Augmented extracellular ATP signaling in bladder urothelial cells from patients with interstitial cystitis

Yan Sun and Toby C. Chai
Division of Urology, Department of Surgery, University of Maryland School of Medicine, Baltimore, Maryland

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Sun, Yan, and Toby C. Chai. Augmented extracellular ATP signaling in bladder urothelial cells from patients with interstitial cystitis. Am J Physiol Cell Physiol 290: C27–C34, 2006. First published August 17, 2005; doi:10.1152/ajpcell.00552.2004.—Interstitial cystitis (IC) is an idiopathic hypersensory condition of the bladder associated with increased urinary ATP and increased stretch-activated ATP release by bladder urothelial cells (BUCs), suggesting augmented purinergic signaling in the bladder. To test this theory further, monolayers of cultured BUCs derived from bladder biopsies obtained from patients with IC and control patients were stimulated with 10–30 μM ATP with subsequent measurement of extracellular ATP levels using the luciferin-luciferase assay. Stimulation with 30 μM ATP resulted in IC supernatant containing several-fold more ATP than control BUCs initially, followed by a slower decrease in ATP levels. This difference in ATP levels was not completely due to activity of extracellular ATP, because blockade with ARL67156 did not normalize the difference. Exposure to hypotonic solutions resulted in similar extracellular ATP concentrations in IC and control BUCs, but there was a slower decrease in ATP levels in IC supernatants. Treatment of IC BUCs with 10–40 μM suramin, a nonspecific P2 receptor antagonist, significantly attenuated the IC BUC response to extracellular ATP, restoring IC BUCs to a control phenotype. Pre-treatment of IC BUCs with 20 ng/ml of heparin-binding EGF-like growth factor (HB-EGF), which previously has been shown to block by suramin and HB-EGF. These findings suggest the possible development of future novel therapeutic techniques.

INTERSTITIAL CYSTITIS (IC) is a clinical syndrome characterized by extreme bladder sensations of urgency and pain in the absence of an identifiable cause. Understanding the sensory signaling pathways is critical to the treatment of this troublesome disease. Although many etiologic theories have been propounded, abnormalities in bladder urothelial cells (BUCs) may play a key pathophysiological role leading to IC (22). Several investigators have hypothesized that epithelial cells lining hollow organs, including the bladder, have neurosensory-like functions because the cells can release ATP in response to stretch (i.e., mechanical deformation) (9, 14). Studies have shown that human BUCs can release ATP in response to mechanical stretch. Furthermore, this release was more pronounced in patients with IC compared with control patients (26, 30). Increased ATP release may result in increased P2X3 expression on the BUCs, suggesting an autocrine role for ATP via this receptor (32, 34). Extracellular ATP has been demonstrated to stimulate the release of intracellular ATP from human umbilical vein endothelial cells (6). Interestingly, an animal model of IC, feline IC, has also been shown to have an augmented ATP release by BUCs and also altered purinergic receptor expression (4, 5). Therefore, it has been suggested that the BUC can mediate sensation as well as protect the bladder stroma (1).

Because there is evidence that IC urothelial cells in vitro are phenotypically different than controls, we queried how exogenous ATP stimulation, mimicking increased urinary ATP in IC urine specimens (30), affects explanted cultured IC and control BUCs. The findings from this study support the hypothesis that augmented purinergic mechanisms in BUCs could help to explain hypersensory dysfunction in patients with IC. Newer approaches to treatment for hypersensory bladder dysfunction such as IC could target the purinergic signaling pathways in BUCs.

MATERIALS AND METHODS

Materials

All chemicals and buffers were purchased from Sigma (St. Louis, MO). An ECL kit was purchased from (Amersham Pharmacia Biotech, Piscataway, NJ). MAb to uroplakin III (clone AUI) was purchased from (Boston, MA). MAb anti-actin was purchased from Oncogene (MO). An ECL kit was purchased from (Amersham Pharmacia Bio-LT). All chemicals and buffers were purchased from Sigma (St. Louis, MO). An ECL kit was purchased from (Amersham Pharmacia Biotech, Piscataway, NJ). MAb to uroplakin III (clone AUI) was purchased from (Boston, MA). MAb anti-actin was purchased from Oncogene (MO). An ECL kit was purchased from (Amersham Pharmacia Biotech, Piscataway, NJ).
and control patients while the patients were under general or regional anesthesia. The techniques of urothelial cell culture from biopsies have been established and described previously (35). Explanted cells obtained from patients with IC and control subjects have been shown to stain positively for pan-cytokeratin, consistent with their epithelial origin (30). Further experimentation to confirm that cultured cells were truly urothelial cells is detailed in the next section. For each experiment, \( 1 \times 10^6 \) cells were plated 24 h before the start of the experiment. All experiments were performed in 10% FBS-supplemented medium, except for the experiment shown in Figs. 4 and 8, in which serum-free medium was used.

