Functional atropine sensitive purinergic responses in the healthy rat bladder

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ABSTRACT

While acetylcholine is regarded to be the main directly contractile transmitter substance in the urinary bladder, interactions with other transmitters likely occur. Presently, the interplay between purinergic and cholinergic signalling was investigated to unravel the involvement of the urothelium and efferent neurons in the functionally important purinergically evoked release of acetylcholine in vitro. Functional characterization of receptor subtypes involved in this interplay was also performed.

In vitro organ bath experiments with electrical field stimulation (EFS) or administration of agonist were performed in the absence and presence of the neurotoxin tetrodotoxin (TTX; 5 × 10⁻⁷ M) and/or receptor antagonists, in intact and urothelium-denuded full thickness rat bladder strip preparations.

Interestingly, functional contractions to ATP (10⁻⁶–10⁻³ M) remained unaffected by TTX, but were significantly lowered in the presence of the muscarinic antagonist atropine (10⁻⁶ M). However, in urothelium-denuded strip preparations, this latter phenomenon was not present and the ATP response remained unaltered. To rule out purinergic interference caused by break-down of ATP, experiments were performed in which the stable ATP-analogue αβMeATP (10⁻⁷–10⁻⁵ M) gave rise to functional atropine-sensitive contractions. Furthermore, contractions to ATP were not affected by P2Y6 purinoceptor blockade (by MRS2578; 10⁻⁷, 10⁻⁵ M), nor were relaxatory responses to ATP sensitive to atropine, PPADS (3 × 10⁻⁵ M) or αβMeATP. Lastly, relaxations to ADP (10⁻⁶–10⁻³ M) or NECA (10⁻⁶–10⁻⁵ M) were unaltered by the presence of atropine. To conclude, purinergic functional contractile, but not relaxatory, responses are supported by the cholinergic transmitter system in vitro, through non-neuronal mechanisms in the urothelium. Involved purinoceptors are of the P2X-subtype, most likely P2X1 and/or P2X3.

1. Introduction

The parasympathetic innervation of the urinary bladder transmits the signals in the micturition phase, and acetylcholine acting on muscarinic M₄ receptors evokes the major part of the contractile detrusor responses in most species (Chess-Williams et al., 2001; Matsui et al., 2000). However, the non-adrenergic, non-cholinergic (NANC) transmitter ATP supports this parasympathetically mediated contraction (Burnstock, 1995; D’Agostino et al., 2012). The contribution of the purinergic transmitter system in the contractile response, mainly mediated by the purinoceptor P2X1, varies between species (O’Reilly et al., 2001; Vial and Evans, 2000). For instance, ATP does not appear to play a significant role in the parasympathetically mediated direct smooth muscle contraction of the healthy human bladder (Birder and Andersson, 2013). The purinergic contribution to bladder control is often regarded to be more prominent during various disorders, where ATP may regulate the micturition reflex on other levels as well (Fowler et al., 2008). For instance, ATP has been shown to be important for initiating the micturition reflex arc (Ford et al., 2006; Kanai, 2011). This activation of sensory afferents has been accredited to P2X3 and/or P2X2/3 receptors (Cockayne et al., 2005). The hypersensitivity of afferents observed during disorders such as bladder pain syndrome/interstitial cystitis (BPS/IC) has been suggested to stem from an increased expression of those receptors in combination with an increased release of non-neuronal ATP (Sun et al., 2001).

Furthermore, ATP has paracrine functions and may stimulate the release of other neuromodulators from the bladder urothelium (Winder et al., 2012). These substances may either directly stimulate smooth muscle cells, suburothelial cells, afferent or efferent neurons, or even trigger the release of other transmitters from the urothelium (Andersson, 2002; Birder and Andersson, 2013).

