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Pre- and Postjunctional Effects of Diadenosine Polyphosphates in the Guinea-pig Vas Deferens

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Abstract

The pre- and postjunctional activities of a number of diadenosine polyphosphates were examined in the guinea-pig isolated vas deferens at the level of the membrane potential, using a modified sucrose-gap technique.

P^1,P^3-Di(adenosine 5′)triphosphate (Ap_3A), P^1,P^4-di(adenosine 5′)tetraphosphate (Ap_4A) and P^1,P^5-di(adenosine 5′)pentaphosphate (Ap_5A) all caused concentration-dependent depolarization of the smooth muscle membrane. The potency order was: $Ap_5A > Ap_4A \geq Ap_3A$. P^1,P^2–Di(adenosine 5′)pyrophosphate (Ap_2A) did not evoke depolarization even at the highest concentration tested (1 mM). All the dinucleotides caused a reduction in the amplitude of evoked excitatory junction potentials (e.j.ps). The potency order was: $Ap_5A = Ap_4A > Ap_3A > Ap_2A$. The depolarizations evoked by the dinucleotides were markedly reduced by the selective P_{2X}-purinoceptor antagonist, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS, 10 μM), as was the amplitude of the fully facilitated e.j.p. The inhibition of the e.j.p. evoked by Ap_3A and Ap_2A was reduced by the P_1-purinoceptor antagonist, 8-*p*-sulphophenyltheophylline (8-*p*SPT, 50 μM), but that evoked by Ap_5A and Ap_4A was not.

Thus, Ap_3A, Ap_4A and Ap_5A evoke depolarization of the guinea-pig vas deferens via P_{2X}-purinoceptors, and additionally Ap_2A and Ap_3A exert a prejunctional effect via P_1-purinoceptors. The prejunctional activity of Ap_4A and Ap_5A is mediated via an undefined purinoceptor, which is neither P_1 nor P_{2X}.

With the increasing awareness of the biological activity of adenine dinucleotides as intracellular or extracellular signal molecules, and the appreciation that they may in fact have roles as neurotransmitters or neuromodulators (Hoyle 1990; Pintor et al 1992a, 1993b) it has become necessary to examine their pharmacological activity in greater detail. The hypothesis that adenine dinucleotides, such as diadenosine tetraphosphate (Ap_4A), diadenosine pentaphosphate (Ap_5A) and diadenosine hexaphosphate (Ap_6A) might be neurotransmitters or neuromodulators arises from the determination of their presence in central nerve terminals (Pintor et al 1992a, 1993b), secretory granules in adrenal medullary chromaffin cells (Rodriguez del Castillo et al 1988; Castillo et al 1992; Pintor et al 1992b) and platelets (Flodgaard & Klenow 1982; Luthje & Ogilvie 1983; Fijnheer et al 1992; Schluter et al 1994), and that they can be released by appropriate stimuli (Pintor et al 1991b, 1992b, 1993b; Castillo et al 1992; Schluter et al 1994).

In autonomically innervated organs, adenine dinucleotides have many actions. In the guinea-pig vas deferens (MacKenzie et al 1988), human urinary bladder (Hoyle et al 1989) and rat mesenteric artery (Ralevic et al 1995) they evoke contractions that are probably mediated via P_{2X}-purinoceptors. In the human colon and in some blood vessels, dinucleotides have inhibitory activity mediated via P_{2Y}-purinoceptors on smooth muscle cells and endothelial cells, respectively (Busse et al 1988; Nees 1989; Hoyle & Burnstock 1992; Ralevic et al 1995), and in adrenal chromaffin cells they also appear to bind to P_{2Y}-purinoceptors (Pintor et al 1991a; Castro et al 1992). In several organs, including guinea-pig vas deferens, human colon and rat brain, adenine dinucleotides can act on nerve terminals to inhibit the release of neurotransmitters (Stone 1981; Stone & Perkins 1981; Hoyle & Burnstock 1992; Klishin et al 1994), but in these cases the receptors are largely undefined. Although diadenosine polyphosphates appear to act via P_2-purinoceptors the possibility that they act via their own specific receptors, or an as yet unrecognized subclass of P_2-purinoceptor cannot be ruled out (Stone 1981, 1991; Hoyle 1990, 1992; Pintor et al 1993a).

