Role of ATP in DNA synthesis of renal proximal tubule cells: involvement of calcium, MAPKs, and CDKs

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Role of ATP in DNA synthesis of renal proximal tubule cells: involvement of calcium, MAPKs, and CDKs. Am J Physiol Renal Physiol 291: F98–F106, 2006. First published January 17, 2006; doi:10.1152/ajprenal.00486.2005. —Although ATP has been shown to act as a modulator in various kidney functions, its effect on renal proximal tubule cell (PTC) proliferation has not been elucidated. This study investigated the effect of ATP on cell proliferation and the effect of its related signal pathways on primary cultured PTCs. Treatment with >10^{-5} M ATP for 1 h stimulated incorporation of thymidine and bromodeoxyuridine. ATP (10^{-4} M)-induced stimulation of thymidine incorporation was blocked by suramin (a P2X and P2Y receptor antagonist), reactive blue 2 (a P2Y receptor antagonist), MRS-2159 (a P2X<sub>1</sub> receptor antagonist), and MRS-2179 (a P2Y<sub>1</sub> receptor antagonist). ATP increased intracellular Ca<sup>2+</sup> concentration, which was blocked by suramin, methoxyverapamil, and EGTA. ATP-induced stimulation of cell proliferation was also blocked by EGTA (an extracellular Ca<sup>2+</sup> chelator), methoxyverapamil (a Ca<sup>2+</sup>-channel blocker), and nifedipine (an L-type Ca<sup>2+</sup>-channel blocker), suggesting a role for Ca<sup>2+</sup> influx. ATP-induced phosphorylation of p38 and p44/42 MAPKs was blocked by nifedipine. ATP increased expression levels of cyclin-dependent kinase (CDK)-2, CDK-4, and cyclin E, which were blocked by suramin, methoxyverapamil, and EGTA. ATP-induced stimulation of cell proliferation was also blocked by EGTA (an extracellular Ca<sup>2+</sup>-chelator), methoxyverapamil (a Ca<sup>2+</sup>-antagonist), and nifedipine (an L-type Ca<sup>2+</sup>-channel blocker), suggesting a role for Ca<sup>2+</sup> influx.

ATP-induced phosphorylation of p38 and p44/42 MAPKs was blocked by nifedipine. ATP increased expression levels of cyclin-dependent kinase (CDK)-2, CDK-4, and cyclin E, which were blocked by suramin, reactive blue 2, MRS-2159, MRS-2179, and nifedipine. However, ATP decreased expression levels of p21<sup>WAF1/Cip1</sup> and p27<sup>kip1</sup>. ATP-induced stimulation of thymidine incorporation and increase of CDK-2 and CDK-4 expression were blocked by SB-203580 (a p38 MAPK inhibitor) and PD-98059 (an MEK inhibitor), but not by SP-600125 (a JNK inhibitor). In conclusion, ATP stimulates proliferation by increasing intracellular Ca<sup>2+</sup> concentration and activating p38, p44/42 MAPKs, and CDKs in PTCs.

Therefore, an ATP-induced elevation of intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]i) might be of particular importance in regulation of cell proliferation (4). However, there is little information on the regulatory mechanisms of cell proliferation by ATP in renal PTCs.

In addition to [Ca<sup>2+</sup>], mitogen-activated protein kinase (MAPK) is also believed to have a role in induction of cell proliferation. Extracellular ATP induces Ca<sup>2+</sup>-dependent MAPK activation via the P2 purinergic receptors in neonatal rat astrocytes (38). On the other hand, cell proliferation is associated with activation of the diverse proteins. Positive regulators include the cyclins and their catalytic partners cyclin-dependent kinases (CDKs), which are essential for progression of the cells through each phase of the cell cycle and various cell cycle checkpoints (49, 50). Negative regulators include the cyclin kinase inhibitors (CKI), which inhibit the cell cycle at multiple checkpoints through the inactivation of cyclin-CDK complexes (48). In rat hepatocytes, ATP stimulated cell cycle progression and proliferation by activating cyclin D1 (51). It is becoming increasingly known that primary cultured rabbit PTCs play a key role in the pathogenesis of the renal complications of ischemic injury. The protective effect of ATP on ischemic injury in PTCs has been reported (31). Moreover, purinergic receptors mediate cell proliferation and enhance recovery from ATP-induced renal ischemic injury in rats, suggesting that ATP is an important regulator of PTC proliferation (41). However, little is known about ATP-induced alterations in cell proliferation and ATP-related signal pathways in PTCs.

