Connexin 30 Deficiency Impairs Renal Tubular ATP Release and Pressure Natriuresis

Arnold Sipos,*† Sarah L. Vargas,* Ildikó Toma,* Fiona Hanner,* Klaus Willecke,‡ and János Peti-Peterdi*†

*Departments of Physiology and Biophysics and Medicine, Zilkha Neurogenetic Institute, University of Southern California, Los Angeles, California; †Hungarian Academy of Sciences, Research Group for Pediatrics and Nephrology, and Institute of Pathophysiology, Semmelweis University, Faculty of Medicine, Budapest, Hungary; and ‡Institute of Genetics, Division of Molecular Genetics, University of Bonn, Bonn, Germany

ABSTRACT

In the renal tubule, ATP is an important regulator of salt and water reabsorption, but the mechanism of ATP release is unknown. Several connexin (Cx) isoforms form mechanosensitive, ATP-permeable hemichannels. We localized Cx30 to the nonjunctional apical membrane of cells in the distal nephron and tested whether Cx30 participates in physiologically important release of ATP. We dissected, partially split open, and microperfused cortical collecting ducts from wild-type and Cx30-deficient mice in vitro. We used PC12 cells as ATP biosensors by loading them with Fluo-4/Fura Red to measure cytosolic calcium and positioning them in direct contact with the apical surface of either intercalated or principal cells. ATP biosensor responses, triggered by increased tubular flow or by bath hypotonicity, were approximately three-fold greater when positioned next to intercalated cells than next to principal cells. In addition, these responses did not occur in preparations from Cx30-deficient mice or with purinergic receptor blockade. After inducing step increases in mean arterial pressure by ligating the distal aorta followed by the mesenteric and celiac arteries, urine output increased 4.2-fold in wild-type mice compared with 2.6-fold in Cx30-deficient mice, and urinary Na⁺/H⁺ excretion increased 5.2-fold in wild-type mice compared with 2.8-fold in Cx30-deficient mice. Furthermore, Cx30-deficient mice developed endothelial sodium channel–dependent, salt-sensitive elevations in mean arterial pressure. Taken together, we suggest that mechanosensitive Cx30 hemichannels have an integral role in pressure natriuresis by releasing ATP into the tubular fluid, which inhibits salt and water reabsorption.

It is well established that renal tubular epithelial cells release ATP,1–4 which then binds to purinergic receptors along the nephron and collecting ducts to regulate salt and water reabsorption5–12; however, the molecular mechanism of ATP release and the identity of ATP channels are less clear. The possible mechanisms include vesicular release,1,2 pannexin or connexin hemichannels,13–16 ATP-permeable anion channels such as the cystic fibrosis transmembrane conductance regulator,17 or the maxi anion channel.18 Previous work also suggested that the release of ATP in epithelia may be triggered by mechanical stimulation,19–21 including elevations in tubular fluid flow rates. For example, tubular flow–dependent ATP release resulting in purinergic calcium signaling has been well characterized in the cortical collecting duct (CCD)21–23; however, the ATP release mechanism has not been identified.

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Correspondence: Dr. János Peti-Peterdi, Department of Physiology and Biophysics and Department of Medicine, Zilkha Neurogenetic Institute, University of Southern California, 1501 San Pablo Street, ZNI 335, Los Angeles, CA 90033. Phone: (323) 442-4337, Fax: (323) 442-4466; E-mail: petipete@usc.edu

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Connexin 30 (Cx30) is a member of the connexin (Cx) family of transmembrane proteins (more than 20 isoforms). Various Cx proteins can form large pores in the nonjunctional plasma membrane of cells (gap junction hemichannels) before the assembly of two Cx hemichannels into complete gap junction channels between adjacent cells.14 Cx hemichannels are large, mechanosensitive ion channels that allow the passage of a variety of small molecules and metabolites, including ATP.14–16 Our laboratory localized Cx30 in the nonjunctional apical plasma membrane of cells in the distal nephron,24 suggesting that Cx30 may function as an ATP-releasing, luminal membrane hemichannel in this nephron segment. To address this hypothesis, we applied a recently developed ATP biosensor technique18 combined with a Cx30 knockout mouse model25 to show tubular flow–induced ATP release through luminal Cx30 hemichannels in the renal collecting duct. The physiologic significance of this mechanically induced Cx30-mediated luminal ATP release was further studied by assessing its involvement in pressure natriuresis, a multifactorial, mechanistic intrarenal phenomenon that causes diuresis and natriuresis in response to elevations in systemic BP.26 Pressure natriuresis involves proximal and distal nephron components; hemodynamic factors such as medullary blood flow and renal interstitial hydrostatic pressure; and renal autocoids such as nitric oxide, prostaglandins, kinins, and angiotensin II.26–32 Because pressure natriuresis is related to mechanical factors and Cx hemichannels are mechanosensitive, we hypothesized that Cx30-mediated ATP release is involved, at least in part, in pressure natriuresis, a critically important phenomenon in the maintenance of body fluid balance and BP.