The aim of the present study was to examine the pharmacological activity of an homologous series of diadenosine polyphosphates: diadenosine pyrophosphate (Ap_2A), diadenosine triphosphate (Ap_3A), Ap_4A and Ap_5A in the guinea-pig vas deferens. This series of compounds includes those with a short chain length (Ap_2A, Ap_3A) and those with a long chain length (Ap_4A, Ap_5A). The length of the phosphate chain seems to be an important determinant in the selectivity, or even specificity, of these compounds for P_{2X}- or P_{2Y}-purinoceptors in certain tissues (Hoyle & Burnstock 1992; Ralevic et al 1995). A modified sucrose-gap technique (Hoyle 1987) was used to examine the effects of these compounds on membrane potential and purinergic neuromuscular transmission in isolated smooth muscle pre-

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parations of the guinea-pig vas deferens. This preparation was chosen because the postjunctional P_2-purinoceptor has been subclassified as P_{2X} (Burnstock & Kennedy 1985), the transmitter responsible for non-adrenergic excitation from sympathetic nerves has been identified as ATP (Sneddon et al 1982; Sneddon & Burnstock 1984; Sneddon & Westfall 1984), and the transmitter release can be modulated by activation of P_1-purinoceptors (Sneddon et al 1984).

Materials and Methods

Male guinea-pigs (300–600 g) were stunned and bled from the neck. The vasa deferentia were removed and placed in modified Krebs solution of the following composition (mM): NaCl 133, KCl 4·7, NaH_2PO_4 1·4, $NaHCO_3$ 16·3, $MgSO_4$ 0·6, $CaCl_2$ 2·5, glucose 7·7. Phentolamine (1 μM) was routinely included in this solution to prevent adrenergic effects. Whole vasa deferentia were gently pinned out at their resting length on a wax surface immersed in Krebs solution; the connective tissue sheath was removed and strips of smooth muscle approximately 1 mm wide and 1 cm long were dissected from the prostatic and middle regions (the strips also contained adhering underlying mucosa). The preparations were allowed to equilibrate in Krebs solution at room temperature (21°C) for at least 2 h before being mounted in the sucrose-gap apparatus (Hoyle 1987). In the recording chamber the superfusing Krebs solution (flow rate approx. 1·5 mL min^{-1}) was gassed with 95% O_2–5% CO_2, and was maintained at 35 ± 0·5°C. Electrical field stimulation (EFS) was applied via parallel Pt wire electrodes approximately 3 mm apart, situated above and below the preparation (Hoyle 1987), using a Grass S48 stimulator and a stimulus isolation unit (Grass SIU5). Stimuli were applied as trains of 10 pulses at a frequency of 1 Hz, and the trains were applied at approximately 1 min intervals. EFS evoked excitatory junction potentials (e.j.ps) that facilitated during the train. The strength of the field was kept constant (100 V), but the pulse-width was selected for each preparation (in the range of 0·015–0·05 ms) so that the fully facilitated e.j.ps were of maximal amplitude, but did not trigger action potential discharge. Concentration–response relationships were determined by applying diadenosine polyphosphates at a given concentration for 60 s, followed by a period of washout for 15 min before the subsequent dose to avoid desensitization. Desensitization was purposefully induced by applying the dinucleotide for 20 min. The P_1-purinoceptor antagonist 8-*p*-sulphophenyltheophylline (8-*p*SPT) and the P_2-purinoceptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) were allowed to equilibrate with preparations for at least 20–25 min before a dose of diadenosine polyphosphate was applied.

Drugs

The following chemicals were all obtained from Sigma Chemicals Co.: adenosine 5′-triphosphate sodium salt (ATP); P^1,P^2-di(adenosine 5′)pyrophosphate sodium salt (Ap_2A); P^1,P^3-di(adenosine 5′)triphosphate ammonium salt (Ap_3A); P^1,P^4-di(adenosine 5′)tetraphosphate ammonium salt (Ap_4A); P^1,P^5-di(adenosine 5′)pentaphosphate sodium salt (Ap_5A). 8-*p*-Sulphophenyltheophylline (8-*p*SPT) was obtained from Research Biochemicals Incorporated (RBI), and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) was obtained from Cookson Chemicals. All drugs were dissolved in distilled water to produce stock solutions of 10–100 mM, and except for 8-*p*SPT and PPADS were stored frozen. Further dilutions were made in Krebs solution as required.

Statistical analysis

Results are expressed as a mean ± s.e. (n). Differences between means were tested using Student's *t*-test for paired or unpaired data, as appropriate. Concentration–response relationships were compared using analysis of variance. A probability level of 5% was taken as the fiducial for significance.

