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Inhibition of stretching-evoked ATP release from bladder mucosa by anticholinergic agents

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OBJECTIVE

- To determine whether muscarinic receptor antagonism affects stretching-induced release of ATP.

MATERIALS AND METHODS

- Mucosal strips, dissected from guinea pig (male, 450g; $n = 10$) urinary bladders, were placed in horizontal organ baths and superfused with Ca^{2+}-free Tyrode's solution.
- Superfusate samples were taken pre- and post- intervention (rapid stretching or relaxation) and ATP concentration was quantified using a luciferin–luciferase assay.
- The effect of muscarinic acetylcholine receptor antagonism on ATP release was assessed by addition of methoctramine (1 μM) and 4-DAMP (10 nM).

RESULTS

- Rapid stretching (0 to 13.3 ± 1.2 mN; no. strips = 20) increased ATP in the superfusate to a median threefold increase over basal levels.
- After a period of equilibration, tension in the mucosal strips relaxed until it had reached a new steady-state after 60 min and stretching was repeated. In the presence of 4-DAMP (10 nM) or methoctramine (1 μM), ATP concentrations after stretching reduced to 61% or 20%, respectively. By contrast, ATP concentrations in mucosa-matched controls, perfused with vehicle, increased in response to stretching by 391% and 1500%, respectively.
- Rapid relaxation also stimulated ATP release. This release did not appear to be sensitive to 4-DAMP or methoctramine.

CONCLUSIONS

- An alteration of resting mucosal tension is the key determinant of ATP release, as ATP is released from the mucosa in response to both stretching and relaxation.
- Muscarinic receptor antagonism inhibits stretching-evoked ATP release from bladder mucosa, suggesting that anticholinergic agents used to treat human lower urinary tract pathologies act on urothelial muscarinic receptors.

What's known on the subject? and What does the study add?
ATP and acetylcholine (ACh) are released from the urothelium or from cultured urothelial cells in response to mechanical stretching or perturbations that mimic stretching, such as exposure to hypotonic solutions. Limited evidence suggests that ATP and ACh may interact, as the muscarinic agonist carbachol evokes ATP release from cultured urothelial cells. Our aim was to determine whether antimuscarinics, shown previously to reduce sensory nerve firing during bladder filling, might function by reducing mucosal ATP release.

By stretching mucosa strips and measuring superfusate ATP using a luciferin–luciferase assay, we showed that muscarinic receptor antagonism (4-DAMP, 10 nM or methoctramine, 1 μM) inhibits stretching-evoked ATP release, suggesting anticholinergic agents used to treat human lower urinary tract pathologies act on urothelial muscarinic receptors. We also showed that alteration of resting mucosal tension is the key determinant of ATP release, as ATP is released from the mucosa in response to relaxation, as well to stretching.

KEYWORDS

bladder, urothelium, mucosa, stretching, ATP, anticholinergics

INTRODUCTION

Stress-evoked release of ATP from the basolateral surface of the bladder wall was initially shown by a change in transmural pressure [1]. Subsequently, ATP release has been measured upon lateral stretch of mucosal strips or urothelial cell cultures [2–4], as well as exposure of urothelial cells to hypotonic solutions to cell swelling [5,6]. ATP release is greater in cells from overactive bladders [2,3,7], and reduced after botulinum toxin treatment [8]. Furthermore, exposure to ATP itself promotes release, suggesting an autocatalytic process [6]. It has been proposed that this ATP pool eventually activates adjacent afferent nerves, thus providing a sensory mechanism for relaying information about bladder volume [9,10]. This hypothesis is supported by a reduction of afferent nerve firing on bladder filling in P2X3 knockout mice [11]. A number of key issues remain unknown, however, including the processes that regulate stress-induced ATP release, as well as the cellular pathways that mediate ATP release. An involvement of cholinergic pathways is possible as acetylcholine (ACh) is also released from the bladder wall on stretching [12] and from cultured urothelial cells

exposed to a hypotonic solution [13]. Anticholinergic agents, used clinically to treat the overactive bladder, are effective in the filling phase of the micturition cycle as they increase bladder capacity and decrease sensations of urgency [14,15]. The non-subtype-specific anticholinergic oxybutynin and M3-specific anticholinergic darifenacin reduce afferent firing during bladder filling [16,17]. This may be through direct desensitization of afferents, but an alternative hypothesis is that these drugs reduce the release of ATP from mucosa (urothelium and sub-urothelium) when the tissue is stressed. This hypothesis is supported by two lines of evidence: (1) muscarinic receptor activation by non-subtype-specific agonists increased intracellular calcium and evoked ATP release in cultured rat urothelial cells [18]; and (2) intravesical instillation of a non-subtype-specific agonist increased voiding frequency in an *in vivo* rat preparation [19]. To bridge the gap between isolated cells and whole animal, we measured stretching-induced ATP release from mucosal preparations in the absence and presence of two muscarinic receptor antagonists that have some selectivity for M3 or M2 subtypes.

