The Luminal P2Y Receptor in the Isolated Perfused Mouse Cortical Collecting Duct

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Abstract. Extracellular nucleotides regulate renal ion transport. With the use of in vitro perfusion and [Ca\textsuperscript{2+}] imaging, this study investigated whether mouse and rabbit cortical collecting ducts (CCD) respond to luminal nucleotides. In mouse CCD, luminal ATP (EC\textsubscript{50}: 10 \(\mu\)M) and UTP (EC\textsubscript{50}: 9.7 \(\mu\)M) increased [Ca\textsuperscript{2+}], with an initial peak and a plateau. To make certain that basolateral P2 receptors were not activated by luminal nucleotides via leak diffusion, luminal trypsin (1 \(\mu\)M), a known agonist for basolateral proteinase-activated receptors, was perfused. Mouse CCD that were responsive to luminal ATP were nonresponsive to luminal trypsin but always showed [Ca\textsuperscript{2+}] elevations by basolateral trypsin (10 or 100 nM). Luminal \(\alpha\),\(\beta\)- and \(\beta\),\(\gamma\)-methylene ATP, 2-methyl-S-ATP, ADP, UDP, and 2',3'-O-4-benzoylbenzoyl ATP had no effect (100 \(\mu\)M, \(n = 9\)). Without external Ca\textsuperscript{2+}, luminal ATP still stimulated a [Ca\textsuperscript{2+}], increase. Mouse CCD also responded to basolateral ATP (EC\textsubscript{50}: 23 \(\mu\)M) and UTP (EC\textsubscript{50}: 23 \(\mu\)M) with smaller [Ca\textsuperscript{2+}] elevations. Confocal microscopy of perfused CCD showed that luminal ATP (100 \(\mu\)M) rapidly increased [Ca\textsuperscript{2+}] in nearly all cells (\(n = 6\)) and the same cells that responded to luminal ATP responded to basolateral ATP (100 \(\mu\)M). In contrast, rabbit CCD did not respond to luminal ATP/UTP (\(n = 8\)) despite ATP's known effect from the basolateral side (EC\textsubscript{50}: 34 \(\mu\)M). These data indicate the expression of luminal P2Y receptors (probably P2Y\textsubscript{2}) in principal cells of mouse CCD but not in rabbit CCD.

Nucleotides have been shown to regulate ion transport processes in epithelial cells via a large variety of different P2 receptors. Mammalian P2 receptors are subdivided into metabotropic (G-protein coupled) P2Y (P2Y\textsubscript{1}, P2Y\textsubscript{2}, P2Y\textsubscript{4}, P2Y\textsubscript{11}) and ionotropic (nonselective Ca\textsuperscript{2+} permeable cation channel) P2X (P2X\textsubscript{1–7}) receptors (1). A unique characteristic of epithelial tissue is the expression of P2 receptors in the luminal and/or basolateral membrane. ATP release from epithelial cells into the luminal fluid space has recently been demonstrated (2). This suggests not only that specific cellular functions are regulated by a proposed release of ATP from basolateral neuronal sources but also that nucleotides released from epithelial cells may regulate epithelial functions in a paracrine/autocrine mode via luminal P2 receptors.

It is important to note that each epithelial tissue seems to be equipped with an individual expression pattern of P2 receptors. Recent studies indicate that a variety of different P2Y and P2X receptors are frequently expressed in the same epithelial tissue (3–5). Given these numerous different receptors in the same preparation and the lack of specific pharmacologic agonists, it is often difficult to assign a specific P2-receptor subtype to an observed effect. It is possible to resolve this problem through the generation of P2-receptor knockout mice. Recent data from P2Y\textsubscript{2}-receptor (−/−) mice have established that this receptor is the dominant P2 receptor that mediates tracheal NaCl secretion stimulated by luminal ATP (6). This study has also confirmed that in gallbladder, for example, a luminal P2Y\textsubscript{6} receptor seems much more important than the P2Y\textsubscript{2} receptor in the activation of luminal nucleotide-mediated NaCl secretion.

