**In vivo paracrine effects of ATP-induced urothelial acetylcholine in the rat urinary bladder**

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**ABSTRACT**

Mechanical stretch of the urothelium induces the release of ATP that activates bladder afferent nerves. In the rat urinary bladder, ATP is also a contractile co-transmitter in the parasympathetic innervation. In isolated preparations, ATP evokes a urothelial release of acetylcholine that substantially contributes to ATP-evoked contractile responses. Currently we aimed to further examine the interactions of ATP and acetylcholine in the rat urinary bladder in two *in vivo* models. In the whole bladder preparation, atropine reduced ATP-evoked responses by about 50% in intact but denervated bladders, while atropine had no effect after denudation of the urothelium. In a split bladder preparation, reflex-evoked responses of the contralateral half were studied by applying stimuli (agonists or stretch) to the ipsilateral half. Topical administration of ATP and methacholine as well as of stretch induced contralateral reflex-evoked contractions. While topical administration of atropine ipsilaterally reduced the ATP- and stretch-induced contralateral contractions by 27 and 39%, respectively, the P2X purinoceptor antagonist PPADS reduced them by 74 and 84%. In contrary, the muscarinic M2-(M4)-selective receptor antagonist methoctramine increased the responses by 38% (ATP) and 75% (stretch). Pirenzipine (M1-selective antagonist) had no effect on the reflex. *In vitro*, in the absence of the reflex, methochotamine did not affect the ATP-induced responses. It is concluded that urothelial ATP potently induces the micturition reflex and stimulates urothelial release of acetylcholine. Acetylcholine subsequently acts on afferents and on the detrusor muscle. While muscarinic M2 and/or M4 receptors in the sensory innervation exert inhibitory modulation, muscarinic M3 receptors cause excitation.

1. **Introduction**

The urothelium has important signaling and sensory functions and plays a crucial role for the initiation of the micturition reflex. ATP released from the urothelium induced by mechanical stretch has been considered to be an important signaling substance for activating bladder afferent nerves (Ford et al., 2006). Thus, the urothelium not only serves as a passive barrier, but also generates and integrates the sensory input for the micturition reflex. Purinoceptors of the P2X, P2X3 and/or P2X2/3 have been described on the afferent nerves (Cockayne et al., 2000; Cockayne et al., 2005) as the targets for ATP when inducing bladder sensation. The significance of the P2X3 purinoceptor is elucidated by the findings in knockout mice, which exhibit urinary bladder hyporeflexia (Cockayne et al., 2005). On the other hand, seemingly contrasting findings have been reported for P2X2/3−/− and P2X3−/− mice, which exhibited a normal bladder function under non-pathological conditions (Takezawa et al., 2016). Other signaling substances thus seem to be involved and, notably, acetylcholine, nitric oxide and neurokinin A have been shown to be released from the urothelium when stretched (Fowler et al., 2008). The exact roles of the different urothelium-derived substances are far from unraveled, but indicate the importance of the urothelium for bladder function. The complexity of the possible roles of the urothelium is also underlined by the fact that all five muscarinic and all seven P2X purinergic receptors, as well as some P2Y purinergic and nicotinic receptor subtypes are expressed in the tissue (Bschleipfer et al., 2007; Birder et al., 2004; Beckel and Birder, 2012).

Recently, we have shown in *in vitro* studies that ATP evokes a release of acetylcholine from the urothelium and that this release substantially contributes to the ATP-evoked detrusor contractile response (Stenqvist et al., 2017; Stenqvist et al., 2018). So, urothelial acetylcholine may act as a paracrine hormone in the bladder. But does acetylcholine affect the afferent nerve fibers as well? Namely, the afferent nerve fibers exhibit, in addition to the purinoceptors, muscarinic M2, M3 and M4 receptors (Nandigama et al., 2010) and activation and/or modulation of the initiation of the micturition reflex by acetylcholine...
may be possible. Also, results from in vivo cystometry in the rat indicate cholinergic effects on the afferent C-fibers (Kullmann et al., 2008).