RESULTS

Localization of Cx30 in the Mouse Kidney

Immunolocalization studies found intense Cx30 labeling in the luminal, nonjunctional cell membrane of a select population of cells in the connecting tubule (CNT) and CCD (Figure 1A). Vascular structures or other tubule segments were devoid of Cx30 labeling. Previous studies identified the Cx30-positive cells in the CNT-CCD as intercalated cells (ICs) and established that in the mouse kidney, it is the only cell type that expresses Cx30.24 No immunolabeling was found in Cx30−/− kidney sections, confirming specificity of the Cx30 antibody (Figure 1B).

Luminal ATP Release Measurements

Because ATP release through Cx hemichannels has been established14–16 and the unusual, nonjunctional membrane localization of Cx30 (Figure 1) suggested hemichannel function, we measured Cx30-mediated ATP release into the tubular fluid using freshly dissected, in vitro microperfused CCDs from wild-type and Cx30−/− mice and an established ATP biosensor technique (Figure 2).18 We used cell-specific fluorescence markers to identify ICs and principal cells (PCs), the two main cell types of the CCD (Figure 2, A and B). As described previously, the luminal surface of ICs was labeled using rhodamine-conjugated peanut lectin,33 whereas quinacrine identified acidic vesicles in PCs.34 With the help of a glass micropipette, a single ATP-sensing PC12 cell loaded with calcium fluorophores was positioned through the opening of the CCD in direct contact with the apical surface of ICs or PCs (Figure 2B). In response to increasing tubular flow rate from 2 to 20 nl/min, the biosensor PC12 cells produced large elevations in \([\text{Ca}^{2+}]_c\) when positioned next to ICs (\(\Delta[\text{Ca}^{2+}]_c = 432 \pm 14 \text{nM} \); \(n = 8\)), indicating ATP release from these cells (Figure 2C). Substantial but significantly reduced flow-induced \([\text{Ca}^{2+}]_c\) responses were observed when the biosensor cell was attached to PCs’ apical membrane (\(\Delta[\text{Ca}^{2+}]_c = 146 \pm 10 \text{nM} \); \(n = 7\)). Importantly, the ATP biosensor signal was almost completely abolished when ICs in preparations dissected from Cx30−/− mice were used (Figure 2, C and F). Preincubation of the biosensor cells with the purinergic receptor inhibitor suramin served to ensure ATP specificity, and it completely blocked the biosensor \([\text{Ca}^{2+}]_c\) signal. Similarly, no response was detected when the biosensor PC12 cell was positioned at the exit of the open CCD without any direct cell membrane contact with either ICs or PCs.

Similar to the effect of increased tubular flow, reducing bath osmolality from 300 to 270 mOsm/kg (creating an interstitium-to-lumen pressure gradient) caused significant ATP release from ICs in a Cx30-dependent manner (Figure 2F). Analysis of the dose-response relationship of exogenous ATP-induced elevations in PC12 cells \([\text{Ca}^{2+}]_c\) resulted in an \(EC_{50}\) value of 45.94 \(\mu\text{M}\) (Figure 2D). On the basis of these measurements that translated biosensor cell \([\text{Ca}^{2+}]_c\) responses to ATP levels, the relationship between tubular flow rate and luminal ATP release was established (Figure 2E). The tubular flow rate with the half-maximal effect was 6.97 nl/min. The maximum luminal ATP release was in the 50-\(\mu\text{M}\) range.