The PTC culture system used in this study retains the differentiated phenotype in vitro that is typical of a renal proximal tubule, including a polarized morphology and a distinctive proximal tubule transport and hormone response (9, 22, 23). Our recent report that oxalate and high glucose inhibited cell proliferation in primary cultured PTCs is consistent with the results obtained from intact renal tissue (21, 42). Therefore, PTCs in hormonally defined, serum-free culture conditions would be a powerful tool for studying the effect of ATP on cell proliferation. Here, we investigated the effect of ATP on cell proliferation and ATP-related signal pathways in PTCs.

MATERIALS AND METHODS

Materials. Adult New Zealand White male rabbits (1.5–2.0 kg) were purchased from Dae Han Experimental Animals (Chungju, Korea). Class IV collagenase, soybean trypsin inhibitor, DMEM (1,000 mg/l glucose), and Ham’s F-12 nutrient mixture (1,800 mg/l glutamine) were purchased from Sigma-Aldrich (St. Louis, MO). DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin was used for cell culture. MDCK cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Invitrogen, Carlsbad, CA). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Three growth supplements (5 mM HEPES buffer (pH 7.4) and 20 mM sodium bicarbonate (pH 7.4)) were grown in 2 ml of a 1:1 mixture of DMEM-F-12 with 15 mM glucose) were purchased from Life Technologies (Grand Island, NY); ATP, adenosine, UTP, α,β-methyleneadenosine 5′-triphosphate (AMP-CPP), 2-(methylthio)adenosine 5′-triphosphate (2-methylthio-ATP), EGTA, BAPTA-AM, 4,5-trimethoxybenzoate (TMB-8), nifedipine, methoxyverapamil, reactive blue 2 (RB-2), MRS-2179, MRS-2159, and β-actin from Sigma Chemical (St. Louis, MO); butyrolactone I from Biomol (Butler Pike, PA); PD-98059 and SP-600125 from Calbiochem (La Jolla, CA); [3H]thymidine from DuPont/NE (Boston, MA); phospho-p38 MAPK, p38 MAPK, phospho-p44/42 MAPK, and p44/42 MAPK antibodies from Cell Signaling Technology (Herts, UK); antibody to CDK-2, CDK-4, cyclin E, p21WAF1/Cip1, and p27kip1 from Santa Cruz Biotechnology (Santa Cruz, CA); goat anti-rabbit IgG from Jackson ImmunoResearch (West Grove, PA); and Liquiscint from National Diagnostics (Parsippany, NY). All other reagents were of the highest purity commercially available.

Isolation of rabbit renal proximal tubules and culture conditions. Primary rabbit kidney PTC cultures were prepared using a modification of the method reported by Chung et al. (9). All procedures for animal management followed the standard operation protocols of Chonnam National University. An Institutional Review Board at Chonnam National University approved our research proposal and Chonnam National University. An Institutional Review Board at Chonnam National University. All experiments were performed in triplicate. The values were converted from absolute counts to percentage of control to allow comparison between experiments.

The cells were counted using the following methodology: the cells were washed twice with PBS and trypsinized from the culture dishes. The cell suspension was mixed with a 0.4% (wt/vol) trypan blue solution, and the number of live cells was determined using a hemocytometer. Cells that failed to exclude the dye were considered nonviable.

Bromodeoxyuridine incorporation. The level of incorporation of the thymidine analog 5-bromo-2-deoxyuridine (BrdU) was measured to determine the level of DNA synthesis. The PTCs were maintained at 37°C in a 5% CO2-humidified environment and the level of radioactivity was determined using a liquid scintillation counter (model LS 6500, Beckman Instruments, Fullerton, CA).

