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The ecto-ATPase inhibitor ARL 67156 enhances parasympathetic neurotransmission in the guinea-pig urinary bladder

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Abstract

The influence of enzymatic degradation on the neurotransmitter actions of ATP was studied using the ecto-ATPase inhibitor 6-*N*,*N*-diethyl-D-β,γ-dibromomethyleneATP (ARL 67156). Field stimulation of the parasympathetic nerves innervating guinea-pig urinary bladder muscle strips (1–8 Hz for 20 s) produced characteristic biphasic contractions, the peak magnitudes of which were significantly increased by 29–32% by ARL 67156 (100 μM). A similar degree of enhancement was seen in the presence of atropine (1 μM), consistent with ARL 67156 acting to enhance the action of neuronally released ATP. The effects of ARL 67156 reversed rapidly on washout of the drug. Contractions evoked by exogenous ATP (100 μM) were also potentiated by ARL 67156 (100 μM), but those to the stable analogue α,β-methyleneATP (5 μM) were unaffected. ARL 67156 (100 μM) also enhanced contractions to exogenous acetylcholine (1 μM) and histamine (3 μM), but this potentiation was abolished by pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) (100 μM). It is concluded that when ATP acts as a neurotransmitter its postjunctional actions are attenuated by enzymatic degradation. ARL 67156 inhibits this breakdown. © 1997 Elsevier Science B.V.

Keywords: ATP; Ecto-ATPase; ARL 67156; Purinergic; Urinary bladder

1. Introduction

Parasympathetic nerve stimulation causes contraction of the smooth muscle of the urinary bladder and so voiding of urine. In most species studied these neurogenic contractions are partially resistant to blockade of muscarinic receptors by atropine and substantial evidence supports the proposal that adenosine 5'-triphosphate (ATP) is the non-cholinergic excitatory neurotransmitter (see Anderson, 1993; Hoyle, 1996 for reviews). In the guinea-pig urinary bladder, excitatory junction potentials and a substantial portion of the initial, phasic component of neurogenic contractions are inhibited by selective desensitisation of P_{2X}-purinoceptors by α,β-methyleneATP (Kasakov and Burnstock, 1983; Moss and Burnstock, 1985; Fujji, 1988; Brading and Mostwin, 1989; Brading and Williams, 1990; Bramich and Brading, 1996) or by the P_{2X}-purinoceptor antagonist suramin (Hoyle et al., 1990; Creed et al., 1994; Hashitani and Suzuki, 1995). In contrast, acetylcholine contributes more to the smaller secondary, tonic phase of neurogenic contractions by acting at muscarinic M_3 receptors (Noronha-Blob et al., 1991) to stimulate production of 1,4,5-inositol trisphosphate and release of internal calcium stores (Iacovou et al., 1990).

Both acetylcholine and ATP are inactivated by breakdown. Ecto-nucleotidases which sequentially degrade ATP to adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate and adenosine are present in many tissues (see Ziganshin et al., 1994; Plesner, 1995), including smooth muscle cells of the guinea-pig urinary bladder (Cusack and Hourani, 1984; Welford et al., 1987; Hourani and Chown, 1989). The most important step in inactivation of ATP is the initial hydrolysis to produce ADP, which is less active at P_{2X}-purinoceptors. The extent to which this limits the neurotransmitter actions of ATP is not clear as, until recently, no selective inhibitor of ATP breakdown was available. However, a recently developed analogue of ATP, 6-*N*,*N*-diethyl-D-β,γ-dibromomethyleneATP (ARL 67156, formerly known as FPL 67156) inhibits ecto-ATPase in human blood cells (Crack et al., 1995) and the rat vas deferens (Khakh et al., 1995) and enhances exogenous

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ATP-evoked contractions of the rabbit ear artery and guinea-pig vas deferens, but not responses to the stable analogue α,β-methyleneATP (Crack et al., 1995; Westfall et al., 1996).

Recently we reported that ARL 67156 potentiates the purinergic component of neurogenic contractions of the guinea-pig vas deferens by up to twofold (Westfall et al., 1996), suggesting that the sympathetic neurotransmitter actions of ATP in this tissue are limited by extracellular breakdown. The aim of the present study was to use ARL 67156 to investigate the influence of breakdown of ATP on parasympathetic neurotransmission in the guinea-pig isolated urinary bladder. A preliminary account of these results has been published (Westfall et al., 1995).

