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The interaction of diadenosine polyphosphates with P_{2X}-receptors in the guinea-pig isolated vas deferens

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1 The site(s) at which diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AP_4A) and diadenosine 5′, 5‴-P^1,P^5-pentaphosphate (AP_5A) act to evoke contraction of the guinea-pig isolated vas deferens was studied by use of a series of P_2-receptor antagonists and the ecto-ATPase inhibitor 6-N,N-diethyl-D-β,γ-dibromomethyleneATP (ARL 67156).

2 Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) (300 nM – 30 μM), suramin (3 – 100 μM) and pyridoxal-5′-phosphate (P-5-P) (3 – 1000 μM) inhibited contractions evoked by equi-effective concentrations of AP_5A (3 μM), AP_4A (30 μM) and α,β-methyleneATP (α,β-meATP) (1 μM), in a concentration-dependent manner and abolished them at the highest concentrations used.

3 PPADS was more potent than suramin, which in turn was more potent than P-5-P. PPADS inhibited AP_5A, AP_4A and α,β-meATP with similar IC_{50} values. No significant difference was found between IC_{50} values for suramin against α,β-meATP and AP_5A or α,β-meATP and AP_4A, but suramin was more than 2.5 times more potent against AP_4A than AP_5A. P-5-P showed the same pattern of antagonism.

4 Desensitization of the P_{2X1}-receptor by α,β-meATP abolished contractions evoked by AP_5A (3 μM) and AP_4A (30 μM), but had no effect on those elicited by noradrenaline (100 μM).

5 ARL 67156 (100 μM) reversibly potentiated contractions evoked by AP_4A (30 μM) by 61%, but caused a small, significant decrease in the mean response to AP_5A (3 μM).

6 It is concluded that AP_4A and AP_5A act at the P_{2X1}-receptor, or a site similar to the P_{2X1}-receptor, to evoke contraction of the guinea-pig isolated vas deferens. Furthermore, the potency of AP_4A, but not AP_5A, appears to be inhibited by an ecto-enzyme which is sensitive to ARL 67156.

Keywords: AP_4A; AP_5A; ARL 67156; P_{2X}-receptor; vas deferens

Introduction

Diadenosine polyphosphates are naturally occurring molecules which are involved in numerous intracellular biochemical pathways. However, they may also be important extracellular signalling agents as the release of micromolar concentrations of compounds such as diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AP_4A) and diadenosine 5′,5‴-P^1,P^5-pentaphosphate (AP_5A) into the extracellular space can be measured from platelets (Flodgaard & Klenow, 1982; Schlüter *et al*., 1994), chromaffin cells (Pintor *et al*., 1991; Castillo *et al*., 1992) and neurones (Pintor *et al*., 1992). Furthermore, at these concentrations AP_4A and AP_5A have widespread extracellular actions, causing contraction of visceral and vascular smooth muscle, excitation of neurones, release of catecholamines from chromaffin cells and inhibition of platelet aggregation (see Ogilvie, 1992 for review).

AP_4A and AP_5A appear to produce their effects through a number of different receptor types and may act as agonists at P_{2X1}- (Evans *et al*., 1995; Ralevic *et al*., 1995), P_{2Y}- (Castro *et al*., 1992) and P_{2U}-receptors (Lazarowski *et al*., 1995) and antagonists at P_{2T}-receptors (see Cusack, 1993). The existence of a separate site which recognises di-, but not mono-adenosine nucleotides such as adenosine 5′-triphosphate (ATP), has also been proposed (Hilderman *et al*., 1991; Walker *et al*., 1993; Pintor *et al*., 1993; Pinto & Miras-Portugal, 1995) and the name P_{2D}-purinoceptor suggested (Pintor *et al*., 1993), (see Burnstock & King, 1996 for discussion of nomenclature). A further complicating factor is that some actions of AP_4A and AP_5A are inhibited by selective P_1-receptor antagonists (Klishin *et al*., 1994; Ziganshin *et al*., 1995; Hoyle *et al*., 1996; Rubino & Burnstock, 1996). It is not clear if this is due to a direct action of AP_4A and AP_5A on P_1-receptors or if it depends upon their breakdown to adenosine. Thus, great care must be taken when trying to classify the receptors at which AP_4A and AP_5A act.