Evidence for luminal P2 receptors has been presented from numerous epithelial tissues, such as bronchus (7), colon (8), epididymis (9), sweat duct (10), and inner ear (11). In all of these tissues, the luminal P2Y\textsubscript{2}-receptor subtype seemed to be functionally the most relevant P2 receptor expressed. Other epithelia, such as rat submandibular gland duct cells, do not show functional expression of luminal P2Y\textsubscript{2} receptors. They do, however, respond to luminal 2',3'-O-4-benzoylbenzoyl ATP (Bz-ATP), which suggests the presence of luminal ionotropic P2X\textsubscript{7} receptors (12). The expression of luminal P2X\textsubscript{7} receptors has also been proposed in rat pancreatic ducts (4,5).

In kidney epithelial cells, our knowledge of P2-receptor expression and functional responses to extracellular nucleotides is still very limited. In cultured renal tubular cells, a number of studies indicate that P2 receptors are expressed in the luminal and basolateral membrane. Our own studies in MDCK-C7 (13) and M-1 cells (14) or, for example, work from A6 cells (15) suggest that responses to luminal and basolateral ATP on [Ca\textsuperscript{2+}] are mediated via the metabotropic P2Y\textsubscript{2} receptor subtype. In mouse inner medullary collecting duct cells (mIMCD-K2), evidence for multiple expression of P2Y (P2Y\textsubscript{1} and P2Y\textsubscript{2}) and P2X (P2X\textsubscript{3}, P2X\textsubscript{4}) receptors has been presented (16). In a variety of distal tubular cell lines, extracellular nucleotides inhibit Na\textsuperscript{+} absorption and activate NaCl secretion (14,16,17). Studies with intact mammalian kidney
tubules indicate the presence of basolateral P2 receptors along the entire nephron (18–21). Basolateral ATP has been shown to inhibit the adiuretin-induced increase in H₂O permeability in rat inner medullary collecting duct (20) and rabbit cortical collecting duct (CCD), probably via a P₂Y₂ receptor (22). In rat inner medullary collecting duct, the inhibition of H₂O transport was attributed to a P₂-receptor–mediated decrease of cAMP (20). In addition, basolateral extracellular nucleotides most generally elevate cytosolic Ca⁺⁺ in renal tubules (19,21,23).

A preliminary study of perfused rabbit CCD suggested the functional expression of luminal P₂ receptors (24). On the basis of immunohistochemistry, recent work proposed the expression of P₂Y₂ receptors in the luminal and basolateral membrane of rat inner medullary collecting duct principal cells (18). The present study was undertaken to investigate whether intact perfused CCD tubules of mouse and rabbit show functional expression of luminal P₂ receptors.

**Materials and Methods**

**Microdissection of Isolated CCD**

Experiments were carried out in mouse and rabbit isolated CCD segments. Nephron segments of both sexes of mice (30 to 40 g) and rabbits (800 g) were microdissected at 4°C from kidney slices in carbogen (98.5% O₂ and 1.5% CO₂) gassed dissection solution (see below) using ultrafine watchmaker forceps. The kidney tubules were transferred into a specialized perfusion chamber mounted on an inverted microscope.