Pressure Natriuresis and Diuresis in Cx30−/− Mice

To test the significance of Cx30-mediated ATP release from the CCD in vivo, we instrumented wild-type and Cx30−/− mice for luminal ATP release measurements. Mice with no detectable luminal ATP release were considered pressure natriuretic, and those with detectable luminal ATP release were considered pressure diuretic. The prevalence of pressure natriuresis in wild-type mice (87%) was significantly higher than that in Cx30−/− mice (25%) (\(P < 0.001\); Figure 2G). These results strongly implicate Cx30-mediated ATP release in the maintenance of renal pressure natriuresis.

Figure 1. Immunofluorescence localization of Cx30 in the mouse kidney (red). (A) In the wild-type kidney, intense Cx30 labeling (arrowheads) was found at the apical membrane of select cells in the CCD, and the labeling showed a continuous linear pattern. Other structures, including the proximal tubule (PT), were devoid of Cx30 labeling. (B) No labeling was found in Cx30−/− kidneys. Cell nuclei were labeled with DAPI (blue). Bar = 20 \(\mu\text{m}\).
pressure natriuresis measurements. This included the ligation of the distal aorta first and then the mesenteric and celiac arteries together that caused significant, two-step elevations in mean arterial pressure from the resting value of 115.0 ± 4.5 to 131.0 ± 4.1 and then to 151 ± 4.8 mmHg. There were no significant differences in BP levels between Cx30+/- and Cx30−/− mice at any given time during these experiments (Figure 3, Table 1). The pressure natriuresis/kaliuresis/diuresis relationships are plotted in Figure 3, A through C. As shown in Figure 3A, the increased renal perfusion pressure-induced natriuresis was significantly blunted in Cx30−/− mice (Cx30+/+ 1.60 ± 0.08, Cx30−/− 1.00 ± 0.15 μEq/min during the highest pressure interval; P < 0.05). There was no difference in pressure-induced kaliuresis in wild-type and Cx30−/− mice (Figure 3B). Similar to natriuresis, the urine output increased in response to elevations in renal perfusion pressure in both groups (Figure 3C); however, the magnitude of pressure diuresis was markedly blunted in Cx30−/− mice (Cx30+/+ 10.6 ± 1.6, Cx30−/− 6.1 ± 1.2 μl/min during the highest pressure interval; P < 0.05). GFR was maintained at a constant level during these studies and was not different between wild-type and Cx30−/− mice (Figure 3D). Also, hematocrit did not change during these experiments, indicating that fluid balance was generally well maintained in each experimental period (Table 1).

**Collecting Duct Specificity**

To ascertain that the aforementioned salt retention phenotype of Cx30−/− mice was due to the altered function of the CNT-CCD where Cx30 is exclusively expressed, we treated mice with benzamil, a selective inhibitor of the collecting duct–specific epithelial sodium channel (ENaC). Because there was no difference in BP between wild-type and Cx30−/− mice under normal conditions, we placed mice on high-salt diet for 2 wk. BP in the wild-type mice (75.0 ± 1.7 mmHg) did not change; however, high-salt diet caused a significant BP elevation in Cx30−/− mice (103.0 ± 2.4 mmHg; P < 0.0001; Figure 4). Administration of the ENaC blocker benzamil had no effect in Cx30+/+ mice, but it returned BP to normal levels in Cx30−/− mice on high-salt diet (Figure 4).

**Expression of Renal Salt Transporters and ENaC in Cx30−/− Mice**

We studied the expression of the main renal salt transporters, Na+:H+ exchanger, Na+:2Cl−:K+ cotransporter, Na+:Cl− co-transporter (NCC), and ENaC, in wild-type and Cx30−/− mice using immunoblotting of whole-kidney homogenates, as shown in Figure 5. There was no statistically significant dif-
ference in the expression of these salt transporters or ENaC between Cx30+/− and Cx30−/− mice, with the exception of NCC, which was significantly less abundant (76 ± 6% of that in the wild-type mice) in kidneys of Cx30−/− mice (Figure 5).

**Cx and Purinergic Receptor Expression Profile in Cx30−/− Mice**

The expression profile of well-established renal Cx isoforms in kidneys of Cx30+/+ and Cx30−/− mice were compared using reverse transcriptase–PCR (RT-PCR) of whole kidney samples, as shown in Figure 6A. Expression of Cx30.3, Cx37, Cx40, Cx43, and Cx45 was found in both wild-type and Cx30−/− mice, with the exception that Cx30−/− mice were Cx30 deficient, as expected (Figure 6A). Similarly, a variety of P2X and P2Y purinergic receptors, including the main luminal membrane isoform P2Y2, seemed to be expressed in kidneys of both Cx30+/+ and Cx30−/− mice (Figure 6B).

