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Behavioral/Systems/Cognitive

Identification of Atropine- and P2X1 Receptor Antagonist-Resistant, Neurogenic Contractions of the Urinary Bladder

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Acetylcholine and ATP are excitatory cotransmitters in parasympathetic nerves. We used P2X1 receptor antagonists to further characterize the purinergic component of neurotransmission in isolated detrusor muscle of guinea pig urinary bladder. In the presence of atropine (1 μM) and prazosin (100 nM), pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) (0.1–100 μM) and suramin (1–300 μM) inhibited contractions evoked by 4 Hz nerve stimulation in a concentration-dependent manner (IC50 of 6.9 and 13.4 μM, respectively). Maximum inhibition was 50 – 60%, which was unaffected by coadministration of the ectonucleotidase inhibitor ARL67156 (1–300 μM). PPADS and suramin also reduced contractions to exogenous ATP (300 μM) by 40 – 50%, but abolished those to the P2X1 agonist α,β-methyleneATP (α,β-meATP) (1 μM). The P2X1 antagonists reactive blue 2, NF279 (8,8′-[carbonylbis(imino-4,1-phenylenecarboxyliminoo)-4,1-phenylenecarboxylinocarbonyl]) bis-1,3,5-naphthalenetrisulfonic acid), MRS2159 (pyridoxal-α5-phosphate-6-phencylazo-4-carboxylic acid) (100 μM), and NF449 [(carbonylbis(5,1,3-benzenetriylbis(carbonylimino)))tetrakis-benzene-1,3-disulfonic acid] (3 μM) abolished contractions to α,β-meATP (1 μM; n = 4 – 5), but only reduced contractions evoked by 4 Hz nerve stimulation by ~40 – 60% (n = 4 – 6) and ATP by 30 – 60% (n = 4 – 7). However, prolonged exposure to α,β-meATP (50 μM) abolished contractions evoked by all three stimuli (n = 5 – 12). PPADS (100 μM) and suramin (300 μM) reduced the peak neurogenic contraction of the mouse urinary bladder to 30 – 40% of control. At the same concentrations, the P2X1 antagonists abolished the nonadrenergic, purinergic component of neurogenic contractions in the guinea pig vas deferens (n = 4 – 5). Thus, P2X1 receptor antagonists inhibit, but do not abolish, the noncholinergic component of neurogenic contractions of guinea pig and mouse urinary bladder, indicating a second mode of action of neuronally released ATP. This has important implications for treatment of dysfunctional urinary bladder, for which this atropine- and P2X1 receptor-resistant site represents a novel therapeutic target.

Key words: parasympathetic; urinary bladder; noncholinergic; ATP; P2X1 receptor; P2X4 receptor

Introduction

Dysfunction of the urinary bladder is a major and ever expanding condition for which highly effective therapeutic options are still limited (de Groat and Yoshimura, 2001; Andersson and Wein, 2004; Fry et al., 2005). Parasympathetic nerves provide the major excitatory innervation to the urinary bladder and mediate contraction of the detrusor smooth muscle and voiding of urine (Brading, 1987). The classical neurotransmitter released by these nerves is acetylcholine (ACh), which acts at postjunctional muscarinic receptors, and these are the main target for drugs currently used to treat dysfunctional bladder. However, as long as ago as the 19th century, a large component of parasympathetic contractions of the urinary bladder was recognized to be resistant to the muscarinic receptor antagonist atropine in many species (Langley and Anderson, 1895), and it is now accepted to be mediated by a non-adrenergic, noncholinergic (NANC) neurotransmitter (Burnstock, 2001). In 1983, Kasakov and Burnstock showed that the atropine-resistant contractions in the guinea pig were abolished by desensitization of purinergic P2 receptors by the ATP analog α,β-methyleneATP (α,β-meATP) and so proposed ATP as the NANC cotransmitter. The inhibitory effect of α,β-meATP has since been confirmed in a variety of species, and the P2X1 receptor, a ligand-gated cation channel, has been identified as its site of action (Burnstock, 2001; Kennedy, 2001). Thus, ACh, acting via muscarinic receptors, and ATP, acting through P2X1 receptors, are widely accepted to be excitatory cotransmitters in the urinary bladder.