2. Materials and methods

2.1. Tissue preparation and stimulation

Albino male guinea-pigs (250–400 g) were killed by asphyxiation with CO_2 and subsequent cervical dislocation. The bladder was removed and cleaned of connective tissue. From each bladder 2–4 longitudinal strips (approximately 12 mm long and 3 mm wide) consisting of smooth muscle and urothelium were prepared as described by Ambache and Zar (1970) and mounted in 2 ml horizontal baths. The tissues were allowed to equilibrate under a resting tension of 1 g at 35°C for 1 h in a physiological salt solution of the following composition (mM): NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 11; and bubbled with 95% O_2/5% CO_2. Tension was recorded using Scaime transducers connected via Universal amplifiers to a Gould pen recorder.

Intramural nerves were stimulated by electrical field stimulation for 20 s at 15 min intervals with a pulse width of 0.5 ms and supramaximal voltage, via parallel platinum electrodes, using a Grass S44 stimulator and SIU5F stimulus isolation unit. Neurogenic contractions were greatly depressed by tetrodotoxin (1 μM). The tetrodotoxin-resistant component could be inhibited by desensitisation of the P_{2X}-purinoceptor by α,β-methyleneATP, suggesting that it was due to direct depolarisation of nerve varicosities, causing neuronal release of ATP (unpublished observations, see also Brading and Williams, 1990).

Drugs were added directly to the tissue bathing solution and washed out by replacement with drug-free solution. When studying the effects of ARL 67156 or atropine on neurogenic contractions, three reproducible control responses were obtained before addition of the drug. Preliminary studies showed that ARL 67156 (100 μM) rapidly increased the amplitude of neurogenic contractions with maximal effect after 15 min. The potentiation was rapidly reversible, with responses returning close to control levels 15 min after washout of ARL 67156.

When examining the effects of ARL 67156 on responses to exogenous agonists, three reproducible control responses to ATP (100 μM), acetylcholine (1 μM) or histamine (3 μM) were obtained at 20 min intervals or 30 min intervals for α,β-methyleneATP (5 μM). ARL 67156 (100 μM) was then added 15 min before the fourth application of agonist. The concentrations of each agonist were determined in preliminary experiments to produce sub-maximal responses, of similar magnitude, on the linear portion of the concentration–effect curve. Similarly, when using pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) to study the interaction between ARL 67156 and exogenous acetylcholine or histamine, three reproducible control responses to acetylcholine or histamine were obtained, then PPADS (100 μM) was added for 40 min, before further agonist responses were evoked. In the continued presence of PPADS, ARL 67156 (100 μM) was added and 15 min later a further response to acetylcholine or histamine obtained.

2.2. Statistics

Values in the text refer to mean ± S.E.M. Statistical significance of the raw data was tested by Student's paired *t*-test and differences considered significant when $P < 0.05$.

2.3. Drugs

ATP (disodium salt), α,β-methyleneATP (lithium salt), atropine sulphate, acetylcholine chloride and histamine diphosphate (all Sigma), ARL 67156, provided by Astra, and PPADS (Tocris Cookson) were dissolved in distilled water and kept as 10^{-1} M stock solutions.

3. Results

3.1. Effects of ARL 67156 on neurogenic contractions

Field stimulation of the parasympathetic nerves of the guinea-pig urinary bladder with trains of pulses for 20 s at 1–8 Hz produced characteristic biphasic contractions (see Fig. 1a and Fig. 2), in a frequency-dependent manner. The initial, transient peak reached a maximum within 5 s, then fell to a lower level. In all cases the results reported below refer to the peak magnitude of the contraction.

We have previously shown that neurogenic contractions of the guinea-pig vas deferens are potentiated in a concentration-dependent manner by ARL 67156 from 5–100 μM (Westfall et al., 1996). ARL 67156 (100 μM) significantly increased contractions of the guinea-pig urinary bladder evoked by field stimulation at 1, 2, 4 and 8 Hz (Fig. 1a, b). The percentage increase of responses in the presence of ARL 67156 was approximately the same at all stimulation frequencies (1 Hz, 32 ± 6%; 2 Hz, 30 ± 6%; 4 Hz, 31 ± 6%; 8 Hz, 29 ± 6%).

