearly emerging as a distinct field and discipline in renal physiology and pathophysiology that has the potential to develop new drug treatments. In this e-book, we bring together a spectrum of excellent papers by leading experts in the field which present and discuss the latest developments and state-of-the-art technologies. The papers broadly cover three areas. The first two articles deal with ATP releasing mechanisms, wherein Bjaelde and associates show that spontaneous and induced ATP release can occur via exocytosis in renal epithelial cells; and Svenningsen and associates demonstrate that mechanosensitive connexin 30 hemichannels mediate tubular ATP release and purinergic calcium signaling in the collecting duct, which may play an important role in regulation of salt and water reabsorption in the collecting duct.

Articles 3 to 7 cover a variety of physiological aspects of purinergic signaling and its interactions with other intrarenal systems. Persson and associates demonstrate how the interactions between adenosine, angiotensin II and nitric oxide influence the afferent arteriole sensitivity and thus the tubuloglomerular feedback, which is critical for efficient function of the nephron as a unit. Craigie and associates reviewed our current knowledge of relationship between P2X4 and P2X7 receptors and its impact on renal function. Menzies and associates present data showing the effect of P2X4 and P2X7 receptor antagonism on the pressure diuresis relationship in rats, which may play a role in hypertension-related kidney damage. Looking from a different perspective, Crawford and associates show that ATP released from sympathetic nerves regulate renal medullary vasa recta diameter through pericytes, which may potentially regulate medullary blood flow. Marques and associates, document that genetic deletion of P2Y2 receptor, a widely studied purinergic receptor, does not affect NaCl absorption in medullary thick ascending limb.

Finally, articles 8 to 10 deal with the potential roles of purinergic signaling in pathophysiological conditions. Sebastian and associates show that deficiency of P2Y2 receptor aggravates chronic kidney disease by accelerating the disease progression. Rangan reviewed our current knowledge on the role of extracellular ATP and P2 receptor signaling in regulating cyst growth and interstitial inflammation in polycystic kidney disease, one of the most common kidney diseases. Birch and associates summarized the current evidence for the involvement of P2X receptors in the regulation of renal tubular and vascular function, and highlighted the novel data describing their putative roles in regulating the physiological and pathophysiological processes in the kidney.

Last but not least, we thank all the authors for contributing their valuable work and the Frontiers in Physiology Editorial Office for bringing out this e-book.

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# Renal epithelial cells can release ATP by vesicular fusion

***Randi G. Bjaelde, Sigrid S. Arnadottir, Morten T. Overgaard, Jens Leipziger and Helle A. Praetorius****

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Renal epithelial cells have the ability to release nucleotides as paracrine factors. In the intercalated cells of the collecting duct, ATP is released by connexin30 (cx30), which is selectively expressed in this cell type. However, ATP is released by virtually all renal epithelia and the aim of the present study was to identify possible alternative nucleotide release pathways in a renal epithelial cell model. We used MDCK (type1) cells to screen for various potential ATP release pathways. In these cells, inhibition of the vesicular $H^+$-ATPases (bafilomycin) reduced both the spontaneous and hypotonically (80%)-induced nucleotide release. Interference with vesicular fusion using N-ethylamide markedly reduced the spontaneous nucleotide release, as did interference with trafficking from the endoplasmic reticulum to the Golgi apparatus (brefeldin A1) and vesicular transport (nocodazole). These findings were substantiated using a siRNA directed against SNAP-23, which significantly reduced spontaneous ATP release. Inhibition of pannexin and connexins did not affect the spontaneous ATP release in this cell type, which consists of ∼90% principal cells. TIRF-microscopy of either fluorescently-labeled ATP (MANT-ATP) or quinacrine-loaded vesicles, revealed that spontaneous release of single vesicles could be promoted by either hypoosmolality (50%) or ionomycin. This vesicular release decreased the overall cellular fluorescence by 5.8 and 7.6% respectively. In summary, this study supports the notion that spontaneous and induced ATP release can occur via exocytosis in renal epithelial cells.

**Keywords: vesicles, ATP, flow, hypotonic swelling, $Ca^{2+}$, MDCK**

## INTRODUCTION

P2 receptor activation substantially influences the overall function of renal epithelia. In general, P2 receptor activation dampens transepithelial transport. For example extracellular P2 receptor agonists reduce the $HCO_3^-$ reabsorption in the proximal tubule (Bailey, 2004), and $P2Y_2$ receptor activation markedly restrains the arginine vasopressin (AVP)-induced water permeability in the collecting duct (Kishore et al., 1995). Moreover, $P2Y_2$ receptor activation has also been shown to impair the activity of epithelial $Na^+$ channels (ENaC) in the collecting duct (Pochynyuk et al., 2010). The two mechanisms in the collecting duct underlie the suggested distal hyper-reabsorption documented in $P2Y_2$ receptor-deficient mice (Pochynyuk et al., 2010), which also have been associated to the hypertension observed in these animals (Rieg et al., 2007, 2011). Recently, basolateral application of ATP has been shown to significantly inhibit the transepithelial transport in the thick ascending limb via activation of P2X receptors (Silva and Garvin, 2009; Marques et al., 2012). This general pattern of P2 receptor-mediated transport inhibition prompts the suggestion that epithelial ATP release, and the subsequent P2 receptor activation may constitute a negative feedback system that protects renal epithelial cells from overstimulation. This hypothesis should be considered in the light of the type of stimuli known to induce ATP release from renal epithelial cells. These include many types of mechanical perturbations, such as osmotic stress (Boudreault and Grygorczyk, 2004), flow-dependent force on the primary cilium (Praetorius and Leipziger, 2009) and pressure-induced stretching of the epithelium (Praetorius et al., 2004a; Jensen et al., 2007). Moreover, AVP has been shown to induce the release of ATP as detected using a biosensor (Odgaard et al., 2009). It is possible to speculate that the release of ATP from renal epithelia is a self-protection mechanism to avoid either mechanical or hormonal overstimulation of the renal epithelial cells.

The mechanism for regulated ATP release is still only settled for a subset of renal epithelial cells. With the existing data, it is unlikely that there is a single common pathway for ATP release from all types of renal epithelial cells. A recent study demonstrated the importance of connexin30 (cx30)-hemichannels as a pathway for ATP release in the intercalated cells of the murine collecting duct (Sipos et al., 2009), where cx30 is expressed exclusively in the apical domain (McCulloch et al., 2005). As at least some ATP-release pathways are cell type-specific, it would be reasonable to assume that, in general, renal epithelia use several pathways to release this paracrine factor. Other epithelia have been shown to possess cell type-specific ATP release mechanisms, such as the respiratory epithelium where a pore-mediated (pannexin 1) release mechanism predominates in ciliated epithelial cells (Seminario-Vidal et al., 2011) and exocytosis/vesicular release is responsible for the ATP release from the goblet cells (Jones et al., 2012).

Here, we show that both the constitutive and stimulated ATP release by MDCK cells are reduced by interfering with either vesicular acidification or vesicular release, whereas inhibitors

of connexins/pannexins did not. Moreover, using total internal reflection fluorescence (TIRF) microscopy we detected the spontaneous and stimulated release of vesicles, which could take up quinacrine and N-methylanthraniloyl-ATP (MANT-ATP), and the content of ATP in the quinacrine-loaded vesicles was confirmed by fluorescence activated cell sorting (FACS) and luminometry. Based on our findings, we conclude that ATP can be released via exocytosis from renal epithelial cells.

## MATERIALS AND METHODS

### CELL CULTURE

Wild-type Madin-Darby Canine Kidney (MDCK) type 1 cells (passages 54–70; American Type Culture Collection, Rockville, MD, USA) were cultured to confluence in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum, 2 mM glutamine, 100 U $ml^{-1}$ penicillin, and 100 $\mu g$ $ml^{-1}$ streptomycin (Gibco, Grand Island, NY, USA), but without riboflavin and phenol red as previously described (Praetorius and Spring, 2001; Praetorius et al., 2004b). For microscopy the cells were cultured on 25 mm diameter cover slips (VWR, Herlev, Denmark, for wide field microscopy or 1001/25 (Glaswarenfabrik Karl Hecht KG, Sondheim, Germany) for TIRF microscopy to either confluence or non-confluence depending on the protocol. For all other experiments, MDCK cells were cultured to confluence on 25-mm diameter filter inserts (0.4 $\mu m$) HD PET membrane (high pore density polyethylene terephthalate), in 6-well plates, in 25 $cm^2$ Falcon tissue culture flasks or in 100 mm petri dishes (all from Becton Dickinson Labware Europe, Le Pont de Claix, France).

### MICROSCOPY AND PERFUSION

MDCK cell mono-layers cultured on coverslips were viewed at 37°C on the stage of an inverted microscope (TE-2000, Nikon) equipped with differential interference contrast (DIC) combined with low-level fluorescent light provided by a xenon lamp and monochromator (Visitech International, Sunderland, UK). Imaging was performed using either a plan Fluo 20X, 0.75 NA or a 60X, 1.4 NA Plan Apo objective (Nikon), an intensified SVGA charged coupled device (CCD) camera and imaging software (Quanticell 2000/Image Pro, VisiTech). The cellular fluorescence was sampled at the rate of 1 Hz and measurements were initiated 60 s prior to the start of perfusion. We used TIRF microscopy to visualize vesicles just beneath the plasma membrane. The TIRF set-up was provided by Bio-Science ApS, Gilleleje, Denmark and consisted of an iMIC stage (Till-Photonics, Munich, Germany) equipped with three lasers (405 nm iWave Toptica, 488 nm iWave Toptica (Toptica Photonics AG, Graefelfing, Germany), and 532 nm Cobolt Jive (Cobolt AB, Solna, Sweden). A Yanus scan-head combined with the Polytrope imaging-mode switch the laser beams via a vanometric mirror so that they focused on the back focal plane of the objective. The lasers were adjusted to an angle of ∼64° to create an evanescence field around the glass-salt solution interface. The preparation was imaged with a 60X, 1.45 NA Plan Apo (Olympus) objective and a CCD camera (Sensicam qe, PCO, Kelheim, Germany). The cells were mounted in a semi-open (covered by only half a cover-slip) chamber modified from the chamber (RC-21BRFS) available from Warner Instruments, Hamden, CT, USA. The semi-open chamber avoids the build-up of pressure in the system, reduces evaporation compared to a completely open chamber and retains the good optical properties of a closed chamber. The solutions were superfused at constant flow rates of 12 $\mu l$ $s^{-1}$, which corresponds to a bulk flow velocity of 820 $\mu m$ $s^{-1}$ equaling 0.103 dynes $cm^{-2}$ (assuming $6.97 \times 10^{-3}$ poise as the dynamic viscosity of water at 37°C). All antagonist and agonist solutions were prepared from frozen stock-solutions immediately prior to each experiments.

### ANALYSIS OF $[Ca^{2+}]_i$ IMAGING

On average, 300 fluo-4 loaded MDCK cells were imaged in time-lapse (60 images at 1 Hz), and the cells that displayed spontaneous increases in the fluorescence intensity were identified as follows: the fluorescence intensity of each pixel in the first frame of the image sequence was multiplied by 1.05 and subsequently subtracted from the entire sequence. Using this procedure, cells that displayed an increase in florescence intensity greater than 5% were observed as bright areas on a black background. These bright areas were marked as regions of interest (ROI) in all of the frames in the modified sequence. Subsequently, the ROIs were transferred to the original image sequence, and the average fluorescence intensities for the ROIs were extracted from this sequence. The data were imported to the analysis program Igor Pro (Wavemetics, Lake Oswego, OR, USA) and the multi-peak finding feature of the program was used to identify and quantify each computed $Ca^{2+}$ event. Thus, a $Ca^{2+}$ event was defined as a single increase in fluo 4 fluorescence intensity greater than 5%. For each experiment, his procedure was used to determine the number of responding cells, the number of $[Ca^{2+}]_i$ events per second in the field of observation (∼300 cells), as well as the amplitude and duration of the each event.

### siRNA-MEDIATED KNOCKDOWN OF CANINE SNAP-23

The protocol used was as previously described by Ge et al. (2003) with minor modifications. Briefly, confluent MDCK cells were re-suspended in serum-free RPMI-1640 medium (Sigma-Aldrich) at a cell density of approximately $5 \times 10^6$ $ml^{-1}$. A scrambled siRNA or a siRNA directed against canine SNAP-23 (scrambled control and SASI_Mm01_00176533 and scrambled control from Sigma) or TE-buffer as a control (mock transfection) were added to the cell suspensions, at a final concentration of 2 $\mu M$. The mixtures were incubated on ice for 5 min before the cells were transferred to a 4 mm Gene Pulser cuvette (Bio-Rad-Laboratories, Copenhagen, Denmark) and subjected to a single electroporation pulse (400 V and 975 $\mu F$, Gene Pulser Xcell (Bio-Rad). Immediately after the pulse, 500 $\mu l$ DMEM containing 10% fetal bovine serum was added. Each group of cells was divided into three subgroups for analysis at 24, 48, or 72 h. The effectiveness of the siRNA silencing was investigated using quantitative PCR (see below), immunoblotting (see below) and live cell $[Ca^{2+}]_i$ imaging (see above) for all three time groups.

### QUANTITATIVE RT-PCR

The RNeasy MiniKit from QIAGEN GmbH (Hilden, Germany) was used to isolate RNA according to the vendor's protocol. The RNA concentration was determined by spectrophotometery. RNA

(50 ng $\mu l^{-1}$) was incubated with 0.33 μM Random Decamers (MWG Biotech, Ebersberg, Germany) for 3 min at 85°C, then the reverse transcriptase reagents (0.46 mM dNTP [TaKaRa Bio Inc., Shiga, Japan], 4.25 μl of 5x First Strand Buffer [Invitrogen], 4.76 U $l^{-1}$ SuperScript III Reverse Transcriptase [Invitrogen] and 0.24 U $l^{-1}$ SUPERase-ln [Ambion, Austin, TX, USA]) were added. As negative controls, water was added instead of reverse transcriptase or RNA. The reverse transcriptase program was as follows: 5 min at 55°C, 60 min at 45°C, 15 min at 70°C, and 10 min at 4°C. Reverse transcription was confirmed by electrophoresis on a 2% agarose gels (Low EEO; AppliChem GmbH, Darmstadt, Germany) using Tris-borate-EDTA (*TBE*) buffer. Qualitative RT-PCR (qPCR) was performed to determine the relative mRNA levels. The primers used were: SNAP-23-F 5′-GCA TAG AAG AAG GCA TGG AC-3′ (100 nM), SNAP-23-R, 5′-GTT GTT GAG GCT GCC CAT TT-3′ (500 nM), G3PDH-F, 5′-CAC GGC AAA TTC CAC GGC ACA G-3′ (500 nM) and G3PDH-R, 5′-ATG ACC ACC GTC CAT GCCA A-3′ (100 nM). The probes used were; SNAP-23-P 5′-CAT GGG GAG ATG GTG AAG ACA ACT-3′ and G3PDH-P 5′-TTG TCA GCA ATG CCT CCT GCA CCA CCA ACT (both 100 nM, 5′ Fluorescein, 3′ Blackhole Quencher 1). The master mix consisted of: 0.2 mM dNTPs (Invitrogen), 1x Ex Taq buffer (TaKaRa), 5 mM $MgCl_2$, 0.025 U Taq $\mu l^{-1}$ (TaKaRa). The cDNA was diluted two times and the PCR program (1 cycle of 10 min at 95°C; 40 cycles of 30 s at 95°C, 1 min at 60°C, and 1 min 72°C) was run on a Mx3000, Stratagene thermocycler (Agilent Technologies inc., Santa Clara, CA, USA). The data were analyzed using MxPro ver. 4.0 software (Stratagene).

#### PROTEIN PURIFICATION

For protein purification, MDCK cells were resuspended in lysis-buffer (50 mM Tris-HCl, 250 mM NaCl, 0.5% nonyl phenoxypolyethoxylethanol-40, 5 mM EDTA, 20 mM NaF, 0.5 M Phenylmethanesulfonyl fluoride [dissolved in EtOH], phosphatase inhibitor cocktail mix II [diluted 1:100, Sigma], and one protease inhibitor tablet [Roche]), incubated on ice for 15 min with a gentle vortexing every 2 min and then centrifuged for 15 min at 14200 g. The supernatant was transferred to a new tube and the pellet was re-suspended in 500 μl lysis-buffer and stored at −80°C. As control, a piece of fresh mouse kidney was homogenized and treated as described above using lysis-buffer. After 30 min incubation on ice, with intermittent gentle vortexing, the tissue lysate was centrifuged at 14,200 *g* for 30 min, the supernatant was transferred to a new tube, and the pellet was re-suspended in lysis buffer and stored at −80°C. The protein concentrations of the lysates were determined using the Pierce BCA, Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's protocol.

#### SDS-PAGE AND WESTERN BLOTTING

Protein samples (10 μg) were loaded on two identical ready gels (Bio-Rad) and electrophoresed at 125 V performed for 1–1.5 h at room temperature. BenchMark Pre-Stained Protein Ladder (Invitrogen) or Spectra Multicolor Broad Range Protein Ladder (Fermentas, Burlington, Ontario, Canada) were used as molecular weight markers. The proteins were transferred onto ethanol-activated Immobilion-FL PVDF membranes (pore size 0.45 μm, Millipore, Billerica, MA, USA) at 100 V for 1 h at 4°C. After overnight blocking at 4°C with 2% skimmed-milk (ARLA, Viby, Denmark) in 0.1 M PBS, the first membrane was incubated with primary antibody against SNAP-23 (1:1000, Synaptic Systems, Goettingen, Germany) diluted in 0.1 M PBS containing 0.1% Tween-20 (PBS-T) for 1 h at room temperature. As a control, the second membrane was incubated with SNAP-23 primary antibody (1:1000), with SNAP-23 control peptide (1:1000 Synaptic Systems, Goettingen, Germany). The membranes were then incubated with Donkey-anti-rabbit IRDye680 secondary antibody (1:12000, LI-COR GmbH, Bad Homburg, Germany) in the dark, and the bands were visualized using a LI-COR Odyssey scanner.

#### HYPOTONIC STRESS ASSAY

The MDCK cells were cultured to confluence on 25-mm diameter filter inserts, which allowed samples to be taken both from the apical and basolateral sides of the epithelium. The cells were equilibrated in HEPES buffered salt solution (HBS) for 1 h at 37°C prior to the experiment. At the end of this incubation time, samples for the baseline ATP release were carefully taken and replaced with fresh HBS. Hypotonic stress was induced by replacing half of the HBS on the basolateral side of the filters with water. After 10 min at 37°C, samples were carefully taken from both sides of the filter. All samples were boiled for 1 min immediately after sampling (to prevent potential enzyme dependent ATP degradation) and stores on ice before analysis.

#### ISOLATION OF INTACT VESICLES FROM MDCK CELLS

For isolation of intact vesicles we used a cell cracker developed at European Molecular Biology Laboratory (Heidelberg, Germany) with an 8.01 mm diameter ball. The cell cracker mechanically disrupts the cells and releases the contents of the cells. The cell cracker was kept on ice for the entire protocol. The MDCK cells loaded with quinacrine (5 μM, 30 min) were passed through the cell cracker 20 times and then briefly centrifuged and re-suspended (repeated 10 times); at this time a significant amount of free quinacrine-loaded vesicles could be observed in the suspension by wide field microscopy (60X, 1.4 NA Plan Apo objective [Nikon]). After the last centrifugation step, the supernatant containing the vesicles was transferred to a new tube and fluorescence-activated cell sorting (FACS) was used to sort the cell debris into four populations, one of which contained only small vesicles (defined by the size and 488 nm fluorescence). A high $K^+$ solution (pH 7.2, 125 mM KCl, 0.8 mM $MgSO_4$, 14 mM Na-HEPES, 5.6 mM *D*-glucose) was used as the re-suspension media during cell cracking to mimic the cytosolic environment. Cell sorting was performed at the FACS Core Facility, Health, Aarhus University, Denmark using a FACSAria III (Becton Dickinson) high speed cell sorter equipped with a 488 nm laser and a (530/30 nm) emission filter. The vesicles were sorted on the basis of the 488 nm fluorescence, and then size gated to include only the smallest quinacrine positive particles. After sorting, an aliquot of the sample was taken for microscopy and the reminder was centrifuged using an Air-Driven Ultracentrifuge (Airfuge, Beckman) running at 20 psi (corresponds to 100,000 g) for 10 min. The vesicle pellet was re-suspended in lysis-buffer (170 mM $NH_4^+$, 170 mM $Cl^-$, 110 μM EDTA, 220 μM $Na^+$, 1 mM $K^+$, 1 mM

$HCO_3^-$) and the ATP concentration was determined using the luciferin-luciferase assay as described below.

### LUCIFERIN-LUCIFERASE ASSAY

The ATP Determination Kit (A22066, Invitrogen) was employed for the luciferin-luciferase assay using a modified version of the vendor's protocol. To each well in a 96-well plate, the kit reaction buffer was added to the samples or standard solution provided by the kit at room temperature. The samples and standards was read immediately after in an Enspire 2300 Multilabel Reader (PerkinElmer, Waltham, MA, USA) for the hypotonic stress assay and a Mithras LB940 Multimode Reader (Berthold Technologies, Bad Wildbad, Germany) for measurement of the ATP content of intact vesicles. In the hypotonic stress assay, four different sets of ATP standards were run alongside the samples from each experiment to account for the effects of hypotonic dilution and DMSO, in which bafilomycin A1 was dissolved. In the vesicle assay, two different sets of standards were run to account for the high $K^+$ buffer and the lysis-buffer.

### SOLUTIONS

The HEPES-buffered salt solution had the following composition, in mM: [$Na^+$] 138, [$K^+$] 5.3, [$Ca^{2+}$] 1.8, [$Mg^{2+}$] 0.8, [$Cl^-$] 126.9, [$SO_4^{2-}$] 0.8, HEPES 14, glucose 5.6, probenecid 5, pH 7.4 (37°C, 300 mOsmol $l^{-1}$). The $Ca^{2+}$-free solution had the following composition, in mM: [$Na^+$] 139, [$K^+$] 5.3, [$Mg^{2+}$] 0.8, [$Cl^-$] 125.3, [$SO_4^{2-}$] 0.8, EGTA 1, Hepes 14, glucose 5.6, probenecid 5, pH 7.4 (37°C, 300 mOsmol $l^{-1}$). TE-buffer, in mM: Tris-HCl 10, EDTA 1, pH 7.5. Lysis buffer for vesicle lysis had the following composition, in mM: [$NH_4^+$] 170, [$Cl^-$] 170, EDTA 0.1, [$Na^+$] 0.2, [$K^+$] 1, [$HCO_3^-$] 1. High $K^+$-solution had the following composition, in mM: [$Na^+$] 14, [$K^+$] 125 mM, [$Cl^-$] 125, [$Mg^{2+}$] 0.8, [$SO_4^{2-}$] 0.8, HEPES 14, glucose 5.6, pH 7.2.

Sources of chemicals were: fluo 4-AM and BAPTA-AM (Invitrogen), probenecid, quinacrine from Sigma, and MANT-ATP from Jena Bioscience (Jena, Germany). The solution used in fluo 4 experiments contained 5 mM probenecid to inhibit extrusion of the dye. All experiments were carried out at 37°C, pH 7.4.

The following substances were diluted in HBS: ATP, probenecid, N-ethylmalemide; ethanol: Brefeldin A and ionomycin. The following substrances were diluted in DMSO: bafilomycin A1, cytochalacin B, 18α-glucyrrhetinic acid and nocodazol. The content of vehicle in all experiments did not exceed 0.1% (*v/v*), which does not influence the [$Ca^{2+}$] oscillations.

### STATISTICCAL ANALYSIS

All values are shown as the mean ± s.e.m. Statistical significance was determined using the Mann-Whitney-Wilcoxon non-parametric test for comparison of two groups and the One-Way ANOVA followed by a Tukey-Kramer multiple comparison test for comparison of more than two groups. In both cases a *p*-value less than 0.05 was considered significant. The number of observations refers to the number of preparations (independent experiments) analyzed.

## RESULTS

### SPONTANEOUS AND STIMULATED $[Ca^{2+}]_i$ INCREASE IN MDCK CELLS

Previously, we have shown that renal epithelial cells spontaneously release ATP. By carefully comparing the spontaneous $[Ca^{2+}]_i$ oscillations in MDCK cells and perfused renal tubules with extracellular ATP, as measured using the luciferin/luciferase assay, we provided evidence that these spontaneous $[Ca^{2+}]_i$ oscillations were indicative of the spontaneous release of extracellular ATP by the renal epithelia (Geyti et al., 2008). We used this association to screen for potential ATP release pathways in type 1 MDCK cells, which in our laboratory consist of approximately 90% principal- and 10% intercalated-like cells. We previously established a protocol to detect incremental increases in the $[Ca^{2+}]_i$ of greater than 5% above baseline (Geyti et al., 2008). Changing to an 80% hypotonic solution on the apical side triggered a significant increase in the number and amplitude of the $[Ca^{2+}]_i$ oscillations observed in MDCK cells (**Figure 1A**).

### BAFILOMYCIN A1 REDUCES SPONTANEOUS AND STIMULATED ATP RELEASE

Bafilomycin A1 inhibits vesicular $H^+$-ATPases, which in addition to maintaining a continuously low intravesicular pH, also provide the driving force for vesicular accumulation of various transmitters such as glutamate, 5-hydroxytryptamine (5-HT), γ-Aminobutyric acid (GABA) (Moriyama and Futai, 1990), and modified amino acids (Al-Damluji and Kopin, 1996). Moreover, the $H^+$-ATPases and thus, bafilomycin A1 have previously been demonstrated to be essential for the accumulation of ATP in zymogen granules in the exocrine pancreas (Haanes and Novak, 2010). **Figure 1B** illustrates the effect of bafilomycin A1 on spontaneous [$Ca^{2+}$] oscillatory activity in MDCK cells as an original trace, the summarized number of events and the number of responding cells. Bafilomycin A1 significantly reduced spontaneous $[Ca^{2+}]_i$ oscillations in terms of both the total number of events and the number of cells that showed spontaneous $[Ca^{2+}]_i$ oscillations (**Figure 1C**). With respect to stimulated $[Ca^{2+}]_i$ elevations, bafilomycin A1 significantly reduced the $[Ca^{2+}]_i$ response to hypotonic stress, whereas the response to externally applied ATP was unaffected by the treatment (**Figure 1D**). We also confirmed that bafilomycin A1 reduced the spontaneous ATP release from the apical side of MDCK cells ($p < 0.05$, **Figure 2A**). The level of spontaneous ATP-release to the basolateral side of the cells was under the detection-limit in our assay. Therefore the ATP release from MDCK cells was stimulated by hypotonic stress (50%), and this ATP release showed distinct bafilomycin sensitivity ($p < 0.05$, **Figure 2B**). Bafilomycin A1 did not influence the ATP determination assay, but standard curves were included for all ion-compositions as the ion content has dramatic effect on the assay. These results are consistent with a hypothesis that vesicular acidification is involved in both spontaneous and stimulated $[Ca^{2+}]_i$ increase.

### INTERFERENCE WITH THE VESICULAR RELEASE PATHWAY INHIBITS SPONTANEOUS AND STIMULATED $[Ca^{2+}]_i$ INCREASE

To investigate if ATP was released by exocytosis, we tested various substances that interfere at different points of the exocytosis pathway. **Figure 3** shows the effect of *N*-ethylmaleimide

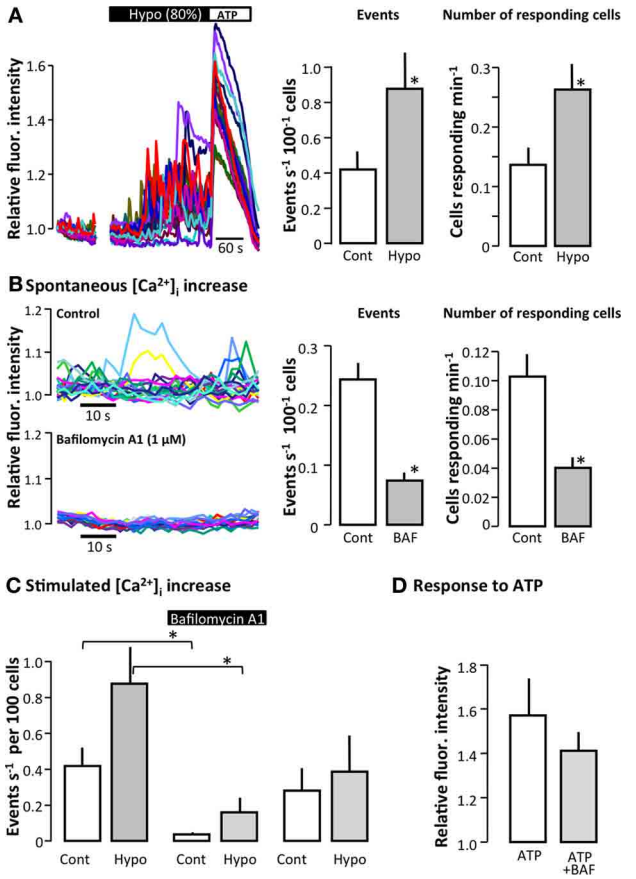


**FIGURE 1 | The effect of bafilomycin A1 on the spontaneous and stimulated $[Ca^{2+}]_i$ increase in MDCK cells. (A)** The left part of the figure shows the original trace of the fluo 4 fluorescence in MDCK cells. The initial part of the trace illustrates the small number of spontaneous $[Ca^{2+}]_i$ events in confluent MDCK cells during baseline, followed by the effect of reducing osmolality to 80%. The right part of the figure shows the summarized data in terms of the total number of $[Ca^{2+}]_i$ events greater than 5% above baseline and the number of responding cells; values are mean ± s.e.m. ($n = 16$). **(B)** The left part of the figure shows original traces of spontaneous $[Ca^{2+}]_i$ events in a control situation and after the addition of bafilomycin A1 (1 µM, 30 min). To the right, the summarized data are shown as the total number of spontaneous $[Ca^{2+}]_i$ events and the number of responding cells; values are mean ± s.e.m. ($n = 9$). **(C)** The left part of the figure shows the effect of bafilomycin A1 (Baf, 1 µM, 30 min) on the $[Ca^{2+}]_i$ events induced by reducing the osmolality to 80% of the control. The right part shows the $[Ca^{2+}]_i$ activity 30 min after return to control solution after washout of bafilomycin A1; values are mean ± s.e.m. ($n = 15$). **(D)** Effect of bafilomycin A1 on the ability of MDCK cells to react to extracellular ATP (100 µM); values are mean ± s.e.m. ($n = 16$). The asterisks indicate statistical significance.

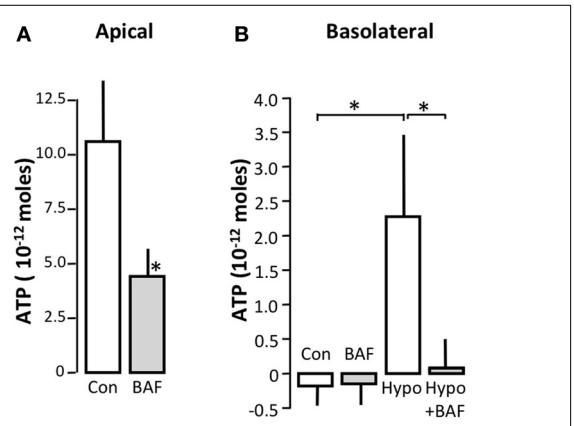


**FIGURE 2 | The effect of bafilomycin A1 on the spontaneous and stimulated ATP release from MDCK cells. (A)** Effect of bafilomycin A1 (1 µM) on ATP release from the apical side under baseline conditions. **(B)** Effect of bafilomycin A1 on the ATP release from the basolateral side under baseline conditions and after reducing the osmolality to 50% of the original value on the basolateral side in the absence or presence of bafilomycin A1 (1 µM). The values are shown as mean ± s.e.m. ($n = 8$). The asterisks indicate statistical significance.

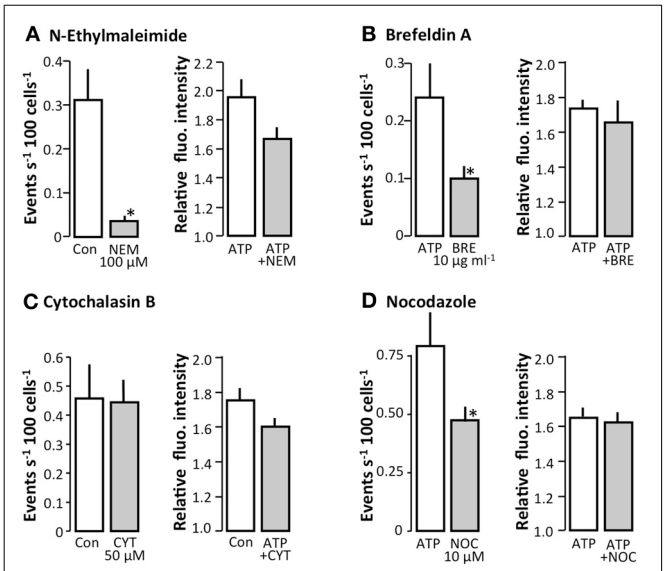


**FIGURE 3 | The effect of various inhibitors of the exocytotic pathway on spontaneous $[Ca^{2+}]_i$ events in MDCK cells. (A)** The effect of N-ethylmalimide (NEM, 100 µM, $n = 6$), **(B)** Brefeldin A (BRE, 10 µg ml$^{-1}$, $n = 13$), **(C)** Cytochalasin B (CYT, 50 µM, $n = 5$), and **(D)** Nocodazole (NOC, 10 µM, $n = 7$). The left hand side of the figures show the spontaneous $[Ca^{2+}]_i$ events and the right hand side shows the response to the application of ATP (100 µM). All values are mean ± s.e.m. The asterisks indicate statistical significance.

(NEM), brefeldin A, cytochalasin B, and nocodazole on spontaneous $[Ca^{2+}]_i$ increments, shown as the number of events. NEM interferes with the ability of vesicles to dock to the plasma membrane by inhibiting the function of Soluble *N*-ethylmaleimide-sensitive factor Attachment Protein receptors (SNAREs) and Soluble *N*-ethylmaleimide-sensitive factor Attachment Proteins (SNAPs), which are required for this process. We observed that NEM (100 µM) and significantly reduced the spontaneous $[Ca^{2+}]_i$ oscillations in MDCK cells ($p = 0.0022$). Similar results were found with brefeldin A (10 µg ml$^{-1}$), which interferes with the vesicular movement of proteins from the ER to the Golgi apparatus and within the Golgi apparatus ($p = 0.042$). Nocodazole (10 µM) inhibits vesicles mobility by interfering with microtubule polymerization, along which the vesicles are transported (Bjaelde et al., 2011). When nocodazole (10 µM)

was added, we observed a significant inhibition of spontaneous $[Ca^{2+}]_i$ oscillations in MDCK cells ($p = 0.017$). However, the number of responding cells per min was not affected by nocodazole. Cytochalasin B (50 μM), which inhibits the assembly of actin filaments, had no effect on spontaneous $[Ca^{2+}]_i$ oscillations. None of the drugs interfered with the ability of the cells to react to agonist stimulation, as indicated by the response of the cells to the positive control ATP (100 μM, see the right hand side of **Figures 3A–D**).

We also screened for effect of blocking connexin hemi-channels and pannexins to determine the roles of these pathways in the release of ATP. Carbenoxolone (100 μM) and 18β-glycyrrhetinic acid (50 μM) had no effect on the spontaneous $[Ca^{2+}]_i$ events, whereas mefloquine (100 μM) promoted an increase in the spontaneous $[Ca^{2+}]_i$ events (**Figure 4**). In this context, it should be emphasized that $[Ca^{2+}]_i$ imaging requires addition of probenecid to inhibit export of probe via multidrug resistance-associated protein 2. Probenecid is also known to inhibit pannexins, but has no effect on connexins-hemichannels at 5 mM (Silverman et al., 2008). Probenecid (5 mM) did not inhibit either the spontaneous or the stimulated (hypotonic) ATP release from MDCK cells as measured by the luciferin-luciferase assay (data not shown); rather, probenecid induced a slight increase in the spontaneous ATP release from the cells.

### siRNA-MEDIATED SILENCING OF THE CANINE VESICULAR DOCKING PROTEIN SNAP-23 INTERFERES WITH VESICULAR RELEASE

So far, our data support the hypothesis that ATP may be released by vesicular fusion from renal epithelial cells. To further test this notion, we silenced canine SNAP-23, a protein which is an important part of the docking mechanism responsible for docking of vesicles to the target membrane (Ravichandran et al., 1996; Wong et al., 1997). **Figure 5A** shows representative original traces of the changes in $[Ca^{2+}]_i$ using scrambled siRNA (left) or siRNA directed against SNAP-23 (right). Transient knockdown of the SNAP-23 protein in MDCK cells reduced the spontaneous $[Ca^{2+}]_i$ oscillations significantly to 47% at 48 h after siRNA transfection, whereas transfection with the siRNA had no effect compared to the control mock-transfected cells (**Figure 5B**). The transfection process did not affect ability of the cells to react to 100 μM ATP (**Figure 5C**). Quantitative rtPCR confirmed a significant reduction in relative amounts of mRNA of 56%

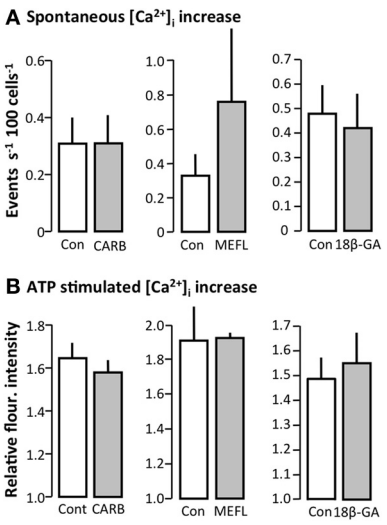


**FIGURE 4 | Effect of gap junction and pannexin inhibitors on spontaneous $[Ca^{2+}]_i$ events in MDCK cells. (A)** Carbenoxelone (CARB, 100 μM, $n = 7$), mefloquine (MEFL, 100 μM, $n = 2$) and 18β-Glychyrritinic acid (18β-GA, 50 μM, $n = 12$). **(B)** Corresponding reactivity to ATP (100 μM). All values are given as mean ± s.e.m.

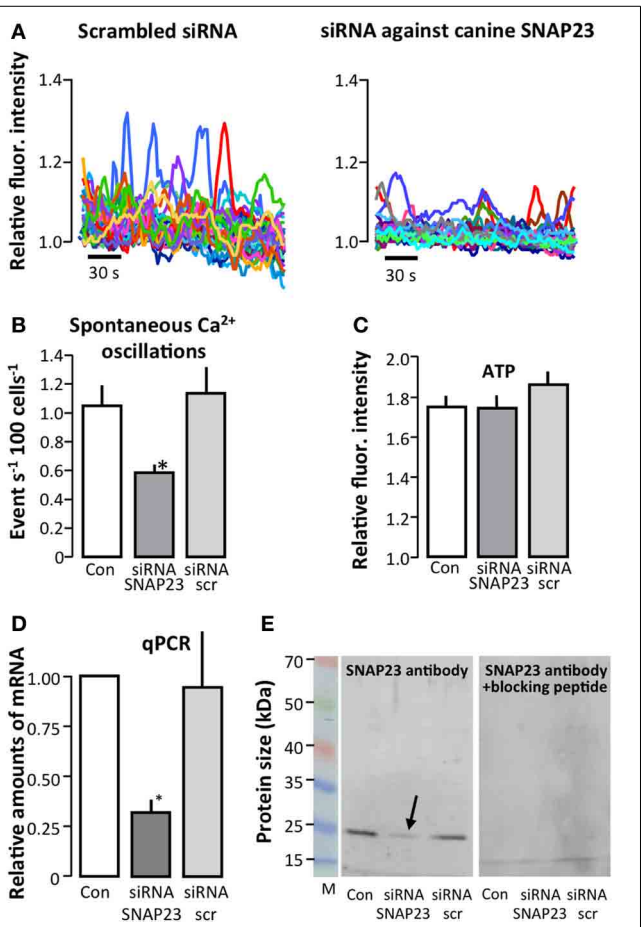


**FIGURE 5 | Effect of siRNA-mediated knockdown of canine SNAP-23 in MDCK cells.** The effect of transfection of MDCK cells with scrambled siRNA (siRNA scr) or siRNA directed against SNAP-23 on spontaneous $[Ca^{2+}]_i$ events; representative original traces shown in **(A)** and **(B)** shows the summarized data as number of events $s^{-1}$ as mean ± s.e.m. (Con: $n = 12$, siRNA SNAP-23: $n = 9$, siRNA scr: $n = 8$). **(C)** The effect of knocking down canine SNAP-23 on the ability of MDCK cells to respond to ATP (100 μM, Con: $n = 12$, siRNA SNAP-23: $n = 9$, siRNA scr: $n = 8$). Please note that preparations, which did not respond to extracellular ATP were not included in the analysis. **(D,E)** Confirmation of siRNA-mediated knockdown of SNAP-23 by qPCR **(D)** and immunoblotting **(E)**. The arrow indicates the reduced protein expression of SNAP-23 (~25 kDa, expected size ~23 kDa) in the lysate of cells transfected with siRNA directed against SNAP-23 (left). The control blot incubated with primary antibody, which had been pre-incubated with a SNAP-23 peptide is shown to the right. Con: $n = 4$, siRNA SNAP-23: $n = 4$, siRNA scr: $n = 3$. All experiments were performed at 48 h after transfection of the MDCK cells. The asterisks indicate statistical significance.