The parasympathetic innervation of the bladder represents the efferent link of the micturition reflex arc, which terminates on the detrusor muscle. In the rat, the contractile response is evoked by the parasympathetic release not only of acetylcholine but of ATP as well (Ambache and Zar, 1970; Burnstock et al., 1972; Giglio et al., 2007). While acetylcholine elicits the contraction by mainly acting on muscarinic M3 receptors (Tobin, 1995; Longhurst et al., 1995), ATP acts on P2X1 and possibly P2X3 purinoceptors as well (King et al., 2004). Furthermore, the parasympathetic release of transmitters is modulated by muscarinic inhibitory (muscarinic M2 (or M4) receptors) and facilitatory (M1) autoreceptors (Tobin and Sjogren, 1995; Tobin and Sjogren, 1998; Somogyi et al., 1994).

The current study aimed to further examine the interactions of ATP and acetylcholine in the rat urinary bladder in two in vivo bladder models. In a well-established model, cystometry of the whole bladder was performed aiming for the confirmation of the previous in vitro findings, which, as mentioned above, have shown that a substantial part of the ATP-induced detrusor contraction depends on urothelial release of acetylcholine (Stenqvist et al., 2017). The studies in the other model, a split bladder model, aimed for examining purinergic and muscarinic effects at the afferent level of the micturition reflex arc. In order to discriminate between afferent and efferent effects, we have developed an in vivo model in the rat (Aronsson et al., 2014). Presently using this model, we split the bladder in situ and measure the tension of the contralateral half of the bladder and use the ipsilateral half for stimulation (stretch, electrical nerve stimulation, topical administration of agonists or antagonists). In order to establish the site of receptor interactions, some complementary in vitro examinations were performed.

2. Materials and methods

2.1. Animal procedures

The Animal Ethics Committee in Gothenburg approved the experiments conducted (1794/18). 47 adult male rats (approximately 250–350 g) of the Sprague-Dawley strain were used for the in vivo and in vitro experiments. The in vivo experiments were carried out under 1–2% isoflurane (in air; Forene Abbott, Wiesbaden, Germany) general anesthesia. For in vitro experiments, or at the end of the in vivo experiments, rats were given an overdose of sodium pentobarbital (100–150 mg/kg, APL, Stockholm, Sweden) and the urinary bladder was excised.

In the in vivo whole bladder experiments, the isoflurane concentration in the inspired air was kept at 1.5–2.0%. In the experiments studying afferent effects, a relay via the central nervous system is required. In order to achieve an active micturition reflex, isoflurane anesthesia was lowered to 1.0–1.2%. The absence of changes in blood pressure and heart rate after strong paw-pinches was used for controlling an adequate depth of anesthesia. Body temperature was maintained at about 38 °C by means of a thermostatically controlled blanket connected to a thermistor inserted into the rectum. A cannula was placed into the femoral vein for drug administration and the blood pressure was monitored continuously via a catheter placed into the femoral artery. The urinary bladder was exposed ventrally, and after splitting the prostatic glands, the urethra and the pelvic nerve were identified and separated from the surrounding tissue.

2.2. Whole bladder cystometry

In the whole bladder experiments, bladder pressure was measured continuously via a catheter inserted through a small incision and fixated with a ligature at the top of the bladder, as previously described (Modiri et al., 2002; Andersson et al., 2008). An additional catheter was inserted through the same incision and fixated next to the other catheter.

The basal pressure was maintained at 10–15 mm Hg by adding small volumes of saline (0.05–2.0 ml) when necessary. Methacholine (1, 2 and 5 μg/kg IV; 6, 12 and 30 nmol/kg) and ATP (5, 10 and 100 μg/kg IV; 9, 18 and 180 μmol/kg) were administered intravenously in three subsequent rounds; first in the absence of any other intervention, then after both pelvic nerves had been cut and finally in the presence of atropine (1 mg/kg IV; 3.5 μmol/kg). The experiments were performed in normal, intact urinary bladders and in urothelium-denuded bladders. The denudation was performed by filling the bladder via the apex with collagenase type 1 (0.1% in saline; 0.2–0.5 ml) for 30 min (Andersson et al., 2008). Thereafter, the bladder was gently rubbed in order to detach urothelial cells on the inside of the bladder. Before the onset of the experiment, the bladder was rinsed with saline in order to remove collagenase and loose urothelial cells.