In order to investigate the effect of ARL 67156 on

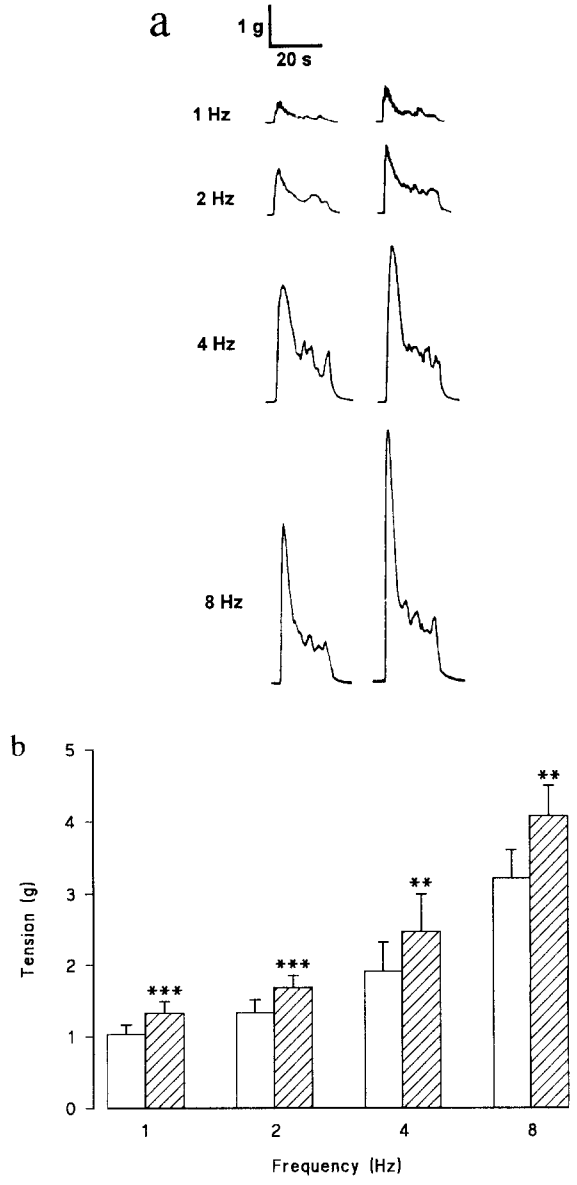

Fig. 1. The effect of ARL 67156 on neurogenic contractions evoked at 1, 2, 4 and 8 Hz for 20 s. (a) The traces on the left show control responses, and those on the right show responses after 15 min in the presence of ARL 67156 (100 μM). All responses are from the same muscle strip. (b) Open bars represent control responses and the cross-hatched bars those obtained in the presence of ARL 67156 (100 μM; $n = 8$). $^{**}P < 0.01$, $^{***}P < 0.001$.

neurogenic contractions in the absence of the cholinergic component, further experiments were performed in the presence of the muscarinic receptor antagonist atropine (Fig. 2). Atropine (1 μM) abolished contractions to acetylcholine (1 μM, not shown) and reduced the phasic component of neurogenic contraction at 4 Hz from 2.50 ± 0.38 g to 1.43 ± 0.23 g ($n = 6$; $P < 0.05$) (note that the secondary, predominantly cholinergic component was inhibited to a greater extent than the initial, predominantly purinergic component). The response to nerve stimulation in the presence of atropine was significantly increased by subsequent addition of ARL 67156 (100 μM) from 1.43 ± 0.23 g to 1.85 ± 0.36 g ($n = 6$, $P < 0.05$). The effect of ARL 67156 was rapidly reversible with responses returning close to control levels 15 min after washout.

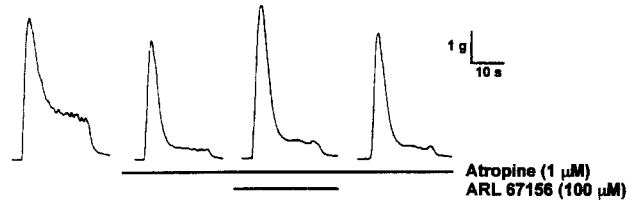

Fig. 2. The effect of ARL 67156 on neurogenic contractions at 4 Hz for 20 s in the presence of atropine. After the control response was obtained (first panel), atropine (1 μM) was added, and was then present throughout. 20 min later, nerve stimulation was repeated (second panel). ARL 67156 (100 μM) was then added and another neurogenic contraction obtained 15 min later (third panel). Finally, ARL 67156 was washed out and 15 min later nerve stimulation repeated (fourth panel).

