Br. J. Pharmac. (1983), **79**, 907–913

Studies on the stereoselectivity of the P_2-purinoceptor

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1 ATP, 2-chloro-ATP, 2-methylthio-ATP, and their unnatural L-enantiomers, were synthesized and their effects tested on the guinea-pig taenia coli and urinary bladder, and the stimulated frog ventricle.

2 The potent P_2-purinoceptor agonists, 2-chloro-ATP and 2-methylthio-ATP were, respectively, 30 and 200 times more effective than ATP in relaxing the guinea-pig taenia, but approximately as effective as ATP in contracting the guinea-pig bladder and augmenting the force of contraction of the frog ventricle.

3 A high degree of stereoselectivity was observed for relaxations of the guinea-pig taenia coli produced by the P_2-purinoceptoragonists, and 2-methylthio-ATP was over 700 times more effective than its L-enantiomer. In contrast, stereoselectivity for contraction of the guinea-pig bladder was observed only at low concentrations with each pair of enantiomers, and a similar low stereoselectivity was displayed by the frog ventricle.

4 These results show that P_2-purinoceptors mediating inhibitory responses in the guinea-pig taenia coli can show a high degree of stereoselectivity, while P_2-purinoceptors mediating excitatory responses in the guinea-pig bladder and in the frog ventricle show little stereoselectivity.

5 The partial stereoselectivity of the P_2-purinoceptor in smooth muscle contrasts with the absolute stereospecificity of P_1-purinoceptors for adenosine on smooth muscle and autonomic nerve terminals and the absolute stereospecificity of the receptor for ADP on the human platelet.

Introduction

Adenosine and adenine nucleotides exert diverse and potent pharmacological activities on a variety of tissues innervated by autonomic neurones, and there is evidence that adenosine 5′-triphosphate (ATP) may be a neurotransmitter released from non-adrenergic, non-cholinergic nerves supplying the guinea-pig taenia coli and urinary bladder, acting on receptors that have been termed 'purinergic' (for reviews see Burnstock, 1979; 1981). Differential responses elicited by exogenous adenosine and by ATP have led to the subdivision of these receptors into P_1-purinoceptors that respond maximally to adenosine and to adenosine 5′-monophosphate (AMP), and P_2-purinoceptors that respond maximally to ATP and to adenosine 5′-diphosphate (ADP) (Burnstock, 1978; Brown & Burnstock, 1981). Support for this classification comes from the selective competitive inhibition by methylxanthines of the effects of adenosine but not of ATP (Burnstock, 1978; Maguire & Satchell, 1981), and from the opposing effects of adenosine and ATP on tissues such as the mammalian urinary bladder (Burnstock, Dumsday & Smythe, 1972). A comparison of the relaxation of the guinea-pig taenia coli elicited by the enantiomers of adenosine, AMP, ADP and ATP revealed another difference between P_1- and P_2-purinoceptor, in that the P_1-purinoceptor exhibited an absolute stereospecificity for the naturally occurring D-enantiomer of adenosine, while the P_2-purinoceptor exhibited relatively poor stereoselectivity for the naturally occurring D-enantiomer of ATP over the unnatural L-enantiomer of ATP, 9-(β-L-ribofuranosyl)adenine 5′-triphosphate (L-ATP) (Cusack & Planker, 1979). Recently, further evidence for stereospecificity of the pre-and postjunctional P_1-purinoceptor in a variety of tissues was provided by comparing the responses to adenosine and to several adenosine analogues with those of their L-enantiomers (Cusack, Hickman &

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Born, 1979; Brown, Burnstock, Cusack, Meghji & Moody, 1982).

In the present study we have investigated the stereoselectivity of the postjunctional P_2-purinoceptors in the guinea-pig taenia coli (where ATP induces relaxation), the guinea-pig urinary bladder (where ATP causes contraction), and in the electrically driven frog ventricle (where ATP increases the force of contraction), by comparing the responses to ATP and to two potent P_2-purinoceptor agonists 2-chloro-ATP and 2-methylthio-ATP (Gough, Maguire & Satchell, 1973), with those of the corresponding unnatural L-enantiomers.