Fig. 1. Time- and dose-dependent effects of ATP on incorporation of thymidine and 5-bromo-2-deoxyuridine (BrdU) and cell count. A and D: thymidine incorporation in proximal tubule cells (PTCs) treated with 0–10^{-3} M ATP for 4 h or 10^{-4} M ATP for 0–24 h and pulsed with 1 μCi of [methyl-3H]thymidine for 24 h at 37°C. The cells were then washed twice with PBS, fixed in 10% TCA for 15 min, and washed twice in 5% TCA. The acid-insoluble material was dissolved in 2 N NaOH at room temperature, and the level of radioactivity was determined using a liquid scintillation counter (model LS 6500, Beckman Instruments, Fullerton, CA).

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OR) in 2% BSA-PBS overnight at 4°C. After they were washed with PBS, the coverslips were mounted with Dako fluorescent mounting medium onto glass slides using Gelvatol and examined under a microscope (Fluoview 300, Olympus). The number of BrdU-labeled cells relative to the total number of cells per field of vision was determined. At least 10 fields of vision per coverslip were counted.

**ATP assay.** The intracellular ATP content of neutralized acid extracts of cells was measured using a luciferin-luciferase assay kit according to the manufacturer’s instructions (Roche, Penzberg, Germany). The method was used to determine the ATP dependency of the light-emitting luciferase-catalyzed oxidation of luciferin (35). At 0 – 4 h, samples were diluted in a buffer containing 100 mM Tris and 4 mM EDTA (pH 7.75) and mixed immediately with equal amounts of the luciferase reagent. The light emitted from the luciferase was measured using a luminometer (Berthold Detection Systems, Pforzheim, Germany), and the values were calibrated against a standard ATP curve. The ATP content of the control cells in the culture medium (1.6 nmol/mg of protein) was defined as 100%.

**RNA isolation and RT-PCR.** The total RNA was extracted from the PTCs using the method described in the literature (8). The PTCs were homogenized with STAT-60, a monophasic solution of phenol and guanidine isothiocyanate (TEL-TEST, Friendwood, TX). Two micrograms of the purified RNA were synthesized into the cDNA using avian leukemia virus RT with the oligo(dT)18 primers. PCR amplification was performed using 5 µl of the RT product, 10 pmol of each primer, 1.25 U of Taq polymerase (Promega, Madison, WI), and 1 mM dNTP. After an initial incubation at 95°C for 5 min, 28 amplification cycles were performed: 95°C for 40 s, annealing at 55°C for 1 min, and extension at 72°C for 40 s. The rabbit-specific sense and antisense primers used in the study are as follows: P2Y1, 5’-GCATCTCGGTTGATACATGTTCC-3’ (forward) and 5’-GCTGTTGAGACTTGCTAGACCT-3’ (reverse); P2Y2, 5’TACAGCTCTGTCATGCTGG-3’ (forward) and 5’-GCCAGAAGTATGACAGCAGG-3’ (reverse); P2Y4, 5’TCTTTGCAAGTTTTGCCCTTC-3’ (forward) and 5’-CCGCGGGCTAGTACTATCCA-3’ (reverse); P2Y6, 5’-CTGTGCTGTCATGCAGATACAGGC-3’ (forward) and 5’-GTTGGCCCGGGAACTTC-3’ (reverse); P2X1, 5’-TCATCATCACAAAGTCAAGT-3’ (forward) and 5’-TGAAGTTGAGACTCTGGAGAC-3’ (reverse); P2X2, 5’-TCCATCATCACAAAGTCAAGT-3’ (forward) and 5’-TGGTTGCCGCCGGAACTTC-3’ (reverse); P2X3, 5’-TCCAATACAAAGTCAAGT-3’ (forward) and 5’-TTGGTTGCCGCCGGAACTTC-3’ (reverse).

**Fig. 2.** Effect of ATP analogs on incorporation of thymidine (A) and BrdU (B). PTCs were treated with ATP, adenosine, CTP, and UTP (10^{-4} M) for 4 h. Values are means ± SE of 6 independent experiments with triplicate dishes. *P < 0.05 vs. control.