**Digital Video Imaging**

The setup consisted of an inverted microscope (Axiovert 100 TV, Zeiss, Jena, Germany) with a 40× objective (Fluar 40×, 1.3 oil, Zeiss), a monochromator (Till Photonics, Planegg, Germany), and a GEN 3 intensified CCD camera (ICCD 350, Videoscope, Washington). Image acquisition and data analysis were performed with the software package Metamorph/Metafluor (Universal Imaging, West Chester, PA). Freshly dissected CCD were mounted into the perfusion system previously built and used in this laboratory (25). Measurement of [Ca⁺⁺]i was performed with the Ca⁺⁺-dye fura-2. Tubules were incubated in 10 μM basolateral fura-2/AM for 15 min at room temperature in Ringer’s solution to which 1.6 μmol/L pluronic F127 had been added. Pluronic F127 is a surfactant polyol that helps to reduce fura-2 dye loss during the experiment. To demonstrate successful luminal agonists perfusion, we added 10 μM Lucifer Yellow (LY; excitation, 430 nm; emission, >500 nm) to the luminal perfusate. LY is not excitable with the two fura-2 wavelengths (345 nm and 380 nm), and fura-2 is not excited with 430 nm of light. Thus, the two fluorescence signals could be observed without significant cross contamination. Images were recorded sequentially after 345 nm, 380 nm, and 430 nm excitation. One fura-2 ratio and the LY fluorescence were recorded every 2 s, respectively.

**Confocal Microscopy**

The perfusion system was adapted to an inverted confocal microscope (LSM410, Zeiss), equipped with 63× objective (C-Apochromat 63×/1.2 water, Zeiss). [Ca⁺⁺]i changes were measured with the Ca⁺⁺ dye fluo-3. After mounting, the tubules were incubated in 20 μM basolateral fluo-3/AM for 45 min at room temperature in Ringer’s solution to which 1.6 μmol/L pluronic F127 had been added. Fluo-3 was excited at 488 nm with a blue-enhanced argon laser. The scanning speed was set to 2.08 s for a 512 × 512-pixel image. The pinhole was set to achieve a full-width half-maximum z-resolution of approximately 1 μm. This has previously been verified by fluorescence latex beads. Image series were analyzed by measuring mean pixel intensity versus time in individual cells/areas using the Metafluor imaging software (Universal Imaging).

**Solutions and Chemicals**

Pluronic F127, fluo-3/AM, LY, and fura-2/AM were obtained from Molecular Probes (Eugene, OR). All other chemicals were of the highest grade of purity available and were obtained from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany). The experiments of continuous basolateral and luminal perfusion of kidney tubules were performed with the following solutions: 145 mM NaCl, 1 mM MgCl₂, 1.3 mM Ca-gluconate, 5 mM D-glucose, 0.4 mM KH₂PO₄, 1.6 mM K₃HPO₄, 0.5 mM probenecid, pH 7.4. The 1 μM Ca⁺⁺ solution contained 145 mM NaCl, 1 mM MgCl₂, 1 mM ethyl-α-aminooethyl ether)-N,N′,N′′-tetraacetate acid, 0.96 mM Ca-gluconate, 5 mM D-glucose, 0.4 mM KH₂PO₄, 1.6 mM K₃HPO₄, and 0.5 mM probenecid. All solutions were titrated to a pH of 7.4. As kidney tubule dissection solution, we used F12 (HAM) medium (Life Technologies, Karlsruhe, Germany) to which 5 mmol/L glycine was added.

Commercially available nucleotidediphosphates are frequently contaminated with nucleotidetriphosphates. To circumvent this problem, we treated the ADP and UDP stock solution as described by Nicholas et al. (26) for 1 h in 10 U/ml hexokinase and 22 mM glucose, which resulted in the complete conversion of UTP to UDP and ATP to ADP.

**Statistical Analyses**

The data shown are either original traces or mean values ± SEM (n), where n refers to the number of experiments. Paired t test was used to compare mean values within one experimental series. A P value of less than 0.05 was accepted to indicate statistical significance.