**Other Systemic and Renal Parameters in Cx30−/− Mice**

All mice were age matched, and body weight was not different between Cx30+/+ and Cx30−/− groups (Table 1). Also, plasma electrolyte levels were the same. Because Cx30 hemichannels were localized to the apical membrane of ICs, which regulate pH homeostasis, plasma [Cl−] and [HCO3−] were measured but did not show any difference between the two groups (Table 1). Plasma aldosterone and vasopressin levels were not different between Cx30+/+ and Cx30−/− mice (Table 1).

**DISCUSSION**

This study identified functional Cx30 hemichannels in the intact kidney tissue as an important source of luminally released ATP in the collecting duct. Cx30 hemichannel opening at the luminal cell membrane of the CCD was triggered mechanically by elevations in tubular fluid flow rate or an interstitium-to-lumen osmotic pressure gradient, resulting in significant amounts of released ATP (up to 50 μM) in the luminal microenvironment. Physiologic significance of the Cx30-mediated luminal ATP release was confirmed in vivo in a mouse model of pressure natriuresis, an important renal physiologic mechanism that maintains body fluid and electrolyte balance and BP. Cx30−/− mice expressed a salt retention phenotype indicated by their reduced ability to excrete urinary salt and water in response to acute elevations in BP. This was due to the dysfunction of the Cx30-expressing CCD. This report describes a mechanically induced ATP-releasing mechanism in the intact CCD and its partial involvement in the pressure-natriuresis phenomenon. Also, this article further emphasizes the in vivo physiologic significance of purinergic regulation of renal tubular salt and water reabsorption.

These studies were inspired by our laboratory’s localization of Cx30 in cells of the distal nephron in the mouse, rat, and rabbit kidney.24 High expression of Cx30 was found at the luminal membrane of the medullary and cortical thick ascending limbs, distal tubule, and the cortical and medullary collecting ducts.24 As shown in Figure 1A and discussed previously,24 among these species, the mouse kidney has the lowest level of renal Cx30 expression, where it is restricted to ICs of the CNT.
CCD. In contrast, in the rat kidney, Cx30 was found in PCs of the CCD as well. This cell-specific expression of Cx30 in the mouse kidney allowed us to detect Cx30-mediated ATP release from ICs of the intact, in vitro microperfused CCD using a biosensor approach (Figure 2). PCs served as control and consistent with Cx30 expression, significantly reduced biosensor cell responses were detected from these cells (Figure 2, C and F). ATP specificity of the biosensor technique was confirmed using purinergic receptor inhibition as previously established. In the Cx30−/− tissue, ATP biosensor responses were almost completely abolished, suggesting that Cx30 hemichannel activity is a major ATP-releasing pathway in the tubular lumen. Significantly reduced or absent ATP levels around PCs and at the open exit of the CCD, respectively (Figure 2F), indicate rapid ATP degradation after release. This is consistent with the high levels of ATP-degrading enzymes co-localizing with Cx30 at the luminal membrane of ICs in the CCD and in other nephron segments. It should be mentioned that another Cx isoform, Cx30.3, was recently localized also at the nonjunctional luminal membrane of select renal epithelial cells, partially overlapping with Cx30. Nevertheless, luminal Cx30 hemichannel-mediated ATP release is likely not an isolated and CCD-specific phenomenon as it is expected to be present in other nephron segments and may involve other Cx isoforms as well.

It is well established that elevations in tubular flow triggers ATP release through both luminal and basolateral cell membranes of the CCD and that the released ATP binds to a
This study provides further mechanistic details of the renal tubular purinergic signaling system by identifying Cx30 hemichannels as a major releasing mechanism of luminal ATP. Because Cx30 hemichannel activity was triggered by mechanical stimulation of the apical cell membrane (increased tubular flow rate or an interstitium-to-lumen osmotic pressure gradient), in subsequent in vivo experiments, we applied the pressure-natriuresis model, which also involves mechanical factors.27–30 We speculate that high BP-induced elevations in intrarenal interstitial hydrostatic pressure, similar to the interstitium-to-lumen osmotic pressure gradient applied in the in vitro experiments (Figure 2F), initiated the Cx30-mediated release of ATP in vivo. Purinergic inhibition of tubular salt and water reabsorption then caused elevations in CCD fluid flow rate, leading to more ATP release and augmentation of pressure natriuresis. It is also possible that upstream, proximal tubular mechanisms31,32 initiated the elevations in CCD flow rate that were further augmented by triggering Cx30-mediated ATP release in the collecting duct system. The 40% reduced pressure natriuresis and diuresis in Cx30−/− mice (Figure 3, A and C) may represent the Cx30-dependent collecting duct component of this phenomenon.