**Fig. 3.** Response of PTCs to 0.5–4 h of treatment with 10^{-4} M ATP and effect of purinoceptor analogs on ATP-induced increase in [3H]thymidine incorporation. A: ATP assay in PTCs treated with 10^{-4} M ATP for 0.5, 1, and 4 h. p < 0.05 vs. control. bP < 0.05 vs. 1 h. B: thymidine incorporation in PTCs preincubated with suramin (10^{-6} M), reactive blue 2 (RB-2, 5 x 10^{-5} M), MRS-2179 (10^{-7} M), or MRS-2159 (10^{-7} M) for 30 min and then treated with ATP for 4 h. PTCs were treated with ATP (10^{-4} M), α,β-methyleneadenosine 5’-triphosphate (AMP-CPP), or 2-(methylthio)adenosine 5’-triphosphate (2-methylthio-ATP, 10^{-5} M) for 4 h. Values are means ± SE of 6 independent experiments with triplicate dishes. *P < 0.05 vs. control. **P < 0.05 vs. ATP alone. C: expression of P2X and P2Y receptor subtypes in PTCs. RT-PCR results are representative of 1 of 3 experiments.
GTTAGTGGATGCTGTT-3′ (reverse); P2X4, 5′-CTCCTGCAAGGACATTTAGAAGGACCCACAG-3′ (forward) and CTAGTGACCAATAGAATAGGCCCCTGAGTCTGTAGACTG-3′ (reverse); P2X5, 5′-GGGGCTGCTCGTCCTGATGAAC-3′ (forward) and CCTAGGAGCGCCAAGCCAGAGC-3′ (reverse). This sequence has been used successfully to clone the P2Y receptors from rabbits and mice (28). RT-PCR was performed using the β-actin primers as a control for the amount of cDNA. The PCR products were visualized using the ethidium bromide staining method.

Measurement of [Ca2+]i by confocal microscopy. The changes in [Ca2+]i, were monitored by fluo 3-AM, which had initially been dissolved in dimethylsulfoxide and stored at −20°C. The PTCs in the 35-mm culture dishes were rinsed twice with a bath solution (140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 0.5 mM MgCl2, 10 mM glucose, and 5.5 mM HEPES, pH 7.4), incubated in bath solution containing 3 μM fluo 3-AM with 5% CO2-95% O2 at 37°C for 40 min, rinsed twice with bath solution, mounted on a perfusion chamber, and scanned at 1-s intervals using confocal microscopy (×400; Fluoview 300, Olympus). The fluorescence was excited at 488 nm, and the emitted light was read at 515 nm. All the analyses of [Ca2+]i were processed on a computer, and the results are expressed as relative fluorescence intensity.

Western blot analysis. The cell homogenates (20 μg of protein) were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose paper. The blots were then washed with H2O, blocked with 5% skim milk powder in 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.05% Tween 20 for 1 h, and incubated with the appropriate primary antibody. The primary antibodies were detected using goat anti-rabbit IgG (1:5,000 dilution) conjugated to horseradish peroxidase. The bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech).

Statistical analysis. Values are means ± SE. The difference between two mean values was analyzed by Wilcoxon’s nonparametric sign test or ANOVA. The difference was considered statistically significant when P < 0.05.

RESULTS

Effect of ATP on cell proliferation. The time- and dose-response effect of ATP on thymidine and BrdU incorporation was determined by incubation of the PTCs that had reached 70–80% confluence in 0–10−3 M ATP for 4 h (Fig. 1, A–C). The level of thymidine and BrdU incorporation, as well as the number of cells, was increased at >10−5 M ATP, with the maximal stimulatory effect at 10−4 M. To determine the time course of ATP, PTCs were treated with 10−4 M ATP for 0–24 h. Thymidine incorporation was stimulated beginning at 1 h. This effect peaked at 4 h and gradually decreased to the control level over a 24-h period (Fig. 1D).