**Results**

**Luminal ATP Increases [Ca⁺⁺]i in Isolated Perfused Mouse CCD**

To investigate the effect of luminal nucleotides, we perfused isolated mouse CCD in a fluorescence video-imaging setup. Effective luminal perfusion was verified in each experiment with LY (10 μM) added to the luminal perfusate. A single experiment is shown in Figure 1. The upper three pictures show sequential fura-2 pseudocolor ratio images before, during, and after perfusion with 100 μM luminal ATP. As presented here, luminal ATP led to an increase in the fura-2 fluorescence ratio. The lower three images show corresponding LY fluorescence images, indicating the successful luminal perfusion of the agonist. The addition of LY alone never altered [Ca⁺⁺]i.
Epithelial cells frequently express multiple P2 receptors in their luminal and basolateral membranes (4,5,12). It was therefore a prerequisite to demonstrate in a paired approach that the investigated tubule was intact and that no cross contamination of the luminal and basolateral fluid spaces occurred. Our approach to this problem was to use trypsin as a pharmacologic indicator of a strictly luminal perfusion. Trypsin has recently been shown to activate exclusively basolateral proteinase-activated receptors (PAR-2) coupled to the generation of \( \text{InsP}_3 / \text{Ca}^{2+} \) in M-1 mouse CCD cells (27). Our data suggest that this is similarly true for the freshly isolated perfused mouse CCD.

In 17 of 20 tubules, luminal trypsin (1 \( \mu \)M) had no effect on \([\text{Ca}^{2+}]_i\), whereas the same tubules always responded to 10 or 100 nM basolateral trypsin. Figure 2 shows an original recording of the fura-2 fluorescence ratio measured from the entire tubule (upper trace) and the luminal perfusion of LY (lower trace) measured from the luminal area of the tubule. Luminal ATP (100 \( \mu \)M) rapidly increased \([\text{Ca}^{2+}]_i\) with an initial sharp peak (mean \( \Delta \) fluorescence ratio peak increase, 1.33 \( \pm \) 0.33; \( n = 16 \)) and a secondary plateau (mean \( \Delta \) fluorescence ratio increase, 0.44 \( \pm \) 0.06; \( n = 37 \) of 24 tubules). Thereafter, trypsin (1 \( \mu \)M) was added to the luminal side, leaving \([\text{Ca}^{2+}]_i\) unchanged. Figure 2 also indicates that basolateral ATP (100 \( \mu \)mol/L) induced a reversible \([\text{Ca}^{2+}]_i\) increase. Finally, we show that basolateral trypsin (100 nM) induced a reversible \([\text{Ca}^{2+}]_i\) increase (mean \( \Delta \) fluorescence ratio increase, 0.93 \( \pm \) 0.19; \( n = 19 \)). Only those tubules that did not respond to luminal trypsin at the end of each experiment were included in the analysis.

**Luminal P2X-Receptor Agonists Do Not Increase \([\text{Ca}^{2+}]_i\), in Isolated Perfused Mouse CCD**

Subsequently, we tested other luminal nucleotides, especially UTP and a large variety of known P2X agonists. Figure 3 represents our findings, which indicate that luminal UTP (100 \( \mu \)M) rapidly and reversibly increased \([\text{Ca}^{2+}]_i\), as well (mean \( \Delta \) fluorescence ratio peak increase, 1.33 \( \pm \) 0.33; \( n = 16 \)). At a concentration of 100 \( \mu \)M, the following other luminal nucleotides were without effect: \( \alpha,\beta\)-methylen ATP (\( n = 10 \)), \( \beta,\gamma\)-methylen ATP (\( n = 9 \)), 2-methyl-S-ATP (\( n = 9 \)), ADP (\( n = 9 \)), UDP (\( n = 10 \)), and Bz-ATP (\( n = 10 \)). The concentration-response curve of a single experiment for luminal UTP is shown in the upper panel of Figure 4. At all concentrations, UTP and ATP induced an initial sharp peak increase and a secondary \([\text{Ca}^{2+}]_i\) plateau. The lower panel summarizes these data, indicating that luminal UTP (EC\( _{50} \): 10 \( \mu \)M) and ATP (EC\( _{50} \): 10 \( \mu \)M) with similar potency raised cytosolic \( \text{Ca}^{2+} \) in isolated perfused mouse CCD (\( n = 5 \)).

