Extracellular ATP-induced calcium signaling in mIMCD-3 cells requires both P2X and P2Y purinoceptors

Shen-Ling Xia, Lanjun Wang, Melanie N. Cash, Xueling Teng, Ruth A. Schwalbe, and Charles S. Wingo. Extracellular ATP-induced calcium signaling in mIMCD-3 cells requires both P2X and P2Y purinoceptors. Am J Physiol Renal Physiol 287: F204–F214, 2004. First published April 6, 2004; 10.1152/ajprenal.00281.2003.—Kidney tubules are targets for the activation of locally released nucleotides through multiple P2 receptor types. Activation of these P2 receptors modulates cellular Ca2+ signaling and downstream cellular function. The purpose of this study was to determine whether P2 receptors were present in mIMCD-3 cells, a mouse inner medullary collecting duct cell line, and if so, to examine their link with intracellular Ca2+ homeostasis. To monitor intracellular Ca2+ concentration ([Ca2+]i), experiments were conducted using the fluorescent dye fura 2. ATP (0.1–100 μM) produced a dose-dependent increase in [Ca2+]i in a physiological Ca2+-containing solution, with an EC50 of 2.5 μM. The P2-receptor antagonist PPADS reduced the effect of ATP on [Ca2+]i, and the P1-receptor agonist adenosine caused only a small increase in [Ca2+]i. Preincubation of cells with the phospholipase C antagonist U-73122 blocked the ATP-induced increase in [Ca2+]i, indicating P2Y receptors were involved in this process. In a Ca2+-free bath solution, thapsigargin and ATP induced intracellular Ca2+ release from an identical pool. Nucleotides caused an increase in [Ca2+]i, in the potency order of UTP > ATP > ATPγS > ADP > UDP that is best fitted with the P2Y2 subtype profile. Although the P2Y agonist UTP induced a similar large transient increase in [Ca2+]i as did ATP, a small but sustained increase in [Ca2+]i occurred only in ATP-stimulated cells, suggesting the role of P2X receptors in Ca2+ influx. The sustained increase in [Ca2+]i could be blocked by either nonselective cation channel blockers Gd3+ or P2X antagonists PPADS and PPND. Furthermore, when either Gd3+ or PPNS was applied to the bath solution before ATP application, the ATP-induced increase in [Ca2+]i was significantly reduced. Both RT-PCR and Western blotting corroborated the presence of P2X1 and P2Y2 receptors. These studies demonstrate that mIMCD-3 cells have both P2X and P2Y subtype receptors and that the activation of both P2X and P2Y receptors by extracellular ATP appears to be required to regulate intracellular Ca2+ signaling.

epithelia; purinergic receptors; collecting duct; calcium channel; kidney

RENAL EPITHELIAL CELLS RELEASE a significant number of nucleotides into the nephron tubule. The released ATP acts as autocrine and paracrine regulators and thus influences many physiological processes along the tubules via purinergic receptors (for a review, see Refs. 10, 26, 38). Purinergic receptors include P1 receptor families that are sensitive to adenosine and P2 receptor families that are sensitive to ATP, ADP, and UTP (18). The latter can be further divided into two major membrane-bound receptor subtypes: P2X and P2Y. The P2X receptors are ATP-gated ion channels (31). The P2Y receptors are coupled to G proteins linked to PLC signaling transduction pathways that can lead to the release of intracellular Ca2+ from inositol 1,4,5-trisphosphate (IP3)-sensitive Ca2+ stores (14). Both ionotropic P2X receptors and metabotropic P2Y receptors are found along the renal epithelium and in many cell lines of renal origin (26, 38, 46).

The inner medullary collecting duct (IMCD) is the final renal segment responsible for the regulation of solute and ionic composition of the urine. There is increasing evidence that extracellular nucleotides play an important role in the regulation of IMCD function. For example, extracellular ATP is involved in the inhibition of AVP-stimulated water permeability in rat IMCD cells (22), inhibition of Na+ short-circuit current, stimulation of Cl− short-circuit current in the mouse IMCD cell line mIMCD-K2 (5, 28), and modulation of whole cell Cl− current in the mouse IMCD cell line mIMCD-3 (41). Nucleotide actions in IMCD cells are Ca2+ dependent. Extracellular application of ATP has shown to induce an increase in intracellular Ca2+ concentration ([Ca2+]i) (5, 15, 42) through cell membrane P2Y receptor subtypes. Several studies show that G protein-coupled P2Y2 receptors are widely expressed in mIMCD-K2 cells (28) and in rat IMCD cells (15, 23). In addition, G protein-coupled P2Y1 receptors are also expressed in mIMCD-K2 cells (28). In contrast, to date only one IMCD cell line, mIMCD-K2, is reported to have P2X receptor subtypes, i.e., P2X3 and P2X4 (28). The role of P2X receptors in the regulation of Ca2+ homeostasis in IMCD cells, however, is largely unknown. Moreover, the relationship between ionotropic P2X receptors, which may cause Ca2+ influx, and metabotropic P2Y G protein-coupled receptors, which can lead to the release of intracellular Ca2+, in the mobilization of intracellular Ca2+ in response to ATP stimulation has not been determined.

The mIMCD-3 cell line is widely used as a model in the study of renal transport physiology (2, 4, 12, 13, 29, 32, 33, 35, 36, 40, 41, 51). Although several laboratories have shown that ATP increases intracellular Ca2+ in mIMCD-3 cells (5, 41, 51), the source of Ca2+ that causes the increase has not been analyzed thoroughly. Moreover, the P2 receptor subtypes in response to extracellular ATP have not been defined.

Address for reprint requests and other correspondence: S.-L. Xia, PO Box 100224, Dept. of Medicine, Univ. of Florida College of Medicine, Gainesville, FL 32610-0224 (E-mail: xiasl@medicine.ufl.edu).
The objective of this study was to determine whether P2 receptors were functionally expressed in mLmCD-3 cells, and if so, to examine their roles in cellular Ca\(^{2+}\) mobilization in response to extracellular ATP and to provide an initial characterization of the receptor subtypes.