Receptor dependency of ATP on thymidine incorporation. Adenosine (a nonselective agonist), UTP (a P2Y receptor agonist), or CTP (a P2 receptor agonist) was added to the PTCs to determine which purinergic receptor is involved in the ATP-induced stimulation of cell proliferation. UTP increased the level of thymidine and BrdU incorporation (Fig. 2). However, adenosine and CTP had no effect on thymidine and BrdU incorporation. The cellular ATP content was measured at different times after addition of the extracellular ATP. The significant increase in the cellular ATP content 1 h after addition of ATP correlated with the result of thymidine incorporation (Fig. 3A). To determine which subtypes of the P2 receptors mediate the effect of ATP on thymidine incorporation, the PTCs were treated with suramin (a P2X and P2Y receptor antagonist), RB-2 (a P2Y receptor antagonist), MRS-2179 (a P2Y1 receptor antagonist), or MRS-2159 (a P2X7 receptor antagonist) for 30 min before the ATP treatment (32).
Pretreatment with suramin, RB-2, MRS-2179, or MRS-2159 prevented significantly the ATP-induced increase in thymidine incorporation (Fig. 3B). However, there was no statistical significance among suramin vs. RB-2, MRS-2179, or MRS-2159. Furthermore, treatment of PTCs with AMP-CPP (a P2X receptor agonist) or 2-methylthio-ATP (a P2Y receptor agonist) increased the level of thymidine incorporation, suggesting a role for the P2 purinoceptors. RT-PCR was used to examine the distribution of mRNA, because no specific antibodies directed against the P2 purinoceptors are available. The RT-PCR results also suggest that P2Y1, P2Y2, P2Y4, and P2Y6, as well as P2X1 and P2X4, receptors are expressed in renal PTCs (Fig. 3C).

Effect of ATP on [Ca\(^{2+}\)]. Confocal microscopy was used to examine the effect of ATP on [Ca\(^{2+}\)]. Figure 4A shows that 10\(^{-4}\) M, but not 10\(^{-6}\) M, ATP increased [Ca\(^{2+}\)], in the PTCs. This ATP-induced increase in [Ca\(^{2+}\)] was not observed in the presence of suramin (Fig. 4B). To determine the source of the cytosolic free Ca\(^{2+}\), the PTCs were preincubated with nifedipine (an L-type Ca\(^{2+}\) channel blocker) and EGTA (an extracellular Ca\(^{2+}\) chelator) for 30 min before ATP treatment. ATP did not increase [Ca\(^{2+}\)], in the presence of nifedipine or EGTA (Fig. 4, C and D). In addition, the involvement of Ca\(^{2+}\) in the effect of ATP on cell proliferation was examined. Pretreatment of methoxyverapamil, nifedipine, and EGTA significantly blocked the ATP-induced increase in thymidine incorporation (Fig. 5). However, there was no statistical significance among control vs. methoxyverapamil, nifedipine, or EGTA treatment groups. On the other hand, TMB-8 and BAPTA-AM (intracellular Ca\(^{2+}\) antagonists) did not prevent the ATP-induced stimulation of thymidine incorporation. Methoxyverapamil, nifedipine, EGTA, TMB-8, and BAPTA-AM alone did not alter cell proliferation or viability (data not shown).

Effect of ATP on MAPK phosphorylation. The involvement of MAPKs in the effect of ATP on cell proliferation was examined. Pretreatment with SB-203580 (a p38 MAPK inhibitor) and PD-98059 (an MEK inhibitor), but not SP-600125 (a JNK inhibitor), blocked the ATP-induced stimulation of thymidine incorporation (Fig. 6A). As shown in Fig. 6B, ATP-induced p38 and p44/42 MAPK phosphorylation was attenuated by pretreatment with suramin, RB-2, MRS 2179, or MRS 2159. Nifedipine or BAPTA-AM was then added to the PTCs to confirm the involvement of Ca\(^{2+}\) in the ATP-induced phosphorylation of MAPKs. As shown
in Fig. 6C, the ATP-induced increase in p38 and p44/42 MAPK phosphorylation was blocked by nifedipine, but not by BAPTA-AM.

