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The mechanism of action of AMP-induced inhibition of sympathetic neurotransmission in the isolated vas deferens of the rat and guinea-pig

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1 The proposal that adenosine 5'-monophosphate (AMP) can be used as a selective antagonist of ATP at P_2-purinoceptors on smooth muscle was investigated by examining the electrical and mechanical responses of guinea-pig and rat vasa deferentia to stimulation of sympathetic nerves and to exposure to exogenous agonists.

2 The magnitude of the contractile response of the rat vas deferens to field stimulation of the sympathetic nerves was reduced by addition of AMP. This effect was rapid in onset and readily reversed by washout.

3 The action of AMP on these contractile responses was reversed by the subsequent addition of the specific P_1-purinoceptor antagonist 8-phenyltheophylline (8-PT). 8-PT on its own had no significant effect on contractile responses to nerve stimulation.

4 The magnitude of excitatory junction potentials (e.j.ps) in the guinea-pig vas deferens evoked by a train of stimuli at 0.5 Hz was reduced in a dose-dependent manner by introduction of AMP (10^{-6}–10^{-3} M). The inhibitory effect of 10^{-5} M AMP on e.j.p. magnitude was completely and rapidly reversed by introduction of 10^{-5} M 8-PT. The effect of 10^{-4} M AMP was partially reversed by 10^{-5} 8-PT.

5 The contractile responses of the guinea-pig vas deferens to exogenously applied adenosine 5'-triphosphate (ATP) were not reduced by AMP, even at a concentration of 2.5×10^{-4} M. Similarly in the rat vas deferens, contractile responses to exogenously applied α,β-methylene ATP (a more potent P_2-purinoceptor agonist) were reduced by only 27.2%. The same concentration of AMP did not affect the contractile responses of the rat vas deferens to noradrenaline.

6 We conclude that the primary mechanism of action of AMP is to inhibit sympathetic neurotransmission by an agonist action at P_1-purinoceptors on the sympathetic nerve terminal reducing the release of neurotransmitter, and therefore AMP cannot be used as a selective P_2-purinoceptor antagonist.

Introduction

There is now considerable evidence that the sympathetic nerves innervating many smooth muscles, including the vas deferens and arteries of various species, release adenosine 5'-triphosphate (ATP) as a co-transmitter with noradrenaline (see for example Westfall *et al.*, 1978; Fedan *et al.*, 1981; Burnstock, 1982; Sneddon *et al.*, 1982; Meldrum & Burnstock, 1983; Sneddon & Burnstock, 1984a,b; Sneddon & Westfall, 1984; Stjarne & Astrand, 1984; Allcorn *et al.*, 1986; Burnstock & Warland, 1987a).

The most useful substances used so far to block responses of smooth muscle mediated by ATP have been arylazidoaminopropionyl ATP, $ANAPP_3$ (Fedan *et al.*, 1981), and the stable analogue of ATP, α,β-methylene ATP used to produce selective desensitization of the P_2-purinoceptors (Kasakov & Burnstock, 1983). However, both of these substances are potent agonists, which means that it is not possible to use them for continuous electrophysiological recordings. They are also relatively expensive and do not antagonize all responses mediated by ATP on smooth muscle, particularly those thought to be mediated by the P_{2y} subclass of purinoceptor (see

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Kennedy & Burnstock, 1985; Burnstock & Warland, 1987b). Therefore, the discovery of an inexpensive, selective, competitive antagonist of ATP-mediated responses on smooth muscle would be a major advance for testing the purinergic neurotransmission hypothesis. A recent report (Satchell, 1986) indicated that adenosine 5'-monophosphate (AMP) inhibits the twitch ('purinergic') component of the contractile response of the rat vas deferens to sympathetic nerve stimulation and the contractile response to exogenous ATP, but not that to exogenous noradrenaline (NA) or high K^+ solution. Since AMP had no agonist action on its own, and its effects were not reversed by the P_1-purinoceptor antagonist theophylline, the author suggested that AMP could be useful as a reversible, selective antagonist of P_2-purinoceptors. However, previous investigators have attributed the inhibitory effect of AMP on neurotransmission in the vas deferens to the prejunctional agonist action on P_1-purinoceptors, reducing transmitter release (Clanachan *et al.*, 1977; Wakade & Wakade, 1978; Stone, 1981).