AP_4A and AP_5A cause contraction of the guinea-pig vas deferens (Stone, 1981; MacKenzie *et al*., 1988; Bailey & Hourani, 1995; Hoyle *et al*., 1995b). It has been assumed that they act through the P_{2X1}-receptor as the contractions were similar to those elicited by α,β-methyleneATP (α,β-meATP), which is thought to act in this tissue via the recently cloned P_{2X1}-receptor (Evans *et al*., 1995). Also, the P_2-receptor antagonists suramin (Bailey & Hourani, 1995) and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) (Hoyle *et al*., 1995b) inhibited AP_4A and AP_5A, but only single or high antagonist concentrations were used. Thus, there is little quantitative data with which to characterize their site of action.

Another approach often used is agonist cross-desensitization. In mechanical studies AP_5A, but not AP_4A, mimicked the ability of α,β-meATP to desensitize the P_{2X1}-receptor and so inhibit the purinergic component of neurogenic contractions (MacKenzie *et al*., 1988). However, desensitization produced by AP_5A and α,β-meATP had different time-courses. Thus, it is not clear if AP_4A , AP_5A and α,β-meATP act at the same or separate receptors to elicit contraction in this tissue. In contrast, in an electrophysiological study, desensitization to AP_4A or AP_5A attenuated depolarization evoked by ATP (Hoyle *et al*., 1995a). The reason for the discrepancy between the two types of study is not known.

It is clear from the above that previous studies on the site(s) through which AP_4A and AP_5A act in the guinea-pig isolated vas deferens have been largely qualitative and have not reached the same conclusion. Therefore, the aim of this study was to characterize these site(s) quantitatively, by comparing the ability of a full range of concentrations of the P_2-receptor antagonists suramin, PPADS and pyridoxal-5′-phosphate (P-5-P) to inhibit their contractions and those to α,β-meATP. The

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influence of breakdown of AP_4A and AP_5A on their potency was also investigated with the ecto-ATPase inhibitor 6-N,N-diethyl-D-β,γ-dibromomethyleneATP (ARL 67156) (Crack *et al*., 1995; Westfall *et al*., 1996a). A preliminary account of these results has been published (Westfall *et al*., 1996b).

Methods

Albino male guinea-pigs (250–400 g) were killed by asphyxiation with CO_2 and subsequent cervical dislocation. The vasa deferentia were removed, cleaned of connective tissue 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; bubbled with 95% O_2, 5% CO_2. Tension was recorded by Scaime transducers connected via Universal amplifiers (Gould) to a pen recorder (Gould 2200S).

Concentration-response curves to AP_4A, AP_5A and ATP were obtained in individual tissues by addition of the drugs to the bath in a non-cumulative manner. Shortly after the contraction had reached a peak the agonist was washed out by several changes of the bathing solution. Care was taken to avoid desensitization by leaving 30 min before the next concentration of agonist was added.

The effects of suramin, PPADS and P-5-P on responses to exogenous agonists were examined in individual tissues. Three reproducible control responses to equi-effective concentrations of AP_5A (3 μM), AP_4A (30 μM) or α,β-meATP (1 μM) were obtained at 30 min intervals. The lowest concentration of suramin, PPADS or P-5-P used was then applied to the tissue for 30 min and the agonist readded. This procedure was repeated until steady state inhibition was reached. Thereafter, progressively higher concentrations of antagonist were administered in the same manner.

The effect of desensitization of the P_{2X}-receptor on contractions to AP_4A or AP_5A was determined as follows. Control responses to AP_5A (3 μM), AP_4A (30 μM) or noradrenaline (100 μM) were first obtained, then α,β-meATP (50 μM) was applied, evoking a large transient contraction. Once tension had returned to the baseline level a further 50 μM α,β-meATP was added. This procedure was repeated until the tissue had been exposed to a cumulative concentration of 200 μM α,β-meATP. The drug was washed out and when α,β-meATP (50 μM) was added two min later no contraction was seen, confirming that the P_{2X}-receptors had been desensitized. α,β-meATP was again washed out and two min later the test agonist administered.

Similarly, when the effects of ARL 67156 were studied, three reproducible control responses to AP_5A (3 μM) or AP_4A (30 μM) were obtained at 30 min intervals and ARL 67156 (100 μM) was then added 10 min before the fourth application of agonist. We have previously shown ARL 67156 to equilibrate within 10 min (Westfall *et al*., 1996a).

