Inhibition of stretch-evoked ATP release from bladder mucosa by anti-cholinergic agents.

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2313 Words

ABSTRACT

Objective:

- To determine whether muscarinic receptor antagonism affected stretch-induced release of ATP.

Materials and methods:

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

Results:

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

Conclusion:

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

243 words

INTRODUCTION

Stress-evoked release of ATP from the basolateral surface of the bladder wall was initially shown by a change to the transmural pressure (1). Subsequently, ATP release has been measured due to 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 increased in cells from overactive bladders (2,3,7), itself reduced after botulinum toxin treatment (8), and exposure to ATP itself suggesting an autocatalytic process (6). It has been proposed that this ATP pool eventually activates adjacent afferent nerves thus providing a sensory mechanism to relay 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).

However, a number of key questions remain unanswered 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 is also released from the bladder wall on stretch (12) and from cultured urothelial cells exposed to a hypotonic solution (13). Anti-cholinergic 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 anti-cholinergic oxybutynin and M3-specific anti-cholinergic darifenacin reduce afferent firing during bladder filling (16,17). This may result from direct desensitisation of afferents, but an alternative hypothesis that they reduce the release of ATP from mucosa (urothelium and sub-urothelium) when the tissue is stressed is supported by two lines of evidence: i. Muscarinic receptor activation by non-subtype specific agonists increased intracellular calcium and evoked ATP release in cultured rat urothelial cells (18); ii. Intravesical instillation of a non-subtype specific agonist increased voiding frequency in an *in vivo* rat preparation (19). In order to bridge the gap between isolated cells and whole animal, we measured stretch-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. Efforts were made to minimise the number of animals used and their suffering. 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 stretch or damage. Two strips (6 – 8 mm long and 2 – 3 mm wide) were cut along the craniocaudal axis.

Stretch of mucosa strips. Strips were tied at either end using fine silk suture (7/0; Pearsalls, 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, USA) connected to a bridge amplifier (TBM-4M; WPI, UK). Preparations were continuously superfused (10 $ml.min^{-1}$) with fresh, gassed Ca^{2+}-free Tyrode's and allowed to equilibrate at a slack length (0 mN tension) for one hour prior to each experiment. Following equilibration, preparations were stretched to a constant tension over <5 seconds by driving the micromanipulator arm holding the force transducer. Preparations equilibrated for a further one hour during which time the tension relaxed to near pre-stretch 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 minutes, after which preparations were again stretched to a similar tension. After a further 30 minutes' 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 time points during the above protocol, immediately prior to, and following, mechanical (stretch, relaxation) or pharmacological (4-DAMP, methoctramine or vehicle control) interventions, as well as at regular time points in between. Samples of superfusate were taken 0.5 cm downstream from the arm of 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 dry weight of the strips at the end of each experiment was 10.2±0.7 mg, range 4.7-15.7 mg; n=20.

Measurement of ATP concentration using luciferin-luciferase assay. ATP in the superfusate was measured using a luciferin-luciferase assay (FL-AAM; Sigma-Aldrich, UK) and a luminometer (GloMax 20/20; Promega, UK). The relationship between ATP concentration over the range 0.1 pM-100 nM and luminescence was determined and 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] vs. luminescence relationships if the ATP was dissolved in distilled water or in gassed Ca^{2+}-free Tyrode's. The ATP content of each sample was calculated relative to the standard curve and expressed as $pmol.g^{-1}$ dry weight of tissue.

Statistical analysis. n_a refers to the number of animals 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 was tested for statistical significance using the Wilcoxon or Student's *t*-test, as appropriate; the null hypothesis was rejected at $P<0.05$.

RESULTS

Stretch-evoked release of ATP from mucosa strips. The stretch protocol was validated before any pharmacological interventions on stretch-evoked ATP release were made. After 60 minutes 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] in the superfusate, from 1.63 [0.05, 2.40] to 6.60 [2.16, 24.74] pmol.g^{-1}, i.e. to a median of 404% of basal levels (Figure 1A). The ATP concentration was measured approximately 5 seconds after the stretch, as preliminary experiments showed that at this time the rise of [ATP] was maximum. To achieve the relatively limited range of tensions, preparations were stretched from an initial length of 7.7±0.5 mm (range: 6-10 mm) to 13.1±1.1 mm (range: 8-20 mm); a mean increase in length of 71% (range: 22-122%; n_s=20, n_a=10). Following the initial stretch, tension in the mucosal strips relaxed until it had reached a new steady-state after 60 minutes. At this time the tension had reduced to 1.03±0.23 mN and [ATP] was reduced to 2.17 [0.24, 3.8] pmol.g^{-1}; not significantly different from pre-stretch values (Figure 1B).