**METHODS**

**Cell culture.** Cells from a continuous cell line, mLmCD-3, were purchased from the American Type Culture Collection (ATCC, Manassas, VA), mLmCD-3 cells at passage 15–35 were grown on glass coverslips in Dulbecco’s modified Eagle’s medium and Ham’s nutrient mixture F-12 (1:1) with 10% (vol/vol) fetal bovine serum and 0.1% gentamicin (stock concentration of gentamicin: 10 mg/ml). The cell glasses were placed in an incubator in the presence of humidified 95% air-5% CO\(_2\). Cells in confluence 2–5 days after plating were used for intracellular Ca\(^{2+}\) measurement. For RNA extraction and Western blot analysis, the cells were grown in 100-mm petri dishes using the same conditions as above. Another continuous cell line, mLmCD-K2 (a gift from Dr. Bruce A. Stanton, Dartmouth Medical School, Hanover, NH), was also cultured under similar conditions.

**Solutions.** For intracellular Ca\(^{2+}\) measurement, a Ca\(^{2+}\)-containing Ringer solution contained (in mM) 140 NaCl, 5 KCl, 1.5 CaCl\(_2\), 1 MgCl\(_2\), 10 glucose, and 10 HEPES. A Ca\(^{2+}\)-free Ringer solution contained (in mM) 140 NaCl, 5 KCl, 1 MgCl\(_2\), 10 glucose, 10 HEPES, and 1 EGTA. Cells were continuously superfused with experimental solutions through a gravity-fed system at a rate of 3–6 ml/min. Solutions were evacuated by suction. All solutions were adjusted to pH 7.4 with NaOH. The dyes and drugs, as needed, were dissolved in DMSO (5–10% vol/vol). Chemicals used in this study were purchased from Sigma (St. Louis, MO) or as indicated.

**Fura 2-AM loading and intracellular Ca\(^{2+}\) measurement.** Cells were loaded at room temperature for ~2 h in HEPES-buffered Ringer solution containing 5–10 \(\mu\)M Ca\(^{2+}\)-containing indicator fura 2-AM (Molecular Probes, Eugene, OR), then washed at least three times, and incubated for an additional 15–20 min in dye-free Ringer solution to reduce the possibility of incomplete hydrolysis of the AM esters by intracellular esterases. In some experiments, the detergent Pluronic F-127 (20% solution in DMSO) was added to the loading solution (0.05% vol/vol) to facilitate the uptake of fura 2. The cells in this group had no detectable difference in Ca\(^{2+}\) responses to ATP compared with cells in the group without the detergent.

The [Ca\(^{2+}\)]\(_i\) measurements were made with a ratiometric imaging system (InCyt Im2, Intracellular Imaging, Cincinnati, OH), including a PC, a filter wheel of conventional design, a charge-coupled device (CCD) camera, and a Nikon TE 300 microscope with \(\times 40\) air objective (0.9 numerical aperture). In each experiment, either a number of single cells or a group of cells [i.e., region of interest (ROI)] was selected using the software. The fluorescent emissions as paired signals, at a wavelength of 510 nm from the ROI, were measured accordingly to the software. The [Ca\(^{2+}\)]\(_i\) was done in a Bio-Rad iCycler thermal cycler for 1 cycle of denaturation at 94°C for 4 min; 30 cycles at 94°C denaturation for 30 s, 60 and 68°C annealing for 30 s for P2Y\(_2\) and P2X\(_1\), respectively, 72°C extension for 1 min; and 1 cycle for a final extension at 72°C for 3 min.

**Cloning and sequencing of purinergic receptors.** Gel-excised PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. The PCR products were then ligated into the TA cloning site of pCR 2.1 using a TA Cloning Kit (Invitrogen) according to the manufacturer’s instructions. The transformation was performed using One Shot TOP10 Chemically Competent Escherichia coli cells. The transformed colonies were plated on LB ampicillin plates (50 \(\mu\)g/ml) containing 5-bromo-4-chloro-3-indolyl-\(\beta\)-galactopyranoside (40 \(\mu\)g/ml) to allow blue/white screening of positives containing the insert of interest. The following day, white colonies were picked and each was incubated in 2 ml of LB media containing ampicillin (50 \(\mu\)g/ml) overnight at 240 rpm at 37°C. One milliliter of the overnight culture was pipetted into a 1.5-ml Eppendorf tube for plasmid isolation using a PerfectPrep Miniprep Kit (Brinkmann) according to the manufacturer’s instructions. The purified DNA was visualized under 0.1% ethidium bromide on a 0.8% agarose gel using a 500-base pair ladder as a standard. Two distinct positive clones were sent for sequencing (DNA Sequencing Core Laboratory, Gainesville, FL). The resulting sequences were compared with the published sequences using the basic alignment research tool (BLAST).

**Preparation of protein samples and Western blot analysis.** A confluent monolayer of mLmCD-3 cells was rinsed twice with ice-cold PBS (150 mM NaCl, 4.52 mM NaH\(_2\)PO\(_4\), 15.48 mM Na\(_2\)HPO\(_4\), pH 7.4) and then harvested using a rubber policeman. After a brief centrifugation at 660 \(g\) (Eppendorf 5417R; 2,500 rpm) for 10 min, the cells were resuspended in a hypotonic lysis buffer containing 20 mM NaH\(_2\)PO\(_4\), 0.5 M NaCl (pH 7.4), and a protease inhibitor cocktail (Complete, Roche Molecular Biochemicals). The mixture was left on ice for 50 min, with gentle shaking every 10 min. The cells were disrupted by dounce homogenization using glass duncers, and the undisrupted cells and cellular debris were removed by centrifugation at 2,060 \(g\) (4,400 rpm) for 15 min at 4°C. The supernatant was then divided and with lysis buffer and subjected to high-speed centrifugation at 30,000 \(g\) at 4°C for 30 min. The resulting pellet was resuspended in 500 \(\mu\)l of lysis buffer. The sample was then diluted 1:1 with Laemmli buffer using a Eppendorf Perfect RNA Extraction Kit (Brinkman, Westbury, NY) according to the manufacturer’s instructions. Quantification of the purified RNA was performed using a Bio-Rad SmartSpec 3000 spectrophotometer with wavelengths of 260 and 280 nm (Bio-Rad, Hercules, CA).