**Immunostaining and Western blot analysis for uroplakin III.** To further confirm that cultured cells were urothelial in origin, we examined these cells for expression of uroplakin III using commercially available antibody clone AU1. According to the manufacturer’s protocol, cells were grown until they formed a confluent monolayer on coverslips. The cells were rinsed with PBS buffer and fixed in ice-cold acetone for 10 min. Endogenous peroxidase activity was blocked in 0.1% \( \text{H}_2\text{O}_2 \) for 20 min. The cells were incubated in mouse uroplakin III antibody clone AU1 (1:100 dilution) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:1,000 dilution; Amersham Pharmacia Biotech) for 2 h at room temperature. Cultured cells were also incubated in the absence of either primary or secondary antibody as negative controls. After being washed several times with PBS, the coverslips were mounted and cultured cells were visualized using 3,3′-diaminobenzidine (Vestin stain ABC kit; Vector Laboratories, Burlingame, CA). Paraffinized bladder biopsy specimens were deparaffinized by being washed three times with xylene and three times each with 100%, 95%, and 70% ethanol alcohol. Sections were then incubated overnight at 4°C with the same mouse antibody uroplakin III clone AU1 at 1:100 dilution. The next day, the sections were rinsed and incubated with the appropriate HRP-labeled secondary antibody (1:1,000 dilution) for 2 h at room temperature. The slides were then rinsed and mounted as described above.

For Western blot analysis for uroplakin III, cultured cells were trypsinized (0.25% trypsin) off the plates. SDS-PAGE and Western blot analysis were performed according to established protocols (32). Briefly, the cells were retrieved by centrifugation and washed three times with cold PBS buffer. Cell pellets were then lysed with ice-cold denaturing buffer and incubated on ice for 1 h. The entire cell lysate was used for this experiment. Protein quantification was determined using the Bio-Rad protein assay kit according to the manufacturer’s protocol. SDS-PAGE (10% gel) was performed with 50–70 \( \mu \)g of protein loaded for each sample. Once SDS-PAGE was finished, the gel was electrotransferred to PVDF membranes and blotted for 1 h with 5% nonfat milk blocking solution in Tris-buffered saline with Tween 20 (TBST) buffer. The membrane was incubated overnight at 4°C with 1:100 uroplakin III antibody in TBST buffer containing 5% nonfat milk. The next day, the membranes were washed with TBST, incubated for 2 h at room temperature with corresponding anti-mouse secondary antibody (1:1,000 dilution), and then visualized with ECL reagents. For molecular mass determination, SDS-PAGE was performed together with prestained standard protein markers with molecular mass ranging between 10 and 250 kDa. Membranes were subsequently incubated with a 1:25,000 dilution of actin antibody to validate the technique.

**ATP stimulation assay.** ATP at 10, 20, or 30 \( \mu \)M concentration was added to a monolayer of \( 1 \times 10^6 \) cells plated 24 h previously in culture medium plus 10% FBS. Trypan blue staining confirmed that cell viability was not affected by these ATP concentrations at the time points sampled. The supernatant was collected during a 3-h period (**time 0**, immediately after adding ATP; and 1, 2, and 3 h after addition of ATP). For these experiments, cells from eight patients with IC \((n = 8)\) and eight control patients \((n = 8)\) were used. In one set of experiments, the cells were stimulated repetitively with 30 \( \mu \)M ATP at **time 0** and at 24, 48, and 72 h. Supernatant was collected for 3 h after each ATP addition. The sample sizes for the long-term exposure to ATP experiments were \( n = 2 \) for IC and \( n = 2 \) for control cells.