Methods

Guinea-pig taenia coli

Guinea-pigs of either sex (300–650 g) were stunned and bled. The longitudinal muscle of the caecum (taenia coli), together with its underlying myenteric plexus, was dissected out and kept moist with modified Krebs solution of the following ionic composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.3, $NaHCO_3$ 16.3, $MgSO_4$ 0.6, $CaCl_2$ 2.5 and glucose 7.7. Strips of taenia coli approximately 1.5 cm in length were attached by thread to a rigid support and then transferred to overflow organ baths, where they were continually gassed by 95% O_2 and 5% CO_2 and maintained at 36.5 ± 0.5°C. Guanethidine (3.4 μM), phentolamine (1 μM) and propranolol (1 μM) were present throughout. The preparations were initially placed under a resting tension of 1 g, and mechanical activity was recorded under isometric conditions with a Dynamometer UF1 force transducer and displayed on a Grass polygraph. The tone of the preparations was standardized by the addition of carbachol (50 nM routinely during an 8 min cycle, but in the range 50–65 nM as preparations aged) to allow quantification of the magnitude of inhibitory responses (Brown & Burnstock, 1981). Concentration-response curves for ATP and one other analogue were obtained for each preparation.

Guinea-pig bladder

Mucosa-free detrusor muscle strips (20 × 2 mm) were prepared from the urinary bladder and were connected by thread to a rigid support. The preparations were superfused with modified Krebs solution (as described above) containing guanethidine (3.4 μM) and atropine (1 μM). The Krebs solution was maintained at 35°C, and a flow rate of 1.5–2 ml per min was controlled by a Watson-Marlow peristaltic pump. The preparations were initially mounted under 0.5 g resting tension, and the mechanical activity was recorded isometrically with a Dynamometer UF1 force transducer and displayed on a Grass polygraph. Electrical field stimulation was achieved by passing square-wave pulses (4 Hz, 0.2 ms duration and supramaximal voltage) to platinum ring electrodes (separated by 20 mm) from a Grass SD9 stimulator. The duration of a period of electrical stimulation was sufficient to allow the neurogenic excitatory response to decay to one third of the maximal amplitude attained (usually 7–10 s), and the neurogenic responses were elicited every 5 min. Drugs were also superfused over the preparations in such a way that exposure to the chemical stimuli was discontinued after the excitatory responses had decayed to one third of the maximum response. Since it is known that ATP and some other spasmogens produce a prolonged increase in the sensitivity of the bladder smooth muscle membrane to excitatory stimuli, an interval of 30 min separated subsequent additions of drugs. Additionally, drug-induced contractions were expressed as a percentage of the mean of two successive responses to field stimulation elicited prior to superfusion of the drug, since these responses would reflect any increased excitability of the smooth muscle. Concentration-response relationships were compared for ATP and one other analogue on each preparation.

Frog ventricle strip

Frogs (*Rana pipiens*) were stunned by a blow to the back of the head, decapitated and pithed. The hearts were removed and placed in oxygenated Ringer solution (Gambhir & Tripathi, 1978). Strips of ventricle were mounted on platinum strip electrodes (separated by 7 mm) and then transferred to 10 ml overflow organ baths at room temperature (17–20°C). A resting tension of 0.5 g was initially applied. The preparations were electrically stimulated (continuously at 0.5 Hz, 5 ms duration) at twice threshold voltage. The mechanical activity was recorded isometrically with a Grass FT03C force transducer and displayed on a Grass polygraph. Log concentration-response curves were obtained for the cumulative addition of individual agonists. A concentration-response curve to ATP was obtained before each analogue was tested and as many as 5 analogues were tested on each preparation. The positive inotropic effects elicited by these drugs were measured as percentages of basal activity and were then expressed as percentages of maximal responses to ATP.

All preparations were allowed to equilibrate for 60 min before the use of drugs. The bathing solution in the organ bath experiments was changed periodically during that time.