2.3. Split bladder in situ experiments

In the split bladder experiments, threads were ligated at the top, sides and below the entrance of the pelvic nerve on each side of the bladder. Thereafter the bladder was divided into two completely separated parts along the midline from the top of the bladder all the way to the urethra (for detailed description, see Aronsson et al., 2014). The lower ligatures were fixated to the underlying tissue. The thread ligated to the top of one of the bladder halves was connected to an adjustable isometric force transducer (contralateral half; Linton Instrumentation, Norfolk, UK), while the other half was used for force application (80 mN; via the thread from the top of the bladder half) and for topical administration of drugs (ipsilateral half; schematic illustration in Fig. 1). In some experiments the topical administration was performed directly on the contralateral half in order to induce reflex independent responses. The ipsilateral pelvic nerve, exposed distally to the major pelvic ganglion, was placed on a bipolar platinum electrode and a basal

![Fig. 1. Schematic illustration of in situ half bladder preparation. The dashed circle indicates the whole bladder before cutting it into two halves. The left arrow and the arrow within the circle in the top represent the applied stretch (ipsi; ipsilateral side) and the tension measurement (contra; contralateral side). The drop represents the administration of solution (on either side), and the bar to the left, the electrode for electrical stimulation of the pelvic nerve. Finally, the black strings represent ligations for stabilizing the tissue and for the stretch response, while the two dashed lines the sites for pelvic nerve disruption.](image-url)
tension of 10 mN was applied on the contralateral side. The isoflurane concentration in the inspired air was then reduced from 2% to 1.2%, and further if necessary (to 1.0%), and electrical stimulation at 20 Hz (15 V square wave (supramaximal voltage); 0.8 ms pulse width) was delivered continuously for about 30 s at approximately 5 min intervals. No response to the stimulations occurred initially when the reflex stimulation was inhibited by the deep anesthesia, but after a period of 20–30 min responses generally appeared. If not, the isoflurane concentration was further reduced and strong paw-pinches was used for controlling that the depth of anesthesia was still adequate. The electrical nerve stimulations were repeated until stable and repeatable responses occurred.

Methacholine (5 μg/kg (30 nmol/kg) IV) was injected and the contraction of the contralateral bladder half was monitored. This injection of methacholine as well as the electrical pelvic nerve stimulations at the start and at the end of the experiments was used to verify that the application of drugs on the ipsilateral side had not affected the contralateral side. After the onset of stable reflex-evoked responses to the electrical nerve stimulations, stretch and topical agonist stimulation of the ipsilateral side took place. For comparison, drugs were in some experiments also applied on the contralateral half (non-reflex stimulation). The drugs, agonists and antagonists, were topically administered on the urothelial side of the bladder half in volumes (20–30 μl) that fully covered the surface of the tissue. The bladder half was meticulously insulated with filter papers so that no solution could reach the opposite bladder half or the surrounding tissue. Weights of 8 g (80 mN; (Aronsson et al., 2014)) were used to apply a force that stretched the ipsilateral bladder half. Methacholine and ATP were examined at 10 and 100 (0.05 and 0.5 mM) μg/ml and at 10, 100 and 1000 μg/ml (0.018, 0.18 and 1.8 mM), respectively. The largest concentrations evoked substantial reflex independent (contralateral administration) and reflex-evoked (ipsilateral administration) responses. Methacholine at 100 μg/ml and ATP at 1000 μg/ml were thus selected for further examinations in the presence of antagonists. The pH of the methacholine (100 μg/ml) and the ATP (1000 μg/ml) solutions were 6.6 and 4.1, respectively. In view of observations showing that a low pH may activate bladder afferents (Zagorodnyuk et al., 2009), the effect of citric acid (0.005 and 0.5 M, pH 2.7 and 1.8, respectively) was examined. While the response to ipsilateral citric acid at 0.005 M was minute (0.2 ± 0.1 mN (n = 4)), it was 1.5 ± 0.6 mN at 0.5 M. The inhibitory effects of the antagonists at concentrations previously used in in vivo studies were validated in pilot experiments (Aronsson et al., 2015). In pilot experiments the P2X purinoceptor antagonist suramin (10 mg/ml; 7.0 mM) was examined and found not to cause any further inhibition to that evoked by PPADS (2 mg/ml; 3.3 mM). The stimulations with the selected concentrations and with stretch were performed before and after the administration of antagonists. The antagonists were administered in the same way as the agonists were, but were present for 2 min before any further procedure was performed. In order to avoid excessive systemic absorption, the substances were allowed to act for no longer than 5 min before washing the exposed bladder half.