**Basolateral ATP and UTP Increase \([\text{Ca}^{2+}]_i\), in Isolated Perfused Mouse CCD**

Our data from M-1 CCD cells (14) indicated that basolateral ATP and UTP also increased \([\text{Ca}^{2+}]_i\). This is similarly true for the freshly isolated intact tissue. Figure 2 shows a single example of the effect of 100 \( \mu \)M basolateral ATP. Similar
effects were observed with UTP; this series of experiments is summarized in Figure 4. It is interesting that those nucleotides given from the basolateral side led to threefold smaller \([\text{Ca}^{2+}]_i\) elevations with similar EC\(_{50}\) values (ATP, EC\(_{50}\): 23 \(\mu\)M; UTP, EC\(_{50}\): 23 \(\mu\)M, \(n = 9\)).

**Luminal ATP Activates \([\text{Ca}^{2+}]_i\), Store Release in Isolated Perfused Mouse CCD**

Obviously, the above data indicate that luminal ATP and UTP mediate their effect via P2Y receptors in the luminal membrane of CCD cells. Because P2Y receptors most generally couple to the InsP\(_3\)/Ca\(^{2+}\) signaling pathway (1), it must be assumed that these nucleotides activate \([\text{Ca}^{2+}]_i\), store release. This is indeed true, as shown in Figure 5. In the presence of extracellular Ca\(^{2+}\) (luminal and basolateral), ATP induced the known biphasic \([\text{Ca}^{2+}]_i\) increase. After lowering extracellular Ca\(^{2+}\) to 1 \(\mu\)M, luminal ATP still induced a transient \([\text{Ca}^{2+}]_i\) elevation without a plateau (\(\Delta\) fluorescence ratio 345 nm/380 nm increase, 1.08 \(\pm\) 0.63; \(n = 4\)). Thus, the initial peak and plateau were due to release of Ca\(^{2+}\) from intracellular stores and subsequent activation of transmembranous Ca\(^{2+}\) influx, respectively. Figure 5 also shows that re-addition of basolateral Ca\(^{2+}\) resulted in a substantial \([\text{Ca}^{2+}]_i\) increase, consistent with reloading of \([\text{Ca}^{2+}]_i\) via the basolateral membrane.

**Figure 2. Original recording of \([\text{Ca}^{2+}]_i\), measurement in an isolated perfused mouse CCD (upper panel).** The lower panel indicates LY fluorescence measured as luminal perfusion marker in an area of interest corresponding to the tubular lumen. Luminal and basolateral ATP (100 \(\mu\)M) reversibly elevate \([\text{Ca}^{2+}]_i\), measured over the entire tubule. A consistent finding is the very rapid initial \([\text{Ca}^{2+}]_i\) increase by luminal ATP. Note also the substantial quantitative difference of the luminal and basolateral ATP effect. Luminal trypsin (1 \(\mu\)M) does not elevate \([\text{Ca}^{2+}]_i\). In contrast, 100 nM basolateral trypsin markedly elevated \([\text{Ca}^{2+}]_i\).

**Figure 3.** (A) Original recording of \([\text{Ca}^{2+}]_i\), measurement in an isolated perfused mouse CCD (upper panel). The lower panel indicates LY fluorescence measured as luminal perfusion marker in an area of interest corresponding to the tubular lumen. Luminal ATP and UTP (100 \(\mu\)M) reversibly elevate \([\text{Ca}^{2+}]_i\), measured over the entire tubule. 2-Me-S-ATP and \(\beta\)-methylene ATP do not increase \([\text{Ca}^{2+}]_i\). (B) Original recording of \([\text{Ca}^{2+}]_i\), measurement in an isolated perfused mouse CCD (upper panel). The lower panel indicates LY fluorescence measured as luminal perfusion. Luminal ATP (100 \(\mu\)M) reversibly elevates \([\text{Ca}^{2+}]_i\), measured over the entire tubule. UDP, ADP, \(\alpha\), \(\beta\)-methylene ATP, and 2',3'-O-4-benzoylbenzoyl ATP do not increase tubular \([\text{Ca}^{2+}]_i\).
Localization of Luminal ATP-Mediated \([Ca^{2+}]_i\) Increase in Isolated Perfused Mouse CCD

