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Investigation of the actions of PPADS, a novel P_{2X}-purinoceptor antagonist, in the guinea-pig isolated vas deferens

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1 Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) was investigated for its ability to act as an antagonist at P_{2X}-purinoceptors which mediate neurogenic excitatory junction potentials (e.j.ps) and contractions in the guinea-pig isolated vas deferens.

2 PPADS (10^{-7} M) caused a small potentiation of the phasic, predominantly purinergic component of contractions evoked by sympathetic nerve stimulation, but higher concentrations of PPADS (3×10^{-6}–3×10^{-5} M) elicited a substantial and significant concentration-dependent inhibition. In contrast, over the same concentration-range, PPADS had no effect on the tonic, predominantly noradrenergic phase.

3 PPADS (3×10^{-5} M) also inhibited contractile responses to exogenous α,β-methyleneATP (10^{-8}–10^{-3} M), a P_{2X}-purinoceptor agonist, without affecting the responses to exogenous noradrenaline (10^{-8}–10^{-3} M), carbachol (10^{-5} M) or histamine (10^{-4} M).

4 PPADS (10^{-7}–3×10^{-5} M) produced a concentration-dependent reduction in e.j.p. magnitude and resting membrane potential. The maximum effect was seen at 10^{-5} M PPADS, which reduced e.j.p. magnitude from 13.7 ± 0.6 mV ($n=12$) to 1.8 ± 0.7 mV ($n=12$) and membrane potential from -64.8 ± 0.6 mV ($n=51$) to -55.0 ± 1.8 mV ($n=12$).

5 The PPADS-induced depolarization was not inhibited by the P_{2X}-purinoceptor antagonist, suramin (10^{-4} M). This indicates that the depolarization was not due to an agonist action of PPADS at P_{2X}-purinoceptors.

6 The results support the proposal that PPADS is a selective antagonist at P_{2X} purinoceptors as opposed to non-P_2-purinoceptors in the guinea-pig vas deferens, but its ability to cause membrane depolarization independently of P_{2X}-purinoceptors and also, at a low concentration, to potentiate the phasic component of the neurogenic contraction indicates that it has other actions.

Keywords: Pyridoxalphosphate-6-azophenyl-2′-4′-disulphonic acid (PPADS); P_{2X}-purinoceptor; vas deferens; cotransmitter; purinergic; sympathetic

Introduction

There is considerable evidence to indicate that in many tissues adenosine 5′-triphosphate (ATP) is released from sympathetic nerves and acts as a cotransmitter with noradrenaline (for recent reviews see Burnstock, 1990; von Kügelgen & Starke, 1991). For example, stimulation of the sympathetic nerves in the vas deferens of the mouse, rat and guinea-pig produces a biphasic contractile response which is thought to result from the combined action of ATP and noradrenaline released as cotransmitters. The initial phase of the neurogenic contraction is blocked by arylazidoaminopropyionyl-ATP ($ANAPP_3$) (guinea-pig) (Fedan *et al.*, 1981), by selective desensitization of P_{2X}-purinoceptors with α,β-methyleneATP (guinea-pig, rat and mouse) (Meldrum & Burnstock, 1983; Allcorn *et al.*, 1986) and by the P_2-purinoceptor antagonist, suramin (mouse and rat) (Dunn & Blakeley, 1988; von Kügelgen *et al.*, 1989; Blakeley *et al.*, 1991; Mallard *et al.*, 1992) and is therefore considered to be mediated by ATP acting on P_{2X}-purinoceptors (Burnstock & Kennedy, 1985; Kennedy, 1990). The secondary component of the neurogenic response is blocked by α-adrenoceptor antagonists such as prazosin and so is thought to be due to the action of noradrenaline acting on α_1-adrenoceptors.

The underlying electrical response to sympathetic nerve stimulation in the vas deferens is a rapid, transient excitatory junction potential (e.j.p.) which is also blocked by $ANAPP_3$ (Sneddon *et al.*, 1982), α,β-methyleneATP (Sneddon & Burnstock, 1984; Stjärne & Åstrand, 1984; Allcorn *et al.*, 1986) and suramin (Sneddon, 1992; Sneddon & Machaly, 1992), but not by α-adrenoceptor antagonists. Indeed the e.j.p. is particularly useful for monitoring P_{2X}-purinoceptor-mediated responses since it appears to be produced solely by ATP, whereas the mechanical responses are composed of two phases which can be described as predominantly noradrenergic or predominantly purinergic, but each of which has some contribution from both transmitters (for details see Amobi & Smith, 1990).