**ATP assay.** ATP assays were performed using luciferin-luciferase assay kit (Sigma). A 100-\( \mu \)l sample of supernatant was added to the 100 \( \mu \)l of reaction mix of luciferin-luciferase. Flash luminescence was read for 5 s using a Victor II 1420 multilabel counter (Wallac PerkinElmer, Foster City, CA). For each experiment, a standard curve using known concentrations of ATP was constructed with \( R^2 \) linear correlation coefficients of 0.992–0.999 across a \( 1 \times 10^5 \) range of concentrations. Control cell culture media served as blank values. The ATP concentration was calculated using corresponding standard curves with a detection limit of 2 \( \text{fM} \) ATP.

Because different agents [e.g., ARL 67156, suramin, KCl, heparin-binding EGF-like growth factor (HB-EGF)] were added to BUCs to determine their effects on extracellular ATP, it was necessary to determine whether each of these agents affected the luciferin-luciferase ATP assay. Only ARL67156 affected the ATP standard curve. Therefore, different standard curves were separately obtained for each of the concentration of ARL67156 used. With ARL67156, only the slope of the standard curves, not the linear correlation between ATP concentration and luminescence, was affected.

**Blockade of ecto-ATPase activity with ARL67156.** Three concentrations of ARL67156 were used (1, 10, and 100 \( \mu \)M) to block degradation of extracellular ATP by ecto-ATPase. This procedure was performed to determine whether differences in extracellular ATP measured between IC and control cells would reflect differences in ecto-ATPase activities. In these experiments, cells from three patients with IC and two control patients were used. These cells were stimulated with only 30 \( \mu \)M ATP, because this dose had the most differential effect between IC and control cells. ARL67156 was added simultaneously with the 30 \( \mu \)M ATP-stimulated plates.

**Osmolality measurements.** To determine whether the added exogenous ATP might have changed the toxicity (i.e., osmolality) of the medium, the osmolalities of 10, 20, and 30 \( \mu \)M ATP in cell culture medium were measured using the Advanced Osmometer model 3900 (Advanced Instruments, Norwood, MA), which uses freezing-point depression to determine osmolality. We put 200 \( \mu \)l of sample into the machine’s automatic sampling system, and each sample was tested in duplicate. The osmolalities of the 0.0375 M and 0.075 M KCl solutions also were determined.

**Effect of hypotonicity on extracellular ATP.** To evaluate the effect of hypotonic solution on extracellular ATP, we added 0.0375 M of KCl and 0.075 M of KCl to the BUCs and measured ATP in the supernatant at 0, 1, 2, and 3 h. In this experiment, cells from three patients with IC and two control patients were used.

**Suramin treatment.** Suramin, a nonspecific P2 receptor antagonist, was used at 10, 20, and 40 \( \mu \)M concentrations to evaluate effect on extracellular ATP in response to ATP stimulation. The concentrations of suramin used did not cause cell death on the basis of Trypan blue staining of cells. Coadministration of suramin with ATP was not effective in decreasing extracellular ATP (data not shown), so all experiments were conducted with a 24-h preincubation with the various doses of suramin before stimulation with ATP. Only 30 \( \mu \)M ATP stimulation was used because of the maximum stimulatory effect. In this experiment, cells from three patients with IC and four control patients were used.

**HB-EGF treatment.** IC cells were pretreated with 20 ng/ml HB-EGF for 48 h before the start of 30 \( \mu \)M ATP stimulation, then the supernatant was collected at **time 0** (immediately after ATP addition) and at 1-, 2-, and 3-h time points. Cells from two patients with IC were used in this experiment.

**Statistical analysis.** The results are presented as means ± SE. Student’s \( t \)-test was used to compare the means, and \( P < 0.05 \) was considered statistically significant. In all experiments, each cell line was plated at least in duplicate and the measurements were averaged, but the sample size was still considered as 1. Therefore, all data were
calculated on the basis of sample sizes in which each individual patient was considered \( n = 1 \).

RESULTS

Uroplakin III is Present in Cultured BUCs

Uroplakin III is a specific urothelial differentiation marker. Representative results of immunohistochemical staining are shown in Fig. 1. All biopsy specimens and cell populations were immunohistochemically positive for uroplakin III (brown coloration). Western blot analysis of cultured cells detected uroplakin III at the expected molecular mass (47 kDa) (Fig. 2). It has been shown previously that these cultured cells also stain positively for cytokeratin (30).

