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The activity of phosphorothioate analogues of ATP in various smooth muscle systems

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1 Phosphorothioate analogues of adenosine 5′-triphosphate (ATP) have been tested on the rat and guinea-pig vas deferens, the guinea-pig taenia coli and urinary bladder.

2 Adenosine 5′0-(2-thiotriphosphate) (ATPβS) was more active than adenosine 5′0(1-thiotriphosphate) (ATPαS) and ATP in producing contractile responses on the vas deferens of rat and guinea-pig, and guinea-pig bladder, though the difference of potency was less marked for producing relaxation of the carbachol-contracted taenia coli. No differences were observed between the A and B diastereoisomers of ATPαS or ATPβS.

3 Contractions of the vas deferens produced by ATPαS were of much longer duration than those produced by ATPβS.

4 When tested against electrically-evoked twitch responses of the vas deferens the order of potencies was reversed with ATP being most active and ATPβS least active. These inhibitory effects were blocked by 8-phenyl-theophylline. The calculated pA_2 values for ATP, adenosine, β, γ-methylene ATP (APPCP) and ATPαS were similar, suggesting a common site of action.

5 The results do not reveal any stereoselectivity among the tissues tested, for the diastereoisomers of ATP phosphorothioates; the observed differences of potency may be due to differences between ATPαS and ATPβS in their rates of metabolism to adenosine. The different response profiles to the phosphorothioates may however reflect some differences of receptor mechanisms.

Introduction

Purine compounds such as adenosine and its triphosphate derivative (ATP) exert a variety of effects on different organs and tissues of the body (Burnstock, 1972; 1981; Stone 1981a; Su, 1983). However, adenosine exerts many of its actions on the presynaptic nerve terminal, giving rise to the speculation that this compound functions primarily as a regulator of neuroeffector interaction (DeMey *et al.*, 1979; Stone, 1981a; Burnstock, 1983; Fredholm *et al.*, 1983). ATP on the other hand has most of its direct actions at the postjunctional membrane, giving rise to the suggestion that some neurones may release sufficient ATP either alone (Burnstock, 1972) or as a cotransmitter (Sneddon *et al.*, 1982; Burnstock, 1983) to alter directly the activity of the effector tissue, possibly by activating a specific set of calcium channels (Stone, 1981b).

Although arylazidoaminopropionyl ATP analogues are ATP antagonists (Fedan *et al.*, 1981; Sneddon *et al.*, 1982; Westfall *et al.*, 1983) these compounds cannot block all the actions of ATP (Fedan *et al.*, 1982; Frew & Lundy, 1982), possibly indicating subtypes of receptor. It has even been suggested that ATP and ADP may act on distinct populations of receptors in the same tissue (Schwartzman *et al.*, 1981).

Another approach to the study of ATP receptors is to use analogues with agonist activity which may clarify the possible existence of receptor subtypes. In the present study, the effects of a series of phosphorothioate analogues of ATP have been examined in a variety of smooth muscles: guinea-pig taenia coli, urinary bladder and vas deferens, and the rat vas deferens. In these analogues (Figure 1) one non-bridging oxygen atom of a phosphate group has been replaced by sulphur (Eckstein, 1980; 1983). The effects of these compounds have been compared with ATP, β, γ-methylene ATP and adenosine 5′-O-(3-thiotriphosphate) (ATPγS).

Methods

Male guinea-pigs weighing 400–600 g were killed by stunning and cervical dislocation and the taenia coli dissected out as described by Burnstock *et al.* (1966)