As previously mentioned, acetylcholine has a vital role in normal bladder function and may affect signalling at various levels of the micturition arc (Andersson, 2002; Kullmann et al., 2008). Therefore, anticholinergic drugs are a common treatment for lower urinary tract symptoms (LUTS), present in disorders such as overactive bladder

Abbreviations: ATP, adenosine-5’-triphosphate; ACh, acetylcholine; LUTS, lower urinary tract symptoms; NO, nitric oxide; αβMeATP, alpha-beta-methylene-adenosine-5’-triphosphate; ADP, adenosine-5’-diphosphate; NECA, 5’-(N-ethylcarboxamido)adenosine; TTX, tetrodotoxin; PPADS, pyridoxalphosphate-6-azophenyl-2’,4’-disulfonic acid; BPS/IC, bladder pain syndrome/interstitial cystitis; EFS, electrical field stimulation

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the P2Y6 purinoceptor has been suggested to be involved in urothelial release studies in both urothelial cells and urinary bladder tissue, where ATP appears to induce the release of acetylcholine, e.g. from the urothelium or suburothelium (Andersson and Yoshida, 2003; Yokoyama et al., 2005). Hence, interactions between the cholinergic transmitter system and other signalling systems that may stimulate the release of non-neuronal acetylcholine is of scientific interest.

Crosstalk between the purinergic and cholinergic transmitter systems, where ATP appears to induce the release of acetylcholine, supporting purinergic bladder contraction both in vivo and in vitro, has been reported previously (Aronsson et al., 2014; Stenqvist et al., 2017). In vitro studies suggest this atropine sensitive part of the ATP-induced contractile response to emanate from the urothelium (Stenqvist et al., 2018; Stenqvist et al., 2017). This is further supported by transmitter release studies in both urothelial cells and urinary bladder tissue, where the P2Y6 purinoceptor has been suggested to be involved in urothelial release of acetylcholine (Hanna-Mitchell et al., 2007; Silva et al., 2015). However, receptors functionally involved in this interaction are yet to be identified and possible neuronal involvement ought to be further examined.

The present study aims to further unravel the involvement of the urothelium and efferent neurons in the functional purinoergically evoked release of acetylcholine in vitro. Additionally, relevant receptor subtypes in this functional interaction will be investigated.

2. Materials and methodology

2.1. Animal procedures

The following experimental procedures were approved by the Animal Ethics Committee in Gothenburg (permit numbers 196-13 and 1794/18). A total number of 33 male Sprague Dawley rats (400–700 g) were presently used.

2.2. In vitro organ bath experiments

The rats were euthanized using an intraperitoneal overdose of pentobarbitaline (> 60 mg/kg; APL, Stockholm, Sweden) followed by excision of the heart. Subsequently, the urinary bladders were excised and kept in Krebs solution (CaCl₂ 1.25 mM, glucose 5.5 mM, KCl 4.6 mM, KH₂PO₄ 1.15 mM, MgSO₄ 1.15 mM, NaCl 118 mM and NaHCO₃ 25 mM) at all times. The experiments were carried out using an organ bath set-up with full-thickness bladder strips (6 × 2 mm), excised proximal to the ureters and above the trigone. The bladder strip preparations were mounted in 25 mL organ baths between a fixed hook and an adjustable steel rod coupled to an isometric force transducer. AcqKnowledge software (Biopac Systems, Goleta, USA) and a MP100WSW data acquisition system were used to record bladder contractions and relaxations. The temperature of the baths was kept at a constant level of 38 °C using a warm-water circuit. Continuous gassing with 5% carbon dioxide in oxygen kept the pH of the Krebs solution at a constant level of 7.4. The bladder strip preparations were pre-stretched to 10 mN and a stable baseline at approximately 5 mN was achieved after 45 min. Administration of high potassium Krebs solution (124 mM, sodium exchanged for an equimolar amount of potassium) was used as a reference for maximal contraction and was administered at the beginning and at the end of each experimental session. All drugs were administered in a volume of 125 μL, thus being diluted 200 times resulting in the concentration specified. Agonists were administered cumulatively, and antagonists and tetrodotoxin (TTX, Tocris Bioscience, Bristol, United Kingdom) were allowed to equilibrate for 20 min before further stimulation.

2.2.1. Neuronal and urothelial involvement

Electrical field stimulation (EFS; 2–40 Hz, at a supramaximal voltage of 50 V and a square wave pulse duration of 0.8 ms), and pharmacologically active substances (ATP; 10⁻⁶–10⁻³ M or methacholine; 10⁻⁸–10⁻³ M, performed in separate groups) administered directly into the organ bath in a cumulative manner, were employed. Stimulation was repeated after blockage of voltage-gated sodium channels using the neurotoxin TTX (5 × 10⁻⁷ M). Finally, agonist stimulation was performed in the presence of both the muscarinic antagonist atropine (10⁻⁶ M) and TTX.