Statistics

Values in the text refer to mean ± s.e.mean or geometric mean with 95% confidence limits for IC_{50} values (after Fleming *et al*., 1972). IC_{50} values were calculated for each individual concentration-inhibition curve. Data were compared by Student's paired *t* test or one way analysis of variance and Tukey's comparison as appropriate. Differences were considered significant when $P<0.05$. Concentration-inhibitory response curves for the antagonists were fitted to the data by logistic (Hill equation), non-linear regression analysis (FigP, Biosoft, Cambridge, U.K.).

Drugs

α,β-meATP (lithium salt), AP_4A (ammonium salt), AP_5A (sodium salt), ATP (disodium salt), P-5-P (all Sigma), ARL 67156 (provided by Astra Charnwood) and suramin (Bayer) were dissolved in distilled water and stored as 100 mM stock solutions. PPADS, a gift from Dr G. Lambrecht, University of Frankfurt, was dissolved in distilled water as a 10 mM stock solution and stored frozen in darkness. (−)-Noradrenaline bitartrate (Sigma) was dissolved in acid saline and frozen as a 100 mM stock solution. Potassium chloride (Sigma) was kept as a 2 M stock. The firefly luciferin-luciferase assay (Sigma) was used to analyse the ATP content of the AP_4A and AP_5A solutions.

Results

Concentration-response relationships of agonists and antagonists

AP_5A (30 nM–100 μM) ($n=7$), AP_4A (300 nM–100 μM) ($n=7$) and ATP (1 μM–1 mM) ($n=6$–10) evoked concentration-dependent, rapid, transient contractions, which reached a peak in about 5 s and then subsided rapidly, even in the continued presence of the agonist (see Figure 3). We have previously shown that α,β-meATP evokes similar contractions (McLaren *et al*., 1994) and comparing the data showed that the rank order of agonist potency was α,β-meATP > AP_5A > AP_4A > ATP. However, none of the curves had a clear maximum and so it was not possible to calculate EC_{50} values.

Suramin, PPADS and P-5-P (all 100 μM) shifted the concentration-response curves to AP_4A and AP_5A to the right such that the response to most concentrations of agonists was abolished (not shown). This flattening of the curves limited the range of antagonist concentrations which could be meaningfully studied. Therefore, in order to characterize the actions of the antagonists over as wide a concentration range as possible, we studied their effects against equi-effective concentrations of α,β-meATP (1 μM), AP_5A (3 μM) and AP_4A (30 μM). These each evoked contractions of approximately 50% amplitude of that obtained to 1 mM ATP. α,β-meATP was used rather than ATP as ATP may act at more than one site to evoke contraction in this tissue (Bailey & Hourani, 1995) and the potency of ATP, but not α,β-meATP, is decreased by breakdown by ecto-ATPase (Westfall *et al*., 1996a).

PPADS (300 nM–30 μM), suramin (3–100 μM) and P-5-P (3–1000 μM) inhibited the contractions evoked by α,β-meATP (1 μM), AP_5A (3 μM) and AP_4A (300 μM) in a concentration-dependent manner and abolished them at the highest concentrations used (Figure 1). In each case, PPADS was significantly more potent than suramin, which in turn was significantly more potent than P-5-P ($P<0.05$, Table 1). PPADS had a similar antagonist potency against α,β-meATP, AP_5A and AP_4A. Likewise, no significant difference was found between the IC_{50} values for suramin against α,β-meATP and AP_5A or α,β-meATP and AP_4A. However, suramin was significantly more potent against AP_4A than AP_5A ($P<0.05$). The same pattern of relative potency was also seen with P-5-P.

Effects of αβ-meATP-induced desensitization

To further characterize the site(s) of action of AP_4A and AP_5A we studied the effect of desensitization of the P_{2X1}-receptor (see Methods). This procedure abolished contractions evoked by AP_5A (3 μM) (control = 2.27 ± 0.12 g, $n=5$, Figure 2a) and AP_4A (10 μM) (control = 2.48 ± 0.19 g, $n=5$, Figure 2b). Higher concentrations of AP_5A (10 μM) and AP_4A (100 μM) were now also ineffective (not shown). In contrast, contractions evoked by noradrenaline (100 μM) were unaffected (control = 4.11 ± 1.03 g, test = 3.71 ± 0.52 g, $n=6$, Figure 2c). This suggests that α,β-meATP selectively desensitized the P_{2X1}-receptor and that both AP_4A and AP_5A act through this receptor to evoke contraction.