Drugs used

Atropine sulphate was obtained from Antigen International Ltd, Roscrea, and guanethidine monosulphate and phentolamine mesylate from Ciba Laboratories, Horsham. Propranolol hydrochloride, carbamylcholine chloride (carbachol), L-xylose, 2-chloroadenosine and adenosine 5'-triphosphate (ATP) were supplied by Sigma, London. 9-β-L-Ribofuranosyladenine (L-adenosine) was synthesized from L-xylose (Acton, Ryan & Goodman, 1964) and phosphorylated to 9-β-L-ribofuranosyladenine 5'-triphosphate (L-ATP) as described by Holý & Šorm (1971). 2-Chloro-9-β-L-ribofuranosyladenine (2-chloro-L-adenosine) was synthesized as previously described (Cusack *et al.* 1979). 2-Methylthioadenosine and 2-methylthio-9-β-L-ribofuranosyladenine (2-methylthio-L-adenosine) were obtained by displacement of chloride from 2-chloroadenosine (Maguire, Nobbs, Einstein & Middleton, 1971) and 2-chloro-L-adenosine (Cusack *et al.*, 1979) respectively with methanethiol. 2-Chloroadenosine 5'-triphosphate (2-chloro-ATP) and 2-methylthioadenosine 5'-triphosphate (2-methylthio-ATP) were synthesized by phosphorylation followed by pyrophosphorylation of 2-chloradenosine and 2-methylthioadenosine respectively (Gough *et al.*, 1973), and 2-chloro-9-β-L-ribofuranosyladenine 5'-triphosphate (2-chloro-L-ATP) and 2-methylthio-9-β-L-ribofuranosyladenine 5'-triphosphate (2-methylthio-L-ATP) were synthesized in an identical manner from 2-chloro-L-adenosine and 2-methylthio-L-adenosine. The purity of the nucleoside triphosphates was examined by high pressure liquid chromatography and stock solutions were assayed by ultraviolet spectroscopy.

Tabulated results are presented for relaxations of the guinea-pig taenia coli. However, since maxima are not obtainable for the guinea-pig bladder and frog ventricle responses, EC_{50} values could not be estimated.

Statistical methods

Results given are expressed as mean ± s.e.mean. Statistical analysis of data was carried out using Student's *t* test for paired and unpaired samples. A probability of < 0.05 was considered significant.

Results

Guinea-pig taenia coli

ATP, 2-chloro-ATP, 2 methylthio-ATP and their L-enantiomers each induced relaxation of the carbachol-contracted taenia coli in a concentration-dependent manner (Figure 1). ATP was significantly more effective than L-ATP in eliciting relaxation up to 30 μM, although they were approximately equally effective in producing maximal inhibition of tone (Figure 1a). Also, 2-chloro-ATP was significantly more effective than 2-chloro-L-ATP over a wide concentration range (up to 3 μM) but the difference in the effectiveness of the enantiomers was again less marked at higher concentrations (Figure 1b). 2-Chloro-ATP exerted prolonged inhibitory effects on the smooth muscle at concentrations of 60 μM and