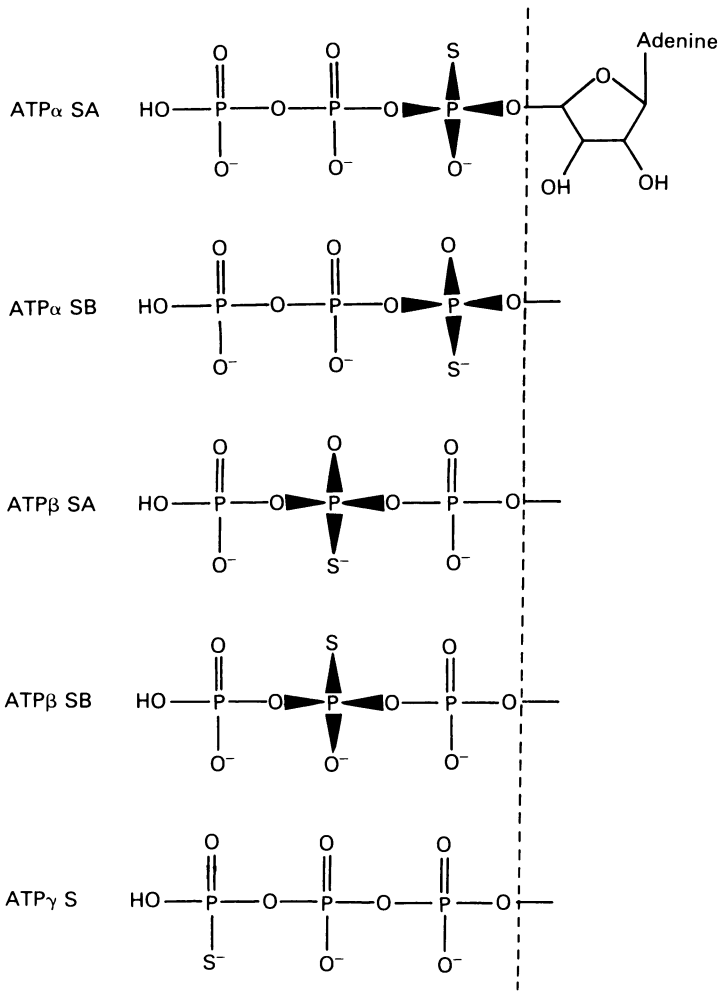

Figure 1 Structural formulae of the phosphorothioate analogues of adenosine 5′-triphosphate (ATP) used in this study.

(taenia strip preparation). Segments of taenia 1–2 cm in length were then suspended in 6 ml organ baths containing Krebs solution (mM composition: NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.3, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 11) gassed with 95% O_2/5% CO_2 and maintained at 37°C. In all experiments the taenia strips were allowed a 10 min period of equilibration in the organ bath before being connected to a force displacement transducer to measure isometric tension. The baseline tension (in the absence of carbachol) was adjusted to approximately 1 g. A further period of 30 to 60 min elapsed before drugs were added to the bath.

In some experiments the bathing medium also contained atropine, 1 μM and guanethidine, 10 μM, in order to examine nucleotide effects on resting tone. In other cases atropine was omitted and the tone of the preparation raised by administering carbachol, 0.1 μM, into the organ bath 60 s before the administration of nucleotide.

The Krebs solution was perfused continuously at a rate of approximately 3 ml min^{-1}. Drugs were administered directly into the organ bath in a volume of 50 or 100 μl. The concentrations of compounds quoted throughout this paper are the calculated final bath concentrations.

Guinea-pig vasa deferentia cleaned of connective tissue, blood vessels and semen and strips of bladder (from the same animals) from which the mucosa was carefully cut away (Ambache & Zar, 1970) were

suspended in the medium described above, but always with atropine 1 μM and guanethidine 10 μM in the organ baths. Tension was recorded isometrically with a baseline tension of 0.5 g imposed on the bladder preparation and 1 g on the vas deferens.

The prostatic portion of vasa deferentia from 200 g rats were suspended in the same apparatus and Krebs solution (but without atropine or guanethidine) but were in addition stimulated to contract by a pair of parallel platinum wires positioned alongside the preparation. Stimuli were delivered at a frequency of 0.1 or 0.05 Hz, duration 1 ms and 80 V amplitude.

Most tissues were exposed to no more than two of the phosphorothioates and the order of presentation was varied both with respect to the compound and its concentration. Some tissues however were tested with all the analogues to confirm their relative activities and their antagonism by 8-phenyltheophylline, for example, under identical conditions. Again care was taken to ensure a varied order of presentation.

Materials

The following drugs have been used; atropine sulphate (Sigma); adenosine 5′-triphosphate disodium salt (ATP) (Sigma); β,γ-methylene ATP (APPCP) (Sigma); adenosine 5′-O-(3-thiotriphosphate) lithium salt (ATPγS) (P-L Bio-chemicals); guanethidine sulphate (CIBA); 6-(2-hydroxy-5-nitrobenzyl)-thioguanosine (HNBTG) (Calbiochem); erythro-9-(2-hydroxy-3-nonyl)-adenine HCl (EHNA) (Wellcome Research Laboratories).