Effects of ARL 67156 on contractions

We have previously shown that 100 μM ARL 67156 potentiates the peak amplitude of contractions of the guinea-pig vas deferens evoked by ATP (100 μM) by approximately 60%, but has no effect on those to α,β-meATP (Westfall *et al*., 1996a), consistent with ARL 67156 inhibiting ecto-ATPase. ARL 67156 (100 μM) potentiated the peak response to AP_4A (30 μM) by 61% (control = 3.55 ± 0.27 g, test = 5.71 ± 0.39 g, $n = 8$), but caused a small decrease in the peak amplitude of contractions evoked by AP_5A (3 μM) (control = 3.22 ± 0.14 g, test = 2.54 ± 0.24 g, $n = 6$) (Figure 3). In either case the effect of ARL 67156 reversed rapidly on washout of the drug.

AP_4A and AP_5A as provided by Sigma are only 95% pure, but analysis with the firefly luciferin-luciferase assay for ATP showed that it accounted for only 1% and 0.5% of the AP_5A and AP_4A solutions respectively. As both AP_4A and AP_5A are more potent than ATP in this tissue, it is unlikely that the effects of ARL 67156 on AP_4A and AP_5A are due to the presence of this small amount of ATP.

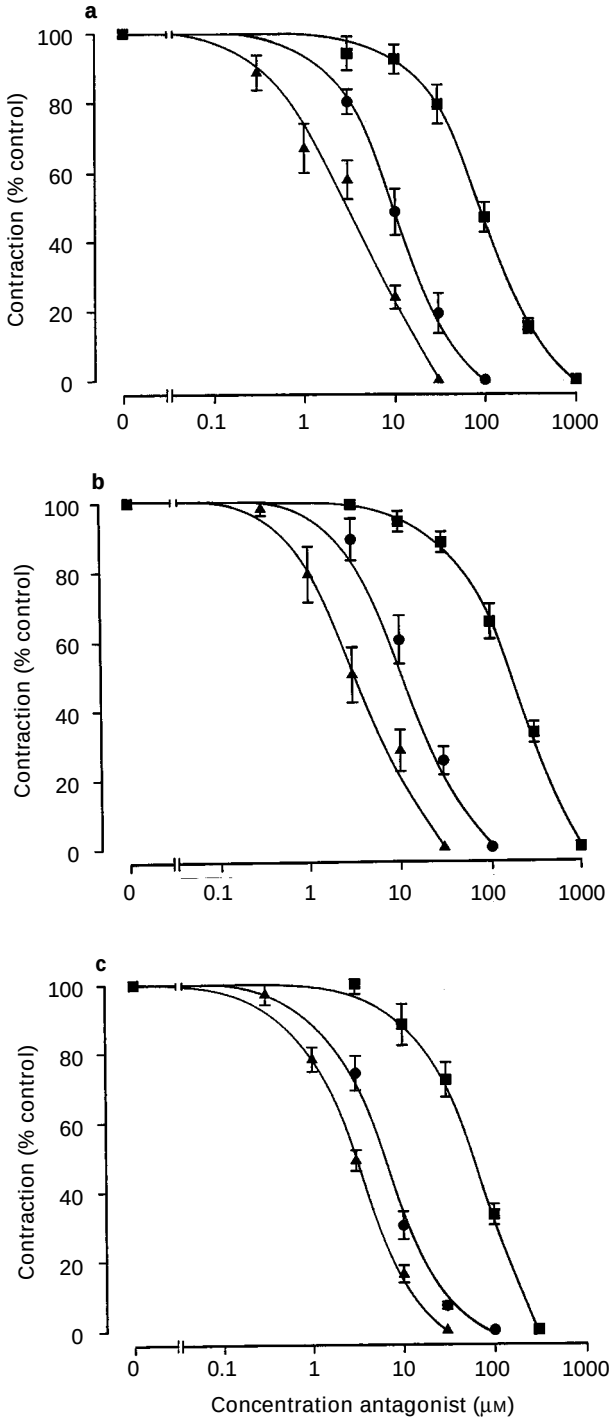

Figure 1 Inhibition of contractions evoked by (a) α,β-meATP (1 μM), (b) AP_5A (3 μM) and (c) AP_4A (30 μM) by PPADS (▲), suramin (●) and P-5-P (■). The graphs show mean data ($n = 6$);