In order to test these two possibilities, we have examined the effect of AMP on neurotransmission in the vas deferens in two ways. Firstly, we examined the effect of AMP on contractile responses of rat and guinea-pig vas deferens to nerve stimulation with trains of pulses, and exogenous agonists. Secondly, we examined the effect of AMP on the electrical response of the guinea-pig vas deferens to sympathetic nerve stimulation, the excitatory junction potential (e.j.p.). We used the selective P_1-purinoceptor antagonist 8-phenyltheophylline (8-PT) to determine whether the AMP was acting by postjunctional antagonism of P_2-purinoceptors or a prejunctional stimulation of P_1-purinoceptors (Clanachan, 1981). The electrophysiological model was particularly suitable since we have previously characterized in this tissue the inhibitory effect on e.j.ps of established P_1-purinoceptor agonists acting prejunctionally, and postjunctional P_2-purinoceptor inhibition. We found that changes in e.j.p. magnitude are a much more sensitive indicator of drug action than the corresponding changes in contractile responses (Sneddon *et al.*, 1984a; Sneddon & Westfall, 1984; Sneddon & Burnstock, 1984b).

Methods

Contractile responses to sympathetic nerve stimulation and exogenous agonists

Albino male rats or guinea-pigs 250–500 g were killed by a blow to the head and exsanguinated. The whole vas deferens was removed and pinned out on a Sylgard dish in modified Krebs solution. The surrounding connective sheath was carefully removed and the tissue opened up by a single longitudinal cut up one side of the muscle, exposing the lumen to the bathing solution. Each tissue was mounted under 1.5 g resting tension in a 5 ml organ bath. Tension was monitored with an isometric tension transducer (either Grass FT03 or Gould Metrigram), and recorded on a four channel pen recorder (either Grass 79D or Gould 2400S). Contractile responses to nerve stimulation were obtained at 10 min intervals by 20 s trains of pulses, 8 Hz, 0.5 ms pulse width, supramaximal voltage, from a stimulator (Grass S44) connected via a stimulus isolation unit (Grass SIU5) to platinum electrodes which ran parallel to the tissue, on either side, to ensure uniform stimulation of the nerves along the whole length of the tissue.

After the magnitude of the contractile responses had reached a steady level, usually after about 40 min, 4–6 control responses were obtained. After introduction of drugs into the bathing solution a further 4–6 responses were obtained, without interruption of the 10 min stimulating cycle. A similar protocol was adopted to examine the effects of AMP on contractile responses to exogenously applied ATP, α,β,methylene-ATP and NA, except that the time cycle of stimulation was increased as necessary, to allow complete recovery of the tissue before the next agonist application. Suitable agonist concentrations were determined from preliminary experiments so that the concentration of each agonist lay in the mid-range of the response curve. The Krebs solution used throughout was of the following composition (mM): NaCl 118, KCl 5.4, NaH_2PO_4 1.16, $NaHCO_3$ 25, $MgSO_4$ 1.16, $CaCl_2$ 2.5, glucose 11.1. The solution was bubbled continuously with 95% O_2/5% CO_2 gas and maintained at 36.5 ± 0.5°C. This applies also to the electrophysiological experiments described below.

Electrophysiology experiments

The tissue was dissected and mounted in an electrophysiology recording chamber in the same way as described above. Intracellular recordings were made with glass microelectrodes (20–40 MΩ) and the signal recorded onto a storage oscilloscope (Tektronix) and magnetic tape recorder (Racal) via a preamplifier (Cell Explorer 800, Dagan). Recordings were made only from cells with stable resting membrane potentials more negative than −60 mV. E.j.ps were evoked at a frequency of 0.5 Hz, with 0.5 ms pulses at a voltage which was below that required to initiate a muscle action potential. All quantitative data concerning drug effects on e.j.p. magnitude are based on continuous recordings from the same cell, taking the

average of at least five representative e.j.ps from the train before and after introduction of a drug, and after its washout.