The diastereoisomers of adenosine 5′O-(2-thiotriphosphate) (ATPβS) and adenosine 5′O-(1-thiotriphosphate) (ATPαS) were synthesized as described previously (Yee *et al.*, 1979). The isomers are referred to as A and B isomers (Eckstein & Goody, 1976) the A isomers having the $\mathbf{R}_p$ configuration and the B isomers the $\mathbf{S}_p$ configuration (Eckstein, 1983) see Figure 1). ATPγS does not exist in isomeric forms.

As the synthetic compounds ATPαS and ATPβS contained an unknown quantity of water of crystallization, a solution of approximately 10^{-3} M was made and the concentration of nucleotide in that solution or a dilution of it was determined by u.v. absorption spectrophotometry at 259 nm. The concentration was read from a standard curve plotted for ATP, as the phosphorothioate analogues show the same spectrophotometric properties as ATP itself. Dilutions of the concentrated solution were then made so as to provide equivalent standard solutions (1, 10, 100 μM etc) of the various agonists. Because of the expense or limited quantities available several compounds were tested to a maximum concentration of only 100 μM.

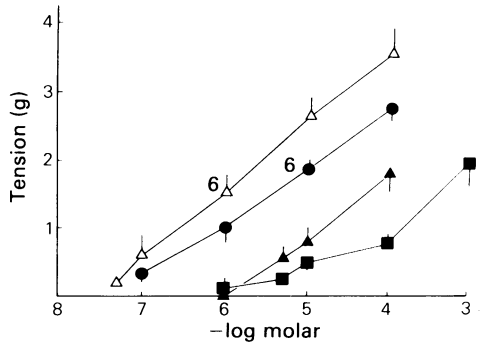

Figure 2 log Concentration-response curves for some of the ATP analogues producing contraction of the guinea-pig vas deferens: ATP (■), ATPαSB (▲), APPCP (●) and ATPβSB (△) (for abbreviations, see text). In this and all subsequent figures the symbols indicate the mean, and vertical lines the standard error of the mean, of results from five preparations unless indicated otherwise by a number alongside the symbol.

Results

Guinea-pig vas deferens

Both ATPαS and ATPβS produced contraction of the isolated guinea-pig vas, though the βS compounds were substantially more potent than the αS compounds. As can be seen in Figure 2 there is an approximately 100 fold difference of potency at all points on the dose-response curves obtained: full dose-response curves could not be produced because of the limited quantities available. The concentrations producing a 1.5 g increase of tension were 1.02 ± 0.1 μM (s.e.mean., $n = 5$) and 44 ± 13 μM for the ATPβSB and αSB isomers respectively. APPCP and ATPγS were intermediate in potency, but so

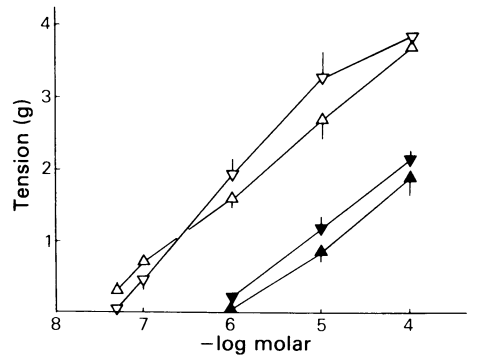

Figure 3 log Concentration-response curves for the diastereoisomers of ATPαS and ATPβS in producing contraction of the guinea-pig vas deferens: ATPαSA (▼), ATPαSB (▲), ATPβSA (▽), ATPβSB (△). Details as for Figure 2.

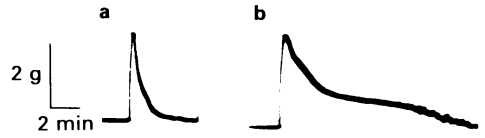

Figure 4 Representative responses of the vas deferens, of similar amplitude, to (a) ATPβSB, 1 μM and (b) ATPαSB, 100 μM.

similar that only the dose-response curve of the former is shown in Figure 2 (also Figures 5 and 7). ATP itself was less active than any of the modified compounds, producing a 1.5 g increase of tension at 341 ± 32 μM ($n = 5$).

As illustrated in Figure 3 the A and B isomers of ATPαS or ATPβS were equally active. None of the pairs of points was significantly different (Student's t test).