Recently, the compound pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), has been proposed as a selective P_{2X}-purinoceptor antagonist in the rabbit isolated vas deferens (Lambrecht *et al.*, 1992). PPADS was found to inhibit the contractile response to exogenous α,β-methylene-ATP, a selective P_{2X}-purinoceptor agonist, but not that to exogenous noradrenaline. PPADS also inhibited selectively the initial phasic contraction to sympathetic nerve stimulation, thought to be predominantly mediated by ATP acting through P_{2X}-purinoceptors, without having any effect on the later, secondary component, which is thought to be mainly due to noradrenaline acting at α_1-adrenoceptors. Responses mediated through muscarinic cholinoceptors, P_1-purinoceptors and H_1-histamine receptors were also unaffected by PPADS. In addition, PPADS was found to inhibit selectively P_{2X}-purinoceptor-mediated contractions in guinea-pig submucosal arterioles (Bungardt *et al.*, 1992). Thus, as far as it has been tested, PPADS appears to display selectivity for the P_{2X}-purinoceptor.

In this study we have examined the effect of PPADS on mechanical and electrical responses of the guinea-pig vas deferens in order to investigate further its selectivity in blocking responses to P_{2X}-purinoceptors. A preliminary account of these results has been published (McLaren *et al.*, 1993).

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Methods

Albino male guinea-pigs (300–400 g) were killed with CO_2 and subsequent exanguination. The vasa deferentia were dissected out, cleaned of connective tissue, mounted for recording and allowed to equilibrate at 35°C for 1 h. A physiological salt solution of the following composition was used throughout (mM): NaCl 118, KCl 5.4, NaH_2PO_4 1.16, $NaHCO_3$ 25, $MgSO_4$ 1.16, $CaCl_2$ 2.5 and glucose 11.1, bubbled with 95% O_2, 5% CO_2.

In isometric tension recording experiments, the vas deferens was suspended in a 2 ml organ bath, under a resting tension of 0.5–0.75 g and connected to a Gould Metrigram transducer which was coupled to a Gould 2200s pen recorder via a Gould universal preamplifier. The sympathetic axons in the tissue were stimulated by electrical field stimulation using a Scientific Research Instruments stimulator giving a stimulus of 4 Hz for 20 s with a pulse width of 0.5 ms and supramaximal voltage every 10 min. In individual tissues the effect of a range of concentrations of PPADS (10^{-7}–3×10^{-5} M) on neurogenic contractions were examined. Initially, sympathetic nerves were stimulated until two consecutive contractions of similar magnitude were evoked. The lowest concentration of PPADS used was then applied to the tissue for 15 min, in which time steady state inhibition was reached. Thereafter, progressively higher concentrations of PPADS were administered and the inhibition allowed to reach equilibrium. Each concentration of PPADs was tested in each of six tissues.

Concentration-response curves to α,β-methyleneATP were obtained by direct addition to the bath. Shortly after the contraction had reached a peak the drug was washed out by several changes of the bathing solution. Care was taken to avoid desensitization of the muscle to α,β-methyleneATP by leaving a time interval of 30 to 45 min before the next concentration of agonist was added. Concentration-response curves to noradrenaline were obtained in a similar manner. For either agonist, a concentration-response curve was first obtained followed by a test curve in the presence of PPADS. Contractile responses to histamine and carbachol were obtained with concentrations that had been shown in preliminary experiments to be near EC_{50} for either agonist. PPADS was allowed to equilibrate for at least 15 min before addition of agonists.

In the electrophysiological studies the vasa deferentia were pinned out in a 2 ml organ bath and maintained as described above for the mechanical experiments. Intracellular recording of the transmembrane potential was carried out with glass microelectrodes of 30–50 MΩ resistance. The signal was recorded on a storage oscilloscope (Tektronix) and a tape recorder (Racal) via a preamplifier (Cell Explorer 800, Dagan). Impalements were accepted if the resting potential maintained a stable level of at least −60 mV. E.j.ps were evoked continuously by field stimulation at 0.5 Hz, 0.2 ms pulse width and at a voltage lower than that necessary to initiate a contraction. As with the mechanical recordings, each tissue was exposed to increasing concentrations of PPADS (10^{-7}–3×10^{-5} M). Initially, the lowest concentration used was applied for 15 min before measurements of the e.j.p. magnitude and membrane potential were taken. Progressively higher concentrations were then administered and steady state e.j.p. magnitude and membrane potential recorded. In several experiments suramin (10^{-4} M) was added to the bath 30 min before the addition of PPADS (10^{-5} M).