ATP Stimulation Results in Significantly Higher Extracellular ATP in IC compared with Control Supernatants

Without any exogenous ATP, there were no differences in extracellular ATP concentrations from IC and control BUC cultures (Fig. 3A). The concentrations were stable and low (\(~0.02\, \mu M\)) for 3 h. ATP concentrations in IC and control BUC supernatants were not different from that measured in supernatants with no cells present (Fig. 3A).

Addition of 10 \( \mu M \) ATP resulted in an increase in ATP concentrations in the supernatant of both control and IC BUC cultures (Fig. 3B), but never to the added level of 10 \( \mu M \) (dotted line, maximal ATP in Fig. 3). There was a significantly higher level of instantaneous ATP (time 0) in IC compared with control BUC supernatants. There was a rapid decrease in extracellular ATP after time 0 in both IC and control cells.

Addition of 20 \( \mu M \) ATP (Fig. 3C) resulted in the same pattern as 10 \( \mu M \) ATP. There was a higher level of ATP measured instantaneously (immediately after addition of ATP) in IC compared with control BUC supernatants. At no time point did the extracellular ATP concentration approach the concentration of 20 \( \mu M \) ATP added (dotted line). Again, there was a rapid decrease in extracellular ATP after time 0.

In a separate experiment, the stability of 10 and 20 \( \mu M \) ATP in cell media at 37°C with no BUCs present was determined. There was not any measurable degradation of ATP during a 3-h time period (data not shown). Therefore, it was deduced that IC and control BUCs degrade or take up extracellular ATP at these two concentrations but that IC cells take up significantly less ATP at the early time points.

Stimulation of the BUCs with 30 \( \mu M \) ATP resulted in significantly higher measured extracellular ATP from IC compared with control supernatants at time 0 and at 1 h (Fig. 3D). However, a different pattern emerged. ATP in IC supernatant, but not in control supernatant, measured at time 0 exceeded the amount of ATP added (dotted line in Fig. 3), suggesting release from intracellular stores that overcame ATP degradative or reuptake ability.

When the cells were repetitively stimulated with exogenous 30 \( \mu M \) ATP at 0, 24, 48, and 72 h (Fig. 4), ATP levels in the IC supernatant were consistently higher compared with control supernatant (maximum 2- to 5-fold greater at various time points). This occurred each time ATP was added. Furthermore, IC supernatant consistently contained ATP >30 \( \mu M \) added each time. This did not occur with the control cells.

Elevated Levels of ATP in IC Supernatant Is Not Completely Due to Differences in Ecto-ATPase Activity

To determine whether differences in ecto-ATPase activities might explain differences detected in the supernatant ATP between IC and control BUCs, ARL67156, a blocker of ecto-ATPases, was used. When 1 \( \mu M \) ARL67156 was used (Fig. 5A), stimulation with 30 \( \mu M \) ATP resulted in similar ATP levels at time 0 between IC and control supernatants; however, at the 1- and 3-h time points, IC supernatant still contained significantly higher ATP levels than control supernatant. With 10 \( \mu M \) ARL67156, a similar effect was demonstrated (Fig. 5B), except that there was no statistical difference at the 3-h time point. With 100 \( \mu M \) ARL67156, IC supernatants had significantly higher ATP levels at the 1 and 3 h time points. These data suggest that the initial difference in ATP levels at time 0 (Fig. 3D) may be due to differences in ecto-ATPase activity. However, even with ecto-ATPase blocked, IC ATP levels at later time points were still higher than those of controls, suggesting that these differences in ATP levels were not due entirely to differences in ecto-ATPase activity.

Hypotonicity Results in Higher ATP Levels in IC Supernatant

Previously published data on the feline IC model showed that BUCs from these animals released more ATP in response
to hypotonic stress compared with control BUCs (4). Hypotonic stress can induce swelling in cells. We tested whether hypotonic solutions can cause increased ATP release in human IC BUCs. The osmolalities of the different cell conditions used in these experiments were determined and are listed in Table 1. Although increasing ATP concentrations in cell media (10, 20, and 30 mM KCl) had a decreasing tonicity, 30 mM ATP was still higher in tonicity compared with 0.0375 M KCl, which caused no release of ATP (Fig. 6). Only the most hypotonic 0.075 M KCl solution induced ATP release from both IC and control BUCs (Fig. 6). IC supernatant, however, still contained significantly more ATP at the 1-h time point. Note that the absolute levels of ATP released by 0.075 M KCl were low, with ATP concentration 1 mM.