The tubular fluid flow rates applied in vitro (2 to 20 nl/min; Figure 2) are in the range of previously established free-flow collections from early distal tubules in vivo.32 Interestingly, the earlier in vivo data in control (7.5 nl/min) and acute hypertension (8.3 nl/min)32 well correspond to the most sensitive range of the tubular flow rate–ATP release relationship established in this study (6.97 nl/min; Figure 2E).

The development of a salt retention phenotype in Cx30−/− mice that lack luminal ATP release in the CCD is also in agreement with the phenotype in mice deficient in the main luminal ATP receptor of the nephron, P2Y2; therefore, the similar phenotype of Cx30 and P2Y2 knockout mice emphasizes the physiologic importance of renal tubular ATP (see the summarizing scheme in Figure 7). Also, the mechanosensitive, Cx30-mediated ATP release and subsequent purinergic calcium signaling in the nephron22,23 fit with the previously identified downstream signaling steps of pressure natriuresis. These steps involve calcium-dependent synthesis of arachidonic acid metabolites,27–29 nitric oxide, and cGMP30 and ultimately the reduced salt reabsorption in both proximal and distal nephron segments.26–32 That pressure kaliuresis was not different between wild-type and Cx30−/− mice (Figure 3B) may suggest the involvement of ATP-insensitive K+ secretion pathways, for example the maxi-K+ (or BK) channel rather than the low conductance ROMK channel.21,38,39

To connect further these in vivo findings to the Cx30-expressing CCD segment and to emphasize the importance of Cx30-mediated ATP release in BP control, we placed mice on high-salt diet for 2 wk. Systemic BP was significantly elevated in Cx30−/− versus wild-type mice, which was corrected by the administration of a pharmacologic inhibitor of ENaC, a CCD-specific ion channel that reabsorbs sodium (Figure 4). Also consistent with the uninhibited salt reabsorption in the CNT-CCD in Cx30−/− mice (Figure 7) was the reduced expression of the NCC (Figure 5). This is most likely a compensatory change caused by the increased ENaC-mediated salt retention in Cx30−/− mice, which is reminiscent of the findings in mineralocorticoid escape.40 Cx30−/− mice on normal salt intake were normotensive, which may be explained by the effects of anesthesia or downregulation of NCC expression. Importantly, the impaired pressure natriuresis and diuresis in Cx30−/− mice were not attributed to alterations in other renal Cxs or purinergic receptors, because the expression of these genes, at least on the mRNA level, was preserved and seemed to be similar between the two groups (Figure 6). Likewise, there was no difference in systemic parameters, including aldosterone and vasopressin levels (Table 1), that could have influenced renal salt and water reabsorption.

Tubular flow–dependent [Ca2+]i elevations in the Cx30−/− mice have been demonstrated both in vitro22,23 and in vivo1 and, importantly, in both PCs and ICs.22 Because it is well established that cells of the CCD lack direct coupling through gap junctions,23 on the basis of our data, we propose both autocrine (on ICs) and paracrine (on PCs) effects of luminal ATP released through Cx30 hemichannels, at least in the mouse kidney (Figure 7). As stated already, the expression of luminal membrane Cx30 hemichannels is more ubiquitous in the distal nephron in other species,24 suggesting their generalized role in luminal ATP release and salt and water reabsorption. Also, the hemichannel function may help to resolve the apparent controversy that cells of the CCD express various Cx isoforms24,37 even though they lack direct cell-to-cell coupling through gap junctions.23 Furthermore, we speculate that Cx30 hemichannel opening induced by mechanical stimulation (interstitial pressure, tubular flow) may involve primary cilia (in PCs) and microvilli (in ICs) that are well-established sensors of shear and hydrodynamic impulses.22