Response profile The shapes of contractile responses of the guinea-pig vas to applied nucleotides have been described in some detail by Fedan *et al.* (1982). In particular it was possible to confirm the appearance of a second phase to responses to ATP when applied at concentrations of about 100 μM and above, seen as an increased slope of the concentration-response curve (Figure 2). However, a marked difference was found between responses to ATPαS and ATPβS, as illustrated in Figure 4. At concentrations which produced initial peak contractions of similar amplitude, the response to ATPαS was much more prolonged in duration.

Guinea-pig bladder

In producing contraction of the guinea-pig bladder strip to a tension of 1 g, the order of potency was the same as for contraction of the vas,

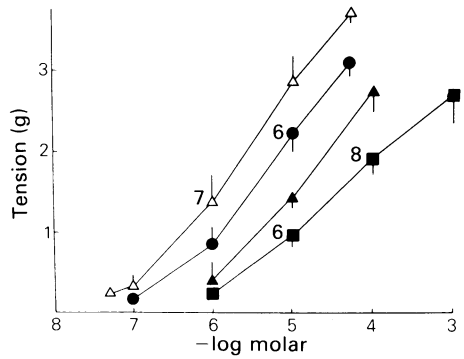

Figure 5 log Concentration-response curves for some ATP analogues producing contraction of the guinea-pig bladder: ATP (■), ATPαSB (▲), APPCP (●) and ATPβSB (△). Details as for Figure 2.

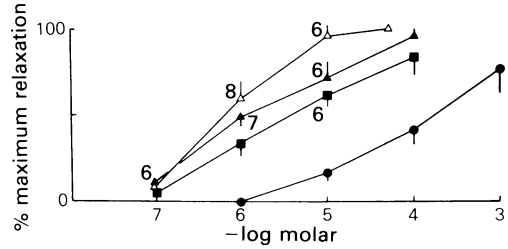

Figure 6 log Concentration-response curves for some ATP analogues producing relaxation of the guinea-pig taenia coli: ATP (■), APPCP (●), ATPαSB (▲) and ATPβSB (△). Details as for Figure 2.

ATPβS > APPCP > ATPαS > ATP (Figure 5). Again the A and B isomers of ATPβS and αS were not significantly different (Student's t test), the concentrations producing a 1.5 g increase of tension being respectively 1.16 ± 0.06 μM (s.e.mean, $n = 5$) and 1.05 ± 0.11 μM ($n = 5$) for ATPβSA and B, and 10.6 ± 1.8 μM ($n = 5$) and 12.1 ± 2.0 μM ($n = 5$) for ATPαSA and B.

Guinea-pig taenia coli

In producing relaxation of the carbachol-contracted taenia, ATPβS was again the most potent compound tested, ATPβSB having an EC_{50} of 0.6 ± 0.04 μM ($n = 5$). However, in this preparation ATPαS showed almost the same potency for ATPαSB, $EC_{50} = 1.08 \pm 0.14$ μM ($n = 6$) (Figure 6) with ATP itself being very active (EC_{50} 4.1 ± 0.31 μM, $n = 5$). Indeed none of the points on the ATP and ATPαS dose-response curves was significantly different (Student's t test). APPCP on the other hand was only weakly active in this system, being at least one hundred times less potent than ATPβS with an EC_{50}

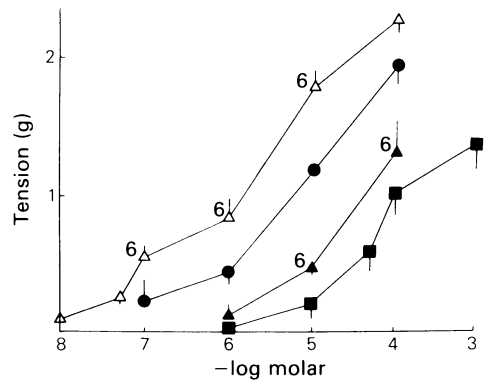

Figure 7 log Concentration-response curves for some ATP analogues producing contraction of the rat vas deferens: ATP (■), ATPαSB (▲), APPCP (●) and ATPβSB (△). Details as for Figure 2.