Results

The diadenosine polyphosphates, Ap_3A, Ap_4A and Ap_5A all caused concentration-dependent depolarization of the smooth-muscle membrane in the guinea-pig vas deferens, with a potency order of $Ap_5A > Ap_4A \geq Ap_3A$ (Fig. 1). Ap_2A did not evoke depolarization at the highest concentration tested (1 mM). The three active dinucleotides were all more potent than ATP, which was tested at concentrations of 100 μM and 1 mM. At the higher concentrations tested the active dinucleotides evoked action potential discharge during the phase of depolarization. Usually action potential discharge was only transient, and stopped even though the smooth-muscle membrane remained depolarized, or was continuing to depolarize (Fig. 2A).

In response to EFS (10 pulses, 1 Hz, 100 V, 0·015–0·05 ms pulse-width) e.j.ps were generated that summated fully before the final (tenth) e.j.p. in the train. The mean ampli-

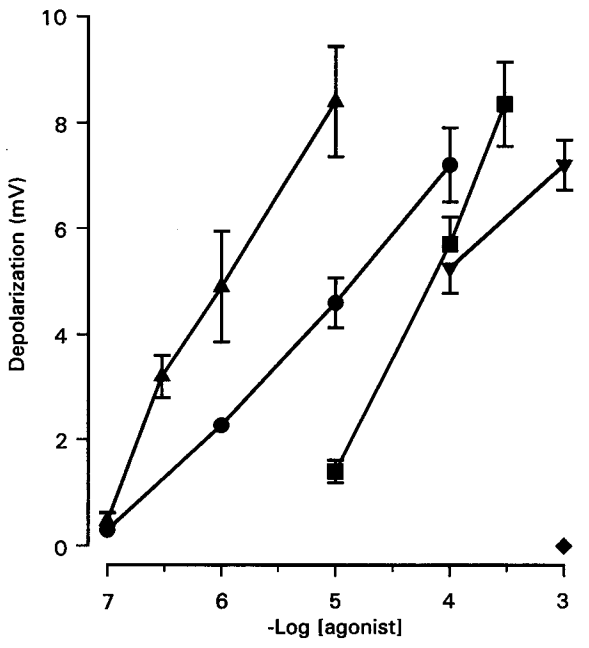

FIG. 1. Concentration–response relationships for depolarization of the smooth muscle cells of the guinea-pig vas deferens evoked by diadenosine pyrophosphate (Ap_2A,◆), diadenosine triphosphate (Ap_3A, ■), diadenosine tetraphosphate (Ap_4A, ●), diadenosine pentaphosphate (Ap_5A, ▲) and ATP (▼), recorded using the sucrose-gap technique.

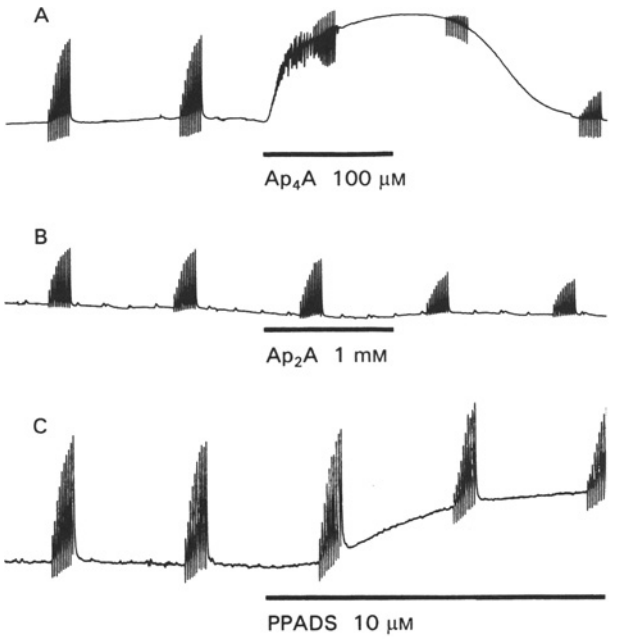

FIG. 2. Sucrose-gap recording of changes in membrane potential, and in response to electrical field stimulation evoked by A. diadenosine tetraphosphate (Ap_4A, 10 μM); B. diadenosine pyrophosphate (Ap_2A, 1 mM); and C. pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS, 10 μM). In each preparation trains of ten pulses of electrical field stimulation were applied at a frequency of 1 Hz every 60 s (100 V, 0·02–0·04 ms), and evoked excitatory junction potentials (e.j.ps, upward transients). The downward transients associated with the e.j.ps are stimulus artefacts. The transients occurring during the depolarization induced by Ap_4A are action potentials. Ap_4A and Ap_2A were applied for 60 s, as indicated by the bar. PPADS was applied for the duration indicated by the bar. The calibration bar in panel A also applies to panels B and C.