In the pilot experiments, it was observed that long experiments including several topical administrations of antagonists affected the reference responses (methacholine IV and electrical nerve stimulation) indicating systemic absorption. In order to avoid this, the examinations were separated into shorter protocols. However, when more than one antagonist was included, new stimulations in the absence of any antagonist was performed in order to establish that baseline responses were not affected. In order to establish that true reflex-evoked responses had occurred, the pelvic nerve was cut at the end of a few experiments in which the reflex mechanism was not inhibited by previous administration of antagonists.

The split bladder experiments were performed in four different protocols. The first protocol (n = 4–5) included stimulations before and after topical administration of atropine (1 mg/ml; 3.5 mM), and PPADS (2 mg/ml; 3.3 mM), respectively. The second protocol (n = 5) included stimulations before and after atropine, followed by atropine + PPADS. The third protocol (n = 4–5) included stimulations before and after methoctramine (1 mg/ml; 1.4 mM) and pirenzepine (1 mg/ml; 2.4 mM). In a fourth protocol (n = 5), topical administrations (ipsi- or contralaterally) of methacholine and ATP were examined before and after topical PPADS and atropine, respectively. An additional part of the fourth protocol included the examination of contractile effects of intravenous administration of ATP (5, 10 and 100 μg/kg IV) before and after the topical administration of atropine. This last part aimed for examining ATP responses not including any reflex contribution. Therefore, the experiments were performed after the disruption of the pelvic innervations.

2.4. In vitro

The excised bladder was opened sagittally and two approximately 6 × 3 mm tissue strips were cut out, and each strip was mounted in an organ bath (Linton Instrumentation, Norfolk, UK); one end attached to a mounting hook and the other to an isometric force transducer (TSD125C, Biopac systems Inc., Goleta, CA). The organ baths contained Krebs solution (NaCl, 118 mM; KCl, 4.6 mM; KH 2PO4, 1.15 mM; MgSO4, 1.15 mM; NaHCO3, 25 mM; CaCl2, CaCl2, 1.25 mM; and glucose, 5.5 mM), gassed by 95% O2 and 5% CO2, and heated to 38 °C. The tissue strips were stretched to a basal tension of about 5 mN, and left to equilibrate for 45 min. Following the equilibration phase, a high potassium Krebs solution (containing 124 mM K+) obtained by exchanging Na+ for equimolar amounts of K+ was administered, in order to evaluate the viability of the bladder tissue and also to be used as a reference contractile response. ATP (1 × 10−6 -1 × 10−5 M; final bath concentrations) was cumulatively administered in 125 μl volumes to the baths (25 ml). The ATP administrations were repeated in the presence of increasing concentrations of methoctramine (125 μl; 1 × 10−7, 1 × 10−6 and 1 × 10−5 M). At the end of the experiments, the high potassium Krebs solution was administered once again. When the contractile responses were normalized to the reference high potassium responses, the first high potassium response was used for the first part of the experiments (ATP and ATP + 1 × 10−7 M methoctramine) and the last high potassium response for the latter part (ATP + 1 × 10−6 M methoctramine and ATP + 1 × 10−5 M methoctramine).

2.5. Compounds and solutions

The drugs employed were adenosine 5′-triphosphate disodium salt hydrate (ATP; P2 purinoceptor-agonist; (Ralevic and Burnstock, 1998)), acetyl-β-methylcholine chloride (methacholine; muscarinic receptor agonist), atropine (muscarinic antagonist), collagenase type I, isoflurane (Forene Abbott, Wiesbaden, Germany), pentobarbitone (APL, Stockholm, Sweden), pirenzepine dihydrochloride (muscarinic M1 receptor-selective antagonist; (Eglen and Watson, 1996)), pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium (PPADS; P2 purinoceptor antagonist; (Ralevic and Burnstock, 1998)), methoctramine hydrate (muscarinic M2 (M4) receptor-selective antagonist; (Melchiorre, 1988)) and suramin sodium salt (P2 purinoceptor antagonist; (Dunn and Blakeley, 1988)). Unless otherwise stated, all drugs were purchased from Sigma-Aldrich Sweden AB, Stockholm, Sweden.

2.6. Statistics and calculations

Statistical significance was determined by Student’s t-test for paired data. For multiple comparisons, statistical significance was determined by one-way or two-way analysis of variance (ANOVA) followed by the Holm-Sidak multiple comparison test. P-values < .05 were regarded as statistically significant. Graphs were generated and parameters computed using the GraphPad Prism program (GraphPad Software, Inc., San Diego, US). Responses are presented as change of tension. Data are
presented in the form of means ± standard error of the mean.