Figure 7. Mechanically induced, Cx30-mediated ATP release into the tubular lumen in the CCD and its effects on salt and water reabsorption, natriuresis, and diuresis. Increases in intrarenal pressure and/or tubular flow rate that accompany BP elevations result in the opening of Cx30 hemichannels at the luminal membrane of CCD ICs and the release of ATP. Stimulation of luminal purinergic receptors (mainly P2Y3) results in the inhibition of salt and water reabsorption and increased natriuresis and diuresis. This inhibitory mechanism is absent in Cx30−/− mice, resulting in a salt retention phenotype.
at the Cx30-expressing apical membrane. Supporting this hypothesis is the recent finding that the loss of apical monocilia on collecting duct PCs impairs ATP secretion across the apical cell surface.\(^\text{42}\)

It should be mentioned that, as established previously,\(^\text{25}\) Cx30\(^{-/-}\) mice are deaf as a result of dysfunction of the inner ear. We find it intriguing that these studies identified another phenotype in these mice, namely renal salt retention as a result of dysfunction of the collecting duct system. Similarities in regulatory mechanism and common ion transporters in the renal collecting duct and the inner ear are widely known,\(^\text{43}\) and, very recently, a genetic link between Bartter syndrome (dysfunction of the Cx30-expressing nephron segment) and sensorineural deafness in humans has been established.\(^\text{44}\) This suggests that Cx30-mediated ATP release is an important physiologic regulatory mechanism in many organs and species, including humans. Mediation of ATP release by other hemichannels and their role in various organ functions have been described in many other cell types, including the glomerular endothelium,\(^\text{45}\) red blood cells,\(^\text{46}\) osteocytes,\(^\text{47}\) and taste buds.\(^\text{48}\)

In summary, this is the first report on the expression of functional Cx30 hemichannels in renal tubules that release ATP into the tubular fluid in response to mechanical stimulation. Autocrine/paracrine effects of luminal ATP released via Cx30 hemichannels involve inhibition of renal salt and water reabsorption, and this novel mechanism seems to be connected to at least one part (the distal nephron–collecting duct component) of pressure natriuresis and diuresis.

CONCISE METHODS

Mice
The Cx30\(^{-/-}\) mouse model was established and described previously.\(^\text{25}\) Wild-type and Cx30\(^{-/-}\) mice (C57BL6 background) were bred at the University of Southern California. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Southern California. Genotype was confirmed by PCR of tail biopsies.

Immunohistochemistry
Rabbit polyclonal anti-Cx30 antibodies were purchased from Zymed Laboratories (San Francisco, CA) and used as described previously.\(^\text{24}\) Briefly, after fixation and blocking, wild-type and Cx30\(^{-/-}\) mouse kidney sections were incubated with Cx30 antibodies at a 1:50 dilution overnight and washed in PBS. Sections were then incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG and enhanced with Alexa Fluor 594–labeled tyramide signal amplification according to the manufacturer’s instructions (Molecular Probes, Eugene, OR). After a wash step, sections were mounted with Vectashield mounting medium containing the nuclear stain DAPI (Vector Laboratories, Burlingame, CA) and examined with a Leica TCS SP2 confocal microscope (Leica Microsystems, Heidelberg, Germany).

ATP Biosensor Technique
Animals were anesthetized. After midline, abdominal incision, one kidney was removed; placed into ice-cold dissection solution containing DME mixture F12, 1.2 g/L H\(_2\)CO\(_3\), and 3% FBS; and sliced into 2- to 4-mm slices. CCDs were dissected freehand at 4°C and partially split open to provide access to the luminal cell surface and placed into a temperature-controlled chamber containing oxygenated Krebs–Ringer solution. Then, tubules were perfused in the 0- to 20-nl/min range\(^\text{46}\) with a solution containing (in mM) 25 NaCl, 5 KCl, 1 MgSO\(_4\), 1.6 NaHPO\(_4\), 0.4 NaH\(_2\)PO\(_4\), 5 glucose, 1.5 CaCl\(_2\), 110 NMDG-cyclamate, and 10 HEPES. In some experiments, bath osmolality was reduced from 300 to 270 mOsm/kg by adding 10% volume distilled water. ICs were labeled by 0.02 mg/ml Alexa Fluor 594–peanut lectin\(^\text{39}\) and PCs by 25 μM quinacrine.\(^\text{34}\) After cannulation and perfusion of a microdissected CCD, a small group of Fluo-4– and Fura Red–loaded (1 and 3 μM, respectively, 25°C, 15 min + 250 μM sulfinpyrazone) PC12 cells (American Type Culture Collection, Manassas, VA) were gently positioned in direct contact with the apical surface of PCs or ICs. After positioning the biosensor cells, a short (10 min) equilibration period was given. These PC12 cells were used as biosensors of freshly released extracellular ATP on the basis of changes in their [Ca\(^{2+}\)]\(_{ic}\) as described previously.\(^\text{18}\) 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\(^{2+}\)]\(_{ic}\) as described previously.\(^\text{49}\) In some experiments, PC12 cells were preincubated with the purinergic receptor blocker suramin (50 μM). Each perfused CCD was dissected from a different mouse.