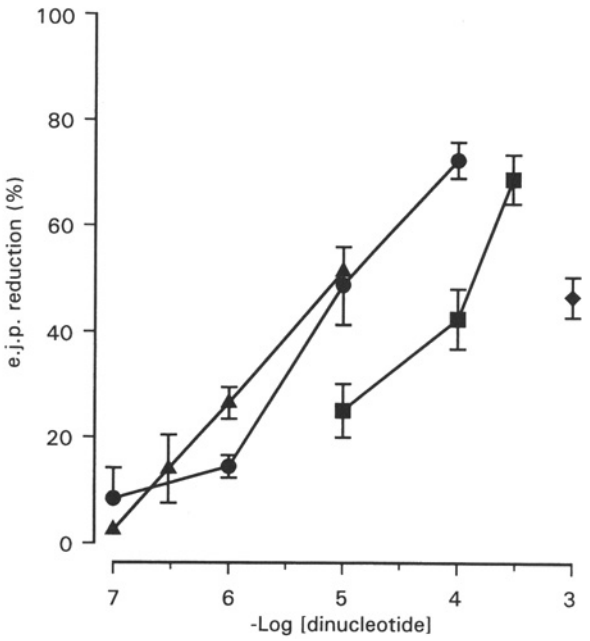

FIG. 3. Concentration–response relationships for inhibition of fully facilitated e.j.ps by diadenosine pyrophosphate (Ap_2A, ◆), diadenosine triphosphate (Ap_3A, ■), diadenosine tetraphosphate (Ap_4A, ●) and diadenosine pentaphosphate (Ap_5A, ▲) in the guinea-pig vas deferens. E.j.ps were measured 60–90 s after application of the dinucleotide had ended, and when the membrane potential had returned close to its control level.

tude of fully summated e.j.ps was 4·4 ± 0·27 mV (30). During depolarization evoked by the dinucleotides the amplitude of the e.j.ps tended to diminish, and remained diminished during the early stages of the wash-out, even though the membrane potential had returned to its control level (Fig. 2A). The reduction in e.j.p. amplitude evoked by the dinucleotides was concentration-dependent (Fig. 3). Ap_2A, which did not cause depolarization, also caused the e.j.p. amplitude to decrease (Fig. 2B). During perfusion with Ap_2A (mM) the fully facilitated e.j.p. was significantly reduced by 53·6 ± 3·79% (7) ($P < 0·01$) of its control value.

To test whether the decrease in e.j.p. amplitude was due to a postjunctional or prejunctional action, ATP, at a concentration that evoked a depolarization which approximated the amplitude of the fully facilitated e.j.p. (100 μM) was applied 1 min after wash-out of the dinucleotide had begun. There was no significant difference for any dinucleotide tested between the control response to ATP and that observed when applied at a time when e.j.ps would have been severely attenuated. For example, ATP at 100 μM produced a depolarization of 4·5 ± 0·41 (6) and 4·8 ± 0·84 mV (6) before and after application of Ap_4A (10 μM), respectively.

When Ap_3A (100 μM), Ap_4A (10 μM) or Ap_5A (1 μM) was applied for 20 min to induce tachyphylaxis, the response to EFS almost disappeared and the response to ATP (1 mM) was severely attenuated (Table 1), but depolarizations evoked by applied KCl (160 mM) were maintained.

PPADS (10 μM) caused a slow depolarization which reached a maximum of 4·6 ± 0·90 mV (15) after approximately 10 min, and a significant reduction in e.j.p. amplitude (Fig. 2C). After 25 min in PPADS (10 μM), the fully facilitated e.j.p. was reduced to 49·9 ± 6·76% (15) of its control value. In the presence of PPADS (10 μM), the depolarizing responses to Ap_3A, Ap_4A and Ap_5A were attenuated, but these compounds were still able to reduce the e.j.p. amplitude (Table 2). Ap_2A also inhibited e.j.p. generation in the presence of PPADS, with the fully facilitated e.j.p. being reduced to 48·5 ± 8·32% (4). This was not significantly different from the effect of Ap_2A in the absence of PPADS.