Statistics

Values in the text refer to the mean ± s.e. mean. Statistical sigificance of results was tested with Student's t test for paired or unpaired data, as appropriate, and differences in the means of two groups were considered to be significant when $P < 0.05$.

Drugs

All drugs were made up immediately before each experiment except α,β-methylene ATP which was stored frozen as a 10^{-1} M solution in distilled water. 8-Phenyltheophylline (8-PT) was dissolved in 80% (v/v) methanol containing 0.2 M NaOH, and aqueous dilutions added to the Krebs solution. Drugs were obtained from the Sigma Chemical Co Ltd and included: adenosine-5'-triphosphate, disodium salt (ATP), α,β-methylene ATP, noradrenaline ((−)-arterenol hydrochloride, NA), adenosine 5'-monophosphate, sodium salt (AMP), and 8-phenyltheophylline (8-PT).

Results

Contractile responses to sympathetic nerve stimulation

The contractile response of the rat vas deferens to field stimulation of the sympathetic nerves with a train of pulses for 20 s at 8 Hz, produced the characteristic biphasic response which is illustrated in the control responses within Figures 1a and 1b. The timecourse and magnitude of the responses obtained at 10 min intervals was consistent over a period of at least 5 h. Addition to the bathing medium of AMP produced a reduction in the magnitude of the neurogenic response which was rapid in onset, less than 10 min, and lasted for as long as the AMP was present (up to 1 h). The inhibitory action of AMP was readily reversed by washout of the drug. The action of AMP was antagonized by subsequent introduction of 8-PT (10^{-5} M). Figure 1a illustrates the effects of 2.5×10^{-5} M AMP and Figure 1b the effects of 2.5×10^{-4} M AMP on the responses of the rat vas deferens to electrical stimulation of the sympathetic nerves, and the subsequent reversal of this effect by 8-PT (10^{-5} M).

The effects of AMP and 8-PT illustrated in Figure 1a and b were estimated quantitatively. The magnitude of the responses after drug treatment, expressed as a percentage of control, were as follows: (a) after AMP 2.5×10^{-5} M, 57.1 ± 3.6% ($n = 6$, $P < 0.01$);

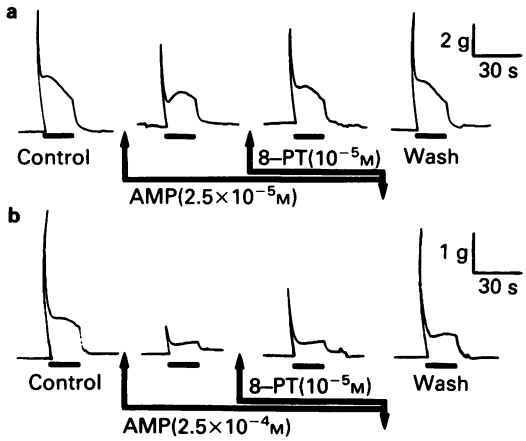

Figure 1 Effect of adenosine 5'monophosphate (AMP) and subsequent addition of 8-phenyltheopylline (8-PT) on contractile responses of rat vas deferens to sympathetic nerve stimulation (8 Hz, 0.5 ms pulse width, supramaximal voltage, for 20 s at 10 min intervals. The solid bar immediately below each response indicates a period of nerve stimulation). Note that addition of AMP did not have any effect on the resting tension of the vas deferens. (a) Effect of 2.5×10^{-5} M AMP followed by addition of 10^{-5} M 8-PT. (b) Effect of 2.5×10^{-4} M AMP followed by addition of 10^{-5} M 8-PT. These representative responses were taken from stages of the experiment where full equilibration of each drug effect had been reached. See Methods for details.

and after AMP 2.5×10^{-5} M + 8-PT 10^{-5} M, 75.0 ± 2.4% ($n = 6$, $P < 0.01$); (b) after AMP 2.5×10^{-4} M, 21.1 ± 3.0% ($n = 6$, $P < 0.01$); AMP 2.5×10^{-4} M + 8-PT 10^{-5} M, 48.0 ± 4.6% ($n = 6$, $P < 0.01$). Thus in each case the degree of reduction by AMP, and reversal by 8-PT, were statistically significant.

