Extracellular nucleotide signaling along the renal epithelium

ERIK M. SCHWIEBERT1 AND BELLAMKONDA K. KISHORE2

1Departments of Physiology and Biophysics and of Cell Biology, University of Alabama at Birmingham, Birmingham, Alabama, 35294-0005; and 2Division of Nephrology and Hypertension, Department of Internal Medicine, University of Cincinnati Medical Center, Cincinnati, Ohio, 45267-0585

Schwiebert, Erik M., and Bellamkonda K. Kishore. Extracellular nucleotide signaling along the renal epithelium. Am J Physiol Renal Physiol 280: F945–F963, 2001.—During the past two decades, several cell membrane receptors, which preferentially bind extracellular nucleotides, and their analogs have been identified. These receptors, collectively known as nucleotide receptors or “purinergic” receptors, have been characterized and classified on the basis of their biological actions, their pharmacology, their molecular biology, and their tissue and cell distribution. For these receptors to have biological and physiological relevance, nucleotides must be released from cells. The field of extracellular ATP release and signaling is exploding, as assays to detect this biological process increase in number and ingenuity. Studies of ATP release have revealed a myriad of roles in local regulatory (autocrine or paracrine) processes in almost every tissue in the body. The regulatory mechanisms that these receptors control or modulate have physiological and pathophysiologic roles and potential therapeutic applications. Only recently, however, have ATP release and nucleotide receptors been identified along the renal epithelium of the nephron. This work has set the stage for the study of their physiological and pathophysiological roles in the kidney. This review provides a comprehensive presentation of these issues, with a focus on the renal epithelium.

purinergic; adenosine 5’-triphosphate; kidney; receptors; epithelia

HISTORIC ASPECTS OF PURINERGIC SIGNALING

SEVENTY YEARS AGO, Drury and Szent-Gyorgyi (41) were the first to recognize the potent extracellular actions of purine nucleotides and nucleosides in mammalian heart. After this initial report, research was limited to the actions of adenosine and ATP on the cardiovascular system (37, 70, 123). In the early 1960s, a component of the autonomic nervous system that was neither adrenergic nor cholinergic was identified in several tissues. Moreover, aspects of the cardiovascular system also had an unknown neurotransmitter that was neither norepinephrine nor acetylcholine (21, 24). In the early 1970s, Burnstock (21, 24) proposed that the principal neurotransmitter that is released from these specialized nerves was ATP. On the basis of the accepted criteria for neurotransmitters, Burnstock (21, 24) proposed the term “purinergic” for these nonadrenergic and noncholinergic nerves. After Burnstock presented his proposal, the concept of extracellular purinergic neurotransmission was strengthened by a larger body of experimental evidence demonstrating the role of ATP as a neurotransmitter or cotransmitter with norepinephrine, acetylcholine, or other chemical mediators. In addition, knowledge about specific extracellular receptors that mediate the physiological effects of purine nucleotides and nucleosides began to emerge.

In 1980, Burnstock proposed the classification of purinergic receptors into two major groups, P1 and P2, depending on the preferential affinity to adenosine or ATP, respectively (22, 23). This classification also took
into account the selective activation of adenylate cyclase by adenosine and the induction of prostaglandin synthesis by ATP. Subsequently, Lodos et al. (86) and Van Calker et al. (147) identified two subclasses of adenosine (P1) receptors. These subclasses were later termed “A1” and “A2” in the nomenclature. By using molecular homology cloning methods (157) and receptor binding assays (33), A3 and A4 subtypes of P1 receptors were also identified. The A4 subtype is still not validated fully. These adenosine receptor subtypes couple to different heterotrimeric G proteins and effectors; adenosine receptors are not the major focus of this review (for reviews on adenosine receptors, see Refs. 100, 101, 108, and 133).

On the basis of the differences in rank-potency order profiles of nucleotides and nucleotide analogs, Burnstock and Kennedy suggested a subclassification of P2 receptors into P2X and P2Y subtypes (25). Later studies revealed that P2X and P2Y receptors also differ in their transduction mechanisms (55). P2X receptors appeared to have an intrinsic ion channel that increased the permeability of the plasma membrane to Na\(^{+}\), K\(^{+}\), and Ca\(^{2+}\) (and, possibly, anions), whereas P2Y receptors were traditional G protein-coupled receptors that coupled to heterotrimeric G proteins, phospholipases, and phosphoinositol signaling pathways. Gordon (50) further delineated the existence of P2T and P2Z subtypes of receptors into platelets and mast cells, respectively. These were originally thought to be distinctly different from the P2X and P2Y receptor subtypes. In his seminal review, Gordon also emphasized the concept of biologically relevant release of nucleotides and nucleosides, discussing the sources, effects, and fates of these purinergic agonists. Subsequently, another subtype of P2 receptor that responds to the pyrimidine nucleotide UTP as well as to ATP was identified and termed “P2U receptor” or the pyrimidine receptor. In addition to the P2 receptor subtypes discussed above, receptors that bind adenosine dinucleotide polyphosphates (Ap\(_{2}\)A, Ap\(_{3}\)A, and Ap\(_{5}\)A) were identified and classified as P2D receptors (27, 59). Although possible functions as neurotransmitters or cotransmitters have been suggested, the exact physiological roles of the diadenosine polyphosphates (Ap\(_{n}\)A) are not yet well established.

**PURINERGIC RECEPTORS: “METABOTROPIC” P2Y G PROTEIN-COUPLED RECEPTORS AND “IONOTROPIC” ATP-GATED P2X RECEPTOR CHANNELS**

Figure 1 shows the probable topologies of P2Y and P2X purinergic receptors that we discuss below in this section.

**Metabotropic P2Y G Protein-Coupled Receptors**

P2Y receptors are G protein-coupled receptors that bind purine and/or pyrimidine nucleotides and their derivatives. This class of receptors includes the cloned mammalian P2Y\(_{1}\), P2Y\(_{2}\), P2Y\(_{4}\), P2Y\(_{6}\), and P2Y\(_{11}\) receptors. The P2Y\(_{1}\) receptor subtype is the original P2Y receptor of the old nomenclature, whereas the P2Y\(_{2}\) receptor is the P2U receptor of the old classification (46). In addition, the pharmacologically characterized, but not yet cloned, P2Y\(_{ADP}\) (or P2T) receptor from platelets is also included in this class (reviewed in Refs. 1, 2, 8, 13, and 82). The cloned P2Y\(_{3}\) receptor represents a species homolog of the P2Y\(_{6}\) receptor. Moreover, the mammalian equivalent of the *Xenopus laevis* P2Y\(_{5}\) receptor has not yet been cloned (108). Receptors that were initially numbered P2Y\(_{5}\), P2Y\(_{7}\), P2Y\(_{9}\), and P2Y\(_{10}\) were subsequently found not to be receptors for nucleotides (despite their cloning by homology to other P2Y receptor genes) and, therefore, have been deleted from this class (108). Table 1 shows the properties of different subtypes of cloned mammalian P2Y receptors, their agonist rank-potency order, sources of cDNA, and the cell and tissue distribution. P2Y receptor expression in the kidney has been reviewed recently (4, 26, 63).

P2Y receptors vary in length from 308 to 379 amino acids, with a molecular mass of 41–53 kDa in glycosylated form. These receptors have seven transmembrane-spanning α-helical hydrophobic regions typical of G protein-coupled receptors. The NH\(_{2}\) terminus of the receptor proteins is on the extracellular side of the plasma membrane, whereas the COOH terminus lies on the cytosolic side of the membrane. The three-dimensional orientation of the transmembrane domains creates a pocket with positively charged amino acids that interact with the phosphate groups of the nucleotide ligands (ATP/ADP/UTP/diadenosine polyphosphates). These proteins are usually N-glycosylated on their second extracellular loop. Although the exact roles of glycosylation are not known, it has been suggested that the carbohydrate moieties stabilize the protein conformation, protect the receptors from the action of proteases, and modulate receptor function (for recent and exhaustive reviews, see Refs. 2 and 108).
The different subtypes of P2Y receptors interact with different types of G proteins through their intracellular loops. These G proteins usually activate a membrane-bound phosphatidylinositol-specific phospholipase C (PLC), resulting in enhanced formation of inositol 1,4,5-trisphosphate (IP$_3$) and mobilization of cytosolic Ca$^{2+}$ (Ca$^{2+}$). In addition, activation of both P2Y$_1$ and P2Y$_2$ receptors leads to subsequent inhibition of adenylate cyclase through a G$_i$-dependent mechanism. Furthermore, the P2Y$_{11}$ receptor is unique among P2Y receptors because it couples to the stimulation of both phosphatidylinositol and adenylate cyclase signaling pathways. On the other hand, the activation of platelet P2Y$_{ADP}$ receptor results predominantly in the inhibition of adenylate cyclase. Depending on the receptor subtype and on the cell type that expresses the receptor, the formation of IP$_3$ and mobilization of Ca$^{2+}$ can stimulate several other intracellular signaling pathways. These pathways include activation of protein kinase C (PKC), phospholipase A$_2$ (PLA$_2$), Ca$^{2+}$-dependent K$^+$ channels, or nitric oxide (NO) production. In addition, the diacylglycerol (DAG), formed as a result of PLC action, stimulates PKC, which, in turn, may activate phosphatidylcholine-specific PLC, phospholipase D (PLD), the MAP kinase pathway, and/or Ca$^{2+}$ influx via voltage-operated Ca$^{2+}$ channels. The specific downstream events seem to be dependent on the particular cell type in which the P2Y receptor is expressed. Thus in endothelial cells, the activation of P2Y$_1$ or P2Y$_2$ receptors results in the activation of PKC, without detectable elevation in IP$_3$ or Ca$^{2+}$, and with subsequent rapid tyrosine phosphorylation of MAP kinase, resulting in the production of prostacyclins. Because of this complex nature of signal transduction mechanisms triggered by P2Y receptors, the response time of P2Y receptors is longer than the response time of P2X receptors (for recent and exhaustive reviews, see Refs. 2, 26, and 108).

