Flow regulation of endothelin-1 production in the inner medullary collecting duct

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Pandit MM, Inscho EW, Zhang S, Seki T, Rohatgi R, Gusella L, Kishore B, Kohan DE. Flow regulation of endothelin-1 production in the inner medullary collecting duct. Am J Physiol Renal Physiol 308: F541–F552, 2015. First published January 13, 2015; doi:10.1152/ajprenal.00456.2014.—Collecting duct-derived endothelin (ET)-1 is an autocrine inhibitor of Na+ and water reabsorption; its deficiency causes hypertension and water retention. Extracellular fluid volume expansion increases collecting duct ET-1, thereby promoting natriuresis and diuresis; however, how this coupling between volume expansion and collecting duct ET-1 occurs is incompletely understood. One possibility is that volume expansion increases tubular fluid flow. To investigate this, cultured IMCD3 cells were subjected to static or flow conditions. Exposure to a shear stress of 2 dyn/cm² for 2 h increased ET-1 mRNA content by ~2.3-fold. Absence of perfluorocarbon (PFC) or phospholipase C prevented the flow response. Evaluation of possible flow-activated Ca2+ entry pathways revealed no role for transient receptor potential (TRP)-C3, TRPC6, and TRPV4; however, cells with TRPP2 (polycystin-2) knockdown had no ET-1 flow response. Flow increased intracellular Ca2+ but was blunted in TRPP2 knockdown cells. Nonspecific blockade of P2 receptors, as well as specific inhibition of P2X2 and P2Y2 receptors, prevented the ET-1 flow response. The ET-1 flow response was not affected by inhibition of either epithelial Na+ channels or the mitochondrial Na+/Ca2+ exchanger. Taken together, these findings provide evidence that in IMCD3 cells, flow, via polycystin-2 and P2 receptors, engages Ca2+-dependent signaling pathways that stimulate ET-1 synthesis.

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IMCD produces substantially more ET-1 and expresses a higher density of ET receptors than the CCD (27, 53). Consequently, to address this question, the present study was undertaken to examine the regulation of IMCD ET-1 production. We report flow-stimulated IMCD ET-1 production and, of greatest importance, describe two key findings: 1) an interaction between the purinergic and ET systems that may combine to elicit a sustained diuretic and natriuretic response and 2) a role for polycystin-2 in this flow effect.

MATERIALS AND METHODS

Reagents. Calcineurin inhibitory peptide, calphostin C, Pyr3, SKF-96365, 5-BDBD, A-74003, and A-438079 hydrochloride were obtained from Tocris Bioscience (Ellisville, MO). Dinolosin pentaphosphate was obtained from Timtec (Newark, DE). ARC-118925 was generously provided by Prof. Dr. Christa E. Müller (Pharmaceutical Institute, University of Bonn, Bonn, Germany). All other drugs and chemicals was obtained from Sigma (St. Louis, MO) unless stated otherwise.

Na\(^+\) and water loading experiments. Male Sprague-Dawley rats (200–250 g, Harlan Laboratories, Indianapolis, IN) and C57BL/6 mice (25 g) were handled in accordance with University of Utah Institutional Animal Care and Use Committee requirements. For salt loading experiments, all rats and mice were fed normal NaCl (0.25%) or high-NaCl (8%) diets and given free access to drinking water for 3 days each. For water loading, rats and mice were given free access to a regular NaCl diet plus water or water containing 1% sucrose for 3 days each. IMCDs were isolated using previously described procedures (47). Briefly, renal inner medullas were minced and incubated in HBSS (containing 1.26 mM CaCl\(_2\), 0.49 mM MgCl\(_2\), 0.41 mM MgSO\(_4\), 4.3 mM KCl, 0.44 mM KH\(_2\)PO\(_4\), 4.2 mM NaH\(_2\)PO\(_4\), 138 mM NaCl, 0.34 mM NaH\(_2\)PO\(_4\), and 5.6 mM dextrose) supplemented with 15 mM HEPES (pH 7.4) at 37°C. The digest was filtered through a 96-μm mesh screen to remove any residual tissue. The suspension was centrifuged for 5 min at 1,500 rpm, and the cell pellet was washed with HBSS. The final cell pellet was used for RNA analysis as described below.

RNA analysis and real-time PCR. RNA from acutely isolated and cultured cells was obtained using the RNeasy Mini Kit and reverse transcribed using an Omniscript RT Kit (Qiagen, Valencia, CA). GAPDH and ET-1 mRNA levels were determined by real-time PCR (StepOne Plus, Applied Biosystems, Foster City, CA) using primers (Integrated DNA Technologies, Coralville, IA) for the P2Y1 and P2Y14 receptor genes to detect all P2 receptor mRNA. Sense and antisense primers (Integrated DNA Technologies, Coralville, IA) were designed and tested for the P2Y14 receptor gene to detect all P2 receptor mRNA. Sense and antisense primers (Integrated DNA Technologies, Coralville, IA) were designed and tested for the P2Y14 receptor gene to detect all P2 receptor mRNA.