The introduction of AMP had no effect on the resting tension of the vas deferens. This was true of all the concentrations of AMP used in this study, 10^{-6}–10^{-3} M. 8-PT on its own did not significantly change the responses of the tissue to nerve stimulation (not shown). Notice that AMP reduced both phases of the contraction to a similar degree and that subsequent addition of 8-PT restored both of the responses equally well (see Discussion).

Electrophysiological investigations

Field stimulation of the guinea-pig vas deferens at a frequency of 0.5 Hz produced a train of e.j.ps which were fully facilitated after the 5th stimulus, and showed no sign of summation. The magnitude of the fully facilitated e.j.p. was reduced in a dose-dependent manner by concentrations of AMP from 10^{-6}–10^{-3} M. Figure 2a shows a representative

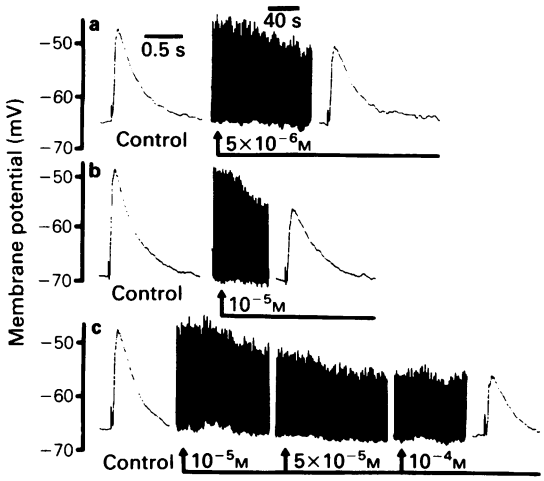

Figure 2 Effect of adenosine 5'-monophosphate (AMP) on the magnitude of intracellularly recorded e.j.ps in smooth muscle cells of the guinea-pig vas deferens. The e.j.ps were evoked by stimulation of the sympathetic nerves at 0.5 Hz, 0.1 ms pulse width at a voltage which was subthreshold for the initiation of an action potential. Fast time scale examples are shown before and after addition of AMP. Note that addition of AMP at any of the concentrations used did not have any effect on the resting membrane potential of the cell. (a) Effect of 5×10^{-6} M AMP on e.j.p. magnitude. (b) Effect of 10^{-5} M AMP. (c) Illustrating the dose-dependent decrease in e.j.p. magnitude due to increasing concentrations of AMP. Note that maximum depression of e.j.p. magnitude had occurred at a concentration below 10^{-4} M AMP.