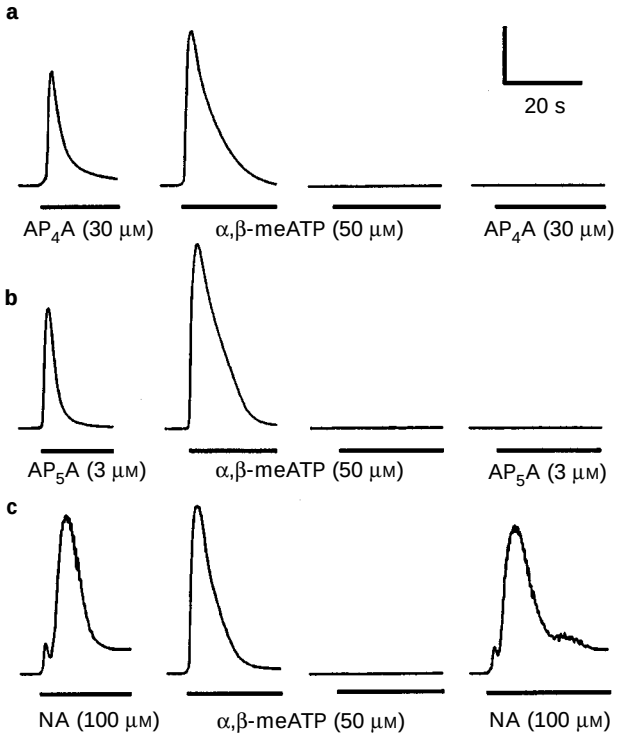

Figure 2 The effect of desensitization of the P_{2X}-receptor by α,β-meATP. Control responses were obtained to (a) AP_4A (30 μM), (b) AP_5A (3 μM) or (c) noradrenaline (NA, 100 μM) (first panel); α,β-meATP (50 μM) was then applied (second panel) and once tension had returned to baseline level further 50 μM aliquots were added until the tissue had been exposed to a cumulative concentration of 200 μM α,β-meATP. The drug was washed out and when α,β-meATP (50 μM) was added 2 min later no contraction was seen (third panel), confirming that the P_{2X}-receptor had been desensitized. α,β-meATP was again washed out and two min later AP_4A and AP_5A no longer evoked contraction, but responses to noradrenaline were unchanged (fourth panel). Note that the vertical scale represents 2 g for contractions evoked by α,β-meATP and noradrenaline, but 1 g for those to AP_4A and AP_5A.

Table 1 Potency of P_2-receptor antagonists in the guinea-pig isolated vas deferens

	α,β-meATP (1 μM)	*AP_5A (3 μM)*	*AP_4A (30 μM)*
PPADS	3.6 (1.6–8.1)	3.4 (1.8–6.4)	3.1 (2.6–3.6)
Suramin	10.6 (6.7–16.8)	14.2 (8.2–24.6)	6.0 (4.7–7.5)
P-5-P	95.9 (76.3–120.6)	166.0 (115.3–238.9)	63.1 (49.1–81.0)

Values shown are IC_{50} and 95% confidence limits (μM) for the antagonists; $n = 6$ for each agonist.