RT-PCR analysis for the detection of P2 receptor mRNA. P2X and P2Y receptor mRNA expression were identified in IMCD3 cell extracts using two-step RT-PCR. C57BL/6 mouse kidney and brain total RNA were used as positive controls. The extracted RNA was quantified by microplate spectrophotometer (Take3, SynergyH1, Bio Tek Instruments, Winooski, VT). P2X and P2Y receptor cDNA sequences were obtained from GenBank. Sense and antisense primers (Integrated DNA Technologies, Coralville, IA) for each gene (Table 1) were designed on different exons on an agarose gel using the Fluorchem E system (Proteinsimple, Santa Clara, CA). PCR products were run on a 2% agarose gel containing 0.5 μg/ml ethidium bromide and visualized using the following primers: (i) Taq polymerase (Taq PCR Core Kit, Quantigen Sciences), and (ii) RT-PCR analysis for the detection of P2 receptor mRNA.

Table 1. List of primers for PCR of purinergic receptor isoforms

<table>
<thead>
<tr>
<th>P2 Isoform</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Size, bp</th>
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<td>P2X1</td>
<td>5'-GCCTGATACGCTTTGCGGTGCTG-3'</td>
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<td>255 bp</td>
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<td>P2X2</td>
<td>5'-GCCTGATACGCTTTGCGGTGCTG-3'</td>
<td>5'-TGACAAATGTCTTGCTGCGTGG-3'</td>
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<td>P2X3</td>
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<tr>
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<tr>
<td>P2x13</td>
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<td>5'-TGACAAATGTCTTGCTGCGTGG-3'</td>
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<td>P2x14</td>
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<td>5'-TGACAAATGTCTTGCTGCGTGG-3'</td>
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Cell culture. The mouse IMCD cell line IMCD3 was used for all experiments unless specified otherwise. IMCD3 cells with transfected receptor potential (TRP)P2 (polycystin-2) knockdown were provided by Dr. Rajeev Rohatgi and Dr. Luca Gusella (Icahn School of Medicine, Mount Sinai, NY) (8). Cells were grown to confluence on 10-cm\(^2\) plastic culture plates in a 5% CO\(_2\) incubator at 37°C; 50-50 DMEM-F-12 supplemented with 10% FBS, 1 mg/ml penicillin, and 1 mg/ml streptomycin was used as the growth medium. For control experiments done under stationary conditions, cells were grown in 12-well plates under identical conditions.

Flow experiments. Rectangular parallel plate polycarbonate flow chambers (catalog no. 31-010, Glycotech, Gaithersburg, MD) were attached to individual 10-cm2 cell culture plates containing confluent IMCD3 cells using vacuum and silicon gaskets to form a channel. The channel had the following dimensions: 0.25 mm depth, 1 cm width, and 5.9 cm length, with a total surface area of 5.9 cm\(^2\) for cells exposed to flow. The flow chamber had two manifolds through which perfuse entered and exited the channel. The liquid was pumped through the channel by a peristaltic pump (Ismatec, Glattburg, Switzerland) to obtain specific shear stresses. HBSS (pH 7.4) was used as the perfuse for control experiments and was supplemented with drugs and/or chemicals for additional experiments. RNA was extracted from cells exposed to flow and from control cells. All experiments were performed at 37°C.

RT-PCR analysis for the detection of P2 receptor mRNA. P2X and P2Y receptor mRNA expression were identified in IMCD3 cell extracts using two-step RT-PCR. C57BL/6 mouse kidney and brain total RNA were used as positive controls. The extracted RNA was reverse transcribed at 42°C for 30 min in a 20-μl reaction volume using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The cDNA template was added to a 50-μl PCR including Taq DNA polymerase (Taq PCR Core Kit, Qiagen Sciences), and PCRs were performed on an iCycler RT-PCR system (Bio-Rad). Amplified PCR products were run on a 2% agarose gel containing 0.5 μg/ml ethidium bromide and visualized using the Fluorchem E system (ProteinSimple, Santa Clara, CA). Product size was estimated with an ExaCTGene 100-bp DNA ladder (Thermo Fisher Scientific, Waltham, MA). Five primer sets were designed and tested for the P2Y14 receptor gene to detect all eight reported splice variants, and we found that the set shown in Table 1 showed specific bands for IMCD3 cells.
Table 2. List of amino acid sequence lengths and predicted molecular masses of purinergic receptor isoforms