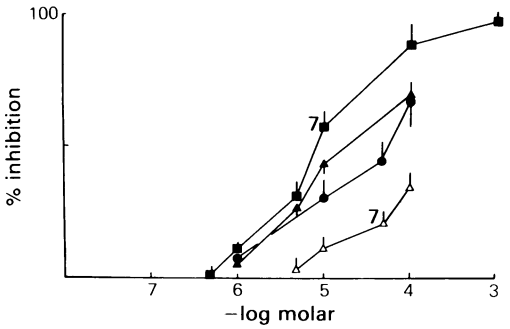

Figure 8 log Concentration-response curves for some ATP analogues producing inhibition of electrically-induced twitch contractions of the rat vas deferens: ATP (■), ATPαSB (▲), APPCP (●) and ATPβSB (△). Details as for Figure 2.

of $165 \pm 24\,\mu M$ ($n=5$). The A and B isomers of ATPαS and ATPβS were equiactive, showing EC_{50} values of 0.96 ± 0.03 ($n=5$) and $1.08 \pm 0.14\,\mu M$ ($n=6$) for ATPαSA and B, and $0.81 \pm 0.16\,\mu M$ ($n=5$) and $0.60 \pm 0.04\,\mu M$ ($n=5$) for the A and B iosmers of ATPβS.

Whereas in four of twelve preparations the relaxant response to ATP was followed by a contraction of the taenia, none of the modified compounds produced such biphasic responses when tested on at least 12 tissues each.

Rat vas deferens

In producing contraction of the unstimulated rat vas deferens the order of potency was similar to that seen on the guinea-pig preparation, ATPβS > APPCP > ATPαS > ATP (Figure 7). A and B isomers were indistinguishable, the concentration producing 1 g of tension being $1.30 \pm 0.21\,\mu M$ ($n=5$) and $1.41 \pm 0.10\,\mu M$ ($n=6$) for ATPβSA and B and $35 \pm 7.1\,\mu M$ ($n=5$) and $44 \pm 6.2\,\mu M$ ($n=6$) for ATPαSA and B.

In producing depression of the electrically evoked twitch contraction of the vas the order of potency was reversed, with ATP > ATPαS > APPCP > ATPβS (Figure 8). The unusual dose-response profiles in Figure 8 are probably the result of examining biphasic responses (direct contraction superimposed upon inhibition of electrically evoked contraction) where the two components have different dose-response relationships and time courses.

Effect of 8-phenyltheophylline

Figure 9 illustrates the effect of 8-phenyltheophylline perfused continuously at a concentration of $10\,\mu M$, on responses of the electrically stimulated rat vas to ATP analogues. It is clear that the depression of evoked activity could be substantially reduced by the xanthine. The parallel shift to the right of the dose-response curves for ATP, adenosine, APPCP and ATPαS is illustrated in Figure 10. 8-Phenyltheophylline alone had no effect on the basal twitch height in 7 of 8 preparations. The exceptional tissue, in which an increase of twitch was seen during perfusion with this xanthine, is illustrated in Figure 9. In 3 experiments clonidine was used as a control agonist but the depression of twitch produced was unchanged by 8-phenyltheophylline (EC_{50} control 82 ± 2 nM, with 8-phenyltheophylline 89 ± 12 nM $n=3$).

The antagonism of purines by 8-phenyltheophylline was also studied at an antagonist concentration of $2\,\mu M$ (which itself did not change basal twitch size in any of 9 preparations), and Schild plots (Arunlakshana & Schild, 1959) constructed for

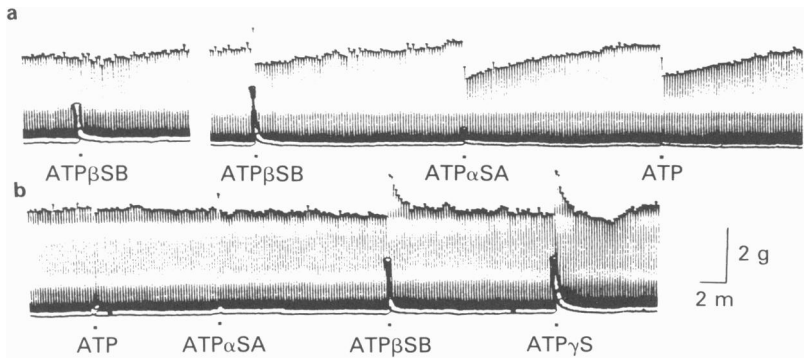

Figure 9 The effects of ATP analogues on the electrically-induced twitch of rat vas deferens. In (a) are shown control records of the inhibitory effects of ATPβSB (1 μM); ATPβSB (5 μM); ATPαSA (10 μM) and ATP (10 μM). In (b) the following responses are illustrated during perfusion with 8-phenyltheophylline (10 μM): ATP (10 μM); ATPαSA (10 μM); ATPβSB (5 μM) and ATPγS (10 μM).