P2Y receptors as a class do not desensitize as readily as other G protein-coupled receptor subfamilies. However, when they do desensitize, the mechanism involves either phosphorylation by protein kinases or uncoupling from the G protein. Suramin is a generalized antagonist of P2 receptors, except for P2Y$_4$. Presently, there are no specific antagonists available to distinguish the different subtypes of cloned mammalian P2 receptors, a void in the field that needs to be filled. Nonselective inhibitors such as suramin, reactive blue 2, and pyrodoxal phosphate-6-azophenyl 2',4'-disulfonic acid (PPADS) can be used to antagonize either or both of the P2Y and P2X receptors (16). PPADS blocks the P2Y$_1$ receptors coupled to PLC but not those coupled to the inhibition of adenylate cyclase. The platelet P2Y$_{ADP}$ receptor is blocked by 2-propylthio-d-$\beta$-$\gamma$-difluoromethylene ATP (FPL 66096) and 2-propylthio-$\beta$-$\gamma$-dichloromethylene-d-ATP (ARL). P2Y receptor expression and function along the nephron will be addressed below (for recent and comprehensive reviews, see Refs. 2 and 108).

### Ionotropic ATP-Gated P2X Receptor Channels

P2X receptors are Ca$^{2+}$-permeable, nonselective cation channels (reviewed in Refs. 1, 8, 13, 16, and 90). To date, eight isoforms have been identified and cloned: P2X$_1$ through P2X$_7$, and P2X$_8$, the most recent isoform cloned from skeletal muscle (146). P2X7 is thought to be the cDNA that corresponds to the P2Z receptor (139) described by Gordon (50) in hematopoietic cells. Like the P2Y receptors, it is likely that multiple P2X receptor channel subtypes remain to be identified. Each isoform consists of two membrane-spanning $\alpha$-helices, a large extracellular domain (comprising at least 50% of the total molecular mass), and intracellular NH$_2$ and COOH termini. From the NH$_2$ terminus to the second transmembrane domain, the isoforms are 37–48% identical, with 10 conserved cysteine residues in the extracellular domain of each isoform. These cysteines may be important in stabilizing the ATP-binding pocket of the extracellular domain. The cysteines may also confer a complex three-dimensional structure. Multiple N-linked glycosylation sites are predicted in the extracellular domain; preliminary immunoblotting results from epithelial membrane protein lysates reveal complex glycosylation that doubles the molecular mass of the P2X receptor channel protein over its predicted size, on the basis of the amino acid sequence (Taylor AL and Schwiebert EM, unpublished observations). The COOH terminus of each of the isoforms is the most variable, and the COOH termini of the different isoforms have been used as antigens whereby the majority of the isoform-specific antibodies have been made.

P2X receptors have no sequence homology to any other ion channel. In over 300 sequences, obtained by PCR amplifications on epithelial and endothelial cDNA
the higher permeability for divalent cations, Ca\(^{2+}\) and Mg\(^{2+}\) also block the channel. Of the seven isoforms, only P2X\(_1\) and P2X\(_7\) desensitize rapidly on binding to the agonist (20, 30). Interestingly, the most abundant isoforms expressed in epithelia (Schwiebert EM, Wallace D, King SR, Braunstein GM, Peti-Peterdi J, Hanaoka K, Guay-Woodford LM, Bell PD, Sullivan L, Grantham JJ, and Taylor AL, unpublished observations; 143) and endothelia (Schwiebert LM, Rice WC, Kudlow BA, Taylor AL, and Schwiebert EM, unpublished observations) are poorly desensitizing members of the P2X receptor family, P2X\(_4\), P2X\(_5\), and, to a lesser extent, P2X\(_2\) and P2X\(_7\). P2X receptor channel expression and function along the nephron will be addressed below.

### ATP Release into the Extracellular Milieu Along the Nephron: Assays, Sources, Mechanisms, and Stimuli

**Extracellular Nucleotides as Agonists**

Cytosolic concentrations of ATP are typically 3–5 mM (possibly as high as 10 mM), whereas its extracellular concentration is very low (50, 108, 124, 142). Despite this huge concentration gradient, ATP and other intracellular nucleotides cannot diffuse out through the lipid bilayer of the cells because of their net negative charge. However, highly controlled and regulated release of intracellular nucleotides occurs in many types of cells under physiological conditions. Pathologically, ATP is released from cells during hypoxia, shear stress, loss of cell viability, or cytolysis. Although metabolism of nucleotides in blood alone is slow and inefficient (in vitro half-time of ATP in whole blood is 10 min and in cell-free plasma is 30 min), nucleotides are rapidly cleared while passing through the vascular bed (half-time of nucleotides in perfused lung is ~0.2 s). This is due to the presence of ecto-ATPase, ecto-apyrase, and 5’-nucleotidase activity on the luminal surface of endothelial cells (50, 108, 124, 142). Thus ATP and its metabolites are traditionally thought to act as autocrine or paracrine factors that act within tissues or tissue microenvironments. The concentration of extracellular nucleotides needed to activate the purinergic receptors is very low (0.1–10 μM)

### Table 2. Properties of different subtypes of cloned mammalian P2X receptors

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Amino Acids</th>
<th>(\gamma)</th>
<th>(pS)</th>
<th>Desens.</th>
<th>EC(_{50}), μM</th>
<th>Tissue Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X(_1)</td>
<td>399</td>
<td>18</td>
<td>Rapid</td>
<td>1</td>
<td>2</td>
<td>SC, VSM</td>
</tr>
<tr>
<td>P2X(_2)</td>
<td>472</td>
<td>21</td>
<td>Poor</td>
<td>8</td>
<td>&gt;100</td>
<td>Br, SC</td>
</tr>
<tr>
<td>P2X(_3)</td>
<td>397</td>
<td>F(^{+})</td>
<td>Rapid</td>
<td>1</td>
<td>1</td>
<td>SC</td>
</tr>
<tr>
<td>P2X(_4)</td>
<td>388</td>
<td>9</td>
<td>Slow</td>
<td>10</td>
<td>&gt;100</td>
<td>Br, SC, L, K, Endo</td>
</tr>
<tr>
<td>P2X(_5)</td>
<td>455</td>
<td>Poor</td>
<td>15</td>
<td>&gt;100</td>
<td>Br, SC, L, K, Endo</td>
<td></td>
</tr>
<tr>
<td>P2X(_6)</td>
<td>379</td>
<td>Slow</td>
<td>12</td>
<td>&gt;100</td>
<td>Br, SC</td>
<td></td>
</tr>
<tr>
<td>P2X(_7)</td>
<td>595</td>
<td>Slow</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>Hemato</td>
<td></td>
</tr>
</tbody>
</table>

EC\(_{50}\) for agonists is representative. Most of the work has been done in heterologous systems. F\(^{+}\) represents the fact that single channels attributed to P2X3 receptor channels were so flickery that a conductance could not be measured reliably. Desens., desensitization; SC, spinal cord (in particular, neurons involved in nociception); VSM, vascular smooth muscle; Br, brain; L, lung epithelium; K, kidney epithelium; Endo, endothelium; Hemato, hematopoietic cells.
compared with their intracellular concentrations. As such, the cells need to release only 0.1% or less of its intracellular ATP pool to trigger autocrine or paracrine ATP signaling (50, 108, 124, 142). Several additional factors must also be considered that may influence the attainment of the effective concentrations of nucleotides in the extracellular milieu. These factors include, but are not limited to, the amount of the nucleotides released, their volume of distribution in extracellular microenvironment, and the presence and activity of ecto-apyrases. Despite these many factors, ATP has been measured in significant concentrations in plasma, bile, and urine (31, 62, 93). Figure 2 integrates the concept of purinergic receptor expression on apical and basolateral membranes, the ATP release mechanisms that may promote ATP release from epithelia (see below), and some examples of epithelial processes that purinergic signaling regulates.