<table>
<thead>
<tr>
<th>P2 Isoform</th>
<th>Sequence Length, amino acids</th>
<th>Predicted Molecular Mass, kDa</th>
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<tr>
<td>P2X1</td>
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<td>44</td>
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<td>P2X2</td>
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<td>P2X3</td>
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<td>P2Y4</td>
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<tr>
<td>P2Y14</td>
<td>338</td>
<td>38</td>
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</table>

Western blot analysis of P2 receptor expression. Mouse IMCD3 cells were lysed and prepared for Western blot analysis as previously described (42). Protein concentration was determined using the Bradford assay (Bio-Rad). Samples were diluted with sample buffer and denatured (10 min, 70°C) using a dry bath incubator. Equal amounts of protein from each sample (run in duplicate) were separated electrophoretically using 4–12% Bolt bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred to Hybond ECL nitrocellulose blotting membranes (GE Healthcare Bio-Sciences, Piscataway, NJ). Membranes were blocked in a solution of 5% nonfat dry milk and PBS + 0.1% Tween 20 (pH 7.4, 60 min) followed by an overnight incubation (4°C) with rabbit polyclonal anti-P2 receptor primary antibody (P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, P2Y1, P2Y2, P2Y4, P2Y5, P2Y12, P2Y13, and P2Y14, Alomone Labs, Jerusalem, Israel). Blots were washed with PBS + 0.1% Tween 20 and incubated with secondary antibody (1:4,000, goat anti-rabbit IgG horseradish peroxidase conjugate) and set on the stage of a Nikon Eclipse TE300 inverted epifluorescence microscope linked to a cooled Pentamax charge-coupled device camera (Princeton Instruments) interfaced with a digital imaging system (MetaFluor, Universal Imaging, Westchester, PA). The chamber temperature was maintained at 37°C with an FCS2 Temperature Controller (Biop tecnics) and perfused using a FCS Micro-Perfusion Pump (Biop tecnics). Cells were viewed and imaged using a x40 epifluorescence objective, which does not require water or oil immersion. Cells were initially kept under static conditions for at least 400 s to ensure that fluorescence was stable. The shear generated across the monolayer was calculated using Poiseuille’s law [τ = μγ = 6μQa2b, where τ is wall stress (in dyn/cm²), γ is shear rate (in s), μ is the apparent viscosity of the fluid (media at 37°C = 0.76 cP), Q is the volumetric rate (in ml/s), a is the channel height (in cm), and b is the channel width (in cm)] to generate a fluid shear stress of ~0.4 dyn/cm². Throughout the experiment, cells were alternately excited at 340 and 380 nm, and images acquired every 1–15 s were digitized for subsequent analysis. Images were acquired every 1 s just before cells were exposed to shear stress and continued up until the peak fluorescence was achieved. At the conclusion of each experiment, an intracellular Ca²⁺ calibration was performed using standard techniques, which included using Ca²⁺-free solution containing EGTA-AM (10 μM) for 10–15 min and a Ca²⁺-containing solution with ionomycin (10 μM). Standard equations were used to calculate experimental values of [Ca²⁺]i for the cells monitored. Four to nine centrally located cells were analyzed in each monolayer per experiment. The mean baseline [Ca²⁺]i value for each cell was calculated by averaging the eight [Ca²⁺]i values measured just before shear was increased. Peak [Ca²⁺]i was taken as the average of the three highest [Ca²⁺]i values after the induction of fluid shear stress (43).

Statistics. Data are presented as means ± SE. One-way ANOVA was used to compare differences between groups. A Shapiro-Wilk test was used to evaluate for normal distribution. A paired t-test was used to evaluate the statistical significance of basal [Ca²⁺]i versus peak [Ca²⁺]i within the same cell. An unpaired t-test was used to compare [Ca²⁺]i between control and TRPP2-deficient cells. P values of <0.05 were considered significant.

RESULTS

Na⁺ and water loading in the IMCD. To determine the effect of salt or water loading on ET-1 mRNA production, acutely isolated IMCDs from mice or rats fed a normal or high NaCl or water diet were evaluated for ET-1 mRNA content. In both mice (Fig. 1A) and rats (Fig. 1B), salt or water loading increased ET-1 mRNA content. The data on NaCl loading have been previously published (28) but are included here for purposes of comparison with water loading. It should be noted that ET-1 mRNA, as opposed to ET-1 protein, was measured in the present study (in both acutely isolated and cultured IMCDs). ET-1 protein release or cell content is not detectable due to the relatively small numbers of cells involved. However, in essentially every condition in which ET-1 mRNA and protein have been measured, ET-1 mRNA reflects ET-1 protein.