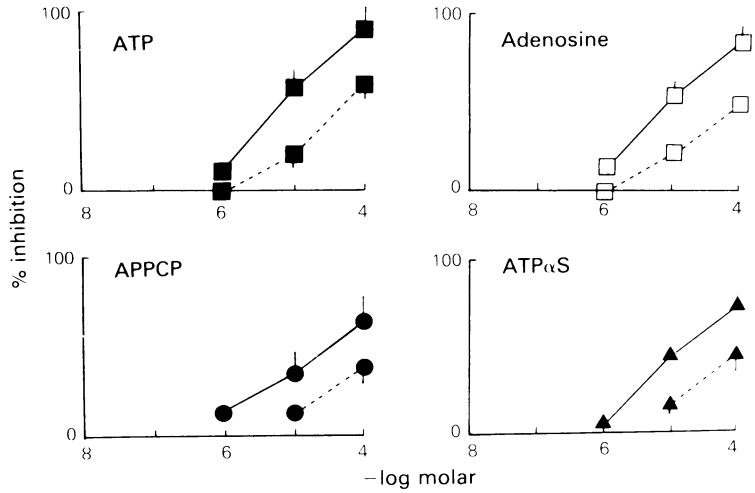

Figure 10 log Concentration-response curves for the inhibitory effects of some purine analogues on the electrically-induced twitch of rat vas deferens: ATP (■), adenosine (□), APPCP (●) and ATPαSB (▲). Solid lines indicate control curves, broken lines indicate responses in the presence of 8-phenyltheophylline (10 μM). Details as for Figure 2.

the same four compounds represented in Figure 10. From these plots an estimate of pA_2 was obtained (Table 1) and, as these did not differ significantly for the four agonists it may be suggested that all are producing their depression of the evoked twitch by acting at similar sites.

In contrast, 8-phenyltheophylline had no effect on the contractile responses of any of the preparations tested here (3 rat vasa, 3 guinea-pig vasa, 2 bladder strips) or on the relaxant responses of the taenia coli (4 preparations).

HNBTG and EHNA

6 - (2 - Hydroxy - 5 - nitrobenzyl) - thioguanosine (HNBTG) is an inhibitor of nucleoside transport processes, and thus inhibits adenosine uptake, and erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) is an inhibitor of adenosine deaminase.

Table 1 pA_2 values for 8-phenyltheophylline against purine inhibition of stimulus-evoked twitches of rat vas deferens

Compound	EC_{50} (μM)	pA_2
Adenosine	9.0 ± 1.3 (6)*	6.08 ± 0.15 (4)
ATP	9.5 ± 2.4 (5)	6.16 ± 0.24 (4)
ATPαS	27 ± 3.2 (5)	6.30 ± 0.18 (4)
APPCP	47 ± 4.5 (6)	6.36 ± 0.20 (4)

*mean ± s.e.mean (n).
The values of pA_2 are not significantly different (Student's t test).

In two experiments a mixture of HNBTG 2 μM and EHNA 2 μM was perfused over preparations of the rat vas. Neither the contractile effects of the nucleotides ATP, APPCP, ATPβS and ATPαS on this tissue nor their inhibitory effects on electrically evoked contractions were affected by this treatment.

Discussion

The most significant finding in the present study was that of a substantial difference of potency between the αS and βS analogues of ATP: the βS compounds are ten to one hundred times more potent in producing contractile responses of the rat and guinea-pig vasa deferentia and guinea-pig bladder. Indeed the potency of ATPβS is comparable with or greater than that of APPCP, an analogue of ATP frequently used to probe ATP receptors because of its high resistance to metabolism.

The question therefore arises of whether the greater potency of ATPβS is due to a higher efficacy, or to reduced metabolism compared with ATP itself. Metabolism normally proceeds to ADP and AMP by the action of ATPases, 5'nucleotidases and pyrophosphatases (Burnstock, 1972; Satchell, 1981), and Burgers & Eckstein (1978) have demonstrated that ATPαS is metabolized by snake phosphodiesterase at one fiftieth or less of the rate of ATP itself. The resistance to metabolism of APCPP and APPCP is normally attributed to the replacement of the oxygen atom linking the phosphorus atoms by a methylene group, since degradation of the molecule

occurs at these points. However, in ATPβS and ATPαS the substitution of oxygen by sulphur is in a non-linking position (Figure 1) which would mean it is unlikely to be a limiting factor in the hydrolysis of the molecule.