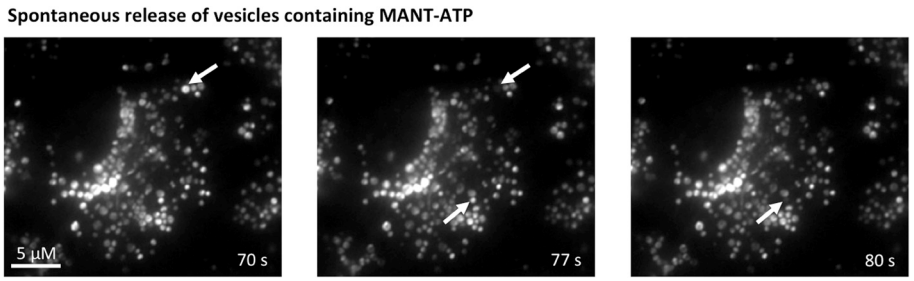


**FIGURE 6 | Spontaneous vesicular fusion in MDCK cells.** Total internal reflection fluorescence (TIRF) microscopy of MDCK cells loaded with MANT-ATP (25 μM, 5 h, 37°C). Arrows indicate vesicles that abruptly disappeared from the evanescence field.

compared to control was seen (**Figure 5D**), and immunoblotting demonstrated that SNAP-23 protein expression was reduced by ∼70% (arrow in **Figure 5E**) in SNAP-23 siRNA transfected cells compared to scrambled control siRNA transfected cells.

### OBSERVATION OF DIRECT VESICLE-FUSION USING TIRF MICROSCOPY

To be able to observe direct vesicle-fusion with the plasma membrane we used two different dyes: quinacrine, which is known to accumulate in acidic vesicles and MANT-ATP for TIRF microscopy. The images obtained the staining patterns of the two dyes seemed very similar, even though quinacrine appeared to stain more vesicles than MANT-ATP. It was not possible to verify whether the two probes stained the same population of vesicles, because of overlapping excitation and emission spectra of the dyes. Moreover, MANT-ATP bleaches very rapidly, making this probe unsuitable for longer time-lapse studies. Using both dyes, it was possible to observe spontaneous disappearance of vesicles (arrows in **Figure 6**) or more consistently after stimulation with the $Ca^{2+}$ ionophore ionomycin (arrows in **Figures 7A,B**). In **Figure 7A**, the arrows indicate vesicles that disappeared abruptly between one frame and the next (sampling rate 1 Hz). With identical intensity histogram profiles for the earlier and later pictures, it is hard to distinguish the vesicle that disappeared rapidly from the evanescence field from the ones that have bleached. By narrowing the histogram, one can still see the remaining bleached MANT-ATP vesicles and confirm that they have not disappeared from the evanescence field. On the other hand, quinacrine was bleached at a negligible rate. Using wide-field fluorescence, we estimated what proportion of the overall quinacrine fluorescence was reduced in response to ionomycin. **Figure 7C** shows the initial stable baseline level of quinacrine fluorescence. After addition of ionomycin, the overall fluorescence is reduced significantly over time. Each line in the original trace on the left-hand side of **Figure 7** represents a region of interest (ROI) placed over the quinacrine-loaded vesicles in the cytosol of a single cell. The right-hand side of the figure summarizes the average reduction in the total fluorescence intensity 8 min after the addition of ionomycin. **Figure 7D** illustrates that ionomycin (1 μM) did stimulate detectable release of ATP to the extracellular space measured by the luciferin/luciferase assay.

Moreover, we observed that reducing the osmolality also accelerated the disappearance of quinacrine-loaded vesicles. **Figure 8A** shows the average change in fluorescence intensity measured by TIRF microscopy. As before, each original trace represents the average fluorescence intensity of the cytoplasm of a single cell. Reducing the osmolality initially increased the overall fluorescence intensity, as apparently more vesicles were recruited into the evanescence field, where after the overall fluorescence intensity subsequently reduced. The impact of reducing the osmolality on the overall fluorescence intensity was of a shorter duration and less extensive than the reduction observed in response to ionomycin (1 μM). **Figure 8B** shows the corresponding release of ATP observed after reducing the osmolality apically as measured by the luciferin/luciferase assay.

### ISOLATION OF INTACT VESICLES AND DETERMINATION OF THEIR ATP CONCENTRATION

To investigate whether or not the quinacrine-loaded vesicles actually contained ATP, we isolated intact vesicles using the EMBL cell cracker and FACS sorting. The cell cracker mechanically disrupts the cell membrane, and by using a buffer resembling the cytosolic environment, the vesicles should remain intact. The isolation of intact vesicles in the cell lysates was confirmed by microscopy (data not shown), before the vesicles were sorted from the rest of the cell debris by FACS. The ATP concentration of the vesicles was calculated to be around 8.5 mM. This value was calculated based on the average diameter of the vesicles (0.9 μm; measured from time-lapse microscopy), together with the FACS data on the number of sorted vesicles per ml and the concentration of ATP, determined by the luciferin-luciferase assay. This experiment was performed twice and 8.5 mM is the average value obtained.

## DISCUSSION

ATP-mediated signaling has a major impact on renal function. Generally, epithelial ATP release will reduce the transepithelial transport (Kishore et al., 1995; Bailey, 2004; Pochynyuk et al., 2008; Marques et al., 2012) directly via activation of P2 receptors on the epithelium itself or indirectly by reducing the demand for transport through the tubulo-glomerular feedback mechanism via $A1_a$ receptors (Sun et al., 2001). Consistent with this, mice deficient in one of the most important P2 receptors in renal epithelial cells, the $P2Y_2$ receptor, exhibit hyper-reabsorption of $Na^+$ in the collecting duct and hypertension. ATP is known to be released from renal epithelial cells in response to mechanical perturbations, such as an increased fluid flow rate (Praetorius and Leipziger, 2009) and hypotonic swelling (Boudreault and

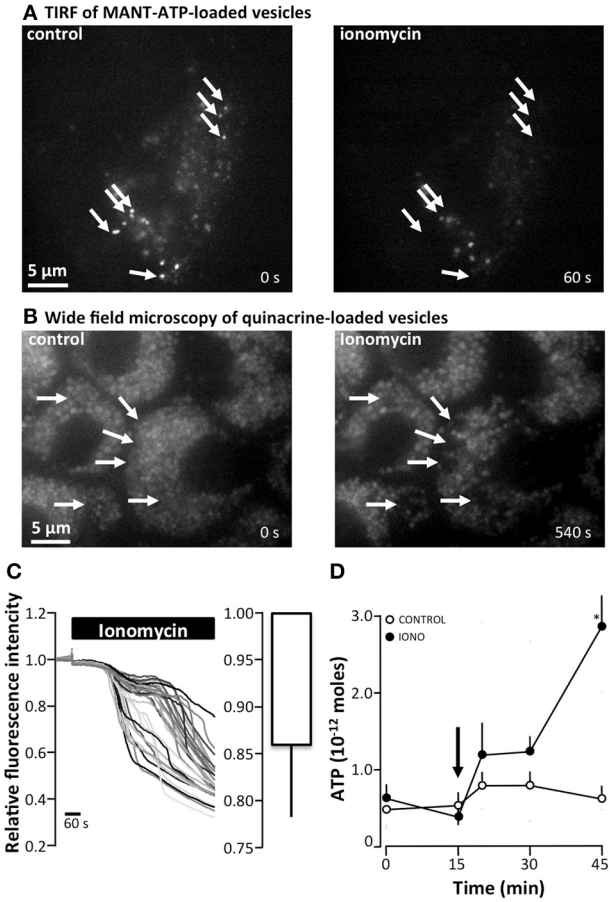


**FIGURE 7 | The effect of ionomycin on ATP release and vesicular fusion in MDCK cells (A) total internal reflection fluorescence (TIRF) microscopy of MDCK cells loaded with MANT-ATP (25 µM, 5 h, 37°C) showing vesicular fusion after the addition of ionomycin (1 µM).** Arrows indicate vesicles that abruptly disappeared from the evanescence field. **(B)** Ionomycin (1 µM)-induced vesicular fusion as indicated by wide-field fluorescence microscopy of MDCK cells loaded with quinacrine (5 µM, 30 min, 37°C). Arrows indicate vesicles that display an abruptly drop in fluorescence. **(C)** Right: Original trace of the effect of ionomycin (1 µM, 37°C) on the relative fluorescence intensity of quinacrine-loaded MDCK cells. The left panel present the mean ± s.e.m. from 6 experiments. **(D)** ATP release from MDCK cells induced by apical application of ionomycin (IONO, 1 µM). The arrow indicates the addition of ionomycin or the control solution; the mean ± s.e.m. values are presented ($n = 10$). The asterisk indicates statistical significance.

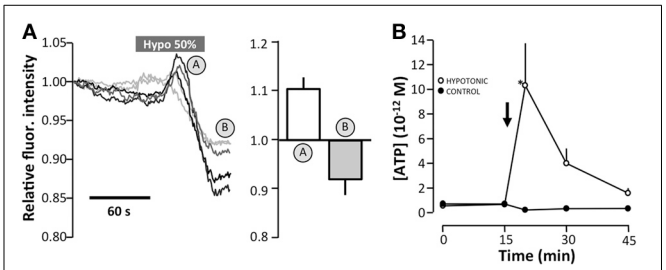


**FIGURE 8 | The effect of hyposmotic stress on vesicular fusion of quinacrine-loaded vesicles by TIRF microscopy of MDCK cells. (A)** Original traces of the total fluorescence intensity of 5 cells as determined by TIRF microscopy. The mean ± s.e.m. values are shown on the right as $n = 17$. **(B)** Effect of apically-induced hypotonic stress (50%) on the level of extracellular ATP. Data are presented as the mean ± s.e.m. ($n = 10$). The asterisk indicates statistical significance.

Grygorczyk, 2004), and is also released in response to AVP (Odgaard et al., 2009).

As a result of the impact that P2 receptor activation has on kidney function, it is unsettling that so little is known about the mechanism of ATP release. Cx30 has been suggested to function as an ATP channel in distal tubular intercalated cells (Sipos et al., 2009), where cx30 is specifically expressed in the apical domain of the cells (McCulloch et al., 2005). Although cx30 is clearly able to form gap junctional channels (Valiunas et al., 1999; Valiunas and Weingart, 2000), the subcellular localization of this protein in the intercalated cells suggests that gab junction formation is not cx30 main function in this cell type. In the collecting duct, the spontaneous and stimulated ATP release are intimately associated with the expression of cx30 (Sipos et al., 2009), which suggests that a hemichannel configuration of cx30 may serve as the main pathway of ATP release. These data are supported by a previous study of cultured HEK cells overexpressing cx30 (Liang et al., 2011), which took advantage of the feature that the open state probability of connexin hemi-channels increases as $[Ca^{2+}]_e$ is reduced. Chelating extracellular $Ca^{2+}$ triggered the cx30-expressing HEK cells to release more ATP compared to wild type, whereas the ATP release at normal $Ca^{2+}$ concentrations was similar in cx30 expressing and wild type HEK cells (Liang et al., 2011). This finding supports the notion that cx30 is an important pathway for ATP release in renal epithelial cells.

As only a select sub-fraction of renal epithelial cells express cx30, cx30 cannot be the only pathway for ATP release. Therefore, we investigated the possibility of alternative pathways of ATP release in renal epithelia. We addressed this issue using a screening procedure for constitutive ATP release in MDCK cells. We found that substances that interfere with vesicular loading and release significantly reduced the spontaneous increases in $[Ca^{2+}]_i$, which we monitored as a proxy for ATP release. This $[Ca^{2+}]_i$ assay has the clear disadvantage that it does not directly measure ATP release; however, on the other hand, the method is very reliable and avoids the interference resulting from changes in the cellular environment or the effect of pharmacological substances on the enzymatic reactions in the luciferein/luciferase method. We have previously validated this method and demonstrated that the degree of spontaneous $[Ca^{2+}]_i$ increase is direct result of constitutive ATP release in both MDCK cells and freshly isolated renal epithelial cells (Geyti et al., 2008). Via this method, we found that bafilomycin A1 significantly reduced both spontaneous and hypo-osmotically-induced $[Ca^{2+}]_i$ increase in MDCK cells. Moreover, we found that substances that potentially interfere with vesicular release (brefeldin A, nocodazole and NEM) reduced the spontaneous $[Ca^{2+}]_i$ events. However, pannexin and connexin blockers, did not have any detectable effect on the spontaneous $[Ca^{2+}]_i$ events. The effect of pharmacological interference with vesicular release was substantiated by siRNA-mediated knockdown of the tSNARE SNAP-23 in MDCK cells. Transient knockdown of SNAP-23 reduced SNAP-23 mRNA and

protein expression by approximately 56 and 70%, respectively, at 48 h. In parallel experiments, spontaneous $[Ca^{2+}]_i$ oscillations were significantly reduced to 47% in cells transfected with the SNAP-23 siRNA compared to cells transfected with a scrambled siRNA. Using bafilomycin A1 as a representative pharmacological inhibitor of vesicular release pathway, we confirmed that inhibition of the vesicular release pathway also reduced constitutive and osmotically-induced ATP release as measured with the luciferin-luciferase assay.

To investigate whether it was possible to detect vesicular release in MDCK cells, we used various approaches to stain ATP-containing vesicles. Quinacrine has been used as a marker for ATP containing vesicles on several occasions (Bodin and Burnstock, 2001; Sorensen and Novak, 2001; Akopova et al., 2012). Quinacrine has also been shown to accumulate in acidic vesicles and has been used as a marker of lysosomes (Bastos et al., 1966). Staining the cells with acridine orange, which has not been associated with ATP content, produced a staining pattern similar to that of cells stained with quinacrine (Bjaelde et al., 2011). Moreover, bafilomycin A1, an inhibitor of vesicular $H^+$-ATPases, has also been shown to prevent the accumulation of quinacrine in intracellular vesicles (Marceau et al., 2009), either as a result of a reduced vesicular pH directly or, in light of our results, as a bafilomycin A1-induced lack of ATP in the granules. Regardless of its shortcomings, quinacrine is at present a reasonable tool to address whether MDCK cells are able to release vesicles, which may potentially contain ATP. To substantiate the release of ATP-containing vesicles, we also used the fluorescently-labeled ATP molecule MANT-ATP, with the expectation that the cell would process this probe in the same manner as endogenous ATP. After prolonged incubation, we observed that MANT-ATP accumulated in vesicles in a similar manner to quinacrine.

We have attempted to confirm whether the quinacrine-stained vesicles actually contained ATP. After mechanically disintegrating the MDCK cells, we used FACS to sort the cellular component on the basis of fluorescence and size, and selectively recovered the smaller particles with a high fluorescence signal when excited at 488 nm. From the number of vesicles retrieved and the ATP content of the vesicle lysate, the vesicles were determined to contain approximately 8.5 mM ATP, compared to the concentration of ATP in the cytosol of 1–3 mM (Ainscow and Rutter, 2002), the concentration of ATP in the vesicles was only slightly higher. We have good reason to assume that the value of 8.5 mM is an underestimation. The vesicles were measured to have an average diameter of 0.9 $\mu$m, which is likely to be an overestimation as a result of the fluorescent blur and the limitations of the resolution in the x-y plane. More importantly, if $H^+$-ATPases are required for the continuous accumulation of ATP in the vesicles then this activity was not supported after the cells were disintegrated and the ATP surrounding the vesicles was washed away, despite the fact that we tried to reduce the leakage of ATP from the vesicles by keeping the preparations on ice until FACS.

Using either quinacrine or MANT-ATP, we were able to observe spontaneous drops in vesicular fluorescence using TIRF microscopy. The sudden fall in vesicular fluorescence were taken as account for direct vesicular fusion with the plasma membrane. This technique has been employed by others to visualize the release of ATP-containing vesicles from lung epithelial cells (Akopova et al., 2012). To stimulate exocytosis, we used either ionomycin or hypotonic stress. Ionomycin is known to induce sustained elevations in $[Ca^{2+}]_i$, and does provoke a substantial release of ATP by MDCK cells. Ionomycin also promoted increased vesicle fusion with the plasma membrane; at longer incubations this effect was so substantial that it was visible as a decrease in the overall fluorescence of the cells in wide-field microscopy. The pattern was slightly different when vesicular release was induced by hypotonic stress. When the osmolality of the solution was reduced, we initially observed increased recruitment of vesicles to the membrane, which was observed as an increase in fluorescence using TIRF microscopy. Following this initial recruitment, subsequent degranulation was observed as sudden drops in vesicular fluorescence. These observations are consistent with the process of vesicular release as one possible mechanism to induce ATP release from renal epithelial cells.

In terms of ATP release through pannexins and connexins, we were unable to detect any evidence for a significant contribution of these pathways to the ATP release from MDCK cells. In many ways, these results are not surprising as the cx30-mediated ATP release is mainly observed in intercalated cells, and in our laboratory, the MDCK type 1 culture contained fewer than 10% intercalated cells, determined as the proportion of non-ciliated cells in the confluent state. However, MDCK type 1 cells, do express connexins, which can form functional gap-junctions between the cells (Cereijido et al., 1984). Regardless of the numerous reports on connexin-hemi-channels as ATP release pathway, the physiological relevance of this pathway has been questioned with regards to the properties of the channel for stimulated ATP release. The main criticism is the inverse dependency of connexin-hemichannels/gap junctions on $[Ca^{2+}]_i$, whereas potential ATP release pathways would require increases in $[Ca^{2+}]_i$ to activate the given pathway (for review see Saez et al., 2003). Connexons are generally known to be closed at physiological $[Ca^{2+}]_e$, a feature, which is in many respects not an ideal one for stimulated ATP release (Li et al., 1996; Contreras et al., 2003; Locovei et al., 2006). This controversy has led to the suggestion of pannexins, which do not form gap-junctions, as a pathway for ATP release (Locovei et al., 2006). Pannexin 1-mediated ATP release was initially demonstrated in erythrocytes (Locovei et al., 2006) and was subsequently shown to be important in many other tissues, including respiratory (Seminario-Vidal et al., 2011) and renal epithelia (Hanner et al., 2012). Taken together, we find it unlikely that connexins and pannexins contribute significantly to the ATP release in this cell culture model of renal epithelia. Whether this finding can be extrapolated to renal epithelial cells in general remains to be established.

In summary, we report that both the spontaneous and stimulated ATP release can be mediated by vesicular release in MDCK cells. Our results are consistent with vesicular release as the principle pathway for ATP release in this renal epithelial cell model.However, the predominant mechanisms of nucleotide release in different segments of the intact renal tubules require further investigation. Our present data suggest that vesicular release of ATP should be considered as a viable possibility in the kidney.

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# ATP Releasing Connexin 30 Hemichannels Mediate Flow-Induced Calcium Signaling in the Collecting Duct

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ATP in the renal tubular fluid is an important regulator of salt and water reabsorption via purinergic calcium signaling that involves the $P2Y_2$ receptor, ENaC, and AQP2. Recently, we have shown that connexin (Cx) 30 hemichannels are localized to the non-junctional apical membrane of cells in the distal nephron-collecting duct (CD) and release ATP into the tubular fluid upon mechanical stimuli, leading to reduced salt and water reabsorption. $Cx30^{-/-}$ mice show salt-dependent elevations in BP and impaired pressure-natriuresis. Thus, we hypothesized that increased tubular flow rate leads to Cx30-dependent purinergic intracellular calcium ($[Ca^{2+}]_i$) signaling in the CD. Cortical CDs (CCDs) from wild type and $Cx30^{-/-}$ mice were freshly dissected and microperfused *in vitro*. Using confocal fluorescence imaging and the calcium-sensitive fluorophore pair Fluo-4 and Fura Red, we found that increasing tubular flow rate from 2 to 20 nl/min caused a significant 2.1-fold elevation in $[Ca^{2+}]_i$ in wild type CCDs. This response was blunted in $Cx30^{-/-}$ CCDs ($[Ca^{2+}]_i$ increased only 1.2-fold, $p < 0.0001$ vs. WT, $n = 6$ each). To further test our hypothesis we performed CD $[Ca^{2+}]_i$ imaging in intact mouse kidneys *in vivo* using multiphoton microscopy and micropuncture delivery of the calcium-sensitive fluorophore Rhod-2. We found intrinsic, spontaneous $[Ca^{2+}]_i$ oscillations in free-flowing CDs of wild type but not $Cx30^{-/-}$ mice. The $[Ca^{2+}]_i$ oscillations were sensitive also to P2-receptor inhibition by suramin. Taken together, these data confirm that mechanosensitive Cx30 hemichannels mediate tubular ATP release and purinergic calcium signaling in the CD which mechanism plays an important role in the regulation of CD salt and water reabsorption.

**Keywords: connexin, hemichannel, ATP release, calcium imaging, intravital microscopy**

## INTRODUCTION

In the kidney, ATP released from the tubular epithelium regulates salt and water transport through activation of purinergic signaling along the nephron and collecting duct (CD). Renal epithelial $[Ca^{2+}]_i$ signaling and ATP release may be elicited by mechanical stimulation e.g., increased tubular fluid flow rate (Satlin et al., 2006; Jensen et al., 2007; Praetorius and Leipziger, 2009; Xu et al., 2009) which shows well-characterized physiological oscillations *in vivo* due to renal hemodynamic feedback mechanisms (Holstein-Rathlou and Leyssac, 1986; Marsh et al., 2005; Kang et al., 2006; Peti-Peterdi et al., 2009). Accordingly, the activation of flow-induced purinergic calcium signaling in renal and tubular epithelial cells including cells of the CCD has been described in detail (Woda et al., 2002; Jensen et al., 2007; Praetorius and Leipziger, 2009; Sipos et al., 2009; Xu et al., 2009). In terms of the luminal ATP release mechanism in the CD, the expression of connexin 30 (Cx30) hemichannels has been shown to be crucial and to play an integral role in regulating CD salt and water transport (Sipos et al., 2009; Stockand et al., 2010; Mironova et al., 2011).

Cx30 is a member of the connexin (Cx) family comprised by 21 structurally similar isoforms (Spray et al., 2006). The Cx family are transmembrane proteins that can form non-selective pores in the plasma membrane, allowing passage of molecules up to 1 kDa in mass (Spray et al., 2006). The classical view is that these pores align and form gap junctions in the junctional membrane, allowing the transfer of molecules between adjacent cells thereby facilitating intercellular communication. However, increasing evidence suggests that Cx pores also reside in non-junctional plasma membrane domains, where they form large, mechanosensitive ion channels which allow the passage of a variety of small molecules and metabolites including ATP (Cotrina et al., 1998; Ebihara, 2003; Bao et al., 2004). Cx isoforms have been identified in nearly all tissues (Willecke et al., 2002). They appear to be involved in a wide variety of physiological functions depending on the isoform expressed. In the kidney, the isoforms Cx37, Cx40 and Cx43 are localized in the vasculature, glomerulus, and tubular segments in a punctuated pattern, typical of classic gap junction channels (Barajas et al., 1994; Arensbak et al., 2001). Interestingly, Cx30 is expressed at the luminal membrane in a subset of cells in the distal nephron-CD in rat, rabbit, and mouse kidney (McCulloch et al., 2005; Sipos et al., 2009).

We recently reported that Cx30 knockout mice showed reduced luminal ATP release both *in vitro* (Sipos et al., 2009) and *in vivo* (Stockand et al., 2010; Mironova et al., 2011) leading to higher CD sodium reabsorption through the epithelial sodium channel (ENaC). Mice lacking the $P2Y_2$ receptor also have

increased renal tubular sodium reabsorption (Rieg et al., 2007). These findings suggest that auto-/paracrine effects of luminal ATP released via Cx30 hemichannels involve regulation of renal salt and water reabsorption and this novel mechanism appears to be integral in several physiological mechanisms including pressure natriuresis and diuresis which maintain body fluid balance and blood pressure (Sipos et al., 2009), and also aldosterone escape (Stockand et al., 2010) via the regulation of ENaC activity (Mironova et al., 2011).

We hypothesized that the high tubular flow-induced $[Ca^{2+}]_i$ signaling which is an established hallmark of the effects of local ATP release in the CD is Cx30-dependent. To test the hypothesis, we used fluorescence imaging techniques to visualize calcium signaling in isolated CCDs *in vitro* as well as in CDs of the living and intact kidney *in vivo* in wild-type or Cx30 knockout mice.

## METHODS

### MICE

The $Cx30^{-/-}$ mouse model was established and described previously (Teubner et al., 2003; Sipos et al., 2009). Wild-type and $Cx30^{-/-}$ mice (C57BL6 background) were bred at the University of Southern California. All animal protocols were conducted in conformity with the Guiding Principles for Research Involving Animals and Human Beings and were approved by the Institutional Animal Care and Use Committee of the University of Southern California. Genotype was confirmed by PCR of tail biopsies.

### COLLECTING DUCT CALCIUM IMAGING *in vitro*

CCDs were freshly dissected from mouse kidneys and loaded with the calcium-sensitive fluorophore pair Fluo-4 and Fura Red as previously described (Sipos et al., 2009). The intact tubules were cannulated and microperfused using a set of concentric glass micropipettes in the 2 to 20 nl/min range with a solution containing (in mM) 25 NaCl, 5 KCl, 1 $MgSO_4$, 1.6 $NaHPO_4$, 0.4 $NaH_2PO_4$, 5 d-glucose, 1.5 $CaCl_2$, 110 NMDG-cyclamate, and 10 HEPES as described and shown previously for renal arteriolar and tubular segments (Peti-Peterdi, 2006; Sipos et al., 2009, 2010). The calibration of perfusion pressure and the resulting tubular flow rate was reported earlier (Peti-Peterdi, 2006). Fluo-4 (excitation at 488 nm, emission at 520 ± 20 nm) and Fura Red (excitation at 488 nm, emission at >600 nm) fluorescence was detected using a Leica TCS SP2 AOBS MP confocal microscope system, and fluorescence was calibrated to $[Ca^{2+}]_i$ as described previously (Peti-Peterdi, 2006).

### MICROPUNCTURE

Mice were anesthetized using ketamine and xylazine ip (100 and 10 mg/kg, respectively) and were surgically prepared for renal micropuncture and tubular microperfusion delivery of the calcium-sensitive fluorophore Rhod-2AM (10 μM, Invitrogen). The mice were placed on a homoeothermic operating table (Vestavia Scientific) and whole body temperature was kept at ~37°C. A ~22 mm dorsal incision on the left subcostal flank and two horizontal midline incisions were made for placement of kidney cup. The left kidney was gently exteriorized and renal pedicle and kidney cleanly dissected of debris and fat. A 22 mm kidney cup (Vestavia Scientific) was placed within the retracted peritoneal cavity opening and the left kidney was placed within the kidney cup and continuously bathed with warm 0.9% saline. Glass capillary tubes ("ID 0.084 × 0.064," Drummond Scientific Company) were pulled with a PP-830 pipette puller (Narishige), tip grinded to an O.D. of ~2 μm, filled with the fluorophore Rhod-2 and put on a micromanipulator (Leitz). Tubule segments were selected using a Stemi 200 stereomicroscope (Zeiss, 250 × magnification), micropunctured with the glass micropipette and microperfused with the fluorescent dye at a rate of 5–10 nl/min for 15–20 min.

### COLLECTING DUCT CALCIUM IMAGING *in vivo*

The mice were placed on the stage of a Leica TCS SP5 AOBS MP confocal microscope system powered by a Chameleon Ultra-II MP laser (Coherent Inc.) and a DMI 6000 inverted microscope and the exposed kidney was placed in a coverslip-bottomed heated chamber bathed in normal saline. The kidney was visualized from below as described before (Kang et al., 2006; Sipos et al., 2007). Rhod-2 was excited at 860 nm and the emitted Rhod-2 fluorescence was collected using a TRITC filter and external detectors. During all procedures, core body temperature was maintained by using a homeothermic table. In some experiments the apparent xy-movements of the kidney during time-lapse imaging were corrected using the TurboReg plug-in for ImageJ (Thévenaz et al., 1998). Analysis of the xyt time-lapse image sequences was performed *post-hoc* by placing ROIs over those CD cells which were intensely labeled by Rhod–2, usually in the cell nucleus. Suramin (10 mg/kg, Sigma) was infused via the cannulated carotid artery at 10 μl/min.

### STATISTICAL ANALYSIS

Data are shown as average ± s.e.m. Students *t*-test was used for statistical comparison of two groups, and 1-Way ANOVA was performed with *post-hoc* Bonferroni multiple comparison test for the comparison of 3 groups. $P < 0.05$ was considered significant.

## RESULTS

### Cx30 DEPENDENCE OF THE FLOW-INDUCED $[Ca^{2+}]_i$ RESPONSE IN THE ISOLATED *in vitro* MICROPERFUSED CCD

To test our hypothesis that increased flow rates in the CD lead to Cx30-mediated increases in $[Ca^{2+}]_i$, we freshly dissected and microperfused CCDs *in vitro* from wild-type and $Cx30^{-/-}$ mice and loaded them with calcium fluorophores (**Figure 1A**). Under conditions of baseline low flow (2 nl/min), fluorescence $[Ca^{2+}]_i$ imaging did not detect significant differences in $[Ca^{2+}]_i$ in CCD cells between wild-type and $Cx30^{-/-}$ mice (286 ± 34 nM and 279 ± 44 nM, respectively, $n = 10$ wild-type and 8 $Cx30^{-/-}$). In response to increasing flow rate to 20 nl/min, CCDs from wild-type mice produced a significant increase in $[Ca^{2+}]_i$ (average $\Delta[Ca^{2+}]_i$ was 177 ± 45 nM, $p < 0.05$, $n = 10$), whereas no significant changes were detected in CCDs from $Cx30^{-/-}$ mice (average $\Delta[Ca^{2+}]_i$ was 9 ± 6 nM, $n = 8$) (**Figures 1A–C**). Thus, our data suggest that the flow-induced increases in $[Ca^{2+}]_i$ *in vitro* microperfused CCDs were dependent on the expression of Cx30.

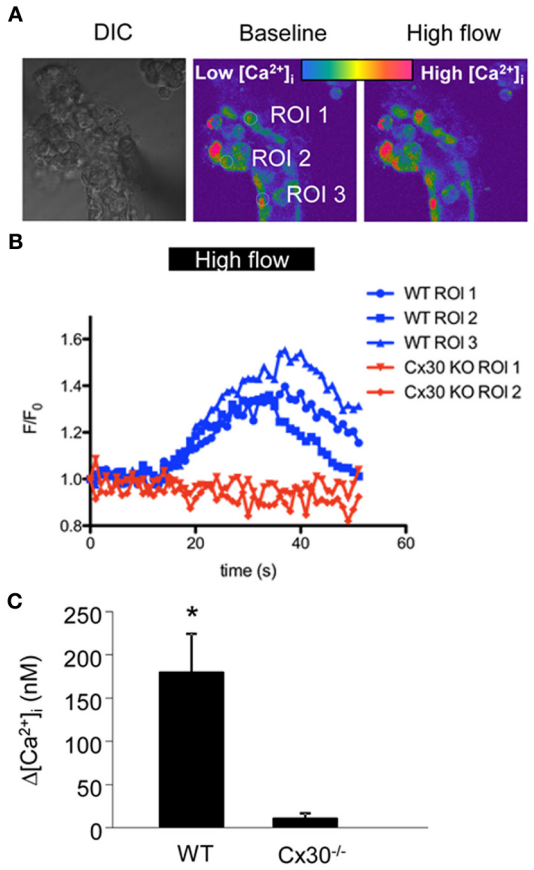


**FIGURE 1 | Flow-induced increases in $[Ca^{2+}]_i$ in cells of the *in vitro* microperfused collecting duct. (A)** CCDs from wild type and $Cx30^{-/-}$ mice were freshly dissected and microperfused *in vitro*, and loaded with the $Ca^{2+}$-sensitive fluorophore pair Fluo-4 and Fura Red. Left: DIC image of a representative preparation. Middle, and right: Pseudo-colored confocal Fluo-4/Fura Red ratio images of the same CCD before and after increasing tubular flow from low (2 nl/min) to high (20 nl/min) rates. **(B)** Representative recordings of Fluo-4/Fura Red ratio from 3 ROIs from wild type (blue, as shown in panel **A**) and 2 ROIs from $Cx30^{-/-}$ CCDs (red) show that Cx30 is essential for flow-induced increases in $[Ca^{2+}]_i$. **(C)** Summary of the CCD $[Ca^{2+}]_i$ responses illustrates that the increase in $[Ca^{2+}]_i$ in response to high flow was almost completely abolished in $Cx30^{-/-}$ mice. ($^*p < 0.001$, $n = 10$ wild-type and 8 $Cx30^{-/-}$).


**FIGURE 2 | Micropuncture delivery of Rhod-2 results in effective loading of the CD *in vivo*.** Loading of the cells of the CD (arrows) with the calcium fluorophore Rhod-2 *in vivo* was established by first injecting Rhod-2 dissolved in DMSO alone **(A)**, or together with Sulfinpyrazone **(B)** under the renal capsule. Neither strategies resulted in cellular dye uptake in the CD. However, loading of CDs (arrow) were achieved by using micropuncture delivery of Rhod-2 dissolved in DMSO **(C)**.

### CD $[Ca^{2+}]_i$ IMAGING *in vivo*

In order to test if Cx30 is involved in CD $[Ca^{2+}]_i$ signaling *in vivo*, we first developed a technique to efficiently load CD cells with the commercially available cell permeable acetoxymethylester (AM) form of fluorophores for confocal fluorescence imaging. The calcium-sensitive fluorophore Rhod-2 was employed in the present experiments, as red fluorescent probes are preferred over green dyes due to the low overlap of their emission with the significant renal tissue autofluorescence. Initially, we used the multicell bolus loading (MCBL) technique that we previously described for real-time imaging of intracellular pH and $[Ca^{2+}]_i$ in the proximal tubules (Sipos et al., 2007; Peti-Peterdi et al., 2009). Injection of Rhod-2 dissolved in DMSO directly under the renal capsule resulted in very poor labeling of the cells of the CD (**Figure 2A**, arrow). To test if the poor loading of the CDs were due to dye leakage via organic anion transporters, we supplemented the Rhod-2/DMSO solution with the organic anion transporter inhibitor sulfinpyrazone to reduce leakage of the de-esterified form of Rhod-2. However, as shown in **Figure 2B** sulfinpyrazone supplementation did not increase the CD loading of Rhod-2. To achieve a more efficient labeling, we next tried tubular/interstitial micropuncture delivery of Rhod-2. As shown in **Figure 2C**, this loading strategy resulted in effective Rhod-2 loading of the cells in the CD, and this loading strategy was employed in the subsequent experiments.

### Cx30 DEPENDENCE OF CD $[CA^{2+}]_i$ SIGNALING *in vivo*

To further support our hypothesis that flow-induced $[Ca^{2+}]_i$ signaling in the CD is dependent on Cx30 expression, we used *in vivo* multiphoton microscopy of calcium fluorophore-loaded intact kidneys in wild-type and $Cx30^{-/-}$ mice. Spontaneous $[Ca^{2+}]_i$ oscillations in the free-flowing CDs were measured which were likely the result of intrinsic tubular flow oscillations caused by tubuloglomerular feedback and the myogenic mechanism as described before (Holstein-Rathlou and Leyssac, 1986; Marsh et al., 2005; Peti-Peterdi et al., 2009). Although tubular flow was not measured or correlated with $[Ca^{2+}]_i$ in the present studies, temporary increases in CD flow were evident by the alterations in CD tubular diameter (not shown). In kidneys from wild-type mice a subset of CD cells showed spontaneous oscillations in Rhod-2 fluorescence reflecting changes in $[Ca^{2+}]_i$ (**Figure 3A**). In contrast, CD cells in $Cx30^{-/-}$ mice showed steady-state Rhod-2 fluorescence with no signs of oscillations (**Figures 3B,D**). To test if the observed CD $[Ca^{2+}]_i$ oscillations in wild-type mice were due to P2-receptor signaling, the purinergic receptor inhibitor suramin was administered in wild-type mice. As shown in **Figures 3C,D**, suramin treatment abolished the oscillations in Rhod-2 fluorescence and resulted in steady-state CD $[Ca^{2+}]_i$ levels (average $\Delta F_{max}/F_0$ was 2.90 ± 0.53, 1.26 ± 0.05, and 1.29 ± 0.04 in wild-type, suramin-treated, and $Cx30^{-/-}$ mice, respectively, $p < 0.001$ $Cx30^{-/-}$ and suramin groups vs. wild-type, $n = 5$ wild-type, 7 suramin, and 11 $Cx30^{-/-}$).

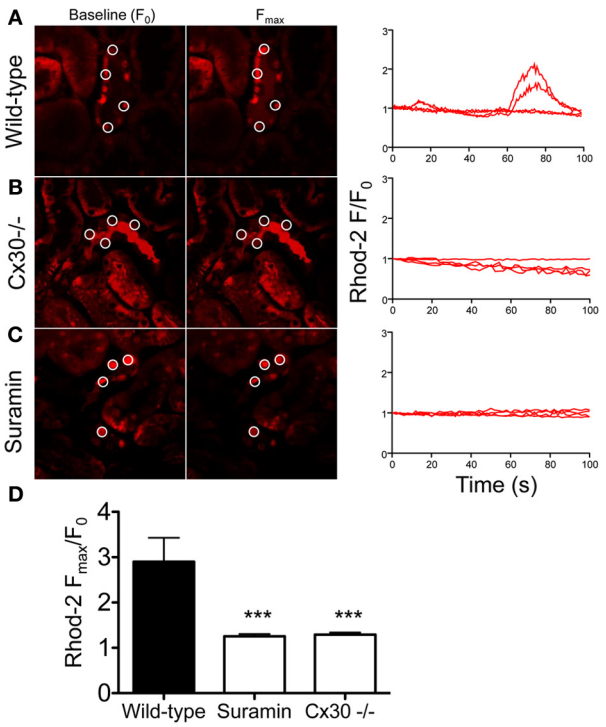


**FIGURE 3 | Multiphoton imaging of CD cell $[Ca^{2+}]_i$ signaling in the intact mouse kidney *in vivo*.** Cells of the CD were loaded with the fluorescent calcium indicator Rhod-2 using micropuncture delivery and its fluorescence intensity (*F*) was recorded within intracellular regions of interest over time, without exogenous stimuli. The CD was identified based on anatomical (heterogenous cell population with typical, bulging luminal surface, downstream from the Y-shaped junction of adjacent connecting segments) and functional (highly concentrated tubular fluid based on its high fluorescence intensity) considerations. Representative images of the same preparation at baseline ($F_0$) and at peak *F* values are shown and selected ROIs of CD cells are indicated by circles. Recordings of normalized Rhod-2 fluorescence ($F/F_0$) demonstrate the presence of spontaneous $[Ca^{2+}]_i$ oscillations in a subset of cells of the CD in wild-type mice **(A)**. In contrast, low and steady-state Rhod-2 fluorescence was found in CD cells from $Cx30^{-/-}$ mice **(B)** or in wild-type mice after suramin treatment **(C)**. Summary of the CD $[Ca^{2+}]_i$ responses **(D)** illustrates that the spontaneous $[Ca^{2+}]_i$ oscillations were almost completely abolished in $Cx30^{-/-}$ mice or after suramin treatment. (***$p < 0.001$ vs. wild-type, $n = 5$ wild-type, 7 suramin, and 11 $Cx30^{-/-}$).

## DISCUSSION

This study is a direct and logical continuation of our recent line of research which aims to characterize ATP releasing mechanisms and their physiological relevance in the kidney. In the present experiments we used confocal fluorescence imaging approaches to further examine the function of one particular ATP conduit, the Cx30 hemichannel in the renal CD. Using *in vitro* microperfused CCDs and ratiometric calcium imaging we first found that the classic phenomenon of tubular flow-induced increases in CD $[Ca^{2+}]_i$ was dependent on the expression of Cx30. In addition, spontaneous $[Ca^{2+}]_i$ oscillations in free-flowing CDs were observed in the intact living kidney of wild type but not $Cx30^{-/-}$ mice and the $[Ca^{2+}]_i$ responses were found to be sensitive to suramin, a P2 purinergic receptor inhibitor. Our results are consistent with the presence of mechanosensitive, ATP releasing Cx30 hemichannels in the distal nephron-CD system and established that CD cell $[Ca^{2+}]_i$ responses are an integral element of the intra-tubular autocrine/paracrine purinergic system in the CD that is driven by Cx30-mediated ATP release. The present studies also established the exciting new technical advance of directly and quantitatively visualizing CD $[Ca^{2+}]_i$ responses in the intact mouse kidney *in vivo*.

