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RESEARCH PAPER

Release of ATP from rat urinary bladder mucosa: role of acid, vanilloids and stretch

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Background and purpose: ATP, released from urothelial cells, modulates afferent nerve firing from the urinary bladder. Here, we have characterized ATP release from the rat bladder mucosa in response to acid, capsaicin, electrical field stimulation (EFS) and stretch, using agonists and antagonists at transient receptor potential vanilloid receptor 1 (TRPV1) and acid-sensing ion channels (ASICs).
Experimental approach: Rat mucosal strips (containing urothelium and lamina propria) in Perspex microbaths were superfused with Krebs solution. ATP was measured after exposure of matched strips to acid (pH 6.6–5.0), capsaicin (0.1–10 μM), EFS or stretch (150% of original length).
Key results: Median basal ATP release was 3.46 nmol·g^{-1}. The mucosal strips responded to stimuli with potency order (median, IQR): acid (pH 5.6–6.0) 286 (103–555) > 10 μM capsaicin 188 (117–431) > 10 Hz EFS 63.0 (13.3–96.4) > stretch 24.4 (6.73–55.1) nmol ATP g^{-1}. ATP release in response to acid was pH dependent ($P < 0.05$). Responses to capsaicin did not desensitize nor were they concentration dependent. TRPV1 antagonist, capsazepine (10 μM) abolished capsaicin-evoked ATP release, and reduced acid-evoked (pH 6.5) release to 30% ($P < 0.001$). The ASIC channel antagonists gadolinium (0.1 mM) and amiloride (0.3 μM) reduced ($P < 0.05$) the acid-evoked (pH 6.5) release to 40 and 6.5% respectively. ASIC (ASIC1, ASIC2a, ASIC2b, ASIC3) and two TRPV1 gene products were detected in mucosal and detrusor extracts.
Conclusions and implications: Capsaicin (at TRPV1) and acid (at both TRPV1 and ASIC) induce ATP release from the rat bladder mucosa. This ATP appears to be principally of urothelial origin. This study highlights the importance of ATP and acid as signalling molecules in modulating bladder function.
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Keywords: bladder; ATP; urothelium; mucosa; acid; ASIC; capsaicin; TRPV1; amiloride; gadolinium

Abbreviations: ASIC, acid-sensing ion channels; DEG/ENaC, degenerin/epithelial Na^+ channel; DRG, dorsal root ganglia; EFS, electrical field stimulation; TRPV1, transient receptor potential vanilloid receptor 1; TTX, tetrodotoxin

Introduction

Adenosine triphosphate (ATP) is a co-transmitter with acetylcholine in parasympathetic nerves innervating the urinary bladder, although recent evidence suggests that it has dual functions. Ferguson *et al.* (1997) showed that pressure changes on the rabbit bladder wall caused a release of ATP, which was thought to originate from urothelial cells in response to bladder distention. It has been postulated that ATP then acts on $P2X_3$ receptors in the sub-urothelial afferent nerve plexus to modulate afferent firing to the CNS (Ferguson *et al.*, 1997). Thus, ATP can influence the bladder volume at which the detrusor contracts (Burnstock, 2008). However, few studies have investigated whether any other stimuli, apart from bladder distention, may also stimulate ATP release, and which receptors are involved.

The vanilloids, capsaicin and resiniferatoxin act via the transient receptor potential vanilloid receptor 1 (TRPV1), a non-selective cation channel that responds to various noxious stimuli, such as heat and low pH, as well as vanilloids. The TRPV1 receptor is involved in thermal and inflammatory pain (Caterina *et al.*, 2000; Davis *et al.*, 2000). The vanilloids have a number of actions in the bladder (see Avelino and Cruz, 2006), and TRPV1 receptor expression has been demonstrated in C-fibres as well as on urothelial cells (Lazzeri *et al.*, 2005). Increased expression of TRPV1 mRNA has been found in the trigonal mucosa of women with symptoms of sensory urgency (Liu *et al.*, 2007). In animal models, TRPV1 is associated with bladder overactivity accompanying cystitis (Sculptoreanu *et al.*, 2005) and spinal cord injury (De Groat, 1997).

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In addition to TRPV1 receptors, there are also other more specialized receptors for detecting changes in pH. Acid-sensing ion channels (ASICs) have been reported in the CNS (Wemmie *et al.*, 2006), as well as in nociceptive fibres innervating other organ systems such as the gastrointestinal tract (Holzer, 2007) and the airways (Faisy *et al.*, 2007), where they are thought to modulate basal tone and contractility. ASICs are members of the degenerin/epithelial Na^+ channel (DEG/ENaC) family of amiloride-sensitive sodium channels, and are located predominantly on sensory nerve endings in the peripheral nervous system (Wemmie *et al.*, 2006).