Statistics

Values in the text refer to mean ± s.e.mean or geometric mean with 95% confidence limits for EC_{50} values (after Fleming *et al.*, 1972). Statistical comparison of the results was tested by Student's *t* test for paired or unpaired data, as appropriate. Differences were considered significant when $P < 0.05$. In some cases (Figure 2b), concentration-response curves were fitted to the data by logistic (Hill equation), non-linear regression analysis (FigP, Biosoft, Cambridge, UK).

Drugs

α,β-methyleneATP (lithium salt), carbachol (carbamylcholine chloride), histamine dihydrochloride (all Sigma) and suramin (Bayer, U.K.) were dissolved in distilled water and kept as 10^{-1} M stock solutions. (−)-Noradrenaline bitartrate (Sigma) was dissolved in acid saline and frozen as a 10^{-1} M stock solution. PPADS was synthesized in one of our laboratories (starting materials aniline-2,4-disulphonic acid and pyridoxal-phosphate). The structure of PPADS was confirmed by ^{1}H n.m.r. spectroscopy and its purity investigated by thin-layer chromatography on high-performance silica gel plates (developing solvent: 1-propanol/aqueous ammonia (33%)/water, 6:3:2 v/v. The final product showed only one spot and the two starting materials could not be detected. PPADS was dissolved in distilled water as a 10^{-2} M stock solution and kept frozen in darkness.

Results

Contractile responses to sympathetic nerve stimulation

Field stimulation of the sympathetic nerves of the guinea-pig vas deferens with trains of pulses for 20 s at 4 Hz, produced a characteristic biphasic response (Figure 1a). PPADS (10^{-7}

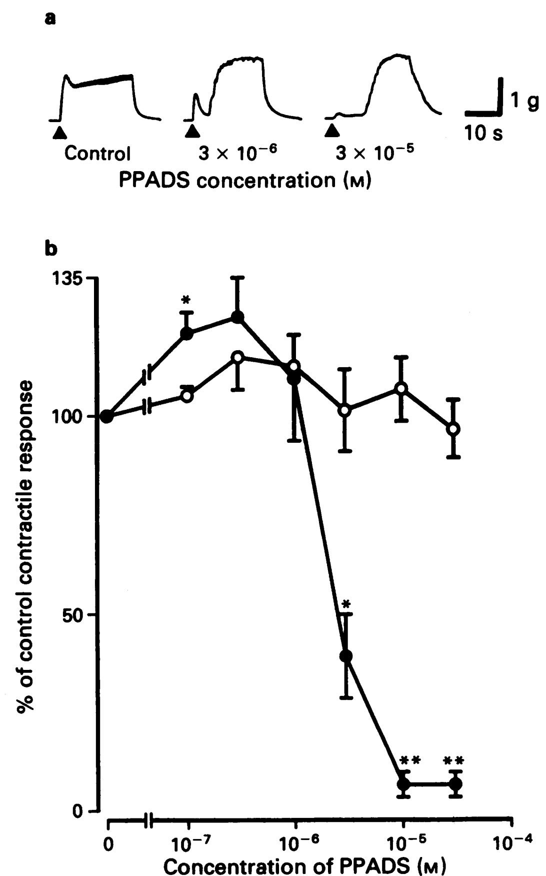

Figure 1 The effects of PPADS on the biphasic contractile response of the guinea-pig vas deferens to sympathetic nerve stimulation elicited by a train of pulses for 20 s at 4 Hz (▲-start of stimulation). (a) The upper panel shows contractions elicited in the same tissue in the absence and presence of PPADS (3×10^{-6} and 3×10^{-5} M). (b) The lower panel shows the mean data ± s.e.mean ($n = 6$) for the effect of PPADS (10^{-7}–3×10^{-5} M) on the initial phasic response (●) and the secondary tonic response (○). $*P < 0.05$, $**P < 0.01$.