In early experiments, desensitization of P2X1 receptors was used because of the lack of P2X receptor antagonists at that time. Suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) were the first useful antagonists to appear, and, although they did inhibit the NANC contractions in the guinea pig (Hoyle et al., 1990; Creed et al., 1994; Ziganshin et al., 2002), they were not as effective as α,β-meATP. This may reflect...
their low potency at and limited selectivity between P2 receptor subtypes (Khakh et al., 2001; Lambrecht et al., 2002) or their ability to inhibit breakdown of ATP by ectonucleotidases (eNTPases) present on the extracellular surface of smooth muscle cells (Hourani and Chown, 1989; Khakh et al., 1995; Ziganshin et al., 1995; Bültmann et al., 1999; Lambrecht et al., 2002). In recent years, more potent and selective P2X antagonists have been developed (Kim et al., 1998, 2001; Lambrecht et al., 2002). Therefore, the aim of the present study was to characterize the purinergic component of neurogenic contractions of the guinea pig isolated urinary bladder, using a range of P2X receptor antagonists.

Materials and Methods

Albino male Dunkin Hartley guinea pigs (250–400 g) were killed by asphyxiation with CO₂ and cervical dislocation. The urinary bladder was removed and cleaned of connective tissue, and four longitudinal strips (~12 mm long and 3 mm wide) consisting of smooth muscle and urothelium were prepared as described by Westfall et al. (1997). These were mounted under isometric conditions in 2 ml horizontal baths and allowed to equilibrate under a resting tension of 1 g at 35°C for 1 h in a physiological salt solution containing the following (in mM): 118.4 NaCl, 25 NaHCO₃, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 2.5 CaCl₂, and 11 glucose (bubbled with 95% O₂, 5% CO₂). Atropine (1 µM) and prazosin (100 nM) were present throughout. Tension was recorded via Dynamometer transducers (Echometer, Wichita Falls, TX) connected to a Grass Instruments (Quincy, MA) polygraph or Powerlab/4e system (ADInstruments, Castle Hill, Australia) using Chart 4.2.4 software (ADInstruments). Intramural nerves were stimulated by electrical field stimulation (EFS) at 4 Hz for 20 s and supramaximal voltage, via two parallel, platinum wire electrodes, using a Grass Instruments S44 stimulator and Grass Instruments SIU5E. Initially, a pulse width of 0.5 ms was used, but the contraction elicited was only inhibited by 85.8 ± 1.3% (n = 9) by tetrodotoxin (TTX) (1 µM), indicating a possible non-neurogenic component to the contractions. Reducing the pulse width to 0.15 ms elicited contractions, which were abolished by TTX (1 µM), and so in all experiments reported below this lower pulse width was used. Time-matched controls showed that the contractions decreased by ~10% on average over the time course of the experiments described. At the end of all experiments, TTX (1 µM) was added to confirm that the responses were neurogenic. In one series of experiments, the urinary bladder of male Olac MF1 mice (4–8 weeks old) was isolated, mounted, stimulated, and recorded from in the same manner as described above, except that a single preparation was obtained from each animal and the urothelial layer was always removed.

Drugs

PPADS (0.1–100 µM) and suramin (1–300 µM) against the atropine-resistant contractions induced by 4 Hz EFS. Both antagonists inhibited the responses in a concentration-dependent manner (Figs. 1, 2, Table 1), but at the highest concentrations of antagonists used, the peak contraction was only depressed by 50–60%. The remaining responses were abolished by TTX (1 µM) (Figs. 1a, 2a), confirming their neurogenic origin. To determine whether the antagonist-resistant component to neurotransmission was specific to 4 Hz stimulation, the effects of antagonists on contractions elicited by EFS at 2 Hz, 0.15 ms, 20 s (mean peak amplitude of 2861 ± 282 mg; n = 7) were studied. Under these conditions, PPADS (0.1–100 µM) and suramin (1–300 µM) again inhibited the neurogenic contractions in a concentration-dependent manner, but by only 40–50% (Fig. 3).