Amiloride, a non-selective blocker of the DEG/ENaC family, has been reported to cause inhibition of bladder afferent activity (Du *et al.*, 2007), and to suppress ATP release induced by hypotonic ('stretch') stimulus in cultured cat urothelial cells (Birder *et al.*, 2003). The ASIC receptors and their primary agonist, low pH, do not appear to have been investigated in the bladder; however, their role as sensory receptors suggests that they may play a part in bladder ATP release, and thus the sensation of fullness and pain.

We hypothesized that low pH would act on ASIC receptors in the bladder mucosa, as well as on urothelial TRPV1 receptors, to cause the release of ATP. The aims of this study were to: (i) develop a method to assess ATP release from rat bladder mucosal strips; (ii) measure and characterize ATP release from the rat bladder mucosa in response to acid and capsaicin; and (iii) investigate ASIC receptor expression in rat bladder extracts. Antagonists of the TRPV1 and ASIC receptors were used to investigate the receptors involved. Stretch and nerve stimulation were used as positive controls.

Methods

Tissue preparation

All animal care and experimental procedures complied with the University Regulations (UNSW Animal Ethics 07/18B). Male rats (Sprague Dawley; ~250 g) were killed by sodium thiopentone (100 $mg \cdot kg^{-1}$ i.p.) followed by cervical dislocation. Approximately 5 min after death, the bladder was removed and placed in cold, gassed Krebs–Henseleit solution. The mucosa was carefully separated from the detrusor. In most instances, the detrusor was discarded, and the mucosa was then cut into approximately four longitudinal strips (average weight 2.0 ± 0.50 mg). In some studies, longitudinal detrusor strips (minus mucosa) were also used.

Further studies were conducted with urothelially denuded mucosal strips, where a blunt scalpel blade was used to scrape off the urothelium before mounting in the microbath. Histological analysis was performed to ensure complete removal of urothelium.

Functional studies

Strips were mounted in Perspex microbaths (Oxford University, Oxford, UK), volume 0.2 mL, and containing inbuilt platinum ring electrodes (Brading and Sibley, 1983). The strips were superfused with carboxygenated Krebs–Henseleit solution at a constant flow rate of 1.8 ± 0.3 $mL \cdot min^{-1}$ via a peristaltic pump. The circuit passed through a heating bath, which maintained the strips at a temperature of 36 ± 0.5°C. The strips were equilibrated under a resting tension of 1.0 g for 60 min, monitored by isometric force transducers and recorded on a Powerlab (ADInstruments, Bella Vista, NSW, Australia).

At the completion of the equilibration period, a sample of perfusate was collected for basal ATP measurement. After 15 min, the agonist, dissolved in Krebs–Henseleit solution to the appropriate concentration, was then exposed to the strip for 30 s. In order to accurately indicate the time of arrival of perfused agonist at each strip, a small air bubble was introduced into the circuit as a time indicator. In preliminary studies, ATP release was measured at 10, 30, 60 and 120 s after agonist stimulation: it was found that ATP release was greater at 10 s than at other times, and in subsequent experiments, only one sample (150 μL) was collected at 10 s.

The agonists tested included acid (hydrochloric acid in Krebs–Henseleit solution; pH range 6.6–5.0) and capsaicin (0.1–10 μM). In initial experiments, three discrete applications of acid or capsaicin were delivered to the strips, usually in increasing concentration/decreasing pH respectively. However, to test the effect of possible desensitization by the first stimulus, they were also tested in reverse order.

For experiments where stretch-evoked ATP release was measured, the strips were stretched to 50% of their length, directly after the 1 h equilibration period. Perfusate (150 μL for ATP measurement) was collected at 10 s after stretch. The same strips were then brought back to their original 1 g tension, and left for a further 1 h before a second stretch stimulus.

Electrical field stimulation (EFS) at 2.5–40 Hz, 0.1 ms pulse width, 40 V was also carried out for 30 s, using a Grass SD9 stimulator (West Warwick, RI, USA). The strips were continuously perfused with normal Krebs–Henseleit solution, and perfusate (150 μL) was collected for ATP measurement 10 and 30 s after onset of EFS.

Inhibitors were used to characterize the responses to acid (pH 6.5 and 5.6), capsaicin (0.1–10 μM) and EFS (2.5–40 Hz). In these experiments, paired adjacent strips were used for the control response and inhibitor-incubated response. The strips were perfused with inhibitors in Krebs–Henseleit for 30 min before agonist stimulation. The antagonists used were capsazepine (1 and 10 μM), amiloride (0.3 μM) and gadolinium (100 μM). Tetrodotoxin (TTX, 1 μM) was used to characterize responses of strips subjected to EFS.

ATP measurement

ATP in the perfusate was measured using the bioluminescence assay kit (Sigma Catalogue: FLAA, Sydney, Australia) by a T20/20n luminometer (Turner BioSystems, Sunnyvale, CA, USA). A standard curve (1 μM–10 pM ATP) was prepared, and the luminescence for each sample perfusate was measured. Changes in pH (range 4.0–7.4) did not affect the luminescence.