Our video-imaging pictures demonstrated a luminal and basolateral ATP-induced homogeneous fura-2 fluorescence ratio increase over the entire tubule. This was similarly true for the trypsin-induced \([Ca^{2+}]_i\) spike. The lower panel summarizes the series of all experiments. It is obvious that ATP and UTP with similar potency elevate \([Ca^{2+}]_i\) from the luminal and basolateral sides. It is apparent that luminal ATP or UTP triggers significantly larger \([Ca^{2+}]_i\) elevations.

No Firm Evidence for Luminal P2Y Receptors in Isolated Perfused Rabbit CCD

In this study, we furthermore investigated whether luminal perfusion of nucleotides increase \([Ca^{2+}]_i\) in rabbit CCD. We were surprised to find that the luminal perfusion of ATP or UTP did not increase \([Ca^{2+}]_i\) in rabbit CCD. A single experiment is shown in Figure 7. It is known that rabbit distal tubules respond to basolateral ATP with a \([Ca^{2+}]_i\) elevation, (22) as is also shown in Figure 7. In eight single perfused CCD tubules, basolateral ATP induced a reversible \([Ca^{2+}]_i\) increase (mean \(\Delta\) fluorescence ratio 345 nm/380 nm increase, 1.04 ± 0.28; \(n = 8\)) with no response to luminal ATP or UTP. The observations can be made: (1) Fluo-3 dye loading or \([Ca^{2+}]_i\) seems partially inhomogeneous in mouse CCD, (2) luminal ATP (100 \(\mu\)M) rapidly increased \([Ca^{2+}]_i\) in nearly all measurable cells, and (3) the same cells that responded to luminal ATP always responded to basolateral ATP (100 \(\mu\)M) as well. Taking into account that approximately 70% of CCD cells are principal cells, these data prove that principal cells respond to luminal ATP. Thus, our data strongly suggest that principal cells express P2Y receptors in their luminal and basolateral membrane. Similar observations were made in six different isolated perfused tubules.

Figure 4. Concentration-response curve of luminal and basolateral ATP and UTP in isolated perfused mouse CCD. The upper panel demonstrates an original recording of luminal UTP (5 to 100 \(\mu\)M) induced \([Ca^{2+}]_i\) elevations. Note the consistently large initial UTP-induced \([Ca^{2+}]_i\) spike. The lower panel summarizes the series of all experiments. It is obvious that ATP and UTP with similar potency elevate \([Ca^{2+}]_i\) from the luminal and basolateral sides. It is apparent that luminal ATP or UTP triggers significantly larger \([Ca^{2+}]_i\) elevations.

Figure 5. Original recording of \([Ca^{2+}]_i\), measurement in an isolated perfused mouse CCD (upper panel). The lower panel indicates LY fluorescence measured as luminal perfusion marker in an area of interest corresponding to the tubular lumen. In the near absence of extracellular \(Ca^{2+}\), luminal ATP (100 \(\mu\)M) induces an increase of \([Ca^{2+}]_i\), indicating the release from intracellular stores.
concentration-response curve for basolateral ATP is shown in the lower panel of Figure 7 with an EC$_{50}$ value of 34 μM. In four other experiments, luminal perfusion of 100 μM ATP induced negligible [Ca$^{2+}$]$_i$ elevations. The same tubules, however, responded to basolateral ATP (100 μM) with large [Ca$^{2+}$]$_i$ increases. Taken together, these data suggest that luminal P2 receptors are not expressed in rabbit CCD.