To confirm that PPADS and suramin fully blocked P2X receptors at the concentrations used, their effects on responses elicited by equi-effective concentrations of the P2X agonists α,β-meATP and ATP were investigated. α,β-meATP (1 µM) and ATP (300 µM) evoked rapid, transient contractions with peak amplitudes of 3136 ± 162 mg (n = 10) and 3365 ± 404 mg (n = 12), respectively. These were inhibited by PPADS (0.1–100 µM) and suramin (1–300 µM) in a concentration-dependent manner (Figs. 1, 2, 4, Table 1). The responses to α,β-meATP were abol-
response to ATP was reduced by only 40–50%. The middle trace shows the response remaining after incubation with PPADS (100 μM) for 40 min, and the right trace shows that the contractions were abolished by TTX (1 μM) and suramin (300 μM; n = 6) (■), and α,β-meATP (1 μM; n = 5) (■). Error bars indicate SEM. Atropine (1 μM) and prazosin (100 nM) were present throughout.

Figure 2. The inhibitory effects of suramin. a, The left trace shows a control, noncholinergic contraction of a guinea pig isolated urinary bladder muscle strip evoked by EFS (4 Hz, 20 s), the middle trace shows the response remaining after incubation with suramin (300 μM) for 40 min, and the right trace shows that the contractions were abolished by TTX (1 μM) and prazosin (100 nM) for 40 min, indicating complete blockade of P2X receptors. These concentrations were then tested against the contractions evoked by 4 Hz EFS and ATP (300 μM). Reactive blue 2 (100 μM; n = 5), NF279 (100 μM; n = 6), MRS2159 (100 μM; n = 5), and NF449 (3 μM; n = 4), like PPADS (100 μM; n = 4) and suramin (300 μM; n = 4), reduced, but did not abolish, the atropine-resistant contractions evoked by EFS at 4 Hz (Fig. 5a). Suramin (p < 0.001) and MRS2159 (p < 0.01) were both significantly more effective than reactive blue 2, NF279, and NF449. The contractions elicited by ATP (300 μM) were also partially resistant to each of the antagonists (n = 4–7) (Fig. 5b), and reactive blue 2 and MRS2159 were significantly more effective than NF449 (p < 0.01). In each case, little reversal of the inhibition was seen on washout of the antagonists.

Genetic deletion of the P2X1 receptor abolishes noncholinergic, neuropathic contractions in mouse urinary bladder (Vial and Evans, 2000), so we next investigated the effects of P2X antagonists in mouse tissue to determine whether our data are species specific. EFS (4 Hz, 0.15 ms, 20 s) elicited contractions of mouse detrusor smooth muscle of 465 ± 68 mg (n = 8) peak amplitude. Atropine (1 μM) reduced this response by 24.3 ± 8.8% (p < 0.05; n = 3), but abolished contractions evoked by exogenous ACh (10 μM) (360 ± 81 mg; n = 6). In the presence of
The inhibitory effects of P2 receptor antagonists. 

The inability of P2X<sub>1</sub> receptor antagonists to abolish the atropine-resistant component of neurogenic contractions is inconsistent with the reported sensitivity of this response to prolonged administration of α,β-meATP; therefore, we reinvestigated the effects of α,β-meATP. α,β-meATP at 50 μM evoked a large, transient contraction of guinea pig detrusor smooth muscle (mean peak of 5513 ± 314 mg; n = 22) that returned to baseline over 5–8 min (Fig. 6). Addition of a further 50 μM α,β-meATP, 10 min after the first, induced a transient contraction that was 22.6 ± 2.2% of the initial response. After a total of 15 min exposure, α,β-meATP was washed out, and, 5 min later, responses to EFS [4 Hz; n = 12 (Fig. 6a)], ATP [300 μM; n = 5 (Fig. 6b)], and α,β-meATP [1 μM; n = 5 (data not shown)] were found to be abolished. However, contractions evoked by histamine (3 μM) were unaffected (mean peak of 3000 ± 15636 mg before and 2875 ± 736 mg after prolonged α,β-meATP; n = 4).