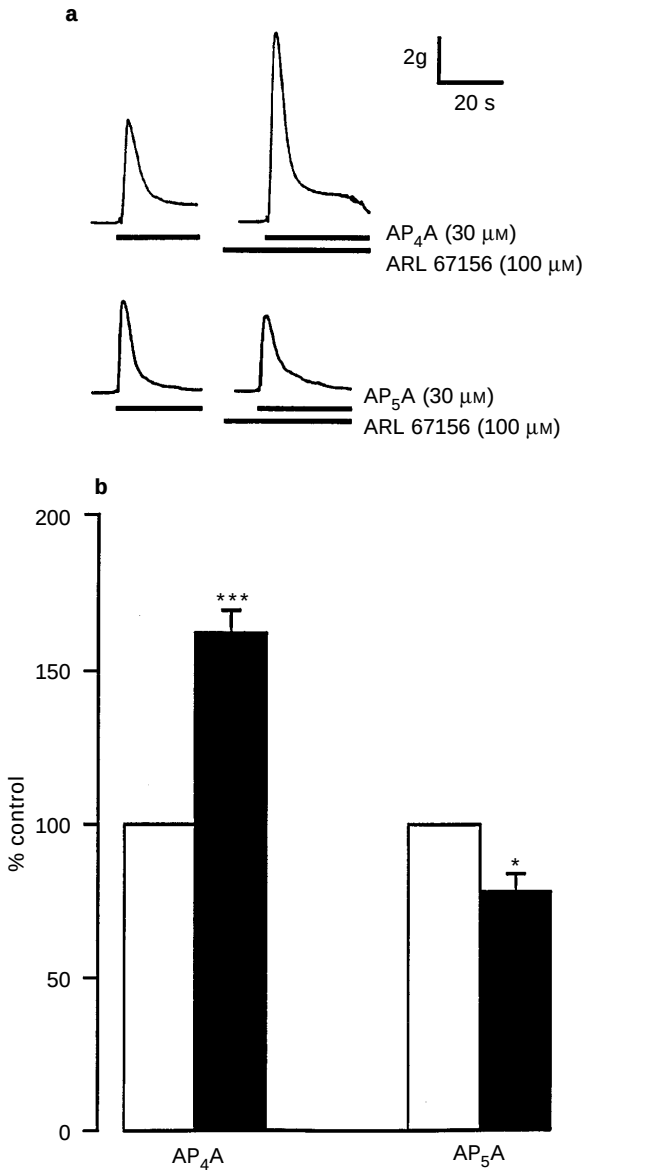

Figure 3 The effect of ARL 67156 on contractions evoked by AP_5A and AP_4A. (a) Typical responses to AP_4A (30 μM) and AP_5A (3 μM) before and 10 min after addition of ARL 67156 (100 μM). (b) The mean responses to AP_4A (30 μM, $n=8$) and AP_5A (3 μM, $n=6$) after addition of ARL 67156 (100 μM, solid columns) expressed as a percentage of control responses (open columns). Statistical significance was determined by analysis of the raw data in g (*$P<0.05$, ***$P<0.001$).

Discussion

The results of this study show that α,β-meATP, AP_4A and AP_5A all evoked contractions of the guinea-pig isolated vas deferens which were inhibited by the P_{2X}-receptor antagonists suramin, PPADS and P-5-P. Of the cloned P_{2X}-receptors, α,β-meATP is a potent agonist at the P_{2X1}- and P_{2X_3}-subtypes only. Whilst the P_{2X1}-receptor is present in many tissues, including visceral smooth muscle, the P_{2X_3}-receptor has a highly restricted distribution, being selectively expressed at high levels in nociceptive sensory neurones (Collo *et al*, 1996). Thus, α,β-meATP is likely to have been acting through the P_{2X1}-receptor to evoke contraction in this study.

These experiments showed that for each antagonist, the potency against AP_4A than AP_5A was not significantly different from that against α,β-meATP, consistent with AP_4A and AP_5A acting at the same site as α,β-meATP, i.e. the P_{2X1}-receptor. This is supported by the finding that AP_5A is an agonist at the cloned P_{2X1}-receptor (Evans *et al*., 1995). However, both suramin and P-5-P were approximately 2.5 times more potent as antagonists against AP_4A than against AP_5A. This could suggest that AP_4A and AP_5A are not acting at the same receptor. This conclusion is difficult to reconcile with the data discussed above, which suggests that both AP_4A and P_5A act at the same site as α,β-meATP. The apparent differences in antagonist potency are small and the potencies of suramin, PPADS and P-5-P seen in this study are similar to those previously found for antagonism at the P_{2X1}-receptor cloned from human urinary bladder (Evans *et al*., 1995) and at the P_{2X1}-receptor in smooth muscle preparations (Hoyle *et al*., 1990; Leff *et al*., 1990; Lambrecht *et al*., 1992; Ziganshin *et al*., 1993; McLaren *et al*., 1994; Trezise *et al*., 1994; Bailey & Hourani, 1995; Ralevic *et al*., 1995; Usune *et al*., 1996). Thus, the small differences seen may simply be due to experimental variability.

Perhaps the strongest evidence for a common site of action is that contractions evoked by AP_4A and AP_5A were abolished by desensitization of the P_{2X1}-receptor by α,β-meATP. Contractions elicited by noradrenaline were unaffected, suggesting that desensitization was selective for the P_{2X1}-receptor. Cross-desensitization between α,β-meATP, AP_4A and AP_5A has also been demonstrated in the urinary bladder of the guinea-pig (Usune *et al*., 1996) and rat (Hashimoto & Kokubun, 1995) and in the rat mesenteric bed (Ralevic *et al*, 1995), again consistent with these agonists acting at the same receptor. AP_4A and AP_5A also show cross-desensitization with ATP in the rat vas deferens (Stone & Paton, 1989). This contrasts with the results of MacKenzie *et al*. (1988) where AP_4A did not mimic the ability of AP_5A and α,β-meATP to inhibit the purinergic (P_{2X1}) component of neurogenic contractions in the guinea-pig vas deferens. However in an electrophysiological study, desensitization to AP_4A or AP_5A attenuated depolarization of the guinea-pig vas deferens evoked by ATP (Hoyle *et al*., 1995a).