3. Results

3.1. In vivo; cystometry

In the whole bladder preparation, intravenous administration of methacholine (1, 2 and 5 μg/kg IV; n = 8–9, Fig. 2) as well as of ATP (5, 10 and 100 μg/kg IV; n = 6–9, Fig. 3) evoked dose-dependent contractile responses in the intact (Fig. 2a and 3a), as well as in the urothelium-denuded bladders (Fig. 2b and 3b). The administration of the drugs resulted in a transient decrease in blood pressure lasting for about 1 min. In the intact bladders, the increase in bladder pressure was 8.9 ± 2.2 mm Hg in response to methacholine 5 μg/kg IV (n = 9, Fig. 2a), while it was 3.1 ± 0.6 mm Hg to ATP 100 μg/kg IV (n = 6, Fig. 3a). Bilateral disruption of the pelvic nerves did not significantly affect the methacholine-evoked response (7.6 ± 1.0 mm Hg at 5 μg/kg IV; n = 7, Fig. 2a) vs 7.6 ± 1.0 mm Hg at 5 μg/kg IV of methacholine). In concordance with the intact bladder preparations the disruption of the pelvic nerves did not affect the methacholine-evoked responses in the urothelium-denuded urinary bladders, while atropine significantly lowered the methacholine-evoked responses (to 1.6 ± 0.2 mm Hg at 100 μg/kg IV; n = 6, Fig. 2b). In the denuded bladders, the ATP-evoked dose-dependent contractile responses were reduced by about 50% in comparison with the responses in intact bladders (1.6 ± 0.2 mm Hg at 100 μg/kg IV; n = 6, Fig. 3b). In these urothelium-denuded bladders, neither the disruption of the pelvic innervation nor the intravenous administration of atropine significantly caused any further reduction of the ATP-induced responses (Fig. 3b).

3.2. In vivo; split bladder responses

Topical administration of methacholine (10 and 100 μg/ml) and ATP (10, 100 and 1000 μg/ml) evoked concentration-dependent responses both when administered ipsilaterally or contralaterally. The direct topical stimulation by the contralateral administration evoked larger responses than the reflex-dependent ipsilateral administration. While the reflex-evoked responses of the opposite bladder half induced by topical administration of ATP (1 mg/ml) and of methacholine

![Fig. 2. Cystometry. Mean contractile responses to intravenous injections of methacholine (1, 2 and 5 μg/kg IV) in intact (a; n = 8–9) and urothelium-denuded (b; n = 7) urinary bladders before (○) and after (▲) disruption of the pelvic nerve and after the subsequent administration of atropine (●; 1 mg/kg IV). The vertical bars represent S.E.M. Two-way ANOVA followed by the Holm-Sidak multiple comparison test was used for the statistical analyses (*p < .05; **p < .01).](image1)

![Fig. 3. Cystometry. Mean contractile responses to intravenous injections of ATP (5, 10 and 100 μg/kg IV) in intact (a; n = 6–9) and urothelium-denuded (b; n = 5–7) urinary bladders before (○) and after (▲; # comparison with control (○)) disruption of the pelvic nerve and after the subsequent administration of atropine (●; 1 mg/kg IV; * comparison with nerve-disrupted (▲)). The vertical bars represent S.E.M. Two-way ANOVA followed by the Holm-Sidak multiple comparison test was used for the statistical analyses (*, # p < .05).](image2)
The atropine-sensitive detrusor contractile response to ATP, which was observed in the cystometry, was further studied in split bladder stretching force (80 mN) were 2.7 ± 0.5, 1.9 ± 0.1 and 3.7 ± 0.6 mN, respectively (Fig. 5). In three experiments, the reflex-evoked responses of the opposite bladder half induced by topical administration (20 Hz) were 6.6 ± 1.6 mN (n = 5), pelvic nerve stimulation at 20 Hz induced a contractile response by 26% (from 2.2 ± 0.2 to 1.6 ± 0.1 mN; p < .05, n = 5, Fig. 7d). Topical administration of methochromine, on the other hand, increased the responses to ATP by 38% (from 2.9 ± 0.2 to 4.0 ± 0.3 mN; p < .05, n = 4, Fig. 7e).