**ATP Release Mechanisms**

There are at least three possible mechanisms for the regulated release of nucleotides under physiological conditions. Exocytosis of ATP-filled vesicles is a major mechanism, especially in platelets, neurons, and neuroendocrine cells, such as adrenal medullary chromaffin cells and mast cells. The dense granules of platelets, mast cells, and chromaffin cells store many agonists; however, they also contain ATP and/or ADP at very high concentrations (mM or higher). Platelet dense granules contain a combined ATP + ADP concentration of ~1 M. Approximately 15% of the dry weight of adrenal medulla is due to ATP. UTP is also released by platelets during platelet aggregation, although the intracellular concentration of UTP is much lower than ATP. As such, ATP and its metabolites are thought to be neurotransmitters or cotransmitters or coagonists with other classic neurotransmitters or histamine (50, 108).

Nonexocytotic release of ATP and other nucleotides likely occurs by passive transport mechanisms, using the large gradient for ATP efflux, secretion, or exit. Nonconductive transport of ATP (passively down its large concentration gradient out of the cell) may also be a route of ATP release. Such adenine nucleotide transporters have been studied intensely in mitochondrial membrane (19, 87, 119) and have been identified recently in endoplasmic and sarcoplasmic reticulum (53), chromaffin granule ghosts (7), and in rat brain synaptic vesicles (51). Nonconductive, bidirectional transporters exist in the plasma membrane for nucleosides primarily in brain (35); therefore, it cannot be discounted that ATP-specific transporters may exist. Indeed, the cystic fibrosis transmembrane conductance regulator (CFTR), once thought to be an ATP channel, may transport ATP as well as other larger organic anions (gluconate, glutathione) at nonconductive rates. By the macropatch-recording method, Lindsell and Hanrahan (85) showed rates of transport of larger organic anions that were on the border between conductive and nonconductive.

ATP-permeable anion channels have been characterized biophysically by many laboratories. Their relationship to CFTR anion channels has been controversial (68, 83, 109, 110, 111, 124, 126); however, recent studies by Engelhart and colleagues (68), Foskett and co-workers (105, 136), and Schwiebert and colleagues (17) suggest that CFTR does not conduct ATP itself but regulates a closely associated anion channel that does

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**Fig. 2.** ATP release mechanisms, extracellular ATP signaling, and emerging role of P2X receptor channels in the transduction of autocrine and paracrine ATP agonist signaling in airway or kidney epithelium. Release mechanisms and the large concentration gradient for ATP release from airway epithelia are shown on the left. P2X receptor channels in the apical or basolateral membrane of the same cell or a neighboring cell, on binding their ATP ligand, may increase cytosolic Ca\(^{2+}\) transiently and trigger Ca\(^{2+}\)-dependent protein kinase signaling in a more sustained manner. These signaling cascades stimulate Cl\(^{-}\) and fluid secretion and ciliary beat frequency (shown in the model) as well as inhibit Na\(^{+}\) absorption and arginine vasopressin (AVP)-induced water reabsorption (not shown in the model).
conduct ATP. Anion channels with less selectivity for Cl− vs. other halides or larger anions are prime candidates for putative ATP channels. These include, but are not limited to, the outwardly rectifying Cl− channel (or ORCC) as well as plasma membrane forms of the voltage-dependent anion channel (VDAC; also called “porin”) (10, 11, 112, 144). Large-conductance or “maxi” Cl− channels that resemble mitochondrial VDAC or porin biophysically have been observed in most if not all cells. Interestingly, the major role of VDAC or porin in the mitochondrial membrane is the transport of newly synthesized ATP from the mitochondrion to the cytoplasm. Schwiebert and co-workers (142) as well as Fitz and colleagues (113, 114, 115, 149) have shown that ATP release is stimulated under conditions of hypotonic stress. This release is immediate (within seconds), and it precedes the time course for regulatory volume decrease after cell swelling (17, 142). Another elegant example of a paracrine ATP signal involves propagation of Ca2+ waves along monolayers of cells in a gap junction-independent manner (49, 120). Thus these are key examples of how ATP works well as a signaling molecule, because it is released rapidly down its large concentration gradient, it acts in an autocrine or paracrine manner, it is rapidly degraded to dissipate the response, and it mediates fast and slow responses, via P2X and P2Y receptors, respectively (16).

Assays to Detect ATP Release and Signaling

Several laboratories have explored ATP release in the context of excitable and nonexcitable cells and have developed different assays to aid this study. Schwiebert and co-workers (142) developed an assay using a luminometer and the firefly substrate and enzyme luciferin-luciferase by which monolayers of epithelial and endothelial cells, which were tight with fluid and had a resistance level >200 Ω/cm2, are lowered into the luminometer and studied in real time. A Turner TD 20/20 luminometer or the equivalent is required to perform these real-time assays, because it is equipped with a large chamber and a platform that can accommodate dish sizes as large as 35 mm or filter diameters as large as 24 mm. Real-time assays have provided better and more reproducible results than assays where aliquots are taken from a preparation and injected into an injection port or cuvette of a luminometer. Excess luciferin-luciferase (1–2 mg/ml of lyophilized material that may not all be the detection enzyme and substrate by mass) is added to monolayers of epithelial cells bathed in a serum-free medium devoid of ATP. Each molecule of ATP released by the monolayer reacts with the luciferin-luciferase and yields 1 photon of light. Light is measured by the luminometer in arbitrary light units that can be calibrated with known concentrations of ATP. Our assay demonstrated that epithelial cells release a basal level of ATP, and under hypotonic conditions, they will release significantly more ATP. This assay has also provided evidence that cells expressing mutant CFTR release significantly less ATP under basal and hypotonic conditions than do wild-type CFTR-expressing cells. Finally, this assay determined that epithelial cells release more ATP apically than basolaterally under all experimental conditions (142).

Another elegant assay determines ATP release by using luciferin-luciferase attached to the surface of a cell. Luciferase is fused to protein A, a protein that binds to IgG antibodies. Cells are incubated first with an IgG antibody to an extracellular epitope of a specific cell surface protein, then with the protein A-luciferase fusion product. The cells are placed in a luminometer to collect photons as an indicator of localized extracellular ATP release at the membrane surface (9). This assay was utilized on hematopoietic cells, where surface antigens are abundant and well characterized. However, it could be adapted to tissue preparation such as isolated and perfused renal tubules, intestinal crypts, or isolated glans or ducts.

The most recent assay involves atomic force microscopy (AFM) using commercially available AFM tips coated with the myosin subfragment S1, which has a high affinity for ATP and changes shape on ATP hydrolysis. Rather than luciferase-luciferin reagent as the readout, the myosin tips are placed next to a cell, bind the ATP as it is released, and the myosin in the tips responds by changing shape (121). Using this assay, Schneider and co-workers (121) showed that cystic fibrosis airway epithelial cells released little ATP (e.g., the vibration of the AFM probe was minimal); however, when the probe was placed in close proximity to the membranes of cystic fibrosis (CF) cells stably complemented with wild-type CFTR, significant vibration of the probe was observed. Adaptation to isolated tubule preparations has not been performed; however, it is a definite possibility.

Creative indirect assays have also been developed, using the P2X receptor channels as a functional readout. Hazama et al. (57) developed an assay that determines ATP release from a single cell. A single PC12 cell, which expresses P2X2, is lifted while in whole-cell patch configuration and placed near a pancreatic β-cell growing on a coverslip. The pancreatic β-cell is then stimulated with glucose, while the PC12 cell is studied for P2X2 current. Current is indicative of the β-cell’s release of ATP (57). This assay is applicable to any cell or dissected tissue preparation of interest (see below), when maintained in culture. Moreover, instead of P2X receptor current as an endpoint, one could load the PC-12 cell with fura 2-acetoxymethyl ester (AM) and measure Ca2+ influx through P2X2 as a different fluorescent endpoint (see below). Hollins and Ikeda (60) used a similar strategy. They transfected rat adrenal chromaffin cells with P2X2 and then stimulated the cells with agents that increase exocytosis. Their patch-clamp data showed that stimulating exocytosis led to P2X1 current, implying that ATP is released in a vesicular manner. Indeed, other laboratories are using these groundbreaking assays or modifying them further with great success. Moreover, their possible application to the kidney and to cell or tissue preparations of the nephron was reviewed recently in more detail (125)
and will be expanded on below (see Hypotheses and Future Directions).