The blank reading (normal Krebs–Henseleit solution, pH 7.4) was subtracted from the luminescence reading of each sample, and the standard curve was plotted using GraphPad Prism 3.0 software (San Diego, CA, USA). ATP released from each strip was then calculated relative to the standard curve,

Table 1 Primer sequences used for gene expression study by RT–PCR

Protein	*Gene*	*Primer sequence*	*Fragment size (bp)*	*GenBank accession no.*
ASIC1	rAccn2	S: 5′-ggtcttcactcggtatggga-3′	366	NM_024154
		A: 5′-caggcagtgatgctgtagga-3′		
		SN:5′-gagagaccgacgagacatcc-3′	223	
ASIC2 (version 1)	rAccn1-V1	S: 5′-tccctgagtcgcactaaattg-3′	238	NM_012892
		A: 5′-tgttgttgcacacggtgac-3′		
		AN:5′-ggaagctgagccagtagagc-3′	166	
ASIC2 (version 2)	rAccn1-V2	S: 5′-cgccaatacctccactctcc-3′	223	NM_001034014
		A: 5′-gaggttgcagagggtcacag-3′		
		AN: 5′-catccaccttggtaacatgc-3′	172	
ASIC3	rAccn3	S: 5′-ttcgctactatggggagttc-3′	309	NM_173135
		A: 5′-ggtatcggcaatccaacaac-3′		
		SN: 5′-ttcccagctgtgactctgtg-3′	244	
TRPV1	Trpv1	S: 5′-gaggctctatgatcgcagga-3′	189	NM_031982
		A: 5′-gtgtcattctgcccattgtg-3′		
		SN:5′-agtaactgccaggagctgga-3′	146	
GAPDH	Gapdh	S: 5′-gtcggtgtgaacggatttg-3′	218	NM_017008
		A: 5′-tggaagatggtgatgggttt-3′		

S, sense primer; A, antisense primer; SN, sense primer for nesting; AN, antisense primer for nesting.

and expressed as nmol per weight (g) of strip. For all data sets except stretch, basal release was subtracted from evoked release. Unless otherwise stated, n = number of rats.

Data analysis

Statistical analysis was performed using GraphPad Prism version 3.0 software. The ATP release data were not normally distributed, and the median with the interquartile range (IQR) was determined for each data set. The Wilcoxon *t*-test was used for paired groups, the Kruskal–Wallis test was used for three or more unpaired groups and the Friedman test was used for three or more paired groups.

Histological studies

After the organ bath procedure, tissue strips were fixed in 10% formalin for histological staining. Briefly, sections were brought down to distilled water, then stained with haematoxylin for 10 min. They were then washed in tap water for 5 min until the sections appeared blue, stained with 1% eosin for 1–2 min, quickly washed with distilled water and then dehydrated with increasing grades of ethanol.

Molecular studies

Total RNA was extracted from rat detrusor, mucosa and intact bladder, as well as dorsal root ganglia (DRG, positive control) using an RNA purification kit (Trizol, Invitrogen, San Diego, CA, USA), and contaminating DNA was removed by a DNase treatment (Burcher *et al*., 2008). Total RNA (2 μg) was reverse transcribed to single-strand cDNA using SuperScriptase III along with random hexamer primers as per manufacturer's instruction. To assess the quality of RNA, expression of the housekeeping gene GAPDH in each sample was verified by PCR amplification of single-strand cDNA using the Access RT–PCR System (Promega, Madison, WI, USA).

Expression of ASIC and TRPV1 receptor subtype transcripts was determined by semi-nested PCR amplification and subtype gene-specific primers. A first-round PCR reaction was performed with 500 nM single-strand cDNA and a pair of sense and antisense primers (Table 1). Products of this first reaction were diluted 10-fold and used as templates for semi-nested PCR amplification using the same sense/antisense primer and a nested sense/antisense primer (Table 1). A standard 25 μL PCR mixture contained 1 μL cDNA (~500 nM), 0.4 μM primers, 0.4 mM dNTPs, 2.5 mM $MgSO_4$ and 2.5 U *Tfl* DNA polymerase. The RT–PCR reaction was conducted at 94°C for 2 min, 30 cycles of 94°C for 30 s, 55°C for 1 min and 70°C for 1 min, followed by a final extension at 70°C for 10 min. PCR products were subjected to electrophoresis on 2% agarose gels containing ethidium bromide, and visualized by Gel Doc XR System (Bio-Rad, Hercules, CA, USA).

Materials

Capsaicin was dissolved in Tween 80, then ethanol, then diluted in 0.9% saline (10:10:80) to give a stock concentration of 10 mM capsaicin. Stock concentrations of capsaicin were then diluted in Krebs. This vehicle, diluted appropriately in Krebs to parallel the capsaicin concentrations used in the study, did not cause significant ATP release, compared with basal. Capsazepine was dissolved in ethanol and distilled water (50:50). Individual aliquots were dissolved in Krebs before use in the experiments. All vehicles, at working concentrations, did not affect luminescence.