The present findings are in line with our previous study showing that Cx30-mediated ATP release in the CD can be triggered by mechanical forces, for example by increases in tubular fluid flow rate (Sipos et al., 2009). In contrast to this previous work in which exogenous biosensor cell $[Ca^{2+}]_i$ responses were measured as an indicator of ATP release (Sipos et al., 2009), the present studies measured Cx30-dependent $[Ca^{2+}]_i$ responses in cells of the intact microperfused CCD (**Figure 1**). Flow-induced $[Ca^{2+}]_i$ responses and ATP release in renal and tubular epithelial cells including the CD are well established (Woda et al., 2002; Liu et al., 2003; Satlin et al., 2006; Jensen et al., 2007; Praetorius and Leipziger, 2009; Sipos et al., 2009; Xu et al., 2009) and the downstream effects of ATP involve purinergic signaling through binding to P2X and P2Y purinergic receptors (Schwiebert and Kishore, 2001; Unwin et al., 2003) that regulate salt and water reabsorption. Consistent with Cx30-mediated mechanosensitive ATP release, recent studies found reduced pressure natriuresis and diuresis in $Cx30^{-/-}$ mice (Sipos et al., 2009). $Cx30^{-/-}$ mice also show reduced ATP release into the tubular fluid and urine and display ENaC hyperactivity in response to a high sodium intake (Sipos et al., 2009; Mironova et al., 2011). It should be noted that $Cx30^{-/-}$ mice appear to express an unaltered level of P2 receptor transcripts (Sipos et al., 2009) as well as functional P2 receptors in the collecting ducts, confirmed by the potent inhibitory effect of exogenous ATP on ENaC activity (Mironova et al., 2011). Collectively, these findings indicate the $Cx30^{-/-}$ mice display a disrupted paracrine feedback inhibition of ENaC by ATP due to a failure in ATP release rather than in downstream ATP signaling. Elevated $[Ca^{2+}]_i$ is one of the many known signaling pathways of purinergic ATP receptors including the activation of the $P2Y_2$ receptor (Vallon and Rieg, 2011) which inhibits CD sodium and water reabsorption (Pochynyuk et al., 2008). The present studies further confirm the functional expression and *in vivo* relevance of ATP releasing Cx30 hemichannels in the CD and suggest the involvement of Cx30-dependent autocrine/paracrine $[Ca^{2+}]_i$ responses in the function of the intra-tubular purinergic system (i.e., inhibition of salt and water transport).

The present study revealed the highly dynamic nature of $[Ca^{2+}]_i$ changes in cells of the CD *in vivo* (**Figure 3**) suggesting rapid, temporary oscillations in the regulation and rate of salt and water reabsorption in cells of the CD. Since no exogenous stimuli were applied in these experiments, the Cx30-dependent spontaneous oscillations in CD $[Ca^{2+}]_i$ were likely the result of the endogenous tubular fluid flow oscillations (triggering mechanosensitive Cx30-mediated ATP release) that are known to exist due to renal hemodynamic feedback mechanisms (Holstein-Rathlou and Leyssac, 1986; Marsh et al., 2005; Kang et al., 2006; Peti-Peterdi et al., 2009). In our previous work we described and visually demonstrated (see supplementary videos 2 and 5) the presence of these endogenous tubular flow oscillations, and the simultaneous, phase-matched oscillations

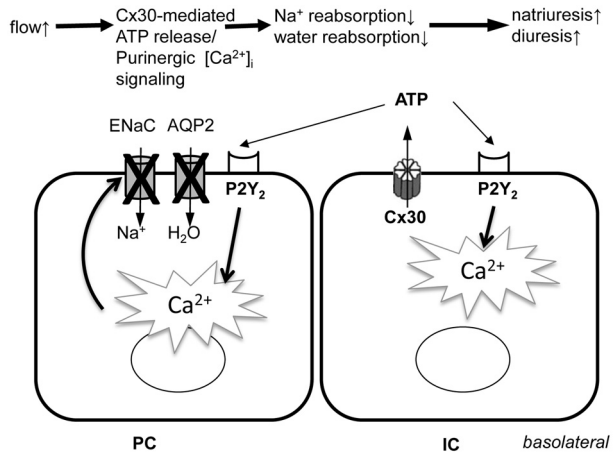


**FIGURE 4 | Illustration of the role of Cx30-mediated ATP release and subsequent purinergic $[Ca^{2+}]_i$ signaling in the function of the intra-tubular autocrine/paracrine purinergic system in the CD.** PC, principal cell; IC, intercalated cell; ENaC, epithelial $Na^+$ channel; AQP2, aquaporin 2 water channel; Cx30, connexin 30 hemichannel; $P2Y_2$, ATP receptor subtype.

in proximal tubule diameter and $[Ca^{2+}]_i$ responses (Peti-Peterdi et al., 2009). In the present work, similar, simultaneous increases in $[Ca^{2+}]_i$ were detected *in vivo* in the CD (**Figure 3A**) and these responses were diminished in $Cx30^{-/-}$ and suramin-treated mice (**Figures 3B–D**). The *in vitro* microperfusion experiments in which equal rates of high flow were applied in both wild-type and $Cx30^{-/-}$ CDs (**Figure 1**) suggested that the diminished CD $[Ca^{2+}]_i$ responses *in vivo* were not due to the lack of flow stimulation but rather the failure in ATP release in $Cx30^{-/-}$ mice. Therefore, the physiological oscillations in tubular fluid flow may function as an important endogenous diuretic mechanism that involves Cx30-mediated ATP release, purinergic $[Ca^{2+}]_i$ signaling and inhibition of tubular salt and water reabsorption in the CD. Consistent with this, $Cx30^{-/-}$ mice which lack this mechanism feature a salt retention phenotype and have salt-sensitive hypertension (Sipos et al., 2009; Mironova et al., 2011). $P2Y_2$ receptor knockout mice display a similar phenotype (Rieg et al., 2007), further supporting the view that dynamic purinergic mechanisms in the CD play an important role in the (patho)physiological regulation of salt and water balance. **Figure 4** illustrates the elements of this local purinergic sensory, signaling and regulatory system in the CD including Cx30-dependent ATP release and $[Ca^{2+}]_i$ signaling that the present studies established.

It should be noted that the present studies detected *in vivo* $[Ca^{2+}]_i$ responses only in a subset of CD cells rather than globally. However, this was likely the result of technical limitations (unequal loading of cells and cortical tissue regions with Rhod-2) since in the *in vitro* dissected and microperfused CD, in which fluorophore loading was very efficient, all cells produced flow-induced $[Ca^{2+}]_i$ responses (**Figure 1**). Also, in the present *in vivo* experiments we did not perform long-term time-lapse $[Ca^{2+}]_i$ imaging due to the above technical limitations, therefore, we did not address the more complex features of CD $[Ca^{2+}]_i$ signals (such as frequency and regularity of oscillations, cell-to-cell propagation, synchronization, etc. and their importance in CD function). However, these important questions need to be addressed in future work using more suitable experimental approaches (e.g., new mouse models with renal tubular expression of genetically encoded calcium indicators).

These studies were carried out in mice, where the expression of Cx30 is restricted to the intercalated cells of the CNT and CCD (McCulloch et al., 2005). However, in kidneys from rat and rabbits Cx30 was also expressed in other nephron segments including the thick ascending limb (McCulloch et al., 2005), indicating that Cx30-mediated ATP release and purinergic $[Ca^{2+}]_i$ signaling could be regulating epithelial transport processes in other tubular segments in other species. In addition, the ATP channel pannexin 1 which was recently localized to the apical plasma membrane of several renal tubule segments including the CD (Hanner et al., 2012) may play a similar role. In terms of other flow-sensing mechanisms in renal tubular cells, in our recent report we speculated (Sipos et al., 2009) that apical membrane Cx30 hemichannel opening induced by mechanical stimulation (interstitial pressure, tubular flow) may involve the supportive function of primary cilia (in principal cells) and microvilli (in intercalated cells) that are well-established sensors of shear and hydrodynamic impulses (Liu et al., 2003). Supporting the existence of interaction between the intra-tubular ATP purinergic system and primary cilia function are the recent findings that the loss of apical monocilia on renal tubular epithelial cells impairs ATP secretion across the apical cell surface (Hovater et al., 2008; Praetorius and Leipziger, 2009; Xu et al., 2009).

In summary, the present studies successfully visualized $[Ca^{2+}]_i$ responses in the CD in the intact kidney *in vivo* and further characterize the function of ATP releasing Cx30 hemichannels in the mouse CD. Our results established that Cx30-dependent $[Ca^{2+}]_i$ responses are an integral element of the intra-tubular autocrine/paracrine purinergic system in the CD that plays an important role in the regulation of renal salt and water reabsorption, the maintenance of body fluid and electrolyte balance and normal blood pressure.

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# Interactions between adenosine, angiotensin II and nitric oxide on the afferent arteriole influence sensitivity of the tubuloglomerular feedback

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Adenosine, via activation of $A_1$ receptors on the afferent arteriole (AA), mediates the tubuloglomerular feedback (TGF) mechanism. Angiotensin II and nitric oxide (NO) can modulate the sensitivity of the TGF mechanism. However, the interaction among these substances in regulating the TGF resetting phenomenon has been debated. Studies in isolated perfused AA have shown a biphasic response to accumulating doses of adenosine alone. In the nanomolar range adenosine has a weak contractile effect (7%), whereas vasodilatation is observed at high concentrations. However, a synergistic interaction between the contractile response by adenosine and that of angiotensin II has been demonstrated. Adenosine in low concentrations strongly enhances the response to angiotensin II. At the same time, angiotensin II in physiological concentrations increases significantly the contractile response to adenosine. Moreover, addition of a NO donor (spermine NONOate) to increase NO bioavailability abolished the contractile response from combined application of angiotensin II and adenosine. These mutual modulating effects of adenosine and angiotensin II, and the effect of NO on the response of AA can contribute to the resetting of the TGF sensitivity.

**Keywords: adenosine, angiotensin II, tubuloglomerular feedback, afferent arteriole, kidney**

The tubuloglomerular feedback (TGF) is a negative-feedback system operating within the juxtaglomerular apparatus that can regulate glomerular filtration rate (GFR) by changing arteriolar resistance and hence blood flow and pressure into the glomerular capillaries. In this control system the tubular load to the distal parts of the nephron is detected via changes in tubular sodium chloride concentration at the macula densa site. This information is then used to determine the contractile state of the afferent arteriole (AA) that is the main effector link of this controller. The sensitivity and reactivity of the TGF system can be modulated via several different factors and via those changing the effector response. Exactly where and how this modulation of the TGF response occurs has not been clear. Recent work from our laboratory has indicated that this modulation to some extent can be carried out by the arterioles themselves.

**Figure 1A** shows signaling pathways of the TGF activated by an increase in NaCl delivery to the macula densa site. Evidence from our laboratory and others indicate that increased NaCl delivery leads to depolarization of the basolateral membrane of the macula densa cells, activation of nitric oxide synthases (NOS) to produce NO, and also activation of NADPH oxidase to produce superoxide (Persson et al., 1991; Liu et al., 2002; Liu and Persson, 2004). This activation of macula densa also leads to release of ATP, possibly via swelling of the macula densa cells that occur following increased uptake of NaCl (Gonzalez et al., 1988a,b).

Results from numerous studies reveal a crucial role of adenosine in the mediation of the TGF (Brown et al., 2001; Schnermann, 2002). Both adenosine $A_1$ and $A_2$ receptors are expressed on afferent arterioles, and can regulate preglomerular resistance. Adenosine in physiological concentrations constricts afferent arterioles via a prominent effect on purinergic $A_1$-receptors (Hansen et al., 2003; Lai et al., 2006). The source for extracellular adenosine in the context of TGF is not fully cleared. Osswald and co-workers first proposed that local generation of adenosine in the juxtaglomerular apparatus may play an important role for signal transmission of TGF (Osswald et al., 1982). More recent experimental studies have demonstrated that ATP release from macula densa cells in response to increased NaCl load in the distal tubule (Bell et al., 2003). ATP itself has a constrictor effect of afferent arterioles via $P_2X$ receptors (Inscho et al., 1992), and therefore it has been debated weather ATP or adenosine is the mediator of TGF signaling. A direct role of ATP in mediating the TGF response may be possible, but the evidence for this is currently not compelling.

Mice lacking $P_2X_1$ receptors, which are present in vascular smooth muscle cells of afferent arteriole, display impaired preglomerular autoregulation (Inscho et al., 2003), but have largely normal TGF responses (Schnermann and Briggs, 2008). Furthermore, pharmacological inhibition of P2 receptors with suramin, did not significantly inhibit TGF of microperfused afferent arterioles with attached macula densas (Ren et al., 2004). ATP

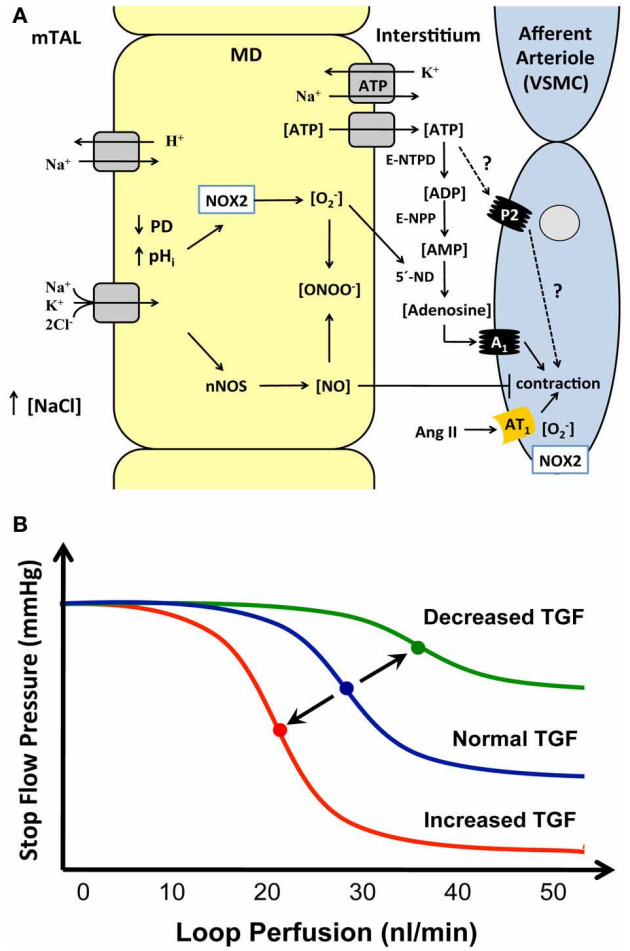


**FIGURE 1 | (A)** Suggested mechanisms by which increased concentration of NaCl at the macula densa site increase apical Na/K/2Cl co-transporter and the Na/H–exchanger that will depolarize basolateral cell membrane potential which will activate NADPH oxidase 2 (NOX2) to increase production of $O_2^-$, but also increase NO production rate from nNOS. At the same time swelling of the macula densa cells can increase ATP leakage. ATP can then be broken down through ecto-5′-nucleotidase (5′-ND) and thereby increase the concentration of adenosine, this process can also be stimulated by $O_2^-$. The NO released could counteract the production of $O_2^-$ by forming peroxynitrite and in this way counteract the sensitization of the TGF. NO could also act directly on the AA to reduce the contractile response elicited by adenosine and angiotensin II (Ang II). Figure modified from that published in Carlstrom and Persson (2009). $A_1$, adenosine receptor type 1; $AT_1$, Angiotensin II receptor type 1; E-NPP, ecto-nucleotide pyrophosphatase/phospho-diesterases; E-NTPD, ecto-nucleoside triphosphate diphosphohydrolase; MD, macula densa; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOX2, NADPH oxidase 2; P2, purinergic receptor type 2; PD, membrane potential; TAL, thick ascending limb of Henle's loop; VSMC, vascular smooth muscle cell. **(B)** The figure shows responses of the stop flow pressure to changes in the proximal tubular perfusion rate during renal micropuncture experiments. Middle curve demonstrates normal TGF response in normo-hydrated animals, the curve to the right shows a decreased TGF response with reduced sensitivity and activity and the curve to the left shows an increased TGF response with increased sensitivity and activity. A rightward shift can occur in situations with volume expansion or increased NO availability whereas a leftward shift can occur during dehydration, increased angiotensin II or ROS concentration or when NO levels are low.

can be hydrolyzed by ecto-ATP diphosphohydrolases, the first step of extracellular degradation of nucleoside 5′-triphosphates and nucleoside 5′-diphosphates. Deficiency of NTPDase1/CD39 went along with impaired TGF responses in mice (Oppermann et al., 2008). Also, mice with ecto-5′-nucleotidase (cd73)-knock out showed decreased TGF responses (Castrop et al., 2004). This enzyme catalyzes the conversion of AMP to adenosine in the interstitium. The observations indicate an important role of extracellular degradation of ATP to adenosine in signaling of the TGF and for adenosine induced constriction of the arteriole. Although, ATP can constrict afferent arterioles, there is not much evidence for mediation of TGF by ATP. Rather, inhibition of $P_2X$ receptors did not influence TGF responses in acutely treated mice (Schnermann, 2011). Thus, most studies support the idea that signal transmission in the TGF starts with a regulated release of ATP from macula densa cells and ends with constriction of afferent arterioles by adenosine via $A_1$ receptors.

One of the ways that the TGF system can be studied is through micropuncture experiments. The stop-flow pressure in the proximal tubule upstream to a wax block is a relative index of glomerular capillary pressure. Stop-flow pressure changes while perfusing the distal nephron, distal from the wax block, with an artificial tubular fluid at different flow rates. There will be a progressive drop in glomerular capillary pressure when flow is increased above a certain level as indicated in the control curve in the **Figure 1B**. Both elevated angiotensin II levels and increased formation of reactive oxygen species (ROS) may sensitize the TGF system, i.e., to be activated at a lower flow rate and respond with a larger reduction in glomerular capillary pressure. Angiotensin II itself can increase the generation of superoxide by activation of the NADPH oxidase, which strengthens the TGF. On the other hand, it also releases NO that desensitizes the TGF system to reduce the TGF response (Liu and Persson, 2004; Patzak et al., 2004). During desensitization, TGF occurs then at a higher tubular flow rate than normal and with a smaller response. An important question that has been debated is how this modulation of TGF by angiotensin II, ROS, and NO can occur and what are the underlying mechanisms(s). The AA is the effector site of TGF, and through microperfusion of isolated afferent arterioles we have determined the contractile responses to angiotensin II and adenosine alone, and their interaction. In one series of experiments, increasing concentrations of adenosine were administered. **Figure 2A** shows a modest arteriolar contraction (7%) to adenosine in low concentrations, whereas high adenosine concentrations induce vasodilation. The dilatory response in the high concentration range is mediated by activation of adenosine $A_2$ receptors (Lai et al., 2006). In the presence of angiotensin II in low concentrations, the arteriolar response to cumulative applications of adenosine was significantly enhanced (**Figure 2A**). Bolus application of angiotensin II alone at $10^{-12}$ and $10^{-10}$ mol/L induced negligible vasoconstrictions (Lai et al., 2009). Therefore, it is clear that the addition of angiotensin II sensitizes the contractile response to increased concentrations of adenosine.

Nonselective inhibition of nitric oxide synthase (NOS) with L-NAME further amplified the contractile response when added to the combined solution of angiotensin II ($10^{-10}$ mol/l) and increasing concentrations of adenosine (**Figure 2B**). In contrast,

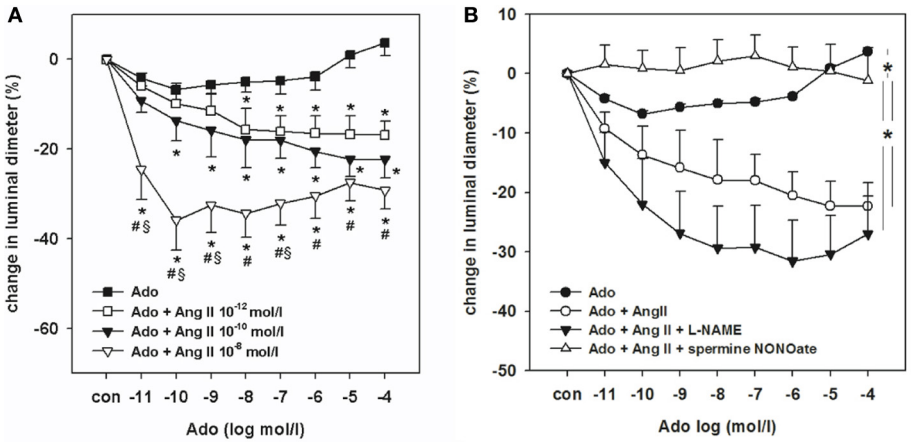


**FIGURE 2 | (A)** Dose response curve for adenosine on afferent arteriolar contraction in increasing concentration from $10^{-12}$ to $10^{-4}$ mol/lwith and without the presence of angiotensin II in three different doses [$10^{-12}$, $10^{-10}$, $10^{-8}$ mol/l, from Lai et al. (2009)] **(B)** Dose response curve for adenosine in increasing concentrations alone or together with angiotensin II ($10^{-10}$ mol/l), or angiotensin II ($10^{-10}$ mol/l) + L-NAME ($10^{-4}$ mol/l) or angiotensin II ($10^{-10}$ mol/l) + L-NAME ($10^{-4}$ mol/l) + spermine-NONOate ($5 \times 10^{-4}$ mol/l) a NO donor. *indicates differences in the course of concentration response curves.

the NO donor spermine NONOate ($5 \times 10^{-4}$ mol/l) completely abolished the contractile response to angiotensin II and adenosine (**Figure 2B**).

Comparing the TGF response curve with the contractions curves from the AA is very interesting. With the present understanding of how the macula densa signal is transmitted we would expect an increased release of adenosine on an increased NaCl load to this segment. The contractile response of the AA will then depend on individual concentrations of angiotensin II, ROS and NO in the juxtaglomerular apparatus. The arteriolar data tells us that if angiotensin II concentration is high there will be a larger response at a lower release of adenosine explaining the increase in TGF sensitivity. On the other hand in a situation with a lot of NO released it will act to reduce the contraction and thereby shifting the TGF sensitivity to a less sensitive level.

We suggest that these interactions of vasoactive substances on the afferent arteriolar contractions can explain at least a part of the phenomenon of resetting of the TGF by angiotensin II, ROS and NO. Increased arteriolar reactivity and TGF responses have been described in several models for hypertension, which may be associated to the abnormal regulation of renal angiotensin II *(elevated)*, oxidative stress *(increased)* and NO bioavailability *(reduced)*.

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# The relationship between P2X4 and P2X7: a physiologically important interaction?

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Purinergic signaling within the kidney is becoming an important focus in the study of renal health and disease. The effectors of ATP signaling, the P2Y and P2X receptors, are expressed to varying extents in and along the nephron. There are many studies demonstrating the importance of the $P2Y_2$ receptor on kidney function, and other P2 receptors are now emerging as participants in renal regulation. The P2X4 receptor has been linked to epithelial sodium transport in the nephron and expression levels of the P2X7 receptor are up-regulated in certain pathophysiological states. P2X7 antagonism has been shown to ameliorate rodent models of DOCA salt-induced hypertension and P2X4 null mice are hypertensive. Interestingly, polymorphisms in the genetic loci of P2X4 and P2X7 have been linked to blood pressure variation in human studies. In addition to the increasing evidence linking these two P2X receptors to renal function and health, a number of studies link the two receptors in terms of physical associations between their subunits, demonstrated both *in vitro* and *in vivo*. This review will analyze the current literature regarding interactions between P2X4 and P2X7 and assess the potential impact of these with respect to renal function.

**Keywords: purinergic, kidney, P2X4, P2X7, receptor**

## INTRODUCTION

ATP is an important extracellular signaling molecule that is the principal agonist for two different types of purinergic receptors: P2Y (G protein-coupled receptors) and P2X (non-selective ion channels). Eight different subtypes have been identified for P2Y (1,2,4,6,11–14) and seven for P2X (1–7), and both P2Y and P2X receptors are found in virtually all mammalian tissues and participate in many different physiological processes [for recent reviews refer to (von Kugelgen and Harden, 2011) and (Surprenant and North, 2009)].

Purinergic signaling within the kidney is emerging as an important focus in the study of renal health and disease, and P2X and P2Y receptors are expressed to varying extents along the nephron; some are more ubiquitously expressed than others, and some alter their expression levels depending on the physiological and pathophysiological state of the kidney (for recent reviews covering P2 receptors in the kidney refer to (Vallon, 2008; Praetorius and Leipziger, 2010; Booth et al., 2012). There are numerous studies comprehensively demonstrating the importance of the $P2Y_2$ receptor on kidney function (Vallon and Rieg, 2011), but P2X receptors are also beginning to emerge as important participants in kidney homeostasis (Bailey et al., 2012).

The recent success in obtaining the first x-ray crystallography structure of a P2X receptor (Kawate et al., 2009) verified previous evidence that functional P2X receptors are composed of three subunits (Nicke et al., 1998; Barrera et al., 2005). All subunits (except for P2X6) assemble to form homotrimeric receptors, and some can be assembled in various configurations to form heterotrimeric P2X receptors (Torres et al., 1999; North, 2002) which display unique pharmacological profiles compared with homotrimers (Lewis et al., 1995; King et al., 2000). P2X7 is distinctive, as in the extensive heterotrimer study by Torres et al. it was the only P2X subunit found not to interact with others to form heterotrimers (Torres et al., 1999); however subsequent studies, as discussed in detail below, have suggested that this subunit may in fact be capable of interacting with other subunits. The amino acid (aa) sequence of P2X7 is considerably longer than all other P2X subunits due to an extended cytoplasmic tail (595 aa vs. 388–471 aa for P2X1-6) and this is reflected in a larger protein mass (P2X1-6 = 43.4–51.7 KDa, P2X7 = 68.6 KDa). The P2X7 receptor also possesses a significantly lower affinity for ATP than all other P2X receptors [$EC_{50}$ = 1–10 μM for P2X1-6 vs. >100 μM for P2X7 (North and Surprenant, 2000)].

The P2X subunit with which P2X7 shares most similarity is P2X4; they are the most closely related of all P2X in terms of aa sequence (48.6% similarity for human sequence and 49.8% for rat), their chromosomal location (only 24 Kb apart on human chromosome 12), and their overlap in tissue

distribution, particularly in immune, endothelial, and epithelial cells (Soto et al., 1996; Murrell-Lagnado and Qureshi, 2008). Given the similarities between these two receptors, researchers have sought to identify if there is a physical and functional interaction between them. Recent reports suggest a close interaction between P2X4 and P2X7, although whether subunits assemble to form functional heterotrimers or interact as homotrimeric receptors to form cooperative receptor complexes is still unclear. We have reviewed the recent literature on the emerging relationship between P2X4 and P2X7, and focus on the potential role of these two receptors in kidney function.

## P2X4 AND P2X7 INTERACTIONS

Evidence from an alveolar cell line suggests that P2X4 and P2X7 receptors can influence the expression of one another; P2X4 receptor expression is up-regulated when P2X7 receptor expression is knocked down, and P2X7 receptor expression is increased when P2X4 receptor expression is decreased (Weinhold et al., 2010). The authors also report alterations in the cellular localization of both receptors during their altered expression profiles, with plasma membrane expression becoming more pronounced in both instances.

Prolonged activation of some P2X receptors can induce formation of non-selective pores that are permeable to large molecules, including fluorescent dyes (North, 2002; Pelegrin, 2011). It was previously believed that channel to pore formation was a unique property of the P2X7 receptor, however, many studies now provide evidence that other subunits can also participate in pore formation e.g., P2X2 and P2X4 (Virginio et al., 1999), P2X4, P2X2 and P2X2/3 heterotrimeric complexes (Khakh et al., 1999), and P2X2/5 heterotrimeric complexes (Compan et al., 2012). The potential for interactions between P2X4 and P2X7 to influence pore formation was explored using a HEK-293 heterologous expression system where co-expression induced an altered response to ATP and fluorescent dye uptake compared with expression of P2X7 alone (Casas-Pruneda et al., 2009). This study provides evidence supporting a functional interaction between P2X4 and P2X7, although it was unable to ascertain the precise mechanism of this interaction; see **Figure 1** for a schematic representation of possible mechanisms.

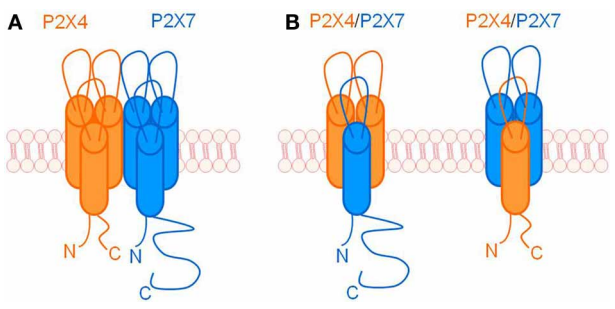


**FIGURE 1 | Schematic representation of ways in which P2X4 and P2X7 may interact. (A)** Individual P2X4 and P2X7 homotrimers physically associating with one another, possibly via C-terminal tail interactions or intermediate scaffolding proteins. **(B)** P2X4 and P2X7 subunits forming heterotrimeric receptors.

The $P2X_{cilia}$ channel receptor is an unidentified P2X receptor expressed in airway ciliated cells; unidentified in that its pharmacology differs from all other previously described P2X homo- or heterotrimers (Korngreen et al., 1998; Ma et al., 1999). Ma et al. presented evidence to suggest that $P2X_{cilia}$ may consist of both P2X4 and P2X7 subunits (Ma et al., 2006), demonstrating that the $P2X_{cilia}$ receptor shares properties in common with P2X4 and P2X7 homotrimers and showing positive immuno-staining for P2X4 in the basal cilia where $P2X_{cilia}$ is expressed. However, the immuno-staining for P2X7 at this location was incomplete and the study was inconclusive as to whether the $P2X_{cilia}$ receptor channel is a P2X4 and P2X7 heterotrimer. Guo et al. subsequently proposed that P2X4 and P2X7 subunits can form functional heterotrimers (Guo et al., 2007). Using a HEK-293 cell heterologous expression system they demonstrated that the surface expression of P2X4 increased more than 2-fold when it was co-expressed with P2X7 and that the subunits physically interacted using immunoprecipitation methods; they were also able to demonstrate that this interaction occurs in native tissue, specifically macrophages. In addition, studies using non-functional mutant P2X4 subunits in the *Xenopus* oocyte heterologous expression system revealed that when they were co-expressed alongside normal P2X7 subunits, the pharmacology of P2X7 receptors was altered. These demonstrations of both physical and functional interactions between P2X4 and P2X7 subunits prompted the authors to suggest that this was evidence for a heterotrimeric assembly.

Subsequent analyses of endogenous P2X4 and P2X7 interactions have indicated a preference for the formation of separate homotrimers that have a close physical interaction, rather than heterotrimers (Nicke, 2008; Boumechache et al., 2009). Chemical cross-linking analysis was used by Antonio et al. in a transfected cell line to show that although P2X4 and P2X7 are in close proximity to each other, no heterotrimeric receptors were formed (Antonio et al., 2011). In addition, atomic force microscopy imaging confirmed that receptor dimers were present and, although the identification of specific homotrimers was not possible, this, along with the results of the close proximity assay, supports the hypothesis that P2X4 and P2X7 homotrimers interact. However, what do these interactions mean for the function of P2X4 and P2X7? A study by Casas-Pruneda et al. shows that P2X4 and P2X7 receptors functionally interact in both a heterologous system (HEK-293 cells) and native epithelia (mouse parotid acinar cells), and that they work together to produce an ATP-activated inward current with functional and pharmacological characteristics that are distinct from either individual homotrimer (Casas-Pruneda et al., 2009). Functional interactions between P2X4 and P2X7 have also been demonstrated in mouse immune cells; normal P2X7 receptor-dependent functions (e.g., P2X7-mediated cell death and release of inflammatory signals) are altered when P2X4 expression levels are reduced (Kawano et al., 2012a,b; Sakaki et al., 2013). The current literature suggests that P2X4 and P2X7 are involved in functionally relevant interactions, both in native and transfected systems, and that the most likely explanation for their close relationship is that they are behaving in a cooperative manner as separate, but physically interacting, homotrimeric receptors.

## PURINERGIC SIGNALING IN THE KIDNEY

All of the key elements of the purinergic signaling system are found within the kidney, including P2 receptors, adenosine receptors, and a range of different ectonucleotidases [for recent review of renal ectonucleotidases see (Shirley et al., 2009)]. In addition, renal tubular cells are capable of releasing nucleotides in response to stimuli such as mechanical stimulation (e.g., stretch, increased flow rate, and osmotic swelling), local acidosis, hypoxia, and various hormones (e.g., vasopressin and aldosterone) (Vekaria et al., 2006; Odgaard et al., 2009). This accumulated evidence suggests a role for nucleotides in regulating renal function.

Functionally, P2 receptors have been shown to have important regulatory effects upon the kidney e.g., P2X1 receptor expression in the afferent arteriole of the glomerulus has been established to have a role in renal blood flow autoregulation (Inscho et al., 2004). With regards to epithelial transport, there are many studies demonstrating the importance of the $P2Y_2$ receptor in the kidney and physiological studies using the $P2Y_2$ null mouse model ($P2Y_2^{-/-}$) have been instrumental in these [for recent overview see (Vallon and Rieg, 2011)]. In the distal nephron $P2Y_2$ receptor activation has been shown to mediate the inhibitory effect of both dietary sodium and aldosterone escape upon epithelial sodium channel (ENaC)-mediated sodium reabsorption (Pochynyuk et al., 2010; Stockand et al., 2010). Additionally, $P2Y_2^{-/-}$ mice have a reduced ability to down regulate ENaC activity in response to nucleotide signaling and this is thought to contribute toward their hypertensive phenotype (Pochynyuk et al., 2008). In the thick ascending limb (TAL), $P2Y_2^{-/-}$ mice have a greater expression of the sodium potassium chloride co-transporter type 2 (NKCC2) and increased furosemide-induced natriuresis, compared with control mice (Rieg et al., 2007; Zhang et al., 2011).

P2X4 and P2X7 are also expressed in the kidney, and genetically modified knockout mice exist for both, enabling researchers to investigate their involvement in the physiology and pathophysiology of tissues of expression, including the kidney. Some of these, along with *in vitro* studies investigating P2X4 and P2X7, will be discussed, with particular emphasis on renal function.

### RENAL P2X4

P2X4 is expressed throughout the kidney (Schwiebert and Kishore, 2001; Turner et al., 2003), and many of the cell-based studies investigating renal P2X4 have focused on its potential to influence epithelial sodium transport. McCoy et al. used a mouse-derived collecting duct (CD) cell line to show that activation of apical P2X, as well as P2Y, receptors had inhibitory effects on ENaC activity; they showed that these cells express P2X4 mRNA (McCoy et al., 1999). This suggested a possible link between P2X receptor activation (tentatively P2X4) and ENaC activity. Following on from this, Gorelik et al. used a *Xenopus*-derived renal epithelial cell line to show that activation of unspecified P2 receptors at the basolateral membrane induced changes in cell structure that altered the surface of the apical membrane in a way that permitted ENaC to become more active (Gorelik et al., 2005). The researchers built on this using a series of P2 receptor agonists and antagonists to show that the effects were most likely P2X4-dependent, and suggested that apical stimulation of P2X receptors has an inhibitory effect up on ENaC, while basolateral stimulation is potentiating (Zhang et al., 2007). In an oocyte heterologous expression system we demonstrated that P2X4 activation was able to inhibit ENaC currents due to a decrease in ENaC surface expression, demonstrating direct apical regulation of ENaC by a P2X receptor (Wildman et al., 2005). Together, these studies suggested a possible role for P2X4 in renal sodium transport, in particular via ENaC, and that there may be opposing actions for apical vs. basolateral activation. This was subsequently corroborated in native tissue using micro-dissected rat CDs; CDs were split-open and whole-cell patch-clamp electrophysiology was performed on the accessible apical membrane (Wildman et al., 2008). Using specific P2 receptor agonists these experiments showed that a P2X receptor was able to inhibit ENaC activity when the sodium concentration of the experimental bathing fluid was 145 mM (standard conditions for these experiments). This P2X receptor was thought to be a P2X4/6 heterotrimer, given the localization of these two receptors, and taking in to consideration the earlier work of Torres and colleagues (Torres et al., 1999). However, in retrospect, there is the possibility of a P2X4 and P2X7 heterotrimer and/or homotrimer interaction. When the sodium concentration of the bathing solution was reduced to 50 mM [more representative of distal tubular fluid sodium concentration (Malnic et al., 1966)], we saw a shift from inhibition to potentiation of ENaC activity by P2X4 activation. We also showed robust expression of the P2X4 receptor at both the apical and basolateral membranes of rat CDs using immunofluorescence. These observations led us to suggest that P2X4 receptors may be capable of responding to the sodium concentration of distal tubular fluid to influence ENaC activity at a local level, and assist the kidneys in maintaining sodium balance.

Most studies published to date using the P2X4 null ($P2X4^{-/-}$) mouse model have not concerned renal function, and have focused predominantly on the receptor's role in pain, inflammation, and synaptic signaling (Sim et al., 2006; Brone et al., 2007; Tsuda et al., 2009; Ulmann et al., 2010). Interestingly, $P2X4^{-/-}$ mice are hypertensive and a study investigating this linked it to endothelial dysfunction and impaired vasodilation (Yamamoto et al., 2006). Renal function was not investigated in this study and so the contribution of lack of P2X4 in the kidney to hypertension is unknown; this possibility should not be discounted, since almost all forms of inheritable hyper- and hypotension identified in humans have evidence of abnormal renal sodium handling (Lifton et al., 2001). Moreover, in a recent series of isolated perfused tubule experiments, Marques et al. demonstrated that $P2X4^{-/-}$ mice had a blunted response to ATP-mediated inhibition of sodium reabsorption in micro-dissected TALs (Marques et al., 2012). This shows a direct effect of P2X4 on epithelial sodium transport in native tissue, and indicates that these $P2X4^{-/-}$ mice have enhanced renal sodium reabsorption which may contribute toward their hypertension.

### RENAL P2X7

P2X7 is constitutively expressed in the majority of immune cells and receptor activation has broad pro-inflammatory effects. Consequently, the P2X7 null ($P2X7^{-/-}$) mouse model has been extensively used to study inflammation in renal pathophysiology,

and expression patterns of P2X7 have been mapped in models of inflammation. A major theme of these studies is that P2X7 expression is up-regulated in diseased/inflamed renal tissue, and that a lack of expression, such as in P2X7$^{-/-}$ mice, offers varying degrees of protection. For example, the induction of unilateral ureteric obstruction is a widely used model of early inflammation and tubulointerstitial fibrosis with progressive kidney injury; this is attenuated in P2X7$^{-/-}$ mice, which show less fibrosis, as well as reduced macrophage infiltration and expression of inflammatory cytokines (Goncalves et al., 2006). A key role for P2X7 in glomerulonephritis was also identified using rodent models of nephrotoxic nephritis in P2X7$^{-/-}$ mice (Turner et al., 2007) and after pharmacological inhibition of P2X7 in rats (Taylor et al., 2009). Moreover, P2X7 expression is up-regulated in the glomerulus and tubular cells in human lupus nephritis, a condition in which glomerular inflammation is an important feature (Turner et al., 2007).

Some studies have found that the P2X7 receptor is expressed in renal epithelial cells (Schwiebert and Kishore, 2001; Hillman et al., 2004), although its function in non-immune cells is less clear. Under *in vitro* conditions, P2X7 receptors can mediate direct renal epithelial cell-fibroblast crosstalk following tubular damage; necrotic tubular cells have been shown to promote interstitial fibroblast cell death by a P2X7-dependent mechanism (Ponnusamy et al., 2011). P2X7 is also involved in polycystic kidney disease (PKD): in a mouse model of autosomal recessive PKD, P2X7 expression is up-regulated in CD cells as they undergo cystogenesis; in addition, P2X7 antagonism can reduce cyst number, but not size, in CD suspension cultures (Hillman et al., 2002, 2004). Contrary to this, P2X7 receptor blockade was shown to prevent cyst enlargement, but not frequency, in a zebrafish model of autosomal dominant PKD (Chang et al., 2011).

P2X7 expression in the kidney has also been linked to rodent models of hypertension, which is interesting, because an association study into human hypertension has shown a link between polymorphisms in the P2X4 and P2X7 gene region and blood pressure regulation (Palomino-Doza et al., 2008). In a renin over-expressing rat model of hypertension the expression of P2X7 is up-regulated in the glomerulus (Vonend et al., 2004). Additionally, hypertension and renal injury are attenuated in P2X7$^{-/-}$ mice with DOCA salt-induced hypertension, and expression of the P2X7 receptor is increased in DOCA salt-treated mice vs. control mice (Ji et al., 2012b). A different study showed an attenuation of blood pressure and renal inflammation in Dahl salt-sensitive rats treated with a P2X7 antagonist (Ji et al., 2012a), suggesting that P2X7 is involved in hypertension and renal injury, potentially via an inflammatory mechanism.

### DO P2X4 AND P2X7 INTERACT IN THE KIDNEY?

Are the proposed interactions between P2X4 and P2X7 relevant in the kidney? Preliminary data from our laboratory suggests that genetic ablation of one may influence the renal expression of the other (**Figure 2**). mRNA measured in microdissected CDs and protein extracted from whole kidneys of P2X4$^{-/-}$ mice revealed that levels of P2X7 were significantly reduced in P2X4$^{-/-}$ mice compared with wild-type controls. P2X4 protein expression was also reduced in P2X7$^{-/-}$ mice. The P2X4 protein decrease observed in P2X7$^{-/-}$ mice was confined to the membrane fraction, whereas the P2X7 protein decrease in P2X4$^{-/-}$ mice was seen in both the membrane and cytosolic fractions. These data suggest cross-talk between P2X4 and P2X7 subunits in the kidney, and certainly in the CD. The nature of the cross-talk between P2X4 and P2X7 observed in the kidney could be one of protein-protein interaction (i.e., a heterotrimeric assembly of P2X4 and P2X7 subunits) or of separate homotrimeric receptor coupling. Although this was not elucidated in this study, previous studies on other native and transfected systems favor the latter mechanism. Will these proposed physical interactions prove to have any functional relevance? They seem to in the immune system (Kawano et al., 2012a,b) and a similar story might hold in the kidney too.