**Putative ATP Release and Release Mechanisms Along the Nephron**

Schwiebert and co-workers (Schwiebert EM, Wallace D, King SR, Braunstein GM, Peti-Peterdi J, Hanaoka K, Guay-Woodford LM, Bell PD, Sullivan L, Grantham JJ, and Taylor AL, unpublished observations) have used the bioluminescence detection assay to detect ATP released from primary cultures and cell lines derived from known nephron segments and grown in vitro to determine the major sources of extracellular ATP, the regulation of ATP release, and, ultimately, the mechanisms of ATP release. This assay has also been applied to human vascular endothelial monolayers in primary cultures derived from different blood vessels throughout the vasculature with promising results (Schwiebert LM, Rice WC, Kudlow BA, Taylor AL, and Schwiebert EM, unpublished observations). Pseudopolarized epithelial cell cultures or polarized epithelial cell monolayers were studied in real time for basal and stimulated ATP release and for the sidedness of ATP release, respectively.

Figure 3 shows estimated concentrations of ATP based on parallel standard curves using the same luciferase-luciferin-containing reagent but with known quantities of ATP. The proximal tubule is the richest source of ATP under basal conditions, at concentrations that reach ~1 μM. On stimulation, this concentration increases; however, maximal ATP release never exceeds 5–10 μM. A study using human renal primary cultures to document ATP concentrations measured under basal and hypotonic conditions with this bioluminescence assay have been published (151). As other cultures are examined along the nephron, the amount of ATP release under basal conditions decreases through the nanomolar range. Collecting duct epithelia release low-nanomolar to picomolar concentrations of ATP under basal conditions, but collecting duct cell lines can be induced to release ATP with hypotonicity (a condition that would be present in diuresis luminally) and with the Ca^{2+} agonists ionomycin and thapsigargin. (Schwiebert EM, Wallace D, King SR, Braunstein GM, Peti-Peterdi J, Hanaoka K, Guay-Woodford LM, Bell PD, Sullivan L, Grantham JJ, and Taylor AL, unpublished observations). As a general principle, ATP release into the apical medium is more robust than basolateral-directed ATP release (151). Hypotonic challenge stimulates ATP release immediately and with transient and sustained components of a time course across the apical and basolateral membranes. Ca^{2+} agonists stimulate a slow, monophasic rise in ATP release that plateaus after several minutes. Ca^{2+} agonists stimulate apical-directed ATP release, whereas they are without effect on basolateral release. Present work is focused on studying the regulation of ATP release as well as ATP release mechanisms in renal epithelial cell models. Similar work is ongoing in human vascular endothelial monolayers (Schwiebert LM, Rice WC, Kudlow BA, Taylor AL, and Schwiebert EM, unpublished observations). The ultimate goal is to adapt this assay to the study of whole kidney or isolated nephron segments (see below).

Bell and colleagues have used the PC-12 cell biosensor method developed by Okada and co-workers to detect ATP release across the basolateral membrane of the macula densa (MD) plaque that lies within the cortical thick ascending limb (cTAL) juxtaposed to the afferent arterioles and the glomerulus (Bell PD, personal communication). It is their hypothesis that ATP is released from macula densa (MD) in response to cTAL transport of Cl⁻ across the MD as an autocrine/paracrine signal for tubuloglomerular (TG) feedback. With the use of whole cell patch-clamp recording of PC-12 cells placed near the basolateral surface of the MD plaque, ATP-gated P2XR current was detected, indicative of ATP release from the plaque. Similarly, PC-12 cells loaded with the Ca^{2+}-sensitive dye fura 2-AM were used as a fluorescence biosensor, and an increase in Ca^{2+} was detected, also indicative of physiologically relevant and sufficient ATP release to stimulate receptors on the biosensor cell in vitro or in the glomerulus in vivo. In both cases, ATP release was stimulated by low NaCl in the lumen of the cTAL and was blocked by luminal and basolateral Cl⁻ transport inhibitors. To address the mechanisms of ATP release, cells of the MD plaque were patch-clamped and the ATP permeability of anion channels recorded in this membrane was examined. The most promising candidate for an ATP-permeable anion channel was a maxi-anion channel of ~300 pS that had an ATP conduc-

![Image of the renal nephron with ATP release](https://example.com/renal-nephron.png)
stance of ~100 pS and also had permeability to other anions such as Cl\(^-\) and gluconate (Bell PD, personal communication). Intriguingly, the voltage dependence of this ATP-permeable channel is similar to the VDAC. As described above, the role of VDAC or porin in mitochondrial membrane is to transport newly synthesized ATP out of the mitochondrion into the cytoplasm (10, 11). If plasma membrane-expressed VDAC channels exist (112, 144), this may be a principle ATP release channel. Nevertheless, this is intriguing preliminary work and is an example of a physiological role for extracellular ATP release and signaling.

Kishore and Knepper incubated freshly microdissected rat inner medullary collecting duct (IMCD) segments in vitro under physiological conditions at 37°C in Terasaki plates under mineral oil and with oxygenation. They measured the release of ATP by the tubules into the medium at different time points using the luciferin-luciferase assay system in a luminometer. Their data showed that that IMCD segments release ATP at a rate of 2.11 ± 0.29 fmol·mm\(^-1\)·30 min\(^-1\), with a range of 1.43–3.3 fmol. This release was confirmed further in microperfused IMCD, which released ATP into the lumen at a rate of 1.52 fmol·mm\(^-1\)·10 min\(^-1\). They also correlated this result with the total ATP content of the IMCD segments. The ATP content was 1 log order of magnitude greater than the rate at which the IMCD released ATP. They concluded that the release was physiological and did not represent a nonspecific leakage due to cell death. These preliminary data indicate that ATP can be released by the collecting duct cells as an autocrine, paracrine, or autoregulatory mediator (Kishore BK and Knepper MA, unpublished observations).

More indirectly, two laboratories have documented a role for endogenous ATP release and signaling as a modulator of intracellular signaling set points. Insel and co-workers (63) have recently published the conclusion that ATP, released endogenously and continuously by Madin-Darby canine kidney (MDCK) cells, modulates phosphatidylinositol signaling and turnover as well as cAMP production in MDCK cells. They showed that removal of the endogenously released ATP with the ATPase/ADPase apyrase or antagonism of P2 receptors significantly decreased arachidonic acid release as well as cAMP production. They also observed that vigorous medium changes on MDCK and other cultures stimulated ATP release mechanically. Similar results have been observed in fura 2-AM Ca\(^{2+}\) imaging experiments by Schwiebert and colleagues (Schwiebert EM, Wallace D, King SR, Braunstein GM, Peti-Peterdi J, Hanaoka K, Guay-Woodford LM, Bell PD, Sullivan L, Grantham JJ, and Taylor AL, unpublished observations). In a circulating system where basal Ca\(^{2+}\) was monitored in normal and polycystic kidney [polycystic kidney disease (PKD)] primary cultures, the ATP scavengers hexokinase and apyrase or the P2Y and P2X receptor antagonist suramin lowered basal Ca\(^{2+}\) significantly and reversibly. In fact, 10 and 100 µM suramin dropped basal Ca\(^{2+}\) significantly in a rapid and reversible manner (Schwiebert EM, Wallace D, King SR, Braunstein GM, Peti-Peterdi J, Hanaoka K, Guay-Woodford LM, Bell PD, Sullivan L, Grantham JJ, and Taylor AL, unpublished observations). Taken together, these two studies suggest that autocrine and paracrine ATP signaling occurs constantly in the extracellular milieu and, at least in part, establishes a “set point” for multiple signal transduction pathways or signaling molecules. Indeed, basal modulation of cell volume regulation and Cl\(^-\) channel activity by endogenous ATP signaling was also shown in hepatocytes and cholangiocytes by Fitz and co-workers (114).

Taken together, for purinergic receptor expression to be relevant physiologically (see next section) there must be biologically relevant ATP release occurring in the same tissue, culture, or microenvironment. Not as much attention has been paid to ATP release in the past; however, interest in this aspect is gaining momentum in the purinergic receptor field. It is likely that these fields will merge and synergize to bring the larger picture of autocrine/paracrine extracellular purinergic signaling into focus.