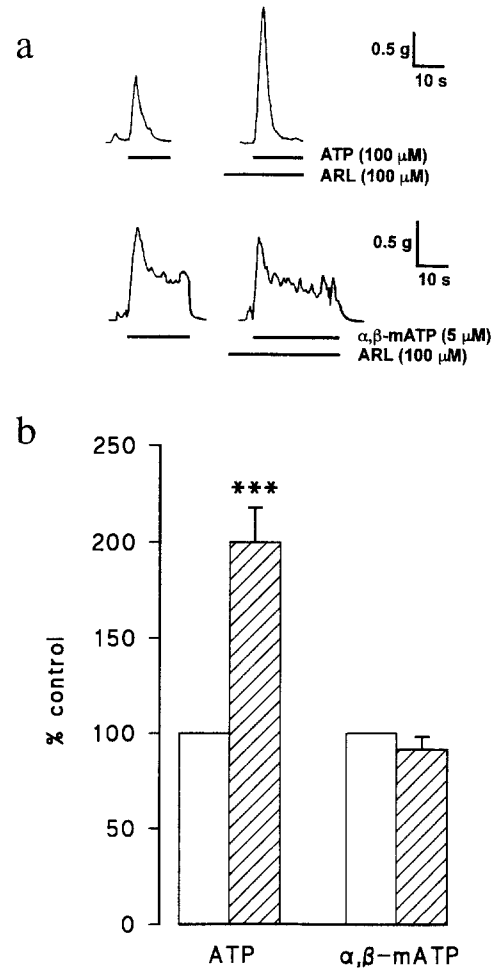

Fig. 3. The effect of ARL 67156 on contractions to exogenous ATP and α,β-methyleneATP. (a) Typical responses to ATP (100 μM) and α,β-methyleneATP (5 μM) before (left-hand traces) and 15 min after (right-hand traces) the addition of ARL 67156 (100 μM). (b) The mean magnitude of responses to ATP (100 μM, $n = 16$) and α,β-methyleneATP (5 μM, $n = 7$) after addition of ARL 67156 (100 μM, cross-hatched bars) expressed as a percentage of control responses (open bars). The statistical significance level shown was determined by analysis of the raw data expressed in grams. $^{***}P < 0.001$.

3.2. Effects of ARL 67156 on exogenous P_{2x}-purinoceptor agonists

To characterise the mode of action of ARL 67156, its effect on responses to sub-maximal concentrations of exogenous ATP (100 μM) and the stable analogue α,β-methyleneATP (5 μM) was tested. Both produced rapid, transient contractions of similar magnitude, which reached a peak in about 5 s (Fig. 3a). The response to exogenous ATP was greatly increased in the presence of ARL 67156 (100 μM), but that to α,β-methyleneATP was unaffected. Fig. 3b shows that the mean magnitude of the responses to ATP was significantly increased, whereas the mean response to α,β-methyleneATP was unchanged.

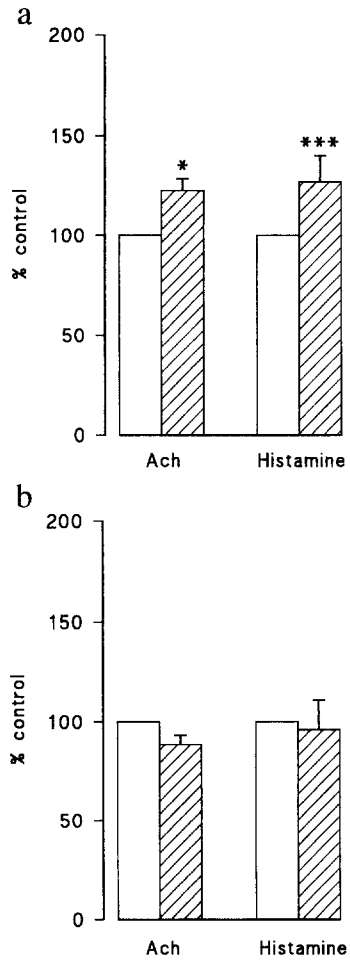

Fig. 4. The effect of ARL 67156 on contractions to acetylcholine and histamine. In each graph the response to acetylcholine (1 μM) and histamine (3 μM) in the presence of ARL 67156 (100 μM) (cross-hatched bar) is expressed as a percentage of the control response (open bar). Responses were obtained either (a) in the absence or (b) in the presence of PPADS (100 μM). The statistical significance level shown was determined by analysis of the raw data expressed in grams. $^{*}P < 0.05$, $^{***}P < 0.001$.