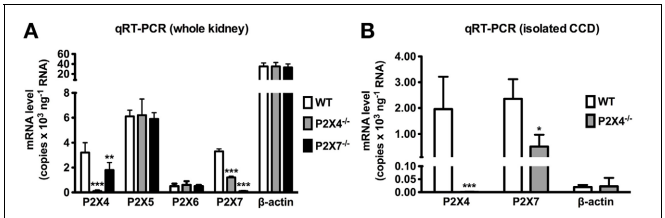


**FIGURE 2 | (A)** P2X4, P2X5, P2X6, P2X7 and β-actin mRNA levels in the whole kidney from wild-type (WT), P2X4 null (P2X4$^{-/-}$) and P2X7 null (P2X7$^{-/-}$) mice. P2X4, 5, 6 and 7 mRNA was readily detected in WT kidney. P2X4 and P2X7 mRNA levels were > 100-fold lower in P2X4$^{-/-}$ and P2X7$^{-/-}$ mice, respectively ($n = 3$). P2X7 mRNA was significantly reduced in P2X4$^{-/-}$ mice ($p < 0.003$; $n = 6$) and P2X4 mRNA was significantly reduced in P2X7$^{-/-}$ mice ($p < 0.03$; $n = 6$). No significant difference was found in P2X5, P2X6 or β-actin between WT and either P2X4$^{-/-}$ or P2X7$^{-/-}$ mice. **(B)** P2X4 and P2X7 mRNA expression in microdissected cortical collecting ducts (CCD) from P2X4$^{-/-}$ mice. P2X7 mRNA was significantly reduced in the CCDs of P2X4$^{-/-}$ mice ($p < 0.05$; $n = 3$). $^{*}P < 0.05$; $^{**}P < 0.03$; $^{***}P < 0.003$.

## DISCUSSION AND FUTURE DIRECTIONS

The existence of physically associated P2X subunits, whether as heterotrimers or interacting homotrimers, implies a complexity to the purinergic signaling system that may serve to adjust the regulation of physiological processes and account for unexpected pharmacological characteristics. Certainly, the altered functional and pharmacological characteristics of P2X4 and P2X7 when they are present together in native tissue and co-expressed in heterologous systems, and of P2X7 when the expression of P2X4 is altered, suggest that the observed interactions are functionally relevant. Moreover, our own findings that the protein levels of each receptor are altered when one is genetically ablated, as well as P2X7 RNA expression in P2X4$^{-/-}$ mice, is consistent with P2X4 and P2X7 functionally interacting in the kidney. However, evidence for functionally relevant P2X4 and P2X7 interactions in the kidney is circumstantial. The generation of a transgenic model that is null for both P2X4 and P2X7 would be useful in revealing the functional interdependence of these receptors, and such a model would also negate the potential of alterations in the expression of one receptor to compensate for loss of the other.

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# Effect of $P2X_4$ and $P2X_7$ receptor antagonism on the pressure diuresis relationship in rats

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Reduced glomerular filtration, hypertension and renal microvascular injury are hallmarks of chronic kidney disease, which has a global prevalence of ~10%. We have shown previously that the Fischer (F344) rat has lower GFR than the Lewis rat, and is more susceptible to renal injury induced by hypertension. In the early stages this injury is limited to the pre-glomerular vasculature. We hypothesized that poor renal hemodynamic function and vulnerability to vascular injury are causally linked and genetically determined. In the present study, normotensive F344 rats had a blunted pressure diuresis relationship, compared with Lewis rats. A kidney microarray was then interrogated using the Endeavour enrichment tool to rank candidate genes for impaired blood pressure control. Two novel candidate genes, *P2rx7* and *P2rx4*, were identified, having a 7- and 3- fold increased expression in F344 rats. Immunohistochemistry localized $P2X_4$ and $P2X_7$ receptor expression to the endothelium of the pre-glomerular vasculature. Expression of both receptors was also found in the renal tubule; however there was no difference in expression profile between strains. Brilliant Blue G (BBG), a relatively selective $P2X_7$ antagonist suitable for use *in vivo*, was administered to both rat strains. In Lewis rats, BBG had no effect on blood pressure, but *increased* renal vascular resistance, consistent with inhibition of some basal vasodilatory tone. In F344 rats BBG caused a significant reduction in blood pressure and a *decrease* in renal vascular resistance, suggesting that $P2X_7$ receptor activation may enhance vasoconstrictor tone in this rat strain. BBG also reduced the pressure diuresis threshold in F344 rats, but did not alter its slope. These preliminary findings suggest a physiological and potential pathophysiological role for $P2X_7$ in controlling renal and/or systemic vascular function, which could in turn affect susceptibility to hypertension-related kidney damage.

**Keywords: purinergic, ATP, kidney disease, renal injury, renal vascular resistance**

## INTRODUCTION

Kidney injury and declining renal function are diagnostic indicators of kidney disease and present a global health burden with high population prevalence (Eckardt et al., 2013). Genetic, epigenetic and environmental factors determine susceptibility to renal injury and the development of chronic kidney disease. Hypertension is a major risk factor for kidney disease (Nakayama et al., 2011) and progression can be slowed if blood pressure is controlled (Hart and Bakris, 2010). Nevertheless, renal injury and fibrosis develop independently of barotrauma and the local actions of agents such as aldosterone (Ashek et al., 2012; Kawarazaki et al., 2012) and angiotensin II (Mori and Cowley, 2004; Polichnowski et al., 2011) have been implicated.

We have previously used the *Cyp1a1-Ren2* transgenic rat to investigate pathways leading to renal injury. In these rats, blood pressure is increased by dietary administration of the non-toxic aryl hydrocarbon, indole-3-carbinol (Kantachuvesiri et al., 2001). The rise in blood pressure can be titrated to study the organ injury associated with slowly developing (Conway et al., 2012) or malignant hypertension (Kantachuvesiri et al., 2001). In the malignant setting, vascular injury predominates, with myocyte vacuolation preceding confluent myocyte cell death and microalbuminuria (Ashek et al., 2012).

Genetic background influences susceptibility to renal injury in several rat models (Churchill et al., 1997; Schulz and Kreutz, 2012), the *Cyp1a1-Ren2* transgenic rat being no exception (Kantachuvesiri et al., 2001). Here, the Fischer (F344) strain is susceptible while *Cyp1a1-Ren2* transgenic rats on the Lewis background are protected from renal injury. We have used these informative strains to identify Quantitative Trait Loci for organ injury (Kantachuvesiri et al., 1999) and the development of reciprocal congenic lines enabled us to validate *Ace*, the gene encoding Angiotensin Converting Enzyme, as a plausible modifier of renal injury (Liu et al., 2009). Although the angiotensin receptor antagonist losartan prevents the blood pressure rise in this model, it is only partially protective against renal vascular injury (Ashek et al., 2012). This suggests that susceptibility to renal injury in this model is governed by the interplay between multiple

pathways. We hypothesized that genes differentially expressed in the *Cyp1a1-Ren2* transgenic rat in the normotensive state would contain candidates contributing to poor renal function and susceptibility to renal injury in the F344 strain or the relative renoprotection observed on the Lewis background.

In the present study we compared the pressure diuresis relationship between the differentially susceptible F344 and Lewis rats. This response being blunted in F344 animals, we re-mined a renal exon-microarray (Liu et al., 2009) identifying the genes encoding the $P2X_4$ receptor and $P2X_7$ receptor as candidates for altered vascular function in F344 rats.

## MATERIALS AND METHODS

### MICROARRAY ANALYSIS

A previously published Affymetrix microarray (Liu et al., 2009) was re-mined to identify differentially expressed probe-sets in the kidney of normotensive *Cyp1a1-Ren2* transgenic rats, i.e., rats in which the *Ren2* transgene was silent. The array was performed on four groups of rats ($n = 4$ per group): the two consomic parental strains (F344, Lewis) and the two reciprocal congenic strains (F344-MOD-Lewis, Lewis-MOD-F344) containing a 14 Mb region of chromosome 10. This congenic region contained the *Ace* locus and the congenics were included in the present analysis to determine whether cis (or trans) regulation occurred. The 16 CEL intensity files were imported into Bioconductor and arrays normalized by the Robust Multi-array Average (RMA) method. The Linear Models for Microarray Data (LIMMA) algorithm was used to calculate fold-change and *p*-value statistics from the normalized intensities.

Differentially expressed genes were imported into the web client online version of the multi-database enrichment tool Endeavour (Aerts et al., 2006, 2009). A list of 157 "training" genes isolated from the rat genome database (Laulederkind et al., 2002) was also imported. The "training" genes used in this study were selected for their association with blood pressure regulation in the rat. They were not tissue specific and assumed no mutual exclusivity with inflammatory, or other disease, processes. The Endeavour method then employed multiple database mining using parallel approaches to enrich the list of differentially regulated genes. These approaches were: (i) published literature text mining; (ii) protein-protein interactions in the STRING database; (iii) transcriptome analysis from the WalkerEtAl database; (iv) sequence comparison with BLAST; and (v) annotations within Gene Ontology, InterPro, KEGG, and Swiss-Prot. Finally, global ranking by Q-statistic generated a list of genes in order of prioritization for the observed phenotype, known as "genomic data fusion."

### ANIMALS

*E*xperiments were performed on male F344 and Lewis rats (Charles River, UK). All rats had access to food and water (Special Diet Services, Witham, Essex, UK) *ad libitum*. Procedures were performed in accordance with the UK Home Office Animals (Scientific Procedures) Act of 1986 after ethical review by The University of Edinburgh.

For Western analysis and immunohistochemistry, F344 and Lewis rats ($n = 3$ per genotype) were killed by decapitation. The kidneys were rapidly excised and the left kidney was snap frozen and stored at −80C for subsequent extraction of total protein. The right kidney was immersion fixed in 10% buffered formalin, transferring to 70% ethanol after 48 h. These kidneys were then paraffin embedded and transverse sections taken for IHC.

### IMMUNOHISTOCHEMISTRY

Primary rabbit polyclonal antibodies against the $P2X_1$ (APR-001, Alomone Labs), $P2X_4$ (APR-002, Alomone Labs), and $P2X_7$ (APR-004, Alomone Labs) receptors were selected based on published validation for use in the rat. Each antibody was then optimized in a dilution series (1:250, 500, 1000, 2000, 4000, 5000, and 7500) using control rat kidney, following heat-induced epitope recovery (HIER) with citrate buffer. The final titers were selected to give minimal background: $P2X_1$ (1:5000), $P2X_4$ (1:7500), and $P2X_7$ (1:2000). All staining was performed on a Leica Bond × immunostaining robot using a refined HRP polymer detection system. Briefly, after HIER and blocking in Peroxidase, the section was incubated in primary antibody for 2 h at room temperature. Following two 5 min washes, sections were exposed to anti-rabbit HRP polymer before being washed. Immunopositive staining was visualized with 3,3′-diaminobenzidine and counterstaining with hematoxylin.

### WESTERN BLOT

Whole kidneys were homogenized in ice-cold buffer containing 250 mmol/l sucrose and 10 mmol/l triethanolamine. Protease inhibitors (Cocktail set III, Calbiochem) and phosphatase/kinase inhibitors (2 mmol/l EDTA, 50 mmol/l NaF, 25 mmol/l sodium glycerophosphate, 5 mmol/l pyrophosphate, and 1 mmol/l sodium orthovanadate) were added and the pH adjusted to 7.6. Following quantification by Bradford assay, protein samples were added to Laemlli buffer and resolved by SDS-PAGE, on a NuPAGE Tris-Acetate gel (8% NovexTM) using a Tris-acetate running buffer (50 mmol/l tricine, 50 mmol/l Tris base, 0.1% SDS, pH 8.24) NuPAGE antioxidant was added to the upper chamber. For the $P2X_4$ studies, 12 μg of total protein was loaded; 20 μg for $P2X_7$ receptor experiments. Following semi-dry transfer the membrane was incubated overnight at 4C with the primary antibody ($P2X_4$ 1:2000; $P2X_7$ 1:1000; Alomone as described above). A goat-antirabbit HRP secondary antibody was then added and the bands visualized by ECL. The $P2X_4$ antibody detected a band of ∼60 kDa; the $P2X_7$ antibody detect a band at ∼75 kDa. The autoradiogram was scanned and band intensity (corrected for background) was quantified by densitometry using ImageJ. Values were normalized to the total protein intensity (Coomasie-Blue) at the appropriate molecular weight.

### RENAL FUNCTIONAL STUDIES

Rats were anaesthetized (Thiobutabarbital 120 mg/kg IP) and prepared surgically for measurement of the pressure-diuresis relationship. The right jugular vein was cannulated and 0.9% NaCl was infused at a rate of 50 μl/min/100 g during abdominal surgery

(to replace surgical losses) and then at 33 μl/min/100 g during the post-surgical equilibration (60 min) and throughout the experimental protocol. The left femoral artery was cannulated and connected to brass transducer (MLT844; Capto) connected to a Powerlab (AD Instruments, UK). Blood pressure was recorded continuously at 1 kHz. A midline laparotomy was performed and a Doppler transit time probe (MA1PRB; Transonic, USA) placed around the left renal artery. Acoustic gel was used to ensure good sonic coupling. Loose silk ties were placed around the superior mesenteric and coeliac arteries: these ligatures were tightened during the experimental procedure to create an acute pressure ramp of two stages above baseline blood pressure. The bladder was catheterized for urine collection under mineral oil with flow rate being determined gravimetrically. The entire procedure was performed under homeostatic temperature control at 37°C.

Pressure-diuresis experiments were performed first on a control group of F344 ($n = 7$) and Lewis ($n = 5$) rats and then on a second cohort of F344 ($n = 5$) and Lewis ($n = 6$) rats receiving an IV infusion (50 μg/min/100 g) of Brilliant Blue G (BBG, Sigma, UK).

### STATISTICAL ANALYSIS

Data are presented as mean ± s.e.m. or as individual data with median. Statistical analysis was performed by Mann-Whitney *U*-test (for Western analysis) or by unpaired *t*-test (physiological data). Comparisons between groups of the pressure-diuresis relationship were made by linear regression.

## RESULTS

### PRESSURE DIURESIS RELATIONSHIP

Compared to Lewis rats, F344 rats had a higher baseline blood pressure (**Figure 1A**) and a lower renal blood flow (**Figure 1B**): renal vascular resistance was significantly higher in F344 rats than in Lewis (**Figure 1C**). The imposition of a pressure ramp evoked an increase in urine flow rate in both strains of rats (**Figure 2A**). The slope of the relationship was significantly different from zero in both groups ($P < 0.001$) but was blunted in the F344 strain compared to the Lewis ($P < 0.01$). There was no significant relationship between blood flow and blood pressure in either strain of animals, indicative of intact auto-regulation (**Figure 2B**).

### RENAL MICROARRAY ANALYSIS

After normalization, 67 probe-sets were differentially regulated on the basis of genetic background: 23 over-expressed and 44 under-expressed (**Table 1**). Endeavour analysis was used to rank the differentially expressed genes enriched against the training genes of blood pressure regulation. The ten highest globally ranked genes are given in **Table 2**. *Ace* was the highest ranked gene, consistent with our previous QTL and congenic studies (Liu et al., 2009), and was not studied further. The 2nd and 3rd ranked genes were *P2rx7* and *P2rx4*, respectively. The expression of both was higher in the F344 rats than in the Lewis rats. This was confirmed by Western analysis: there was a 7-fold increase in total $P2X_7$ receptor protein ($P < 0.05$; **Figure 3A**) and a 3-fold increase in $P2X_4$ receptor abundance ($P < 0.05$; **Figure 3B**).

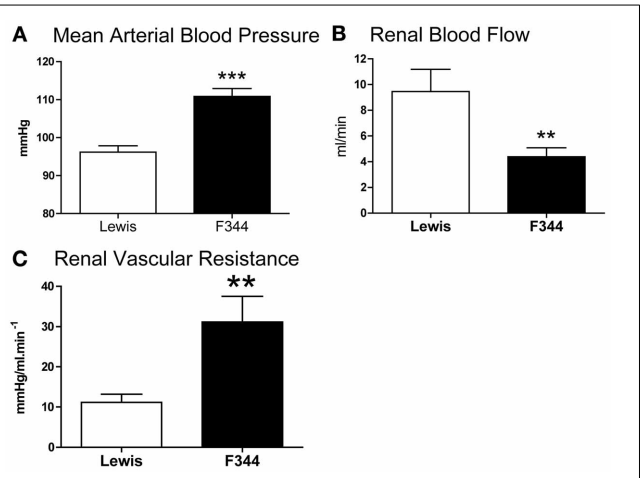


**FIGURE 1 | (A)** Mean arterial blood pressure; **(B)** left renal artery blood flow and **(C)** renal vascular resistance in the left renal artery measured in Lewis ($n = 8$; open bars) and F344 ($n = 7$; black bars) rats. Data are mean ± *SE*. Statistical comparisons were made with unpaired *t*-test. $^{***}P < 0.001$; $^{**}P < 0.01$.

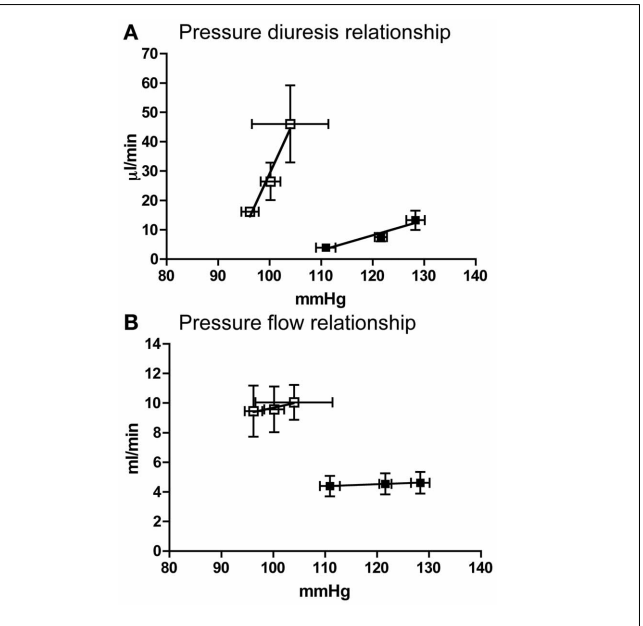


**FIGURE 2 | (A)** Pressure diuresis and **(B)** pressure flow relationship in Lewis ($n = 8$; open squares) and F344 ($n = 7$; black squares) rats. Data are mean ± *SE*. Statistical test was performed by linear regression analysis.

### RENAL LOCALIZATION OF $P2X_{1,4,\text{ and }7}$ RECEPTORS

We observed no differences between strains in the distribution of immunostaining for the P2X receptors. Renal vascular $P2X_4$ immuno-positive staining was restricted to the endothelium throughout the preglomerular vasculature (**Figure 4A**). $P2X_4$ receptor staining was observed in the renal tubules of both strains. In some places this staining was punctate and localized to both the nucleus and cytoplasm (**Figure 4B**).

**Table 1 | Genome wide comparison of gene expression between F344 and Lewis inbred strains listed in order of magnitude of fold change (F344 vs. Lewis, fold > ± 1.2, $p < 0.05$).**

| Over expressed genes (+) | | | Under expressed genes (−) | | |
|---|---|---|---|---|---|
| **Symbol** | **Fold** | ***p*-value** | **Symbol** | **Fold** | ***p*-value** |
| *Rpl30* | +7.6798 | 0.0226 | *Olr1668* | −27.2451 | 0.0123 |
| *Akr1c2* | +7.3466 | 0.0241 | *Olr1680* | −24.6268 | 0.0162 |
| *Spta1* | +5.6906 | 0.0090 | *RGD1309362* | −13.1217 | 0.0162 |
| *Akr1b8* | +4.6613 | 0.0178 | ***Pigzl1*** | **−6.7012** | **0.007** |
| *LOC361914* | +3.6785 | 0.0094 | ***Kif5c*** | **−6.6248** | **0.009** |
| ***Ace*** | **+3.5400** | **0.0178** | *Ces1e* | −5.7903 | 0.0094 |
| *LOC100359585* | +3.3860 | 0.0250 | *Cyp4v3* | −5.2337 | 0.0166 |
| *Guca2b* | +2.7994 | 0.0479 | *Olr1326* | −5.1722 | 0.0336 |
| *Ypel4* | +2.7596 | 0.0253 | *Acsm5* | −4.7035 | 0.0178 |
| *Rtp4* | +2.6916 | 0.0241 | *Hhip* | −4.6118 | 0.0166 |
| *Clstn2* | +2.5879 | 0.0253 | *Hmgcs2* | −4.2039 | 0.0336 |
| ***P2rx4*** | **+2.5327** | **0.0162** | *Cyp2d5* | −3.8624 | 0.0289 |
| ***Klkb1*** | **+2.4303** | **0.0090** | ***Rdh2*** | **−3.4214** | **0.0162** |
| *Exnef* | +2.4073 | 0.0090 | *LOC302192* | −3.3622 | 0.0256 |
| ***Pigr*** | **+2.3473** | **0.0336** | *Lcn2* | −3.097 | 0.0253 |
| ***P2rx7*** | **+2.1586** | **0.0336** | *Csmd1* | −3.019 | 0.0336 |
| *Akr1b7* | +2.1071 | 0.0336 | *Slc10a2* | −2.7769 | 0.0226 |
| *Cd59* | +1.8540 | 0.0256 | *Rxrg* | −2.6987 | 0.0336 |
| *Fam149a* | +1.7008 | 0.0336 | *Cntnap4* | −2.6686 | 0.0192 |
| *P4ha2* | +1.6668 | 0.0336 | *RT1-CE5* | −2.6679 | 0.0336 |
| *Arl4d* | +1.5187 | 0.0336 | ***Erc2*** | **−2.5297** | **0.0253** |
| *Igfbp4* | +1.4873 | 0.0336 | *Ptprq* | −2.4522 | 0.0182 |
| *Col15a1* | +1.2734 | 0.0336 | *RGD1311723* | −2.4244 | 0.0372 |
| | | | *Rbp4* | −2.3816 | 0.0336 |
| | | | *Abcb10* | −2.2669 | 0.0256 |
| | | | *Sult1b1* | −2.2336 | 0.0493 |
| | | | *RGD1563120* | −2.1689 | 0.045 |
| | | | *Mis18a* | −2.1532 | 0.0192 |
| | | | *Slc35f1* | −2.1291 | 0.0372 |
| | | | *Tcerg1l* | −2.0443 | 0.0253 |
| | | | *Acadsb* | −1.9181 | 0.0336 |
| | | | ***Rgs7*** | **−1.8925** | **0.0277** |
| | | | *Retsat* | −1.8721 | 0.0253 |
| | | | *Gas2* | −1.8114 | 0.045 |
| | | | *Ly75* | −1.74 | 0.0442 |
| | | | *Slco1a6* | −1.7194 | 0.031 |
| | | | *Slc26a11* | −1.6736 | 0.0317 |
| | | | *Pfas* | −1.6633 | 0.0178 |
| | | | *Eps8l2* | −1.6505 | 0.0336 |
| | | | ***Dpp6*** | **−1.6382** | **0.0259** |
| | | | *RGD1311575* | −1.5914 | 0.0491 |
| | | | *RGD1564614* | −1.5199 | 0.0344 |
| | | | *Cdc42ep2* | −1.4477 | 0.0372 |
| | | | *Synm* | −1.4011 | 0.0442 |

*Genes identified by enrichment analysis (**Table 2**) shown in bold font.*

Vascular $P2X_7$ receptor staining was observed in the endothelium of the pre-glomerular arteries, including the afferent arterioles of both rat strains (**Figures 4B,C**). Staining was also observed in the glomerulus (**Figure 4C**). In the larger arteries, occasional expression in the vascular smooth muscle was observed but in a given vessel this was limited to a small number of myocytes (**Figure 4D**).

As shown by the low magnification image, $P2X_1$ receptor expression was limited to the vascular network and not expressed in the renal tubules (**Figure 4E**). $P2X_1$ receptor immunopositive staining was observed in the smooth muscle layer of all artery types from lobar to afferent arteriole in both rat strains.

### EFFECT OF INFUSION OF BRILLIANT BLUE G

Under baseline (non-ligated) conditions, acute infusion of BBG caused a significant reduction of mean arterial blood pressure in F344 rats but not in Lewis animals (**Figure 5A**). Blood flow through the left renal artery was not significantly affected by BBG in either group (**Figure 5B**). However, BBG caused a significant *decrease* in renal vascular resistance in F344 rats (**Figures 5C, 6B**).

Acute infusion of BBG did not affect the pressure-diuresis relationship in Lewis rats (**Figure 6A**). In F344 rats, BBG caused a significant leftward shift of the pressure-diuresis intercept (**Figure 6B**), reducing the threshold of this response, but did not alter the gradient of the slope. There was no significant relationship between blood flow and blood pressure in either strain (data not shown).

## DISCUSSION

F344 rats are susceptible to renal vascular injury whereas Lewis rats are relatively protected (Liu et al., 2009). We find that normotensive F344 rats have a blunted pressure diuresis relationship, which would impair blood pressure control and may underpin the susceptibility to vascular injury observed in this strain. At a genetic level, we identified increased renal expression of $P2X_4$ and $P2X_7$ receptors, which may contribute to impaired vascular function in F344 rats, compared to the Lewis strain.

Multiple subtypes of P2X and P2Y receptors are expressed throughout the kidney and extracellular nucleotides regulate renal tubular, endocrine, and vascular functions (Bailey and Shirley, 2009; Bailey et al., 2012; Shirley et al., 2013). Purinergic control of renal vascular tone is complex and the net vasoactive effect depends upon the route of administration/physiological source of the extracellular nucleotide. Thus, ATP applied *in vitro* to the adventitial surface of the renal microvasculature causes contraction (Inscho et al., 1992) mediated by $P2X_1$ receptors (Inscho et al., 2003) in the vascular smooth muscle (Chan et al., 1998). In contrast, infusion of ATP into the renal artery increases blood flow (Tagawa and Vander, 1970) and the vasodilatation is dependent on production of nitric oxide/prostacyclin by the endothelium (Eltze and Ullrich, 1996). The P2 receptor subtype(s) that mediate the vasodilatory response to ATP is not resolved and may vary in different vascular beds. mRNA encoding $P2Y_1$, $P2Y_2$, $P2X_4$, and $P2X_7$ receptors have all been identified in human arterial endothelial cells (Yamamoto et al., 2000; Ray et al., 2002). $P2X_4$ receptors are the most abundantly expressed, followed by $P2X_7$ (∼50%) and then by $P2Y_1$ and $P2Y_2$ (∼20%) receptors (Yamamoto et al., 2000). A similar profile is observed in endothelial cells cultured from the mouse pulmonary artery (Yamamoto et al., 2006) and $P2X_4$ and

**Table 2 | Global prioritization by the Endeavour enrichment method.**

| Gene | Known biological function(s) | Global prioritization | | |
|---|---|---|---|---|
| | | Rank | Score | Rank ratio |
| *Ace* (ENSRNOG00000007467) | BP regulation | 1 | 0.0187 | 0.0909 |
| *P2rx7*(ENSRNOG00000001296) | Ion transport, cell volume, apoptosis | 2 | 0.0624 | 0.182 |
| *P2rx4* (ENSRNOG00000001300) | Ion transport, BP regulation, NOS | 3 | 0.118 | 0.273 |
| *Rgs7* (ENSRNOG00000021984) | G-protein signaling | 4 | 0.583 | 0.364 |
| *Erc2* (ENSRNOG00000015148) | Nerve terminal assembly | 5 | 0.674 | 0.455 |
| *Klkb1* (ENSRNOG00000014118) | Proteolysis, coagulation, inflammation | 6 | 0.787 | 0.545 |
| *Kif5c* (ENSRNOG00000004680) | Motor axon guidance | 7 | 0.796 | 0.636 |
| *Dpp6* (ENSRNOG00000030547) | Proteolysis | 8 | 0.933 | 0.727 |
| *Pigr* (ENSRNOG00000004405) | Antibody receptor | 9 | 0.936 | 0.818 |
| *Rdh2* (ENSRNOG00000029651) | Retinoid metabolism, oxidation reduction | 10 | 0.988 | 0.909 |

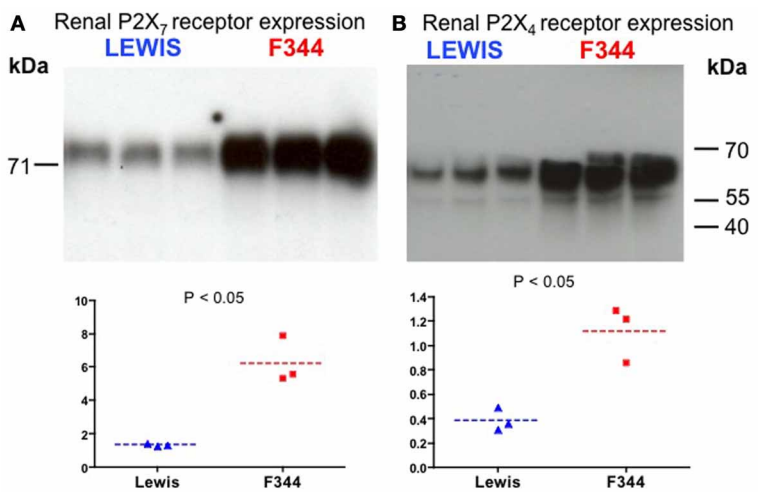


**FIGURE 3 | Western blot analysis of (A) $P2X_7$ receptor and (B) P2X4 receptor expression in whole kidney homogenates.** The top panels show the blot performed in Lewis and F344 rats ($n = 3$ in each). The bottom panels show the blot intensity (normalized to protein loading) quantified by densitometry.

$P2X_7$ receptors have also been immunolocalized to the endothelium of the larger renal arteries of the rat (Lewis and Evans, 2001).

Our studies are largely consistent with this distribution of P2X receptors. $P2X_1$ receptor expression was limited to the vascular smooth muscle of the renal arteries and afferent arteriole. Renal autoregulation is severely attenuated in $P2X_1$ null mice, (Inscho et al., 2004; Guan et al., 2007; Inscho, 2009), illustrating the importance of this receptor for renal vascular function. In the present study, renal autoregulation was intact in both strain of rats and we find no evidence linking differential expression of the $P2X_1$ receptor, or indeed P2X4 or P2X7 receptors to the impaired renal vascular function observed in F344 rats.

We did find increased abundance of $P2X_4$ and $P2X_7$ receptor, both in the microarray analysis and at the protein level. In humans the encoding genes, *P2RX4* and *P2RX7*, are located within 130 kb of each other on chromosome 12. These genes can be regulated independently: the endothelial expression of $P2X_4$ receptors in the human aorta is increased following injury; $P2X_7$ receptor expression is not affected (Pulvirenti et al., 2000). It is possible, however, that these receptors have common promotor elements. Physiological interactions between the receptors are postulated (Craigie et al., 2013) and the locus is associated with human disease. For example, a single nucleotide polymorphism (SNP) in the first intron of *P2RX7* is strongly associated with elevated blood pressure (Palomino-Doza et al., 2008) and a loss-of-function SNP in the *P2RX7* coding region associates with protection against ischemic stroke (Gidlöf et al., 2012). Similarly, a loss of function SNP in the $P2X_4$ receptor has been associated with increased pulse pressure (Stokes et al., 2011).

Consistent with the previous studies described, we localized $P2X_4$ and $P2X_7$ receptors to the endothelium of the preglomerular vasculature. Our bioinformatic ranking analysis associated increased expression with vascular dysfunction and loss of blood pressure control. Both $P2X_4$ (Yamamoto et al., 2006) and $P2X_7$ receptors (Liu et al., 2004) can modulate blood vessel contractility by promoting the release of vasodilators from the endothelium. One interpretation of our data is that

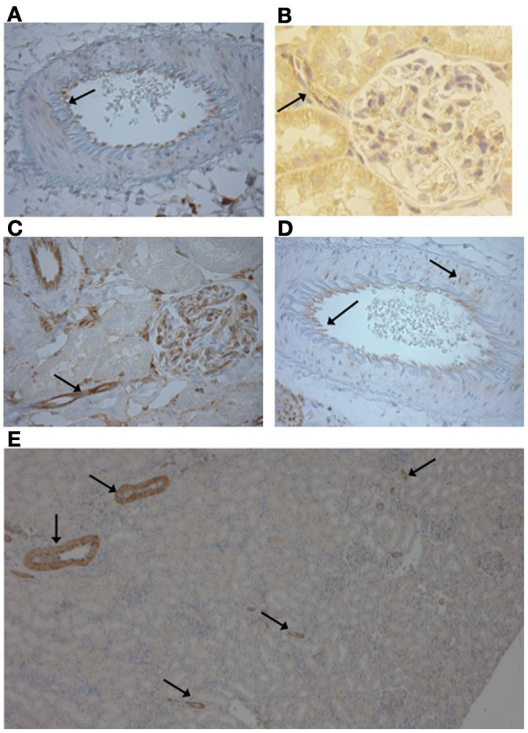


**FIGURE 4 | (A)** Vascular $P2X_4$ receptors were expressed in the endothelium (Image from F344 rat ×400) and **(B)** the afferent arteriole (Image Lewis rat ×500). **(C)** $P2X_7$ receptors were stained in the endothelium of the preglomerular vasculature, including the afferent arteriole (arrow) and cells of the glomerulus (Image F344 rat, ×400). **(D)** Occasional smooth muscle staining of $P2X_7$ was observed (arrow; Lewis rat, ×400). **(E)** $P2X_1$ immunopositive staining was only observed in the vasculature and was limited to the smooth muscle layer of large and small diameter vessels (F344 rat, ×50).

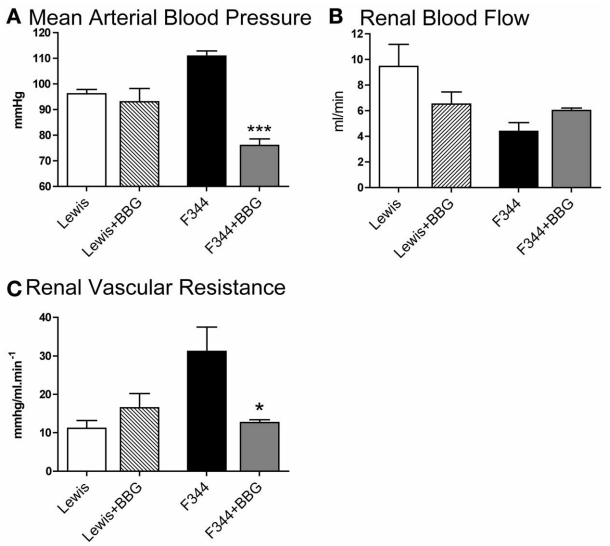


**FIGURE 5 | (A)** Mean arterial blood pressure; **(B)** left renal artery blood flow and **(C)** renal vascular resistance in the left renal artery measured in Lewis and F344 rats receiving either saline or Brilliant Blue G by intravenous infusion. Data are mean ± *SE*. Statistical comparisons were made within strain by unpaired *t*-test. $^{***}P < 0.001$; $^{*}P < 0.05$. Statistical comparisons were made using one way ANOVA with Bonferroni post-test.

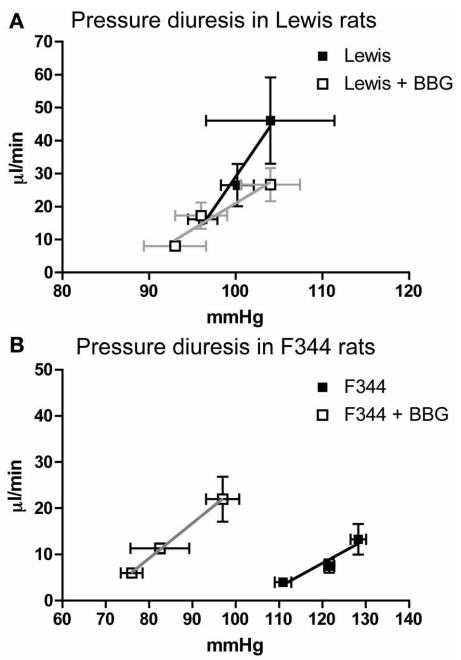


**FIGURE 6 | The Pressure diuresis relationship measured in (A) Lewis and (B) F344 rats receiving either saline (closed symbols) or Brilliant Blue G (open symbols) by intravenous infusion.** Data are mean ± *SE*. Statistical test was performed by linear regression analysis.

the up-regulation of receptors in F344 rats is a compensatory response to improve poor renal blood flow. Thus, acute receptor antagonism *in vivo* should inhibit this tonic vasodilation. There was a trend for this in the Lewis rats but the reduction in blood flow induced by BBG was not statistically different. BBG did induce a significant hemodynamic effect in F344 rats but this was to increase blood flow, rather than to reduce it. One interpretation of this outcome is that in F344 rats $P2X_4/P2X_7$ receptor activation induces a tonic vasoconstriction. It is difficult to reconcile such an effect with the predominantly endothelial location of these receptors. However, the endothelium also releases potent vasoconstrictive mediators, including mono- or di-nucleoside polyphosphates such as adenosine 5′ tetraphosphate (Tolle et al., 2008) and uridine adenosine tetraphosphate is a partial agonist at the rat $P2X_4$ receptor (Wildman et al., 1999) and causes a profound vasoconstriction when perfused via the intravascular route into the isolated rat kidney (Tolle et al., 2008).

An obvious concern in interpreting these results is the selectivity of the antagonist, BBG. This compound is a potent inhibitor of rat $P2X_7$ receptors ($IC_{50} = 10$ nM) and although it can also block the $P2X_4$ receptor, its selectivity for $P2X_7$ receptor is 1000-fold greater. BBG has been used previously *in vivo* to elucidate $P2X_7$ receptor functionality (Jiang et al., 2000; Peng et al., 2009). Indeed, chronic administration of BBG reduces renal injury and lowers blood pressure in the Dahl salt sensitive rat

(Ji et al., 2012a); $P2X_7$ null mice are similarly protected from the renal injury associated with salt-induced hypertension (Ji et al., 2012b). Nevertheless, BBG may also antagonize rat $P2X_4$ receptors and our infusion protocol could inhibit both P2X receptor subtypes. Furthermore, a number of off-target effects of BBG have been reported (Katrahalli et al., 2010), so we cannot exclude the possibility that $P2X_7$-independent effects also contribute to the hemodynamic actions of BBG observed in the F344 rats.

$P2X_4$ and $P2X_7$ receptors were also identified in the renal tubule in both strains of rats. Tubular expression of $P2X_4$ receptor is consistent with several previous studies (Bailey et al., 2012). We found some evidence of intracellular, punctate staining, particularly in the Lewis rats. It is possible that this represents expression of $P2X_4$ receptors in intracellular vesicles, which might act as a reservoir for trafficking of receptors to the apical or basolateral membrane or serve as mediators of vacuolar calcium release (Sivaramakrishnan and Fountain, 2012). P2X receptors, including $P2X_4$ can regulate tubular sodium reabsorption processes (Bailey et al., 2012) but in our studies BBG did not affect urine flow rate. The relationship between $P2X_4$ receptor activation and sodium/water reabsorption is complex, however, and may depend on the local sodium concentration.

In summary, $P2X_7$ and $P2X_4$ receptors are expressed in the vascular endothelium and may contribute to the normal control of renal arterial resistance. Both receptors are attractive candidate genes for impaired renal vascular function and susceptibility to kidney injury. However, their respective roles are not easy to define: the present findings are consistent with a predominant vasoconstrictor effect of $P2X_7$ and vasodilator effect of $P2X_4$, but the relationship is likely to be more complex than this simple dichotomy suggests. For example, endothelial $P2X_7$ receptors can mediate the release of factors that modulate the inflammatory state of the vessel wall (Wilson et al., 2007). Moreover, the encoding gene for $P2X_7$ transcribes a large number of splice variants with reportedly different functionality (Sluyter and Stokes, 2011; Xu et al., 2012), which may also contribute to contrasting vasoactive effects in different strains of rat as observed here.

## AUTHOR CONTRIBUTIONS

Performing experiments: Robert I. Menzies, Data analysis: Robert I. Menzies, Matthew A. Bailey, Data interpretation: Robert I. Menzies, John J. Mullins, Robert J. Unwin, Matthew A. Bailey, Discussion of data and manuscript: all authors, Writing of paper: all authors

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# Sympathetic nerve-derived ATP regulates renal medullary vasa recta diameter via pericyte cells: a role for regulating medullary blood flow?

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Pericyte cells are now known to be a novel locus of blood flow control, being able to regulate capillary diameter via their unique morphology and expression of contractile proteins. We have previously shown that exogenous ATP causes constriction of vasa recta via renal pericytes, acting at a variety of membrane bound P2 receptors on descending vasa recta (DVR), and therefore may be able to regulate medullary blood flow (MBF). Regulation of MBF is essential for appropriate urine concentration and providing essential oxygen and nutrients to this region of high, and variable, metabolic demand. Various sources of endogenous ATP have been proposed, including from epithelial, endothelial, and red blood cells in response to stimuli such as mechanical stimulation, local acidosis, hypoxia, and exposure to various hormones. Extensive sympathetic innervation of the nephron has previously been shown, however the innervation reported has focused around the proximal and distal tubules, and ascending loop of Henle. We hypothesize that sympathetic nerves are an additional source of ATP acting at renal pericytes and therefore regulate MBF. Using a rat live kidney slice model in combination with video imaging and confocal microscopy techniques we firstly show sympathetic nerves in close proximity to vasa recta pericytes in both the outer and inner medulla. Secondly, we demonstrate pharmacological stimulation of sympathetic nerves *in situ* (by tyramine) evokes pericyte-mediated vasoconstriction of vasa recta capillaries; inhibited by the application of the P2 receptor antagonist suramin. Lastly, tyramine-evoked vasoconstriction of vasa recta by pericytes is significantly less than ATP-evoked vasoconstriction. Sympathetic innervation may provide an additional level of functional regulation in the renal medulla that is highly localized. It now needs to be determined under which physiological/pathophysiological circumstances that sympathetic innervation of renal pericytes is important.