**Suramin Decreases Extracellular ATP in IC Supernatant**

Extracellular ATP may play an autocrine role in mediating further release of ATP through activation of the P2X receptor (28). Therefore, the effect of the nonspecific P2 receptor antagonist suramin on extracellular ATP was measured. All concentrations of suramin significantly reduced extracellular ATP in IC (Fig. 7A) and control supernatants (Fig. 7B) at time 0. Suramin reduced ATP release by the IC cells to levels approximately equivalent to those of control cells not treated

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Fig. 3. Effect of ATP stimulation on ATP measured in extracellular space. In all graphs, n = 8 patients with IC and n = 9 control patients; *P < 0.05. A: no ATP stimulation. Supernatant ATP levels without exogenously added ATP: culture medium only (no cells, NB), IC BUCs and control BUCs (NB). B: ATP (10 µM) stimulation. Supernatant ATP levels during 3 h in IC BUC and control BUC after 10 µM ATP stimulation. Dotted line represents maximal supernatant ATP level when no intracellular stores were released (i.e., equivalent to exogenously added ATP). C: ATP (20 µM) stimulation. Supernatant ATP levels during 3 h in IC BUC and control BUC in response to 20 µM ATP stimulation. Dotted line is maximal supernatant ATP level when no intracellular stores were released (i.e., equivalent to exogenously added ATP).

Fig. 4. Repetitive stimulation with 30 µM ATP. Both IC and control BUC were stimulated repetitively with 30 µM ATP at 0, 24, 48, and 72 h with supernatant ATP measured immediately and at 1, 2, and 3 h after each addition of ATP. Arrows indicate when 30 µM ATP was added to the cell supernatant. n = 2 for IC, n = 2 for NB; *P < 0.05.
with suramin; thus suramin appeared to revert the phenotype of IC to that of control BUCs.

**HB-EGF Decreases Extracellular ATP in IC Supernatant**

Previous studies demonstrated that IC urine specimens and IC BUC cultures contained significantly decreased level of the cytokine HB-EGF (21, 23). This experiment tested whether the addition of HB-EGF to IC BUCs normalized the phenotype of the IC BUCs in terms of response to ATP stimulation. Indeed, treatment of IC cells with 20 ng/ml HB-EGF for 48 h before stimulation with 30 μM ATP resulted in significantly less extracellular ATP at time 0 and at 2 and 3 h (Fig. 8). The ATP curve of HB-EGF-treated IC BUC is similar to that of control BUCs (compare Fig. 8 with Fig. 3D).

**DISCUSSION**

Our understanding of the function of the bladder urothelium is undergoing a paradigm shift. Ferguson et al. (14) showed that the bladder urothelium can serve as a sensor of bladder filling by releasing ATP. ATP release by BUC has been shown in multiple species, including humans, mice, and pigs (26, 30, 38). Sensory nerve endings that have been found in close association with bladder urothelial cells have suggested that cross-talk between BUCs and nerves could occur (2). Furthermore, urothelial cell-to-cell cross-talk could also occur. ATP receptors P2X3 and P2X2 have been found in both the nerves and BUCs (8, 32, 34), suggesting that these purinergic receptors could mediate the cross-talk between these cells.

**Table 1. Data Summary**

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Tonicity, mosmol/kgH₂O</th>
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</thead>
<tbody>
<tr>
<td>Cell culture media</td>
<td>290</td>
</tr>
<tr>
<td>Cell culture media + 10 μM ATP</td>
<td>283</td>
</tr>
<tr>
<td>Cell culture media + 20 μM ATP</td>
<td>270.5</td>
</tr>
<tr>
<td>Cell culture media + 30 μM ATP</td>
<td>251</td>
</tr>
<tr>
<td>0.0375 M KCl</td>
<td>220</td>
</tr>
<tr>
<td>0.075 M KCl</td>
<td>146</td>
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**Fig. 6. Effect of hypotonicity on ATP release.** KCl (0.0375 M) had no effect on either type of cell. However, hypotonic solution at 0.075 M KCl caused significant release of ATP from both types of cells. There was significantly more ATP in IC compared with NB supernatant at the 1-h time point. n = 3 patients with IC and n = 2 for control patients; *P < 0.05.