3.3. Effects of ARL 67156 on the responses to acetylcholine and histamine

The effect of ARL 67156 on the responses to sub-maximal concentrations of exogenous acetylcholine and histamine was also tested. ARL 67156 (100 μM) significantly increased the amplitude of contractions to acetylcholine (1 μM) and histamine (3 μM) (Fig. 4a). A similar increase in responses to non-P_{2X}-purinoceptor agonists was also seen in the guinea-pig vas deferens (Westfall et al., 1996). This was inhibited by the P_2-purinoceptor antagonist PPADS. Therefore, we examined the possibility of a similar mechanism occurring in the urinary bladder. In the presence of PPADS (100 μM), neither acetylcholine nor histamine were significantly affected by ARL 67156 (100 μM) (Fig. 4b).

4. Discussion

The results of this study show that ARL 67156 potentiates contractions of the guinea-pig isolated urinary bladder evoked by intramural nerve stimulation, both in the absence and presence of atropine. Exogenous ATP and α,β-methyleneATP both evoked rapid, transient contractions with a similar time-course to the atropine-resistant component of the neurogenic response, but ARL 67156 only potentiated contractions to ATP. This is consistent with the enhancing effect of ARL 67156 being due to inhibition of ATP breakdown and not to non-selective effects on the smooth muscle. If the latter were the case then responses to α,β-methyleneATP would also have been expected to be enhanced. These results suggest that rapid hydrolysis of ATP inhibits its neurotransmitter actions in this tissue.

In these experiments ARL 67156 enhanced neurogenic contractions at all frequencies of stimulation tested by a similar degree (29–32%). This contrasts with our previous data in the guinea-pig vas deferens where the potentiation induced by ARL 67156 was inversely related to frequency of sympathetic nerve stimulation (Westfall et al., 1996). In that study, ARL 67156 approximately doubled the peak amplitude of contractions evoked at 1 Hz, while at 8 Hz the increase was only about 30%, a value similar to that seen here in the urinary bladder. The reason for the smaller potentiation and the lack of frequency dependence in the urinary bladder is not known, but these differences could suggest that breakdown influences the neurotransmitter actions of ATP more in the vas deferens than in the urinary bladder.

In the present study, ARL 67156 could also slightly potentiate contractions evoked by acetylcholine and histamine, an effect that was inhibited by P_2-purinoceptor blockade by PPADS. A similar PPADS-sensitive potentiation of contractions to noradrenaline and KCl by ARL 67156 was seen in the guinea-pig vas deferens (Westfall et al., 1996), where it was suggested that this was due to build up of ATP in the tissue in the presence of ARL

67165, causing a general sensitisation of the smooth muscle cells. A similar mechanism may also be present in the urinary bladder.

In the guinea-pig urinary bladder, exogenous ATP is dephosphorylated by an ecto-ATPase which has a K_m of 532 μM and V_{max} of 1.1 nmol/min per mg (Welford et al., 1987). In our study, we used a concentration (100 μM) of ARL 67156 which is likely to have produced substantial, but not total inhibition of ecto-ATPase activity. ARL 67156 inhibits ecto-ATPase activity with a pIC_{50} of 4.62 in human blood cells (Crack et al., 1995) and 5.1 in the rat vas deferens (Khakh et al., 1995). In the former study, 100 μM ARL 67156 inhibited ecto-ATPase activity by approximately 70–80%. We could not use a range of concentrations of ARL 67156 as only limited amounts of the compound are available and even if this were not the case, it would not be desirable to use higher concentrations. ARL 67156 is a weak antagonist at P_{2X}-purinoceptors ($pA_2 = 3.3$, Crack et al., 1995), so at concentrations above 100 μM the antagonism of P_{2X}-purinoceptors would counter its potentiating action. Clearly, if a compound could be developed that completely inhibited breakdown of ATP, but had no effect at P_{2X}-purinoceptors, then even greater potentiation of the neurotransmitter action of ATP in the guinea-pig urinary bladder is likely.

In conclusion, the results of this study are consistent with the proposal that ATP is a neurotransmitter from parasympathetic nerves which innervate the guinea-pig urinary bladder. Extracellular breakdown of ATP appears to have a physiological role in terminating purinergic neurotransmission in this tissue, though its influence is less than in the guinea-pig vas deferens.

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