**Discussion**

We undertook the current study to investigate whether the CCD of mouse and rabbit express luminal P2 receptors. We found that luminal ATP and UTP equipotently increase [Ca$^{2+}$]$_i$ in perfused mouse CCD but not in rabbit CCD. The pharmacology is consistent with the expression of luminal P2Y$_2$ receptors. A number of different nucleotide agonists known to
activate P2X receptors did not increase $[\text{Ca}^{2+}]_i$ in mouse CCD. The luminal ATP- and UTP-induced $[\text{Ca}^{2+}]_i$ increase was composed of an initial $[\text{Ca}^{2+}]_i$ store release and a subsequent $\text{Ca}^{2+}$ influx. Confocal imaging indicated that principal cells are the ATP-responsive CCD cell subtype. ATP and UTP increased $[\text{Ca}^{2+}]_i$ equipotently, not only from the luminal but also from the basolateral side.

Characterization of Luminal P2 Receptors

To identify luminal P2-receptor subtypes, we tested various selective nucleotide agonists. Only luminal ATP and UTP increased $[\text{Ca}^{2+}]_i$. This agonist profile is indicative of a metabotropic P2Y receptor, either the P2Y$_2$- or the P2Y$_4$-receptor subtype (1). In experiments without extracellular $\text{Ca}^{2+}$, we showed that luminal nucleotides stimulate $\text{Ca}^{2+}$ release from intracellular stores. This is consistent with the activation of a G-protein–coupled P2Y receptor. The ATP-stimulated $[\text{Ca}^{2+}]_i$ peak in low extracellular $\text{Ca}^{2+}$ depicted in Figure 5 was smaller as compared with pre- and postcontrol effect in the presence of extracellular $\text{Ca}^{2+}$. This may suggest that other luminal P2 receptors, namely P2X, are also expressed. However, a large number of different selective P2X-receptor agonists, such as $\alpha$, $\beta$-methylene, $\beta$, $\gamma$-methylene ATP, Bz-ATP, and 2-methyl-S-ATP, failed to increase $[\text{Ca}^{2+}]_i$ (1). We therefore interpret our results to indicate that no luminal P2X receptors are expressed in mouse CCD and that luminal UDP and ADP have no effect. UDP is a known agonist for the P2Y$_6$ receptor (26), which has been demonstrated on the luminal side in some epithelia (6,10). 2-Methyl-S-ATP is a potent agonist of the P2Y$_1$ receptor and P2X$_{1-6}$ (1). On the basis of this pharmacologic evidence, our data indicate that probably only a P2Y$_2$ receptor is functionally expressed in the luminal membrane of CCD principal cells. A recent publication on rat inner medullary collecting duct strongly supports the luminal expression of P2Y$_2$ receptors in the collecting duct (18). The authors used different lines of evidence—reverse transcription–PCR, immunoblotting, and immunocytochemistry—to identify P2Y$_2$-receptor expression in this nephron segment. Their immunocytochemical data demonstrate a predominant expression in the luminal and, to a lesser degree, in the basolateral domain of principal cells. This is in agreement with our functional observation that luminal ATP or UTP induces significantly larger $[\text{Ca}^{2+}]_i$ transients than those stimulated with basolateral ATP or UTP. A definitive answer will be possible through investigation of the recently developed P2Y$_2$-receptor knockout mouse (28).

Effect of Basolateral ATP in Mouse CCD

Previous publications in rat and rabbit collecting duct demonstrated the expression of basolateral P2Y$_2$ receptors (18,22). In this work, we did not specifically address the question of different P2 receptors in the basolateral membrane of CCD. However, preliminary data show that ADP and 2-methyl-S-ATP elevate $[\text{Ca}^{2+}]_i$. Basolateral Bz-ATP, $\alpha$, $\beta$-methylene ATP, and UDP were without effect. The effectiveness of 2-methyl-S-ATP here could be indicative of the presence of P2Y$_1$ or of multiple P2X receptors. Preliminary results indicate that the effect of basolateral ADP was almost completely abolished in the absence of extracellular $\text{Ca}^{2+}$. Mouse CCD could therefore also express basolateral P2X receptors, possibly the P2X$_4$ subtype previously identified in mIMCD-K2 cells (16).