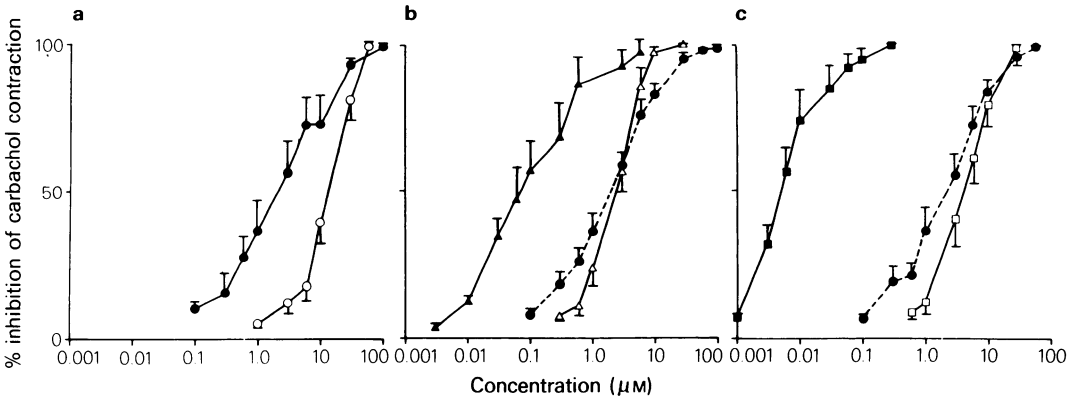

Figure 1 Log concentration-response curves of the guinea-pig taenia coli to the inhibitory effects of (a) ATP (●), ($n = 8$) and L-ATP (○), ($n = 8$), (b) 2-chloro-ATP (▲), ($n = 8$), 2-chloro-L-ATP (△), ($n = 8$) and ATP (●), ($n = 16$) obtained from the same preparations, and (c) 2-methylthio-ATP (■), ($n = 8$), 2-methylthio-L-ATP (□), ($n = 8$) and ATP (●), ($n = 16$) obtained from the same preparations. Each point is the mean of observations from the number of preparations indicated in parentheses. Vertical bars show s.e.mean. Guanethidine (3.4 μM), phentolamine (1 μM) and propranolol (1 μM) were present throughout.

Table 1 Comparison of effects of enantiomers of ATP, 2-chloro-ATP and of 2-methylthio-ATP on guinea-pig taenia coli

Compound	*p(EC50) ± s.e.mean*	*Activity relative to ATP*	*Ratio activity of enantiomers at p(EC50)*
ATP	5.68 ± 0.17	1.0	6.0 (2.6, 14.0)
L-ATP	4.90 ± 0.07***	0.17	
2-Chloro-ATP	7.20 ± 0.17	35.5	
ATP	5.65 ± 0.18***	1.0	35.5 (15.9, 94.2)
ATP	5.83 ± 0.19	1.0	
2-Chloro-L-ATP	5.65 ± 0.08	0.66	
2-Methylthio-ATP	8.25 ± 0.15	195	
ATP	5.96 ± 0.11***	1.0	724 (319, 1610)
ATP	6.05 ± 0.09***	1.0	
2-Methylthio-LATP	5.39 ± 0.10	0.22	

***$P < 0.001$ for the comparison of the enantiomers with ATP in the same preparations. Figures in parentheses show 95% confidence limits for the ratio activity of the enantiomers. The multiple entries for ATP represent controls for each analogue. EC_{50} (values) were calculated according to the method of Waud (1975).

above, as reflected in the reduced amplitude of subsequent carbachol contractions despite thorough washing of the preparations after exposure to this nucleotide. 2-Methylthio-ATP was significantly more effective than 2-methylthio-L-ATP at all concentrations studied (Figure 1c). The rank order of effectiveness was 2-methylthio-ATP > 2-chloro-ATP > ATP, and that for the L-enantiomers was 2-chloro-L-ATP > 2-methylthio-L-ATP > L-ATP (see Table 1). As the concentrations of the L-enantiomers were increased, then the difference in effectiveness between each L-enantiomer and ATP itself was reduced, with 2-chloro-L-ATP being more effective than ATP at concentrations of 6 μM and above.

Guinea-pig bladder

ATP, 2-chloro-ATP, 2-methylthio-ATP and their L-enantiomers each induced contraction of the guinea-pig bladder in a concentration-dependent manner (Figure 2).

At lower concentrations (less than 10 μM), L-ATP was less effective at eliciting contractions than ATP itself ($P < 0.01$ at 0.3 μM) (Figure 2a). However, at higher concentrations (> 10 μM) L-ATP was signific-