An alternative possibility is that AP_4A and AP_5A act at separate receptors, which have similar sensitivity to antagonists as the P_{2X1}-receptor, but the results do not fit the properties of any currently known P_2-receptor. AP_4A, but not AP_5A, is a potent agonist at the P_{2Y_2}- (P_{2U}-) receptor (Lazarowski *et al*., 1995), but PPADS has little effect at this site (Charlton *et al*., 1996). Neither AP_4A nor AP_5A are agonists at the P_{2Z}-receptor (Steinberg *et al*., 1987; Tatham *et al*., 1988) and both are antagonists at the P_{2T}-receptor (see Cusack, 1993). In contrast to our results, suramin is not an antagonist at the proposed separate site for diadenosine nucleotides (Pintor & Miras-Portugal, 1995). Finally, there is no evidence for a functional P_{2Y}-receptor in the guinea-pig vas deferens (Bailey & Hourani, 1995).

In this study the peak amplitude of contractions evoked by AP_4A was potentiated by 61% by ARL 67156. We previously showed that ARL 67156 enhanced contractions to ATP to a similar extent, but had no effect on those to α,β-meATP (Westfall *et al*., 1996a). This suggests that the action of AP_4A in the guinea-pig vas deferens is limited by its breakdown by ecto-enzymes, similar to the coronary bed of the rabbit (Pohl *et al*., 1991), bovine cultured adrenal chromaffin cells (Ramos *et al*., 1995), guinea-pig left atrium (Hoyle *et al*., 1996) and several preparations where the action of AP_4A depends upon its breakdown to adenosine (Klishin *et al*., 1994; Ziganshin *et al*., 1995; Rubino & Burnstock, 1996). In contrast, AP_4A is not broken down in the coronary bed of the guinea-pig (Nees, 1989) and rabbit perfused aorta and mesenteric artery (Busse *et al*., 1988). Thus, the stability of AP_4A is tissue-dependent.

The metabolism of AP_4A by intracellular and plasma enzymes is much better characterized than that by ecto-enzymes (see Ogilvie, 1992). Outside the cell it has been proposed that AP_4A is broken down by ecto-enzymes distinct from ecto-ATPase (Ogilvie, 1992; Ramos *et al*., 1995), but it is not clear if ARL 67156 potentiated AP_4A in the present study because AP_4A was metabolized by ecto-ATPase, or if AP_4A was broken down by a separate ecto-AP_4Aase which is also sensitive to

ARL 67156. To date, biochemical studies on the actions of ARL 67156 have only examined the activity of ecto-ATPase and further studies are required against other nucleotidases. Interestingly, of the three agonists used here, the IC_{50} for suramin was lowest against AP_4A. Suramin, as well as acting as a P_2-antagonist, also inhibits ecto-ATPase (Bailey & Hourani, 1994) and this can decrease its antagonist potency. This would suggest that AP_4A was not metabolized here by a suramin-sensitive enzyme.

In contrast to the potentiation of AP_4A, contractions evoked by AP_5A were inhibited by ARL 67156. Thus, AP_5A appears to be metabolically stable in the guinea-pig vas deferens, unlike in bovine cultured adrenal chromaffin cells where a single ecto-enzyme is thought to break down both AP_4A and AP_5A (Ramos *et al*., 1995). How ARL 67156 inhibited AP_5A is not clear. ARL 67156 has a pA_2 of 3.3 as an antagonist at the P_{2X}-receptor (Crack *et al*., 1995), but the concentration of ARL 67156 used here (100 μM), would not be expected to produce significant antagonism. Furthermore, 100 μM ARL 67156 did not inhibit α,β-meATP when acting through the P_{2X1}-receptor in this tissue (Westfall *et al*., 1996a). An alternative possibility is that part of the action of AP_5A is dependent upon its breakdown by an ecto-enzyme to other active substances such as ATP. However, if this was the case then it would not appear to be an important pathway as ARL 67156 only inhibited the contractions by about 20%.

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