The P_1-purinoceptor antagonist, 8-*p*SPT (50 μM), had no direct effect on the membrane potential of the smooth muscle cells, nor on the responses to EFS: the fully facilitated e.j.p. was 5·2 ± 0·23 (15) and 5·4 ± 0·47 mV before and after incubation with 8-*p*SPT, respectively. 8-*p*SPT did not

Table 1. Inhibition of responses to applied ATP (1 mM) and excitatory junction potentials during tachyphylaxis to diadenosine triphosphate (100 μM), diadenosine tetraphosphate (10 μM) and diadenosine pentaphosphate (1 μM).

	Depolarization (mV)		Excitatory junction potential amplitude (mV)	
	Before	During	Before	During
Triphosphate	7·8 ± 0·1	0·3 ± 0·2**	6·1 ± 1·9	0·9 ± 0·2**
Tetraphosphate	6·6 ± 0·7	0·3 ± 0·2**	4·5 ± 0·3	0·2 ± 0·1**
Pentaphosphate	5·8 ± 1·0	2·2 ± 0·8*	6·8 ± 0·4	2·2 ± 0·8*

*$P < 0·05$, **$P < 0·01$, n = 4·6.

Table 2. Effects of incubation with pyridoxalphosphate-6-azophenyl-2', 4'-disulphonic acid (10 μM) on depolarization and inhibition of excitatory junction potential amplitude evoked by diadenosine triphosphate (100 μM), diadenosine tetraphosphate (10 μM) and diadenosine pentaphosphate (1 μM).

	Depolarization (mV)		Reduction in excitatory junction potential amplitude (%)	
	Before	After	Before	After
Triphosphate	5·7 ± 0·5	1.4 ± 0·8*	42·4 ± 5·6	36·3 ± 5·4
Tetraphosphate	4·7 ± 1·0	0·5 ± 0·6*	48·8 ± 7·5	40·0 ± 7·6
Pentaphosphate	6·6 ± 1·5	2·0 ± 0·8*	26·5 ± 3·0	18·0 ± 6·0

*$P < 0.05$, n = 4·6.

significantly affect the depolarization evoked by any of the nucleotides; however, in the presence of 8-*p*SPT the extent of inhibition of e.j.ps evoked by Ap_3A was significantly depressed, but the inhibitory responses to Ap_4A and Ap_5A were not (Fig. 4). The inhibition of e.j.p.-amplitude induced by Ap_2A (1 mM) was also antagonized by 8-*p*SPT (50 μM), it being 60·5 ± 8·51 (3) and 45·2 ± 6·36% (3) in the absence and presence of the antagonist, respectively ($P < 0.05$, paired *t*-test).

Discussion

The results obtained show that the adenine dinucleotides, Ap_3A, Ap_4A and Ap_5A act at postjunctional and prejunctional sites in isolated preparations of the guinea-pig vas deferens. The postjunctional site is the P_{2X}-purinoceptor, as evidenced by the depolarizations evoked by these compounds being inhibited by PPADS, which is a selective P_{2X}-purinoceptor antagonist (Lambrecht et al 1992; Ziganshin et al 1993, 1994; McLaren et al 1994). Furthermore, prolonged application of Ap_3A, Ap_4A and Ap_5A, which induced tachyphylaxis, also resulted in the responses to endogenous (EFS) and exogenous ATP being blocked in parallel. This is also indicative of an action mediated via P_{2X}-purinoceptors.

The slow depolarization caused by PPADS has been examined previously, and it seems to be a non-specific effect as it is unaffected by other P_2-purinoceptor antagonists such as suramin (McLaren et al 1994).

The rank order of potency of the dinucleotides that was observed in these experiments is similar to that seen at the smooth muscle P_{2X}-purinoceptor in the rat mesenteric vascular bed, but not at the endothelial P_{2Y}-purinoceptor nor the P_{2Y}-purinoceptor in the human colon (Hoyle & Burnstock 1992; Ralevic et al 1995). In rat mesenteric vascular bed, Ap_2A is devoid of P_{2X}-activity, Ap_3A only interacts with P_{2X}-purinoceptors at high concentrations, and Ap_5A is an order of magnitude more potent than Ap_4A, which is similar to what was seen here.

The lack of P_{2X}-purinoceptor-mediated excitation by Ap_2A is consistent with the actions of NAD, ADP-ribose and ADPβF. These compounds are chemically related because they, and Ap_2A, are all essentially derivatives of ADP, differing only in the groups substituted onto the β-phosphate. Pharmacologically, none of these compounds has been shown to have activity at P_{2X}-purinoceptors, while some of them (Ap_2A, ADP-ribose and ADPβF) have activity at P_{2Y}-purinoceptors (Hourani et al 1988; Hoyle & Edwards 1992; Ralevic et al 1995), and some (NAD, ADP-ribose and ADPβF) have activity at P_1-purinoceptors (Burnstock & Hoyle 1985; Hoyle & Edwards 1992; Wood et al 1992).