**SEGMENTAL DISTRIBUTION OF PURINERGIC RECEPTORS ALONG THE NEPHRON EPITHELIUM: PURINERGIC SIGNALING AND REGULATION OF RENAL EPITHELIAL FUNCTION**

The physiological significance of purinergic signaling in different cell types along the nephron and in specific nephron segments is an emerging and complex area of research. The principles of ATP release, ATP degradation, multiple subtypes and subfamilies of ATP receptors, and their coupling to multiple signal transduction cascades (discussed above) are complex in itself. This complexity is magnified by the 20 or more different cell types along the nephron and the 10 or more discrete nephron segments that are in contact with the tubular fluid or interstitium containing nucleotide or nucleoside agonists. In this section, themes will emerge that presently guide this field and will continue to guide it for some time to come. First, purinergic receptors are expressed abundantly in the kidney on all cell types of the glomerulus, on vascular smooth muscle and endothelial cells of the renal vasculature, and on the renal epithelium in all nephron segments where it has been examined. Second, in a given renal epithelial cell model (MDCK, mIMCD-K2, etc.), multiple P2Y receptors and multiple P2X receptor channels are expressed in the same epithelial cell and, often, in the same membrane domain of the epithelial cell. The reason this redundancy in ATP receptors is needed is an important fundamental question. Redundancy may be needed for an essential physiological process in all cells such as cell volume regulation, or the P2Y receptors and P2X receptor channels may fulfill completely different roles in the same epithelial cell. Third, and most important, how does purinergic signaling as an autocrine and paracrine agonist cascade regulate renal function independently of other agonists and how does it modulate the effects of important endocrine hormones such as vasopressin or angiotensin II or aldosterone? Each
of these fundamental questions is just beginning to be addressed.

**P2Y Receptors Along the Renal Epithelium**

The P2Y<sub>2</sub> receptor has been the most often studied P2Y subtype along the nephron. This fact is mainly due to its ubiquitous expression in most, if not, all cells and tissues. Moreover, UTP is the highest affinity agonist for the P2Y<sub>2</sub> receptor, formally defined as the P2U receptor, for this reason. Therefore, laboratories could merely test for the expression of the P2Y<sub>2</sub> receptor with UTP. This has been complicated by the fact that newly cloned subtypes P2Y<sub>4</sub> and, to a lesser extent, P2Y<sub>6</sub>, are also stimulated by UTP or UDP. As such, all previous studies with UTP cannot be solely attributable to the P2Y<sub>2</sub> receptor. P2Y<sub>4</sub> involvement cannot be discounted in studies with UTP cannot be solely attributable to the P2Y<sub>2</sub> receptor, for this reason. Therefore, laboratories could merely test for the expression of the P2Y<sub>2</sub> receptor with UTP. This has been complicated by the fact that newly cloned subtypes P2Y<sub>4</sub> and, to a lesser extent, P2Y<sub>6</sub>, are also stimulated by UTP or UDP. As such, all previous studies with UTP cannot be solely attributable to the P2Y<sub>2</sub> receptor. P2Y<sub>4</sub> involvement cannot be discounted in renal epithelial cell models until its expression in the kidney is ruled out. To our knowledge, expression of P2Y<sub>4</sub> has not been investigated in the kidney. In the context of this section, hints in past literature as well as emerging studies will touch on the expression of P2Y<sub>1</sub>, P2Y<sub>6</sub>, and P2Y<sub>11</sub> in renal epithelial cell models in addition to the extensive work on P2Y<sub>2</sub> (Table 3).

Kishore et al. (73) mapped P2Y<sub>2</sub> receptor expression along the mammalian nephron. Specific antibodies to the P2Y<sub>2</sub> receptor as well as a gene-specific cDNA probe confirmed its expression in terminal IMCD as well as its ubiquitous expression in all regions and nephron segments of the kidney. Apart from the medullary regions and segments, cortical regions and segments also showed strong immunohistochemical signal (Kishore BK, unpublished observations). Of interest, expression of the P2Y<sub>2</sub> receptor on both membrane domains of the IMCD was also found (see Fig. 4), which is similar to the expression of vasopressin V<sub>2</sub> receptor in IMCD (98). This is both interesting and perplexing, and this issue requires further investigation. The present working model for the regulation of collecting duct water permeability that grew out of the work of Kishore and co-workers as well as other investigators is shown in Fig. 4. AVP, acting through its V<sub>2</sub> receptor and the cAMP second messenger system, increases osmotic water permeability of the collecting duct apical membrane by translocating the aquaporin-2 (AQP2) water channel-containing vesicles from a subsapical pool to the apical plasma membrane. The apical membrane is the rate-limiting barrier for transepithelial water transport, as AQP3 and AQP4 are expressed constitutively in the basolateral plasma membrane under normal conditions (for reviews, see Refs. 96 and 97). On the other hand, the agonist activation of the P2Y<sub>2</sub> receptor for extracellular nucleotides, as well as the endothelin receptor (ET<sub>B</sub>R) and the EP<sub>3</sub> subtype receptor of prostaglandin E<sub>2</sub> (EP<sub>3</sub>R), antagonize the AVP-stimulated water transport in collecting duct (18, 43, 58, 71, 79, 116). This antagonism is achieved by virtue of the cross-talk mechanisms that exist between the two mutually opposing intracellular signaling pathways in the collecting ducts, as depicted in Fig. 4 (18, 58, 71, 78, 116).

### Table 3. P2Y receptor subtype distribution along the renal epithelium and in cell lines of renal origin

<table>
<thead>
<tr>
<th>Cell Line or Nephron Segment</th>
<th>Tissue Source or Species</th>
<th>P2 Subtype</th>
<th>Functional Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLC-PK&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Porcine PT</td>
<td>P2Y&lt;sub&gt;2&lt;/sub&gt;</td>
<td>↓ AC</td>
</tr>
<tr>
<td>MDCK</td>
<td>Canine Distal</td>
<td>P2Y&lt;sub&gt;1&lt;/sub&gt;, P2Y&lt;sub&gt;2&lt;/sub&gt;</td>
<td>↑ AA, PGE&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Neprhon</td>
<td>Mouse IMCD</td>
<td>P2Y&lt;sub&gt;3&lt;/sub&gt;, P2Y&lt;sub&gt;2&lt;/sub&gt;</td>
<td>↓ PO&lt;sub&gt;4&lt;/sub&gt; uptake</td>
</tr>
<tr>
<td>OK</td>
<td>Opossum PT</td>
<td>P2Y&lt;sub&gt;2&lt;/sub&gt;</td>
<td>↓ Na&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>mIMCD-K2</td>
<td>Mouse IMCD</td>
<td>P2Y&lt;sub&gt;2&lt;/sub&gt;, P2Y&lt;sub&gt;2&lt;/sub&gt;</td>
<td>↑ Cl&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>NF-5</td>
<td>Mouse early PT</td>
<td>P2Y&lt;sub&gt;1&lt;/sub&gt;, P2Y&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Mitogenic</td>
</tr>
<tr>
<td>Mesangial cells</td>
<td>Rat</td>
<td>P2Y&lt;sub&gt;2&lt;/sub&gt;, P2Y&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Mitogenic</td>
</tr>
<tr>
<td>PT</td>
<td>Rat, Rabbit</td>
<td>P2Y&lt;sub&gt;2&lt;/sub&gt;</td>
<td>↓ Na-K-ATPase</td>
</tr>
<tr>
<td>mTAL</td>
<td>Rat</td>
<td>P2Y&lt;sub&gt;2&lt;/sub&gt;</td>
<td>↑ Ca&lt;sup&gt;2+&lt;/sup&gt;, PI</td>
</tr>
<tr>
<td>cTAL</td>
<td>Rat</td>
<td>P2Y&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>CDG</td>
<td>Mouse</td>
<td>P2Y&lt;sub&gt;2&lt;/sub&gt;</td>
<td>↑ Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
</tr>
<tr>
<td>OMCD</td>
<td>Rabbit</td>
<td>P2Y&lt;sub&gt;2&lt;/sub&gt;, P2Y&lt;sub&gt;1&lt;/sub&gt;</td>
<td>↓ H&lt;sub&gt;2&lt;/sub&gt;O, Cl&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>IMCD</td>
<td>Rat</td>
<td>P2Y&lt;sub&gt;6&lt;/sub&gt;</td>
<td>↑ Ca&lt;sup&gt;2+&lt;/sup&gt;, PI</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>P2Y&lt;sub&gt;6&lt;/sub&gt;, P2Y&lt;sub&gt;1&lt;/sub&gt;</td>
<td>↓ Na&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

In studies where expression was documented only by RT-PCR or antibody staining, no functional significance is listed. The subtypes of P2Y receptors and the present nomenclature are described in the text. LLC-PK<sub>1</sub>, porcine kidney epithelial cell line; MDCK, Madin-Darby canine kidney cell line; A6, *Xenopus laevis* kidney cell line; OK, opossum kidney cell line; PT, proximal tubule; mTAL, medullary thick ascending limb of Henle’s loop; cTAL, cortical thick ascending limb of Henle’s loop; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct; AC, adenylyl cyclase; AA, arachidonic acid production; PGE<sub>2</sub>, prostaglandin E<sub>2</sub> production; PI, phosphoinositide turnover; Ca<sup>2+</sup>, cytosolic calcium; Cl<sup>-</sup>, chloride transport; Na<sup>+</sup>, sodium transport; H<sub>2</sub>O, water transport. The table is constructed on the basis of Refs. 6, 12, 28, 29, 34, 39, 42, 54, 65, 66, 69, 75, 81, 94, 116, 117, 137, 138, 140.