**Fig. 5. Effect of ARL67156 on ATP release.** In all graphs, n = 3 patients with IC and n = 2 control patients; *P < 0.05 in all panels. A: 1 μM ARL67156 treatment. IC supernatant ATP levels remained significantly higher than control cells at 1 h post-ATP stimulation. B: 10 μM ARL67156 treatment. IC supernatant ATP levels remained significantly higher than control at 1 h post-ATP stimulation. The ATP level at time 0 is almost identical between IC and NB supernatants. C: 100 μM ARL67156 treatment. Time 0 ATP levels were essentially the same at this dose of 100 μM ARL67156. However, at 1 and 3 h post-ATP stimulation, IC supernatant ATP levels were significantly higher than NB levels.
Importance of P2X3 receptors in bladder and sensory function was shown on the basis of P2X3-knockout mice having decreased pain thresholds and decreased voiding frequency (13). BUCs can release other neurotransmitters, such as nitric oxide (3) and an uncharacterized smooth muscle relaxant factor (11, 19). Muscarinic receptors, typically thought to be functionally important only in bladder smooth muscle, have also been found in BUCs (12). The function of muscarinic receptors in BUCs is unknown, although extracellular ATP can either affect the cell that released the ATP, and/or another BUC, or a nearby nerve. In the present study, IC BUCs had a consistently higher supernatant ATP secondary to possible augmented release of ATP, decreased degradation of ATP, and/or decreased reuptake of ATP in response to stimulation with extracellular ATP. The differences between IC and control cells depended on the concentration of exogenous ATP added. At lower doses (10 and 20 μM), extracellular ATP never exceeded the amount that was added exogenously (dotted lines in Fig. 3, B and C). At the higher concentration of 30 μM ATP, IC BUCs released intracellular ATP stores (Figs. 3D, 4, and 7A). These findings are similar to those in human umbilical vein endothelial cells showing that endothelial cells stimulated with ATP released ATP (6). The important finding reported herein is that IC BUCs were shown to have an augmented response to ATP stimulation compared with control BUCs. This finding is clinically relevant because patients with IC have increased ATP in their urine (30), and a "vicious cycle" may occur in which increased urinary ATP causes more ATP to be released by the urothelial cells. The increased ATP levels measured in the IC extracellular space was not completely due to differing activities of ecto-ATPases (either lower in IC BUCs or higher in control BUCs). When ecto-ATPases were blocked with ARL67156, there were still significantly higher ATP levels in the IC BUC supernatant from 1 to 3 h after stimulation with 30 μM ATP (Fig. 5, A–C). Therefore, it is possible that the increased extracellular ATP in importance of P2X3 receptors in bladder and sensory function was shown on the basis of P2X3-knockout mice having decreased pain thresholds and decreased voiding frequency (13). BUCs can release other neurotransmitters, such as nitric oxide (3) and an uncharacterized smooth muscle relaxant factor (11, 19). Muscarinic receptors, typically thought to be functionally important only in bladder smooth muscle, have also been found in BUCs (12). The function of muscarinic receptors in BUCs is unknown, although they may be involved in the release of urothelium-derived smooth muscle relaxant factor (11). These studies, taken together, show that BUC, in addition to serving a barrier function, could be involved in the bladder sensory pathway, with the purinergic pathway playing a vital role.

There is also accumulating evidence that cultured BUCs obtained from IC bladder biopsies are intrinsically altered. These defects include alterations in cytokine expression (21, 23, 24) and the unique expression of a recently characterized antiproliferative glycopeptide factor (25). Purinergic regulation in IC BUCs was also found to be augmented (30, 31, 32), suggesting that heightened purinergic signaling may correlate with increased sensation observed clinically in patients with IC. These findings further support the theory that purinergic signaling in bladder urothelial cells are important in bladder sensory signaling.