**Keywords: pericytes, ATP release, P2 receptors, vasa recta, vasoconstriction, kidney slice**

## INTRODUCTION

Dogma suggests that arterioles and precapillary sphincters are the key regulatory mechanisms of blood flow at the tissue level, however it is now becoming widely accepted that an additional locus of local blood flow regulation exists—that being pericyte cells found on the capillaries themselves (Peppiatt-Wildman, 2013). Pericyte cells are smooth muscle-like cells, comprised of a cell body and long processes, which run along and wrap-around the capillary, and possess the contractile proteins α-smooth muscle actin and myosin. Pericytes have the ability to constrict and dilate capillaries, via their processes, following exposure to a number of vasoactive agents including adenosine 5′-triphosphate (ATP) (Peppiatt et al., 2006; Crawford et al., 2012; Peppiatt-Wildman, 2013).

Pericytes have been identified on capillaries in many organs of the body, including the kidney. Within the kidney, pericytes are primarily located on descending vasa recta (DVR) capillaries (Park et al., 1997). DVR extend from the renal cortex toward the outer and then inner medulla, with the highest density of pericytes being observed in the outer medulla (Park et al., 1997; Crawford et al., 2012). It has been suggested that renal pericytes play an integral role in regulating renal medullary blood flow (MBF), which in itself is essential to satisfy the conflicting demands of preserving the cortico-medullary gradients of NaCl and urea, while maintaining adequate oxygen and nutrient delivery, as well as metabolic clearance (Kennedy-Lydon et al., 2013). As such it has been further proposed that dysregulation and/or dysfunction of the pericyte cells themselves may account and/or contribute to a number of renal pathologies (Crawford et al., 2012; Peppiatt-Wildman, 2013).

Using our live tissue slice model (i.e., where vasa recta and pericytes are located *in situ*), we previously demonstrated that exogenous ATP, a well-documented vasoactive transmitter, causes constriction of vasa recta via renal pericytes (Crawford et al., 2011). We further demonstrated that exogenous noradrenaline (NA) causes constriction of DVR via renal pericytes (Crawford et al., 2012), and ATP acts via a variety of pericyte-membrane bound P2 receptors (*nee* purinoceptors), (Crawford et al., 2011).

Consequently, this study added to an already substantial wealth of scientific publications that propose key physiological roles for extracellular ATP and P2 receptors (i.e., purinergic signaling) in the kidney; specifically in this case, a key role for purinergic signaling in regulating renal MBF (Crawford et al., 2011).

Given the increasing interest in renal purinergic signaling it is perhaps not surprising that various sources of endogenous extracellular ATP have been proposed, including renal epithelial, endothelial and red blood cells, in response to stimuli such as: mechanical stimulation (e.g., stretch, increased flow rate, and osmotic swelling), local acidosis, hypoxia, exposure to various hormones (e.g., vasopressin and aldosterone), and to a lesser extent from sympathetic nerve varicosities (Bodin et al., 1991; Sprague et al., 1996; Schwiebert, 2001; Jans et al., 2002; Burnstock and Knight, 2004; Praetorius et al., 2005; Wildman et al., 2009).

Extensive sympathetic innervation of the kidney has previously been shown, however the innervation reported has focused around the proximal tubules, distal tubules and ascending loop of Henle (Dibona, 2000). Sympathetic nerves follow the arterial vasculature from the renal artery to the outer medullary portions of the descending (and ascending) vasa recta, but have not been reported in the inner medulla or papilla regions (Barajas et al., 1992). Electron microscopy has demonstrated that sympathetic nerve varicosities come into close contact with effector cell membranes (both tubular and vascular cell) in the renal cortex, where released transmitters presumably act on receptors expressed by the effector cells. It is likely that a balance between sympathetic nerves and intra-renal effector cells is essential for optimal renal function (Dibona, 2005). Both ATP and NA are co-released from sympathetic nerve varicosities (Burnstock, 1990). Sympathetic nerve stimulation has been shown to induce the release of ATP and NA in human cortical kidney slices (Rump et al., 1996). *In vivo* studies in rats and rabbits have shown that cortical blood flow (CBF) and total renal blood flow (RBF) are regulated in part, by renal sympathetic nerves, whereby electrical stimulation causes constriction of arteries and arterioles at higher frequencies (Rudenstam et al., 1995; Leonard et al., 2000). We now know quite a lot about the effect of sympathetic nerve stimulation on MBF (Walkowska et al., 2005; Eppel et al., 2006a,b; Johns et al., 2011) but some important questions remain, for example are vasa recta pericytes innervated, can neurotransmitter release from nerves associated with said pericytes induce vasoconstriction, and does ATP contribute to this vasoconstriction?

Given that renal pericytes are likely key regulators of MBF, and that purinergic (and noradrenergic) signaling potentially plays a role in the regulatory process, we hypothesize that sympathetic nerves may be an additional source of ATP acting at renal pericytes to regulate MBF. In the current study we use our novel kidney tissue slice model in combination with immunohistochemistry to determine the proximity of vasa recta pericytes to sympathetic nerves in the renal medulla. In addition we investigate the effect of pharmacologically stimulating the release of transmitter substances (ATP and NA) from sympathetic nerves on pericyte activity. Identification of a new pathway (i.e., sympathetic innervation) for the regulation of renal pericytes may inform studies seeking to delineate the elusive mechanisms underlying renal pathology.

## METHODS

### TISSUE SLICING

Animal experiments were conducted in accordance with United Kingdom Home Office Scientific Procedures Act (1986). Kidney tissue slices were obtained as previously described (Crawford et al., 2012). Adult male Sprague Dawley rats (~250 g, purchased from Charles River UK Ltd., Kent, UK) were killed by cervical dislocation; their kidneys were immediately removed and placed in ice-cold physiological saline solution (PSS) bubbled with 95% $O_2$/5% $CO_2$ and prepared for slicing. Prior to slicing, the kidneys were de-capsulated and any renal artery remnants removed. A single kidney was secured on the slicing block of a vibratome tissue slicer (Leica model VT1200S, Leica Microsystems (UK) Ltd, Milton Keynes, UK), and submerged in a bath of ice cold PSS bubbled with 95% $O_2$/5% $CO_2$. PSS contained (mM) 100 NaCl, 5 KCl, 0.24 $NaH_2PO_4$, 0.96 $Na_2HPO_4$, 10 Na acetate, 1 $CaCl_2$, 1.2 $MgSO_4$, 5 glucose, 25 $NaHCO_3$, 5 pyruvate (Sigma-Aldrich Ltd, Dorset, UK). The pH was adjusted to 7.4 using 10 M NaOH. The outer cortical dome region (~3 mm tissue) of the kidney was removed to expose the top of the renal medulla and serial 200 μm-thick coronal kidney slices (in which there was intact cortex and medulla) were cut. Slices were collected and maintained at room temperature in a holding chamber containing PSS, and bubbled with 95% $O_2$/5% $CO_2$ to conserve tissue viability. The slices to be used in "live" experiments were maintained for up to 3 h in the holding chamber, or slices were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich Ltd, Dorset, UK) and used for immunohistochemistry.

### IMMUNOHISTOCHEMISTRY

For experiments in which pericytes and sympathetic nerves were co-labeled, fixed kidney tissue slices were incubated with anti-NG2 (Calbiochem, Merck Millipore, Middlesex, UK) and anti-tyrosine hydroxylase (anti-TH; Vector Laboratories Ltd, Peterborough, UK) primary antibodies, respectively. The anti-NG2-labeled pericytes were probed with a fluorescent Alexa Fluor 555-conjugated secondary antibody (Invitrogen Life Technologies, Paisley, UK), as previously described (Crawford et al., 2012). The tyrosine-hydroxylase signal was amplified with a biotinylated secondary antibody and probed with a FITC-conjugated tertiary antibody (Fluorescent Avidin Kit, Vector Laboratories Ltd, Peterborough, UK). Kidney slices were mounted using Citiflour (Agar Scientific Ltd., Stanstead, UK). The medulla of fixed slices was imaged using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss Ltd., Welwyn Garden City, Hertfordshire, UK). FITC-conjugated secondary antibodies were excited at 488 nm and Alexa Fluor 555-conjugated secondary antibody excited at 543 nm. Emitted light was collected with the following filters: long-pass 560 nm (Alexa Fluor 555) and band-pass 505–550 nm (FITC). Pericytes and sympathetic nerves were imaged in both the inner and outer medulla. Z-stack images (40× magnification) were also obtained in order to measure the distance between nerves and pericytes. At least three z-stacks were obtained in both inner and outer medulla per slice. The distances between sympathetic nerves and the nearest pericyte, in both inner and outer medulla, were measured using Zeiss LSM image

browser software (Carl Zeiss Ltd., Welwyn Garden City, Hertfordshire, UK).

### FUNCTIONAL EXPERIMENTS AND ANALYSIS

Functional DIC imaging experiments in live tissue, investigating *in situ* pericyte-mediated changes in vasa recta diameter, were performed as previously described (Crawford et al., 2011, 2012). Live kidney slices were secured in an open-bath chamber using a purpose-built platinum slice anchor and transferred to the stage of an upright Olympus microscope (model BX51WI, Olympus Microscopy, Southend-on-Sea, UK). Kidney slices were continuously superfused (~2.5 ml/min; 1.25 ml bath volume) with PSS, bubbled with 95% $O_2$/5% $CO_2$ and maintained at room temperature. Pericytes on the vasa recta capillaries (vasa recta were defined as <10 μm in diameter) were identified by their previously established "bump-on-a-log" morphology (Crawford et al., 2012), and DIC images were captured through an Olympus 60× water immersion objective (0.9 NA). Real-time video images of changes in vasa recta diameter were collected every second by an attached Rolera XR Charge Coupled Devise (CCD) camera and recorded using Image Pro Software (Media Cybernetics Inc., Marlow, UK). Kidney slices were superfused with PSS alone for 70 s to establish a baseline vessel diameter at the pericyte and non-pericyte site. Slices were then exposed to tyramine [1 μM; for ~150 s, sufficient time to evoke neurotransmitter release (Glover et al., 1983)], ATP (100 μM), suramin (100 μM), or combinations of the agents, before being subjected to a PSS wash. Compounds were purchased from Sigma-Aldrich Ltd, Dorset, UK, and working solutions were prepared in oxygenated PSS.

Time-series analysis of live kidney slice experiments was carried out using the public domain software ImageJ (NIH, http://rsb.info.nih.gov.ij), as previously described (Crawford et al., 2012). For each experiment, both a pericyte site and a non-pericyte site were identified on a single vasa recta and the diameter of the vasa recta at both locations was measured every five frames for the duration of the experiment (1 frame = 1 s). Each capillary acted as its own control; an average of the first five measurements was taken to represent the resting baseline diameter value, and expressed as 100%, for both pericyte and non-pericyte sites. All subsequent diameter measurements were calculated and expressed as a percentage of the corresponding baseline value for both pericyte and non-pericyte sites as previously described (Crawford et al., 2012).

For all experiments, statistical significance was calculated using Student's *t*-test; $P < 0.05$ (two tailed) was considered significant. Values are expressed as mean ± SEM; sample size ($n$) represents the number of pericytes (1 pericyte and non-pericyte site per kidney slice). All experiments were performed in at least three different animals. One vasa recta per kidney slice was used to ensure all vessels were "naïve" prior to exposure to any drug.

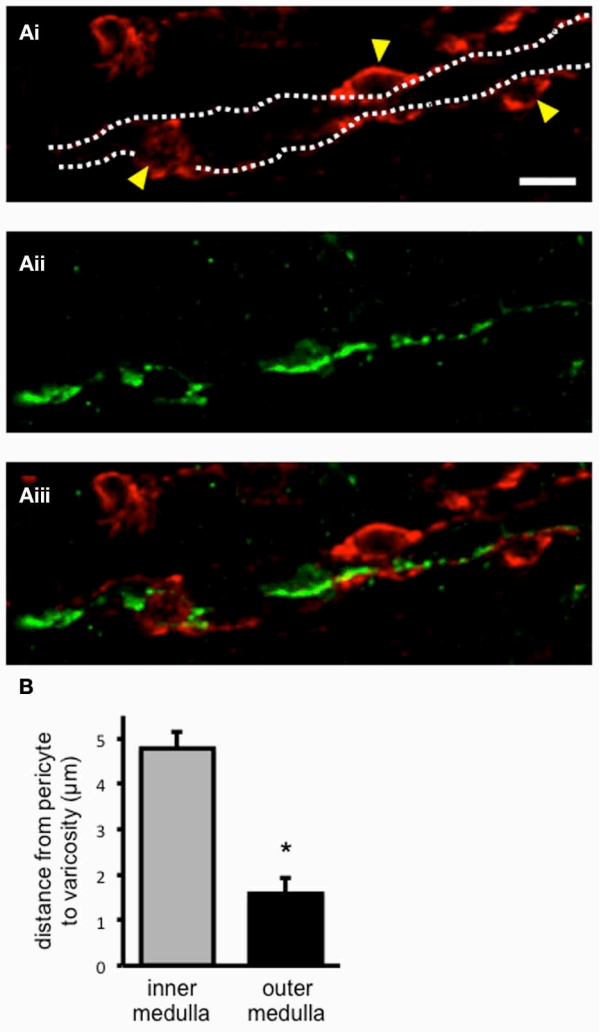


**FIGURE 1 | Sympathetic nerves in close apposition to pericyte cells in the renal medulla.** In **(Ai)**, confocal image of fixed kidney slices (outer medulla region) incubated with anti-NG2, and probed with Alexa 555-conjugated secondary antibody (red) to label vasa recta pericytes and their processes. Pericyte cell bodies are highlighted (arrowheads), and the capillary wall is outlined (dotted lines). In **(Aii)**, from the same image as in **(Ai)**, sympathetic nerves were labeled with anti-tyrosine hydroxylase primary antibody, probed with Alexa 488-conjugated secondary antibody (green). In **(Aiii)**, an overlay of images **(Ai, Aii)** shows sympathetic nerves running along vasa recta capillaries, in close proximity to pericytes. Scale bar = 10 μm. The distance between pericytes and the nearest sympathetic nerve varicosity was measured from confocal images using image browser software. In **(B)**, the distance between pericyte and varicosity was significantly less in the outer medulla region ($n = 21$) when compared to the inner medulla ($n = 21$; $^*P < 0.05$).

## RESULTS

### SYMPATHETIC INNERVATION OF THE RENAL MEDULLA

Pericytes and sympathetic nerves were labeled in the medulla of fixed kidney tissue slices using anti-NG2 and tyrosine hydroxylase (TH) antibodies, respectively, as described in the methods section (**Figures 1Ai, Aii**). Pericytes were clearly defined by their bump-on-a-log morphology (**Figure 1Ai**). Sympathetic nerves were consistently found running through the entire medulla region [i.e., both the outer ($n = 21$) and inner medulla ($n = 19$)]. In all slices analyzed, sympathetic nerves were found, irrespective of localization in the medulla, to be in close proximity to vasa recta perciytes (**Figure 1Aiii**). Confocal z-stack images were used to follow nerves through the tissue, and the distance from pericyte

to the nearest sympathetic nerve varicosity (defined by their nodule-like appearance) was measured using image browser software (see **Supplementary Movie 1** for an example z-stack image). The distance from pericyte to the nearest sympathetic nerve varicosity was significantly less in the outer medulla ($1.58 \pm 0.35\,\mu m$, $n = 21$) compared to the inner medulla ($4.78 \pm 1.08\,\mu m$, $n = 19$, $^*P < 0.05$, **Figure 1B**). Appropriate, parallel control experiments were carried out omitting anti-TH, anti-NG2, and/or biotinylated secondary antibodies, which confirmed an absence of fluorescent labeling.

### EFFECT OF TYROSINE-EVOKED NEUROTRANSMITTER RELEASE ON VASA RECTA DIAMETER

Tyramine was superfused onto live kidney tissue slices in order to stimulate the release of sympathetic neurotransmitters (i.e., ATP and NA; via two distinct mechanisms; displacement from the axoplasm and subsequent vesicular release) from varicosities (Trendelenberg et al., 1987; Kirkpatrick and Burnstock, 1994) and the ability of tyramine-evoked neurotransmitter release to alter the diameter of subsurface vasa recta at both pericyte and non-pericyte sites was measured. Superfusion (~150 s) of tyramine (1 μM) caused a slowly-reversible vasoconstriction of vasa recta at pericyte sites (**Figure 2A**). During exposure to tyramine, vasoconstriction was significantly greater at pericyte sites ($3.9 \pm 0.9\%$, $n = 7$) than at non-pericyte sites ($1.4 \pm 0.3\%$, $n = 7$, $^*P < 0.05$, **Figure 2B**). Maximum tyramine-evoked vasoconstriction occurred ~550 s ($534 \pm 115$ s, $n = 7$) after the cessation of tyramine superfusion (i.e., during tyramine-washout, **Figure 2A**). Maximum vasoconstriction, i.e., during tyramine-washout, at the pericyte site was $11.9 \pm 2.5\%$ as compared to $3.0 \pm 0.6\%$ at the non-pericyte site ($n = 7$, $^*P < 0.05$, **Figure 2B**), and was significantly greater than that measured when tyramine was present in the bath ($^{**}P < 0.05$, $n = 7$, **Figure 2B**). Complete reversibility (i.e., when vessel diameter returned to baseline) was seen ≥600 s from the start of tyramine-washout ($n = 7$). Using Poiseuille's law, the maximum tyramine-evoked vasoconstriction of 12%, equates to a ~1.7-fold increase in vasa recta resistance and thus a ~40% decrease in blood flow (Peppiatt et al., 2006; Crawford et al., 2012).

Tyramine evokes the co-release of ATP and NA from sympathetic nerve varicosities. To investigate the proportion of ATP-mediated vasoconstriction *vs.* NA-mediated vasoconstriction, suramin was superfused onto the tissue slices, for the entirety of each experiment, in order to antagonize ATP-activated P2 receptors, including those present on renal pericytes. Superfusion (for ~150 s) of tyramine (1 μM) following suramin pre-treatment, and in the continued presence of suramin (100 μM), resulted in the maximum tyramine-evoked vasoconstriction (measured during tyramine-washout) being reduced by approximately 50% at pericyte sites ($^{**}P < 0.05$; **Figure 2C**). The maximum tryamine-evoked vasoconstriction at pericyte sites, in the presence of suramin, was reduced to $5.8 \pm 1.7\%$, and was still significantly greater than the change in vessel diameter recorded at non-pericyte sites ($2.1 \pm 0.7\%$, $^*P < 0.05$, $n = 6$; **Figure 2C**). Incidentally, in 6 out of 10 experiments suramin (100 μM) alone caused a modest vasoconstriction of vasa recta

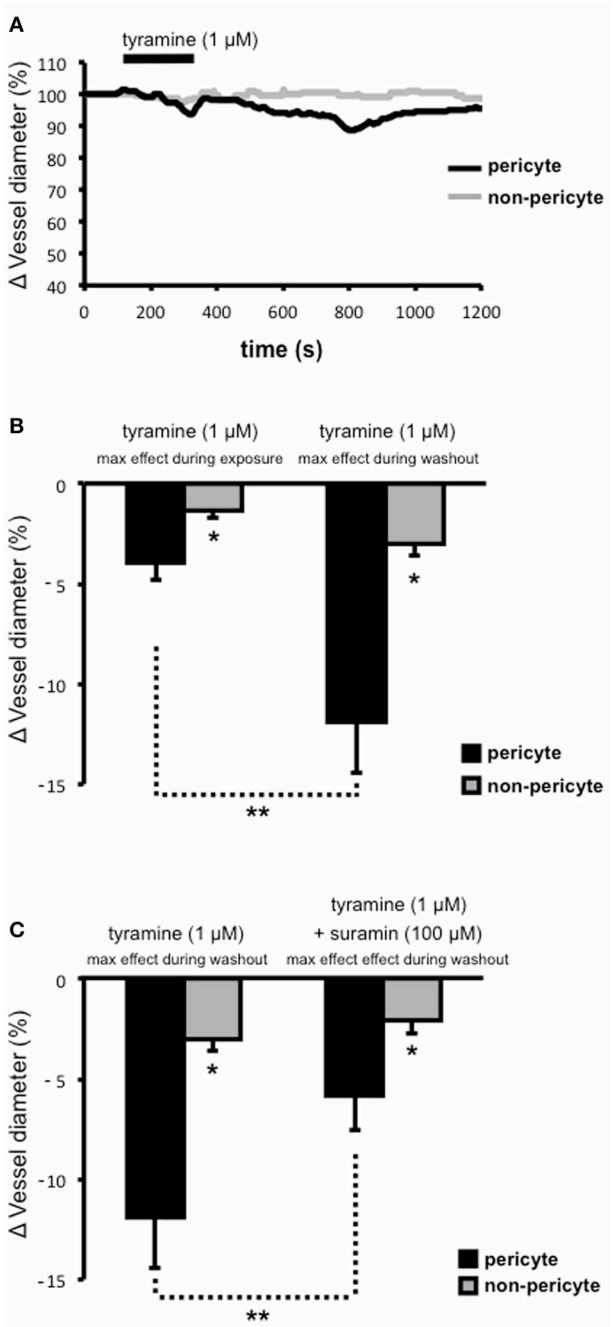


**FIGURE 2 | Tyramine-evoked changes in vasa recta diameter.** Live kidney slices were superfused with tyramine (1 μM). A typical trace from a single experiment shows changes in vessel diameter over time at a pericyte (**A**, black line) and corresponding non-pericyte site (**A**, gray line). Mean data in bar graphs in **(B)**, and **(C)**, show a significantly greater change in vessel diameter at pericyte sites (black bars) compared to non pericyte sites (gray bars), both during tyramine (1 μM) exposure and during the tyramine-washout period (**B**, $^*P < 0.05$, $n = 7$). The constriction measured at pericyte sites was significantly greater during the tyramine washout step than that measured when tyramine was present in the bath (**B**, $^{**}P < 0.05$, $n = 7$). The maximum tyramine-evoked constriction seen at pericyte sites was significantly inhibited by the presence of suramin (**C**, 100 μM, $^{**}P < 0.05$, $n = 6$).

at pericyte sites (2–5%). Whilst suramin significantly altered the maximum tyramine-evoked constriction of vasa recta by pericytes, the presence of suramin did not alter the onset time, time to maximum effect, nor reversibility, of tyramine-evoked vasoconstriction (data not shown, $n = 6$).

Extracellular ATP acts at pericytes to cause vasoconstriction of vasa recta (Crawford et al., 2011) and various sources of endogenous extracellular ATP have been proposed including from renal epithelial cells, endothelial cells, red blood cells, and sympathetic nerve varicosities. To investigate the significance/relative contribution of varicosity-derived ATP to ATP-evoked, pericyte-mediated, vasoconstriction of vasa recta, the additive effects of tyramine and ATP (100 μM), at a concentration previously shown to evoke the greatest constriction of vasa recta (Crawford et al., 2011), were investigated. Prolonged superfusion (~800 s) of tyramine (1 μM) caused a constriction of vasa recta at pericyte sites (**Figure 3A**) culminating in a maximum effect between 400–600 s ($n = 5$). During exposure to tyramine, maximum vasoconstriction was significantly greater at pericyte sites (7.8 ± 3.6%, $n = 5$) than at non-pericyte sites (1.6 ± 0.3%, $n = 5$, $^{*}P < 0.05$, **Figure 3B**). The co-application of ATP (100 μM; for ~200 s) with tyramine (1 μM) during the "maximum tyramine-evoked vasoconstriction period" caused a further increase ($^{**}P < 0.05$, **Figure 3B**) in the constriction of vasa recta at pericyte sites (26.5 ± 4.6%) that was significantly greater than at non-pericyte sites (1.7 ± 0.5%, $n = 5$, $^{*}P < 0.05$, **Figure 3B**). The exogenous ATP-evoked vasoconstriction rapidly desensitized in the presence of exogenous ATP and complete reversibility was seen ≥700 s from the start of tyramine/exogenous ATP-washout (**Figure 3A**). Superfusion (for ~150 s) of ATP (100 μM) alone caused a reversible vasoconstriction of vasa recta at pericyte sites; maximum exogenous ATP-evoked vasoconstriction at the pericyte site was 19.4 ± 2.8% as compared to 3.3 ± 2.9% at the non-pericyte site ($^{*}P < 0.05$, $n = 11$, **Figure 3B**). The exogenous application of ATP (100 μM) alone caused a significantly greater constriction than that observed with tyramine, at pericyte sites (1 μM, $^{***}P < 0.05$, **Figure 3B**).

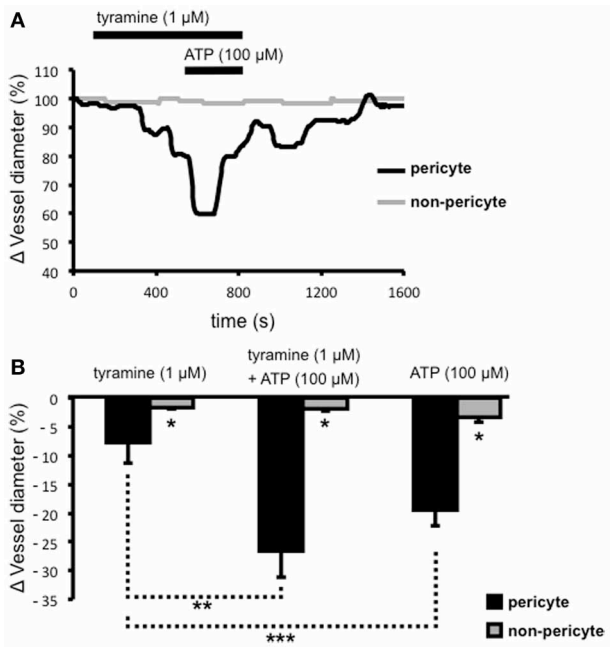


**FIGURE 3 | The additive effects of ATP and tyramine on pericyte-mediated changes in vasa recta diameter.** Live kidney slices were superfused with tyramine (1 μM) and ATP (100 μM); a typical trace from one of these experiments shows changes in vessel diameter over time at a pericyte (**A**, black line) and corresponding non-pericyte site (**A**, gray line). Mean data for these experiments show a significantly greater change in vessel diameter at pericyte sites (black bars) compared with non-pericyte sites (gray bars) under all experimental conditions ($^{*}P < 0.05$, $n = 5$). The combined tyramine (1 μM) and ATP (100 μM)-evoked constriction at pericyte sites was significantly greater than that observed during tyramine (1 μM) incubation alone (**B**, $^{**}P < 0.05$, $n = 5$), and the pericyte-mediated constriction of vasa recta in the presence of exogenous ATP was significantly greater than that evoked by tyramine alone ($^{***}P < 0.05$, $n > 5$).

## DISCUSSION

The main findings of this investigation, using the live kidney slice model, revealed sympathetic nerves in both the outer and inner medulla regions of the kidney, and that pharmacological stimulation of sympathetic nerves *in situ* (by tyramine) evokes pericyte-mediated vasoconstriction of vasa recta capillaries. More specifically, we present evidence that (i) sympathetic nerve varicosities are in closer apposition to pericytes in the outer medulla, (ii) tyramine-evoked vasoconstriction of vasa recta by pericytes is significantly inhibited by the P2 receptor antagonist suramin, (iii) tyramine-evoked vasoconstriction of vasa recta by pericytes is significantly less than exogenous ATP-evoked vasoconstriction. Importantly, these novel findings reveal innervation of renal pericytes by sympathetic nerves, primarily in the outer medulla. This finding being ratified by previous studies, which describe innervation of medullary tubules (Dibona, 2000; Loesch et al., 2009). Moreover, functionally these findings demonstrate that sympathetic nerve-derived ATP, acting as a cotransmitter, is as effective as, or more effective than NA, in its ability to regulate vasa recta diameter, and thus MBF. As such, sympathetic innervation may provide an additional level of functional regulation in the renal medulla that is highly localized (see **Figure 4**).

### SYMPATHETIC NERVE VARICOSITIES IN CLOSE APPOSITION TO PERICYTES IN THE OUTER MEDULLA

The current study identifies, for the first time, TH-labeled, sympathetic varicosities in both the outer and inner medulla regions of fixed rat kidney tissue (**Figure 1**). Our novel live kidney slice model in combination with confocal z-sectional imaging enabled us to observe the path of sympathetic nerves through the tissue, running in parallel to vasa recta capillaries in the renal medulla (see **Supplementary Movie 1**). Furthermore, we demonstrate that sympathetic nerve varicosities are in close apposition to pericyte cells on vasa recta (**Figure 1**). Interestingly, sympathetic nerve varicosities are in closer apposition to pericytes in the outer medulla rather than inner medulla (**Figure 1B**), perhaps supporting a less important role for sympathetic innervation of vasa recta pericytes in the inner medulla where their density is known to be

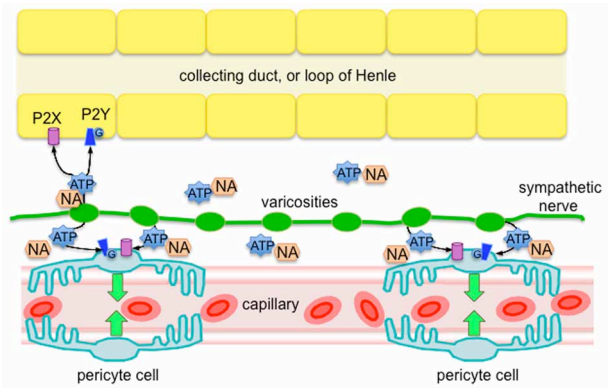


**FIGURE 4 | Diagram of proposed sympathetic innervation of renal pericytes in the outer medulla, whereby ATP is a key neurotransmitter, along with NA, released from sympathetic nerve varicosities.** Released ATP is likely to act via P2 receptors (ionotropic P2X, or metabotropic P2Y) on pericyte cells to evoke localized vasoconstriction of vasa recta capillaries. Based on the findings of others (Loesch et al., 2009), it is likely that epithelial cells of the outer medulla also receive sympathetic innervation that affect an abundance of P2 receptors (Wildman et al., 2008), further supporting the phenomenon of pericyte-mediated tubulovascular crosstalk in the renal medulla (Peppiatt-Wildman, 2013).

significantly less (Crawford et al., 2012). Pericyte cells were found to be ∼1.5 μm from the nearest varicosity in the outer medulla, as opposed to ∼5 μm in the inner medulla. Autonomic nervous system synaptic clefts, which allows delivery of co-transmitters to the site of the effector cells, vary in size with clefts size ranging from 10 nm up to 2 μm (Burnstock, 2008). Varicosities being similar structures, whereby co-transmitters are released "en passage," likely affect effector cells in a 2 μm proximity. Given the close proximity (∼1.5 μm) of sympathetic nerve varicosities to pericytes in the outer medulla, it is therefore plausible that co-transmitters released by apposite sympathetic nerve varicosities could act at contractile vasa recta pericytes to regulate vasa recta capillary diameter and thus MBF, as hypothesized. Similarly, anti-TH antibodies have previously been used to identify sympathetic nerve varicosities in close apposition to proximal tubule and collecting duct epithelial cells of the rat kidney cortex (Loesch et al., 2009). Authors propose that sympathetic neurotransmitters (primarily ATP), released from varicosity vesicles, may regulate tubule function in the renal cortex through the activation of a variety and abundance of ATP-activated P2 receptors; shown to be expressed throughout the nephron by others (Wildman et al., 2009). In accordance with the functional data describing a key role for sympathetic nerve-derived ATP in the regulation of vasa recta diameter via contractile pericytes, we have previously reported that vasa recta and associated pericytes express mRNA for P2X1, 3, and 7 and P2Y4 and 6 (Crawford et al., 2011). Interestingly, *in vivo* studies in rabbits conclude that P2X receptors do not contribute to neurally mediated vasoconstriction (Eppel et al., 2006a,b,c), however this is perhaps not surprising given that we have previously demonstrated much higher levels (40-fold) of P2Y ($P2Y_4$ and $P2Y_6$) than P2X receptor mRNA in rat isolated vasa recta, with pericytes *in situ* (Crawford et al., 2011); supporting sympathetically-derived ATP acting via P2Y receptors on pericytes to mediate vasoconstriction of vasa recta.

It is perhaps interesting to note that whilst all capillary networks are deemed to have a degree of pericyte coverage, capillaries in skeletal muscle of the rat (Saltzman et al., 1992), and peripheral blood vessels of the mouse (Long and Segal, 2009), are not innervated; suggesting that not all pericytes receive sympathetic innervation.

## ATP IS A KEY NEUROTRANSMITTER RELEASED FROM SYMPATHETIC VARICOSITIES

As well as being a substrate for NA synthesis, the ability of tyramine to evoke sympathetic neurotransmitter release is well documented (Kirkpatrick and Burnstock, 1994). Notably in our preparation, the time between application of tyramine and maximum dilation of vasa recta by pericytes (∼550 s) is greater than that seen by the superfusion of a vasoactive agent like NA or ATP (∼100 s; Crawford et al., 2011, 2012). In our slice preparation it is likely that tyramine is both responsible for release, synthesis and subsequent release of newly synthesized co-transmitters (**Figure 2A**). Various neurotransmitters, most notably NA and ATP are contained in a single sympathetic nerve varicosity, all of which are released at one time, the proportion of each released in the current study was unknown. Pharmacological intervention, using a specific ATP-activated P2 receptor antagonist, suramin, allowed an estimation of the proportion of the tyramine-evoked response attributable to ATP released from sympathetic nerves. Tyramine-evoked vasoconstriction of vasa recta by pericytes was significantly inhibited (∼50%) by the P2 receptor antagonist suramin (**Figure 2C**), suggesting a key role for sympathetic purinergic signaling in the regulation of MBF by renal pericytes. Interestingly, others have shown that the α-adrenoceptor antagonist prazosin greatly reduces RBF and CBF in response to renal nerve stimulation, yet MBF was both reduced and increased (Chapman et al., 1982; Eppel et al., 2004, 2006a,b,c). This may of course be due to the relative expression of α-adrenoceptors in the vasculature throughout the kidney, which was not reported by authors of these studies.

## SYMPATHETIC INNERVATION PROVIDES A FINE-TUNING MECHANISM

The current study demonstrates that the vasoconstriction of vasa recta by pericytes evoked by: (i) superfusion of ATP onto live kidney slices, and (ii) tyramine-stimulated release of co-transmitters, are additive. The renal medulla contains numerous potential sources of vasoactive ATP, including from tubular cells, endothelial cells, and red blood cells (Bodin et al., 1991; Sprague et al., 1996; Schwiebert, 2001; Jans et al., 2002; Praetorius et al., 2005; Wildman et al., 2009), in addition to that released by sympathetic nerve varicosities. The additive effects of neuronal co-transmitters and paracrine-released ATP on the renal vasculature, has previously been demonstrated to enhance smooth-muscle cell-mediated vasoconstriction of the glomerular afferent arteriole (Hultstrom et al., 2007). That tyramine-evoked constriction of vasa recta by pericytes is significantly less than exogenous ATP-evoked effects, or indeed maximal exogenous NA-evoked effects (Crawford et al., 2011, 2012), to our minds suggest that sympathetic innervation fine-tuning of MBF can be achieved. However,

it is noteworthy that different proportions of co-transmitter are released in different tissues and the contribution of each co-transmitter can depend on a number of parameters of stimulation, e.g., that short bursts of electrical stimulation favor ATP release, and longer bursts favor NA (Burnstock, 2007).

## CONCLUSIONS

We hypothesized that sympathetic nerves are an additional source of ATP to act upon renal pericytes to therefore regulate MBF (see **Figure 4**). Here we present evidence to support our hypothesis; however acknowledge that *in vivo* studies are still required to provide direct information regarding the control of MBF. What remains to be determined is under which physiological and/or pathophysiological circumstances that sympathetic innervation of renal pericytes, and therefore MBF, is important. Like others we assume that coordination exists between sympathetic nerves and intra-renal effectors such as vessels, tubules and juxtaglomerular cells [neatly termed tubulovascular crosstalk (Dickhout et al., 2002; Kennedy-Lydon et al., 2013; Peppiatt-Wildman, 2013), see **Figure 4**], and that there are functionally specific subgroups of renal nerve fibers mediating specific effects on the renal tubular, vascular or juxtaglomerular granular cells, such as those involved in RBF response and urinary flow rate response (Dibona et al., 1996; Dibona, 2000). A better understanding of these neural sub-populations will determine the significance of renal purinergic innervation and MBF, which is undoubtedly overlooked in light of renal transplantation (denervated kidney) successes, and misconception that kidneys have complete intrinsic ability to regulate blood flow without the need for autonomic input [despite it being well accepted that control of renal hemodynamics involves both intrinsic (myogenic and TGF components of autoregulation) and extrinsic mechanisms]. To contextualize the potential significance of these findings, considering what is now known about the regulation of MBF: despite the hypoxic environment of the renal medulla, regulated MBF serves to provide the vascular and highly metabolic tubular cells with adequate oxygen and nutrients whilst clearing metabolic waste. Imbalances in MBF regulation are detrimental to the health of the kidney as a whole, with localized ischemia leading to papillary necrosis and loss of appropriate sodium and water transport in the loops of Henle. Moreover, it is well established that dysregulation of MBF, acute or chronic, can result in significant pathology. Should sympathetically-derived ATP be involved in said dysregulation, renal pericytes, and indeed purinergic signaling pathways, may represent a novel a therapeutic target in the future.

## ACKNOWLEDGMENTS

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fphys.2013.00307/abstract

**Supplementary Movie 1 | Z-stack series of images showing co-localization of pericytes and sympathetic nerves in the outer medulla.** Pericytes and sympathetic nerves were labeled with anti-NG2, and tyrosine hydroxylase primary antibodies, respectively, and were subsequently probed with fluorescent Alexa-555 (red) and Alexa-488 (green)-conjugated antibodies, respectively. The z-stack series images 5.6 μm through the tissue slice; sympathetic nerves can be seen to run in close proximity to pericytes on vasa recta capillaries. Scale bar = 10 μm.

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# $P2Y_2$ receptor knock-out mice display normal NaCl absorption in medullary thick ascending limb

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Local purinergic signals modulate renal tubular transport. Acute activation of renal epithelial P2 receptors causes inhibition of epithelial transport and thus, should favor increased water and salt excretion by the kidney. So far only a few studies have addressed the effects of extracellular nucleotides on ion transport in the thick ascending limb (TAL). In the medullary thick ascending limb (mTAL), basolateral P2X receptors markedly (~25%) inhibit NaCl absorption. Although this segment does express both apical and basolateral $P2Y_2$ receptors, acute activation of the basolateral $P2Y_2$ receptors had no apparent effect on transepithelial ion transport. Here we studied, if the absence of the $P2Y_2$ receptor causes chronic alterations in mTAL NaCl absorption by comparing basal and AVP-stimulated transepithelial transport rates. We used perfused mouse mTALs to electrically measure NaCl absorption in juvenile (<35 days) and adult (>35 days) male mice. Using microelectrodes, we determined the transepithelial voltage ($V_{te}$) and the transepithelial resistance ($R_{te}$) and thus, transepithelial NaCl absorption (equivalent short circuit current, $I'_{sc}$). We find that mTALs from adult wild type (WT) mice have significantly lower NaCl absorption rates when compared to mTALs from juvenile WT mice. This could be attributed to significantly higher $R_{te}$values in mTALs from adult WT mice. This pattern was not observed in mTALs from $P2Y_2$ receptor knockout (KO) mice. In addition, adult $P2Y_2$ receptor KO mTALs have significantly lower $V_{te}$values compared to the juvenile. No difference in absolute $I'_{sc}$ was observed when comparing mTALs from WT and KO mice. AVP stimulated the mTALs to similar increases of NaCl absorption irrespective of the absence of the $P2Y_2$ receptor. No difference was observed in the medullary expression level of NKCC2 in between the genotypes. These data indicate that the lack of $P2Y_2$ receptors does not cause substantial differences in resting and AVP-stimulated NaCl absorption in mouse mTAL.

**Keywords: purinergic, renal transport, loop of henle, $P2Y_2$ receptors, NKCC2**

## INTRODUCTION

The term purinergic signaling describes the cellular effects mediated by binding of extracellular nucleotides and nucleosides as local signaling molecules to adenosine or to P2 receptors. Nucleotides bind to P2 receptors, which are divided into G-protein coupled receptors (P2Y receptors) and ligand-gated ion channels (P2X receptors). Purinergic signaling and modulation of cell and organ function is ubiquitous involving most areas of physiology and pathophysiology (Burnstock, 2007). In renal epithelia, P2 receptors are expressed in all nephron segments and found both in the apical and basolateral membranes (for review see Rieg and Vallon, 2009; Praetorius and Leipziger, 2010). The renal epithelial cell has been described to release nucleotides under various conditions and is viewed as the primary source of extracellular ATP for paracrine signaling in the kidney (Odgaard et al., 2009; Praetorius and Leipziger, 2010). ATP is also found in trace amounts in the urine (Rieg et al., 2007; Contreras-Sanz et al., 2012). Functionally, extracellular nucleotides inhibit ion transport in the different renal tubular segments. Specifically, in the proximal tubule, ATP decreases of $HCO_3^-$ reabsorption (Bailey, 2004). In the collecting duct (CD), the epithelial sodium channel (ENaC)-dependent $Na^+$ absorption (Lehrmann et al., 2002; Shirley et al., 2005; Pochynyuk et al., 2008) and ROMK channel-dependent $K^+$ secretion is inhibited (Lu et al., 2000). Moreover, extracellular nucleotides reduce the $H_2O$ absorption via Aquaporin-2 (AQP2) in the inner medullary collecting duct (IMCD) (Kishore et al., 2009). In the thick ascending limb (TAL), extracellular ATP mediates an acute decrease in the transepithelial NaCl transport via basolateral P2X receptors (Silva and Garvin, 2009; Marques et al., 2012). Overall, purinergic signaling results in inhibition of water and/or salt transport in the kidney. It is currently viewed that intrarenal purinergic signals tonically dampen absorption and thus, act as an endogenous diuretic system (Praetorius and Leipziger, 2010).