Experiments on purinergic effects were performed using strip preparations from either intact- or denuded (collagenase I, 0.1% in saline, 30 min) rat urinary bladders, according to an established protocol previously shown to remove 80–90% of the urothelium without damaging the suburothelium (Andersson et al., 2008).

2.2.2. Contractile receptor characterization

The stable ATP-analogue α,β-methyleneadenosine 5’-triphosphate (αβMeATP; 10⁻⁷–10⁻³ M; Tocris Bioscience, Bristol, United Kingdom) was used as an agonist, i.e. administered once in a cumulative fashion, in the presence and absence of atropine (10⁻⁶ M). In order to confirm that no desensitization of the purinergic receptors had occurred after the administration of αβMeATP, experiments were conducted where this administration was repeated (in the absence and presence of atropine).

ATP (10⁻⁶–10⁻³ M) was administered directly into the organ baths and repeated in the presence of the P2Y₂ antagonist N,N″-1,4-butane-diyli bis[N’-(3-isothiocyanatophenyl)]thio urea (MRS2578; 10⁻⁷ and 10⁻⁵ M; Tocris Bioscience, Bristol, United Kingdom) alone or in combination with atropine (10⁻⁶ M). Since MRS2578 was diluted in dimethyl sulfoxide (DMSO), a subset of control experiments was conducted where the administrations of ATP were repeated in the presence of a corresponding volume of DMSO (125 μL, present for 20 min).

Also, contractions to ATP (10⁻⁶–10⁻³ M) were studied in the absence and presence of the P₂X₇ antagonist pyridoxal-phosphate-6-azophenyl-2’,4’-disulfonic acid (PPADS; 3 × 10⁻⁵ M) alone or in combination with atropine (10⁻⁶ M).

2.2.3. Relaxatory receptor characterization

Relaxatory responses were examined in bladder strip preparations pre-contracted with medium potassium Krebs solution (50 mM potassium, sodium exchanged for potassium), and the resulting relaxations were measured as change compared to baseline at pre-contraction. Responses to ATP (10⁻⁶–10⁻³ M) were recorded before and after desensitization of the (mainly) P₂X₁/₃-receptors using αβMeATP (10⁻⁵ M, repeated 3–5 times until no further contractile response was observed) (Ralevic and Burnstock, 1998). ATP was subsequently administered in the presence of both αβMeATP (10⁻⁵ M, repeated until no contractile response could be observed) and atropine (10⁻⁶ M).

Relaxation experiments to ADP (10⁻⁶–10⁻³ M) or NECA (10⁻⁸–10⁻⁵ M; Tocris Bioscience, Bristol, United Kingdom) were also conducted and repeated in the presence of atropine (10⁻⁶ M).

All substances used were purchased from Sigma-Aldrich, St Louis, MO, USA unless otherwise stated.

2.3. Statistical analysis

Statistical analysis was performed using repeated measurements analysis of variance (2-way ANOVA) and the Bonferroni post-hoc test. The graphs are presented as sigmoidal regression analysis curves and were generated using the GraphPad Prism software for Windows (GraphPad Software, Inc., San Diego, US). The data is presented as mean ± S.E.M. and p-values < 0.05 were regarded as significantly different.
3. Results

3.1. Neuronal and urothelial involvement

EFS induced frequency dependent contractions that were almost completely abolished in the presence of TTX (5 × 10\(^{-7}\) M) in both intact (control) and urothelium-denuded bladder strip preparations (e.g. from 26.72 ± 2.88 to 1.60 ± 0.11 mN in intact controls at a frequency of 40 Hz, in the absence and presence of TTX respectively, p < 0.001, n = 6, Fig. 1a, c). The remaining response was not further affected by the addition of atropine. Similar responses were seen in urothelium-denuded bladders, where the contraction to EFS was reduced from 23.97 ± 9.01 to 1.41 ± 0.52 mN at 40 Hz (p < 0.001, n = 5, Fig. 1b).