M–3×10^{-5} M) did not alter the resting tension, but had two effects on the phasic, predominantly purinergic, component of the neurogenic contraction. At 10^{-7} M, PPADS caused a small, but significant, potentiation of the phasic response, whilst at higher concentrations PPADS evoked a substantial, concentration-dependent inhibition, which was significant at 3×10^{-6} M and greater, 50% inhibition was seen at approximately 2.5×10^{-6} M PPADS. In contrast, over the same concentration-range, PPADS had no significant effect on the secondary, predominantly noradrenergic phase of the response (Figure 1a,b).

Concentration-response curves to α,β-methyleneATP and noradrenaline

Next, the effect of a concentration of PPADS (3×10^{-5} M), which was maximally effective against the phasic contraction was tested against exogenously applied agonists.

The P_{2X}-purinoceptor agonist, α,β-methyleneATP (10^{-8}–10^{-3} M) evoked rapidly developing, concentration-dependent, transient contractions. Concentration-response curves to α,β-methyleneATP in the absence and presence of PPADS (3×10^{-5} M) are shown in Figure 2a. Since there were was no clear maximum achieved in these curves, it was not possible to calculate EC_{50} values, so the effect of PPADS was examined statistically at each individual concentration of α,β-methyleneATP. PPADS caused a significant inhibition of contractions induced by lower concentrations of α,β-methyleneATP (10^{-8}–10^{-5} M), but this was overcome by the highest concentrations of α,β-methyleneATP (10^{-4}–10^{-3} M). These results suggest that PPADS could be acting as a competitive antagonist here. When measured at the level equal to 50% of the contraction induced by 10^{-3} M α,β-methyleneATP, PPADS (3×10^{-5} M) induced a 13.5-fold shift to the right, giving an apparent pK_B of 5.62.

Noradrenaline (10^{-8}–10^{-3} M) caused concentration-dependent, biphasic contractions consisting of a small initial twitch, followed by a larger, slowly developing maintained contraction. In the absence of PPADS noradrenaline had an EC_{50} of 1.4×10^{-5} M (95% confidence limits = 2.7×10^{-6}–7.4×10^{-5} M) with a maximum response of 2.9 ± 0.1 g ($n = 5$), while in the presence of PPADS (3×10^{-5} M) the EC_{50} was 7.6×10^{-6} M (95% confidence limits = 2.2×10^{-6}–2.6×10^{-5} M) and the maximum contraction 3.5 ± 0.2 g ($n = 5$) (see Figure 2b; values derived from sigmoid curve fitting). Neither value was significantly different from the control.

Responses to carbachol and histamine

Contractions to carbachol and histamine were evoked to determine further the selectivity of PPADS. In the presence of PPADS (3×10^{-5} M) the contraction to 10^{-5} M carbachol (1.6 ± 0.4 g) was not significantly different from the control value (1.2 ± 0.3 g, $n = 6$). Similarly, PPADS (3×10^{-5} M) had no significant effect on contractions evoked by 10^{-4} M histamine (control, 1.1 ± 0.3 g; + PPADS, 0.8 ± 0.2 g, $n = 4$).

Electrophysiological studies

The mean resting membrane potential of control cells was -64.8 ± 0.6 mV and the mean size of fully facilitated e.j.ps recorded from these cells at 0.5 Hz was 13.7 ± 0.6 mV ($n = 51$). PPADS (10^{-7}–3×10^{-5} M) caused a significant and concentration-dependent depolarization and reduction in e.j.p. magnitude (Figure 3a, b); 50% inhibition of e.j.p. magnitude was seen at approximately 7×10^{-7} M PPADS. After washout of PPADS for 1 h the e.j.p. magnitude recovered to 11.2 ± 1.3 mV ($n = 17$) and the resting membrane potential to -65.4 ± 1.4 mV ($n = 17$), which were not significantly different from the control values.

Since activation of P_{2X}-purinoceptors elicits depolarization in the guinea-pig vas deferens (Sneddon & Westfall, 1984), PPADS may be acting here as a partial agonist at the P_{2X}-purinoceptor. To test this possibility we examined the effect of PPADS when present with the P_2-purinoceptor antagonist suramin. In the presence of suramin (10^{-4} M) the resting membrane potential was -66.8 ± 0.9 mV ($n = 5$), which was not significantly different from control cells. In the continued presence of suramin, subsequent addition of PPADS (10^{-5} M) still produced a significant reduction in membrane potential to -54.6 ± 0.6 mV ($n = 8$), which was similar to that seen in the absence of suramin (-55.0 ± 1.8 mV, $n = 12$).