**Preparation of cDNA.** The first-strand RT reaction for mLmCD-3 cells and mLmCD-K2 cells was performed with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) using oligo(dT). The second-strand synthesis was performed with two gene-specific primers (see below) designed to specific sequences in the 3–35-nt region of both genes.

The primers used to amplify a fragment of mouse P2Y\(_2\) (P35383) were designed to anneal to base pairs 411–431 (5′-GGA AGC TCT TTT AGG GAT CG-3′) on the sense strand and base pairs 1365–1384 (5′-GCT CAC CCA CCT TGT TTT G-3′) on the antisense strand. The primers for amplifying mouse P2X\(_1\) (AAF68968) were designed to anneal to base pairs 99–128 (5′-CCG TCT GAT CCA GTT GGT GGT CTT GCT C-3′) on the sense strand and 993–1024 (5′-AGA TGC CAA TTC CAC AGC CGA TGG TAG T-3′) on the antisense strand. All of the primers are from sequences within the coding region. Each PCR reaction was set up in a 0.2-ml Thin Wall Tube (Bio-Rad) with 36 \(\mu\)l of autoclaved water, 5 \(\mu\)l of 10× cDNA PCR buffer (BD Biosciences, Palo Alto, CA), 5 \(\mu\)l of template, 1 \(\mu\)l each of forward and reverse primers at a concentration of 1 \(\mu\)M, 1 \(\mu\)l of 10 mM dNTP, and 1 \(\mu\)l of advantage cDNA polymerase mix (BD Biosciences). The amplification was done in a Bio-Rad iCycler thermal cycler for 1 cycle of denaturation at 94°C for 4 min; 30 cycles at 94°C denaturation for 30 s, 60 and 68°C annealing for 30 s for P2Y\(_2\) and P2X\(_1\), respectively, 72°C extension for 1 min; and 1 cycle for a final extension at 72°C for 3 min.

**Total RNA extraction.** Confluent mLmCD-3 and mLmCD-K2 cells were harvested by carefully scraping off the cell monolayer with a rubber policeman into 10 ml of ice-cold PBS solution (150 mM NaCl, 4.52 mM NaH\(_2\)PO\(_4\), 15.48 mM Na\(_2\)HPO\(_4\), pH 7.4). Cells were then spun at 2,500 rpm for 10 min at 4°C. RNA was extracted from the cell pellet using an Eppendorf Perfect RNA Extraction Kit (Brinkmann, Westbury, NY) according to the manufacturer’s instructions. Quantification of the purified RNA was performed using a Bio-Rad SmartSpec 3000 spectrophotometer with wavelengths of 260 and 280 nm (Bio-Rad, Hercules, CA).
sample buffer (62.5 mM Tris·HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue) containing 20 mM DTT and incubated at 95°C for 5 min before being loaded onto a gel.

Solubilized proteins were separated on a 10% Tris·HCl Ready Gel (Bio-Rad) for 90 min at 20 mA and electrophoretically transferred to polyvinylidene difluoride membranes for 60 min at 225 mA. The membranes were blocked with 5% nonfat dry milk in PBST buffer (50 mM NaH2PO4, 150 mM NaCl, 0.1% Tween 20) for 1 h and then immediately incubated for 1 h with either anti-rP2X1 polyclonal antibody (1:400) or anti-rP2Y2 polyclonal antibody (1:400, Alomone Labs) dissolved in antibody buffer (50 mM NaH2PO4, 150 mM NaCl, 0.1% Tween 20, 3% BSA, 0.01% sodium azide). The antibody used for peptide blocking was treated with 1 µg peptide/µg antibody for 1 h at room temperature immediately before being incubated with the membrane. Dilutions of the antibodies were similar in the presence and absence of peptide. After being washed for 15 min with PBST, the membranes were incubated for 1 h with peroxidase-conjugated goat anti-rabbit antibody (Jackson ImmunoLabs, West Grove, PA) at a 1:2,000 dilution. Membranes were washed and then detected using ECL Plus Western Blotting Detection Reagents (Amersham, Piscataway, NJ) following the manufacturer’s instructions.

The separation of each protein sample was repeated at least three times with the same amount of protein (~20 µg) loaded onto each lane. The Lowry protein assay was used to determine protein concentration (27).

Data analysis. Origin 6.0 (Microcal Software, Northampton, MA) was used for data analysis and graphics. The peak response of each experiment was determined by calculating three to six peak ratios from the ROIs within the experiment. The final results from each group of experiments (n) are reported as the mean peak response (means ± SE). Statistical significance was examined using Student’s t-test. A value of P < 0.05 was considered significant.

RESULTS

Increases in \([Ca^{2+}]_i\), induced by extracellular ATP. The ratio of emitted fluorescence was used as an index of \([Ca^{2+}]_i\) in this report; i.e., an increase in ratio indicates an increase in \([Ca^{2+}]_i\). Figure 1 shows that a short (30 s) time exposure of extracellular ATP (10 µM) could induce a transient increase in \([Ca^{2+}]_i\), in cells in a Ca²⁺-containing bath solution. This transient increase in \([Ca^{2+}]_i\) could be repeatedly induced by reapplying ATP, as long as the previously applied ATP was washed out (Fig. 1A). A 5-min or longer intervening period of washing was required to allow the cells to recover from a 30- to 60-s exposure to ATP, probably from nucleotide receptor desensitization and Ca²⁺ refilling of the store. The amplitude of the average response (peak increase in \([Ca^{2+}]_i\)) from the initial application of ATP to further applications (up to 5) is shown in Fig. 1B. Although the responses of \([Ca^{2+}]_i\) to ATP could be repeated over 10 times in Ca²⁺-containing bath solutions (data not shown), the response seen in the Ca²⁺-free bath solution occurred no more than twice (see note in Fig. 4), indicating that an internal Ca²⁺ release had taken place after ATP application and that an extracellular Ca²⁺ environment or Ca²⁺ influx was needed for the process of repetitive responses.