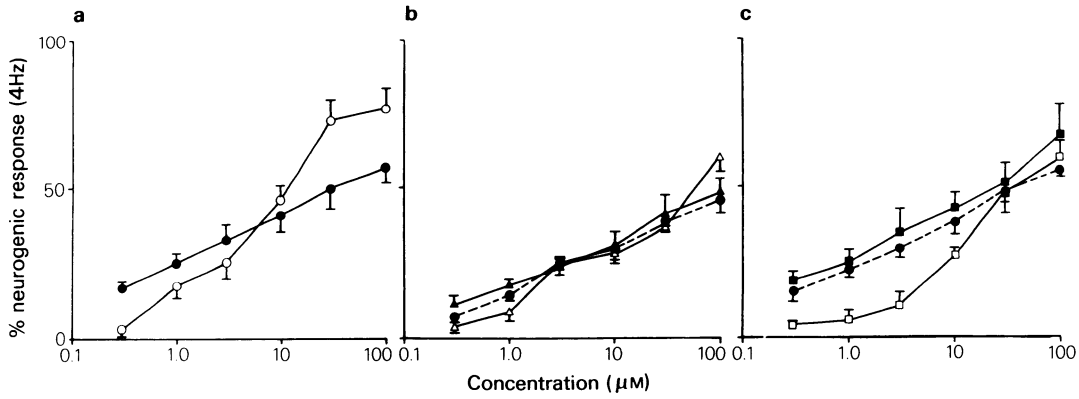

Figure 2 Log concentration-response curves of the guinea-pig bladder to the excitatory effects of (a) ATP (●), ($n = 6$) and L-ATP (○), ($n = 8$), (b) 2-chloro-ATP (▲), ($n = 5$) 2-chloro-L-ATP (△), ($n = 6$) and ATP (●), ($n = 11$) obtained from the same preparations, and (c) 2-methylthio-ATP (■), 2-methylthio-L-ATP (□), ($n = 6$ in each case) and ATP (●), ($n = 12$) obtained from the same preparations. Each point is the mean of observations from the number of preparations indicated in parentheses. Vertical bars show s.e.mean. Atropine (1 μM) and guanethidine (3.4 μM) were present throughout.

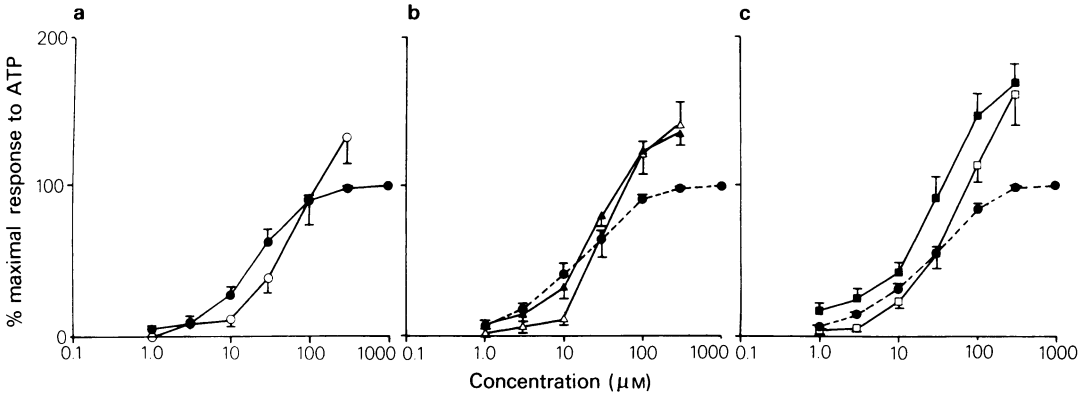

Figure 3 Log concentration-response curves of the electrically stimulated frog ventricle strip to the excitatory effects of (a) ATP (●) and L-ATP (○), ($n = 9$ for both), (b) 2-chloro-ATP (▲), 2-chloro-L-ATP (△), ($n = 8$ in each case) and ATP (●), ($n = 16$) obtained from the same preparations, and (c) 2-methylthio-ATP (■), ($n = 8$), 2-methylthio-L-ATP (□), ($n = 11$) and ATP (●), ($n = 19$) obtained from the same preparations. Each point is the mean of observations from the number of preparations indicated in parentheses. Vertical bars show s.e.mean.

antly more effective than equimolar concentrations of ATP ($P < 0.05$) (Figure 2a). 2-Chloro-ATP was slightly more effective than its L-enantiomer at concentrations up to 10 μM, and less effective at higher concentrations (Figure 2b); these changes were not statistically significant. 2-Methylthio-ATP was more effective than 2-methylthio-L-ATP at lower concentrations (< 10 μM) (Figure 2c); these differences were significant at 1 μM ($P < 0.01$), 3 μM ($P < 0.05$) and 10 μM ($P < 0.05$). In addition, ATP itself was significantly more effective than 2-methylthio-L-ATP at 1 and 3 μM ($P < 0.05$). In each experiment the spontaneous activity of the preparations was increased after the removal of 2-methylthio-ATP at concentrations above 1 μM.