Effect of ATP on cell cycle protein expression. To determine the effect of ATP on the cell cycle proteins, 0–10⁻³ M ATP was added to the PTCs, and the cells were examined by Western blotting. As shown in Fig. 7A, >10⁻⁵ M ATP significantly increased the expression level of cyclin E and CDK-2 in a dose-dependent manner. In contrast, ATP decreased the level of p21WAF1/Cip1, a CDK inhibitory protein, in a dose-dependent manner. Consistent with the results of thymidine incorporation, adenosine and CTP did not affect the CDK-2 expression level, which plays an important role in cell progression. However, the cells were incubated with butyrolactone I (a selective CDK-2 and CDK-1 kinase inhibitor) for 30 min before ATP treatment to confirm that the ATP-induced stimulation of thymidine incorporation was mediated by the activation of CDK-2 (6). Butyrolactone I significantly prevented the ATP-induced increase in thymidine incorporation (Fig. 7C).

Next, the relation between ATP and a P2 purinergic receptor in the expression of CDK-2, CDK-4, p21WAF1/Cip1, and p27kip1 was examined. The ATP-induced increase in the CDK-2 or CDK-4 expression levels and the decrease in the p21WAF1/Cip1 or p27kip1 expression levels were blocked by suramin or RB-2 (Fig. 8A). In addition, ATP and 2-methylthio-ATP increased the level of the cell cycle proteins, and MRS-2179 blocked the ATP-induced CDK-2, CDK-4, and cyclin D1 expression level (Fig. 8B). Western blotting analysis was performed to confirm the involvement of Ca²⁺ in the ATP-induced upregulation of the cell cycle proteins. Figure 8C shows that nifedipine, not BAPTA-AM, blocked the ATP-induced increase in the CDK-2 and CDK-4 expression level. The involvement of MAPKs in the effect of ATP on the CDK-2 and CDK-4 expression levels was examined. As shown in Fig. 8D, SB-203580 (a p38 MAPK inhibitor) and PD-98059 (an MEK inhibitor), but not SP600125 (a JNK inhibitor), blocked the ATP-induced increase in CDK-2 and CDK-4 expression.

DISCUSSION

In this study, ATP was found to stimulate renal PTC proliferation. Under basal conditions, the proximal tubule is the richest source of ATP. As reported by Brana et al. (6), compared with intracellular nucleotide concentrations, only a small concentration (0.1–10 µM) of extracellular nucleotides is required to activate the purinergic receptors. Consistent with this report, in the present study, the minimal effective concentration of ATP to stimulate cell proliferation was 10⁻⁵ M. This suggests that the ATP concentration used in the present study has physiological action on P2 receptor activation. This suggestion is supported by many reports demonstrating that 1–100 µM extracellular ATP augmented cell growth in vitro (37, 38, 44). Differences in the effectiveness of the ATP concentration may be due to an unknown quality of ATP, the cell types, marker indexes, or experimental conditions (in vitro vs. in vivo or serum vs. serum-free media). In this study, ATP stimulated the proliferation of PTCs via activation of the P2 purinergic receptors, as shown by the increase in BrdU and thymidine incorporation, indexes of increased DNA synthesis. In contrast, P2Y receptor activation has been shown to inhibit DNA synthesis in cultured cardiac fibroblasts and myocytes (56, 57). However, there is evidence suggesting that ATP stimulated cell proliferation via the P2 receptors (3, 11, 15). The reason for the different responses to extracellular ATP among the cell types is not clear. However, possible explanations include the potential presence of different P2 receptor subtypes and cell-type-specific differences in postreceptor signal transduction. It is unclear which specific subtypes of the P2 receptors are involved in the effect of ATP on cell proliferation, even though AMP-CPP (a P2X receptor agonist), as well as UTP and 2-methylthio-ATP (P2Y receptor agonists), increased the level of thymidine incorporation. Therefore, the molecular identification of the P2X and P2Y receptor subtypes in rabbit PTCs needs to be examined. In this study, ATP significantly increased the cellular ATP content, which correlated with results of thymidine incorporation. In addition, the ATP-induced stimulation of
Involvement of the P2Y receptor, [Ca\(^{2+}\)], and MAPKs in ATP-induced alteration of cell cycle protein expression. A: response of PTCs to preincubation with suramin or RB-2 for 30 min before ATP treatment for 4 h. B: response of PTCs to preincubation with MRS-2179 for 30 min before ATP treatment or incubation with 2-methylthio-ATP for 4 h. C: response of PTCs to preincubation with BAPTA-AM or nifedipine for 30 min before ATP treatment for 4 h. D: response of PTCs to addition of SB-203580, PD-98059, or SP-600125 (10^(-6) M) for 30 min before 10^(-4) M ATP for 4 h. Each electrophoretogram is representative of 6 different experiments.