**Fig. 1.** Effect of Na⁺ or water loading on inner medullary collecting duct (IMCD) endothelin (ET)-1 mRNA content in rats (A) and mice (B). For Na⁺ loading, rats and mice were fed a normal NaCl (0.25%) or high-NaCl (8%) diet for 3 days. For water loading, rats and mice were fed 1% sucrose in normal drinking water for 3 days. n = 3 for each data point. *P < 0.05 vs. animals fed a normal NaCl and water diet.
levels (27, 48). This may be due, at least in part, to the fact that ET-1 mRNA is very short lived (half life of ~15 min) due to the presence of destabilizing AUUUA sequences in the 3’-untranslated region of the ET-1 message (31, 32).

Effect of flow on IMCD3 ET-1 mRNA. To determine the effect of flow on IMCD3 ET-1 mRNA, an empiric 2-h period was chosen to expose cells to flow at shear stresses from 0–7.5 dyn/cm². All levels of shear stress (0.25, 0.5, 1, 2, 5, and 7.5 dyn/cm²) stimulated ET-1 mRNA: the degree of stimulation (~2–2.5-fold increase) was not significantly different between shear stress magnitudes (Fig. 2A). A shear stress of 2 dyn/cm² was chosen for all further experiments. A time course was performed at a shear stress of 2 dyn/cm² for different lengths of time.

By 4 h, cells had begun to detach. Based on these findings, all subsequent experiments were carried out for 2 h at a shear stress of 2 dyn/cm². Please note that since ET-1 production is largely regulated at the transcriptional level, it is not unexpected for the flow effect to require 1–2 h to detectably alter ET-1 mRNA levels.

Role of Ca²⁺ in flow-stimulated IMCD3 ET-1 mRNA production. Since shear stress-induced cell signaling may depend, at least in part, on changes in [Ca²⁺]i (3, 45), the involvement of Ca²⁺ in flow-regulated ET-1 mRNA accumulation in IMCD3 cells was determined. Cells were exposed to no flow and flow conditions using Ca²⁺-free HBSS (no CaCl₂ added to the formulation and no EGTA added). Absence of media Ca²⁺ prevented the flow-stimulated ET-1 mRNA increase (Fig. 3A). To examine the role of [Ca²⁺]i in the ET-1 flow response, cells were pretreated for 30 min with BAPTA-AM (an intracellular Ca²⁺ chelator, hereafter referred to as BAPTA) and then exposed to static or flow conditions. The ET-1 flow response was completely prevented by treatment with BAPTA (Fig. 3A). In addition, BAPTA reduced basal (no flow) ET-1 mRNA, possibly due to inhibition of autocrine activation of Ca²⁺-dependent ET-1 production.
Thus, both extracellular and intracellular Ca\(^{2+}\) are required for the ET-1 flow response in IMCD3 cells.

**Effect of inhibition of Ca\(^{2+}\) signaling molecules on flow-stimulated IMCD3 ET-1 mRNA production.** Given that Ca\(^{2+}\) is involved in the ET-1 flow response in IMCD3 cells, we next investigated the role of pathways mediating Ca\(^{2+}\) signaling. Inhibition of calmodulin (CaM) with calmidazolium chloride markedly reduced the ET-1 flow response (Fig. 3B). KN-93, an inhibitor of CaM-dependent kinase (CaMK), also greatly inhibited the ET-1 flow response (Fig. 3B). Both CaM and CaMK inhibition reduced basal (no flow) IMCD3 ET-1 mRNA. Since ET-1 in endothelial cells can be modulated by shear stress via Ca\(^{2+}\)-sensitive pathways [phospholipase C (PLC) and PKC], we investigated their role in the ET-1 flow response. Inhibition of PKC (calphostin C) prevented the flow-stimulated ET-1 mRNA increase (Fig. 3C). PLC inhibition (U-73122) also prevented flow-induced ET-1 mRNA accumulation in IMCD3 cells (Fig. 3C). Since the above Ca\(^{2+}\) signaling pathways can modulate calcineurin, the effect of inhibition of calcineurin on the IMCD3 ET-1 flow response was evaluated. Inhibition of calcineurin (cyclosporine A or calcineurin inhibitory peptide) prevented the flow-stimulated ET-1 increase (Fig. 3D). Taken together, these data suggest the involvement of Ca\(^{2+}\)/CaM/CaMK/PLC/PKC/calcineurin in flow-regulated IMCD ET-1 mRNA accumulation.