When examining the ipsilateral effects of the antagonists on stretch-induced contralateral responses, a similar pattern as for topical ATP emerged (Fig. 8). While atropine reduced the response by 39% (from 3.2 ± 0.5 to 2.3 ± 0.5 mN; p < .05, n = 5, Fig. 8a), PPADS reduced it by 84% (from 3.7 ± 0.4 to 0.6 ± 0.2 mN; p < .01, n = 5, Fig. 8b). The combination of atropine and PPADS caused a reduction by 91% (from 3.6 ± 0.3 to 0.3 ± 0.1 mN; p < .01, n = 5, Fig. 8c). In resemblance with the ATP-evoked response, the stretch-induced contractile response increased in the presence of methochromine (by 75%; from 3.2 ± 0.5 to 5.6 ± 0.7 mN; p < .01, n = 5, Fig. 8d). Pirenzepine (1 mg/ml) did not have any effect on the stretch-induced response (3.0 ± 0.4 vs 2.9 ± 0.1 mN; n = 5).

3.3. In vitro; bladder strip responses

In isolated bladder strip preparations with an intact urothelium, ATP (1 × 10⁻⁶–1 × 10⁻³ M; n = 8) evoked concentration-dependent contractions which were not significantly affected by the presence of methochromine (1 × 10⁻⁷–1 × 10⁻⁵ M). In the absence of methochromine, ATP at 1 × 10⁻³ M evoked a contractile response of 2.6 ± 0.8 mN (n = 8; 6.5 ± 1.8% of high potassium response). In the presence of methochromine (1 × 10⁻⁵ M), the corresponding ATP response was 2.9 ± 0.8 mN (n = 8; 6.0 ± 1.0% of high potassium response).

4. Discussion

The current study confirms that ATP exerts effects on the afferent and efferent levels of the micturition reflex arc in the rat urinary bladder. Hence, the induction of the micturition reflex involves ur- othelial ATP, and ATP seems, in the current in vivo models, to be an important transmitter in this respect. Besides activating the afferents, ATP induces a release of acetylcholine from the urothelium, and notably, the paracrine acetylcholine effects are exerted on afferent neu- rons and on the detrusor muscle. Furthermore, at the level of activation of afferent neurons, interactions with acetylcholine seem to be com- posite. In resemblance to ATP, methacholine evokes a reflex-evoked response by its own and causes a release of ATP that subsequently adds a minor ATP-dependent part. The study also shows that the effect of the transmitters on the sensory innervation of the urinary bladder is modulated by an interplay involving different muscarinic receptor subtypes. The physiological significance of the urothelium is apparent by the observations in the urothelium-denuded bladders, in which the ATP-induced reflex responses and the paracrine acetylcholine effects
ATP is an agonist on P2X and P2Y purinoceptors, which are both expressed in the rat urinary bladder in the detrusor as well as in the urothelium (Birder et al., 2004). While P2X purinoceptors are considered mainly to be coupled to the contractile effects of the bladder, P2Y purinoceptors are generally considered to be coupled to relaxation (Aronsson et al., 2010), although suggested to augment spontaneous bladder activity (Fry et al., 2012). In view of P2X purinoceptors exerting purinergic effects at all peripheral levels of the micturition reflex, systemic administration of ATP may cause bladder effects both at the detrusor (P2X1 subtype) and at the urothelium/afferent level (P2X2, P2X3 and P2X2/3 subtypes) (King et al., 2004; Ford et al., 2006). The composite effects of intravenous injections of ATP were apparent in the whole bladder setup; cutting the nerve abolished the reflex-evoked effects, atropine abolished the effects of the subsequent urothelial release of acetylcholine and still a small direct contractile effect existed.

Several P2X purinoceptor agonists exist including ATP itself. A common problem with the agonists, particularly in in vivo studies, is the desensitization that develops at repetitive administrations. While P2X3 purinoceptors, in particular, are rapidly desensitized but are not pH sensitive, P2X2 purinoceptors are sensitive towards pH and desensitize less rapidly (Burnstock, 2000). Noteworthy, the ATP solution is rather acidic (pH 4.1 in the current solutions). In order to determine how this could have influenced the current results, the effect of citric acid at pH 1.8 and 2.7 was examined, since a low pH may induce urothelial release of ATP (Sadananda et al., 2009). While the solution with the low pH caused activation of bladder afferents, the solution with the pH of 2.6 had almost no effect. Furthermore, in the in vivo experiments no desensitization occurred when the ATP administrations were repetitively administered in the absence of any antagonist. So, in the current in vivo model setups, neither any desensitization nor low pH are likely to substantially have caused the observed effects.