Amiloride, capsaicin, capsazepine, gadolinium, TTX and the ATP bioluminescence assay kit were purchased from Sigma-Aldrich Chemical Company (Sydney, Australia). Molecular reagents were from Invitrogen (Mount Waverley, Vic, Australia), except for the Access RT–PCR System which was purchased from Promega (Sydney, Australia). All other reagents were of high analytical grade.

Results

Response to stretch

The median basal ATP release from rat bladder mucosa was 3.46 (IQR 0.25–12.31, $n = 94$ strips) nmol·g^{-1} strip weight. Rat

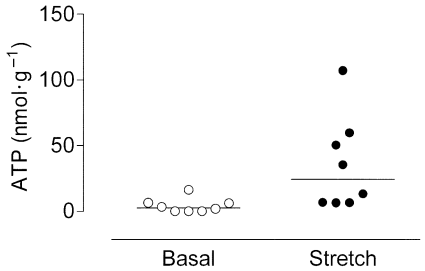

Figure 1 ATP was measured at rest (basal) and then after stretch (to 150% of strip length). Stretch increased release of ATP significantly over the corresponding basal ATP release ($P = 0.0078$; $n = 8$). In this and subsequent figures, the horizontal line shows the median, and paired data were compared using the Wilcoxon test.

Table 2 Summary of ATP release from the bladder mucosa

Stimulus	*ATP release (nmol·g⁻¹ strip weight)*	*Number of strips*
Basal	3.46 (0.25–12.3)	94
Stretch	18.0 (6.73–46.0)	8
Acid (pH 6.5–6.1)	290 (127–485)	33
Acid (pH 6.0–5.6)	286 (103–555)	33
Acid (pH 5.5–5.0)	542 (366–665)	12
Capsaicin (0.1 μM)	142 (86.9–281)	20
Capsaicin (1 μM)	188 (117–431)	20
Capsaicin (10 μM)	114 (74.5–338)	17
Nerve stimulation (10 Hz)	63.0 (13.3–96.4)	14

Values are expressed as median (interquartile range).

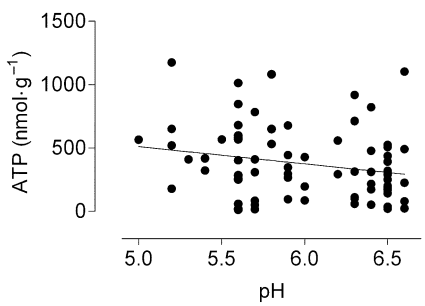

Figure 2 Net ATP release in response to varying pH. Each strip was exposed to three different solutions ranging from 6.6 to 5.0. ATP release was quite variable, but at all pH levels, it was significantly higher ($P < 0.01$; $n = 12$–33) than corresponding basal release (at pH 7.4). There was a significant correlation between pH and ATP release (Spearman's coefficient $r = -0.23$, $P = 0.0453$, $n = 33$). In this and subsequent figures, basal ATP release has been subtracted.

mucosal strips showed a sixfold significant increase in stretch-evoked ATP release (Figure 1, Table 2).

Response to acid

Rat mucosal strips responded to acid ranging from pH 6.6 to pH 5.0 (Figure 2), with ATP release being significantly higher than baseline ATP release (pH 7.4) at all pHs tested (Table 2). There was a small, but significant, pH-dependent increase in ATP release over this range (Figure 2).

To test for desensitization, repetitive acid stimuli (pH 6.6 to pH 5.0), applied in ascending or descending manner, did not significantly affect the level of ATP release ($n = 5$; data not shown). Furthermore, repeated exposure of the same strip to pH 6.6 resulted in ATP release of similar magnitude each time ($n = 3$; data not shown).

Response to acid: effect of antagonists

In initial experiments, 1 μM capsazepine had no significant effect on acid-evoked ATP release (data not shown). However, 10 μM capsazepine significantly reduced the ATP release in response to acid pH 6.5, to 28.9% of control (Figure 3A, $P = 0.0078$) and at pH 5.6, to 32.9% of control (Figure 3B, $P = 0.0078$).

To identify whether the acid-evoked release was mediated by acid receptors, two ASIC blockers, amiloride and gadolinium, were also tested (Figure 3C–F). Gadolinium (100 μM) significantly decreased the acid-evoked ATP release to 40.3% ($P = 0.0134$) at pH 6.5. Although amiloride (0.3 μM) appeared to reduce ATP release at pH 6.5 and 5.6, the result was statistically significant only at pH 6.5 (reduced to 14.4% of control, $P = 0.047$).

There was no change in basal ATP release after the 30 min pre-incubation with capsazepine, gadolinium or amiloride.

Response to capsaicin

Capsaicin produced a significant increase in ATP release from mucosal strips compared with basal level at all three concentrations tested (Figure 4A). However, there was no concentration difference in ATP release (Table 2). Subsequent exposures to the same concentration of capsaicin did not appear to cause desensitization.