**TRP channels and flow-stimulated IMCD3 ET-1 mRNA production.** CD cells express TRP channels on their apical membrane that mediate Ca\(^{2+}\) entry and are flow sensitive, including TRPC3, TRPC6, and TRPV4 (15, 55) as well as TRPP2 (39). Treatment with inhibitors of TRPC3 (Pyr3), TRPC6 (SKF-96365), and TRPV4 (RN-1734) did not significantly alter flow-induced ET-1 mRNA accumulation (Fig. 4). In contrast, an IMCD3 cell line with knockdown of polycystin-2 expression was subjected to flow; no increase in ET-1 mRNA in response to flow was observed (Fig. 4). Taken together, these data suggest a role for polycystin-2, but not TRPC3, TRPC6, or TRPV4, in flow-stimulated ET-1 mRNA accumulation in IMCD3 cells.

**Effect of flow on [Ca\(^{2+}\)] in normal and TRPP2 knockdown cells.** While the above experiments strongly indicated that flow increases [Ca\(^{2+}\)] in IMCD3 cells, this was directly confirmed by measurement of [Ca\(^{2+}\)], using fura-2 AM-loaded cells. Figure 5 shows representative flow-induced [Ca\(^{2+}\)] transients in these two cell types. As shown in Fig. 6, flow (0.4 dyn/cm\(^2\)) increased [Ca\(^{2+}\)] from a basal level of 85.7 ± 8.2 nM to a peak
of 243.4 ± 24.9 nM. To determine whether loss of the ET-1 flow response in IMCD3 TRPP2 knockdown cells was associated with an altered [Ca\(^{2+}\)]\(_i\), response to flow, IMCD3 cells with TRPP2 knockdown were evaluated. These cells had lower basal [Ca\(^{2+}\)]\(_i\) (50.2 ± 4.1 nM) and also had a reduced peak [Ca\(^{2+}\)]\(_i\), response to flow (121.8 ± 11.8 nM; Fig. 6). It should be noted that TRPP2 knockdown is ~80% in these cells, as determined in the present study and as previously reported (8), so it is not possible to say whether incomplete blockade of the [Ca\(^{2+}\)]\(_i\), response to flow was due to remaining TRPP2 or some other mechanism.

Identification of purinergic receptors in flow-stimulated IMCD3 ET-1 mRNA production. In addition to polycystin-2 and other TRP channels, Ca\(^{2+}\) can enter cells via apical purinergic P2X receptors (25). Furthermore, apical purinergic P2Y receptors may modify P2X receptor-mediated Ca\(^{2+}\) entry (25). To assess for a possible role of purinergic receptors, IMCD3 cells were first exposed to a nonspecific purinergic receptor antagonist [pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS)], and the effect on flow-stimulated ET-1 mRNA was assessed. Treatment with PPADS abolished the ET-1 flow response (Fig. 7). To further confirm the role of purinergic receptors in the ET-1 flow response, IMCD3 cells were exposed to P2 receptor agonists to saturate the receptors and mitigate any additional purinergic signaling potentially elicited by flow. As shown in Fig. 6, preincubation with γ-ATP (relatively P2Y specific) or α,β-methylene-ATP (relatively P2X specific) largely prevented additional flow stimulation of ET-1 mRNA content. Notably, both ATP and α,β-methylene-ATP increased basal (no flow) ET-1 mRNA levels, suggesting that pretimulation of these receptors prevented their further activation by flow. Taken together, these data support the notion that purinergic receptors, and possibly both P2X and P2Y receptors, are involved in the flow regulation of IMCD3 ET-1 mRNA accumulation.

To begin to determine which P2 receptors may be involved in the ET-1 flow response, mRNA and protein expression for known P2 receptor isoforms were determined in IMCD3 cells. As shown in Fig. 8, mRNAs for all known isoforms of P2X receptors were detected. Bands were seen on Western blots for P2X isoforms (Fig. 9). However, in most cases, the expected molecular masses for the unmodified proteins were not detected; whether protein modification resulted in different molecular masses or the proteins were simply not present is an open question. As shown in Fig. 10, mRNAs for all known P2Y receptors were detected in IMCD3 cells with the exception of P2Y\(_{12}\) and P2Y\(_{14}\). Bands were seen on Western blots for P2X isoforms (Fig. 11); however, in some cases, the expected molecular masses were not observed. Please note that reverse transcriptase negative controls were run for all PCR samples and showed no bands. Cells were then pretreated with a variety of specific P2 isoform antagonists, and the effect on flow-stimulated ET-1 mRNA was assessed. Please note that attempting to block each purinergic receptor isoform was not feasible due to the lack of specific reagents; it was also impractical to use small interfering RNA for each receptor. Consequently, efforts were focused on P2 receptors that were known to be expressed by the IMCD in vivo and that had been implicated in possibly modifying Ca\(^{2+}\) response to flow. P2Y\(_2\) inhibition with ARC-118925 completely prevented flow-stimulated ET-1 mRNA accumulation (Fig. 7). Treatment with inhibitors of P2X\(_1\), P2X\(_3\) (0.1 μM and 10 μM diinosine pentaphosphate, respectively), or P2X\(_4\) (5-BDBD) did not alter the ET-1 flow response (Fig. 12). In contrast, treatment with the P2X\(_7\) antagonists A-438079 or A-740003 abolished the ET-1 flow response (Fig. 12). Thus, these data suggest that both P2Y\(_2\) and P2X\(_7\) mediate flow-stimulated IMCD ET-1 mRNA accumulation.