Administration of ATP (10\(^{-6}\)–10\(^{-3}\) M) or methacholine (10\(^{-8}\)–10\(^{-3}\) M) evoked concentration-dependent contractile responses. Methacholine-induced responses were not affected by the administration of TTX (maximal contraction of 46.22 ± 1.56 mN and 49.86 ± 2.12 mN before and after TTX-treatment respectively, n.s., n = 6; Fig. 2) but was, as expected, significantly reduced in the presence of atropine (from 46.22 ± 1.56 mN to 9.38 ± 2.93 mN at a concentration of 10\(^{-8}\) M methacholine, in the absence and presence of atropine, 10\(^{-6}\) M, respectively, p < 0.001, n = 6, Fig. 2). Nor were the purinergic responses affected by TTX (1.70 ± 0.60 mN in comparison to 1.54 ± 0.52 mN in the presence and absence of atropine, 10\(^{-6}\) M, respectively, p < 0.001, n = 7; Fig. 3a). However, the ATP-evoked contractile responses were significantly reduced after the subsequent addition of atropine (from 1.70 ± 0.60 mN to 0.68 ± 0.18 mN at a concentration of 10\(^{-8}\) M ATP, in the absence and presence of atropine respectively, p < 0.05, n = 7, Fig. 3a).

Similar to the observations in intact bladder strip preparations, the purinergic responses in urothelium-denuded bladders were unchanged in the presence of TTX (maximal contraction of 1.54 ± 0.59 mN and 2.25 ± 0.90 mN before and after TTX-administration respectively, n.s., n = 7, Fig. 3b). Interestingly, in contrast to controls the subsequent addition of atropine did not significantly affect the ATP-evoked responses in urothelium-denuded bladders (1.54 ± 0.59 mN in comparison to 1.90 ± 0.80 mN in the presence of atropine, at a concentration of 10\(^{-8}\) M ATP, n.s., n = 7, Fig. 3b).

3.2. Contractile receptor characterization

Cumulative administration of the stable ATP analogue αβMeATP (10\(^{-7}\)–10\(^{-5}\) M) yielded concentration-dependent contractions seemingly greater than that of ATP. This purinergic contraction was sensitive to atropine (10\(^{-6}\) M; 13.67 ± 1.75 mN in comparison to 10.33 ± 1.33 mN in the presence of atropine, at a concentration of 10\(^{-5}\) M αβMeATP, p < 0.05, n = 12, Fig. 4). In these experiments αβMeATP was first re-administrated in the absence of atropine to
ascertain that any reduction in contraction could not be ascribed to
desensitizing effects of the agonist itself. No difference was seen be-
tween the first and second applications (αβMeATP 10−5 M; 13.67 ± 1.75 mN in comparison to 13.60 ± 1.80 mN, n.s., n = 12, Fig. 4). In separate controlexperiments, wherenore-administration of αβMeATP was performed, the addition of atropine similarly reduced the αβMeATP-evoked contractile responses (αβMeATP 10 −5 M; from 14.78 ± 3.00 mN to 11.19 ± 3.54 mN in the absence and presence of atropine 10−6 M, p < 0.05, n = 4).

Contractions to ATP (10 −6–10−3 M) were not significantly altered by addition of the P2Y6 purinoceptor antagonist MRS2578 (10−7 or 10 −5 M). Rather, a trend towards increased contractions after P2Y6 blockade could be noted (ATP 10 −3 M; from 2.76 ± 0.60 mN to 4.43 ± 1.2 mN in the absence and presence of MRS2578 10−5 M, respectively, n.s., n = 4, Fig. 5). Furthermore, atropine still significantly lowered contractions to ATP during P2Y6 blockade (ATP 10−3 M; from 2.76 ± 0.60 mN to 1.43 ± 0.37 mN in the absence and presence of MRS2578 10−5 M administered together with atropine 10−6 M, respectively, p < 0.05, n = 10–11, Fig. 5).