In initial experiments, 1 μM capsazepine had no significant effect on ATP release in response to capsaicin (data not shown). However, responses to 0.1, 1 (not shown) and 10 μM capsaicin (Figure 4B) were almost abolished by 10 μM capsazepine. There was no change in baseline ATP release after the 30 min pre-incubation with capsazepine.

Response to EFS

EFS resulted in a frequency-dependent increase in ATP release from mucosal strips (Figure 5A). There was some variability in ATP release at higher frequencies, although release was significantly higher than basal at all frequencies. TTX incubation had no significant effect from 2.5 to 10 Hz; however, it reduced ATP release to 75 and 57% of control value at both 20 and 40 Hz (Figure 5B).

Involvement of the urothelium in ATP release

To assess whether the ATP was of urothelial origin, strips with and without urothelium were stimulated with acid, as low pH appeared to be the strongest stimulus tested thus far. At pH 6.5 ($n = 6$), ATP released from denuded strips was only 16% of that released from matched strips with intact urothelium (Figure 6A). Histological staining (Figure 6B,C) was performed on the strips after the microbath procedure to verify the success of urothelium removal.

Responses in detrusor strips

Basal release from rat detrusor strips (minus mucosa) was 4.35 (0–13.0) nmol·g^{-1}. These strips showed no significant change

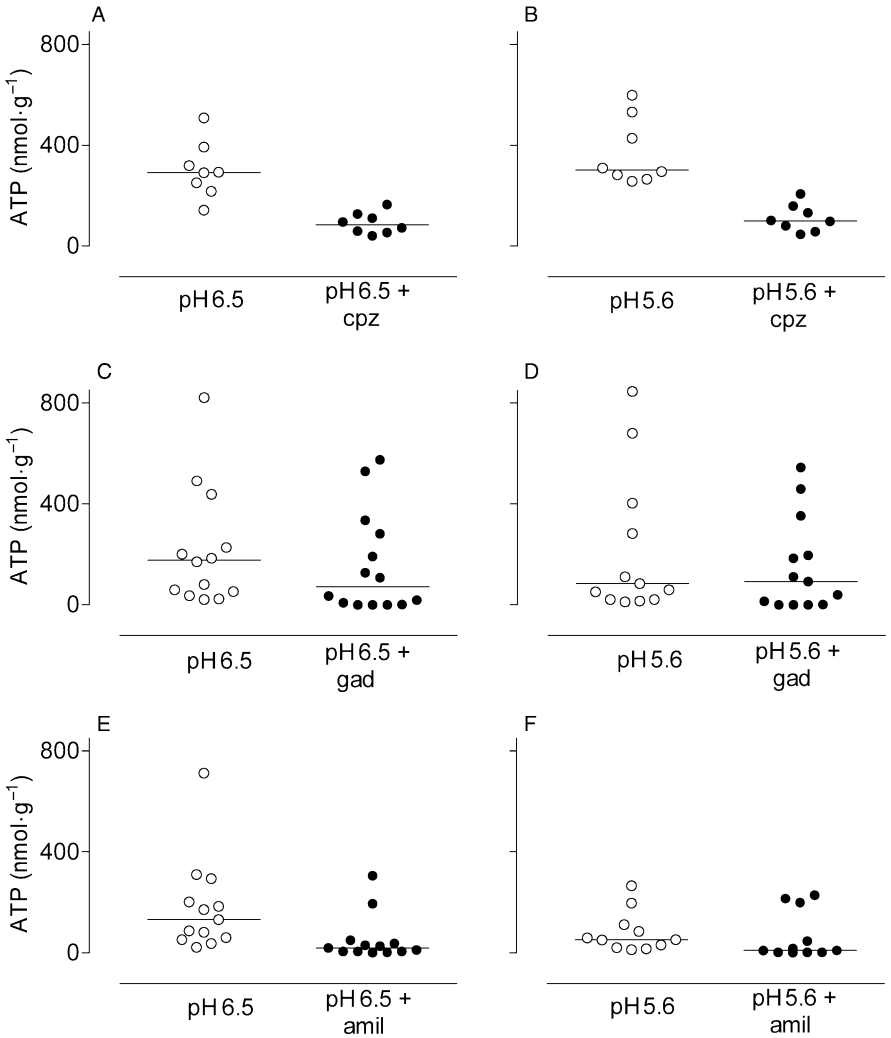

Figure 3 Effect of antagonists on acid-induced ATP release from rat mucosal strips. Paired mucosal strips were studied in the absence/presence of (A,B) 10 μM capsazepine (cpz; $n = 8$), (C,D) 100 μM gadolinium (gad; $n = 13$–14) and (E,F) 0.3 μM amiloride (amil; $n = 12$–13) incubation. The acid-evoked release was significantly reduced by capsazepine ($P = 0.0078$ at pH 6.5 and 5.6) and gadolinium (pH 6.5, $P = 0.0134$). The effects of amiloride were statistically significant only at pH 6.5 ($P = 0.047$).