A substantial part of the ATP-evoked contraction observed in the cystometry experiments depended on the induction of the micturition reflex. After the disruption of the pelvic innervation the reflex-induced ATP effects were abolished, and consequently only the direct effects of ATP on the urothelium and on the detrusor muscle remained. The further addition of atropine indicated that approximately 40–50% of the ATP response in the absence of the reflex depends on the paracrine effects of urothelial acetylcholine, which corresponds well with previous in vitro observations (52%; Stenqvist et al., 2017)). When the same intravenous administration protocol was performed in the split bladder model, atropine reduced the response by 30–50%. In the further examinations in the half bladder model, in which ATP was administered directly on the denervated bladder half, atropine reduced the response by a similar magnitude (39%). Hence, almost half of the ATP-induced contractile response in different experimental models
depends on a subsequent acetylcholine release. Notably, the indirect cholinergic effects of ATP are absent in urothelium-denuded bladders as previous in vitro findings have shown (Stenqvist et al., 2017) and which is confirmed in the current cystometry experiments. Namely, the ATP-induced responses in the urothelium-denuded bladders were not further affected by atropine administration.

The effect of muscarinic receptor agonists has previously been considered more or less exclusively to exert their bladder effects on the detrusor muscle. However, the paradigm that the anticholinergic drugs
Muscarinic receptors may profoundly inhibit or enhance neuronal transmission (Somogyi et al., 1994; Tobin and Sjogren, 1995). Inhibition of the negative feedback in the parasympathetic junction exerted by muscarinic M2 (and/or M4) receptors may result in large increases of nerve-evoked responses (Tobin, 1998). Since inhibitory muscarinic receptors (M2 and M4) exist on afferents as well as in the urothelium, the effect of topical administration of the M2/4-selective antagonist methoctramine was examined in the half bladder model. In contrast to the other antagonists currently used (atropine and PPADS), the presence of methoctramine caused substantially augmented responses to stretch and to topical administration of ATP, while the muscarinic M1-selective antagonist pirenzepine had no effect on the stretch-evoked responses. The current results support previous findings describing that muscarinic receptors may depress afferent signaling in the bladder (Daly et al., 2010). In order to establish whether the muscarinic modulation occurred within the urothelium or at the level of the afferents, experiments on isolated bladder strip preparations were performed. In this setup, the urothelium still functions, while the impact of the sensory nerve fibers, or any reflex, is absent or minute. In the in vitro experiments no effect was observed by methoctramine on the ATP-evoked responses, which favors the idea that the modulation of the induction of the micturition reflex observed in the current model takes place on the afferent nerve fibers in the bladder. The muscarinic receptor effect at this level thus involves inhibition exerted by muscarinic M2 or/and M4 receptors. In view of the selectivity profile of methoctramine (Choppin and Eglen, 2001), it is not possible to discriminate between effects by muscarinic M2 and M4 receptors based on results from the current experimental protocols. Nevertheless, since methacholine evoked reflex responses and atropine inhibited stretch-induced responses, and since pirenzepine had no effect, the muscarinic receptors exerting excitation of afferents in the rat urinary bladder are of the M3 subtype.

The current results may give the impression that urothelial ATP is the key molecule for the onset of the micturition reflex. However, one should bear in mind that the rat is still anesthetized in the current experiments, although at a low isoflurane concentration. In order to evoke the reflex-dependent stretch response, the interaction of several factors, such as urothelial-derived factors and direct stretch-activation of afferents, may be necessary. Elimination of either could be enough for abolishing the response. So, any estimation of the extent of the urothelial ATP contribution in comparisons to other factors should be done with caution.

It is concluded that in the rat urinary bladder, urothelial ATP is an important signaling molecule for the induction of the micturition reflex. Stretch of the urinary bladder wall, and thereby the urothelium, is a stimulus that induces release of ATP and acetylcholine. The release of ATP, in addition to its activation of the afferents, further stimulates urothelial release of acetylcholine that subsequently acts on afferents and directly on the detrusor muscle. However, the ATP and acetylcholine interplay may also be inverse, that is, acetylcholine may induce the release of ATP and also directly activate afferents. Furthermore, muscarinic receptors on sensory nerves modulate the micturition reflex stimulation. While muscarinic M2 and/or M4 receptors inhibit the reflex, muscarinic M3 receptors cause excitation.

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