Purinergic receptor evoked signaling in ATP-stimulated IMCD3 ET-1 mRNA content. To determine whether the effect of ATP on IMCD3 ET-1 mRNA production was mediated by Ca\(^{2+}\) and Ca\(^{2+}\)-regulated signaling pathways, the effect of ATP on ET-1 mRNA in IMCD3 cells under stationary (no flow) conditions was assessed (Fig. 13). First, blockade of P2 receptors with PPADS prevented ATP stimulation of ET-1 mRNA, confirming it was acting via a P2 receptor. Removal of media Ca\(^{2+}\) or chelation of [Ca\(^{2+}\)]\(_i\), with BAPTA markedly reduced ATP-stimulated ET-1 mRNA. Inhibition of CaM or PLC also blocked the ATP response. Hence, ATP stimulation...
of IMCD3 ET-1 mRNA is substantially dependent upon [Ca^{2+}], and extracellular Ca^{2+} concentration and is completely dependent on CaM and PLC.

Role of ENaC on flow-stimulated IMCD ET-1 mRNA production. As described in the Introduction, the ET-1 flow response in mpkCCD cells is mediated by Na^+ delivery via ENaC (36). To determine if this mechanism is operative in IMCD3 cells, the effect of specific ENaC inhibitors (amiloride and benzamil) on the ET-1 flow response was assessed. Neither ENaC inhibitor modified flow-stimulated ET-1 mRNA accumulation in IMCD3 cells (Fig. 14). Finally, blockade of NCLX with CGP-371571 did not alter the ET-1 flow response. Thus, ENaC and NCLX are not involved in the ET-1 flow response in IMCD3 cells.

DISCUSSION

The present study reports that flow increases ET-1 mRNA in IMCD cells and that this response is dependent on 1) extracellular and intracellular Ca^{2+}, 2) CaM/CaMK/calcineurin and PLC/PKC pathways, 3) polycystin-2, and 4) activation of purinergic P2Y_2 and P2X_7 receptors. While these experiments were largely conducted in vitro, they raise a number of intriguing, albeit speculative, possibilities. First, they provide a possible explanation for how BFV expansion, at least in part, induces a natriuretic and diuretic response in the CD independent of circulating hormones: increased tubule fluid flow, by virtue of increased IMCD ET-1 production, could lead to autocrine inhibition of IMCD Na^+ and water transport. Second, these experiments describe, for the first time, polycystin-2 regulation of ET-1 production. Such a relationship may be relevant to renal salt and water excretion under normal physiological conditions as well as in the setting of impaired polycystin-2 function. The latter possibility is of particular interest given that hypertension commonly manifests in patients with polycystic kidney disease before apparent renal functional deterioration (10, 18). Third, the present study reports an interaction between CD purinergic and ET systems. Given that the CD purinergic system elicits rapid and transient inhibition (in minutes) (54), whereas the CD ET system causes delayed and sustained inhibition (in hours), of Na^+ and water transport, these findings suggest the presence of a temporally integrated regulatory system in the CD that provides both rapid onset and sustained inhibition of natriuresis and diuresis in response to BFV expansion. Taken together, our findings identify a novel system in the IMCD wherein flow, via polycystin-2 and purinergic receptor activation, activates Ca^{2+}...
signaling pathways that stimulate the production of ET-1, a highly potent and long-acting inhibitor of CD Na\textsuperscript{+}/H\textsuperscript{+} and water reabsorption.