By measuring Ca<sup>2+</sup> activity in terminal IMCD after exposure to various nucleotide analogs, Ecelbarger et al. (42) demonstrated the existence of a classic P2Y<sub>2</sub> purinoceptor in the rat terminal IMCD (42). Suko et al. (137) reported similar results showing that extracellular ATP induces increases in both Ca<sup>2+</sup> and nuclear Ca<sup>2+</sup> in cultured IMCD cells (137). McCoy et al. (92) showed functional and RT-PCR evidence for expression of P2Y<sub>2</sub> and P2Y<sub>1</sub> receptors in mIMCD-K2 cells. Cha and co-workers (29) also showed evidence for the existence of P2Y<sub>2</sub> as well as P2Y<sub>1</sub> receptors in rat outer medullary collecting duct (OMCD) segments (29). Kishore et al. (71) extended the work of Ecelbarger et al. in Knepper’s group (42) to show that ATP and UTP applied to the basolateral surface of rat terminal IMCD inhibited AVP-induced osmotic water permeability (P<sub>f</sub>) in microperfused IMCD. McCoy et al. (92) also showed functional effects of P2Y<sub>2</sub> receptor activation in IMCD. UTP, ATP, or methylene-ATP analogs that stimulate P2X receptor channels (see section below) stimulated Cl<sup>-</sup> secretion across monolayers of mIMCD-K2 cells. Moreover, in the same monolayers, these nucleotide...
agonists inhibited Na⁺ absorption. Taken together, these studies show that P2Y2 receptor localization and activation in medullary collecting duct segments have a profound effect on cell signaling and modulation of salt and water transport across these segments.

Recently, Deetjen and colleagues (36) have performed elegant fluorescence imaging studies in isolated perfused mouse cortical collecting duct (CCD) showing that luminal UTP triggers an increase in Ca²⁺. Comparison of the response of many nucleotide analogs led them to conclude that the P2Y2 receptor mediated this response. In the course of their studies, they showed that serosal addition of nucleotide agonists also increased Ca²⁺; however, the rank order potency of the agonists was very different. The basolateral P2 receptor in mouse CCD may be a different purinergic receptor entirely. Satlin and co-workers (152) have made similar observations in rabbit CCD; moreover, they have found luminal effects of ATP scavengers and ATP receptor antagonists on ion transport in isolated perfused and impaled rabbit CCD (L. Satlin, personal communication). Rouse and colleagues (116) also showed antagonism of AVP-induced water transport in rabbit CCT. Examination of a large panel of agonists showed that ATP, ADP, UTP, and methylene-ATP analogs attenuated AVP effects, suggesting the expression and involvement of multiple P2Y and P2X receptors in their inhibitory effects (116). Lu and co-workers (88) have shown inhibition of small-conductance, secretory K⁺ channels in freshly isolated, “filleted open” CCD by purinergic agonists (88). In findings to those of Rouse and co-workers (116), ADP and UTP each inhibited the channels, suggesting that multiple P2Y receptors (P2Y2 and, possibly, P2Y1) may underlie this regulation.

Utilization of heterologous renal epithelial models derived from kidney, like the distal nephron models in MDCK cells, A6 Xenopus laevis kidney cells, and LLC-PK₁ porcine kidney cells, has provided ideal models in which to study purinergic signaling. As early as 1979, Simmons (129, 130) showed that exogenous ATP agonists affect ion transport across MDCK monolayers. Soon after this finding, Simmons and co-workers (131) showed that extracellular ATP stimulated Cl⁻ secretion across MDCK monolayers. Very recently, they revisited this work in mIMCD-K2 cells and found that external ATP stimulated Cl⁻ secretion in a similar manner (14). However, it is Insel and co-workers (48, 64, 108) who have led the way in this regard with multiple publications regarding the MDCK model. They have examined purinergic signaling via phosphoinositide signaling cascades. Arguably, their work has triggered present work in isolated nephron segments or in epithelial cell models derived from defined nephron segments. Rather than discuss their work in detail, Insel (63) has recently published a review on his
work that does better justice to this body of literature. Zambon and co-workers (155) have shown recently that P2Y1, P2Y2, and P2Y11 are expressed in MDCK cells, and this laboratory has cloned the canine forms of these P2Y receptor genes (P. A. Insel, personal communication). In addition to work by Insel and co-workers, Kishore, Ecelbarger, and co-workers have done similar work on nucleotide agonist-dependent signaling in IMCD. Ecelbarger et al. (42) demonstrated that prior exposure of rat IMCD to indomethacin, an inhibitor of cyclooxygenase (COX), attenuates Ca\(^{2+}\) response to ATP agonists. This observation suggested that an as yet unidentified COX by-product of arachidonic acid metabolism (e.g., prostaglandin E\(_2\)) is facilitating or mediating the Ca\(^{2+}\) response to ATP. Indeed, preliminary studies by Kishore and co-workers (75) revealed that ATP or UTP, but not ADP, enhanced the production and release of PGE\(_2\) in a COX- and MAP kinase-dependent manner. These studies underscore the theme in purinergic signaling in the kidney and elsewhere that purinergic-triggered signaling pathways are quite complex. This complexity is further enhanced by the presence of multiple P2Y (and P2X) receptors in the same epithelial cell and, in some cases, in the same membrane domain of an epithelial cell.

Indeed, evidence for expression of additional P2Y receptor subtypes is accumulating. In a few studies highlighted above, P2Y1 expression along with P2Y2 was documented. In the S1 segment of PCT as well as in OMCD, stimulation by a panel of nucleotide agonists suggested the concomitant expression of P2Y1 and P2Y2 (29). In a parallel study by the same laboratory, RT-PCR in cell lines derived from S1 PCT and OMCD showed expression of P2Y1, P2Y2, and the P2X receptor channel subtype P2X4 (140). Rank order potency studies of nucleotide agonist effects on Ca\(^{2+}\) in rabbit renal proximal tubule showed that P2Y1, but not P2Y2, was expressed in this segment and mediated ATP-induced increases in Ca\(^{2+}\) (154). Jin and Hopfer (69) found similar results in a renal cell line derived from early proximal tubule of the rat. Specific RT-PCR and functional studies also showed expression of both P2Y1 and P2Y2 in mIMCD-K2 cells (92). Rouse and co-workers (116) had pharmacological evidence that P2Y1, P2Y2, and P2X receptor channels may all mediate ATP-mediated attenuation of AVP-induced water permeability in rabbit renal CCT (116). Bouyer et al. (15) also found a P2Y1-like phenotype in Necturus proximal tubule that increases Ca\(^{2+}\) and stimulated a basolateral Cl\(^{-}\) channel. Finally, Ishikawa et al. (65) found that both P2Y1 and P2Y2 receptors may mediate increased growth rates of rat renal IMCD cells in vitro. Taken together, P2Y1 alone or together with P2Y2 may mediate effects on ATP along the nephron in multiple nephron segments, specifically proximal tubule and collecting duct.

Very recently, Bailey and co-workers (4) have added a third P2Y receptor to the list of those subtypes expressed along the rat renal epithelium. Single-nephron-segment RT-PCR revealed that expression of P2Y6 is high in PCT and in the thick ascending limb and thin descending limb of Henle's loop. Expression was qualitatively lower in OMCD, although RT-PCR is not precisely quantitative. Expression was absent in the thin ascending limb of Henle as well as in IMCD. Functional expression was assessed by using basolateral perfusion of the selective nucleotide UDP, which stimulates P2Y6 receptors more readily than it does other subtypes. UDP-triggered increases in Ca\(^{2+}\) were robust in PCT, weak in OMCD, and absent in other segments. UDP-stimulated PI turnover was also examined and was present in PCT and OMCD but not in other segments. These results are the first to describe molecular and functional evidence for expression of the P2Y6 receptor along the nephron. Taken together, these studies show that multiple P2Y receptors are poised along the nephron to transduce the autocrine and paracrine ATP signal as it travels down the nephron.

**P2X Receptor Channels Along the Renal Epithelium**

Only as recently as 1998 has the expression of P2X receptor channels been assessed in renal epithelial cell models. Filipovic et al. (47) found functional and molecular evidence for a P2X1-like receptor channel in the heterologous renal epithelial cell model LLC-PK1 (47). As mentioned above, RT-PCR in cell lines derived from S1 PCT and OMCD showed expression of the P2X receptor channel subtype P2X4 (140). In 1999, McCoy et al. (92) showed that P2X3 and P2X4 subtypes are expressed specifically in mIMCD-K2 cells and that P2X receptor channels stimulate Cl\(^{-}\) secretion and inhibit Na\(^{+}\) absorption in this model. Schulze-Lohoff et al. (122, 123) have found molecular and functional evidence for P2X7 expression in rat mesangial cells that is involved in apoptosis. Other studies, using a pharmacological approach with methylene-ATP analogs, obtained evidence suggestive of P2X receptor channel expression.