The magnitude of the [ATP] increase generated by stretch was not influenced by the initial [ATP] sampled at the unloaded (0 mN) state (r=-0.25), the change in length of the strip required to generate tension (r=-0.14), nor the change of strip length normalised to its initial length (r=-0.15) or the weight of the animal (mean: 464 g, range: 422-489 g, n_a=10; r=0.22). The latter data are included as animal weight is positively associated with animal age. Stretch-evoked ATP release also 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 bathing 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 itself generated a transient increase of the [ATP]. For example, with superfusate containing vehicle alone the [ATP] increased from 3.5 [3.3, 4.6] pmol.g^{-1} to a maximum of 20.5 [18.4, 559.1] pmol.g^{-1} after 5 minutes (n_s = 5). For superfusate containing 4-DAMP the [ATP] transiently increased from 6.3 [2.0, 12.0] pmol.g^{-1} to a maximum of 43.0 [23.7, 147.7] pmol.g^{-1} after 5 minutes (n_s = 5). Similarly, switching to superfusate containing methoctramine transiently increased the [ATP] from 1.7 [0.05, 2.4] pmol.g^{-1} to a maximum of 6.2 [4.3, 16.9] pmol.g^{-1} after 5 minutes (n_s = 5). The magnitude of the increase (to 585%, 682% and 373% pre-intervention, respectively) was similar in each case. Following 20 minutes 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, stretch increased [ATP] from 4.7 [2.1, 7.8] to 27.8 [20.1, 34.9] pmol.g^{-1}, which represents a median increase to 391% of basal levels (P<0.05; n_s=5, n_a=5). The percentage increase of [ATP] was similar to that at the beginning of the experiments and shows that the stretch protocol and the quantity of ATP release were consistent. In the presence of 10 nM 4-DAMP, the stretch-induced increase of [ATP] was abolished and the concentration was actually significantly reduced to 61% of pre-stretch levels (P<0.05; n_s=5, n_a=5; Figure 2).

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

Rapid relaxation and ATP release. Thirty minutes after the final stretch in the presence or absence of an anti-cholinergic 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 of ATP immediately after tension reduction. Figure 3 shows the percentage increase of ATP 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 seconds) 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 correlation between [ATP] and the larger spontaneous contractions.

DISCUSSION

We show that stretch of guinea pig bladder mucosa strips induced about a three-fold 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 stretch. The mucosa strips were quite sensitive to mechanical disturbance, as bathing with superfusate also evoked some release of ATP, and this was taken into account in our experiments. We therefore have three important observations, each of which is discussed below.

We demonstrate that alteration of resting tension is the key determinant of ATP release: (i) [ATP] increases in response to a rapid tensioning and (ii) returns to baseline concentrations following relaxation approximately one hour 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 stretch-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 for this phenomenon. 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 stretch-dependent modalities 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.

Finally we showed that muscarinic receptor antagonists methoctramine and 4-DAMP inhibit stretch-induced ATP release from guinea pig mucosa. These experiments build on previous demonstrations that muscarinic agonists stimulate ATP release from urothelial cells (13) and increasing voiding frequency in an anaesthetised rat (19), to complete a circle in showing that stretch-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 anti-cholinergic agents on ATP release; each agent was used at a concentration at least 10-fold 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; methoctramine 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 acetylcholine before stretch 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.

Our data add to the evidence that anti-cholinergic agents used to treat human lower urinary tract pathologies act on mucosal muscarinic receptors and is consistent with the clinical observations that these agents act more on OAB 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 desensitisation (16,17), but also limits stress-induced ATP release itself.

Conclusions. M2/M3 muscarinic receptor antagonism inhibits stretch-evoked ATP release from bladder mucosa. This novel interaction of these drugs and ATP has important implications for the treatment of over-active bladder.