The prejunctional site of action of Ap_2A and Ap_3A was probably the P_1-purinoceptor, because inhibition of the e.j.p. appeared to be antagonized by 8-*p*SPT. However, the prejunctional purinocepter on sympathetic nerve terminals in the rat tail artery and vas deferens has been suggested to be a so-called P_3-purinoceptor, which is activated by both adenine nucleosides and adenine nucleotides, and which is sensitive to blockade by 8-*p*SPT (Shinozuka et al 1988, 1990; Forsyth et al 1991; Todorov et al 1994). However, the inhibition of the e.j.p. by Ap_4A and Ap_5A was

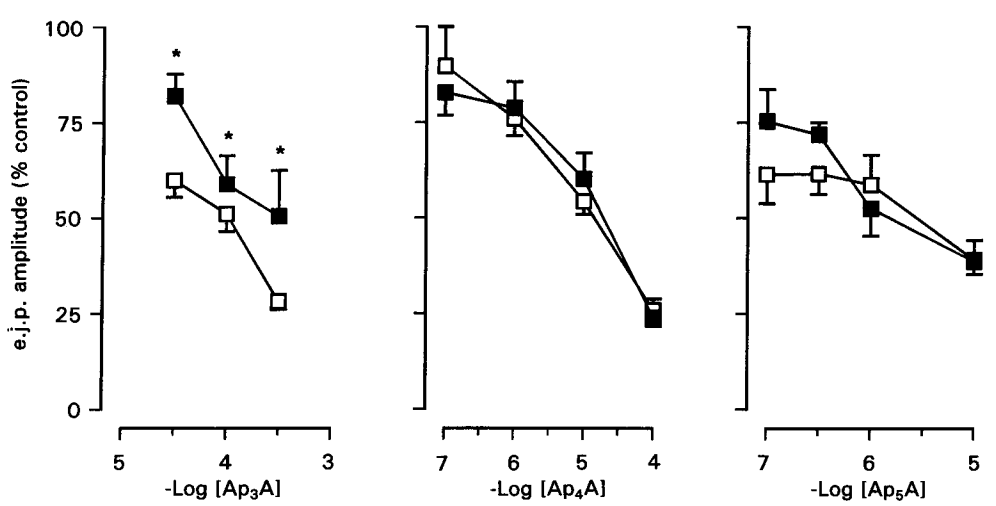

FIG. 4. Concentration–response relationships for inhibition of fully facilitated e.j.ps by diadenosine triphosphate (Ap_3A), diadenosine tetraphosphate (Ap_4A) and diadenosine pentaphosphate (Ap_5A) in the absence (□) and presence (■) of 8-*p*SPT (50 μM). *$P < 0.05$.

not affected by 8-*p*SPT, and thus these two dinucleotides did not appear to be activating a purinocepter like this. The reduction in e.j.p. amplitude seen during perfusion with Ap_4A and Ap_5A, and which persisted during the early stages of the wash-out, was unlikely to be due to desensitization of the postjunctional receptor, because responses to exogenous ATP were maintained at this particular time. The mechanisms underlying this effect remain to be investigated.

It has been suggested that the prejunctional effects of diadenosine polyphosphates in the vas deferens are due to the release of adenosine from the tissue components (Stone & Perkins 1981). The present study did not address this aspect; nevertheless this could be a possibility for the short-chain dinucleotides, but not for the long-chain ones.

The existence of receptors that are specific or selective for diadenosine polyphosphates has been questioned before (Stone 1981, 1991; Hoyle 1990, 1992; Pintor et al 1993a), but until a specific or selective antagonist is available the presence of a true dinucleotide receptor in this preparation must remain a speculation.

In conclusion, diadenosine polyphosphates with a chain that contains three or more phosphates can activate the postjunctional P_{2X}-purinoceptor in the guinea-pig vas deferens. Diadenosine pyrophosphate (Ap_2A) is unable to activate the P_{2X}-purinoceptor. All the dinucleotides act at a prejunctional site resulting in inhibition of release of ATP from the sympathetic nerve terminals. For Ap_2A and Ap_3A this effect is mediated via P_1-purinoceptors, but for Ap_4A and Ap_5A the type of receptor that they activate is unclear.

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