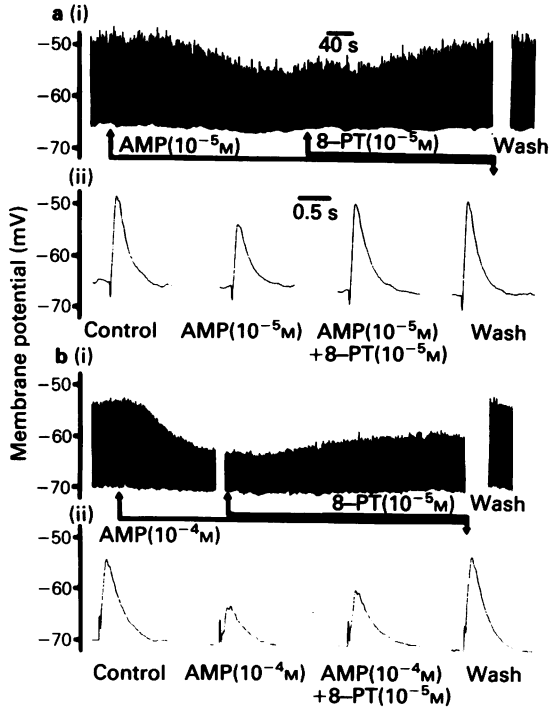

Figure 3 Representative recordings of e.j.ps produced by electrical stimulation of the sympathetic nerves of the guinea-pig vas deferens (Stimulation parameters as for Figure 2). Reversal by the P_1-purinoceptor antagonist, 8-phenyltheophylline (8-PT), of the inhibition of e.j.ps by adenosine 5'-monophosphate (AMP) is illustrated. a(i) Effect of subsequent addition of 10^{-5} M 8-PT on e.j.ps of a preparation which had previously been exposed to 10^{-5} M AMP. a(ii) Representative e.j.ps displayed using a fast time scale from each fully equilibrated stage of the experiment shown in a(i). b(i) Effect of 10^{-5} M 8-PT on e.j.ps of a preparation which had previously been exposed to 10^{-4} M AMP. b(ii) Representative e.j.ps from each stage of the experiment shown in b(i).

example which illustrates that the introduction of AMP did not produce any effect on the control resting membrane potential (70.6 ± 1.6 mV, $n = 18$), and that the onset of action of AMP was rapid. Figure 2c shows that the greatest reduction in e.j.p. magnitude was produced by 10^{-4} M AMP, which produced a reduction of about 50%. The action of AMP was readily reversed by washout of the drug.

Figure 3a is a representative example which shows that 10^{-5} M 8-PT completely reversed the inhibitory effect of 10^{-5} M AMP. The addition of 8-PT did not alter the resting membrane potential of the cells, nor did 8-PT alone affect e.j.p. magnitude (not shown). The effect of a maximally effective concentration of AMP, 10^{-4} M on e.j.ps is shown in Figure 3b. The subsequent addition of 10^{-5} M 8-PT only partially reversed the inhibitory effect of AMP, but washout of both drugs restored e.j.p. magnitude to control values. A higher concentration of 8-PT could not be used due to the limited solubility of this substance in physiological salt solution.

These effects of 8-PT and AMP on e.j.p. magnitude were evaluated quantitatively. AMP 10^{-5} M significantly reduced the e.j.p. magnitude from 19.7 ± 1.0 mV ($n = 18$) to 13.1 ± 0.8 mV ($n = 18$, $P < 0.01$). Subsequent addition of 10^{-5} M 8-PT produced a complete recovery of the e.j.p. magnitude to $21.0 + 1.1$ mV ($n = 7$, $P < 0.01$), which was not significantly different from control. In another group of experiments where 10^{-4} M AMP was used, e.j.p. magnitude declined from a control mean of 20.1 ± 0.8 mV ($n = 21$) to 11.2 ± 0.7 mV ($n = 21$, $P < 0.01$). In this case subsequent addition of 10^{-5} M 8-PT produced a significant recovery of the e.j.p. magnitude to 13.2 ± 0.9 mV ($n = 7$, $P < 0.01$); however, the recovery was partial, since this value was still significantly less than the control ($P < 0.01$).

Effect of AMP on contractile responses to exogenously applied agonists

It is clear from the above results that the inhibitory effects of AMP on neurotransmission in rat vas deferens are reversed by the specific P_1-purinoceptor antagonist 8-PT, indicating that the primary effect of AMP is mediated via its agonist action on prejunctional P_1-purinoceptors. To test for possible postjunctional actions of AMP, we examined its effect on contractile responses to exogenously applied P_2-purinoceptor agonists. ATP was used for the experiments in the guinea-pig vas deferens, and α,β-methylene ATP for the rat vas deferens (ATP could only produce sizeable contractions at high concentrations in the rat, and since these responses were not always reproducible, it was prefereable to use the more potent analogue). The effect of AMP on contractions to exogenous noradrenaline was also examined.