An alternative explanation of the resistance of phosphorothioates to metabolism might be that the non-bridging oxygen atoms are required for attachment to the relevant enzymes, or that the sulphur replacement offers some steric hindrance to the access of enzymes to the bridging oxygen. Whichever explanation is correct, it is clear that the two non-bridging oxygen atoms are functionally equivalent as the A and B isomers show very similar activity.

Nucleotide receptors are generally located postjunctionally, and any presynaptic inhibitory activity of nucleotides is therefore usually attributed to their hydrolysis to adenosine (De Mey *et al.*, 1979). Consistent with this, in the present study the order of potency in inhibiting the electrically-evoked twitch of the rat vas deferens was the reverse of that seen for contractile activity, with ATP > ATPαS > APPCP > ATPβS. Confirmation that this inhibitory activity was due to adenosine was obtained by demonstrating blockade of the inhibition with 8-phenyltheophylline (Griffith *et al.*, 1981) but a shift of dose-response curves by a combination of the adenosine uptake inhibitor HNBTG and the adenosine deaminase inhibitor EHNA was not observed.

The conclusion that the presynaptic inhibitory activity of nucleotides is due to adenosine is consistent with several previous studies (De Mey *et al.*, 1979; Stone, 1981b), but Taylor *et al.* (1983) concluded that presynaptic inhibition of the vas by compounds such as APPCP could not be blocked by theophylline and was therefore due to a presynaptic nucleotide receptor (Taylor *et al.*, 1983). In the present study therefore the pA_2 values for 8-phenyltheophylline against adenosine, ATP, APPCP and ATPαS were compared, but no significant differences were found between the several pA_2 values, implying a common site of action. It is not clear at present, therefore, why Taylor *et al.* (1983) did not observe antagonism in their preparation.

All the evidence discussed so far is therefore compatible with the view that the greater contractile potency of ATPβS and the greater inhibitory potency of ATPαS are due to differences in their ease of metabolism to adenosine. However, it is difficult to account for the different response profiles of ATPαS and ATPβS on this basis. At concentrations which produced on the vas deferens initial twitch-like contractions of similar size, the response to ATPαS was much more prolonged (Figure 4). It is difficult at present to give an explanation of this phenomenon unless the relatively high concentrations of adenosine or AMP produced by the initial hydrolysis of ATPαS have a retarding effect on subsequent metabolism, perhaps by enzyme inhibition.

In the taenia coli it is clear that different rules govern the relaxant action of nucleotides compared with the contractile and presynaptic inhibitory activities discussed above. Thus, the relaxations are not affected by 8-phenyltheophylline, confirming that an adenosine receptor does not mediate these responses (Maguire & Satchell, 1979; Satchell & Maguire, 1982; Brown & Burnstock, 1981; Ferrero & Frischknecht, 1983).

Furthermore the potencies of ATPαS and ATPβS are very similar, and ATP itself is much more active relative to these compounds in relaxing the taenia than when producing contraction of vasa or bladder. It is not clear why this should be so. The relaxant responses to nucleotides can last for 1 or 2 min, even after concentrations less than 1 μM, whereas contractile responses at these concentrations are far more transient. However, it is not possible to say whether these differences of time course and potency are due to receptor phenomena or to a relative absence of metabolic enzymes.

An indication that receptor phenomena may play a significant part in these differences arises from the finding that only ATP, but not ATPαS, ATPβS or APPCP could sometimes produce biphasic responses of the taenia. The secondary contraction has been attributed to prostaglandin synthesis and release as it could be prevented by incubation with indomethacin (Burnstock *et al.*, 1975), but Brown & Burnstock (1981) have previously shown that APPCP was not able to mimic the prostaglandin releasing action of ATP. The failure of ATPαS and ATPβS to mimic this effect only implies a failure to act in a manner identical to ATP itself. The precise structure and conformation of the polyphosphate chain is thus critically important for some actions of ATP (Dipolo, 1976; Brown & Burnstock, 1981; Chapal & Loubatieres-Mariani, 1981; Niedergerke & Page, 1981).

After the completion of this study a report appeared of the effects of ATPαS and ATPβS on the guinea-pig taenia and bladder (Burnstock *et al.*, 1984). The results obtained with the latter preparation were similar to those described here, but in the taenia preparation ATPαS was found to be more active than either ATP or ATPβS. The reasons for this difference are not clear.

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