Possible mechanisms of resistance to P2X<sub>1</sub> blockade

The P2X<sub>1</sub> antagonists used above can also inhibit the breakdown of ATP by ectonucleotidases present on the extracellular surface of smooth muscle cells (Kakh et al., 1995; Bültmann et al., 1999; Lambrecht et al., 2002). By thus increasing the concentration of ATP at the synapse, this could counteract their inhibitory effects and so explain the apparent resistance of the contractions to EFS and exogenous ATP to P2X<sub>1</sub> receptor blockade. To test this hypothesis, the effects of PPADS were reinvestigated in the presence of the ectonucleotidase inhibitor ARL67156 (Westfall et al., 1997). However, PPADS (100 μM) inhibited contractions evoked by EFS at 4 Hz to a similar extent in the absence (47.8 ± 3.9% of control; n = 4) and presence (46.8 ± 4.2% of control; n = 4) of ARL67156 (100 μM).

Finally, the urothelial layer of the guinea-pig urinary bladder was present in all of the experiments described above and so might influence the response to EFS (Maggi, 1993; Fry et al., 2005). However, there was no significant difference in the inhibitory effects of PPADS (100 μM) on contractions evoked by 4 Hz EFS in paired muscle strips from the same animals in which the urothelium was either present (50.2 ± 4.8% of control) or had been dissected away (52.2 ± 3.0% of control; n = 5 each).
Discussion

These results confirm that ATP acting via P2X receptors and ACh acting through muscarinic receptors are excitatory cotransmitters in the guinea pig and mouse urinary bladder. However, they also identify a third component of the neurogenic contraction that is resistant to both atropine and P2X antagonists. This response was not attributable to the following: direct electrical stimulation of smooth muscle, because TTX abolished all contractions evoked by EFS; neuropeptides released from sensory nerves, because they play no role in neurogenic contractions in the guinea pig (Maggi, 1993); or adenosine, produced by breakdown of ATP, because adenosine induces relaxation of detrusor smooth muscle (Brown et al., 1979) and inhibits contractions evoked by exogenous ATP (300 μM) (8). The left traces show a control response to EFS or ATP. In the middle, α,β-meATP (50 μM) was added for 10 min, and then another 50 μM was added for an additional 5 min. The drug was then washed out, and the right traces show that, 5 min later, responses to EFS and ATP were abolished. Atropine (1 μM) and prazosin (100 nM) were present throughout. Note that the response to α,β-meATP (middle traces) are on a slower timescale than those to EFS and ATP (left and right traces) because of the very different durations of the responses evoked by these stimuli.

Figure 6. a, b. The inhibitory effects of prolonged administration of α,β-meATP on contractions of guinea pig isolated urinary bladder muscle strips evoked by EFS (4 Hz, 20 s) (a) and ATP (300 μM) (b). The left traces show a control response to EFS or ATP. In the middle, α,β-meATP (50 μM) was added for 10 min, and then another 50 μM was added for an additional 5 min. The drug was then washed out, and the right traces show that, 5 min later, responses to EFS and ATP were abolished. Atropine (1 μM) and prazosin (100 nM) were present throughout. Note that the response to α,β-meATP (middle traces) are on a slower timescale than those to EFS and ATP (left and right traces) because of the very different durations of the responses evoked by these stimuli.