Extracellular ATP can have an autocrine function in regulating cellular volume (39), gastrointestinal secretion, and hepatobiliary function (29). A review of the exocrine function of extracellular ATP has been published (29). The function of extracellular ATP in the bladder urothelium is currently unknown, although extracellular ATP can either affect the cell that released the ATP, and/or another BUC, or a nearby nerve. In the present study, IC BUCs had a consistently higher supernatant ATP secondary to possible augmented release of ATP, decreased degradation of ATP, and/or decreased reuptake of ATP in response to stimulation with extracellular ATP. The differences between IC and control cells depended on the concentration of exogenous ATP added. At lower doses (10 and 20 μM), extracellular ATP never exceeded the amount that was added exogenously (dotted lines in Fig. 3, B and C). At the higher concentration of 30 μM ATP, IC BUCs released intracellular ATP stores (Figs. 3D, 4, and 7A). These findings are similar to those in human umbilical vein endothelial cells showing that endothelial cells stimulated with ATP released ATP (6). The important finding reported herein is that IC BUCs were shown to have an augmented response to ATP stimulation compared with control BUCs. This finding is clinically relevant because patients with IC have increased ATP in their urine (30), and a "vicious cycle" may occur in which increased urinary ATP causes more ATP to be released by the urothelial cells.

The increased ATP levels measured in the IC extracellular space was not completely due to differing activities of ecto-ATPases (either lower in IC BUCs or higher in control BUCs). When ecto-ATPases were blocked with ARL67156, there were still significantly higher ATP levels in the IC BUC supernatant from 1 to 3 h after stimulation with 30 μM ATP (Fig. 5, A–C). Therefore, it is possible that the increased extracellular ATP in
IC supernatant at these time points is due either to decreased cellular endocytosis of ATP or to decreased receptor-mediated reuptake of ATP into IC cells. However, at time 0, with increasing ARL67156 doses, there is a loss of difference in the ATP levels between IC and control BUCs. This suggests that the early augmentation in IC ATP levels could be due to a decreased ecto-ATPase activity in IC cells (or increased activity in control BUCs). Decreased activity of ecto-ATPase has been detected in overactive bladder smooth muscle, suggesting that increased extracellular ATP activity may mediate the pathophysiology of overactive bladder (18).

Besides exogenous ATP stimulation, several other stimuli can trigger release of ATP in epithelial cells. Mechanical stretch, fluid shear force, physical agitation, and hypotonicity all can increase extracellular ATP (4, 7, 16, 17). Hypotonicity did not result in an initial higher ATP level in IC supernatant, but there was a slower decrease in ATP levels with IC cells (Fig. 6), with the 1-h time point demonstrating significantly higher ATP levels in IC compared with control supernatants. Feline IC bladder urothelium responded to hypotonicity with a higher ATP level compared with control BUCs (4). The difference in amount of ATP released by feline and human BUCs in response to hypotonic stimulation is of the order of several magnitudes (cats, 200 fM; humans, 120,000 fM on the basis of calculations of ATP levels shown in Fig. 6). IC and control BUCs did not release ATP in response to change of growth media, vigorous shaking, and addition of ionomycin (data not shown). These findings suggest that human BUCs do not behave identically to epithelial cells from other organs. The response of IC urothelial cells to extracellular ATP is important to study because urinary ATP levels in patients with IC are significantly elevated (30).

Cellular release of ATP into the extracellular space can occur via three mechanisms: vesicular exocytosis, conductive transport (concentration gradient between intracellular and extracellular concentrations), or specific channels that can be blocked by pretreatment with suramin (Fig. 7, measured extracellular ATP concentration at time 0, with increasing ARL67156 doses, there is a loss of difference in the ATP levels between IC and control BUCs. This suggests that the early augmentation in IC ATP levels could be due to a decreased ecto-ATPase activity in IC cells (or increased activity in control BUCs). Decreased activity of ecto-ATPase has been detected in overactive bladder smooth muscle, suggesting that increased extracellular ATP activity may mediate the pathophysiology of overactive bladder (18).

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Cellular release of ATP into the extracellular space can occur via three mechanisms: vesicular exocytosis, conductive transport (concentration gradient between intracellular and extracellular concentrations), or specific channels that can be regulated by the ATP-binding cassette transporter or CFTR. The mechanisms of epithelial ATP release have been studied in respiratory epithelia (33). Defective extracellular ATP signaling has been thought to be pathophysiological in cystic fibrosis (CF). CF respiratory epithelia have been shown to have diminished ATP release, leading to fluid and ion transport dysfunction typical in patients with CF (33).