MATERIALS AND METHODS

PREPARATION OF SAMPLES

Male guinea pigs (Dunkin-Hartley; approx. 450 g) were killed by cervical dislocation followed by exsanguination. All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and European Communities Council Directive 86/09/EEC.

The urinary bladder was excised and placed in gassed (95% O_2 and 5% CO_2) and chilled (4 °C) Tyrode's solution (composition, mM: NaCl 114, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.7), with no added Ca. The ventral wall was opened longitudinally and the mucosa removed by careful sharp dissection using iris scissors, taking care not to cause stretching or damage. Two strips (6–8 mm long and 2–3 mm wide) were cut along the craniocaudal axis.

STRETCHING OF MUCOSA STRIPS

Strips were tied at either end using fine silk suture (7/0; Pearsalls, Taunton, UK) and then transferred to a horizontal organ bath, where one end was tied to a fixed hook and the other to the arm of an isometric tension transducer (FT03D; Grass Instruments, Quincy, MA, USA) connected to a bridge amplifier (TBM-4M; WPI, Aston, UK). Preparations were continuously superfused (10 mL/min) with fresh, gassed Ca^{2+}-free Tyrode's solution and allowed to equilibrate at a slack length (0 mN tension) for 1 h before each experiment. After equilibration, preparations were stretched to a constant tension over a period of <5 s by driving the micromanipulator arm holding the force transducer. Preparations equilibrated for a further 1 h during which time the tension relaxed to near pre-stretching values. The strips were then superfused with 4-DAMP (10 nM), methoctramine (1 μM) or vehicle (0.1% DMSO or 0.1% water, respectively) for 20 min, after which preparations were again stretched to a similar tension. After a further 30 min of superfusion, preparations were rapidly relaxed by cutting one of the suture ties holding the tissue.

SAMPLING OF SUPERFUSATE

Samples (100 μL) were taken at fixed timepoints during the above protocol, immediately before and after mechanical interventions (stretching, relaxation) or pharmacological interventions (4-DAMP, methoctramine or vehicle control), as well as at regular timepoints in between. Samples of superfusate were taken 0.5 cm downstream from the arm of the tension transducer and immediately frozen on dry ice for later assay. The length of strips was measured before and after the strips were stretched and held under tension. The mean (SEM; range) dry weight of the strips at the end of each experiment was 10.2 (0.7; 4.7–15.7) mg; $n_s = 20$.

MEASUREMENT OF ATP CONCENTRATION USING LUCIFERIN–LUCIFERASE ASSAY

ATP in the superfusate was measured using a luciferin–luciferase assay (FL-AAM; Sigma-Aldrich, Poole, UK) and a luminometer (GloMax 20/20; Promega, Southampton, UK). The relationship between ATP concentration over the range 0.1 pM–100 nM and luminescence was linear on a log–log plot. A blank reading (without superfusate) was subtracted from the luminescence reading of each sample. There was no significant difference in the ATP concentration vs luminescence relationships if the ATP was dissolved in distilled water or in gassed Ca^{2+}-free Tyrode's solution. The ATP concentration of each sample was calculated relative to the standard curve and expressed as pmol/g dry weight of tissue.

STATISTICAL ANALYSIS

n_a refers to the number of guinea pigs and n_s the number of mucosal strips. To calculate the rate of change in tension over time, traces were integrated, taking the minimum value as baseline, using Chart software (v.5 ADInstruments, UK). ATP data are presented as median (25,75% interquartiles), other data as mean (SEM). Differences between mean values were tested for statistical significance using the Wilcoxon or Student's *t*-test, as appropriate; the null hypothesis was rejected at $P < 0.05$.