Their IC50 values at P2X1 receptors (Khakh et al., 2001; Kim et al., 2001; Lambrecht et al., 2002) and abolished contractions of similar amplitude evoked by the P2X1 agonist α,β-meATP. It is also unlikely to be attributable to rapid dissociation of the antagonists from the receptor, so allowing access to ATP, because the antagonists showed little reversibility on washout. The guinea pig P2X1 receptor has yet to be cloned, so it could have different pharmacological properties from the human, rat, and mouse P2X1 receptors. However, the agonist potencies of ATP and α,β-meATP at these isoforms are essentially identical (Khakh et al., 2001; Lambrecht et al., 2002). Antagonist potencies are also very similar, e.g., NF023 inhibited α,β-meATP at recombinant rat and human P2X1 receptors with IC50 values of 0.24 and 0.21 μM, respectively (Soto et al., 1999). In our study, the antagonists also abolished the P2X1 receptor-mediated, purinergic component of sympathetic contractions of the guinea pig vas deferens, indicating that access to the receptors is not restricted. Interestingly, several analogs of PPADS inhibited neurogenic contractions in the guinea pig vas deferens, but not the bladder (Kim et al., 1998), consistent with the differences in antagonist activity in the two tissues seen here.

The P2X1 antagonist-resistant component is also unlikely to be attributable to the antagonists inhibiting ATP breakdown by eNTPDases (Bültmann et al., 1999; Iqbal et al., 2005), leading to an increase in the concentration of ATP at the synapse, such that the inhibition by the P2X1 antagonists was partially reversed (Kennedy and Leff, 1995). The inhibition of neurogenic contractions by PPADS was unchanged by the eNTPDase inhibitor ARL67156, which inhibits ATP breakdown (Westfall et al., 1997). Additionally, suramin has only a small inhibitory effect on ATP breakdown in guinea pig detrusor and reactive blader 2 is ineffective (Hourani and Chown, 1989; Bailey and Hourani, 1994).

Here we constructed full PPADS and suramin concentration-inhibition curves and showed that the depression of the atropine-resistant neurogenic contractions reached a maximum of ~50%. That the responses were not abolished was initially a surprise, because neuronally released ATP is accepted to act via P2X receptors to elicit detrusor contraction (Burnstock, 2001; de Groat and Yoshimura, 2001; Andersson and Wein, 2004; Fry et al., 2005). However, reexamination of previous studies, in which full curves were not constructed, shows that they are consistent with our findings. PPADS at 30 μM (Ziganshin et al., 2002) and 100 μM suramin (Hoyle et al., 1990) inhibited contractions in the guinea pig evoked at 4 Hz EFS by 30–40%. Similar effects were seen in rabbit (Ziganshin et al., 1993; Creed et al., 1994), rat (Tong et al., 1997; Hedge et al., 1998; Benkó et al., 2003; Knight and Burnstock, 2004), and sheep (Creed et al., 1994). The photolysable, irreversible P2X antagonist ANAPP (arylazidoaminopropionyl ATP) also depressed contractions elicited by 4 Hz EFS in guinea pig by approximately only half (Westfall et al., 1983). Thus, 40–50% of the peak noncholinergic, neurogenic contraction of the urinary bladder of a range of species is clearly unaffected by P2X1 receptor antagonists.

Contractions evoked by exogenous ATP were also partly resistant to P2X1 antagonists here, implying that at least two functional P2 receptor subtypes are expressed in guinea pig detrusor smooth muscle. Consistent with this, the concentration-response curve to ATP in this tissue (Welford et al., 1987) (C. Kennedy, unpublished data) and in humans (Hoyle et al., 1989) is biphasic. Similar antagonist-insensitive responses to ATP are also seen in the rat (Bolego et al., 1995; Benkó et al., 2003). In contrast, the response to ATP was abolished by prolonged exposure to α,β-meATP, as reported previously in the guinea pig (Kasakov et al., 1983); and so these data are consistent with ATP mediating the atropine- and P2X1 antagonist-resistant contractions.

The inability of the P2X receptor antagonists to abolish contractions evoked by EFS and ATP is unlikely to reflect incomplete blockade of the P2X1 receptors. The highest antagonist concentrations used were up to three orders of magnitude higher than the concentration of ATP.
and Burnstock, 1983), human (Hoyle et al., 1989), rat (Parija et al., 1991), and rabbit (Chancellor et al., 1992) (but see Chen et al., 1992). Why the P2X antagonist-resistant component is sensitive to inhibition by the P2X agonist α,β-meATP is unclear.