Figure 4a shows a representative record of the effect of AMP on contractions to 3×10^{-6} M ATP. Similar results were obtained with various concentrations of ATP. A concentration of 2.5×10^{-4} M AMP had been shown previously to produce a maximal depression of neurotransmission in the guinea-pig vas deferens, as estimated by the nerve stimulation experiments, but it did not produce any significant reduction in the magnitude of the contractile response of the guinea-pig vas deferens to various concentrations of exogenous ATP. After equilibration for 2 h in the AMP, the average size of ATP-induced contractions, as a percentage of controls, was as follows; 3×10^{-6} M ATP, 116.8 ± 9.9% ($n = 7$); 10^{-5} M ATP, 117.3 ± 17.1% ($n = 6$); 3×10^{-5} M ATP, 109.8 ± 17.4% ($n = 7$). None of these is significantly different from control. In some cases a transient potentiation of the response to ATP was observed after the introduction of AMP, as indicated in Figure 4a. Figure 4b shows a representative record of the effect of the same concentration of AMP on contractile responses of the rat vas deferens to exogenously applied α,β-methylene ATP. It was found that these responses were also largely resistant to the AMP. After 1 h in 2.5×10^{-4} M AMP, responses were still 72.8 ± 3.4% of control ($n = 6$). The same concentration of AMP had no significant effect on the responses of the rat vas deferens to exogenous NA, 3×10^{-5} M (see Figure 4c). After 1 h in 2.5×10^{-5} M AMP, responses to NA were still 105.9 ± 6.0% of control ($n = 6$).

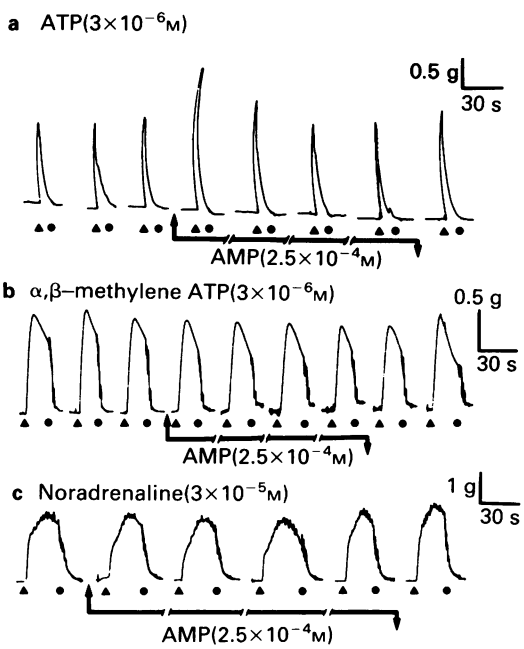

Figure 4 The effect of adenosine 5'-monophosphate (AMP) on contractile responses of rat and guinea-pig vasa deferentia to exogenous agonists. The agonist challenge was repeated at 30 min intervals in each case; (▲) indicates addition of drug; (●) indicates washout of drug. (a) Effect of 2.5×10^{-4} M AMP on responses of guinea-pig vas deferens to 3×10^{-6} M ATP. (b) Effect of 2.5×10^{-4} M AMP on responses of rat vas deferens to 3×10^{-6} M α,β-methylene ATP. (c) Effect of 2.5×10^{-4} M AMP on responses of rat vas deferens to 3×10^{-5} M noradrenaline.

Discussion

The results presented in this paper are consistent with the view that the inhibitory effect of AMP on sympathetic neurotransmission in the vas deferens of rat and guinea-pig is due to a prejunctional agonist action at P_1-purinoceptors mediating a reduction in neurotransmitter release (Clanachan *et al.*, 1977; Wakade & Wakade, 1978; Stone, 1981), and are contrary to the suggestion by Satchell (1986) that the action of AMP could be attributed to a selective antagonism of postjunctional P_2-purinoceptors.