RESULTS

STRETCHING-EVOKED RELEASE OF ATP FROM MUCOSA STRIPS

The stretching protocol was validated before any pharmacological interventions on stretching-evoked ATP release were made. After 60 min of equilibration, unloaded strips (0 mN) were rapidly stretched to generate similar resting tensions (13.3 ± 1.2 mN; $n_s = 20$, $n_a = 10$). This significantly increased ATP concentration in the superfusate, from a median of 1.63 [0.05, 2.40] to 6.60 [2.16, 24.74] pmol/g, i.e. to a median of 404% of basal levels (Fig. 1A). The ATP concentration was measured ≈5 s after the stretch, as preliminary experiments showed that at this time the rise in ATP was at its maximum. To achieve the relatively limited range of tensions, preparations were stretched from a mean (SEM; range) initial length of 7.7 (0.5; 6–10) mm to 13.1 (1.1; 8–20) mm; a mean (range) increase in length of 71 (22–122)%; $n_s = 20$, $n_a = 10$. After the initial stretching, tension in the mucosal strips relaxed until it had reached a new steady-state after 60 min. At this time the mean (SEM) tension had reduced to 1.03 (0.23) mN and median ATP concentration was reduced to 2.17 (0.24, 3.8) pmol/g, i.e. not significantly different from pre-stretching values (Fig. 1B).

The magnitude of the increase in ATP concentration generated by stretching was not influenced by the initial ATP sampled at the unloaded (0 mN) state ($r = –0.25$), the

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*FIG. 1. **A**, stretching-evoked release of ATP. Guinea pig mucosa strips were rapidly stretched (<5s; 0 to 13.3 ± 1.2 mN) and the superfusate ATP measured. **B**, After stretching, mucosal strips relaxed to a steady-state tension after 60 min (left) and ATP concentration was measured following passive relaxation (right). $n_s = 20$, $n_a = 10$.*

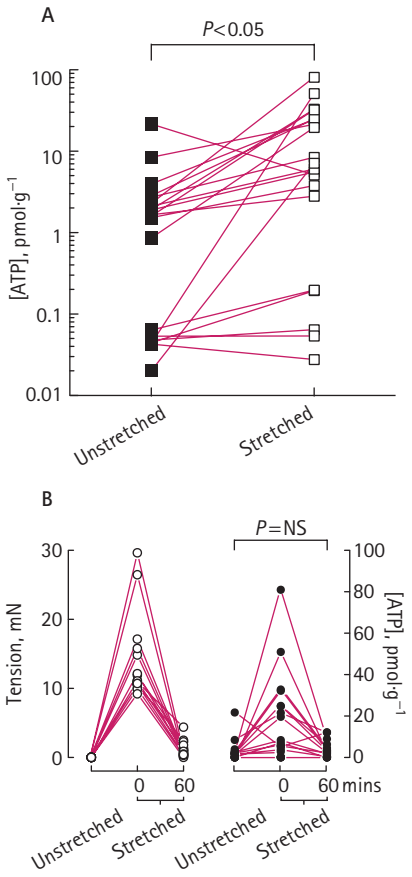
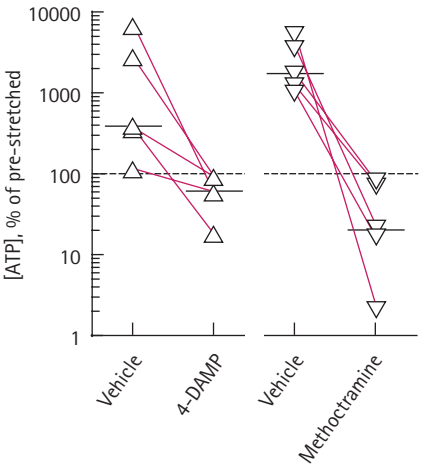


FIG. 2. Effect of 4-DAMP or methoctramine on stretching-evoked ATP release. Mucosa strips were superfused for 20 min with 4-DAMP (10 nM) or methoctramine (1 μM), rapidly stretched and ATP content sampled. Values are relative to superfusate ATP immediately before the rapid stretching (dashed line), i.e. in the presence of the intervention/vehicle. Lines connecting data points denote strips from the same bladder. The short horizontal line for each group denotes median ATP concentration. $n_s = 20$, $n_a = 10$.



change in length of the strip required to generate tension ($r = -0.14$), the change of strip length normalized to its initial length ($r = -0.15$) or the mean (range) weight of the animal (464 [422–489] g; $n_a = 10$; $r = 0.22$). The latter data were included because animal weight was positively associated with animal age. Stretching-evoked ATP release did not correlate with the relatively limited range of tension changes ($r = -0.02$) nor the integral ($r = -0.02$) of the change in tension.