Dose dependence of response. The concentration of ATP used in this study was based on the observation of the dose-response to the application of 1 nM-100 µM extracellular ATP. In the Ca²⁺-containing bath solution, whereas 1 nM ATP had no observable significant effect, the application of 10 nM-100 µM ATP exhibited a dose-dependent effect on intracellular Ca²⁺. Figure 2 shows the dose-response curve of the normalized peak increase in \([Ca^{2+}]_i\), to the concentration of ATP used.

In this curve, 10 µM ATP increased the fluorescence ratio over 70% from baseline (75.9 ± 17.6%), which represents an increase in \([Ca^{2+}]_i\), from a resting level (baseline) of 74 ± 6 nM to a peak level of 620 ± 144 nM (4 paired experiments; 380±30 cells) (see Fig. 2, inset). ATP at doses from 1 to 100 µM induced an increase in \([Ca^{2+}]_i\), in all the experiments performed.

Effect of extracellular ATP on \([Ca^{2+}]_i\), is not via purinergic P1 receptors. ATP can be converted to adenosine by ectonucleotidases and therefore can stimulate purinergic P1 receptors (18). We wanted to determine whether the P1, P2, or both receptors were stimulated by extracellular ATP in mIMCD-3 cells. In four paired experiments (Fig. 3A), the 10 µM ATP-induced transient increase in \([Ca^{2+}]_i\) was significantly reduced (29.7 ± 4% from baseline) in the presence of pyridoxal-5’- phosphosphate-6-phenylazo-2’,4’-disulfonate (PPADS; 100 µM), a nonspecific P2-receptor antagonist, and this block effect to ATP could be reversed on washout of PPADS (63.2 ± 13%). These data indicate that P2 receptors were involved in the response to ATP stimulation. Another four paired experiments were carried out using adenosine (10 µM), a P1-receptor
We further examined the presence of G protein-coupled P2Y receptors by blocking the activation of PLC and IP3 generation with a membrane-permeable PLC inhibitor, U-73122. A 5- to 10-min pretreatment of cells with 10 μM U-73122 blocked the intracellular Ca2+ increase by 10 μM ATP (Fig. 5A), consistent with the blockade of ATP-activated PLC activity. In five such experiments, only a small recovery was observed after the washout of U-73122.

The subtypes of P2Y receptors were functionally characterized by their responses to various nucleotides (Fig. 5B). In a test using a 10-μM dose for all the purinergic agonists, ATP (75.9 ± 17.6%; n = 18), UTP (75.7 ± 7.9%; n = 3), ATPγS (47 ± 8.6%; n = 4), and ADP (40 ± 4.7%; n = 4) produced different levels of increase in [Ca2+]i, whereas UDP (n = 3) had no detectable responses. The functional potencies of P2-receptor agonists in the order of UTP = ATP > ATPγS > ADP >> UDP are best fitted with the established profile of mammalian P2 receptor subtype P2Y2 (38).

agons (Fig. 3B). It caused a much smaller increase in [Ca2+]i, compared with that for ATP (10.3 ± 3.3 vs. 110.7 ± 31.7%). These experiments indicate that although P1 receptors might have some effect, the ATP-induced increase in [Ca2+]i was mainly through P2 receptors in mIMCD-3 cells.

Another nonspecific P2-receptor antagonist, suramin, was also tested in mIMCD-3 cells. The cells treated with suramin (10 μM) showed no significant increase in [Ca2+]i by ATP stimulation. However, suramin itself induced a long, sustained increase in [Ca2+]i, (data not shown).

Evidence for the presence of purinergic P2Y receptors. In the absence of extracellular Ca2+, cells were treated with 5–10 μM thapsigargin, an agent that mobilizes Ca2+ specifically from IP3-sensitive Ca2+ stores by inhibiting endoplasmic reticulum Ca2+-ATPase activity. A transient increase in [Ca2+]i, indicates the release of Ca2+ from intracellular Ca2+ stores (see Fig. 4A). Subsequently, the thapsigargin-treated cells were treated with 10–100 μM ATP, which failed to induce a transient increase in [Ca2+]i. In reverse order, a transient increase in [Ca2+]i was observed with the addition of ATP, but no further increase was observed after the addition of thapsigargin (Fig. 4B). In both cases, the addition of the second reagent failed to cause an increase in [Ca2+]i, indicating that each agent released the full amount of Ca2+ from the same intracellular Ca2+ store. Because it has been well established that thapsigargin releases intracellular Ca2+ from IP3-sensitive Ca2+ stores (34), our results would support the involvement of the P2Y receptor subtype in response to ATP stimulation.

Fig. 2. Dose-response curve of extracellular ATP application in Ca2+-containing bath solution. The vertical coordinate shows the normalized peak increase in [Ca2+]i, (peak response divided by the baseline level of at least 60 s just before addition of ATP). The horizontal coordinate shows the concentration of ATP (in μM) added to induce the increase in [Ca2+]i. The mean (○) and SE (vertical bar) for each experiment are shown. The number of experiments (each experiment represents the response from at least 60 cells) varied from 4 to 18, as indicated in parentheses next to each data point. The dashed line is the sigmoidal curve fitted to the ATP doses, with an EC50 of 2.5 μM. Inset: 10 μM ATP raised [Ca2+]i (data not shown).