2-Chloro-ATP and 2-methylthio-ATP were virtually as effective as ATP over the concentration range (0.3–100 μM) tested.

Frog ventricle

ATP, 2-chloro-ATP, 2-methylthio-ATP and their L-enantiomers each elicited positive inotropic responses of the frog venticle which were concentration-dependent (Figure 3). L-ATP was slightly less effective than equimolar concentrations of ATP itself at concentrations up to 30 μM, but was more effective than ATP at concentrations > 100 μM (Figure 3a). Similarly, 2-chloro-L-ATP was slightly less effective than 2-chloro-ATP at concentrations up to 30 μM, but the enantiomers were equally effective at higher concentrations (Figure 3b). 2-Methylthio-L-ATP was less effective than 2-methylthio-ATP over the entire concentration range (1–300 μM) (Figure 3c). Comparative differences in the effectiveness of the pairs of the enantiomers of each of the three compounds at equimolar concentrations were not statistically significant. At higher concentrations, 2-methylthio-ATP (> 30 μM) ($P < 0.05$) and 2-chloro-ATP (> 100 μM) ($P < 0.001$) were more effective than equimolar concentrations of ATP, whereas the L-enantiomers were equally effective over the entire concentration range. Responses elicited by ATP (> 100 μM) ($P < 0.01$) were significantly smaller than those elicited by equimolar concentrations of all the analogues tested, each achieving a greater maximal increase in the force of contraction, with the exception of L-ATP. The maximum response to ATP was reached by 114 ± 8 s; the analogues took significantly longer ($P < 0.05$), L-ATP taking 174 ± 28 s, 2-chloro-ATP 178 ± 25 s, 2-chloro-L-ATP 153 ± 16 s, 2-methylthio-ATP 191 ± 28 s and 2-methylthio-L-ATP 191 ± 37 s.

Discussion

These results show that 2-chloro-ATP, 2-methylthio-ATP, L-ATP, 2-chloro-L-ATP and 2-methylthio-L-ATP produced characteristic responses of the guinea-pig taenia coli and urinary bladder and of the frog ventricle similar to those established for ATP itself (Ambache & Zar, 1970; Burnstock *et al.*, 1972; Satchell & Burnstock, 1975; Flitney, Lamb & Singh, 1977; Burnstock & Meghji, 1981).

Both 2-chloro-ATP and 2-methylthio-ATP were more effective than ATP in producing relaxation of the taenia coli, as first described by Gough *et al.* (1973), although in our studies these analogues were considerably more effective relative to ATP. For each pair of enantiomers stereoselectivity was observed; ATP was 6 times more effective than L-ATP,

2-chloro-ATP was 36 times more effective than 2-chloro-L-ATP, and 2-methylthio-ATP was over 700 times more effective than 2-methylthio-L-ATP in producing relaxation of the taenia coli. These results for the 2 substituted enantiomers are consistent with those of a previous study (Cusack & Planker, 1979) where 2-azido-substitution markedly increased the potency of the D-but not of the L-enantiomers. The concentration-response curves to the L-enantiomers were all steeper and more sigmoid than those of ATP, 2-chloro-ATP and 2-methylthio-ATP, and this may be due to the lack of activity of the products of their hydrolysis.

Neither 2-chloro-ATP nor 2-methylthio-ATP were significantly more effective than ATP itself at inducing contraction of the guinea-pig bladder. For each pair of enantiomers, some stereoselectivity was observed at low concentrations. At higher concentrations ($> 30\ \mu M$), however, this apparent stereoselectivity was lost or even reversed, because the concentration-response curves for ATP, 2-chloro-ATP and 2-methylthio-ATP were flattened, while those of the L-enantiomers were steeper; L-ATP even produced a higher response than ATP at concentrations above $10\ \mu M$. The rapid dephosphorylation of ATP to adenosine acts to induce relaxation of the bladder (Brown, Burnstock & Cocks, 1979). This may account for the characteristic concentration-response curves for ATP, 2-chloro-ATP and 2-methylthio-ATP, especially at higher nucleotide concentrations. However, dephosphorylation of the L-enantiomers would give rise to products such as L-adenosine and 2-chloro-L-adenosine which are inactive at P_1-purinoceptors (Brown *et al.*, 1982) and therefore would not induce relaxation.