The main difference between our experiments and those of Satchell was that whilst he used theophylline (30–1000 μM) to test for P_1-purinoceptor-mediated actions of AMP, we used 8-PT, which is a more potent and more selective antagonist (Clanachan, 1981). We have previously demonstrated the selectivity of 8-PT in the guinea-pig vas deferens, where it reversed completely the inhibitory effect on e.j.p. magnitude of the specific P_1-purinoceptor agonist 2-chloroadenosine (Sneddon *et al.*, 1984a). Since we obtained a similar reversal of the action of

AMP, using the same protocol, and the same concentration of 8-PT, it is reasonable to conclude that the effect of AMP is like that of the 2-chloroadenosine, i.e. a selective agonist action at P_1-purinoceptors on the sympathetic nerves, to reduce transmitter release.

If AMP is acting only prejunctionally then it follows that it should not reduce the response obtained to P_2-purinoceptor agonists, such as ATP. However, Satchell (1986) reported that 2.5×10^{-4} M AMP reduced contractions to 2.5×10^{-5} M ATP to 8.6% of control value. In contrast to his results, we found that this concentration of AMP (2.5×10^{-4} M) did not reduce contractions to a range of concentrations of exogenous ATP in the guinea-pig vas deferens. In fact, in some cases a significant potentiation of the contractile response was observed (Figure 4a). This potentiation by AMP of contractile response of guinea-pig vas deferens to ATP has recently been reported by Fedan (1987), who observed that even concentrations of AMP as high as 10 mM produced transient enhancement of ATP-induced contractions. Thus, both of these results would seem to rule out any substantial postjunctional antagonist effect of AMP in guinea-pig vas deferens. Similarly, we observed only a small inhibitory effect of AMP on α,β-methylene ATP-induced contractions in the rat vas deferens (Figure 4b). The finding that responses to exogenous NA were unaffected by AMP indicates that it has no non-specific inhibitory effects on the responsiveness of the smooth muscle to agonists. The transient facilitatory effect of AMP on contractions to ATP were not investigated further in this study, but a detailed investigation of this aspect has been published by Fedan (1987).

The cotransmitter hypothesis as described for the vas deferens (e.g. see Sneddon & Westfall, 1984) predicts that any substance that acts prejunctionally to inhibit transmitter release will inhibit both phases of the nerve-mediated contraction, since ATP and NA are thought to be released from the same sympathetic nerve varicosities. Satchell (1986) reported that the initial phasic, 'purinergic' component of the biphasic contraction of the rat vas deferens was inhibited by AMP, but not the secondary, tonic 'adrenergic' component, i.e. a result compatible with a postjunctional antagonist action of P_2-purinoceptors. We did not find any apparent selectivity of AMP for either component of the biphasic contraction of the rat vas deferens in response to nerve stimulation (see Figure 1a and b), which is consistent with our suggestion that AMP acts prejunctionally. We did not make any quantitative assessment of the effect of AMP on the two phases of the biphasic contractile responses, since it is now apparent that to consider the first phase as 'purinergic' and the second as 'adrenergic' is an over simplification, particularly in the rat, where the separation of the two components is not very clear, compared with the responses in other species such as the rabbit (Sneddon *et al.*, 1984b). Nevertheless, it is quite obvious that in our experiments the inhibitory actions of AMP were not confined to the phasic portion of the responses, and are therefore different from the result described by Satchell (1986).

A further consideration relating to the mechanism of action of AMP is that it would be expected that the AMP would be quite rapidly broken down in the tissue to produce adenosine, which could contribute to the inhibitory action of the drug via a prejunctional agonist action at P_1-purinoceptors. It is not possible to tell from our experiments whether the inhibitory effects on neurotransmission which we saw were due to AMP *per se*, or the accumulation of adenosine, since the effect of both would be reversed by 8-PT. However, this consideration does not detract from our conclusion that the inhibitory effects of AMP are ultimately mediated via prejunctional P_1-purinoceptors.

From the results presented in this paper, we conclude that AMP cannot be used as a selective P_2-purinoceptor antagonist for the investigation of ATP as a putative cotransmitter in sympathetic nerves, since its primary action is to inhibit sympathetic neurotransmission by acting as an agonist at prejunctional P_1-purinoceptors.

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