Evidence for the presence of purinergic P2Y receptors. A: in the presence of pyridoxal-5'-phosphate-6-phenylazo-2,4-disulfonate (PPADS; 100 μM), a nonspecific P2-receptor antagonist, the transient increase in [Ca2+]i, by 10 μM ATP was significantly reduced. Subsequently, after PPADS was washed out, 10 μM ATP-induced increase in [Ca2+]i, was renewed. B: adenosine (Ade; 10 μM), a P1-receptor antagonist, failed to cause a significant rise in [Ca2+]i, compared with ATP (see Fig. 4B). Both experiments indicate that the ATP-stimulated rise in [Ca2+]i, is mainly through P2 receptors. Traces in A and B are averaged from >80 cells, respectively; the results are representative of 3 experiments in A and 4 experiments in B. Horizontal solid bars indicate the duration of drug application.
ERs. Because a P2X1-like transcript was detected in mIMCD-3 cells (Gumz ML and Cain BD, personal communication), the large, transient increase in \([\text{Ca}^{2+}]_i\) was blocked (Fig. 6D; \(n = 4\) of 5). P2X-receptor antagonist PPADS (10 \(\mu\text{M}\)) had a similar effect ( \(n = 4\) of 5).

When cells were exposed to the trivalent cation gadolinium (Gd\(^{3+}\); 10–50 \(\mu\text{M}\)), a nonselective Ca\(^{2+}\)-channel blocker that can inhibit ATP-gated channels (30), the ATP-induced sustained increase in \([\text{Ca}^{2+}]_i\) was blocked (Fig. 6D; \(n = 3\)). The blockade process could be reversed after washout. The trivalent cation lanthanum had a similar effect (La\(^{3+}\); 50–100 \(\mu\text{M}\); data not shown).

Because mIMCD-3 cells have been reported to have voltage-dependent Ca\(^{2+}\)-channels (3), nicardipine (10 \(\mu\text{M}\)), an L-type Ca\(^{2+}\)-channel blocker, and pimozide (15 \(\mu\text{M}\)) and amiloride (10 \(\mu\text{M}\)), T-type Ca\(^{2+}\)-channel blockers, were also tested, but no detectable effect was found.

Taken together, our results suggest that Ca\(^{2+}\) entry generated by ATP stimulation occurs via P2X receptors (i.e., ATP-gated cation channels), not voltage-dependent Ca\(^{2+}\) channels.

**Plasma membrane Ca\(^{2+}\) entry via P2X receptors occurs during ATP stimulation.** In the Ca\(^{2+}\)-containing solution, a long exposure of the cells to ATP produced a small but sustained increase in \([\text{Ca}^{2+}]_i\), which was not observed in the Ca\(^{2+}\)-free solution (with Fig. 4B), we wanted to differentiate the role of P2X receptors (i.e., cation channels) in ATP-induced sustained \([\text{Ca}^{2+}]_i\). A stimulation of “only P2Y receptors” was applied via a long exposure of the cells to UTP, a P2Y-receptor agonist. As shown in Fig. 6B, the small, sustained increase in \([\text{Ca}^{2+}]_i\), after the large, transient increase in \([\text{Ca}^{2+}]_i\), did not occur in UTP-stimulated cells ( \(n = 7\) of 7). The absence of UTP stimulation (as well as in ATP stimulation with Ca\(^{2+}\)-free bath solution) supports the notion that P2X receptors are involved in the process of ATP-induced \([\text{Ca}^{2+}]_i\).

We further examined whether the observed sustained increase in \([\text{Ca}^{2+}]_i\) in the Ca\(^{2+}\)-containing solution could be blocked by P2X-receptor antagonists and Ca\(^{2+}\)-channel blockers. Because a P2X1-like transcript was detected in mIMCD-3 cells (Gumz ML and Cain BD, personal communication), pyridoxal-5’-phosphate-6-(2’-naphthylazo-6-nitro-4’,8’-disulphone) (PPND), a P2X1-receptor antagonist (24), was used to test the functional effect. When cells were exposed to PPND (10 \(\mu\text{M}\)), the ATP-induced sustained increase in \([\text{Ca}^{2+}]_i\) was blocked (Fig. 6C; \(n = 4\) of 5). P2X-receptor antagonist PPADS (10 \(\mu\text{M}\)) had a similar effect ( \(n = 4\) of 5).

Because the small, sustained increase in \([\text{Ca}^{2+}]_i\) was not blocked (Fig. 6D; \(n = 3\)), the ATP-induced sustained increase in \([\text{Ca}^{2+}]_i\) could be inhibited by a nonselective Ca\(^{2+}\)-channel blocker, and pimozide (15 \(\mu\text{M}\)) and amiloride (10 \(\mu\text{M}\)), T-type Ca\(^{2+}\)-channel blockers, were also tested, but no detectable effect was found.

Taken together, our results suggest that Ca\(^{2+}\) entry generated by ATP stimulation occurs via P2X receptors (i.e., ATP-gated cation channels), not voltage-dependent Ca\(^{2+}\) channels.

Fig. 4. Activation of purinergic P2 receptors by ATP releases the same intracellular Ca\(^{2+}\) store as does thapsigargin (Tg). A: cells incubated in the absence of extracellular Ca\(^{2+}\) were first treated with 10 \(\mu\text{M}\) Tg, an agent that mobilizes Ca\(^{2+}\) specifically from inositol 1,4,5-trisphosphate (IP\(_3\))-sensitive Ca\(^{2+}\) stores by inhibiting endoplasmic reticulum Ca\(^{2+}\)-ATPase activity. A transient rise in \([\text{Ca}^{2+}]_i\) indicates the release of intracellular Ca\(^{2+}\) stores. Because a P2X1-like transcript was detected in mIMCD-3 cells (Gumz ML and Cain BD, personal communication), pyridoxal-5’-phosphate-6-(2’-naphthylazo-6-nitro-4’,8’-disulphone) (PPND), a P2X1-receptor antagonist (24), was used to test the functional effect. When cells were exposed to PPND (10 \(\mu\text{M}\)), the ATP-induced sustained increase in \([\text{Ca}^{2+}]_i\) was blocked (Fig. 6C; \(n = 4\) of 5). P2X-receptor antagonist PPADS (10 \(\mu\text{M}\)) had a similar effect ( \(n = 4\) of 5). When cells were exposed to the trivalent cation gadolinium (Gd\(^{3+}\); 10–50 \(\mu\text{M}\)), a nonselective Ca\(^{2+}\)-channel blocker that can inhibit ATP-gated channels (30), the ATP-induced sustained increase in \([\text{Ca}^{2+}]_i\) was blocked (Fig. 6D; \(n = 3\)). The blockade process could be reversed after washout. The trivalent cation lanthanum had a similar effect (La\(^{3+}\); 50–100 \(\mu\text{M}\); data not shown).