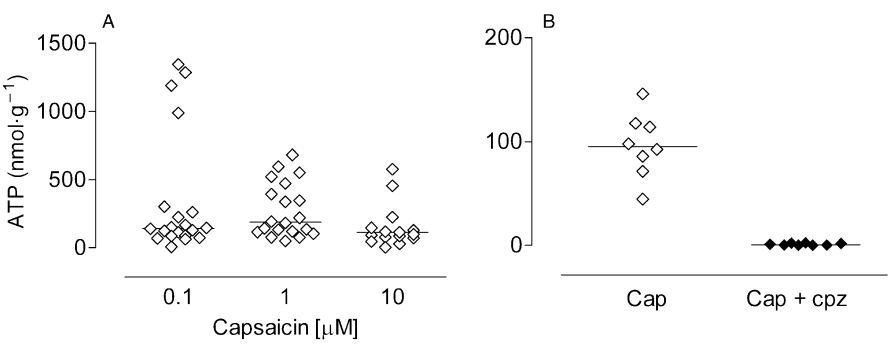

Figure 4 (A) ATP release in response to capsaicin. Each strip was exposed to three different concentrations. There was a significant increase in ATP release from basal levels at all capsaicin concentrations ($P < 0.001$; $n = 17$–18). There was no difference in ATP release between concentrations ($P = 0.354$; Kruskal–Wallis test). (B) Paired experiments showing the pronounced inhibitory effect of capsazepine 10 μM (cpz) on responses to capsaicin (Cap) at 10 μM ($n = 8$; $P = 0.0078$).

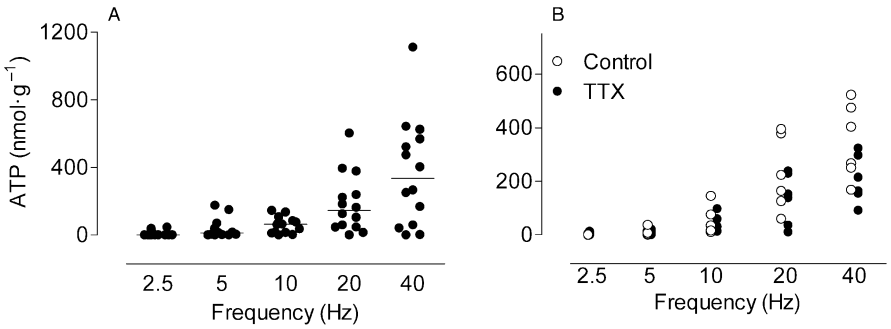

Figure 5 Each strip was exposed to increasing frequencies of electrical field stimulation from 2.5 to 40 Hz. (A) ATP release was frequency dependent ($n = 14$; $P < 0.0001$, Friedman's test). (B) ATP release in the presence of tetrodotoxin (TTX) ($n = 6$) was significantly reduced only at 20 and 40 Hz ($P = 0.031$; Wilcoxon test).

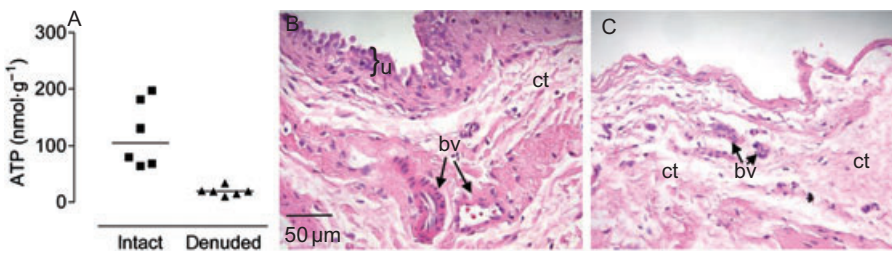

Figure 6 (A) ATP released by acid pH 6.5 from intact mucosal strips was substantially greater than from those with urothelium removed (denuded; $n = 6$, $P = 0.031$, Wilcoxon test). (B,C) H&E staining of rat mucosal strips after an organ bath procedure. (B) Strip with intact urothelium. (C) Strip with urothelium removed. bv, blood vessel; ct, connective tissue; u, urothelium.

in ATP release in response to stretch. Detrusor strips contracted (79 g·g^{-1} strip weight, $n = 6$) in response to capsaicin at 1 µM, but showed no significant ATP release in response to capsaicin. Likewise, detrusor strips showed no significant ATP release in response to EFS (40 Hz), although their mean contractile response to EFS was 24 g·g^{-1} strip weight ($n = 7$).