The TAL is responsible both for the generation of the corticomedullary osmotic gradient and the dilution of the urine. Absorption of $Na^+$ and $Cl^-$ in TAL cells requires normal function of various ion transporters and channels. $Na^+$ and $Cl^-$ are initially transported from the tubular lumen by the apical $Na^+$, $K^+$, $2Cl^-$ cotransporter (NKCC2). $Na^+$ leaves the cytosol via

the basolateral $Na^+$, $K^+$-ATPase, whereas $Cl^-$ passes through the basolateral chloride channel Kb (ClC-Kb) into the interstitium following its electro-chemical gradient. The $K^+$ taken up by NKCC2 is recycled through apical ROMK channels. This active transport process produces a lumen-positive transepithelial voltage, which drives the paracellular transport of $Na^+$, $Ca^{2+}$, and $Mg^{2+}$. Measurements of the transepithelial electrical parameters permits quantification of transepithelial NaCl transport (Greger, 1985).

In addition to basolateral P2X receptors (Marques et al., 2012), the mouse TAL cells expresses $P2Y_2$ receptors in both apical and basolateral membranes (Paulais et al., 1995; Jensen et al., 2007). However, there is currently no evidence for a direct $P2Y_2$ receptor-mediated modulation of the transepithelial ion transport in this tubular segment. We recently described that acute stimulation of the basolateral $P2Y_2$ receptor with UTP (100 μM) had no apparent effect on $Na^+$ and $Cl^-$ absorption in the isolated perfused mTAL (Marques et al., 2012). The present study was conducted to clarify a potential chronic effect of $P2Y_2$ receptor deficiency on NaCl absorption, specifically in the mTAL. To evaluate any changes on NaCl transport, the technique of isolated perfused mTAL tubules was used to determine electrogenic $Na^+$ reabsorption. The main finding of this study is that the resting or AVP-stimulated NaCl absorption is similar in $P2Y_2$ WT and KO mice. Thus, the $P2Y_2$ receptor appears to play no major role in the regulation of mTAL NaCl reabsorption.

## MATERIALS AND METHODS

### ANIMALS

In this study, 3.5–8 weeks old male mice of mixed genetic background (B6D2/SV129) were used. The $P2Y_2$ receptor knockout (KO) mice were bred from heterozygous families and genotyped as described previously (Matos et al., 2005). Animals had free access to standard rodent chow and tap water. The mice were bred in house and handled according to Danish animal welfare regulations.

### TUBULE PERFUSION AND MEASUREMENT OF ION TRANSPORT IN mTAL

Mice were sacrificed by cervical dislocation. The kidneys were removed and placed in ice-cold control solution (see below) before slicing and dissected as described before (Wright et al., 1990). The dissection of mTAL tubules from the inner stripe of the outer medulla was performed in control solution (see below) placed in a chamber cooled to 4°C using fine watchmaker forceps. The isolated nephron segment was transferred to a perfusion chamber at 37°C on an inverted microscope and perfused by a system of concentric glass pipettes (Greger and Hampel, 1981) leaving the opposite end of the tubule open. Ion transport in isolated perfused tubules was measured as previously described using a double-barreled perfusion pipette (Lehrmann et al., 2002). In summary, the transepithelial voltage difference ($V_{te}$) was measured via one barrel between the lumen and a reference electrode placed in the bath. Through the other barrel a small current pulse of 38.7 nA was injected into the tubular lumen and measured as a voltage deflection ($\Delta V_0$). The length and the diameter of the tubules were documented with transmitted light microscopy images to quantify the tubule's cable properties. The cable equation was then used to determine the resistance of the tissue ($R_{te}$ in $\Omega \cdot cm^2$). Applying Ohm's law, the equivalent short circuit current ($I'_{sc}$ in μA/cm²) was then calculated as a measure of NaCl transport in the mTAL. In **Figure 1** an original trace with the used protocol is shown. After mounting the mTAL a stable lumen-positive $V_{te}$ was reached within some 10–15 min. Transport was quantified after stable transport conditions were reached for a period of 10 min. The arrow in **Figure 1** shows the time point from which the $V_{te}$ and $R_{te}$ values were used. The figure also displays an original effect of luminal furosemide (100 μM) indicating complete and reversible inhibition of the transport voltage.

### WESTERN BLOTTING

Age matched (44–45 days) male WT and KO mice were anesthetized with isoflurane, surgically opened and the left ventricle perfused with 20 ml PBS to rinse all blood from the kidneys. The mice were than sacrificed and the kidneys removed. The inner stripe of the outer medullar was dissected from both kidneys and homogenised with a plastic pestle in ice cold 150 μl lysis buffer (200 mM mannitol, 80 mM HEPES, 41 mM KOH, pH 7.5) supplemented with protease and phosphatase inhibitor cocktails (cOmplete ULTRA Roche, Switzerland). Homogenates were then spun (4.000 rpm, 15 min, 4°C). Homogenisation and centrifugation was performed three times. Supernatants (150 μl) were subjected to ultracentrifugation (20.800 *g*, 1 h, 4°C). Pellets (membrane fraction) were dissolved in 50 μl lysis buffer. Protein concentration was determined with a Pierce® BCA Protein Assay Kit (Pierce Biotechnology, USA).

Proteins were separated by 7.5% SDS-PAGE before transferring them to PVDF-plus transfer membranes (MSI, USA). Membranes were blocked with non-fat dried milk (blocking grade blocker, Biorad) overnight at 4°C. Primary antibody incubation was done for 1 h 40 min at room temperature (1:4000 in 5% BSA PBS/0.5% Tween). The polyclonal NKCC2 antibody was a kind gift from J. Loffing, Institute of Anatomy, University of Zürich, Switzerland. Equal lane loading of the protein samples was documented by β-actin detection (Pan Actin AB #4968, Cell Signaling Technology, USA). Blots were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody for

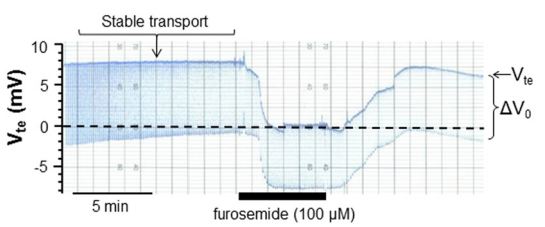


**FIGURE 1 | Original recording of the lumen-positive transepithelial voltage ($V_{te}$) and voltage deflections ($\Delta V_0$) in an isolated, perfused mouse medullary thick ascending limb (mTAL).** Steady state conditions were reached after about 15 min. The arrow indicates the time point used to quantify transport. In addition, the reversible blocking effect of luminal furosemide (100 μ M) is shown.

1h at room temperature. Membranes were washed after both antibody incubations (3 × 10 min in PBS/0.5% Tween and once with PBS 5 min). Blots were analyzed with an enhanced chemiluminescence detection system (FPM-100A, GE Healthcare, USA). Densitometry was performed with the ImageJ (NIH, USA) software.

### SOLUTIONS AND CHEMICALS

Tubule perfusion experiments were performed at 37°C with the following control solution (in mM): 145 NaCl, 1 $MgCl_2$, 1.3 Ca-gluconate, 5 D-glucose, 0.4 $KH_2PO_4$, 1.6 $K_2HPO_4$, 5 HEPES. Solutions were titrated with NaOH to pH 7.4. All chemicals were obtained from Sigma-Aldrich Denmark (Vallensbaek, Denmark) and Merck (Darmstadt, Germany).

### STATISTICS

The data is shown as mean and standard error of the mean (mean ± sem). For experimental series $n$ reflects the number of tubules used. On average 2 tubules were used from each mouse. Comparison of linear regression slope differences was performed in GraphPad Prism (vers. 4.02). Normal distribution was confirmed by the Kolmogorov-Smirnov test. Student's $t$-test was used in normal distribution series while for non-normal distributed series the Mann-Whitney test was used to compare mean values. A $p$-value of $<0.05$ was accepted to indicate statistical significance.

## RESULTS

### BASELINE TRANSPORT PROPERTIES OF mTAL FROM P2Y$_2$ RECEPTOR WT AND KO MICE

To determine if the lack of the P2Y$_2$ receptors influences the basal epithelial transport, we determined the steady state transepithelial transport parameters ($V_{te}$, $R_{te}$ and the derived $I'_{sc}$) in isolated perfused mouse mTALs from WT and KO mice. All baseline transport values were plotted as a function of mouse age (**Figure 2**). This revealed a novel aspect of electrical transport data in mouse mTAL. In WT mice the $V_{te}$ values appear stable, whereas the $R_{te}$ values apparently increase with age. As a consequence of this, the calculated $I'_{sc}$ values in older mice appear lower when compared to younger mice. Thus, this non-homogeneous data set precludes the direct comparison of the whole population from WT and KO mice. To test for possible differences between transport rates in mTAL from WT and KO mice, we divided the mice into two groups. Mice younger than 35 days are still considered juvenile and first viewed as adults after sexual maturation, which occurs around 35 days of age (Safranski et al., 1993). **Figure 3** shows the grouped data, one with juvenile mice (25–35 days) and the other with adult mice (36–55 days). The adult WT mice show a significant increase in $R_{te}$ as compared to juvenile mice ($p = 0.0027$). This results in a significantly lower $Na^+$ and $Cl^-$ absorption in the mTAL of adult WT mice ($p = 0.0014$). Interestingly, this ontogenetic effect on $R_{te}$ is not seen in P2Y$_2$ receptor KO mice.

Surprisingly, in mTALs isolated from KO mice the $V_{te}$ values clearly decreased with age. In contrast, the $V_{te}$ in WT mice did not alter as a function of age (**Figures 2A,D**, significantly different regression lines, $p = 0.046$). Thus, KO tubules of adult mice showed a significantly decreased $V_{te}$ as compared with tubules from juvenile KO mice (**Figure 3**). Subsequently, we compared mTAL transepithelial transport data from juvenile WT with juvenile KO mice and adult WT with adult KO mice, which did not reveal any statistical significant differences (**Figure 3**).

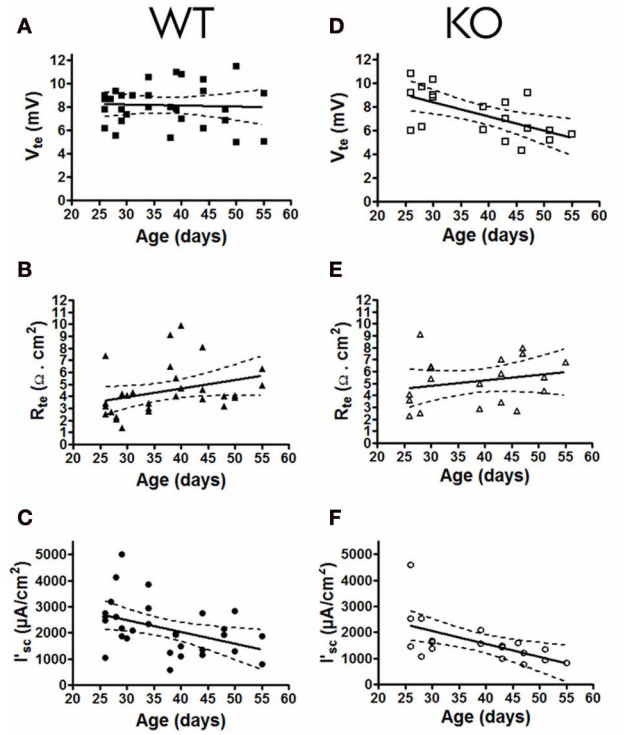


**FIGURE 2 | Basal transport values ($V_{te}$, $R_{te}$, and $I'_{sc}$) of mouse mTALs plotted as a function of age (in days) of P2Y$_2$ receptor WT (A, B, C; $n = 30$) and KO (D, E, F; $n = 19$) mouse.** All 6 data panels include regression line fits where the stippled lines indicate the 95% confidence intervals.

In summary, these data report that (1) mTALs from adult WT mice have significantly lower NaCl absorption rates based on significantly higher $R_{te}$values compared to tubules isolated from juvenile mice. This pattern was not observed in mTALs from KO mice. (2) mTALs from adult KO mice have significantly lower $V_{te}$ values, which leads to a lower but non-significant ($p = 0.08$) reduction of NaCl absorption. (3) No difference was observed between tubules from WT and KO mice when comparing the absolute $I'_{sc}$ data. Importantly, these data do not support higher NaCl absorption rates in the mTAL of P2Y$_2$ receptor KO mice as suggested by elevated NKCC2 protein expression, which was previously documented in these mice (Rieg et al., 2007; Zhang et al., 2011).

### EXPRESSION OF NKCC2 PROTEIN IN MOUSE P2Y$_2$ WT AND KO mTALs

Subsequently, we also performed the analysis of NKCC2 protein expression in membrane fractions from the inner stripe of the outer medullar (ISOM) in 3 P2Y$_2$ WT and 3 P2Y$_2$ KO mice. The data are depicted in **Figure 4**. To our surprise, we found no apparent up-regulation of NKCC2 protein in ISOM of P2Y$_2$ KO mouse

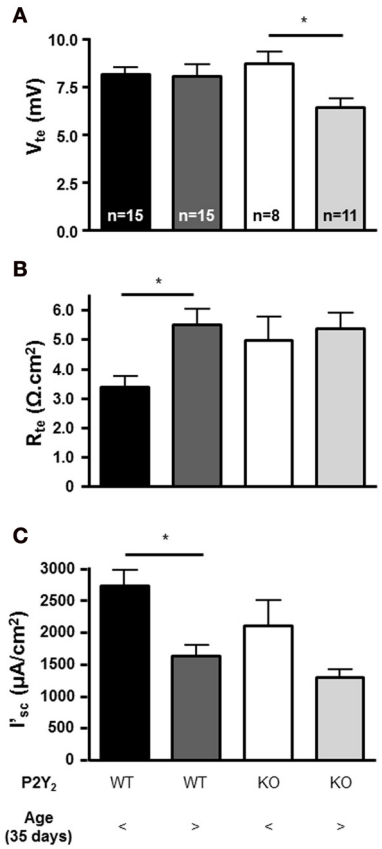


**FIGURE 3 | Ion transport parameters in mTALs (A: $V_{te}$, B: $R_{te}$, and C: $I'_{sc}$) from juvenile (25–35 days) and adult (35–55 days) P2Y$_2$ receptor WT and KO mice (*statistical significance $p < 0.05$).**

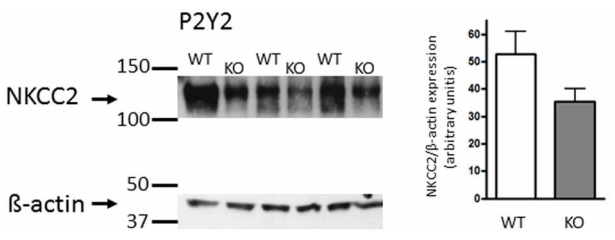


**FIGURE 4 | Western blot revealing similar expression of NKCC2 in P2Y$_2$ receptor WT and KO ($n = 3$ each) inner stripe of outer medulla kidney tissue.** Right side: The NKCC2 densitometric analysis is shown in reference to the equal dye loading control with β-actin.

kidney. Thus, these data are in contrast to those reporting up-regulation of NKCC2 protein expression in P2Y$_2$ KO mice (Rieg et al., 2007; Zhang et al., 2011).

### AVP-STIMULATED NaCl TRANSPORT IN mTALs FROME P2Y$_2$ RECEPTOR WT AND KO MICE

NaCl transport in the mTAL can be stimulated with hormones, such as AVP. This can be observed by a marked increase of $V_{te}$ values after AVP addition in isolated perfused mTALs (Wittner et al., 1988). In this series of experiments, we studied the AVP-stimulated activation of NaCl transport in mTALs from P2Y$_2$ receptor WT and KO mice to determine, if the lack of this purinergic receptor may have influenced the tissues functional responsiveness. An original experiment in a tubule from a WT mouse is shown in **Figure 5A**. In this original experiment, addition of basolateral AVP (10 nM) induced a slow (within minutes) increase of $V_{te}$ from +6 mV to +9.5 mV reaching stable maximum values after about 10 min. The mean $V_{te}$ increase in mTALs from WT mice was 2.5 ± 0.33 mV ($n = 11$), whereas the mean $V_{te}$ increase in mTALs from KO mice was 2.2 ± 0.38 mV ($n = 6$). In **Figure 5B**, this transport activation is depicted for the entire series and shows an increase in $I'_{sc}$ of 814 ± 86 μA/cm$^2$ in mTALs WT mice ($n = 11$). In mTALs from KO mice AVP induced similar increases of transport that amounted to 667 ± 117 μA/cm$^2$ ($n = 6$). These results indicate that the absence of the P2Y$_2$ receptor has no apparent effect on AVP-induced transport activation in mouse mTAL.

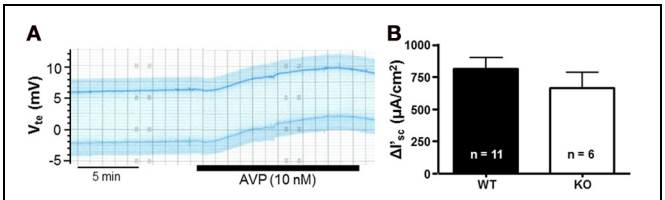


**FIGURE 5 | AVP-stimulated NaCl transport increases in P2Y$_2$ receptor WT and KO mTALs. (A)** An original recording of the AVP-induced (10 nM) NaCl transport stimulation, in a P2Y$_2$ receptor WT mouse mTAL. **(B)** AVP-stimulated NaCl transport was studied in P2Y$_2$ receptor WT ($n = 11$) and KO mice mTALs ($n = 6$). Summary of this results is presented as transport changes ($\Delta I'_{sc}$) in NaCl transport.

## DISCUSSION

The P2Y$_2$ receptor is important in the regulation of transepithelial transport in the distal convoluted tubule (DCT) and CD. The acute stimulation of the receptor inhibits both $Na^+$ and $Ca^{2+}$ reabsorption in the DCT (Koster et al., 1996), ENaC-dependent $Na^+$ reabsorption in the cortical CD (Lehrmann et al., 2002) and water reabsorption in the IMCD (Kishore et al., 2009). In the TAL, the P2Y$_2$ receptor was found in the apical and basolateral membranes, and stimulation of these receptors on either side triggered an increase of $[Ca^{2+}]_i$ (Paulais et al., 1995; Jensen et al., 2007). The flow-induced increase of $[Ca^{2+}]_i$ in mouse mTAL was found to be mediated by the activation of these apical and basolateral P2Y$_2$ receptors (Jensen et al., 2007). However, it is currently not understood, which biological role this receptor may serve in this nephron segment. Insulin is shown to enhance transepithelial transport in this segment in a $Ca^{2+}$-dependent fashion (Ito et al., 1995). Since the P2Y$_2$ receptor stimulation causes significant increments in $[Ca^{2+}]_i$, a straightforward hypothesis would be that the P2Y$_2$ receptor would stimulate transport. However, in a previous study, we did not find any effect of acute addition of basolateral UTP on the transepithelial electrical parameters in mouse mTAL (Marques et al., 2012). This finding is substantiated by another study, where the oxygen consumption was used as a measure of TAL transport activity, which also failed to show major

effects of $P2Y_2$ receptor activation on the transport (Silva and Garvin, 2009). Interestingly, two independent studies have shown a substantial increase in medullary but not cortical NKCC2 protein expression in the TAL of $P2Y_2$ receptor KO mice compared to WT (Rieg et al., 2007; Zhang et al., 2011, 2013). It was therefore likely to assume that NaCl absorption could be increased in mTAL of $P2Y_2$ receptor KO mice. This notion was corroborated by data showing an increased furosemide-induced $Na^+$ excretion in the $P2Y_2$ receptor KO mouse (Rieg et al., 2007; Zhang et al., 2013). In this study we directly addressed whether the NaCl transport rates are actually increased in the KO animals as compared to controls. Surprisingly, we found that the resting $I'_{sc}$ that quantitatively reflects the rate of NaCl absorption was not different in WT and KO mTALs. We therefore repeated the original protein expression experiments using the same NKCC2 antibody as used in the Rieg et al. paper (Rieg et al., 2007). Importantly, we cannot reproduce the results of an increased expression of NKCC2 protein in mTAL (Rieg et al., 2007; Zhang et al., 2011). Our experiments show no apparent difference in NKCC2 protein expression between $P2Y_2$ WT and KO mouse ISOM. A likely explanation for this discrepancy may be found in the different genetic backgrounds of the $P2Y_2$ knock-out mice used in the Rieg et al. and Zhang et al. paper as compared to our study. Whereas the former studies were conducted in mice on a C57BL/J6 and a B6D2 background our results were generated in mice bred on a mixed genetic (B6D2/SV129) background. In our study the unaltered NaCl transport rates therefore correlated with an apparently unaltered level of NKCC2 expression. The conclusion in our study that NKCC2 expression is not different has to be taken with some caution based on the limited number of 3 WT and 3 KO mice, respectively. When inspecting **Figure 4**, one could speculate that NKCC2 expression may be lower in our $P2Y_2$ KO mixed genetic background mouse strain. Seen in conjunction with the transport data, the I'sc was tentatively but not significantly smaller in adult KO mouse mTAL as compared to adult WT mTAL (**Figure 3C**). Eventually, it should be interesting but also a tour de force to revisit this question by also using the $P2Y_2$ receptor knock-out mouse back-crossed into the C57BL/J6 genetic background.

We extended this study to include measurement of transport after stimulation with AVP in WT vs. KO tubules. We found that AVP (10 nM) robustly stimulated NaCl absorption in all studied tubules independent of the presence of the $P2Y_2$ receptor. In summary, we conclude that mTAL tubules from $P2Y_2$ receptor deficient mice show intact resting and AVP-stimulated NaCl absorption, which is not different from WT controls.

Strictly speaking is it not correct to conclude that the transport properties of mTALs were similar in WT and KO mice. The transport of ions in the TAL also involves the paracellular route, which in this tubular segment is cation selective permitting transport of $Na^+$, $Ca^{2+}$, and $Mg^{2+}$. The present study indisputably shows that the driving force for the paracellular transport, i.e., the $V_{te}$ values, is similar in WT and KO mice. The permeability and the tightness of the paracellular shunt are critically dependent of the set of different claudins that are expressed in the epithelial barrier (Gunzel and Yu, 2013). In native respiratory epithelium, P2Y receptors have indeed been reported to acutely regulate of the paracellular shunt permeability (Poulsen et al., 2006). In this study, we were able to detect subtle ontogenetic differences in the $R_{te}$ values in between the $P2Y_2$ WT and KO mice, which may hint to a role of purinergic signaling in the regulation of the paracellular shunt. Moreover, did we detect lower $V_{te}$ values in adult KO mice as compared to juvenile, a finding, which was not seen in the wild type (WT). It is thus fair to speculate that subtle transport differences may indeed exist in the $P2Y_2$ receptor deficient mice.

In conclusion, the mTAL of $P2Y_2$ receptor KO mouse shows an essentially normal transport function in terms of electrogenic transepithelial NaCl absorption during resting or AVP-stimulation. The normal mTAL function in $P2Y_2$ KO mice of mixed genetic background correlated with an unaltered expression level of NKCC2.

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# $P2Y_2$ receptor deficiency aggravates chronic kidney disease progression

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Purinergic signaling is involved in a variety of physiological states. P2 receptors are mainly activated by adenosine triphosphate (ATP). Activation of specific P2Y receptor subtypes might influence progression of kidney disease. To investigate the *in vivo* effect of a particular P2 receptor subtype on chronic kidney disease progression, subtotal nephrectomy was performed on wild type (WT) and $P2Y_2$ receptor knockout (KO) mice. During the observational period of $56 \pm 2$ days, survival of KO mice was inferior compared to WT mice after SNX. Subtotal nephrectomy reduced creatinine clearance in both groups of mice, but the decrease was significantly more pronounced in KO compared to WT mice ($53.9 \pm 7.7$ vs. $84.3 \pm 8.7\mu$l/min at day 56). The KO mice also sustained a greater increase in systolic blood pressure after SNX compared to WT mice ($177 \pm 2$ vs. $156 \pm 7$ mmHg) and a 2.5-fold increase in albuminuria compared to WT. In addition, WT kidneys showed a significant increase in remnant kidney mass 56 days after SNX, but significant attenuation of hypertrophy in KO mice was observed. In line with the observed hypertrophy in WT SNX mice, a significant dose-dependent increase in DNA synthesis, a marker of proliferation, was present in cultured WT glomerular epithelial cells upon ATP stimulation. Markers for tissue damage (TGF-β1, PAI-1) and proinflammatory target genes (MCP1) were significantly upregulated in KO mice after SNX compared to WT SNX mice. In summary, deletion of the $P2Y_2$ receptor leads to greater renal injury after SNX compared to WT mice. Higher systolic blood pressure and inability of compensatory hypertrophy in KO mice are likely causes for the accelerated progression of chronic kidney disease.

**Keywords: purinercic receptors, $P2Y_2$ receptor, subtotal nephrectomy, chronic kidney disease, adenosine triphosphate (ATP)**

## INTRODUCTION

Nucleotides, predominantly adenosine triphosphate (ATP) and uridine triphosphate (UTP), are the ligands for P2 receptors. Intracellular ATP serves as the main source of energy and is crucial for almost all cell functions. Released from either neurons as neurotransmitters or in a paracrine fashion from a variety of cells and tissues, these nucleotides activate P2 receptors to trigger intracellular pathways (Ralevic and Burnstock, 1991; Burnstock, 1995; Oberhauser et al., 1999; Vonend et al., 2002; Ralevic and Burnstock, 2003; Vonend et al., 2003; Burnstock, 2006).

In almost every organsystem expression of several P2 receptor subtypes can be found (Turner et al., 2003; Burnstock and Knight, 2004). Activation of P2 receptors plays a role in transepithelial transport, proliferation, migration, platelet aggregation and has a potential influence on cardiovascular diseases. There are ligand-gated ion channel P2X receptors ($P2X_{1-7}$) and G-protein coupled P2Y receptors ($P2Y_{1,2,4,6,11-14}$) (Abbracchio et al., 2006; Burnstock, 2012). In the kidney in particular, the expression of multiple P2 receptors subtypes can be observed (Bailey et al., 2000; Vonend et al., 2003, 2005).

P2 receptors play a pivotal role within the sympathetic nervous system (Vonend et al., 2005; Gourine et al., 2009). As ATP serves as a co-transmitter from sympathetic nerve endings, activation of these P2 receptors occurs in sympathetic overactivity. It is well-known that patients with chronic kidney disease have an increased sympathetic activity (Rump et al., 2000; Vonend et al., 2002). In addition to ATP release from sympathetic nerve endings, endothelial dysfunction and increased shear stress contribute to a higher extracellular abundance of ATP, released from the endothelium in chronic kidney disease (Wan et al., 2008; Yamamoto et al., 2011).

There is evidence that P2Y receptor subtypes are important for maintaining renal function. In particular the $P2Y_2$ receptor is involved in glomerular cell proliferation and modulation of renal tubule function including natriuresis (Harada et al., 2000; Vonend et al., 2002; O'Mullane et al., 2009; Booth et al., 2012).

**Abbreviations:** ATP, adenosine triphosphate; BP, blood pressure; DNA, deoxyribonucleic acid; FCS, fetal calf serum; h, hour; KO, knock out; NaOH, sodium hydroxide; NS, non-significant; PBS, phosphate buffered saline; PCR, polymerase chain reaction; SNX, subtotal nephrectomy; UTP, uridine triphosphate; WT, wild type.

Due to the role of $P2Y_2$ receptors in vascular and tubule function, inflammation and renal cell proliferation, we hypothesize that activation of $P2Y_2$ receptors plays an important role in the pathophysiology and the progression of chronic kidney disease.

The model of subtotal nephrectomy (SNX) is characterized by increased blood pressure and sympathetic overactivity (Potthoff et al., 2008; Hoch et al., 2011). In this study, for the first time, using this model in $P2Y_2$ receptor knockout mice ($P2Y_2$-R KO), we assessed the effects of $P2Y_2$ receptor on the progression of chronic kidney disease.

## METHODS

### ANIMAL CARE

The $P2Y_2$-R KO mouse was generously provided by Dr B. H. Koller (University of North Carolina, Chapel Hill, USA). Mice were generated by homologous recombination in embryonic stem cell lines. Northern blot RNA analysis from kidney lysates confirmed the complete loss of $P2Y_2$-R expression in these mice (Homolya et al., 1999). The mice were originally on a B6D2 genetic background and back crossed with a SV129 mouse strain for 10 generations before starting the study. Mice homozygous for $P2Y_2$-R KO were used in this study. Genotyping was performed in each individual mouse confirming the presence of the disrupted $P2Y_2$-R gene (Homolya et al., 1999). Wild type (WT) littermates were used as controls. At the date of surgery, all mice were $62 \pm 3$ days old and weighed 21–26 g. Only male mice were used in this study. The investigations and surgery were performed in accordance with institutional guidelines. The animals were housed in type III Makrolon polycarbonate cages at 45% humidity, 20–22°C temperature and a 12 h day-night-cycle with free access to water and food. Standard food was *Altromin* (*Altromin 1314*: 20% protein, 0.4% NaCl, Lage/Lippe, Germany).

### SUBTOTAL NEPHRECTOMY AND SHAM SURGERY

Sham surgery or subtotal nephrectomy (SNX) was performed at $62 \pm 3$ days of age. WT mice and $P2Y_2$-R KO mice were randomly allocated to either sham surgery or SNX. A Kaplan-Meier survival curve was generated from the survival data in the sham-surgery and SNX groups.

The mice were anesthetized with ketamin and xylazine by intraperitoneal injection (0.168 mg/g and 8 mg/g bodyweight, respectively). Subtotal nephrectomy was performed as previously described (Hoch et al., 2011). In brief, both kidneys were exposed by dorsal incision. The right kidney was removed completely. Two-thirds of the functional tissue from the left kidney was removed by ligation of the upper and lower renal poles leading to a subtotal reduction of functional renal tissue. Sham surgery was performed only by skin and abdominal muscle incisions.

### PREPARATION OF URINARY, SERUM AND TISSUE SAMPLES

The mice were sacrificed $56 \pm 2$ days after surgery. Blood samples were gathered by retro orbital vein puncture on day $28 \pm 2$ and on the day of sacrifice. Serum samples were obtained from centrifuged blood samples. Twenty-four hour-urine was collected on day 0, $28 \pm 2$ and on the day of sacrifice using metabolic cages (*Techniplast*, Italy) for urinary albumin, creatinine and urea measurement.

On day $56 \pm 2$, mice were sacrificed, all organs were perfused with ice-cold PBS from the left ventricle and SNX-kidneys and sham-surgery kidneys were removed and embedded in paraffin. Tissue samples for RNA expression analysis were removed from the kidneys prior paraffin embedding.

### SYSTOLIC BLOOD PRESSURE MEASUREMENT

Systolic blood pressure (BP) was measured non-invasively by tail-cuff sphygmomanometer using a *BP-98A* device (*Softron*, Japan). Mice were trained for 4 days prior to evaluation of BP. BP was measured on day 0, $28 \pm 2$ and $56 \pm 2$ prior to sacrifice.

### KIDNEY HYPERTROPHY

Kidney weight was evaluated on the day of surgery (SNX groups) and at the end of study. The remnant kidney weight directly after SNX was estimated by calculating the kidney weight of right kidney minus the weight of the removed tissue from the left kidney. Change in kidney weight from the day of surgery (SNX) until sacrifice was regarded as an index of kidney hypertrophy.

### QUANTIFICATION OF SERUM AND URINE ALBUMIN AND UREA

Measurement of creatinine levels in serum and urine was performed using an enzymatic standard test supplied by *Labor&Technik*, Germany. The analysis was performed following the manufacturer's protocol. Serum and urinary urea and serum albumin were measured using standard tests supplied by *Randox*, Germany. The methods were followed according to the manufacturer's protocol. Urinary albumin levels were measured using the *Albuwell M Kit* (*Exocell*, USA). Urine albumin-to-creatinine ratio (UACR) was calculated for all urine samples (24-h urine collection samples using metabolic cages). Creatinine-clearance was calculated from urine volume, urinary- and serum-creatinine values.

### PROLIFERATION ASSAY

[$^3$H]-thymidine assay was used to estimate proliferative activity in cells (Vonend et al., 2003). WT human glomerular epithelial cells were obtained from *Cambrex Corporation*, USA. For proliferation assays, cell passages ranging from 5 to 12 were used. Cells were housed at 37°C and 5% $CO_2$. Cells were distributed on a 96-well-plate (150.000 cells/well) and grown for 3 days in cell culture medium containing 5% fetal calf serum until 70–80% confluence. At day 3, cells were starved for 24 h by reducing fetal calf serum content to 0.5%. ATP was added to resting cells (4 wells for each concentration) for 24 h (0–100 μM).

The cells were incubated with [$^3$H]-thymidine (1 μCi/ml) during the last 6 h. Cells were washed three times with phosphate buffered saline, twice with ice-cold 10% trichloroacetic acid, and the fixed cellular material was solubilized in 1 ml 0.5 m NaOH for 2 h and mixed with 4 ml scintillation fluid (*Ultima-Gold, Canberra Packard*, Frankfurt, Germany) to measure the amount of radioactivity. Data are expressed as the mean ratio of radioactivity present in the 4 wells with identical concentration divided by control (0 μM ATP) values (% of control).

### RNA EXTRACTION AND REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

WT human glomerular epithelial cells were homogenized and total RNA was isolated with *Trizol-Reagent* (*Invitrogen*, Germany). Following DNA digestion (*Rnase free DNase/Invitrogen*), mRNA was isolated from total RNA with the *PolyATract* mRNA isolation system (*Promega*). cDNA was synthesized according to suppliers' protocol using oligo-dt primer and the reverse transcriptase superscript (*Invitrogen*). The amplification was performed with specific primers for $P2Y_{1,2,4,6,11}$ receptor subtypes with 10% of the first strand cDNA and 1.5 units of *Platinum Taq-Polymerase* (*Invitrogen*) in a volume of 50 µl. After 5 min at 95°C followed by 35 cycles consisting of 1 min at 95°C, 1 min at 52–58°C, 1 min at 72°C and for termination 8 min at 72°C, 10 µl of the reaction products were analyzed on a 1.5% agarose gel containing ethidium bromide.

#### *Quantitative real time PCR (qPCR)*

SNX kidney samples were used to analyze relative expression levels for transforming growth factor beta 1 (TGF-β1), plasminogen activator inhibitor 1 (PAI-1), nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB), monocyte chemoattractant protein 1 (MCP1), cyclooxygenase 1 (COX1) and prostaglandin E synthase 1 (PGES1).

After homogenization of tissue with a *Tissue Ruptor* (*Qiagen*, Germany), total RNA was isolated using a *RNA Micro Kit* (*Qiagen*, Germany) according to the manufacturer's instructions. Quantitative real time PCR was performed with an *ABI PRISM 7300* (*Applied Biosystem*, Germany) and the *SYBR Green master mix* (*Qiagen*, Germany). The PCR reaction was performed in a total volume of 20 µl with 1 µl cDNA corresponding to 50 ng RNA as template.

The two-step PCR conditions were 2 min at 50°C, 15 min at 95°C, followed by 40 cycles (denaturation at 94°C for 15 s; annealing at 55°C for 30 s and extension at 72°C for 34 s). Experiments were performed in triplicate. 18S ribosomal RNA was chosen as the endogenous control (housekeeping gene). The levels of targeted genes were normalized to 18S rRNA expression and analyzed by REST 2008 V2.0.7 software.

The following *Taqman assays* were used: 18S (Mm039 28990_g1), PAI-1 (Mm00435860_m1), TGF-β1 (Mm011788 20_m1), NFκB (Mm01297400_m1), MCP1 (Mm00441242_m1), COX1 (Mm0047214_m1), PGES1 (Mm00452105_m1).

#### *Statistical analysis*

All data are expressed as means ± standard error of mean (SEM). *n* applies to the number of mice/tissue samples used in each group. Datasets were analyzed using SPSS 19.0 software. Multiple comparison of more than two groups was performed by One-Way ANOVA followed by Bonferroni's multiple comparison *post-hoc* test, where applicable. Values of $p < 0.05$ were considered as significant. If applicable, a higher level of statistical significance is stated ($p < 0.01$, $p < 0.001$).

qPCR data was statistically analyzed using REST 2008 V2.0.7 software.

Comparison of survival was calculated using the logrank test (Mantel-Cox test).

## RESULTS

### SURVIVAL

Renal function in the model of subtotal nephrectomy is the major contributing factor determining animal survival. To evaluate a general role of $P2Y_2$ receptor in the progression of chronic kidney disease, we registered the survival of sham surgery and SNX mice.

Observational period for all mice was 56 ± 2 days after surgery. Overall survival was 100% in both sham surgery groups (**Figure 1**). During the observation period, 1 out of 9 WT mice died after SNX compared to 7 out of 19 in the $P2Y_2$-R KO group (survival rate WT vs. $P2Y_2$-R KO: 88.9 vs. 63.1%) (**Figure 1**). Comparing the survival with the WT sham surgery group, survival was not significantly different in WT mice (survival rate 88.9 vs. 100%, $p$ = NS). However, survival was significantly reduced in $P2Y_2$-R KO mice which underwent SNX compared to KO sham surgery mice (survival rate 63.1 vs. 100%, $p < 0.01$, $X^2 = 6.658$, $df = 1$).

### SYSTOLIC BLOOD PRESSURE

Increase in blood pressure occurs in chronic kidney disease. It correlates well with the stage of chronic kidney disease. A high blood pressure also contributes to the progression of kidney disease.

Systolic blood pressure was measured at three different time points (day 0, 28 ± 2, 56 ± 2) using an automated tail-cuff sphygmomanometer. At baseline before surgery (day 0), all four groups showed no difference in systolic blood pressure. Neither sham surgery groups showed a rise in systolic blood pressure during the observation period. In contrast, the SNX groups had a significant elevation of systolic blood pressure (day 28 and 56). In addition, systolic blood pressure was significantly higher in $P2Y_2$-R

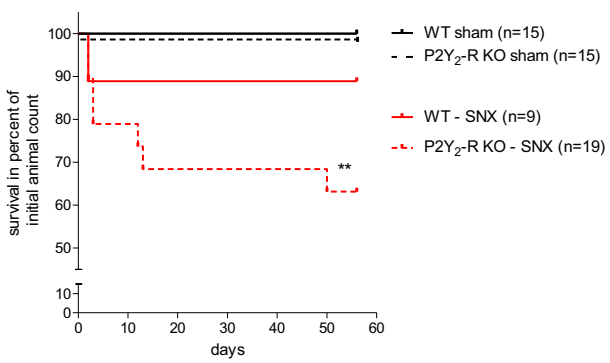


**FIGURE 1 | Kaplan-Meyer-survival curve of all groups: sham surgery groups showed 100% survival after 56 ± 2 days.** In the SNX surgery groups, more WT mice survived during the observation period than $P2Y_2$-R KO mice (88.9 vs. 63.1%). Comparing this data to the sham surgery groups shows that subtotal nephrectomized $P2Y_2$-R KO mice had a significantly reduced survival (**$p < 0.01$) whereas subtotal nephrectomized WT mice showed no significantly reduced survival compared to sham surgery WT mice ($p$ = NS).

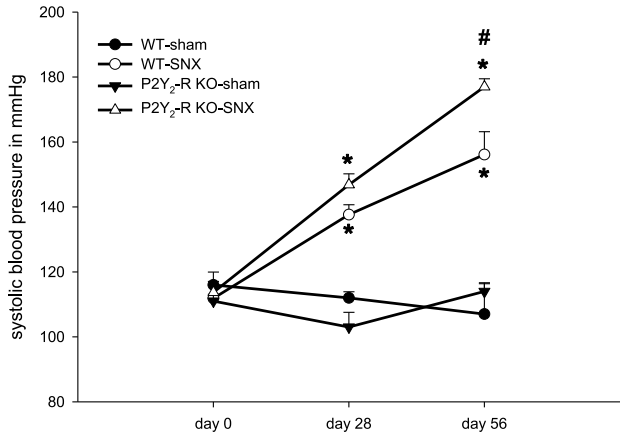


**FIGURE 2 | Systolic blood pressure on day 0 (before surgery), day 28 and day 56 for each group: Sham surgery groups did not show any significant change in systolic blood pressure.** SNX groups showed a significant rise in systolic blood pressure on day 28 and 56 compared to day 0 ($^*p < 0.05$). At day 56, systolic blood pressure was significantly higher in P2Y$_2$-R KO SNX compared to WT SNX mice ($^\#p < 0.05$) (mean ± SEM).