Similarly, 2-chloro-ATP and 2-methylthio-ATP were not significantly more effective than ATP itself at inducing increases in the force of contraction of the frog ventricle (Figure 3). For each pair of enantiomers, some stereoselectivity was observed; 2-methylthio-ATP was more effective than 2-methylthio-L-ATP throughout the concentration-response curve, but 2-chloro-ATP and ATP were more effective than their L-enantiomers only at the lower ($< 10\ \mu M$) concentrations. At higher concentrations, L-ATP and each pair of enantiomers of the analogue achieved a greater maximal response than did ATP, and this possibly reflects differences in rates of hydrolysis of the nucleotides, especially of ATP. The characteristics of this receptor interaction must be interpreted with caution since the concentration-response curves in all 3 preparations studied here were not parallel.

In the present study, the P_2-purinoceptor on the guinea-pig taenia coli displays a marked stereoselectivity, whereas the P_2-purinoceptor in the guinea-pig bladder and frog ventricle does not. Other differences have been reported between tissue responses occurring as a consequence of P_2-purinoceptor activation mediating inhibition and P_2-purinoceptor activation mediating excitation. Apamin, a potassium channel blocker (Banks, Brown, Burgess, Burnstock, Claret, Cocks & Jenkinson, 1979), non-competitively antagonizes the P_2-mediated inhibition of the guinea-pig taenia coli but does not affect the P_2-mediated excitation in the guinea-pig bladder (Shuba & Vladimirova, 1980). In addition, ultraviolet light produces a rapid, reversible and non nerve-mediated relaxation of the guinea-pig taenia coli but has no effect on the guinea-pig bladder (Burnstock & Wong, 1978).

The generally lower stereoselectivity displayed by these P_2-purinoceptors on smooth muscle toward the enantiomers of ATP itself, together with the activity on smooth muscle of nucleoside 5′-triphosphates other than ATP (Lukacsko & Krell, 1982), suggests that binding of the 5′-triphosphate moiety of ATP rather than a sugar or a base, could be the more important requirement for activity, as previously proposed (Cusack & Planker, 1979), particularly in situations where P_2-purinoceptor activation elicits excitatory responses. If the responses to ATP and the analogues are due to more than one type of interaction with P_2-purinoceptors, as is apparently true for the postjunctional responses of the guinea-pig vas deferens to ATP itself (Fedan, Hogaboom, Westfall & O'Donnell, 1982), then our results with the analogues of ATP and their L-enantiomers may reflect differing amounts of participation in one or other of these interactions. The relatively minor influence that replacement of a centre of asymmetry has on the activity of ATP recalls similar low stereoselectivity found for δ-receptors for enkephalin and for substance P receptors (Rossell, Björkroth, Chang, Yamaguchi, Wan, Rackur, Fisher & Folkers, 1977; Kosterlitz, 1980).

The lower stereoselectivity exhibited here by these P_2-purinoceptors on smooth muscle towards the enantiomers of ATP itself is in contrast to the absolute stereospecificity of the receptor for ADP on human platelets (Cusack *et al.* 1979) and for the P_1-purinoceptor for adenosine on platelets, smooth muscle and on autonomic nerve terminals (Cusack *et al.* 1979; Brown *et al.* 1982) and substantiates other recent evidence (Brown & Burnstock, 1981; Satchell & Maguire, 1982) for the existence of separate receptors for adenosine and for ATP on smooth muscle.

This work has been supported by the MRC and the Wellcome Foundation. The help of Dr Stephanie Clark and Miss Mary Sheridan in the preparation of the manuscript is greatly appreciated.

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(Received December 28, 1982. Revised March 25, 1983.)