Because mIMCD-3 cells have been reported to have voltage-dependent Ca\(^{2+}\) channels (3), nicardipine (10 \(\mu\text{M}\)), an L-type Ca\(^{2+}\)-channel blocker, and pimozide (15 \(\mu\text{M}\)) and amiloride (10 \(\mu\text{M}\)), T-type Ca\(^{2+}\)-channel blockers, were also tested, but no detectable effect was found.

Taken together, our results suggest that Ca\(^{2+}\) entry generated by ATP stimulation occurs via P2X receptors (i.e., ATP-gated cation channels), not voltage-dependent Ca\(^{2+}\) channels.

Fig. 5. Evidence for the presence of purinergic P2Y2 receptors. A: ATP and UTP, a P2Y-receptor agonist. As shown in Fig. 6B, the small, sustained increase in \([\text{Ca}^{2+}]_i\), after the large, transient increase in \([\text{Ca}^{2+}]_i\), did not occur in UTP-stimulated cells ( \(n = 7\) of 7). The absence of UTP stimulation (as well as in ATP stimulation with Ca\(^{2+}\)-free bath solution) supports the notion that P2X receptors are involved in the process of ATP-induced \([\text{Ca}^{2+}]_i\).
Further evidence for the presence of purinergic P2X receptors. Figure 7, A and B, shows typical experiments with the ATP-gated channels blockers La³⁺ and Gd³⁺, respectively. When cells were exposed to the bath solution with La³⁺ (50–100 µM; n = 3) or Gd³⁺ (10–50 µM; n = 8), ATP failed to induce a significant increase in [Ca²⁺]. The effect on [Ca²⁺], blocking the action of ATP was reversible, as demonstrated by the [Ca²⁺] response to ATP application after washout of the trivalent cations.

In another series of experiments using similar conditions to those noted above, UTP (10 µM) was observed to induce a significant increase in [Ca²⁺], in one-third of the experiments (n = 4 of 12). The amplitudes of increase in [Ca²⁺], by UTP in those four experiments were 46.5 ± 6.4% in the presence of 10 µM Gd³⁺ vs. 56.5 ± 5.6% in the absence of Gd³⁺ (the averaged amplitudes in the other 8 experiments were 15.9 ± 3.8% in the presence of Gd³⁺ vs. 68.9 ± 5.8% in the absence of Gd³⁺). The difference between the Ca²⁺ responses to ATP and UTP in the presence of Gd³⁺ supports the idea that either the blocking effect of Gd³⁺ has a preference for P2X receptors over P2Y receptors in the current experimental conditions or the blocking of P2X receptors may affect P2Y receptors in response to ATP and UTP.

Following a protocol similar to the above-mentioned Gd³⁺ experiments, we further tested the functional effect of P2X-receptor antagonists. As shown in Fig. 8A, in the presence of 10 µM PPND, the ATP-stimulated increase in [Ca²⁺] was largely inhibited. A partial recovery could be observed after the washout of the antagonist. A summary of four similar experiments is shown in Fig. 8B. The data presented here indicate that the action of ATP through P2X receptor subtypes could be an early event that is necessary for purinergic Ca²⁺ signaling.

Additional P2 receptor agents were tested in mIMCD-3 cells. In a total of 18 experiments with the P2X-receptor agonist α,β-methylene-ATP (α,β-MeATP; 10–100 µM), a significant increase in [Ca²⁺], was observed in four of them. We also tested the P2Y-receptor agonist 2-methylthioadenosine 5′-triphosphate (2-MeSATP; 10–100 µM), and no consistent increase in [Ca²⁺] was found. The P2X₁ antagonist NF-279 (10 µM), a suramin analog, not only failed to inhibit the ATP-induced increase in [Ca²⁺], but also slightly enhanced the response (n = 3); so did TNP-ATP (5 µM; n = 3).

mRNA expression of purinergic P2X₁ and P2Y₂ receptors using RT-PCR. We performed RT-PCR experiments to identify the presence of P2X₁ and P2Y₂ purinoreceptors in mIMCD-3 cells. Previously, it has been established that mIMCD-K2 cells express P2Y₂ but not P2X₁ receptors (28), so these experiments were conducted in parallel as controls. The P2X₁ cDNA with the expected 926-bp band was observed in the mIMCD-3 cells, but not the mIMCD-K2 cells (Fig. 9A). In the second set of experiments regarding P2Y₂, a band was observed at the expected size of 973 bp in mIMCD-3 cells, and, as anticipated, in mIMCD-K2 cells (Fig. 9B). The sequencing data from the positive clones confirmed the expression of both P2X₁ and P2Y₂ receptors in mIMCD-3 cells (Fig. 9, C and D).

Western blot analysis of purinergic P2X₁ and P2Y₂ receptors. Total membranes from mIMCD-3 cells showed a single immunoband at ~55 kDa for the P2X₁ receptors (Fig. 10A, left), and the specificity of the band was demonstrated by peptide-blocking experiments (Fig. 10A, right). The electrophoretic mobility of this band is similar to that reported in human salivary gland epithelial cells as well as transfected human embryonic kidney cells (HEK 293 cells) (44, 48). The calculated molecular mass of mouse P2X₁, 44.9 kDa.

![Graphs A, B, C, D](http://ajprenal.physiology.org/)
The more slowly migrating bands (55 and 67 kDa) are modified in rat lung membranes. Therefore, it is possible that following ATP application without the trivalent cations. Traces in represent the response of a representative of 3 similar experiments in rat brain membranes and two immunobands of the manufacturer to detect a single immunoband near 60 kDa forms of P2Y2 or that the antibody cross-reacts with different P2X1 immunoband found in human platelets (43).