Other key observations in the present study were that flow stimulation of IMCD ET-1 mRNA did not depend on ENaC or mitochondrial NCLX. These findings are in contrast to those observed in mpkCCD cells, a mouse CCD cell line, wherein flow-stimulated ET-1 mRNA accumulation was prevented by blockade of ENaC or NCLX (36). Both mpkCCDC14 and IMCD3 cells express ENaC and exhibit amiloride inhibition of apical-to-basal Na\textsuperscript{+}/H\textsuperscript{+} flux by amiloride (40); hence, the lack of ENaC dependence in IMCD3 is not due to absence of ENaC. However, given that the CCD contains substantially more ENaC than the IMCD (46), it is tempting to speculate that CCD ET-1 production is primarily dependent on Na\textsuperscript{+} delivery, whereas IMCD ET-1 production is primarily regulated by fluid flow (which would be increased during both salt and water loading). In such a scenario, one could envision CCD- and IMCD-derived ET-1 serving different biological roles, wherein the former is primarily intended to respond to salt loads, whereas the latter is primarily intended to respond to volume loads. In this regard, it is notable that in vivo IMCD ET-1 mRNA accumulation was stimulated by both salt and water loading (it is problematic to assess CCD ET-1 production is primarily dependent on Na\textsuperscript{+} delivery, whereas IMCD ET-1 production is primarily regulated by fluid flow (which would be increased during both salt and water loading). In such a scenario, one could envision CCD- and IMCD-derived ET-1 serving different biological roles, wherein the former is primarily intended to respond to salt loads, whereas the latter is primarily intended to respond to volume loads. In this regard, it is notable that in vivo IMCD ET-1 mRNA accumulation was stimulated by both salt and water loading (it is problematic to assess CCD ET-1 mRNA levels in the CCD is very difficult due to the inability to isolate sufficient numbers of cells or tubules).

The present study reports that Ca\textsuperscript{2+} is essential for the ET-1 flow response in IMCD3 cells. These findings are in agreement with a previous study (28) showing that flow-stimulated ET-1 mRNA in mpkCCD cells is dependent on intracellular and extracellular Ca\textsuperscript{2+}. In addition, ET-1 production by primary cultured rat IMCD cells under nonflow (stationary) conditions was Ca\textsuperscript{2+} dependent (48). We also found that the ET-1 flow response is mediated by PLC and PKC; similar findings have been observed in flow-stimulated mpkCCD cells (28) and endothelial cells (48) as well as in stationary rat IMCD cells (48). Notably, PKC regulates ET-1 gene transcription in rat IMCD cells via a nonclassical activator protein-1-like site in the ET-1 promoter (49). Finally, the ET-1 flow response in IMCD3 cells was dependent on CaM, CaMK, and calcineurin, in agreement with a previous study (48) showing a dependence of ET-1 production on these enzymes in stationary cultures of rat IMCD cells. In contrast, the ET-1 flow response in mpkCCD cells was not dependent on CaM-regulated pathways (28), suggesting a difference not only in the initial sensing component between the CCD and IMCD but also the Ca\textsuperscript{2+} signaling pathways involved. Taken together, the results of the present study indicate that Ca\textsuperscript{2+}/CaM/CaMK/calcineurin- and PLC/PKC-dependent pathways are required for flow-stimulated ET-1 mRNA accumulation in the IMCD.

Since extracellular Ca\textsuperscript{2+} is required for the ET-1 flow response in IMCD, we sought to do limited analysis that identify pathways potentially involved in flow-stimulated Ca\textsuperscript{2+} entry. The IMCD contains several flow-regulated apical plasma membrane Ca\textsuperscript{2+}-permeable cation channels (35), which include TRPC3 (14), TRPC6 (16), and TRPV4 (21, 57); however, blockade of these channels did not alter the ET-1 flow response. We cannot exclude that these TRP channels are involved in vivo since our in vitro system may not have normal apical membrane expression of these channels. In contrast, knockdown of polycystin-2, a Ca\textsuperscript{2+}-permeable cation channel (35), completely prevented the ET-1 flow response. Our findings indicate that additional studies would be important to evaluate the role of polycystin-2 in mediating the ET-1 flow response. In addition to examination of such fundamental mechanisms, our findings raise interesting possibilities, as stated earlier, about the early hyperten-
Fig. 10. RT-PCR analysis of P2Y receptor mRNA expression in IMCD3 cells. Results from two separate IMCD3 samples are shown for all blots except P2Y₁₂ and p2Y₁₄. C57BL/6 mouse brain and kidney total RNA were used as positive controls for P2Y₁₂ and/or p2Y₁₄. Primer sequences used for each receptor are shown in Table 1. Arrows indicate the expected band size.