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Figures

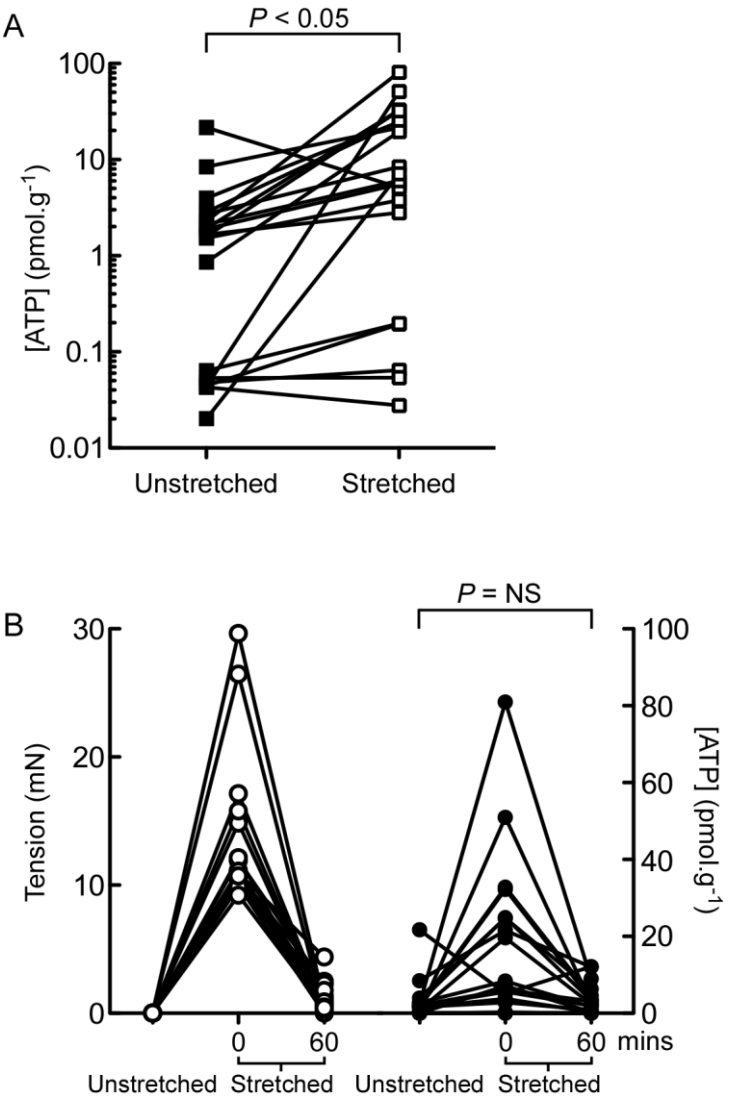

Figure 1. A, stretch-evoked release of ATP. Guinea pig mucosa strips were rapidly stretched (<5s; 0 to 13.3±1.2 mN) and the superfusate [ATP] measured. B, Following stretch, mucosal strips relaxed to a steady-state tension after 60 minutes (left) and [ATP] was measured following passive relaxation (right).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

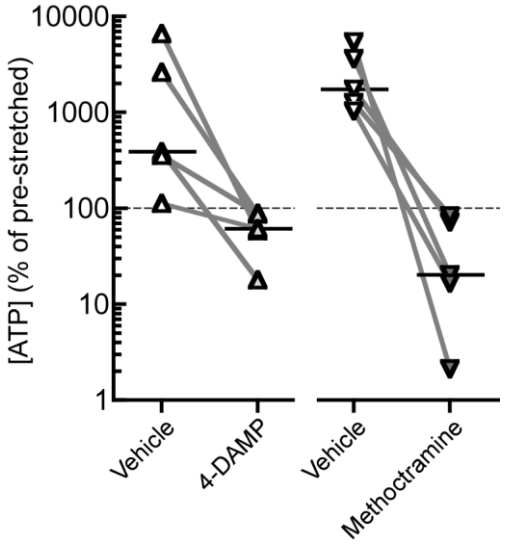

Figure 2. Effect of 4-DAMP or methoctramine on stretch-evoked ATP release. Mucosa strips were superfused for 20 minutes with 4-DAMP (10 nM) or methoctramine (1 μM), rapidly stretched and [ATP] sampled. Values are relative to superfusate [ATP] immediately before the rapid stretch (dashed line), i.e. in 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]. n_s=20, n_a=10.

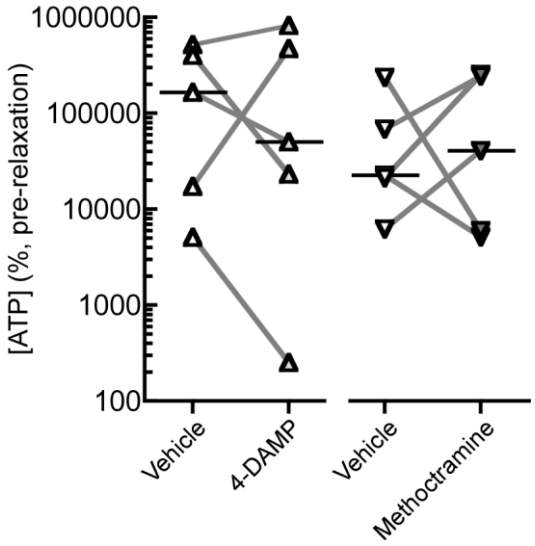

Figure 3. Effect of 4-DAMP and methoctramine on relaxation-evoked ATP release. The suture holding the strip to the force transducer was cut, tension returned to zero and [ATP] was sampled. Values are relative to superfusate [ATP] immediately before the intervention. Lines connecting data points denote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

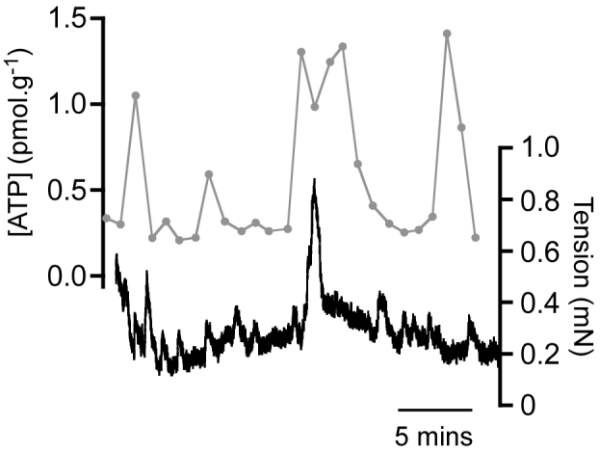

Figure 4. Simultaneous measurement of mucosal strip tension and superfusate [ATP].