The calculated molecular mass for the P2Y2 receptor is 42 kDa (P35383), which is close to the observed 40-kDa band. The calculated molecular mass for the P2X1 receptor antagonist PPNDS (10 μM) was inhibited most of the ATP-stimulated rise in [Ca2+]i. A partial recovery could be observed after washout of the antagonist. Trace represents averaged response of >80 cells.

(AAF68968), is lower than our observed molecular mass as well as that in previous reports (43, 44, 48). The slower electrophoretic migration has been attributed to glycosylation of the receptor in the extracellular domain, like the 70-kDa protein found in human platelets (43).

For the P2Y2 receptor, three immunobands were detected at ~40, ~55, and ~67 kDa for mIMCD-3 cells, as were two other bands at ~35 and ~45 kDa (Fig. 10B, left). Peptide-blocking experiments indicate that the 40-, 55-, and 67-kDa bands are those recognized by the primary antibody (Fig. 10B, right). The calculated molecular mass for the P2Y2 receptor is 42 kDa (P35383), which is close to the observed 40-kDa band in mIMCD-3 cells. In the technical information provided by Alomone Labs, the P2Y2 receptor antibody has been shown by the manufacturer to detect a single immunoband near 60 kDa in rat brain membranes and two immunobands of ~42 and ~60 kDa in rat lung membranes. Therefore, it is possible that the more slowly migrating bands (55 and 67 kDa) are modified forms of P2Y2 or that the antibody cross-reacts with different proteins. The band pattern of interest in mIMCD3 cells was similar to that in mIMCD-K2 cells, and in both cases the 40-kDa band appears to be the most prominent. The calculated mass, peptide-blocking studies, and the presence of a 40-kDa band in both mIMCD-3 and mIMCD-K2 cells would suggest that the 40-kDa band is most likely P2Y2.

**DISCUSSION**

This study provides functional evidence that both P2X and P2Y receptors are present in mIMCD-3 cells and that these receptors participate in the elevation of intracellular Ca2+ signals via ATP stimulation. The pharmacological data on intracellular [Ca2+]i measurements and molecular data demonstrate the presence of P2 receptor subtypes P2X1 and P2Y2 in this cell line. Furthermore, this study suggests that P2X receptors appear to work with P2Y receptors, through an unidentified cellular signaling pathway, to regulate the intracellular Ca2+ response to extracellular ATP stimulation.

It is well known that intracellular Ca2+ signaling is a focal point of many cellular signal transduction pathways and that it regulates a variety of physiological activities (6). The activation of different P2 receptors (i.e., P2X and P2Y) has been shown to cause different physiological responses. For example, extracellular ATP can influence either vasoconstriction via a P2X-dependent mechanism or vasodilatation via a P2Y-dependent mechanism in renal blood vessels (11, 16). More recently, it has been demonstrated that activation of P2X can induce cell apoptotic death, whereas activation of P2Y can induce cell proliferation (20). It is clear that a full understanding of ionotropic P2X and metabotropic P2Y signal transduction pathways in the renal epithelium will help to better define the roles of P2 receptors in the regulation of electrolyte, water, and acid-base in renal tubules.

Fig. 7. Purinergic P2X receptors involve ATP-stimulated Ca2+ signaling. Cellular Ca2+ response to ATP application (10 μM) was inhibited in bath solution with trivalent cations, either La3+ (50–100 μM), a nonspecific calcium channel blocker (A), or Gd3+ (10–50 μM), a nonspecific cation channel blocker (B), indicating that P2X signaling is necessary for ATP-induced Ca2+ signaling. The block effect was reversible as shown by the following ATP application without the trivalent cations. Traces in A and B represent the response of >80 cells, respectively. The experiments are representative of 3 similar experiments in A and 8 in B.

Fig. 8. ATP-stimulated Ca2+ signaling is inhibited by P2X antagonist. A: P2X1-receptor antagonist PPNDS (10 μM) inhibited most of the ATP-stimulated rise in [Ca2+]i. A partial recovery could be observed after washout of the antagonist. Trace represents averaged response of >40 cells. B: summary of a total of 4 similar experiments from 16 regions of interest containing >160 cells. Values are means ± SE. *P < 0.03 compared with control and P = 0.08 compared with washout (paired t-test).
In the kidney, P2Y receptors have been shown in almost all cells, and P2Y2 appears to be ubiquitous (26, 38). Studies using native cells or cultured cells from rats, rabbits, and mice show that the renal collecting duct has P2Y2 subtype receptors (8, 15, 23, 24, 28, 45, 46). The order of P2-receptor agonist potency in those studies is very similar to that in the current study (Fig. 5B). In addition, previous studies have also shown that P2Y1 receptors are expressed in rat IMCD cells (15, 23), rat outer medullary collecting tubule (8), mIMCD-K2 cells (28), and rabbit cortical collecting duct (47). Our data that the Ca2+/H11001 response induced by ATP could be inhibited by PPADS (Fig. 3A) and that the cells had a reasonable Ca2+/H11001 response to ADP at a dose of 10 μM (Fig. 5B) suggest that other P2Y subtype receptors, P2Y1 and P2Y4, may be present.

In contrast to the P2Y receptor, however, few studies have documented the expression of P2X receptors within the kidney. Filipovic et al. (17) demonstrated a P2X1-like purinergic receptor in LLC-PK1 cells, a widely used renal epithelial cell line. Schulze-Lohoff et al. (37) showed the expression of P2X7 in cultured mesangial cells. Chan et al. (9) investigated localization of the P2X1 receptor subtype in rat kidneys, and the receptor subtype has been detected in the vascular smooth muscle of intrarenal arteries, but not renal tubules. McCoy et al. (28) detected the presence of P2X3 and P2X4 receptors in mIMCD-K2 cells, but not other P2X receptor subtypes, including P2X1 receptors. More recently, Schwiebert et al. (39) reported that the P2X1 subtype sequence was detected by degenerate RT-PCR in mouse B6 collecting duct primary cultures. In the current study, the functional expression of P2X1 receptors in mIMCD-3 cells has been shown by pharmacological analysis (Figs. 6–8) and molecular analysis (Figs. 9 and 10). Although P2X1 (as well as P2X3) receptors are generally thought to rapidly desensitize after binding of ATP (usually within 1–2 s in excitable cells) (31), the actual desen-