Recently, Schwiebert and co-workers (Schwiebert EM, Wallace D, King SR, Braunstein GM, Peti-Peterdi J, Hanaoka K, Guay-Woodford LM, Bell PD, Sullivan L, Grantham JJ, and Taylor AL, unpublished observations) have attempted to map P2X receptor channel expression in cell line and primary cultures derived from defined nephron segments. Degenerate RT-PCR with primers that amplify all seven isoforms of the P2X receptor gene family was performed. Verification of this work is ongoing with subtype-specific antibodies. The amplified product was cloned for sequencing, and 12–32 white bacterial colonies bearing a PCR product insert were sequenced for each epithelial cell mRNA sample. Each sequence derived from each epithelial cell mRNA sample was subcloned for sequencing, and 12–32 white bacterial colonies bearing a PCR product insert were sequenced for each epithelial cell mRNA sample. Each sequence derived from each white bacterial colony was subjected to the BLAST algorithm to define and confirm the identity of the P2X receptor subtype found (3). In human mixed renal epithelial primary cultures, two different proximal tubule primary cultures, in human mesangial cell primary cultures, and in human autosomal dominant polycystic kidney disease (ADPKD) primary cultures, P2X4...
and P2X5 are expressed abundantly to the exclusion of all other isoforms. Only human renal mixed epithelial and human proximal tubule primary cultures expressed a third isoform, P2X7, and only with low incidence. In the RCCT-28A cell line, a model of A-type intercalated cells, P2X5 was the predominant subtype expressed, whereas, in mIMCD-K2 cells, P2X3 and P2X4 were expressed at equal incidence to the exclusion of other isoforms. In collecting duct primary cultures from wild-type mice and the cpk autosomal recessive PKD mouse, P2X5 was the most abundant sequence found, whereas a smattering of P2X1, P2X2, and P2X4 was also found in much lower incidence. These recent and novel findings are shown in a schematic fashion in Fig. 5. They need to be confirmed by biochemical methods as well as single-nephron RT-PCR with degenerate primers and with primers specific to each subtype; however, they agree with studies in epithelial cell models from other tissues as well as the early studies described above. Taken together, these results suggest that multiple P2X receptor channels, in addition to multiple P2Y receptors, are poised to receive autocrine/paracrine nucleotide agonist signals as they travel along the renal epithelium.

*Pathophysiological Paradigms in Which Purinergic Signaling May Be Beneficial or Detrimental*

The pathophysiological significance of purinergic signaling in different cell types along the nephron and in specific nephron segments is also being appreciated.

**Hypertension.** One major area in which purinergic signaling may have import is that of hypertension. Because extracellular purinergic signaling modulates NaCl and water handling along the nephron, this signaling could affect certain hypertensive states. Moreover, because salt and water reabsorption is inhibited by extracellular ATP in the tubular lumen or apical environment, delivery of ATP as a therapeutic agent could lower salt and water reabsorption, blood volume, and blood pressure. Certainly, studies by Kishore et al. (71) as well as McCoy et al. (92) agree and underscore this hypothesis. On the other hand, Churchill and Ellis (32) demonstrated that, in rat renal cortical slice preparation, ATP (100–500 μM) and its analogs stimulate renin secretion in a concentration-dependent manner. They also demonstrated that this effect is mediated by a subtype of P2Y receptor via nitric acid (32). Thus it is likely that the multiple purinergic mechanisms may play a role in blood pressure regulation through kidney.

**Water balance.** Kishore and co-workers (72) have extended their work on the involvement of purinergic signaling in rat models of hypo- and hypervolemia and polycythemia of ischemic-reperfusion injury. Preliminary data indicate that the abundance of the P2Y2 purinoceptor protein in the renal medulla is increased significantly in the hydrated state (hypervolemia) compared with dehydrated state (hypovolemia). Furthermore, there is an apparent shift in the subcellular localization of this protein in the medullary collecting duct cells, as revealed by immunoperoxidase labeling with P2Y2 receptor-specific antibody. In the hypovolemic condition, a predominantly basal labeling is seen as opposed to a predominantly apical labeling in the hypervolemic state (72). Interestingly, Kishore and associates (74) also observed that the distribution of immunoreactive cytosolic phospholipase A2 (cPLA2) in the medullary collecting duct cells is also altered under these conditions, with an intense labeling for cPLA2 on the basal aspect of the cells. These preliminary observations, when established further by more extensive studies, will underscore the importance of the association between the purinergic signaling and arachidonic acid metabolism in the long-term conditioning of collecting duct water permeability and thus offer a basis for the vasopressin-independent regulatory mechanisms. Furthermore, in a preliminary study Kishore and colleagues (76) observed that, in the renal medulla of rats during the reperfusion phase after bilateral renal pedicle clamping, P2Y2 purinoceptor mRNA was markedly increased with a concomitant decrease in AQP2 water channel mRNA. Because it has been well documented by Fernandez-Llama and co-workers (45) that decreased aquaporin protein abundance in collecting duct cells is a contributing factor in the increased urine flow seen in moderate postischemic acute renal failure, the preliminary observations of Kishore et al. (76) on P2Y2 expression suggest the probable involvement of this receptor in the diuretic condition of ischemic reperfusion injury (IRI). Moreover, release of ATP and adenosine in other tissues during IRI has been shown and could play additional modulatory roles beyond regulation of water balance by the medulla.

**Tubuloglomerular feedback.** Studies by Bell and colleagues have shown that ATP signaling in the interstitium between the macula densa plaque in the cTAL and the afferent arteriole-glomerulus complex may aid in tubuloglomerular feedback mechanisms that regu-
late renal blood flow and glomerular filtration rate (Bell PD, personal communication). In pathophysiological states where these parameters change dramatically, autocrine/paracrine ATP signaling may be affected.

**PKD.** Alternatively, in recessive and dominant forms of PKD, extracellular ATP signaling could prove detrimental. Wilson et al. (151) showed that human PKD epithelial monolayers release as much or more ATP than do normal epithelial controls. They also showed that a subset of human PKD cyst fluid samples had nanomolar to micromolar ATP concentrations (some as high as 10 μM ATP). All samples had measurable ATP levels above background, suggesting that ATP release into the PKD cyst lumen and extracellular ATP signaling within the cyst occur in vivo. They have expanded this data set into other PKD models more recently (Schwiebert EM, Wallace D, King SR, Braunstein GM, Peti-Peterdi J, Hanaoka K, Guay-Woodford LM, Bell PD, Sullivan L, Grantham JJ, and Taylor AL, unpublished observations). Figure 6 shows our general working hypothesis concerning the possible detrimental impact of extracellular purinergic signaling. ADPKD cysts, as fluid-filled structures or spheres lined by a single layer of epithelial cells, present a physiological enigma. Ion and fluid transport become encapsulated, especially secretory transport into the cyst. As such, any secretagogue may be detrimental. Indeed, growth factors are released and trapped with the cyst lumen where they interact with growth factor receptors, creating a vicious autocrine/paracrine growth loop (95, 150). Purinergic agonists are mitogens or comitogens with growth factors in some cell models (44, 49, 103, 106, 120, 123, 148).

As with normal renal function, the role of extracellular ATP signaling in renal disease states is only beginning to be explored. It is our hope that many other laboratories will become interested in purinergic signaling in the kidney, adapt some of these ATP assay methodologies, and study purinergic receptors and their effects on renal function.

**LESSONS FROM THE STUDY OF EPITHELIA DERIVED FROM OTHER TISSUES**

A multitude of laboratories have studied purinergic agonists and their effects of epithelial cell models. In particular, purinergic agonists were among a large panel of agonists tested for their ability to stimulate Cl\(^{-}\) and fluid secretion from cystic fibrosis (CF) tissues and epithelial cell models from the lung and airways and from the gastrointestinal (GI) system. In CF, Cl\(^{-}\) and fluid secretion are lacking, whereas sodium absorption is augmented. Purinergic agonists were among the few agonists tested that were successful in correcting this defective NaCl transport in CF cells (77, 80, 134, 135). As such, UTP and UTP analogs are being developed to target the P2Y\(_2\) receptor as well as the