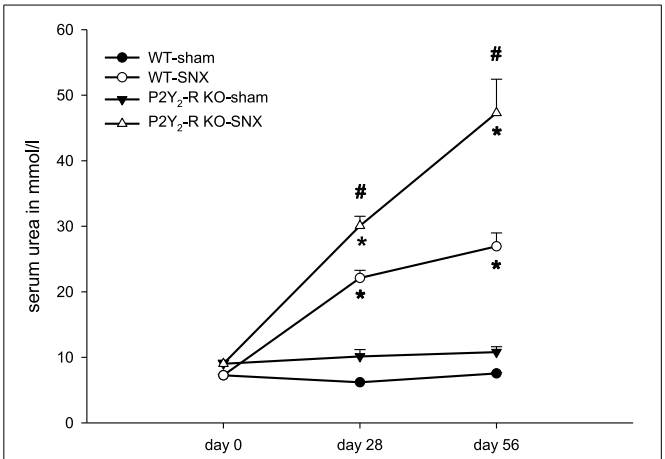


**FIGURE 3 | Serum urea on day 0 (before surgery), day 28 and day 56 for each group: sham surgery groups did not show any significant change in serum urea levels.** SNX groups showed a significant rise in serum urea levels on day 28 and 56 compared to day 0 ($^*p < 0.05$). At day 28 and 56, serum urea levels were significantly higher in P2Y$_2$-R KO SNX compared to WT SNX mice ($^\#p < 0.05$) (mean ± SEM).

KO mice after SNX compared to WT SNX mice (day 56, WT vs. P2Y$_2$-R KO: 156 ± 7 vs. 177 ± 2 mmHg) (**Figure 2**).

## RENAL FUNCTION

In order to evaluate renal function, measurement of renal retention parameters and endogenous creatinine clearance was performed in all groups.

During the observation period (day 28 ± 2, 56 ± 2) there was a significant increase in serum urea in both SNX groups compared to baseline (day 0). There was no significant change of serum urea in sham surgery groups (**Figure 3**). At day 56 ± 2, serum urea was 3.6-fold higher in WT SNX compared to WT sham surgery mice and 4.4-fold higher in P2Y$_2$-R KO SNX compared to KO sham surgery mice. Accordingly, serum urea was significantly higher in P2Y$_2$-R KO SNX mice compared to WT SNX mice. This is in line with the observed creatinine clearance at day 56: creatinine clearance was significantly higher in WT SNX mice compared to P2Y$_2$-R KO SNX mice.

All assessed physiological parameters are summarized in **Table 1**.

## ALBUMINURIA

Mice that underwent subtotal nephrectomy are characterized by the progression of chronic kidney disease. The histologic equivalent in tissue injury is the onset and progression of glomerulosclerosis and is accompanied by albuminuria.

Albuminuria, estimated by UACR, was almost absent in sham surgery groups at all time points. In contrast, compared to baseline, there was a significant rise in albuminuria in the SNX groups (**Figure 4**). In addition, the rise in UACR was more pronounced in P2Y$_2$-R KO SNX mice. After 56 days, the UACR was more than 2.5-fold higher in KO SNX compared to WT SNX mice (**Table 1**, **Figure 4**).

Marked differences in glomerulosclerosis between P2Y$_2$-R KO SNX and WT SNX mice could not be detected (data not shown).

## GENE EXPRESSION

Kidney cortex tissue from SNX mice at day 56 was assessed for the expression of specific genes which are associated with inflammatory response and which are related to the function of P2Y$_2$-receptor in the immune system. In addition, gene expression was assessed for genes related to kidney injury.

In qPCR analysis, wecompared the expression level of specific target genes in P2Y$_2$-R KO SNX mice to the expression in WT SNX mice. qPCR results were normalized to 18S ribosomal RNA expression. Comparison revealed a significant increase in expression ratio for TGF-β1, PAI-1 and MCP1 in P2Y$_2$-R KO SNX mice (**Table 2**, **Figure 5**).

## HYPERTROPHY OF KIDNEY TISSUE

Loss of kidney tissue results in the hypertrophy of the healthy remnant kidney, partially compensating for the loss of functional tissue. Since the P2Y$_2$-receptor is involved in cell proliferation, we assessed the increase in kidney weight.

In SNX mice, at day 0, total kidney weight (two kidneys) was reduced to a similar extent in WT and KO mice (WT: −69.3 ± 1.35%; P2Y$_2$-R KO: −68.7 ± 1.1%).

At day 56, SNX kidney weight was measured and compared to the estimated weight at day 0. Comparison of the SNX groups showed a significant difference at day 56 (**Figure 6A**). Remnant kidney weight in WT SNX mice increased significantly during the observational period (day 56, 150 ± 6% compared to day 0, $p < 0.05$). In contrast, SNX in P2Y$_2$-R KO mice led to a minor, non-significant increase in remnant kidney weight (day 56, 113 ± 6% compared to day 0, $p =$ NS).

**Table 1 | Serum and urine parameters from all groups after 56 ± 2 days.**

| Parameter | Units (SI) | WT-SNX | $P2Y_2$–R KO-SNX | WT-sham | $P2Y_2$-R KO-sham |
|---|---|---|---|---|---|
| Serum creatinine | μmol/l | 19.5 ± 3.5 | 24.8 ± 2.7 | 9.7 ± 0.7 | 4.4 ± 0.4 |
| Serum urea* | mmol/l | 27.1 ± 2.1 | 47.4 ± 5.2 | 7.5 ± 0.1 | 10.8 ± 0.8 |
| Urine volume (24 h) | ml | 1.8 ± 0.2 | 2.6 ± 0.4 | 0.4 ± 0.1 | 0.2 ± 0.1 |
| Creatinine clearance* | μl/min | 84 ± 9 | 54 ± 8 | 114 ± 14 | 112 ± 29 |
| Urine albumin-to-creatinine ratio* | mg/mg | 2.0 ± 0.5 | 5.1 ± 0.8 | 0.02 ± 0.00 | 0.05 ± 0.02 |

*Parameters marked with "*" show a significant difference ($p < 0.05$) comparing WT and $P2Y_2$-R KO SNX groups (mean ± SEM).*

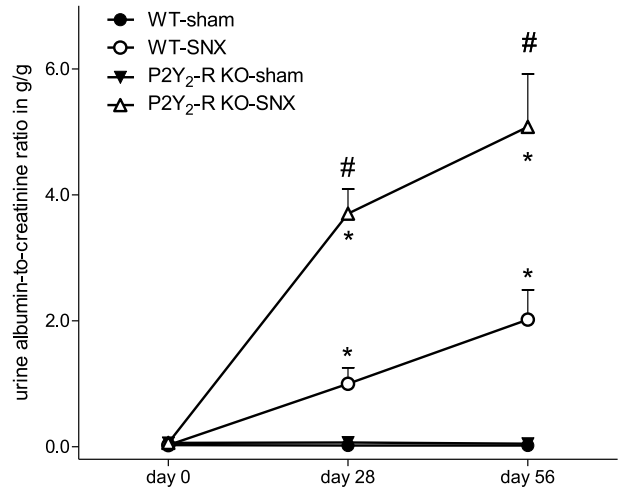


**FIGURE 4 | During the observation period, UACR significantly increased in the SNX groups compared to the sham surgery groups (day 28 and 56, $*p < 0.05$).** In addition, UACR was significantly higher in $P2Y_2$-R KO SNX compared to WT SNX ($^{\#}p < 0.05$) (mean ± SEM).

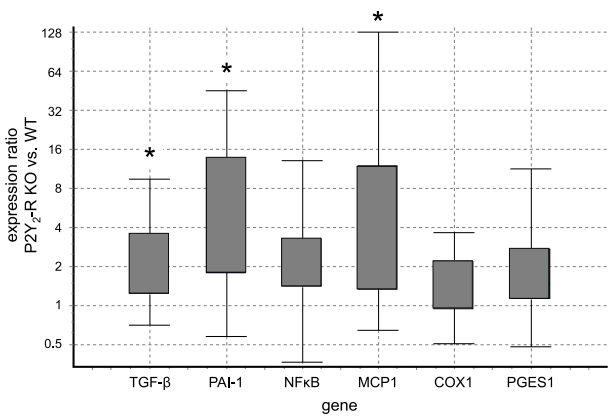


**FIGURE 5 | Gene expression ratio of target genes indicating tissue damage and inflammation in WT SNX vs. $P2Y_2$-R KO SNX mice assessed by qPCR: positive values indicate fold-change in $P2Y_2$-R KO SNX over WT SNX expression levels.** TGF-β1, PAI-1 and MCP1 showed a significantly higher expression in $P2Y_2$-R KO SNX than WT SNX mice ($*p < 0.05$). (Boxes represent the interquartile range. Whiskers represent the minimum and maximum observations. Analysis was performed with REST 2008 V2.0.7 software).

**Table 2 | Relative gene expression normalized to 18S expression levels comparing WT and $P2Y_2$-R KO SNX mice.**

| Gene | Expression | *SE* | *p*-value | Result |
|---|---|---|---|---|
| 18S | 1.000 | | | |
| TGF-β1 | 2.191 | 0.999–4.524 | 0.047 | *(UP) |
| PAI-1 | 4.332 | 1.369–22.287 | 0.010 | *(UP) |
| NF-κB | 2.067 | 1.096–4.861 | 0.070 | |
| MCP1 | 4.187 | 1.102–32.674 | 0.024 | *(UP) |
| COX1 | 1.443 | 0.762–2.621 | 0.165 | |
| PGSE1 | 1.778 | 0.858–3.297 | 0.122 | |

*Values higher than 1 indicate higher expression in $P2Y_2$-R KO mice. Data analysis was performed using REST 2008 V2.0.7 software.*

*$*p < 0.05$.*

### P2Y RECEPTOR EXPRESSION AND PROLIFERATION ASSAY IN HUMAN GLOMERULAR EPITHELIAL CELLS

To illustrate the proliferative effect explaining the observed hypertrophy in the WT mice with functional $P2Y_2$ receptor after SNX, we assessed P2Y-receptor status in glomerular epithelial cells and the measured insertion of [$^3$H]-thymidine upon ATP stimulation in order to estimate DNA synthesis which is an indicator of cell proliferation. RT-PCR with specific primers for $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ receptors confimed the expression of these receptor subtypes in WT human glomerular epithelial cells (data not shown).

Human glomerular epithelial cells were stimulated with increasing doses of ATP (1–100 μM) in an *ex vivo* assay. Compared to non-stimulated controls, ATP induced a significant, dose dependent increase in DNA synthesis up to 180.1 ± 20.5% (**Figure 6B**).

## DISCUSSION

Chronic kidney disease is a major health burden and has a major impact on cardiovascular morbidity and mortality (Matsushita et al., 2010). Progression of kidney disease is vastly dependent on two factors: inflammatory response and fibrotic remodeling. The progression of chronic kidney disease is linked to a progressive scarring of glomeruli and subsequent development of tubulointerstitial fibrosis (Ma and Fogo, 2003). This is accompanied by albuminuria, an independent predictor for cardiovascular events (Matsushita et al., 2010). Diseased kidneys activate

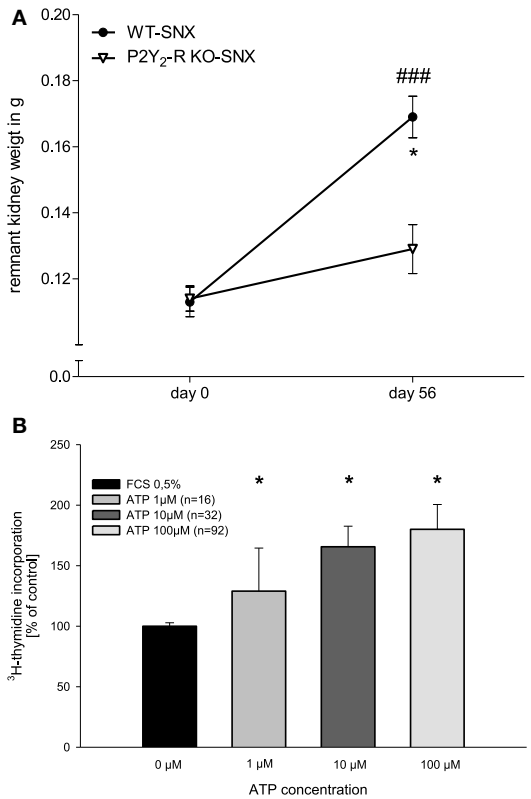


**FIGURE 6 | (A)** Remnant kidney weight in SNX groups at day 0 and day 56 ($^{*}p < 0.05$ compared to day 0; $^{\#\#\#}p < 0.001$ WT SNX vs. $P2Y_2$-R KO SNX). **(B)** [$^3$H]-thymidine insertion in WT human glomerular epithelial cells in dependence of ATP stimulation (0–100 µM) ($^{*}p < 0.05$ compared to 0 µM ATP).

afferent sensory nerves, which project to the sympathetic nuclei in the central nervous system. This leads to increased sympathetic activity in organs like the heart, blood vessels but also kidneys (Rump et al., 2000). In chronic kidney disease, sympathetic overactivity, mechanical stress and inflammatory responses from various cell types within the kidney induce the release of extracellular ATP (Brecht et al., 1976; Gerasimovskaya et al., 2002; Oberg et al., 2004; Kotanko, 2006; Suliman and Stenvinkel, 2008; Masuo et al., 2010). This excess of extracellular ATP likely leads to activation of various P2 receptor subtypes. However, it remains unclear whether activation of P2 receptors modulates the outcome in chronic kidney disease. A P2 receptor subtype, which has been associated with nephron function and renal cell proliferation, is the $P2Y_2$ receptor (Harada et al., 2000; Vonend et al., 2003; Pochynyuk et al., 2008). This study elucidates the $P2Y_2$ receptor's role in a model of chronic kidney disease.

The decreased survival in $P2Y_2$-R KO mice after SNX is one of the most striking findings, which indicates a general role of the $P2Y_2$ receptor in chronic kidney disease. To evaluate the underlying cause of this difference, we assessed blood pressure, physiological parameters and histology throughout the study.

Chronic kidney disease is associated with increased blood pressure due to increased sympathetic activity and activation of the renin angiotensin system (Mailloux, 2001; Vonend et al., 2008; U.S. Renal Data System, 2011). It has been shown previously that mice develop hypertension after SNX (Ma and Fogo, 2003). In our study, sham surgery and SNX mice showed a similar systolic blood pressure at baseline. In sham surgery mice, systolic blood pressure remained stable during the observation period. In the SNX groups, blood pressure significantly increased over time. Interestingly, $P2Y_2$-R KO mice developed a significantly higher blood pressure compared to WT SNX mice.

$P2Y_2$ receptor activation is a crucial element in a variety of physiological mechanisms that influence blood pressure. These mechanisms include endothelium function and vasoconstriction in the vasculature, modulation of inflammatory response of immune cells and modulation of epithelial function. The $P2Y_2$ receptor is expressed along the nephron and plays an important role in tubule epithelial function (Unwin et al., 2003). In the distal nephron, Ponchynyuk et al. showed that $P2Y_2$ receptor activation decreases the open probability of the epithelial sodium channel (ENaC) leading to increased natriuresis and water excretion (Pochynyuk et al., 2008, 2010). Rieg et al. then demonstrated that $P2Y_2$ receptor activation causes an immediate drop in blood pressure and increases the fractional sodium excretion. In $P2Y_2$-R KO mice, sodium excretion was reduced compared to WT mice. This also caused a decrease in free water excretion. Rieg et al. could not observe any difference in glomerular filtration rate at baseline or upon stimulation of $P2Y_2$ receptor, nor could they detect any difference in mean blood pressure at baseline comparing WT and KO mice (Rieg et al., 2011). In this study, Rieg et al. used $P2Y_2$-R KO mice on a SV129 background, using mice from the same source as mice used in our study. The results are consistent with our findings, which show no significant difference in systolic blood pressure of $P2Y_2$-R KO compared to WT mice at baseline. However, in an earlier study, using mice on a C57Bl/6 background, significantly higher baseline systolic blood pressure in these KO mice was observed (Rieg et al., 2007). Rieg et al. assumed that the genetic background might explain this difference. Even though basal systolic blood pressure did not differ in our study, in $P2Y_2$-R KO SNX mice, systolic blood pressure was significantly higher at the end of the observational period compared to WT SNX mice. This might be explained by the lack of $P2Y_2$ receptor activation and its effects on natriuresis and water excretion.

Another factor that might contribute to the elevated blood pressure in $P2Y_2$-R KO mice is the impact of endothelial vasodilatation upon $P2Y_2$ receptor activation. This response is driven by endothelium-derived hyperpolarizing factor and nitric oxide production (Marrelli, 2001; Buvinic et al., 2002; da Silva et al., 2009; Raqeeb et al., 2011). Impaired endothelial vasorelaxation is a hallmark of endothelial dysfunction which occurs in the setting of chronic kidney disease. Therefore, it is feasible that $P2Y_2$ receptor deficiency in chronic kidney disease contributes to the impaired endothelial function resulting in elevated systolic blood pressure as observed in our study.

In our study, progression of kidney disease after SNX was more pronounced in $P2Y_2$-R KO mice than in WT controls. Creatinine clearance was significantly reduced, and both albuminuria and serum urea were significantly higher in $P2Y_2$-R KO mice after SNX. Since increased blood pressure accelerates the decline of kidney function and increases proteinuria, the difference in systolic blood pressure is likely to be one of the contributing factors for the observed outcome (Young et al., 2002; Matsushita et al., 2010).

However, if increased systolic blood pressure was the sole cause for worse outcome in the $P2Y_2$ receptor KO group, a marked difference in glomerulosclerosis should be present in these mice. Histological assessment of the kidneys could not reveal any difference in glomerulosclerosis (data not shown). This finding suggests that mechanisms other than increased blood pressure contribute to the marked difference in renal function between $P2Y_2$-R KO and WT SNX mice.

As stated above, progression of kidney disease is also dependent on the inflammatory response to kidney injury. It has been shown that $P2Y_2$ receptors have a direct impact on inflammation and proliferation.

In order to delineate mechanisms other than increased systolic blood pressure, mRNA expression was analyzed at day 56 after SNX. Markers indicating increased maladaptive changes were significantly increased in $P2Y_2$-R KO SNX mice. TGF-β1 and PAI-1 showed a significant increase at day 56 after SNX in the $P2Y_2$-R KO group (**Figure 5**). TGF-β1 not only reflects increased tissue damage but can act as a strong inducer of inflammation (Lan and Chung, 2012).

The progressive decline in kidney function and the differences between WT and KO SNX mice in our study, might be associated with modulation of the inflammatory response (Suliman and Stenvinkel, 2008). $P2Y_2$ receptors are among the P2 receptors that show markedly increased expression in macrophages after induction of inflammatory response indicating a potential role of the $P2Y_2$ receptor in the regulation of inflammation (Luttikhuizen et al., 2004). Others showed that $P2Y_2$ receptor activation increases the expression of cyclooxygenase-1/2 and release of prostaglandin $E_2$ (PGE2) which inhibits the release of pro-inflammatory cytokines from macrophages and T-lymphocytes (Welch et al., 2003; Degagne et al., 2009; Kalinski, 2012). Furthermore, administration of the $P2Y_2$ receptor agonist UTP greatly reduced the infiltration of neutrophils after myocardial infarction, an effect which was abolished in $P2Y_2$-R KO mice (Cohen et al., 2011). Therefore, activation of $P2Y_2$ receptors in an inflammatory setup can limit the extent of inflammatory response. Since a sustained inflammation contributes to the progression of chronic kidney disease, deficiency of $P2Y_2$ receptors might be detrimental for kidney function.

In order to assess effects on inflammation, we analyzed mRNA expression of NfκB, MCP1, PGSE1, and COX1 in SNX kidney cortex samples. NfκB, PGSE1, and COX1 showed a statistically non-significant tendency toward higher expression in $P2Y_2$-R KO SNX mice. It is well-known that in this model, differences in mRNA levels in late observational periods tend to be subtle, since a steady state with slow progression of kidney disease has been established (Rumberger et al., 2007). This might partially explain the non-significant differences in some of the inflammatory markers in qPCR assessment. Interestingly, at day 56, MCP1 expression was higher in $P2Y_2$-R KO SNX compared to WT SNX mice, which indicates an increased inflammatory response in the KO SNX group. Since sustained inflammation accelerates the progression of chronic kidney disease, this might partially explain the worse outcome in $P2X_2$-R KO mice after SNX.

In addition to modulating inflammation, P2Y receptors are also involved in the regulation of proliferation and hypertrophy. Vonend et al. showed that P2Y receptor activation promotes proliferation of human mesangial cells (Vonend et al., 2003). In our study, WT SNX mice showed a significant increase in remnant kidney weight whereas remnant kidneys in $P2Y_2$-R KO SNX mice failed to show a significant increase in renal mass. These adaptive processes are sustained by a proliferation of different renal cell types, leading to an increase in glomerular size and increase in tubular tissue, which can partially compensate for the reduced GFR and therefore counteract the decline in kidney function (Waldherr and Gretz, 1988; Hoy et al., 2003; Alperovich et al., 2004). In the absence of the $P2Y_2$ receptor, these adaptive mechanisms seem to be abolished and could partially explain the lower creatinine clearance in the KO SNX mice.

The balance between proliferation and apoptosis depends upon the concentration of extracellular ATP and on the subtype of stimulated P2-receptors (Harada et al., 2000). Extracellular ATP leads to a dose-dependent proliferation of mesangial cells in a manner that is $P2Y_2$ receptor dependent (Harada et al., 2000). In our study, deficiency of $P2Y_2$ receptor abolished most of the remnant kidney hypertrophy following SNX. In addition, albuminuria was significantly higher in the $P2Y_2$-R KO group. To illustrate the proliferative effect explaining the observed hypertrophy in the WT mice with functional $P2Y_2$ receptor after SNX, we observed a dose dependent increase in cell proliferation by ATP stimulation in glomerular epithelial cells. These cells are essential for the structure of the glomerular filtration barrier and their integrity is important to prevent albuminuria. The lack of compensatory hypertrophy might contribute to the worse outcome after subtotal nephrectomy in the $P2Y_2$ receptor deficient group.

In summary, $P2Y_2$ receptor deficiency is detrimental in chronic kidney disease. This is most likely caused by increased elevation of systolic blood pressure. The $P2Y_2$ receptor also modulates inflammation and proliferation which may also contribute to the progression of chronic kidney disease in our model. We conclude that the $P2Y_2$ receptor is crucial for blood pressure regulation and response to renal injury after induction of chronic kidney disease. Therefore, the $P2Y_2$ receptor stimulation might be a potential therapeutic target.

## ACKNOWLEDGMENTS

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# Role of extracellular ATP and P2 receptor signaling in regulating renal cyst growth and interstitial inflammation in polycystic kidney disease

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Polycystic kidney diseases (PKD) are a group of inherited ciliopathies in which the formation and growth of multiple cysts derived from the distal nephron and collecting duct leads to the disruption of normal kidney architecture, chronic interstitial inflammation/fibrosis and hypertension. Kidney failure is the most life-threatening complication of PKD, and is the consequence of cyst expansion, renal interstitial disease and loss of normal kidney tissue. Over the last decade, accumulating evidence suggests that the autocrine and paracrine effects of ATP (through its receptor family P2X and P2Y), could be detrimental for the progression of PKD. (2009). *In vitro,* ATP-P2 signaling promotes cystic epithelial cell proliferation, chloride-driven fluid secretion and apoptosis. Furthermore, dysfunction of the polycystin signal transduction pathways promotes the secretagogue activity of extracellular ATP by activating a calcium-activated chloride channel *via* purinergic receptors. Finally, ATP is a danger signal and could potentially contribute to interstitial inflammation associated with PKD. These data suggest that ATP-P2 signaling worsens the progression of cyst enlargement and interstitial inflammation in PKD.

**Keywords: polycystic kidney disease, cyst, interstitial inflammation, purinergic, ATP**

## INTRODUCTION

Polycystic kidney diseases (PKD) are a group of inherited ciliopathies in which the formation and growth of multiple fluid-filled renal cysts leads to disruption of normal kidney architecture, chronic renal interstitial inflammation and fibrosis, and hypertension (Harris and Torres, 2009). Kidney failure is the most serious and life-threatening complication of PKD (Rangan et al., 2005), and is the consequence of cyst expansion, renal interstitial disease and loss of normal kidney tissue (Grantham et al., 2011). Cyst growth in PKD is due to a combination of proliferation of cyst-lining epithelial cells of the distal nephron and collecting ducts, chloride-driven fluid secretion and extracellular matrix defects (Grantham et al., 1989). Although there is no treatment to currently prevent kidney failure due to PKD, rapid progress is being made (Harris and Torres, 2009). For example, in 2012, a 3-year Phase 3 clinical trial using a small molecule inhibitor (tolvaptan, a vasopressin type 2 receptor antagonist) successfully attenuated the rate of kidney enlargement in humans with PKD (Kelsey, 2013). The premise for the use of vasopressin receptor antagonism was based on preclinical data showing that vasopressin altered in intracellular purinergic (cAMP) signaling in PKD (Wang et al., 2005).

It has been hypothesized that a multi-drug approach, targeting cyst growth and interstitial inflammation, will be needed to effectively prevent kidney failure in PKD (Aguiari et al., 2013), and therefore further therapies are needed (Chang and Ong, 2013). For over a decade, it has been postulated that the autocrine and paracrine effects of extracellular nucleotides and their metabolites could be detrimental for the progression of PKD, and that strategies to attenuate this activation using inhibitors of nucleotide release, nucleotide scavengers or nucleotide antagonists could be useful for the therapy of PKD (Wilson et al., 1999; Schwiebert, 2001; Leipziger, 2003; Hillman et al., 2005; Hovater et al., 2008; Xu et al., 2009). Currently, knowledge regarding the role of extracellular nucleotides in the pathogenesis of kidney failure due to PKD remains in the early phase of clinical translation. This is highlighted by the paucity of published articles, as a PubMed search performed on the 29th April 2013, using the terms "ATP and polycystic kidney disease," revealed 42 publications, only 15 of which were original scientific articles and 6 were review articles that were directly relevant to this field of research. Nevertheless, significant insights have been gained from this limited information to support that extracellular nucleotide signaling is worthy of further pursuit in PKD. In fact, it has been hypothesized that this approach might act synergistically with vasopressin receptor antagonism (Buchholz et al., 2011; Luft, 2011). Extracellular nucleotides and metabolites can be classified according to the base from which they are derived and the number of associated number of phosphate groups (adenosine-derived; ATP, ADP; AMP; guanosine-derived: GTP, GDP, GMP; cytidine-derived: CTP, CDP, CMP; 5-methyluridine- and uridine-derived). The majority of the available data regarding extracellular nucleotides in PKD is based on ATP-P2 receptor signaling. Therefore, this mini-review will focus primarily on the role of ATP and its P2 receptors, in the pathogenesis and progression of cyst enlargement and interstitial inflammation in PKD.

## OVERVIEW OF PKD

PKD consists of several variants which differ according to their mode of inheritance, underlying genetic mutation and pattern of cyst formation. These variants include automosal dominant PKD (ADPKD), autosomal recessive PKD (ARPKD) and other autosomal recessive cystic renal diseases, including nephronophthisis (NPHP). ADPKD is the most common type of cystic renal disease, with an incidence of approximately one in every 1000 live births, and estimated to affect 6.5 million people world-wide (Bisceglia et al., 2006; Harris and Torres, 2009). Structurally, in ADPKD, the cysts are spherical in shape and occur in both kidneys. Cyst growth is not synchronized. Therefore the end-stage kidney in ADPKD contains thousands of cysts that vary in size from 100 μm to several centimeters in diameter, resulting in a large (weighing up to 3–5 kg, compared to 125–170 g in normal males) and irregularly shaped organ. In ADPKD, the cysts start to form in early life (possibly *in utero* or during the early postnatal period) due to the clonal proliferation of focal epithelial cells lining the distal nephron and collecting duct, which leads to diverticular-like protrusions extending into interstitium (Baert, 1978). With continued growth, the latter detach from the parent nephron when their diameter exceeds 100 μm, and form "encapsulated cysts" (Hovater et al., 2008). Once in the interstitium the cysts continue to slowly expand over many decades (Grantham et al., 2011). Kidney failure therefore occurs after a long latent period, usually by the 5th decade of life (Baert, 1978), when a sufficient number of cysts (possibly > 1000) (Luft, 2011) have collectively grown to disrupt normal kidney architecture and function. In contrast, ARPKD is a less frequent, childhood disease (1:20 000 live births) (Sweeney and Avner, 2006). It is characterized by the synchronized microcystic dilation of collecting ducts. Detachment of the dilated cystic collecting ducts from the nephron does not occur (Baxter, 1961). The kidneys are large but maintain their reniform shape, and kidney failure typically occurs during the neonatal period (Sweeney and Avner, 2006). NPHP has an autosomal recessive mode of inheritance, and is characterized by tubulointerstitial nephropathy and corticomedullary duct ectasia, but kidney enlargement does not occur (Wolf and Hildebrandt, 2011). NPHP is one of the most frequent genetic disorders causing kidney failure in children and adolescents (Wolf and Hildebrandt, 2011).

ADPKD, ARPKD and NPHP are caused by mutations in the *Pkd1/Pkd2*, *Pkhd1* and *Nphp* (Grantham et al., 1989, 2011; Wilson et al., 1999; Schwiebert, 2001; Rangan et al., 2005; Wang et al., 2005; Harris and Torres, 2009; Xu et al., 2009; Aguiari et al., 2013; Chang and Ong, 2013; Kelsey, 2013) genes respectively, which encode the proteins, polycystin (PC)-1/PC-2, fibrocystin and nephrocystin (Sweeney and Avner, 2006; Harris and Torres, 2009). These so-called "cystproteins" (Hildebrandt and Otto, 2005) have all been found to co-localize to the primary renal cilia (an antenna-like sensory organelle involved in mechanosensation), and interact with each other at a molecular and functional level (Kaimori and Germino, 2008; Fedeles et al., 2011). In physiological states, the intact cystoprotein complex maintains the normal function of the cilium, negatively regulates the cell-cycle (Bhunia et al., 2002) and promotes intracellular calcium transport (Cowley, 2008) and cellular differentiation as well as normal renal tubular morphogenesis (Boletta and Germino, 2003). The intracellular level of PC-1 plays a central role in both ADPKD and ARPKD, as it is the rate-limiting component that ultimately determines cyst formation (Fedeles et al., 2011). Interestingly, ADPKD is a focal disease, as only 1–2% of nephrons in a kidney develop cysts (Martinez and Grantham, 1995). It has therefore been postulated that a heterozygote germ-line mutation in *Pkd1* or *Pkd2,* combined with postnatal disruption of the second normal allele is required for cyst formation (the two-hit mechanism of the Knudson theory) (Nauli et al., 2006). An additional molecular explanation for the postnatal onset of ADPKD is an age-related decline in the dosage of functional PC-1 protein (Rossetti et al., 2009). In contrast to ADPKD, postnatal somatic mutations do not have a role in cyst formation in ARPKD (Sweeney and Avner, 2006). Despite their importance in PKD, therapeutic approaches to modulate the genetic expression of PCs has been problematic because: (1) there is significant heterogeneity in the genetic mutations of PC-1 (Harris and Torres, 2009); (2) over- as well as under-expression of PC-1 can cause PKD (Harris and Torres, 2009); (3) there are difficulties with the delivery of viral vectors for gene therapy due to the poor endocytosis capability of the epithelial cells lining cysts (Witzgall et al., 2002). Therefore, treatments to prevent kidney failure due to PKD have focused on the abnormalities in cellular function and signal transduction arising from the dysfunction of cystproteins (Torres et al., 2007). These abnormalities are largely a consequence of reduced intracellular calcium which leads to increased intracellular levels of cAMP. As discussed earlier, cAMP levels are also increased by circulating vasopressin.

Halting the growth of cysts is one of the main therapeutic goals in preventing the decline of kidney function in PKD. The mechanisms of cyst growth involve three factors (Torres et al., 2007; Terryn et al., 2011): (i) increased proliferation of CECs: (ii) the gradual and incremental accumulation of fluid inside cysts; and (iii) abnormalities in extracellular matrix formation and degradation which are permissive for cyst expansion. The accumulation of fluid in cysts is an important mechanism of disease progression in PKD, and as discussed below, may be induced by purinergic signaling (Terryn et al., 2011). It is due to the net transepithelial secretion of chloride across apical membranes of CECs *via* chloride channels [cystic fibrosis transmembrane conductance regulator in response to the induction of cAMP-mediated protein kinase A (CFTR-PKA); and calcium-activated chloride channels]. This chloride efflux then induces Na+ (as a result electric coupling) and water (as a result of osmotic coupling via aquaporin channels) efflux, resulting in the progressive accumulation of fluid within the cysts (Terryn et al., 2011).

## POTENTIAL MECHANISMS OF EXTRACELLULAR ATP RELEASE AND P2 RECEPTOR SIGNALING IN PKD

The intracellular concentration of ATP ranges between 1 to 10 mM (Beis and Newsholme, 1975), and ∼0.1% of this reservoir (Schwiebert, 2001) may theoretically be released into the extracellular space (up to 10 μmol/L) of the renal microenvironment in PKD, which includes the interior of encapsulated cysts (as in ADPKD) (Schwiebert, 2001), the renal interstitial space and/or the nephron lumen. The mechanisms of the extracellular release could hypothetically involve (Jacobson and

Boeynaems, 2010): (1) apoptosis and necrosis of cystic epithelial cells (CECs) as well as destruction of normal renal epithelial cells with disease progression (Goilav, 2011); (2) non-lytic mechanisms (Bowler et al., 2001) involving CECs, that requires the exocytosis of secretary granules, vesicular transport and membrane channels and include ATP-binding cassette transporters, pannexins and connexins (Lohman et al., 2012), possibly in response to mechanical stretch [as shown in bladder epithelial cells (Ferguson et al., 1997)], hypoxia (Bergfeld and Forrester, 1992), dysfunction of cystoproteins (Schwiebert et al., 2002), increased cellular metabolism (Wilson, 1997; Sullivan et al., 1998) and other ATP release mechanisms that may be abnormal in PKD (Schwiebert et al., 2002); and (3) release of ATP from other local sources such as infiltrating inflammatory cells and renal nerves (Bailey et al., 2000).

Once in the extracellular fluid, ATP is capable of activating purinergic receptors, which are one of the most abundant receptor families in mammalian tissues (Abbracchio et al., 2009). There are two groups of ATP-responsive (P2) receptors: (i) P2Y receptors are G-protein coupled which act through a second messenger, and respond to a wide variety of nucleotides (ATP, ADP, UTP, UDP and UDP-glucose) They consist of eight members (subtypes 1, 2, 4, 6, 11, 12, 13, and 14, which vary depending on the type of G protein involved and specificity of the ligand) (Jacobson and Boeynaems, 2010) and modulate intracellular calcium and cAMP; and (ii) P2X receptors are rapidly acting non-selective cation channels that are calcium permeable, and which open primarily after binding to ATP (Schwiebert, 2001). They consist of eight subtypes, and in response to ATP-ligand binding, cause plasma membrane depolarization which allows calcium influx from external stores (Schwiebert, 2001).

Extracellular ATP is rapidly degraded in the plasma (Gorman et al., 2007) and in order for it to have a physiological or pathological function, it requires accumulation in the local microenvironment. The net build-up of ATP in PKD is likely to be dependent on a number of factors (Di Virgilio, 2012): (i) the pattern of P2 receptor expression in cystic and non-cystic renal tissue as well as in infiltrating inflammatory cells; (ii) the level of expression of nucleotide hydrolyzing enzymes (CD39 and CD73), which breakdown ATP and generate adenosine, which has anti-inflammatory and anti-tumor effects (Di Virgilio, 2012).

## EVIDENCE FOR ATP RELEASE INTO THE LOCAL MICROENVIRONMENT AND P2 RECEPTOR ALTERATION IN PKD

Fluid extracted from micro-dissected cysts from ADPKD patients was found to contain pharmacologically relevant levels of ATP (up to 10 μM) (Wilson et al., 1999), which is a thousand times higher than that found in peripheral blood (Lazarowski et al., 2001). Primary cultures of renal epithelial cells from ADPKD and ARPKD patients release between two to five times more ATP, both across the apical and basolateral membrane, under resting conditions and with hypotonic challenge, compared to non-ADPKD cells (Wilson et al., 1999; Schwiebert et al., 2002). This might, in part, be due to the slower degradation of ATP in ADPKD cells due to a reduced expression of CD39 (Xu et al., 2009) or other mechanisms. ATP, in concentrations of 4 nM, was also detectable in cyst fluid from *cpk* mice (a murine model that phenotypically resembles ARPKD, and is due to a mutation in *cystin*) (Hillman et al., 2004). However, the presence of ATP in the interstitial space, nephron lumen or urine of patients and animals with PKD has not been reported and is not known. In the microenvironment of tumors (which are thought to share some analogy to the pathogenesis of PKD) (Ghiringhelli et al., 2009), ATP has been shown to accumulate, and under these circumstances, thought to be primarily due to increased cancer cell metabolism and tumor-associated inflammation (Ghiringhelli et al., 2009; Di Virgilio, 2012). This could potentially also be the case in PKD.

Several studies have examined the expression of P2 receptors *in vitro* and *in vivo* in the cyst lining epithelium in PKD. In primary cultures of ADPKD and ARPKD cells from humans, mRNA for both P2X and P2Y receptors was found to be expressed (Xu et al., 2009). In *cpk* mice, immunohistology showed that collecting duct cysts were positive for $P2X_7$ protein (Hillman et al., 2002). Similarly, in human ARPKD, $P2X_7$ was also expressed in the epithelial cells of dilated collecting ducts and cysts but not present in normal human fetal kidneys (Hillman et al., 2004). In the Han:Sprd rat model of PKD, CECs from heterozygous rats were positive for P2Y ($Y_2$ and $Y_6$), $P2X_5$ and $P2X_7$ (Turner et al., 2004). Taken together, these data suggest that ATP accumulates in the PKD microenvironment, and that CECs possess the appropriate receptors to be able to respond to the presence of the ligand.

## CILIARY DYSFUNCTION IN PKD ALTERS THE AUTOCRINE ATP-P2 AXIS, INTRACELLULAR CALCIUM AND CHLORIDE SECRETION IN CYSTIC EPITHELIAL CELLS

Growing evidence suggests that extracellular ATP has a physiological role in maintaining the health and function of renal epithelial cells, and that this is dependent on an intact and functioning cilium. In immortalized normal human kidney tubular epithelial cells (HK-2 cell line), $P2X_7$ receptors were localized to the primary cilia, supporting the possibility that ATP-P2 signaling is involved in mechanosensation and cyst formation (Chang and Ong, 2013). In addition, in *C. Elegans*, ATP synthase (which produces ATP from ADP) physically associates with the polycystins, LOV-1 and PKD2, suggesting that ATP synthase activity could be dysfunctional in PKD and might be a factor contributing to extracellular accumulation of ATP (Hu and Barr, 2005). The importance of a functioning cilia for ATP-purinergic signaling was further revealed from *in vitro* studies of collecting duct principal cells from Oak Ridge Polycystic kidney disease ($orpk^{tg737}$) mice [a model of ARPKD in which cells lack a cilium due to a mutation in *polaris* (Hovater et al., 2008)]. These studies showed that the complete absence of an apical cilia impairs ATP secretion in response to mechanical, chemical and osmotic stimuli, in collecting duct principal cells (Hovater et al., 2008).

In normal renal epithelial cells extracellular ATP promotes chloride secretion and regulates intracellular calcium (Wildman et al., 2003). In PKD mutant cells, accumulating evidence suggests that chloride secretion in response to ATP is exacerbated. For example, over-expression of the cytoplasmic COOH-terminus of PC-1 in mouse cortical collecting duct cells prolonged the duration of chloride conductance in response to ATP, supporting

the hypothesis that PKD mutant cells are more sensitive to some of the physiological effects of ATP (Hooper et al., 2003; Wildman et al., 2003). Therefore, it has been hypothesized that the dysfunction of the PC signal transduction pathways promotes the "secretagogue activity" of extracellular ATP by stimulating a calcium-activated chloride channel *via* purinergic receptors (Hooper et al., 2003; Wildman et al., 2003). In addition to chloride, ATP also acts in an autocrine fashion to stimulate intracellular calcium *via* purinergic receptor signaling. However, the intracellular calcium response to ATP is impaired in both murine and human ADPKD cells (Hovater et al., 2008; Xu et al., 2009). The latter was also associated with a reduced expression $P2X_7$ and CD39 (Xu et al., 2009).

## FUNCTIONAL EVIDENCE SUGGESTING THAT EXTRACELLULAR ATP-P2 SIGNALING DIRECTLY PROMOTES CYST EXPANSION AND INTERSTITIAL INFLAMMATION IN PKD

### CYST EXPANSION (CHLORIDE-DRIVEN FLUID SECRETION AND PROLIFERATION OF CECs)

In normal inner medullary collecting duct cells extracellular purinergic agonists can be mitogenic or co-stimulatory with other growth factors (Ishikawa et al., 1997). Therefore, in PKD, ATP has been postulated to have a role in the expansion of encapsulated renal cysts (as in ADPKD) or cystic tubular expansion (as in the ARPKD). It has been suggested that because cysts in the former are "encapsulated," ATP accumulation may have a more important pathogenic role because of sequestration within the cyst interior, but this has not been proven (Schwiebert, 2001).