---

**Fig. 9.** mRNA expression of P2X1 and P2Y2 receptors using RT-PCR. A and B: PCR products of both P2X1 and P2Y2 receptors, respectively, were seen in mIMCD-K2 cells, but only that of P2Y2 was observed in mIMCD-K2 cells. Left lane: 500-bp DNA ladder. Gels are representative of at least 3 separate experiments. C and D: ClustalW alignments of RT-PCR products from isolated RNA of mIMCD-3 cells with the cloned mouse P2X1 (BC015084) and P2Y2 (XM_285737). Dotted lines indicate identical cDNA sequences.
sustained phase of the [Ca^{2+}] response to ATP continues even longer than the aforementioned elicited whole cell current response. In conjunction, the P2X channels (perhaps more than 1 subtype) in mIMCD-3 cells appear to provide an appropriate Ca^{2+} influx in response to ATP (see Fig. 6).

Ca^{2+} influx plays an important role in signal transduction and in replenishing depleted Ca^{2+} stores. Voltage-dependent Ca^{2+} channels can provide a Ca^{2+} influx, and T-type Ca^{2+} channel subunit-α1G mRNA is actually expressed in mIMCD-3 cells (3). However, it is unlikely that ATP-elicited Ca^{2+} influx occurs through this mechanism, because of the lack of effect of nifedipine and pimozide, L- and T-type Ca^{2+} channel blockers, respectively. Nonexcitable mIMCD-3 cells may employ other Ca^{2+} influx mechanisms, such as capacitative Ca^{2+} entry (50) and Ca^{2+} entry via ATP-gated nonspecific cation channels (the current study). The former mechanism is activated by the depletion of internal Ca^{2+} stores, resulting in activation of a Ca^{2+} influx (34). The latter mechanism is provided by agonist binding to a receptor to form ion channels (i.e., ATP-gated P2X ion channels) (10, 31). The two mechanisms may work together, but dissection of the separate actions is difficult. However, it is conceivable that each Ca^{2+} influx pathway has played its role in response to ATP. For example, our data show that Ca^{2+} entry to refill intracellular Ca^{2+} stores is essential for the cells to maintain the consecutive responses to repeated ATP stimulations (compare Figs. 1 and 4). The process of refilling most of the intracellular Ca^{2+} stores that are releasable via P2Y signaling pathways may be very rapid, because the repeated Ca^{2+} responses could be achieved within a few minutes (to recover the desensitization of the ATP receptor and to replenish the stores with Ca^{2+}; see Fig. 1). In contrast, the long, sustained phase of the [Ca^{2+}]_{i} response in the presence of ATP could be contributed to by the combination of capacitative Ca^{2+} entry and Ca^{2+} entry through ATP-gated nonspecific cation channels. However, the sustained phase that has not been observed in the presence of UTP (Fig. 6B) supports that notion that the sustained phase of the [Ca^{2+}]_{i} is due to Ca^{2+} entry largely via ATP-gated ion channels.

The precise function of coexisting multiple P2X and P2Y receptor subtypes remains to be understood. Although P2X channels may provide an appropriate Ca^{2+} influx to facilitate the intracellular Ca^{2+} response to ATP, the data on ATP-stimulated intracellular Ca^{2+} release in a Ca^{2+}-free bath solution (see Fig. 4B) indicate that the Ca^{2+} influx is not a prerequisite for internal Ca^{2+} release. While it might be possible that other ions in the Ca^{2+}-free bath solution could still move in or out of the cell through ATP-gated nonspecific cation channels and positively affect G protein-coupled P2Y receptor signaling pathways (needs to be determined), our data from Figs. 7 and 8 suggest that the functional P2X receptor itself plays a pivotal role in the P2Y receptor signaling pathway, at least in the intracellular Ca^{2+} in response to ATP action. However, the data cannot rule out the possibility that the P2X antagonists used in the study also affected P2Y receptors. A previous study shows that in the presence of trivalent cations, the ATP-elicited nonspecific cation current became much smaller (30). Exactly how P2X-receptor antagonists block the increase in intracellular Ca^{2+} by ATP is subject to future investigation.

The mIMCD-3 cell line retains many cellular characteristics of the intact collecting duct, including formation of polarized functional epithelia (35), and has been used as a model for cellular biological and physiological studies (13, 36, 40, 41). The current study shows that cells were responsive to even submicromolar concentrations of ATP (Fig. 2), which is very close to the in vivo condition of the collecting duct (38). Our study also provides evidence of the presence of P2X and P2Y receptor subtypes in the cell line. Taking these results into consideration, the mIMCD-3 cell line can be used as a valuable model for defining nucleotides in the physiological and pathological processes of renal epithelial cells.

In summary, the present study has showed that Ca^{2+} entry to refill intracellular Ca^{2+} stores is an essential process for the cells to maintain the consecutive responses to repeated ATP...
stimulations. The ATP-induced increase in [Ca\(^{2+}\)] involves both P2X and P2Y subtype receptor signaling pathways. ATP failed to induce a significant increase in [Ca\(^{2+}\)] in the presence of P2X-receptor antagonists, indicating that P2X receptor-related signaling pathways, which usually cause extracellular Ca\(^{2+}\) influx, could interact with P2Y receptor-related signaling pathways that usually lead to internal Ca\(^{2+}\) release. The finding that inhibition of P2X signaling pathways affects the P2Y signaling pathways may provide a new regulatory mechanism of intracellular Ca\(^{2+}\) homeostasis in mIMCD-3 cells.

ACKNOWLEDGMENTS

We thank Lee Ann Day and Joyce Merritt for technical support and Alicia Rudin for comments on the manuscript. We thank Dr. Bruce A. Stanton, Dartmouth Medical School, for providing us with mIMCD-K2 cells.

GRANTS

This work was supported by grants from the Medical Research Service of the Department of Veterans Affairs and by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-49750.

REFERENCES