Fig. 11. Western blot analysis of P2Y receptor expression in IMCD3 cells. Results are shown in duplicate for each receptor subtype along with β-actin loading controls. Each of the two lanes was loaded with IMCD3 cell lysates (20 μg protein/lane). Predicted molecular sizes are shown in Table 2. Arrows represent the approximate predicted protein size based on the Accession Number database (http://www.uniprot.org/uniprot).
receptors are expressed on IMCD cells in vivo (52); we found response to flow is via purinergic-gated P2X receptors. P2X PPADS (a P2X inhibitor), media lacking Ca\textsuperscript{2+} under stationary conditions. Cells were pretreated for 30 min with 30 μM diinosine pentaphosphate [IP\textsubscript{51} (LD); a P2X\textsubscript{i} inhibitor], 10 μM diinosine pentaphosphate [IP\textsubscript{51} (HD); a P2X\textsubscript{i} inhibitor], 15 μM 5-BDBD (a P2X\textsubscript{4} inhibitor), 50 μM A-438079 (a P2X\textsubscript{7} inhibitor), or 20 μM A-74003 (a P2X\textsubscript{7} inhibitor) and exposed to static or flow (2 h at 2 dyn/cm\textsuperscript{2}) conditions followed by the determination of ET-1/GAPDH mRNA levels. n = 10 for each data point. *P < 0.05 vs. cells treated identically but not exposed to flow.

Fig. 12. Effect of P2X receptor isomorph inhibition on flow-stimulated ET-1 mRNA levels in IMCD3 cells. Cells were pretreated for 30 min with 0.1 μM diinosine pentaphosphate [IP\textsubscript{51} (LD); a P2X\textsubscript{i} inhibitor], 10 μM diinosine pentaphosphate [IP\textsubscript{51} (HD); a P2X\textsubscript{i} inhibitor], 15 μM 5-BDBD (a P2X\textsubscript{4} inhibitor), 50 μM A-438079 (a P2X\textsubscript{7} inhibitor), or 20 μM A-74003 (a P2X\textsubscript{7} inhibitor) and exposed to static or flow (2 h at 2 dyn/cm\textsuperscript{2}) conditions followed by the determination of ET-1/GAPDH mRNA levels. n = 10 for each data point. *P < 0.05 vs. cells treated identically but not exposed to flow.

Another mechanism by which Ca\textsuperscript{2+} could enter cells in response to flow is via purinergic-gated P2X receptors. P2X receptors are expressed on IMCD cells in vivo (52); we found that IMCD cells express all known P2X isoform mRNA and possibly several of the proteins. Blockade of P2X\textsubscript{7}, but not P2X\textsubscript{1}, P2X\textsubscript{3}, or P2X\textsubscript{4}, receptors prevented the ET-1 flow response. Blockade of P2X\textsubscript{7} receptors reduces ATP modulation of salt and water transport in pronephric ducts (20, 22). Since P2Y receptors have been reported to interact with P2X receptors in elevating [Ca\textsuperscript{2+}]\textsubscript{i} levels (25), the role of P2Y receptors in modifying the ET-1 flow response was investigated.

Fig. 13. Effect of modulation of Ca\textsuperscript{2+}, Ca\textsuperscript{2+}-regulated pathways, or P2 receptor blockade on ATP-stimulated ET-1 mRNA levels in IMCD3 cells under stationary conditions. Cells were pretreated for 30 min with 30 μM PPADS (a P2X inhibitor), media lacking Ca\textsuperscript{2+}, 50 μM BAPTA-AM (an intracellular Ca\textsuperscript{2+} chelator), 20 μM calmidazolium chloride (an inhibitor of Ca\textsuperscript{2+}), or 2 μM U-73122 (a PLC inhibitor) followed by exposure to vehicle or 30 μM γ-ATP for 2 h and then the determination of ET-1/GAPDH mRNA levels. n = 8–10 per data point. *P < 0.05 vs. no ATP. **P < 0.05 vs. no ATP and vs. the ATP control (without inhibitor or no Ca\textsuperscript{2+} media); #P < 0.05 vs. the no ATP control.

In summary, the present study makes several novel and potentially important observations. First, we demonstrated that polycystin-2 is necessary for flow-stimulated ET-1 synthesis in IMCD3 cells. Second, we reported that P2X\textsubscript{7} and P2Y\textsubscript{2} receptors are required for the flow response and that their activity depended on polycystin-2. Third, these systems ultimately...
modulate Ca$^{2+}$-dependent signaling pathways. While further studies are needed to validate this proposed system in vivo, we speculate that the polycystin-2/P2 receptor/ET-1 system constitutes a complex interactive pathway through which the CD can detect tubule fluid flow and ultimately achieve both immediate and sustained regulation of CD salt and water reabsorption. This system may be of physiological relevance and has at least the theoretical potential to be involved in hypertensive states characterized by impaired CD polycystin-2, purinergic, or ET system dysfunction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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