According to these results, ATP increased [Ca\(^{2+}\)], which was blocked by suramin (a P2X and P2Y receptor antagonist). This suggests that the ATP-activated cell proliferation and the intracellular Ca\(^{2+}\) store was not determined in this study. However, the extracellular Ca\(^{2+}\) influx plays a direct role in ATP-induced PTC proliferation.

The role of ATP as an extracellular signaling molecule involved in cell proliferation was examined. Accordingly, ATP was examined to determine whether it activates the MAPK signaling cascades and whether ATP-triggered proliferation is involved in the phosphorylation of MAPKs. These signal pathways were confirmed by the blockade effect of p38 and the p44/42 MAPK inhibitor on ATP-induced cell proliferation. However, the JNK inhibitor did not block ATP-dependent cell proliferation. Pretreatment with the P2X and P2Y receptor antagonists abolished the ATP-induced p38 and p44/42 MAPK phosphorylation, suggesting that P2 purinoceptors mediated MAPK signaling. Similar results showing that ATP mediated by P2Y receptor activation stimulates cell proliferation in human mesangial cells via p44/42 MAPK activation or in rat mesangial cells via p38 MAPK activation have been reported by other investigators (27, 52). However, we are aware of no report on the regulatory mechanisms of ATP in PTC proliferation.

This study showed that rabbit PTCs express CDK-2, CDK-4, p21\(^{WAF1/Cip1}\), and p27\(^{kip1}\). Cyclin D1 is associated with preexisting CDK-4 and cyclin A or E with preexisting CDK-2 to form an active complex (30). Furthermore, ATP can activate CDK-2 and CDK-4 expression, which is believed to play a key role in PTC growth (1). Although the ATP-activated expression of cyclin A or E was not measured, the regulation of CDK-2, CDK-4, and cyclin D1 may provide sufficient information for understanding the effect of ATP on cell proliferation.

There is evidence implicating p21\(^{WAF1/Cip1}\) and p27\(^{kip1}\) expression level decreased
in response to ATP, which was in inverse proportion to the change in the CDK-2 and CDK-4 level. These results show that the decrease in the p21 or p27 level is associated with an increase in proliferation in response to mitogenic stimuli and that the forced overexpression of p21 or p27 inhibits proliferation (7, 26).

Interestingly, MAPKs are upstream regulators of CDK-2 and CDK-4 expression. These results are similar to the report showing that p44/p42 MAPK phosphorylation is essential and sufficient for the increase in CDK-2 (36) and decrease in p27kip1 expression (29). However, Delmas et al. (10) reported that the p42/p44 MAPK activation triggers p27kip1 degradation independently of CDK-2/cyclin E in NIH/3T3 cells. As described above, ATP regulation of the MAPK and CDK-cyclin complex has not been elucidated in other types of cells (55). One of the upstream regulators must be the influx of Ca\textsuperscript{2+}, because [Ca\textsuperscript{2+}], peaked at ~10 s and the activation of the MAPKs peaked at 5–30 min. Moreover, these results show that extracellular Ca\textsuperscript{2+} influx triggers the activation of p44/p42 and p38 MAPKs. This study identified a new mechanism showing that ATP increased [Ca\textsuperscript{2+}], leading to activation of p38 and p44/p42 MAPKs. This was followed by an alteration in the CDK-cyclin complex, p21, and p27, which are involved in stimulation of PTC proliferation. In conclusion, ATP stimulates proliferation via an increase in [Ca\textsuperscript{2+}], and activation of MAPKs and CDKs in the PTCs. The results suggest a role for extracellular ATP, P2 purinoceptors, and the Ca\textsuperscript{2+}-dependent MAPK signaling pathway, which can provide the basis for a detailed investigation of the upstream signaling components and the downstream targets of ATP-induced cell proliferation. The possible role of the P2X and P2Y receptor-MAPK signaling pathways in renal pathogenesis requires further study.

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