3.3. Relaxatory receptor characterization

Relaxatory responses were investigated in tissues pre-contracted with medium potassium Krebs solution (50 mM). Concentration-dep-
dendent relaxations were observed to ATP (10−6–10−3 M), which re-
mained unchanged after desensitization of P2X purinoceptors by αβMeATP (repeated additions 3–5 times until no contractile response left). These purinergic relaxations were not sensitive to the muscarinic antagonist atropine (10−6 M; n = 6, Fig. 6a). Similar findings were made in the presence of the P2X purinoceptor antagonist PPADS (3 × 10−8 M) alone, as well as together with atropine (10−8 M; n = 6, Fig. 6b).

Similar to the observations for ATP, relaxatory responses to ADP or the P1 adenosine purinoceptor agonist NECA were unaffected by the administration of atropine (10−6 M; n = 6, Fig. 7a and b).

4. Discussion

In the current study, the involvement of the urothelium and efferent
neurons in the purinergically evoked release of acetylcholine in vitro
was examined. Furthermore, the contribution of different receptor
subtypes in this functional interplay was characterized.

The findings show that an ATP-induced release of acetylcholine with functional implications exists in the rat urinary bladder in vitro, resulting in purinergic contractions being significantly reduced by the muscarinic antagonist atropine. Similar observations in the rat and guinea pig bladder have previously been made in vivo (Aronsson et al., 2014; Sjogren and Andersson, 1979), but this phenomenon seems to be species specific and has not been observed in bladders from cat or rabbit (Liu et al., 1998; Theobald Jr., 1983). Interestingly, similar inter-
actions between the purinergic and the cholinergic transmitter sys-
tems appear to occur in different parts of the intestine for some species, for instance in the mouse distal colon (Moody and Burnstock, 1982; Zizzo et al., 2007).

Previous functional studies of the ATP-evoked release of acet-
ylcholine have not fully clarified from where this release emanates, although suggestions of an urothelial origin have been presented pre-
viously (Stenqvist et al., 2017). This is supported by the current results.
Namely, the presence of TTX, in a concentration abolishing EFS-evoked contractions, still allowed for atropine to cause a significant reduction in contractile response not only to cholinergic stimulation, but also to purinergic. The fact that the ATP-induced in vitro responses were TTX-insensitive strengthens the notion that ATP can exert its effects by stimulating the release of another neuromodulator from a non-neuronal source, in addition to direct stimulation of the detrusor (Aronsson et al., 2010; McMurray et al., 1998). Consequently, in urothelium-denuded bladders the ATP-induced release of acetylcholine appeared to be abolished, supporting our previous finding that this interaction may emanate from the urothelium (Stenqvist et al., 2018; Stenqvist et al., 2017). Furthermore, an urothelial origin seems likely as the enzymes responsible for synthesis of acetylcholine; namely choline acetyltransferase (ChAT) and carnitine acetyltransferase (CRAT) respectively, are expressed both in detrusor and urothelial cells. Moreover, urothelial cells express the organic cation transporter OCT3, which is important for non-neuronal release of acetylcholine (Hanna-Mitchell et al., 2007). Previous studies have also shown an ATP-induced release of acetylcholine to occur from urothelial cells (Silva et al., 2015). However, the suburothelium cannot be excluded as a complementary source for purinergic release of acetylcholine as the myofibroblasts have been shown to express purinergic receptors and positive stainings for ChAT in the lamina propria have been presented previously (Sui et al., 2006; Yoshida et al., 2006).

In order to characterize possible receptors involved in ATP-evoked release of acetylcholine the effects of various purinoreceptor ligands were examined. When administered as an agonist, the stable ATP-analogue αβMeATP evoked contractions that, similar to ATP, were sensitive to atropine. In the concentrations presently administered, this reduction in contraction can seem to be smaller than the relative effect on ATP-evoked functional response. This could be due to a number of reasons, such as possible differences between the two agonists in terms of affinity, receptor activation, magnitude of response, relaxatory components etc. It clearly shows, however, that atropine-sensitive purinergic contractions are, at least to a large extent, mediated by ATP rather than its metabolites. This since αβMeATP acts exclusively on P2X purinoreceptors, with P2X1 and P2X3 being regarded as principal targets (Ralevic and Burnstock, 1998). The potential importance of other purinoreceptors stimulated by metabolites of ATP was further investigated by employing P2Y and P1 (i.e. “non-P2X”) selective agonists and/or antagonists. P2Y6 purinoreceptor blockade with MRS2578 did not alter functional contractile responses to ATP, in contrast to what could possibly have been expected based on transmitter release data from previous studies (Hanna-Mitchell et al., 2007). Since atropine could readily affect the functional response to ATP, even under P2Y6 blockade, this purinoreceptor cannot currently be shown to alone play a functionally important role in the purinergic-cholinergic interaction. Control experiments where ATP was administered in the presence of DMSO, to rule out any interference due to the dilution of MRS2578 in DMSO, showed no effect. Thus, DMSO itself was deemed to have no impact on these results. Noteworthy, a tendency towards an increase in purinergic contractile response was observed in the presence of MRS2578, which indicates that the P2Y6-purinoreceptor may be involved in bladder relaxation.