Pressure-Natriuresis Measurements
Ten- to 13-wk-old Cx30\(^{-/-}\) mice (n = 8) and Cx30\(^{-/-}\) littermates (n = 6) were anesthetized using the combination of ketamine and inactin (10 mg/100 g body wt each). The surgery was carried out on a temperature-controlled mouse operating pad (Vestavia Scientific, Birmingham, AL). Trachea was cannulated to facilitate breathing, and cannulas were inserted into the carotid artery to perform continuous BP measurements by BP-1 Blood Pressure Monitor (World Precision Instruments, Sarasota, FL) and into the femoral vein for intravenous infusion. The abdominal aorta (distal to the renal artery) and then the mesenteric artery were ligated to elevate BP in a stepwise manner. A catheter was placed into the bladder to collect urine. The operation was followed by a 15-min equilibration period. Renal functions were then determined during three consecutive 20-min periods: (1) Control, (2) first elevation period in which the abdominal aorta was compressed, and (3) the second elevation period in which the mesenteric artery was additionally ligated. In each period, urine and blood samples were collected to perform volume, Na\(^+\) and K\(^+\) measurements. Immediately after blood collection (70 μl/sample), mice received an equivalent amount of donor mouse whole blood, collected a few hours before the experiment.

In Vivo Parameters
Mice had free access to drinking water until the day of the experiment. After femoral vein cannulation, the mice were given a 0.35-μl/g body
High-Salt Diet
Wild-type (n = 19) and Cx30−/− (n = 19) mice were kept on normal (0.3%) or high-salt (8% NaCl + 0.45% NaCl in drinking water) diet for 2 wk. Some mice received benzamil (1.4 mg/kg per d intraperitoneally), a selective inhibitor of the ENaC, during the second week. Mice were anesthetized, and BP was measured through the cannulated carotid artery, as described above in Pressure-Natriuresis Measurements.

Western Blot Analysis of Renal Epithelial Salt Transporters
Manually dissected slices of kidney cortex were homogenized in a buffer containing 20 mM Tris–HCl, 1 mM EGTA (pH 7.0), and a protease inhibitor cocktail (BD Biosciences, San Jose, CA). A total of 40 μg of protein was separated on a 4 to 20% SDS-PAGE and transferred onto polyvinylidene difluoride membrane. The blots were blocked for a minimum of 1 h with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) at room temperature. This was followed by an incubation of the primary antibody (1:3000 dilution of rabbit anti-Cx30; gift from Dr. Alicia McDonough, Los Angeles, CA) for 4°C overnight, NaCl 0.9%, and an incubation of the secondary antibody (1:15,000; LI-COR Biosciences) and visualized with Odyssey Infrared Imaging System, Western Blot Analysis (LI-COR Biosciences).

RT-PCR
Total RNA was purified from whole kidneys of Cx30+/+ and Cx30−/− mice using a Total RNA Mini Kit (Bio-Rad, Hercules, CA). RNA was quantified using spectrophotometry and reverse- transcribed to single-stranded cDNA using avian reverse transcriptase and random hexamers according to the manufacturer’s instructions (ThermoScript RT-PCR system; Invitrogen, Carlsbad, CA). Two microliters of cDNA was amplified using a master mix containing Taq polymerase (Invitrogen) and the primers each at a final concentration of 100 μM. The primer sequences and expected band sizes for various Cx and ATP receptors were described previously.24,37,45 The PCR reaction was carried out for 30 cycles of the following: 94.0°C for 30 s, 55.4°C for 30 s, and 72.0°C for 30 s. The PCR product was analyzed on a 2% agarose gel.

Statistical Analysis
Statistical analysis was performed by ANOVA and post hoc comparison by Bonferroni test. P < 0.05 was considered significant.

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DISCLOSURES
None.


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