Molecular studies

Using a single round of RT–PCR, expression of gene transcripts of ASIC 1, ASIC 2 (version 1), ASIC2 (version 2), ASIC 3 and TRPV1 could only be detected in DRG (positive control). Semi-nested RT–PCR amplification of mRNA was used to increase sensitivity and specificity in intact, detrusor and mucosal bladder tissue. Following nesting, all genes of interest were expressed in rat bladder mucosa and detrusor ($n = 4$) (Figure 7). There was some variability in the expression of ASIC2 (version 2) between individual rat bladders. For TRPV1, two PCR product bands at 146 bp and approximately 220 bp were observed in all bladder samples. The smaller fragment matched with the rat TRPV1 mRNA sequence (GenBank accession no. NM_031982), which was also detected in the DRG. The larger fragment with much weaker intensity matched with the rat TRPV1 alternatively spliced variant (accession no. AF327067), and was negligible in the DRG. This spliced variant may represent functional molecules exclusive to the bladder.

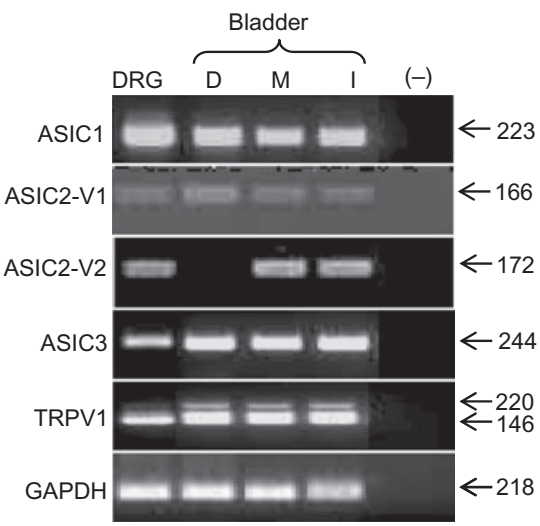

Figure 7 Expression of ASIC1, ASIC2 (version 1), ASIC2 (version 2), ASIC 3 and transient receptor potential vanilloid receptor 1 (TRPV1) was found on dorsal root ganglia (DRG), detrusor (D), mucosal (M) and intact bladder (I) homogenates. ASIC2 (version 1) showed weaker expression, while ASIC2 (version 2) displayed some variability between individual rats. TRPV1 expression was found in one band (146 bp) in DRG, and in two distinct bands (146, 220 bp) in detrusor, mucosal and intact tissue.

Discussion

Urothelial cells are currently an important focus of bladder research and express several receptor types (Birder, 2006). In these experiments, we investigated TRPV1 (discussed below) and the ASIC receptor system (see Wemmie *et al.*, 2006; Lingueglia 2007). The microbath was a suitable apparatus for quantifying ATP release. Our novel findings show that acidified Krebs (pH 5.0–6.6) was a potent stimulus for ATP release

Table 3 Brief summary of acid-sensing ion channels (ASICs) characteristics[a]

Isoform	*pH range for activation*	*Blockers*
ASIC1a	6.2–6.8	Amiloride (0.3 μM), NSAIDs, psalmotoxin (30 nM)
ASIC1b	5.1–6.2	Amiloride (0.3 μM)
ASIC2a	4.1–5.0	Amiloride (0.3 μM), gadolinium (100 μM)
ASIC2b	n/a (modulates ASIC2a and 3)	n/a
ASIC3	6.2–6.7	Amiloride (0.3 μM), gadolinium (100 μM), NSAIDs

[a]Table modified from Chen *et al.* (2005), Faisy *et al.* (2007), Lingueglia (2007) and Alexander *et al.* (2008).

from the rat bladder mucosa. The release appears to be mediated via both TRPV1 and ASIC receptors, as responses were inhibited by the TRPV1 antagonist, capsazepine inhibited responses at a range of pH, whereas gadolinium and amiloride were effective only at the higher pH (6.5). Although gadolinium and amiloride are known to interact with ion channels other than ASICs, the concentrations used here (taken from Faisy *et al.*, 2007; Holzer, 2007) were orders of magnitude lower than those required for diuretic action, or to inhibit stretch-activated channels (Du *et al.*, 2007).

Several different ASICs have been described, sensitive to varying pH levels (Table 3; Lingueglia 2007). It is likely that cells co-express several ASIC subunits (Benson *et al.*, 2002), and therefore responses from our experiments may represent a combination from more than one subunit. A very recent report has demonstrated that acetic acid can activate biphasic currents in cultured rat urothelial cells, which are blocked by 10 μM amiloride (Kullmann *et al.*, 2009). ASIC1a, ASIC1b and ASIC3 operate in the pH range of our study (Table 3). Our molecular data confirmed the presence of ASIC1, ASIC2 (version 1), ASIC2 (version 2), ASIC3, as well as TRPV1 receptors in the rat DRG, detrusor and mucosal tissue. The weak bands for all ASIC receptors, which became more apparent after semi-nested PCR amplification, suggest a low level of expression as compared to the DRG and also compared to the housekeeping gene, GAPDH.