Identification of ATP-Responsive Cell in Mouse CCD

Our video-imaging data (Figure 1) indicated that the entire tubule responded to luminal ATP or UTP. However, this does not allow us to conclude which cell type (intercalated or principal) responded to luminal ATP. On the basis of the confocal imaging experiments, we deduce that principal cells are ATP-responsive cells because most cells responded to luminal ATP and approximately 70% of all cells in the mammalian collecting duct are principal cells (29). In addition, the typical flat-shaped geometry of the responsive cells seem to underscore this argument. Principal cells have a typical cell architecture in the collecting duct. It is interesting to note that...
those cells that were responsive to luminal ATP also responded with smaller \([\text{Ca}^{2+}]_i\), elevation to basolateral ATP. This suggests that principal cells express P2 receptors in their luminal and basolateral membranes. Our data do not allow us to conclude whether intercalated cells also responded to luminal ATP or UTP.

**Basolateral PAR-2 in Mouse CCD**

A recent publication identified PAR-2 coupled to InsP₃/Ca²⁺ in the basolateral membrane of M-1 CCD cells (27). Trypsin can act as a specific activator of this receptor. As we have shown here, very low concentrations of trypsin (10 and 100 nmol/L) in intact mouse CCD induced large \([\text{Ca}^{2+}]_i\) increases only from the basolateral side. To make certain that luminally added ATP or UTP did not activate a basolateral P2 receptor, we perfused luminal trypsin (1 μM) in the same tubule at the end of each experiment. In most investigated tubules, the luminal perfusion of trypsin (1 μM) did not elevate \([\text{Ca}^{2+}]_i\), although the same tubule responded to a 10- or 100-fold lower concentration of basolateral trypsin. This indicates that luminally added ATP or UTP activates P2Y receptors in the luminal membrane. In addition, these results show that the intact CCD responds exclusively to basolateral trypsin, which is consistent with the expression of PAR-2 receptors in mouse CCD.

**Functional Effects of Extracellular Nucleotides in the Collecting Duct**

Functional studies addressing the effect of extracellular nucleotides in the renal tubules are limited. Two very interesting articles showed that basolateral ATP inhibited ADH-stimulated H₂O transport via P2Y₂ receptors (20,22), suggesting a role of extracellular ATP in the urinary concentration process. Extracellular ATP decreased the ADH-stimulated cAMP elevation (20). Our study raises the question of whether luminal ATP and UTP may have similar effects on aquaporin 2–mediated water uptake. By these means, ATP could play an important role in short-term regulation of cellular volume as suggested for biliary epithelial cells (30). Another interesting line of evidence suggests that extracellular ATP could mediate the stimulation of cell proliferation in renal tubular cells (31).

Our study further encompassed the species comparison of luminal P2Y-receptor effects in mouse and rabbit CCD. The results strongly suggest that the luminal P2Y receptor in rabbits does not show significant expression in the collecting duct, pointing to marked species difference.

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Errata

The article by Monkawa et al., “Novel Mutation in Thiazide-Sensitive Na-Cl Cotransporter Gene of Patients with Gitelman’s Syndrome” (J Am Soc Nephrol 11: 65–70, 2000) contained an error in the amino acid substitution caused by a mutation. In patient E, the C-T amino acid change caused the mutation Ala569Val, rather than Ala569Glu. As a result, the following corrections should be noted: (1) In the Abstract, column 2, line 9, “Ala569Glu” should read “Ala569Val.” (2) In Table 1, “A569E” should read “A569V.” (3) In the Results section, p. 68, column 2, lines 22 and 25, “Ala to Glu” should read “Ala to Val”; p. 69, column 1, line 1, and p. 70, column 1, lines 5 and 7, “Ala569Glu” should read “Ala569Val.” (4) In Figure 4 legend, “Ala to Glu” should read “Ala to Val.” The authors regret any inconvenience caused by this error.