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**Fig. 6.** ATP release, purinergic receptors, and purinergic receptor-mediated signaling may be detrimental in polycystic kidney disease (PKD). This schematic shows how an autosomal dominant (AD) PKD cyst arises slowly from a normal renal tubule to become a fluid-filled sphere encapsulated by a single cell layer of renal epithelial cells. ADPKD cysts arise heterogeneously from the nephron but mostly from distal nephron. Like growth factors, ATP and its metabolites are “trapped” as nucleotide and nucleoside agonists in the cyst fluid. Trapped ATP and ATP metabolites, as fluid and Cl\(^{-}\) secretagogues and mitogens or co-mitogens with already trapped growth factors, may exacerbate fluid accumulation and volume expansion in ADPKD cysts and proliferation of epithelial cells that line ADPKD cysts. The hypothesized result is slowly accelerated cyst expansion and growth. Purinergic receptors of all types are expressed on renal epithelium; thus the autocrine signaling cascade may be intact in the lumen of the cyst. Finally, recent work from Schwiebert and co-workers has shown that nucleotide agonists increase cytosolic Ca\(^{2+}\) in ADPKD cells, whereas collaborative work with Wallace, Sullivan, and Grantham shows that nucleotide agonists stimulate Cl\(^{-}\) secretion across human ADPKD monolayers grown in primary culture. Clearly, all of the elements exist for purinergic stimulation of Cl\(^{-}\) secretion via cytosolic Ca\(^{2+}\) signaling, a function that is detrimental when the nephron architecture has changed from a tubule to a cyst. Ado, adenosine.
P2Y$_4$ and P2Y$_6$ receptors to stimulate Cl$^-$ and fluid secretion in CF airways (77, 80, 126, 134, 135). Hwang and colleagues (61) demonstrated that the addition of purinergic agonists to the apical or basolateral membrane of rat primary tracheal epithelial cell cultures stimulates Cl$^-$ secretion as a downstream result of activating purinergic receptors. This study also concluded that the P2Y receptors stimulated were different on the apical side vs. the basolateral side. Paradiso and colleagues (104) showed that purinergic agonists increase Ca$^{2+}$ when added to either side of an airway epithelium. Iwase et al. (67) also showed stimulation of Cl$^-$ secretion in rabbit tracheal epithelium, whereas they also observed that ATP suppressed sodium absorption. This result was confirmed by Devor and Pilewski (38), who showed that UTP and ATP inhibited sodium absorption in human non-CF and CF airway epithelium. Taylor et al. (143) described how P2X receptor activation stimulated Cl$^-$ secretion across the nasal epithelium of anesthetized mice, as well as across mouse and human primary epithelial cell monolayers. In IMCD monolayers, multiple P2Y and P2X receptors have been found (92). In the MDCK kidney epithelial model, Insel (63) found multiple P2Y receptors (P2X receptor expression in MDCK models has not been examined). Luo and colleagues (89) also found that multiple P2Y and P2X receptors were expressed on the luminal and serosal membranes of pancreatic duct, where they stimulated Cl$^-$ secretion and increased Ca$^{2+}$. Taken together, no matter what epithelium is studied, the same theme of multiple P2Y and P2X receptors being expressed in a given epithelial model rings true.

Collectively, these studies also describe ATP stimulation of Cl$^-$ secretion and inhibition of sodium absorption across airway and kidney epithelia. Because Cl$^-$ secretion is lacking and sodium absorption is elevated in CF, and depending whether P2Y and/or P2X receptors were targeted with agonists, purinergic agonist therapy may serve to correct abnormal handling of salt and water by the respiratory epithelium. Figure 7 shows a working hypothesis concerning what may happen as a consequence of a lack of extracellular ATP signaling. Schwiebert and co-workers as well as other laboratories (68, 121, 136, 143) have shown that ATP release and signaling are lost in the apical medium bathing CF airway epithelium. CFTR regulates this process via multiple mechanisms (68, 105, 121, 126, 136, 143), by virtue of its ability to be a conductance regulator and a regulator of other cellular processes (see Fig. 7). Extracellular ATP release and signaling also control cell volume regulation (17, 56, 149). CFTR potentiates this process as well. In either case, however, CFTR does not conduct ATP as an anion itself (17, 56, 83, 109, 110, 136). That does not mean that it may transport ATP at nonconductive rates or regulate channel-mediated, transporter-mediated, or exocytic mechanisms of ATP release (84, 111). Under hypotonic stress, cells swell, release more ATP, and return to their normal cell volume. In the presence of ATP scavengers (hexokinase, apyrase) or purinergic receptor antagonists (suramin, reactive blue 2), cells are not capable of regulatory volume decrease. Additionally, under isotonic conditions, ATP scavengers cause cells to swell spontaneously, whereas ATP agonists like ATP$_y$S cause cells to shrink. These data demonstrate the important role ATP and purinergic receptors play in maintaining cell volume. Indeed, given the altered ion transport in CFTR mutant cells, it has been shown

![Fig. 7](http://ajprenal.physiology.org/)

**Fig. 7.** Loss of ATP release and signaling may be detrimental in cystic fibrosis (CF) of the lung and airways. In a published study and a study in press, Schwiebert and co-workers have found that CF airway epithelial cell models have lost the ability to release ATP into the apical medium. The mechanism of why the defective CF transmembrane conductance regulator (CFTR) leads to defective ATP signaling is being examined; however, it appears that CFTR regulates positively a separate, yet closely associated ATP-permeable anion channel. CFTR may also regulate adenine nucleotide transporters or modulate the insertion or exocytosis of ATP-filled vesicles. A lack of extracellular ATP signaling in the apical microenvironment may lead to a lack of Cl$^-$ and fluid secretion as well as Na$^+$ and fluid hyperabsorption. As such, impaired osmotic or ionic strength or volume of the apical surface fluid may result.
that the airway surface liquid (ASL) salt content and/or volume bathing CF airway epithelia is abnormal compared with non-CF epithelia. With the use of two different techniques, the two most recently published articles on ASL have demonstrated that there is a decreased ASL volume in CF airways that leads to the inability to clear mucous. The two groups disagree on the salt composition of the ASL (52, 91, 132, 156). This phenotype may reflect a more fundamental abnormality in cell volume regulation of the CF airway epithelium (Fig. 7).

To further enhance the important biological role of ATP in CF, Korngreen, Priel, and colleagues (80, 141) demonstrated that ATP increases ciliary beat frequency in freshly isolated rabbit ciliated airway epithelia, whereas Wong and Yeates (153) described the same in vivo on the tracheal lumen of anesthetized dogs. Together, these studies illustrate the importance of extracellular ATP and luminal purinergic receptors in mucus clearance along the airway. An impaired process in CF, enhancement of mucociliary clearance by purinergic agonist therapy would only benefit this disease phenotype as well.

HYPOTHESES AND FUTURE DIRECTIONS

As this review underscores, the elements of ATP release, ATP receptors, and nucleotide-regulated functions along the nephron are in place for autocrine/paracrine signaling along the renal epithelium. Because the tubular lumen of the nephron is a closed system with regard to ATP filtered at the glomerulus or released into the lumen, this environment is ideal for extracellular purinergic signaling. The complexities of multiple ATP release mechanisms and multiple types of P2Y and P2X receptors expressed by a given renal epithelial cell along the nephron have been delineated by this review. Moreover, ATP release mechanisms and ATP receptors are also present in glomerular mesangium, vascular endothelium, and vascular smooth muscle surrounding renal vessels. This arrangement could allow elaborate purinergic cross-talk among renal nephron, glomerulus, and vasculature that tightly controls kidney function.

Challenges to renal investigators involve simplifying the system to an isolated nephron segment or epithelial cell derived from that nephron segment to dissect out ATP release mechanisms that are expressed. For ATP release to be relevant biologically, ATP receptors must be expressed. They also need to be defined in a given renal epithelial model or in a tubule preparation. Finally, the precise roles of P2Y receptors and P2X receptor channels need definition. It is not as simple as the notion that P2Y receptors expressed luminally and P2X receptors expressed basolaterally. There are mixtures in each membrane of a renal epithelial cell model. Why does the nephron need so many purinergic receptors? This is an open question that requires an answer.

Finally, emerging ATP agonist detection technology needs to be applied to the kidney. Figure 8 shows some hypothetical applications of a luciferase-luciferin agent in solution, luciferase conjugated to protein A, and an atomic force microscopy (AFM) probe coated with myosin fragments. Each can be applied to the kidney, in the right hands and with some ingenuity. The PC-12 biosensor method has already been applied to the macula densa by Bell and co-workers, as described above. It is the hope of the authors of this review that more technology, hard work, and ingenuity be applied to the concept of purinergic signaling along the nephron.

Fig. 8. Application of ATP release assay methods to the renal nephron. These schematics are possible applications of ATP detection assays to the renal nephron. Isolated tubule preparations are highlighted here; however, micropuncture assays or whole kidney perfusion assays may also be utilized with extracellular ATP agonist detection as the desired endpoint. In A, use of a protein A (proA)-luciferase (LUC) conjugate may be applicable to isolated segments. For example, if the IgG to an external epitope can be perfused, followed by perfusion of the proA-LUC conjugate, ATP detection may be possible. In C, AFM probes coated with myosin could be placed on the trailing end of an isolated perfused nephron segment or it could be placed immediately above a “filleted-open” nephron segment. (See Refs. 9, 121, and 142.)
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