Capsaicin is known to induce ATP release from rat cultured urothelial cells (Birder *et al.*, 2002). Here, we have shown that capsaicin can also induce ATP release from rat bladder mucosal strips. The responses were abolished by capsazepine, a somewhat non-selective antagonist of TRPV1. TRPV channels are important for mucosal ATP release via stretch (Birder *et al.*, 2002; Gevaert *et al.*, 2007). In particular, the TRPV1 channel is associated with normal bladder sensation of fullness and is thus indirectly contributes to bladder contraction.

Acid in the pH range used here was a more potent stimulus than capsaicin. Although acid-induced release was significantly pH dependent, responses to capsaicin were not concentration dependent. Neither stimulus was subject to desensitization, in agreement with recent studies on capsaicin in cultured urothelial cells (Birder *et al.*, 2003; Kullmann *et al.*, 2009). It is noteworthy that our molecular studies showed two TRPV1 PCR bands in the detrusor and mucosa, but only one band was seen in DRG. It is probable that the more abundant TRPV1 transcript represents the 'neural' isoform, whereas the splicing variant may be the urothelial-specific isoform, which was not desensitized by capsaicin. This is further evidence for a difference in TRPV1 receptor function between afferent fibres and urothelial cells.

Stretch is an established stimulus for ATP release in bladder strips from several species including human and pig (Kumar *et al.*, 2004), rabbit (Ferguson *et al.*, 1997) and guinea pig (Zagorodnyuk *et al.*, 2007), although there is little data from the rat. Our findings show that acid and capsaicin were the most effective stimuli for ATP release in the normal rat bladder mucosa (Table 2). Notably, in the present study using rat mucosal strips, stretch produced only a marginally higher response than basal release. Behavioural differences between species may explain our finding with stretch. For example, a recent study on rat voiding behaviour found that abdominal pressure contraction is necessary for complete bladder emptying in the rat (Smith *et al.*, 2007).

The mucosal strips used in our study contain multiple cell types. After removal of urothelium from mucosal strips, acid-evoked ATP was greatly reduced, suggesting that ATP is likely to originate from urothelial cells. There is also a possibility that some suburothelial structures might also have been removed during 'urothelial' denudation. Thus, a component of the acid-evoked ATP might be released from other cell types within the mucosa (e.g. suburothelial nerves and/or myofibroblasts). ATP release, due to EFS, is known to be partly neuronal (Yoshida *et al.*, 2004). In our experiments, we verified that ATP can be released by EFS. EFS-evoked ATP release was frequency dependent, and unaffected by TTX at frequencies up to 10 Hz. This may indicate non-neuronal sources of ATP, although we cannot exclude the involvement of TTX-insensitive Na^+ channels. At higher frequencies (20 and 40 Hz), TTX reduced, but did not abolish the EFS-induced ATP release, although afferent discharge at such frequencies is unlikely to be achieved *in vivo* (Häbler *et al.*, 1993).

ASICs in the urothelial cells may be involved in bladder sensation of fullness, and implicated in the pathophysiology of bladder dysfunctions. Further clarification of the roles of individual ASIC subunits involved would require the use of highly specific antagonists, when available. In addition, studies to localize the cell types expressing ASIC receptors would be extremely valuable. However, there are limitations in the specificity and reliability of many receptor antibodies. A recent paper showed that three commercially available TRPV1 antibodies resulted in unspecific staining on the urothelium (Everaerts *et al.*, 2009).

Our findings have important implications for bladder function and pathophysiology. ATP administered intravesically has been demonstrated to induce bladder hyperactivity in conscious rats (Pandita and Andersson, 2002). In humans, urine pH ranges from approximately pH 5 to 8. Systematic studies of urine pH and bladder functions do not appear to exist, although it has been observed that lower urine pH is associated with female bladder dysfunctions (K.H. Moore, unpubl. data). Conversely, alkaline solutions, such as sodium bicarbonate, appear to increase bladder capacity (Sethia and Smith, 1987), and alkalinized lignocaine can relieve symptoms of painful bladder/interstitial cystitis (Nickel *et al.*, 2009).

In conclusion, this study provides compelling evidence for acid as an important stimulus in modulating bladder

function. This is the first study to make a comparative analysis of stretch, capsaicin and acid (a completely novel stimulus in the bladder), in strips of rat bladder mucosa. While stretch was weak in the rat, acid (at physiological pH) was the most potent stimulus tested, and appears to be acting via both TRPV1 and ASIC receptors, whose transcripts were demonstrated in the bladder mucosa and detrusor. Acid of varying pH may be acting on different ASIC subunits in the bladder mucosa, causing ATP release and modulating bladder tone and contractility. Thus, release of ATP, a key signalling molecule, is governed not only by distension, but by several other stimuli, including low pH. Given the acidity of urine, this may have important implications in basic bladder physiology. With the focus of current bladder research shifting towards the sensory pathways, the ASIC receptor system offers new prospects in bladder research and therapeutics.

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