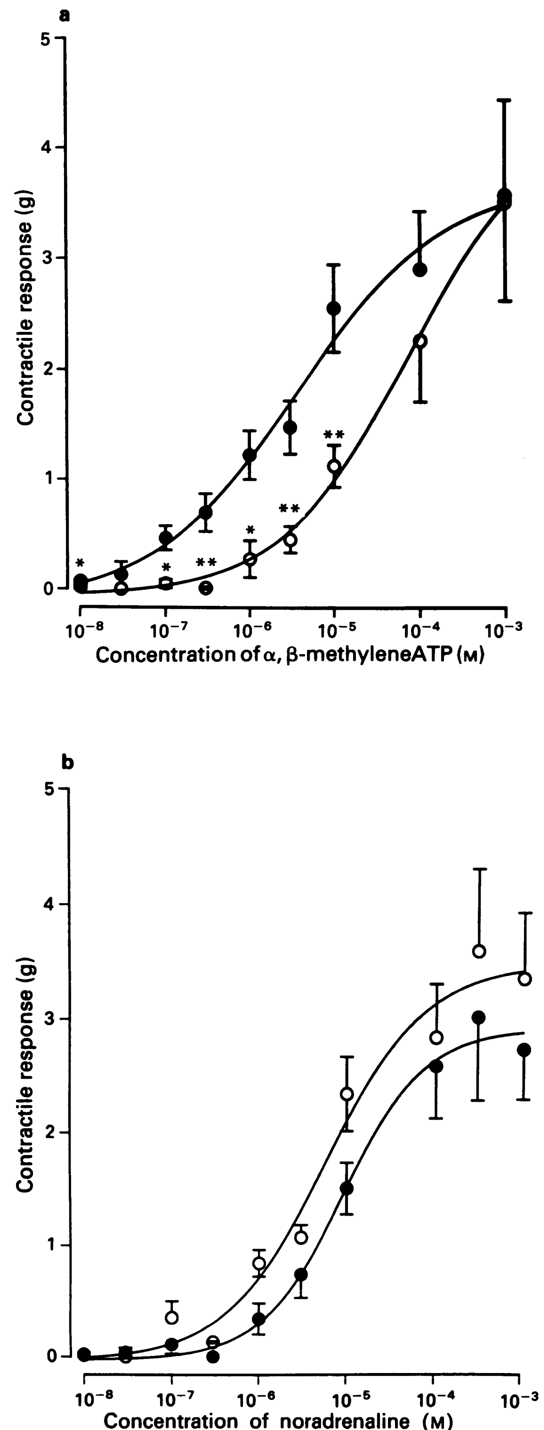

Figure 2 The effect of PPADS on the concentration-response curves to exogenously applied (a), α,β-methyleneATP (10^{-8}–10^{-3} M, $n = 7$) an (b) noradrenaline (10^{-8}–10^{-3} M, $n = 5$) in the absence (●) and presence (○) of PPADS (3×10^{-5} M). Mean with s.e.mean, $*P < 0.05$, $**P < 0.01$.

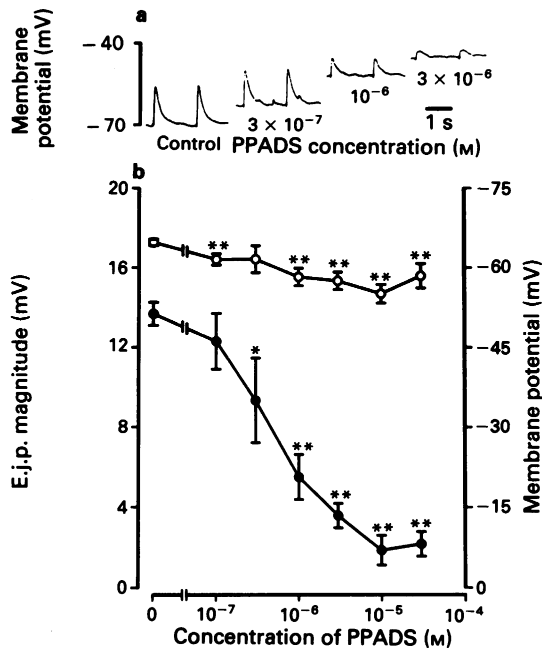

Figure 3 The effect of PPADS on the magnitude of fully facilitated e.j.ps (0.5 Hz, 0.2 ms pulse width, submaximal voltage) and the membrane potential. (a) The upper panel shows recordings in a single cell of the membrane potential and e.j.ps, initially in the absence and then in the presence of increasing concentrations of PPADS (3×10^{-7}–3×10^{-6} M). (b) The lower panel shows the mean data ± s.e.mean ($n = 5$–51) for the effects of PPADS (10^{-7}–3×10^{-5} M) on e.j.p. magnitude (●) and membrane potential (○). *$P < 0.05$, **$P < 0.01$.