The augmented responses of IC BUC to 30 μM ATP could be blocked by pretreatment with suramin (Fig. 7, A and B). Measured extracellular ATP concentration at time 0 was ~17 μM in suramin-treated control BUCs (Fig. 7B). This level was about twofold lower than that achieved for suramin-treated IC BUCs, which was about 35 μM (Fig. 7A). Lack of ATP level normalization at time 0 between suramin-treated IC and control BUCs could be due to diminished ecto-ATPase activity in IC cells (Fig. 5, A–C, time 0), because suramin should not affect ecto-ATPase activity. However, the drop in ATP levels that did occur at time 0 in suramin-treated IC cells (from ~80 μM to 35 μM) could be explained by antagonism of P2 receptor-mediated regulation of extracellular ATP levels.

Suramin’s only clinical use currently is as an antiparasitic agent against Trypanosoma gambiense or Trypanosoma rhodesiense (African sleeping sickness) and Onchocerca volvulus (onchocerciasis). In laboratories, suramin has been used as a pharmacological agent to block P2X and P2Y receptors (20, 27). The use of suramin as an antineoplastic agent for bladder urothelial carcinoma has been investigated (36). This agent has been shown to inhibit urothelial carcinoma in vitro through inhibition of EGF binding (15). Interestingly, EGF has been shown to be secreted in significantly higher concentrations by IC compared with control BUCs (23). Intravesicular suramin has been studied in a phase I trial for treatment of superficial bladder cancer (36) and demonstrated acceptable safety and tolerability. Furthermore, intravesicular suramin was shown to diminish bladder instability in a bladder outlet obstruction model in rats (37), suggesting that purinergic mechanisms play a role in bladder overactivity. Therefore, intravesicular suramin could be an effective treatment for patients with IC, and a clinical trial should be considered.

One of the abnormalities of the IC BUC phenotype is the decreased in vitro and in vivo expression of HB-EGF, possibly as a result of the inhibition of HB-EGF by antiproliferative factor (21, 23). Treatment of IC BUCs with 20 ng/ml HB-EGF, which is the upper limit found in urine from asymptomatic control subjects (21), reversed the augmented ATP release of IC BUCs in response to 30 μM ATP (Fig. 8). The mechanism underlying HB-EGF’s effect on ATP release remains to be investigated.

Burnstock (10) reviewed the role of targeting the purinergic signaling pathway in clinical medicine. Diseases that can be targeted include supraventricular tachycardia, cancer, dry eye, bladder hyperactivity, erectile dysfunction, CF, osteoporosis, diabetes, gut motility, and vascular disorders. The data presented herein suggest that reducing the augmented urothelial purinergic signaling in IC could be considered for clinical testing.

Extracellular ATP signaling is augmented in IC BUCs. At low concentrations of ATP stimulation, IC supernatant contained significantly higher ATP compared with control BUCs. At higher concentrations of ATP stimulation, there was a significantly higher ATP concentrations of extracellular ATP in IC compared with control BUC supernatants. Treatment IC BUCs with suramin, a nonspecific P2 antagonist, significantly reduced the amount of ATP released from IC BUCs. Similarly, but presumably through a different mechanism, HB-EGF treatment of IC BUCs also reduced the augmented extracellular ATP. Differences in ecto-ATPase activity could not completely explain the higher ATP levels in IC supernatant. Hypotonic stimulus of BUCs also caused the IC supernatant to have significantly more ATP, but only 1 h after exposure to the hypotonic stimulus. These findings suggest that purinergic activity, specifically extracellular ATP signaling, is significantly augmented in IC BUCs and may play a role in the pathophysiologic of bladder hypersensation in IC.

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REFERENCES
11. Chaiyaprasithi B, Mang CF, Kilbinger H, and Hohenfellner M. Burnstock G.

13. Chess-Williams R. Ferguson DR, Kennedy I, and Burton TJ.

15. Guyot A and Hanrahan JW.


16. Grygorczyk R and Hanrahan JW.

19. Hawthorn MH, Chapple CR, Cock M, and Chess-Williams R. Urothe-