To date the functional roles of ATP-P2 signaling on the mechanisms of cyst expansion has been examined *in vitro* in cultured cells and in a zebrafish model. Collectively, these studies have indicated that ATP-P2 signaling may either accelerate or attenuate cyst growth. Similar to normal tubular epithelial cells (Kishore et al., 1995, 2000), in primary cultures of human PKD cells, ATP agonists (ATP, Bz-ATP and UTP) increased intracellular calcium levels and secretory anion transport by activating chloride-dependent secretion (that was independent of cAMP/PKA/CFTR) (Schwiebert et al., 2002). In contrast, Hillman and colleagues examined the role of ATP-P2 signaling in the initial steps of cyst formation, by growing CEC aggregates *ex vivo* from *cpk* mice (Hillman et al., 2004). In this study, the exposure of these cells to 2′- and 3′-*O*-(4-benzoylbenozoyl)-adensosine 5′-triphosphate (BzATP, a $P2X_7$ agonist) reduced the number of cysts that formed by approximately one-third (Hillman et al., 2004). Cyst size and proliferation was not altered but there was a non-significant increase in caspase-3 activity (Lazarowski et al., 2001). Exogenous ATP and UTP also reduced cyst number in this model but to a much lesser extent (nearly 10%) (Lazarowski et al., 2001). In comparison to *cpk* mice CECs, in MDCK cell-derived cysts, treatment with non-specific P2 receptor antagonists (Reactive Blue 2, suramin) or removal of ATP from culture medium with apyrase (by 50%) attenuated the cAMP-ERK-dependant growth by ∼50% (Turner et al., 2007). In this model, cyst growth was not affected by treatment with a non-selective P2X inhibitor, Coomassie Brilliant Blue G), suggesting a role for P2Y receptors (Turner et al., 2007). Similarly, in a separate report, in MDCK cysts derived from principal cells (Clone C7), ATP-dependent cyst growth was driven by fluid secretion rather than cell proliferation and was synergistic with cAMP (Buchholz et al., 2011). The latter was largely dependent on extracellular ATP and attenuated by the P2 receptor antagonist, suramin (Buchholz et al., 2011), and the effects were not evident in MDCK cells derived from intercalated cells (Buchholz et al., 2011). Consistent with the data in MDCK cells, in a zebrafish model of ADPKD (morpholino induced knockdown of *Pkd2*), a $P2X_7$ antagonist (oxidized ATP) markedly reduced the cystic dilatation and peritubular oedema in pronephric ducts compared to the control (no treatment) or to a $P2X_7$ agonist (Bz-ATP) (Chang et al., 2011). This was associated with a reduction in cell proliferation and p-ERK activity (Chang et al., 2011).

The contrasting results mentioned above are most likely due to differences in the experimental design (particularly the stage of cyst growth), the cellular model and the type of PKD examined. Perhaps, ATP-P2 signaling reduces cyst *formation* (as in *cpk* CEC aggregates) but promotes expansion once the cysts have actually formed. Further *in vivo* data using genetically orthologous models of PKD are awaiting to provide clarification regarding the role of ATP-P2 signaling in initiation and growth of cysts in PKD.

### RENAL INTERSTITIAL INFLAMMATION

Renal interstitial inflammation is recognized as an important factor in the progression in PKD (Ta et al., 2013). Macrophage accumulation may promote cyst growth and interstitial inflammation, and be detrimental to the progression of PKD (Swenson-Fields et al., 2013; Ta et al., 2013). The release of excess extracellular ATP is a "danger signal" that is likely to lead to interstitial inflammation *via* inflammatory signaling pathways (Idzko et al., 2007; Ivison et al., 2011). To date, the role of ATP-P2 signaling in mediating interstitial inflammation associated with PKD has not been examined. Based on data from other experimental models, one might hypothesize that extracellular ATP (released into the renal interstitium by inflammatory cells and CECs) could have a proinflammatory effect (Deplano et al., 2013). For example, in antibody-mediated autoimmune glomerulonephritis, renal injury, glomerular macrophage accumulation and the urinary excretion of the monocyte chemoattractant protein-1 was attenuated by $P2X_7$ deficiency (in mice) or by administration of a $P2X_7$ antagonist (A-438079) (in rats) (Taylor et al., 2009) Similarly, in a murine model of renal interstitial fibrosis (unilateral ureteral obstruction), interstitial macrophage and myofibroblast accumulation, interstitial fibrosis and tubular cell apoptosis was also attenuated by $P2X_7$ deficiency (Goncalves et al., 2006). Lastly, preliminary data shows that treatment of rats with unilateral ureteral obstruction with a $P2X_7$ antagonist (Brilliant Blue G) also attenuated interstitial inflammation and fibrosis but increased tubule cell proliferation (Leite et al., 2012). The latter raises the possibility that the ATP-P2 system could have complex and divergent effects *in vivo* in PKD.

## FUTURE DIRECTIONS AND POTENTIAL FOR ATP-P2 SIGNALING IN THE THERAPY OF HUMAN PKD

Clinical trials (Phase 1 and 2) to determine the safety and efficacy of $P2X_7$ receptor antagonists in chronic inflammatory

diseases (rheumatoid arthritis, inflammatory bowel disease) are presently in progress [reviewed in Arulkumaran et al. (2011)]. In PKD, further preclinical evidence is required before clinical trials of $P2X_7$ receptor antagonists can be considered. Experiments comparing disease progression in compound/double knockout mice using $P2X_7^{-/-}$ mice and genetically orthologous/non-orthologous murine models of PKD are required (Hillman et al., 2005). In addition, in experimental models of cancer, the overexpression of either CD39 or CD73 promotes tumor progression due to loss of tumor-associated inflammation (Synnestvedt et al., 2002; Eltzschig et al., 2009). On the other hand, in PKD, the loss of interstitial inflammation due to the transgenic expression of CD39 or CD73, might be renoprotective, as shown in experimental renal ischaemia (Crikis et al., 2010). Studies to determine the effects small molecule inhibitors (such as Brilliant Blue G, suramin, A-438079 or preferably those used in clinical trials) during the early as well as the established phases of PKD in small animal models are also needed. In particular, the stage- and disease-specific effects (relevant to human disease) of ATP-purinergic signaling in preclinical models needs to be understood (Schwiebert et al., 2002). The effects of natural and existing small molecule modulators of ATP-P2 signaling in the pathogenesis of PKD should not be forgotten, as this may accelerate translation to the clinic. For example, the methylxanthine, caffeine, induced ATP release in smooth muscle cells (Katsuragi et al., 2008), and it is possible that effects on P2 receptors underlie its ability to promote chloride driven fluid secretion in human ADPKD cells (Belibi et al., 2002). Similarly, the effects of existing pharmacological inhibitors, such as clopidogrel and ticlopidine which affect $P2Y_{12}$ receptors (Savi and Herbert, 2005), on cyst growth in *in vitro* models, could be screened.

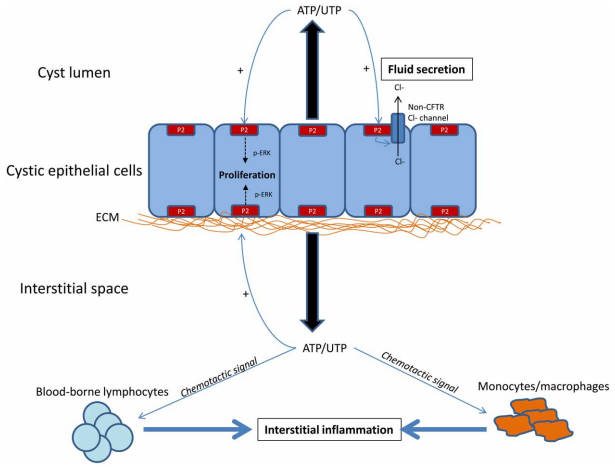


**FIGURE 1 | Hypothetical diagram showing the potential role of ATP-P2 signaling in the pathogenesis of cyst growth and interstitial inflammation in PKD.** Current data suggests that extracellular ATP is increased in the microenvironment of the kidney affected by PKD, due to apical/basolateral release by cystic epithelial cells. Reduced degradation or increased cellular sensitivity to ATP may also contribute to net accumulation of ATP in the local environment. The presence of extracellular ATP has paracrine and autocrine effects on numerous cells, including cystic epithelial cells and interstitial inflammatory cells. In cystic epithelial cells, *in vitro* data suggests that P2 receptor activation promotes chloride-driven fluid secretion (independent of PKA-cAMP-CFTR mechanism) and proliferation (via p-ERK), both of which lead to cyst growth. In addition, preclinical data from non-PKD animal models suggests that P2 signaling promotes the chemotaxis of macrophages and lymphocytes, promoting interstitial inflammation. However, the hierarchy by which ATP-P2 signaling mediates proliferation, chloride secretion and interstitial inflammation in PKD is not known. In addition, the localization of P2 receptors (basolateral vs. apical distribution) or whether specific receptors have unique functions is not known.

## CONCLUSION

Evidence accumulated to date supports that ATP-P2 signaling is potentially important in the pathogenesis of PKD. However, the study of ATP-P2 signaling in PKD is presently in an early phase of investigation (**Figure 1**) and further work, particularly preclinical *in vivo* studies, are needed before randomized controlled trials using small molecular inhibitors of P2 receptor antagonists in humans with PKD can be considered.

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# Emerging key roles for P2X receptors in the kidney

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P2X ionotropic non-selective cation channels are expressed throughout the kidney and are activated in a paracrine or autocrine manner following the binding of extracellular ATP and related extracellular nucleotides. Whilst there is a wealth of literature describing a regulatory role of P2 receptors (P2R) in the kidney, there are significantly less data on the regulatory role of P2X receptors (P2XR) compared with that described for metabotropic P2Y. Much of the historical literature describing a role for P2XR in the kidney has focused heavily on the role of P2X1R in the autoregulation of renal blood flow. More recently, however, there has been a plethora of manuscripts providing compelling evidence for additional roles for P2XR in both kidney health and disease. This review summarizes the current evidence for the involvement of P2XR in the regulation of renal tubular and vascular function, and highlights the novel data describing their putative roles in regulating physiological and pathophysiological processes in the kidney.

**Keywords: P2X receptor, P2 receptors, kidney, renal circulation, renal tubular transport, inborn errors, P2X7 receptor, pathology**

## INTRODUCTION

Extracellular ATP and related nucleotides have been shown to contribute to complex autocrine and/or paracrine signaling networks throughout the body following their activation of P2 receptors (P2R; formerly termed purinoceptor; Burnstock and Knight, 2004). The P2 family of receptors is divided into metabotropic G protein-coupled P2Y receptors (P2YR) and ionotropic ligand-gated P2X receptors (P2XR), which act as non-selective cation channels (Fredholm et al., 1994). There are eight pharmacologically distinct P2YR ($P2Y_{1,2,4,6,11-14}$) and seven unique P2XR subunits (P2X1-7), which can form seven homomeric assemblies, seven established heteromeric assemblies (P2X1/2, 1/4, 1/5, 2/3, 2/6, 4/6, 4/7), as well as several predicted heteromeric assemblies. Heterologous expression systems have been used to demonstrate that P2XR can readily form functional trimeric or hexameric assemblies (North, 2002). The actual number of subunits present in native cell multimeric assemblies is still debated, however, the trimer is favored (North, 2002). It is established that there are an abundance of P2R in a variety of cell types throughout the body, linked to numerous physiological processes such as regulation of other membrane-located ion channels, cell-to-cell communication, secretion of cytokines, metabolic processes such as glugoneogenesis, cell proliferation, and cell death (Burnstock and Knight, 2004).

It is perhaps not surprising that P2R are ubiquitously expressed throughout the kidney, in both cortical and medullary vascular and tubular compartments. They contribute to a diverse range of physiological and pathophysiological processes and yet the localization of specific P2R subtypes can be highly regional, overlapping, and often debated. Much of the renal literature published to date focuses on the role of metabotropic P2YR, and in particular in their regulation of sodium (Na) and water transport in the collecting duct (CD; Kishore et al., 2009; Wildman et al., 2009; Vallon and Rieg, 2011), with P2XR being somewhat overlooked (with the exception of their role in regulating afferent arteriole diameter; see summary **Figure 1**). Arguably a dogma exists that P2XR play little or no role in kidney function. However, a recent glut of manuscripts provides compelling evidence for P2XR playing key roles in the kidney. This review will discuss the most recent advances in the field of "renal P2XR" [i.e., the key literature of the last 3-years (2010–2013)], focusing on the tubular and vascular localization of P2XR and their function(s) in both the physiological and pathological setting (see summary **Figure 2**).

## NEW ROLES FOR P2XR IN RENAL TUBULAR TRANSPORT

Expression of P2XR varies throughout the nephron. P2X4R and P2X6R are expressed in the proximal tubule, distal tubule, loop of Henle and CD, making these receptor subtypes the most widely distributed (Unwin et al., 2003). P2X1R and P2X7R are localized predominantly in Bowman's capsule and the CD and are more widely distributed in vascular networks (as discussed in the next section; Inscho et al., 2003; Vitzthum et al., 2004; Osmond and Inscho, 2010; Crawford et al., 2011). Immunohistochemical studies have also demonstrated low levels of expression for P2X2R and P2X5R in the cortical and medullary CDs (Turner et al., 2003; Wildman et al., 2008). The putative roles previously described for P2XR in the nephron have included inhibition of fluid reabsorption in the proximal tubule, inhibition of magnesium absorption in the distal tubule, and inhibition of AQP2-mediated water absorption and modulation (inhibiting and potentiating) of ENaC-mediated $Na^+$ absorption/reabsorption in the CD (Bailey et al., 2012; see summary **Figure 1**).

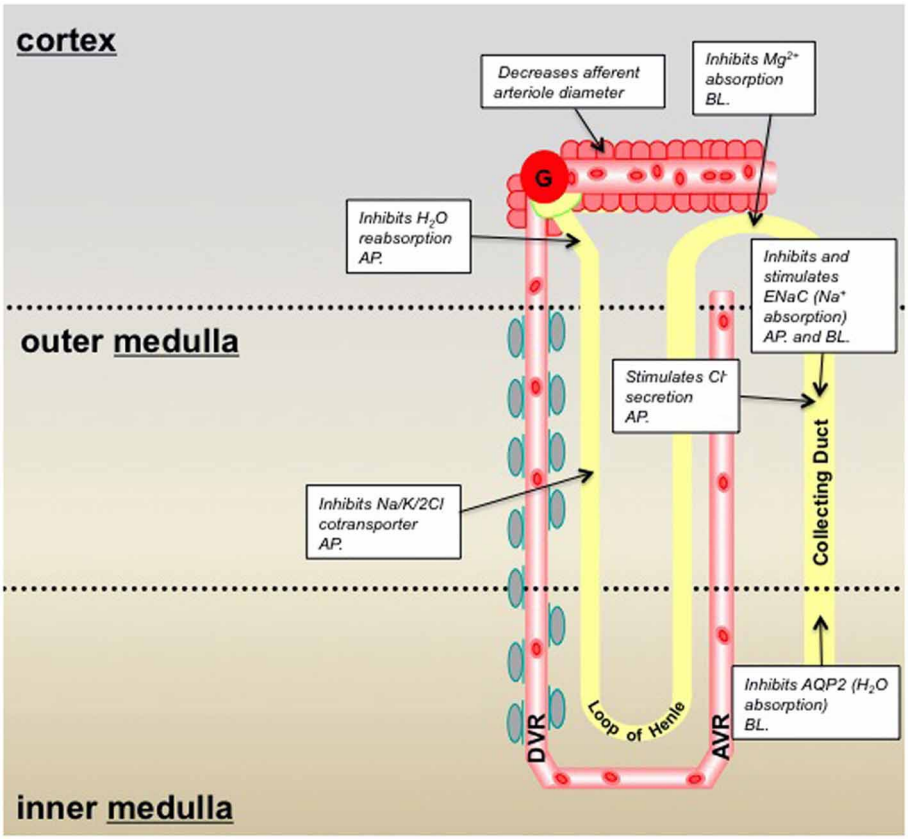


**FIGURE 1 | Summary of P2X receptor-mediated effects in the kidney and epithelial cell lines derived from specific nephron segments; pre-2010/11.** Information taken from: Wildman and King, 2008; Bailey et al., 2012. Key: *AP,* denotes that the P2X receptors are localized on the epithelial cell *apical* membrane; *BL,* denotes localization on the *basolateral* membrane.

Novel data obtained from pharmacological experiments that utilized P2XR selective agonists, complimented by studies in knockout mice ($^{-/-}$), now provide compelling evidence for a functional role for P2XR in the medullary thick ascending limb (mTAL) (Marques et al., 2012). It is well-established that NaCl enters cells of the TAL via the apical Na/H exchanger and Na-K-2Cl co-transporter and leaves the cell via basolateral Na-K-ATPase, and nitric oxide (NO) inhibits both the Na/H exchanger and Na-K-ATPase to regulate ion transport. Experiments to determine the effect of basolateral ATP on NaCl absorption in isolated perfused mouse mTALs, using the electrical measurement of equivalent short-circuit current, demonstrated that basolateral ATP attenuated the absorptive short-circuit current (Marques et al., 2012). Authors used P2XR selective agonists and antagonists to demonstrate the effect was mediated, not via P2YR as might have been expected, but via P2XR. Experiments reproduced in both P2X7R$^{-/-}$ and P2X4R$^{-/-}$ concluded that the ATP-inhibition of transport was reduced in the P2X4R$^{-/-}$ animals thus indicating a key role for P2X4R. This finding was further corroborated by PCR experiments, which confirmed the presence of P2X4R mRNA, together with P2X1R and P2X5R mRNA in isolated mTAL (Marques et al., 2012). In addition, complimentary studies seeking to determine the factors responsible for flow-mediated NO production in the mTAL identified ATP as a candidate. Researchers used P2YR and P2XR selective antagonists to demonstrate a role for basolateral P2XR (and apical P2YR) in ATP-mediated, flow-induced production of NO in the mTAL (Cabral et al., 2012). Collectively, data from these studies suggest that ATP, released by increased tubular flow rate, acts on basolaterally-expressed P2X4R (potentially a heteromeric assembly, with either P2X1R or P2X5R), to increase NO production, which ultimately inhibits NaCl reabsorption in the mTAL. However, the mechanism by which luminal ATP activates basolaterally-expressed P2X4 receptors is yet to be elucidated.

Studies performed in our laboratory and with collaborators have similarly identified roles for P2X4R, and potentially P2X7R, in the regulation of Na reabsorption. However, our studies have focused on the CD rather than the TAL. Initially using M1 cells, an immortalized mouse cortical CD cell line, in combination with electrophysiology techniques we identified ionotropic P2XR-mediated channel activity (Birch et al., 2013a). Application of ATP to outside-out patches activated single-channel openings, from multiple receptor subtypes, with single-channel properties similar to those of P2XR previously identified in other cell types (Evans, 1996; Birch et al., 2013a). Characterization of the exact

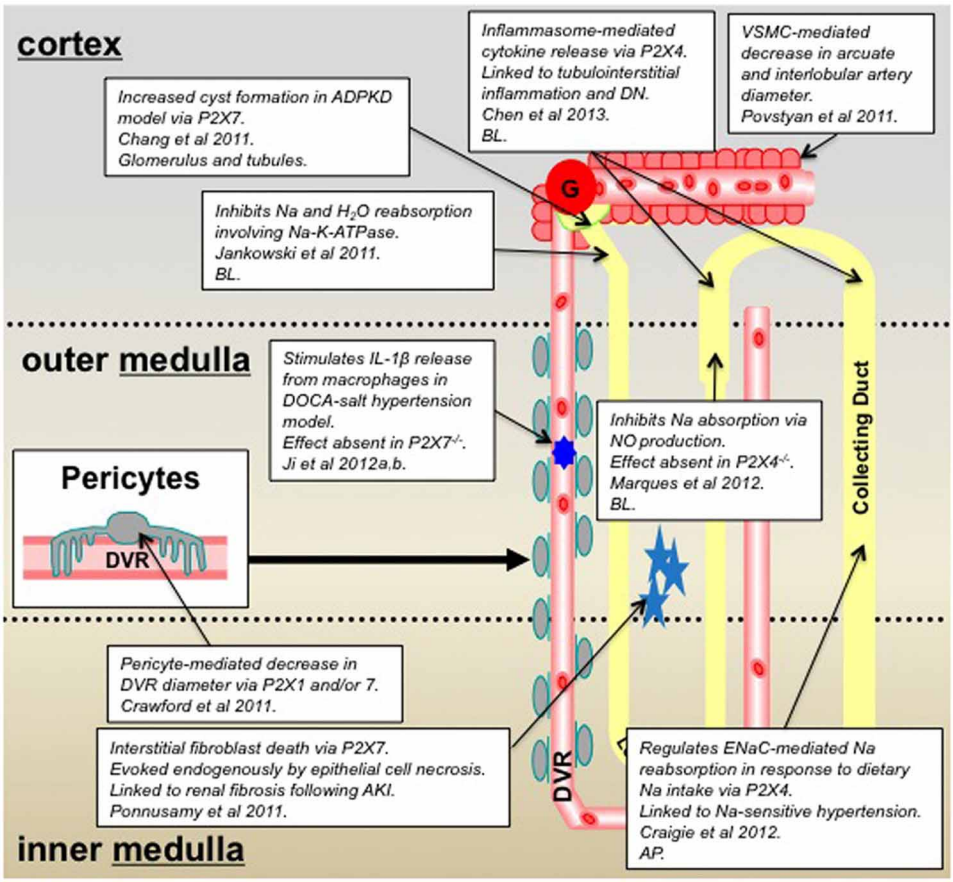


**FIGURE 2 | Summary of P2X receptor-mediated effects in the kidney and epithelial cell lines derived from specific nephron segments; 2011–2013 inclusive.** Key: *AP*, denotes that the P2X receptors are localized on the epithelial cell *apical* membrane; *BL*, denotes localization on the *basolateral* membrane; ADPKD, autosomal dominant polycystic kidney disease; DN, diabetic nephropathy; VSMC, vascular smooth muscle cell; DVR, descending vasa recta.

P2XR subtypes mediating the single-channel activity is a current focus of the group. Initial observations in rat CD principal cells *in vitro* demonstrated that apical P2X4R modulates ENaC (epithelial Na channel) activity: high concentrations of luminal ATP evoked P2X4R-mediated inhibition of ENaC activity, whereas low concentrations conversely potentiated ENaC activity (Wildman et al., 2008). In addition to our *in vitro* functional data we have used P2X4R$^{-/-}$ mice to demonstrate an *in vivo* role for P2X4R in Na reabsorption and salt sensitivity (Craigie et al., 2012). Data from our most recent *in vivo* renal clearance studies have revealed a raised mean arterial blood pressure (MABP) in P2X4R$^{-/-}$ when compared with wild-type (WT) littermates. When dietary Na was altered to what is termed a low Na diet (0.03% compared to 0.3% in a standard diet), the reduction in dietary Na significantly reduced MABP in the P2X4R$^{-/-}$ but not in WT. Interestingly, when comparing the ENaC-mediated reabsorption for WT and P2X4R$^{-/-}$ on a standard diet there was no significant difference. However, when on a low Na diet the P2X4R$^{-/-}$ mice exhibited a significantly reduced fractional Na excretion compared with WT, indicating this group of animals were unable to increase ENaC-mediated Na reabsorption in the absence of P2X4R. These data collectively suggest that P2X4R$^{-/-}$ mice have salt-sensitive blood pressure and are unable to increase their ENaC-mediated sodium reabsorption when dietary sodium is low. The latter finding is compatible with our previous *in vitro* data that describes a P2X4-mediated potentiation of ENaC activity when extracellular sodium is low. Thus, P2X4 may act as an intrinsic regulator of ENaC activity in the aldosterone-sensitive distal nephron to moderate Na reabsorption in response to changes is Na load. Perhaps of note, *in vivo* pharmacological studies performed in rats, whereby P2XR agonists were intravenously infused showed a marked, progressively-increasing diuresis, together with an increase in the Na excretion rate. Authors concluded that activation of P2XR increased renal Na and water excretion in rats via a Na-K-ATPase-dependent mechanism (Jankowski et al., 2011). Evidently, P2X4R in the CD may feature in the complexity of essential hypertension.

Whilst the precise mechanism(s) responsible for P2XR-mediated increases in Na excretion require clarification, the evidence in favor of significant functional roles for P2XR in the nephron is increasing. This is further confirmed by pathophysiological findings that provide evidence for the involvement of

P2XR in kidney dysfunction. P2X4R have been highlighted as key receptors in inflammasome activation and have been linked to diabetic nephropathy (DN) (Chen et al., 2013). It is well-established that tubulointerstitial inflammation is involved in the development of DN, and is mediated by key inflammatory cytokines. Chen and colleagues demonstrated that extracellular ATP causes P2X4R to activate the NOD-like receptor 3 (NLRP3) inflammasome, causing cytokine maturation and release (Chen et al., 2013). In light of the potential link between P2X4R, inflammasome activation, and DN, P2X4R signaling in NLRP3 inflammasomes was investigated using renal biopsies from patients with type-2 diabetes. Authors reported increased expression of P2X4R in renal tubular epithelial cells of patients with type-2 diabetes compared with non-diabetic patients, and that P2X4R expression correlated with expression of IL-1 and IL-18 (Chen et al., 2013). This finding is perhaps not surprising since ATP is one of the best established damage-associated molecular patterns (DAMPs), and as such plays an important role in inflammation and immunity by functioning as a signal of cell damage, stress or death during injury and disease (Chen and Nunez, 2010). A cautionary note: whilst these studies substantiate the hypothesis that P2X4R blockade would be a successful therapeutic strategy in attenuating DN, there is evidence to suggest this may lead to hypertension by disrupting sodium transport in the distal nephron (as previously discussed). This highlights the fact that although P2XR have emerged as attractive therapeutic targets for many pathophysiological conditions, their widespread expression and diverse roles means the manipulation of these receptors is extremely complex.

Activation of P2X7R (albeit via a more established mechanism), like P2X4R, induces downstream inflammatory events, including the NALP3 inflammasome/caspase-1-dependent maturation of IL-1β and IL-18, and their subsequent release from various myeloid cell types (Idzko et al., 2007). There is now also evidence for Madin-Darby canine kidney (MDCK) epithelial cells expressing functional P2X7R, as well as TLR4 and molecules associated with the NALP3 inflammasome (Jalilian et al., 2012), perhaps highlighting their importance in *renal* inflammatory injury and *renal* disease. P2X7R are perhaps the P2XR subtype most linked to pathophysiology of the kidney. Historically, P2X7R (which can form pores under certain circumstances) are known to be pro-inflammatory, and whilst they are extensively expressed in cells of the immune system they are not *highly* expressed in healthy kidney tissue (Hillman et al., 2005). Recent additional lines of evidence that describe alternative roles for P2X7R in kidney dysfunction have revealed a role for P2X7R in: the disrupted calcium homeostasis in peripheral blood mononuclear cells of chronic kidney disease patients (Lajdova et al., 2012), P2XR-mediated cytogenesis in polycystic kidney disease (Chang et al., 2011), interstitial fibroblast cell death following ATP release from necrotic renal epithelial cells (Ponnusamy et al., 2011), and a crucial role in driving hypertension and renal injury (Ji et al., 2012b). It seems that both P2X4R and P2X7R are evermore commonly associated with kidney dysfunction. With this in mind we have utilized P2X4R$^{-/-}$ and P2X7R$^{-/-}$ mouse models and noted that in the P2X4R$^{-/-}$, mRNA for P2X7R was reduced and vice versa, which collectively indicates an interaction of P2X4R with P2X7R at the expressional level to (patho)physiologically regulate renal function (Birch et al., 2013b; Craigie et al., 2013). The specific role of P2XR in renal pathophysiology will be discussed below in greater detail.

## NEW ROLES FOR P2XR AND RENAL VASCULATURE

In addition to tubular epithelial cell expression, P2R are expressed in renal vascular and glomerular cells of the medulla and cortex (Bailey et al., 2012). The role of P2R in renal vasculature is complex and dependent on many factors including species, vascular tone, and variable P2R expression patterns. Despite this, numerous functional studies have previously established that P2R have a regulatory role in the renal vasculature (Inscho et al., 1992; Weihprecht et al., 1992; Eltze and Ullrich, 1996; Inscho, 2001; Rost et al., 2002; Eppel et al., 2006a). Much of the historical evidence surrounding a role for P2XR in the regulation of renal blood flow focuses on the P2X1R, most likely a reflection of this being the key receptor expressed by vascular smooth muscle cells (VSMCs). P2X1R are expressed in afferent arterioles but not in efferent arterioles (Osmond and Inscho, 2010) and functional data suggest that ATP-mediated activation of P2X1R represents an important mechanism for regulating renal blood flow and glomerular capillary pressure by regulating afferent arteriolar resistance and in pressure-dependent autoregulatory adjustments in afferent arteriolar diameter (Inscho et al., 2003). *In vivo* studies in which rabbits were infused intrarenally with α, β-methylene ATP described a reduction in both cortical and medullary blood flow that implicated P2XR in the regulation of regional renal blood flow (Eppel et al., 2006b). The interpretation of these former studies have, however, been challenged by recent mouse micropuncture experiments, that have raised questions as to the role of P2XR specifically in tubuloglomerular feedback (TGF), which is the mechanism responsible for matching glomerular filtration to perfusion in the loop of Henle (Schnermann, 2011). The author of this study reported that both intravenous infusion and luminal perfusion of inhibitors of P2R (at a dose that significantly reduced the blood pressure response to α, β-methylene ATP), failed to affect the TGF response to saturating increases in loop perfusion rate. Moreover, macula densa release of nucleotides appeared not to be involved in the vasoconstriction that accompanies increased loop flow *in vivo* (Schnermann, 2011). These findings are in contrast to an *in vivo* study in which anaesthetized rats were treated with $Ip_5I$, a potent P2X1R antagonist, and showed a significant loss of autoregulatory ability, whereas in anaesthetized rats treated with DPCPX, a potent A1R antagonist, autoregulatory control of renal blood flow remained intact (Osmond and Inscho, 2010). The disparity noted above may reflect the acknowledged differences in the relative expression of P2XR between species. Collectively these data suggest that P2X1R are implicated in renal autoregulatory (and maybe not TGF) responses, although the relative contribution of P2XR to these complex physiological processes requires further clarification and may be species dependent. Furthermore, it is possible that ATP and adenosine signaling pathways integrate to mediate TGF. ATP released in response to increased perfusion pressure and/or increases in tubular concentrations could mediate vasoconstriction of the afferent arteriole via P2XR, but

might also be dephosphorylated to form adenosine, which may compound the afferent arteriole vasoconstriction.

In a recent study utilizing the live kidney slice model to investigate regulation of medullary blood flow (MBF) we reported a role for P2XR (and P2YR) in the regulation of vasa recta diameter via contractile pericytes (Crawford et al., 2011). Data described an ATP-mediated constriction of *in situ* vasa recta via contractile pericytes and also demonstrated attenuation of the ATP-mediated constriction in the presence of the P2XR antagonist PPADS. Additionally pericytes constricted vasa recta diameter when live slices were superfused with 2meSATP and BzATP, which act at P2XR. PCR experiments performed on isolated vasa recta to investigate P2R mRNA expression combined with the pharmacological data acquired in functional experiments indicated a putative role for both P2X1R and P2X7R in the regulation of MBF (Crawford et al., 2011). Since pericytes are considered to be relatively undifferentiated having the ability to differentiate further in to VSMCs, it is interesting to note that calcium entry via P2XR has also recently been implicated in the contractile mechanism of renal VSMCs isolated from arcuate arteries and interlobular arteries (Povstyan et al., 2011), thus providing additional evidence for P2XR having a functional role in mediating VSMC contraction in other vascular cells of the kidney. Regarding the functional role of P2XR in pericytes *per se*, P2XR (particularly P2X7R) have also been implicated in the regulation of the retinal pericytes (Sugiyama et al., 2004, 2005) and in the regulation of pericytes of spiral ligament capillaries in the cochlea (Wu et al., 2011).

Given the established role for P2XR in the regulation of vascular cells throughout the body it is not surprising that there are emergent studies in the renal field also highlighting their significance in renal blood flow regulation. What is striking, however, is the accumulating evidence that points toward P2XR playing a critical role in renal pathophysiology, whether the etiology is vascular or tubular in origin.

## P2XR AND RENAL PATHOPHYSIOLOGY

With current funding bodies placing ever increasing importance on translational "bench to bedside" research it is particularly exciting when a molecule or group of molecules emerge as having prominent roles in organ pathology. As eluded to already in this article, there is a compelling mass of evidence that indicates P2XR play crucial and divergent roles in renal pathophysiology. As such, P2XR now represent novel putative targets for renal disease intervention and treatment strategies (North and Jarvis, 2013), and are currently the subject of avid investigation by multiple major international pharmaceutical companies.

Much of the current focus on the potential role of P2XR in renal disease is focused on the homomeric P2X7R, this ligand-gated cation channel being unique in terms of both its structure and function. Unlike its other family members it is thought to exist only as a homomer and has an extended C-terminus with 200 extra amino acid residues, which is thought to be pivotal in regulating its function, being involved in: determining cellular localization, stimulation of various signaling cascades, protein-protein interactions, and post-translational modification of the receptor itself (Costa-Junior et al., 2011). Sustained stimulation of P2X7R with high agonist concentrations (mM) is classically associated with the formation of large transmembrane pores that disrupt the ion gradients within cells resulting in subsequent cell death by either apoptosis or necrosis (Surprenant et al., 1996). The P2X7R is typically expressed by immune cells and mediates release of pro-inflammatory cytokines, prominently interleukin-1 beta (IL-1β), via activation of caspase 1 (Ferrari et al., 1997; Costa-Junior et al., 2011). In contrast to the healthy kidney in which P2X7R is virtually undetected, P2X7R expression is readily detected in inflammatory renal diseases, such as glomerulonephritis (Vonend et al., 2004). The role of P2X7R in renal pathophysiology is not however limited to inflammatory disease. Indeed current topical studies demonstrate that P2X7R plays a detrimental role in a broad range of renal pathologies that will be discussed below.

A recent study has suggested that the pro-inflammatory nature of P2X7R could contribute to the development of hypertension and consequential renal injury. The functional role of P2X7R in hypertension was investigated using WT and P2X7$^{-/-}$ mice rendered hypertensive by a high salt diet and deoxycorticosterone acetate (DOCA) treatment (Ji et al., 2012b). After DOCA-salt treatment, P2X7R mRNA and protein expression was increased in WT mice, accompanied by a greater increase in systolic blood pressure (SBP), significantly greater than that of P2X7$^{-/-}$ mice. Inflammatory cell infiltration was reduced in P2X7$^{-/-}$ mice, and IL-1β release from macrophages was inhibited. Furthermore, the downstream effectors of IL-1β signaling, COX-2 and ROS were also affected; COX-2 expression being reduced in P2X7$^{-/-}$ mice and anti-oxidant levels being increased in their serum. Authors of the study suggested that blockade of the P2X7R-mediated inflammatory responses protect renal function against P2X7R-associated renal injury, this being reflected by reduced albuminuria and renal interstitial fibrosis, and increased creatinine clearance (Ji et al., 2012b). With regard to hypertension, both WT and P2X7$^{-/-}$ exhibited initial increases in SBP following DOCA-salt treatment, and only at day-4 of treatment were differences in SBP observed between the two animal groups; P2X7$^{-/-}$ mice having significantly lower SBP than WT mice. This study built on previous observations demonstrating that immune cell infiltration was pivotal to the development of salt-sensitive hypertension (Franco et al., 2007), authors of this preceding study reported P2X7R expression by immune cells exacerbated renal injury by instigating a vicious cycle of inflammation. Additional *in vivo* evidence to support this has been attained from pharmacological studies performed in Dahl salt-sensitive (DS) rats (Ji et al., 2012a). Hypertensive rats were infused with two P2X7R selective antagonists (brilliant blue G and A-438079); the salt-sensitive hypertension, urinary protein or albumin excretion, renal interstitial fibrosis, and macrophage and T-cell infiltration in the DS rats was markedly reduced, whilst creatinine clearance significantly improved (Ji et al., 2012a). Collectively these novel studies provide persuasive evidence that the P2X7R is critical in hypertension and renal injury progression, thus highlighting the therapeutic potential for this receptor in a number of disease states underlying salt-sensitive hypertension, including but not limited to, hyperaldosteronism.

As previously mentioned, blockade of P2X7R has been shown to inhibit IL-1β release from renal macrophages (Ji et al., 2012b),

and this may have significant consequences for the underlying mechanisms of certain forms of renal disease. Renal fibroblasts are activated by IL-1β amongst other cytokines and due to the P2X7R/IL-1β axis it seems pertinent to assume P2X7R-mediated attenuation of fibroblast activation could occur. Whilst this may have beneficial effects in terms of reducing renal interstitial fibrosis, activation of renal fibroblasts post acute kidney injury (AKI) is vital for normal renal repair. AKI is typically characterized by renal tubular cell death and associated release of high concentrations of ATP into the interstitium, combined with the associated damage to the tubular basement membrane, to which interstitial fibroblasts are directly connected; this milieu provides an ideal setting for P2X7R to play a prominent role in either AKI associated fibrosis or renal repair. To investigate this paradigm, a recent study investigated the effects of supernatant from necrotic renal proximal tubular cells (RPTC) on the viability of renal fibroblasts (Ponnusamy et al., 2011). The supernatant from necrotic RPTC was shown to induce the expression of P2X7R in cultured rat renal interstitial fibroblast cells (NRK-49F), and the ATP released from necrotic RPTC induced NRK-49F cell death by a P2X7R-mediated mechanism, therefore demonstrating a deleterious direct renal epithelial-fibroblast cross-talk pathway (Ponnusamy et al., 2011). It was concluded that damaged renal epithelial cells could directly induce the death of renal interstitial fibroblasts by ATP activation of P2X7R. This evidence was not, however, in accordance with a previous study which showed that the number of Epo-TAg (a genetic marker for cortical interstitial fibroblasts) expressing fibroblasts is reduced in the injured kidney, but upon intense stimulation such as severe anemia or hypoxia, there was a greater increase in Epo-TAg expressing fibroblasts in the injured kidney which consequently narrowed the disproportion between the injured and uninjured kidney. Authors conclude that rather than fibroblasts being destroyed in the event of AKI, the threshold for stimulated gene expression is increased (Maxwell et al., 1997), which could possibly be mediated by P2X7R. So it is unclear if AKI results in a reduced number of renal interstitial fibroblasts or an alteration in cell properties.

Renal interstitial fibroblasts have both beneficial and deleterious effects on the kidney by playing a key role in renal repair and renal fibrosis, respectively. Consequently, interstitial fibroblast cell proliferation, activation, and death are tightly regulated by numerous factors. It is certainly plausible that ATP is one such factor and P2X7R is an important mediator. It has been shown *in vitro* that P2X7R activation directly influences interstitial fibroblast cell death (Ponnusamy et al., 2011). However, the possibility exists that P2X7R also indirectly influences fibroblast activation by inducing IL-1β release from infiltrating immune cells (Ji et al., 2012b). Both studies propose P2X7R as a therapeutic target. Although pharmacological blockade of P2X7R was highly effective at attenuating renal fibrosis, the implications this may have on the repair response post-AKI need to be considered.

Lastly, in addition to the pro-inflammatory and pro-death functions of P2X7R discussed in preceding paragraphs there seems also to be a role for P2X7R in autosomal dominant polycystic kidney disease (ADPKD). This common, yet complex, genetic renal disease occurs in response to mutations in either the PKD1 or PKD2 gene (Mochizuki et al., 2013), and is characterized by progressive cyst enlargement due to excess fluid secretion and proliferation of renal tubular epithelial cells (Torres et al., 2009). Whilst this condition is a major cause of end stage renal disease, there still lacks a clinically approved therapy, primarily due to the slow progression of the disease and complexity of its development. The current focus is to assess the potential of combination therapies targeting either cyst development through inhibition of cell proliferation, and/or cyst growth through attenuation of fluid secretion. Recent data yielded from the zebrafish model of ADPKD where PKD2 is knocked-down, described increased P2X7R mRNA expression at an early stage, which coincided with cyst formation in the glomerulus and tubular regions in 75% of PKD2 morphants. Pharmacological blockade with a P2X7R antagonist OxATP reduced the frequency of cyst formation to 35%, suggesting that P2X7R contributes in some way to cyst formation in ADPKD (Chang et al., 2011). PKD2 morphants demonstrate increased ERK activation in primary cilia, which is linked to enhanced cell proliferation and fluid secretion and hence cyst enlargement in PKD. Previous studies have demonstrated that P2X7R acts as a flow sensor for ERK activation in osteoblasts (Okumura et al., 2008). Given that authors describe inhibition of P2X7R as suppressing the elevated ERK activation in PKD2 morphants by over 50%, coupled with the fact cilium bending has been shown to trigger ATP release, suggests a similar P2X7R-coupled mechanism may be present in renal epithelial cells. This hypothesis is further substantiated by findings that demonstrated inhibition of P2X7R did not suppress cyst progression through the traditional anti-inflammatory mechanism (Chang et al., 2011; Praetorius and Leipziger, 2013). Whilst extremely novel, these data are, however, at odds with previously published data collected from studies using the MDCK model of cyst formation. Here authors agreed that ERK activation is an important factor in cyst growth. However, they found no evidence for the involvement of a P2XR, and concluded that a P2YR is responsible for cAMP-dependent activation of the ERK pathway and cyst growth (Turner et al., 2007).

## SUMMARY

P2XR represent a receptor group important in the regulation of tubular and vascular function in both kidney health and disease (see **Figures 1**, **2** for a summary). Much of the recent data providing compelling functional evidence that substantiate this phenomenon has been generated from knockout mouse studies, which further attests to the importance of these models in improving our knowledge of how kidney function is regulated. As a result of these studies there appear to be three key P2XR subunits/subtypes, P2X1, 4, and 7, which have emerged as having numerous roles in the regulation of kidney function. As such, these receptor subtypes not only represent favorable novel therapeutic targets in multiple pathological settings, but also represent a novel focus for future research on the role of nucleotides in the kidney.

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