Discussion

The results of this study show that PPADS selectively inhibits those responses in the guinea-pig vas deferens which are mediated by P_{2X}-purinoceptors, but has no effect on contractions mediated by α_1-adrenoceptors, muscarinic cholinoceptors or histamine receptors. In general, this is consistent with the initial reports of the actions of PPADS in the rabbit vas deferens (Lambrecht *et al.*, 1992) and guinea-pig submucosal arterioles (Bungardt *et al.*, 1992), where it was found that PPADS selectively inhibited responses mediated by P_{2X}-purinoceptors. However, in the present study we also found that PPADS has additional excitatory actions, depolarization of the smooth muscle cells, which is independent of P_{2X}-purinoceptors and, at a low concentration, a small potentiation of the phasic component of the neurogenic contraction through an unknown mechanism.

PPADS selectively modulated the phasic, predominantly purinergic component of neurogenic contractions of the guinea-pig vas deferens while having no effect on the tonic, mainly noradrenergic component. PPADS also selectively inhibited contractions to α,β-methyleneATP, but had no effect on those elicited by noradrenaline, carbachol or histamine. The PPADS-induced inhibition of contractions evoked by α,β-methyleneATP was overcome by increasing the concentration of the agonist, suggesting that PPADS could be acting here as a competitive antagonist at P_{2X}-purinoceptors. This contrasts with the study in rabbit vas deferens (Lambrecht *et al.*, 1992) where, at lower concentrations than used here, PPADS appeared to cause a non-competitive antagonism of contractions evoked by α,β-methyleneATP.

In the electrophysiological studies PPADS caused a reduction in the magnitude of e.j.ps which underlie the phasic, purinergic contraction in the guinea-pig vas deferens (Sneddon *et al.*, 1982) and this is consistent with the view that PPADS is an antagonist at P_{2X}-purinoceptors. However, PPADS also induced a membrane depolarization, which may also contribute to the reduction in the amplitude of the e.j.ps due to a decrease in driving force (but see Sjöstrand, 1973). One possible explanation for this depolarization is that PPADS is a partial agonist at P_{2X}-purinoceptors, which are known to mediate depolarization in this tissue (Sneddon & Westfall, 1984). We investigated this by determining whether suramin, which is also a P_{2X}-purinoceptor antagonist (Dunn & Blakeley, 1988; Blakeley *et al.*, 1991; Mallard *et al.*, 1992; Sneddon, 1992), would alter the depolarization caused by PPADS. PPADS still caused a depolarization when suramin was present, indicating that the depolarization was not due to an excitatory action of PPADS at P_{2X}-purinoceptors.

Although PPADS depolarized the smooth muscle cells, this did not lead to a contraction. Presumably, the depolarization was not great enough to induce sufficient calcium influx to initiate contraction. How PPADS depolarized the smooth muscle cells was not studied further here. One possibility is that PPADS was acting non-selectively to disrupt the membrane of the cell. However, this is unlikely as the effects of PPADS on the contractile responses to noradrenaline, histamine and carbachol were not reduced. Another possibility is that PPADS could modify the activity of ion channels in the membrane of the smooth muscle, e.g. by blocking potassium channels, or less likely, by activating sodium, calcium or chloride channels. However, this was not examined here and the exact mechanism requires further investigation. Likewise, the mechanism through which a low concentration of PPADS potentiated the phasic component of the neurogenic contraction is not clear. One possibility is that the potentiation was due to the small depolarization induced by PPADS, as has been found for other agonists in this tissue (Sjöstrand, 1973).

In conclusion, PPADS selectively inhibits responses in the guinea-pig vas deferens which are mediated by the P_{2X}-purinoceptor as opposed to responses mediated by non-P_2-purinoceptors, but its ability to depolarize the smooth muscle cells independently of P_{2X}-purinoceptors and also, at a low concentration, to potentiate the phasic component of neurogenic contractions, shows that it has other actions.

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