The identity of the P2X antagonist-resistant receptor is also unclear, but all other known P2X receptors, except for the P2X4, are sensitive to at least one of the antagonists used in this study (Khakh et al., 2001; Kim et al., 2001; Lambrecht et al., 2002). The P2X7 homomultimer is probably not involved, because suramin abolishes the purinergic excitatory junction potentials in the guinea pig (Hashitani and Suzuki, 1995) and genetic deletion of the P2X7 subunit abolishes noncholinergic, neurogenic contractions and responses to exogenous ATP in the mouse (Vial and Evans, 2000) (although a crucial role of P2X7 subunits in trafficking of P2X7 subunits to the plasma membrane cannot be ruled out). A role for a P2X4,α heteromultimer can, however, be considered. Rat P2X2 and P2X4 subunits coexpressed in Xenopus oocytes copurify and form a novel functional phenotype, at which α,β-meATP is a weak partial agonist (Nicke et al., 2005). This could explain the ability of α,β-meATP to inhibit the P2X7 antagonist-insensitive component of contractions to EFS and ATP in the urinary bladder. However, the sensitivity of the P2X7,α,β heteromer to suramin was much greater than that of the P2X7 homomer, although a full concentration–inhibition curve was not constructed. Clearly, more studies are required to determine the involvement of P2X7 subunits in parasympathetic neurotransmission.

It is unlikely that P2Y receptors mediate the P2X antagonist-resistant responses seen in the present study, although P2Y receptor agonists induce contraction of guinea pig (Bailey and Hou rani, 1994) and rat (Bolego et al., 1995; Naramatsu et al., 1997) bladder. Of the eight cloned P2Y receptors, ATP is an agonist at the P2Y1, P2Y2, P2Y4, and P2Y11 subtypes, but suramin and/or PPADS antagonize each of these (Abbracchio et al., 2006). Consistent with this, suramin abolished contractions in the guinea pig evoked by the P2Y agonists cytidine 5′-triphosphate, inosine 5′-triphosphate, and uridine 5′-triphosphate (UTP) but only partially inhibited those to ATP (Bailey and Hourani, 1994). Additionally, UTP-evoked contractions of rat detrusor were unaffected by prolonged exposure to α,β-meATP (Bolego et al., 1995). Additional experiments on guinea pig recombinant P2Y receptors, once cloned, are needed to confirm this conclusion.

In contrast to non-primates, ACh is the sole excitatory neurotransmitter in the healthy human urinary bladder (Fry et al., 2005), so what is the relevance of the present data to humans? The potential importance is attributable to the appearance of atropine-resistant contractions in unhealthy human bladder conditions, such as interstitial cystitis and idiopathic detrusor instability (IDI) (Fry et al., 2005). These contractions are abolished by prolonged exposure to α,β-meATP and so assumed to be mediated by ATP and P2X2 receptors, but this has not been confirmed using P2X2 receptor antagonists. Furthermore, although the P2X2 is the main P2X2 subunit present in human detrusor smooth muscle, its expression is unchanged in dysfunctional bladder (Moore et al., 2001; O’Reilly et al., 2002). It is notable that the P2X2 subunit is also expressed, and its colocalization with nerve varicosities more than doubles in tissue from adults with IDI (Moore et al., 2001; O’Reilly et al., 2002). Interestingly, pregnancy in rats is also associated with an increase in P2X2 subunit junctional clustering (Yunaev et al., 2000), and the PPADS-sensitive component of neurogenic contractions is smaller in pregnant rats (Knight and Burnstock, 2004).

Other proposed explanations for the appearance of a purinergic component of parasympathetic neurotransmission in human urinary bladder include that more ATP is released from motor nerves and that ATP is broken down less effectively in the synapse (Fry et al., 2005). To these must be added the possibility that the purinergic component is not mediated by the P2X2 receptor alone, and the novel component postulated here plays a role. This has important implications for the search for new drugs to treat dysfunctional bladder because it identifies a new therapeutic target for what is a major and expanding therapeutic problem. Furthermore, a receptor that is only functional in dysfunctional urinary bladder is an attractive target for drug development.

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