THE EFFECT OF MUSCARINIC RECEPTOR ANTAGONISM ON ATP RELEASE FROM MUCOSA

The superfusate used to bathe the mucosa strips was changed to a solution containing either 4-DAMP (10 nM), methoctramine (1 μM) or vehicle. The mechanical effect of this switch generated a transient increase in ATP concentration. For example, with superfusate containing vehicle alone, median ATP increased from 3.5 (3.3, 4.6) pmol/g to a maximum of 20.5 (18.4, 559.1) pmol/g after 5 min ($n_s = 5$). For superfusate containing 4-DAMP median ATP concentration transiently increased from 6.3 (2.0, 12.0) pmol/g to a maximum of 43.0 (23.7, 147.7) pmol/g after 5 min ($n_s = 5$). Similarly, switching to superfusate containing methoctramine transiently increased the median ATP concentration from 1.7 (0.05, 2.4) pmol/g to a maximum of 6.2 (4.3, 16.9) pmol/g after 5 min ($n_s = 5$). The magnitude of the increase (to 585%, 682% and 373% pre-intervention, respectively) was similar in each case. After 20-min superfusion of drug or vehicle, ATP returned to a level not significantly different to that before the superfusate change. The strips were therefore ready to stretch again and tension in the preparations was once more increased to previous values using the same stretching protocol.

4-DAMP VS VEHICLE

With mucosal strips superfused by vehicle, stretching increased median ATP concentration from 4.7 (2.1, 7.8) to 27.8 (20.1, 34.9) pmol/g, which represents a median increase to 391% of basal levels ($P < 0.05$; $n_s = 5$, $n_a = 5$). The percentage increase in ATP was similar to that at the beginning of the experiments and shows that the stretching protocol and the quantity of ATP release were consistent. In the presence of 10 nM 4-DAMP, the stretching-induced increase in ATP was abolished and the concentration was actually significantly reduced to 61% of pre-stretching levels ($P < 0.05$; $n_s = 5$, $n_a = 5$; Fig. 2).

METHOCTRAMINE VS VEHICLE

A similar finding was recorded in the presence of 1 μM methoctramine. In the presence of vehicle alone, stretching increased ATP to 1750% of pre-stretching levels. Methoctramine abolished the stretching-induced increase of ATP and reduced ATP to 20% of control ($P < 0.05$; $n_s = 5$, $n_a = 5$; Fig. 2).

RAPID RELAXATION AND ATP RELEASE

Thirty minutes after the final stretching, in the presence or absence of an anticholinergic agent, the suture holding the mucosa strip to the force transducer arm was cut and tension reduced rapidly to zero. In all preparations there was a large increase in ATP concentration immediately after tension reduction. Figure 3 shows the percentage increase in ATP concentration in the presence of vehicle, 4-DAMP or methoctramine. There was no difference in the percentage changes in any group.

RELATIONSHIP BETWEEN ATP AND SPONTANEOUS CONTRACTIONS OF MUCOSAL SHEETS

Mucosal sheets developed small spontaneous contractions that might distort the tissue sufficiently to release ATP. Figure 4 shows a sample experiment when ATP was sampled at regular intervals (every 60 s) and tension recorded simultaneously from a preparation that had been previously stretched to about 10 mN resting tension. During this time, three large and one smaller surges of ATP were recorded, but there was no strict temporal

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correlation between ATP and the larger spontaneous contractions.

DISCUSSION

We showed that stretching of guinea pig bladder mucosa strips induced about a threefold release of ATP, which was abolished by the muscarinic receptor antagonists 4-DAMP and methoctramine. Relaxation of the strips also released ATP, to a far greater extent than stretching. The mucosa strips were sensitive to mechanical disturbance, as bathing with superfusate also evoked some release of ATP, and this was taken into account in our experiments. As a result of the present study, we made three important observations, each of which is discussed below.

Firstly, we showed that alteration of resting tension is the key determinant of ATP release: (i) ATP concentration increases in response to rapid tension and (ii) returns to baseline concentrations after relaxation ≈1 h later. We chose to stretch mucosal strips to a consistent increase of resting tension rather than to a similar percentage of resting length [4,20]. The large range of length changes (122–222% of original) required to achieve consistent tension changes implies that the passive mechanical properties of the mucosa will influence stretching-induced ATP release. Thus, there was no correlation between percentage change in strip length and the amount of ATP released.