The finding that neither P2X purinoreceptor desensitization with αβMeATP, nor the further addition of atropine altered the relaxatory response in pre-contracted bladder strip preparations confirms that the P2X purinoreceptors (likely mainly of the P2X1 and P2X3 subtypes) are not fundamental for functional relaxations to ATP, which was expected as the P2X-purinoreceptors are generally considered to mediate contractile responses (Bolego et al., 1995; Ralevic and Burnstock, 1998). Similar observations were made after a more general P2X purinoreceptor blockade with PPADS. Thus, any purinergically mediated release of acetylcholine seems to lack importance in the relaxatory response to ATP, and there is no obvious indication that other receptors than the P2X purinoreceptors are involved in the interaction between ATP and acetylcholine. However to rule this out, the direct functional relaxatory effects to the metabolites of ATP, namely ADP and the P1 adenosine

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**Fig. 6.** Mean relaxatory responses to ATP (10^{-5}–10^{-3} M) a) before (open symbols) and after (closed symbols) desensitization of P2X purinoceptors with αβMeATP (10^{-5} M), administered alone () and in combination with atropine (•; 10^{-5} M). b) Before (open symbols) and after (closed symbols) the blockade of P2X purinoceptors with PPADS (30 μM) administered alone (▲) and in combination with atropine (▼; 10^{-5} M). The data is presented as mean difference in relaxatory responses to the baseline at pre-contraction. n = 6 for each group. Vertical bars indicate S.E.M.

**Fig. 7.** Mean P2Y and P1 relaxatory responses to a) ADP (10^{-5}–10^{-3} M, or b) NECA (10^{-5}–10^{-5} M) in the absence (square) and presence (circle) of atropine (10^{-5} M). The data is presented as mean difference in relaxatory responses to the baseline at pre-contraction. n = 6 for each group. Vertical bars indicate S.E.M.
purinoreceptor agonist NECA, were examined. Neither were sensitive to atropine.

The fact that ATP may act as a modulator on the urothelium, initiating the release of other neurotransmitters, has previously been shown in cell cultures and urinary bladder tissue (Hanna-Mitchell et al., 2007; Silva et al., 2015). However, this is, to our knowledge, the first study that sheds some light on what receptor subtypes that are involved in the ATP-induced release of urothelial acetylcholine to such an extent that it may functionally stimulate smooth muscle contraction in vitro. Furthermore, urothelial acetylcholine is of great interest in the mictrition reflex arc as it may exert its effect on afferent or efferent nerve endings located near the urothelium or suburothelium (de Groat, 2006). It may also affect myofibroblasts of the lamina propria as well as stimulate muscarinic and nicotinic receptors directly on the urothelium, which in turn might lead to the release of other neuromodulators such as nitric oxide and ATP (M. C. Andersson et al., 2008; Sui et al., 2014). Thus, the interaction between the purinergic and the cholinergic transmitter system may exist at several levels of the mictrition reflex arc and further studies are needed in order to fully characterize this important functional link.

5. Conclusions

The purinergic contractile response is supported by the cholinergic transmitter system in vitro. This ATP-induced release of acetylcholine seems to emanate from the urothelium (or possibly the suburothelium) and appears to be independent of nerve transmission (TTX-insensitive). Several purinergic receptors involved in this interaction seem to be the P2X2-subtype, most likely P2X1 and/or P2X3.

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