Secondly, rapid relaxation of mucosal strips also stimulated significant ATP release, suggesting that *change* in tension, rather than the magnitude of change, is at least as important to evoke ATP release. Application of negative pressure to isolated detrusor myocytes increased the opening probability of a Gd^{3+}-sensitive ion channel, but there was no indication of an 'off' response [21] and thus is different from the observations above with the mucosal preparations. However, urothelium has a range of stretching-dependent mechanisms that regulate ATP release, including epithelial Na channels (ENaC; [1,22,23]), Gd^{3+} sensitive pathways [24] and TRP channels [24,25]. The directional sensitivity and relative importance of these different routes remain to be evaluated.

Thirdly, we showed that muscarinic receptor antagonists methoctramine and 4-DAMP inhibit stretching-induced ATP release from guinea pig mucosa. These experiments build on previous findings that muscarinic agonists stimulate ATP release from urothelial cells [13] and increase voiding frequency in an anaesthetized rat [19], to complete a circle in showing that stretching-induced release of ATP from the mucosa is muscarinic receptor-dependent.

The concentrations used were chosen to demonstrate a proof-of-principle for an effect of anticholinergic agents on ATP release; each agent was used at a concentration at least tenfold greater than the pK_i value for the primary subtype and approximately equal to the pK_i for the secondary subtype, i.e. 4-DAMP was used at 10 nM with M3 and M2 pK_i values 9.3 and 8.1 and methoctramine was used at 1 μM with M2 and M3 pK_i values 7.7 and 6.0 [26,27]. Further experiments will be required to determine the subtype that exerts a more significant effect on ATP release.

Of interest was the observation that in several experiments ATP release in the presence of 4-DAMP or methoctramine was less than baseline levels and consistent with an endogenous release of ACh before stretching was induced. This finding is in disagreement with that of Kullmann *et al.* [19] in which intravesical instillation of atropine had no effect on voiding parameters in rats, suggesting inter-preparation or inter-species differences.

The present data add to the evidence that anticholinergic agents used to treat human lower urinary tract pathologies act on mucosal muscarinic receptors and are consistent with the clinical observations that these agents act more on overactive bladder symptoms associated with filling rather than voiding [15]. They also support the hypothesis that their action in reducing bladder sensations of bladder filling is not only on afferent desensitization [16,17], but also on limiting stress-induced ATP release itself.

In conclusion, M2/M3 muscarinic receptor antagonism inhibits stretching-evoked ATP release from bladder mucosa. This novel interaction of these drugs and ATP has important implications for the treatment of overactive bladder.

FIG. 3. Effect of 4-DAMP and methoctramine on relaxation-evoked ATP release. The suture holding the strip to the force transducer was cut, tension returned to zero and ATP content was sampled. Values are relative to superfusate ATP immediately before the intervention. Lines connecting data points denote strips from the same bladder. The short horizontal line for each group denotes median ATP concentration. $n_s = 20$, $n_a = 10$.

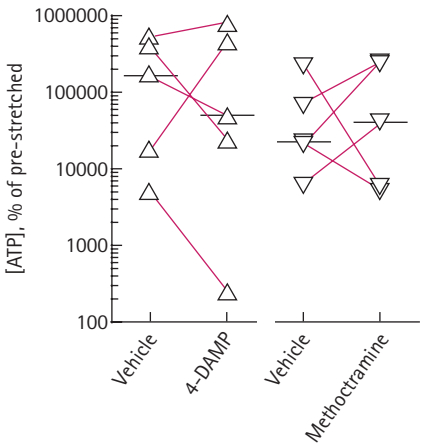
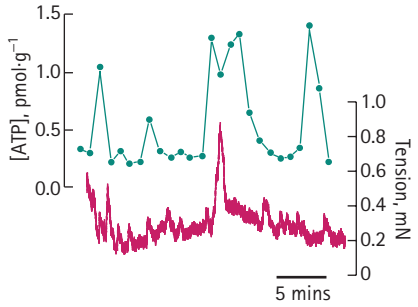


FIG. 4. Simultaneous measurement of mucosal strip tension and superfusate ATP.



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CONFLICT OF INTEREST

Christopher H. Fry is an advisor to Takeda, Boston Scientific.

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Abbreviations: **ACh**, acetylcholine; na, number of guinea pigs; ns, number of mucosal strips.

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