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Pharmacological effects of isopolar phosphonate analogues of ATP on P_2-purinoceptors in guinea-pig taenia coli and urinary bladder

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1 Isopolar methylene phosphonate analogues of adenosine triphosphate (ATP) were synthesized and tested on the guinea-pig isolated taenia coli (where ATP causes relaxation) and urinary bladder (where ATP causes contraction), to see if restoration of the electronegativity of the methylene linkage would enhance pharmacological potency. The compounds used were the dichloromethylene and difluoromethylene analogues of adenosine 5'-(β,γ-methylene)triphosphonate (AMP-PCP), L-adenosine 5'-(β,γ-methylene)triphosphonate (L-AMP-PCP) and 2-methylthioadenosine 5'-(β,γ-methylene)-triphosphonate (2-methylthio-AMP-PCP).

2 The order of potency of the analogues depended on the tissue, and was independent of the nature of the purine or ribose moieties. None of the analogues was degraded by ectonucleotidases on either tissue.

3 In the taenia coli the order of potency for relaxation was difluoromethylene > dichloromethylene > methylene, and this reflected the order of electronegativity of the analogues. The isopolar analogues of L-AMP-PCP were inactive in the taenia coli.

4 In the bladder the order of potency for contraction was difluoromethylene > methylene > dichloromethylene, suggesting that electronegativity is of lesser importance here. The isopolar analogues of L-AMP-PCP were active in this tissue.

5 The differences between the two tissues in the order of potency for these non-degradable analogues supports suggestions that P_2-purinoceptors in the taenia coli (P_{2Y}) are different from those in the bladder (P_{2X}). The isopolar analogues of L-AMP-PCP, like L-AMP-PCP itself, were selective agonists at the P_{2X}-purinoceptor.

Introduction

Adenosine triphosphate (ATP) has potent pharmacological effects on a variety of tissues including vascular and visceral smooth muscle (for review see Gordon, 1986). In particular, ATP induces relaxation of the guinea-pig taenia coli and contraction of the guinea-pig urinary bladder, apparently by actions at specific P_2-purinoceptors (Burnstock, 1978). ATP is also rapidly and sequentially dephosphorylated by ectonucleotidases present on both these tissues ultimately to adenosine (Cusack & Hourani, 1984; Hourani *et al.*, 1985), which causes relaxation by acting at separate P_1-purinoceptors (Burnstock, 1978).

ATP is not very potent on the bladder, and this has been attributed to rapid degradation (Brown *et al.*, 1979). A recent study using a range of ATP analogues has shown that, in general, analogues resistant to dephosphorylation are more potent at causing contraction of the bladder (Cusack *et al.*, 1986). In particular, analogues of ATP such as adenosine 5'-(β,γ-methylene)triphosphonate (AMP-PCP), in which a bridging oxygen in the triphosphate chain has been replaced by a non-hydrolysable methylene group, are much more potent than ATP (Brown *et al.*, 1979; Cusack & Hourani, 1984).

In the taenia coli, however, the methylene analogues of ATP such as AMP-PCP are much less potent than ATP (Satchell & Maguire, 1975), even though they are not broken down (Hourani *et al.*, 1985). In a more detailed study, no relationship was found in the taenia coli between potency and dephosphorylation for a range of ATP analogues (Welford *et al.*, 1986). Indeed, L-adenosine 5'-(β,γ-methylene)triphosphonate (LAMP-

PCP), is completely inactive in the taenia coli (Hourani *et al.*, 1985), even though it is the most potent known analogue on the bladder (Cusack & Hourani, 1984). This provides evidence for the proposed subclassification of P_2-purinoceptors into P_{2X} (which mediate contraction) and P_{2Y} (which mediate relaxation) (Burnstock & Kennedy, 1985), and L-AMP-PCP has been used to define P_2-purinoceptors in a range of tissues (Hourani *et al.*, 1986).

The lack of potency of the methylene analogues on the taenia coli could be due to their failure to mimic adequately the electronic nature of the triphosphate chain of ATP. Thus the replacement of an electronegative oxygen by an electropositive methylene group means that AMP-PCP is not as fully ionized as ATP at physiological pH (Myers *et al.*, 1963). In an ingenious attempt to overcome this deficiency, Blackburn *et al.* (1981b) used dihalomethylene groups instead of methylene to restore the electronegative character of this linkage. We decided to test such 'isopolar' analogues of ATP on the taenia coli to see if restoration of the electronegativity of the linkage would enhance pharmacological potency.

As well as the dichloro- and difluoromethylene analogues of AMP-PCP described by Blackburn *et al.* (1981b), we synthesized the corresponding 2- methylthio derivatives, and the dichloro- and difluoromethylene analogues of L- AMP-PCP. As the P_2-purinoceptors on the bladder appear to be different from those on the taenia coli, we also tested these analogues on the guinea-pig bladder.

Methods

Pharmacological studies

Male albino guinea-pigs (200–800 g) were killed by a blow to the head and exsanguination, and the urinary bladder or taenia coli were dissected free. The bladder was freed of connective tissue and the mucosal layer, and the apical region was cut into strips about 2 mm wide. Lengths of taenia coli or of bladder strips (approximately 10 mm) were attached by thread to a rigid support and superfused (1 ml min^{-1}) with modified Krebs solution of the following composition (mM): NaCl 120, KCl 5.9, $MgCl_2$ 1.2, $NaHCO_3$ 15.4, NaH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 11.5, gassed with 95% O_2 and 5% CO_2 and warmed to 35°C.

In addition the Krebs solution contained guanethidine (3.4 μM) for studies on both tissues, and also atropine (1 μM) for studies on the bladder. The tissues were initially mounted under 1 g tension, and mechanical activity was recorded isometrically with a Grass FT10 transducer and displayed on a Grass 79C polygraph. The preparations were equilibrated for 60 min before addition of drugs or nerve stimulation.

The tone of the taenia coli was raised by carbachol (usually 100 nM) before addition of nucleotides, and relaxations were expressed as percentage inhibition of the contraction induced by carbachol. EC_{50} values were calculated from the linear part of the log concentration-response curves by least squares linear regression analysis. For the studies on the bladder, contractions in response to the nucleotides were expressed as a percentage of the contraction caused by nerve stimulation. Non-adrenergic, non-cholinergic nerves were stimulated via platinum ring electrodes by means of a Grass SD9 stimulator at a voltage of 40V with a pulse duration of 0.3 ms and a frequency of 50 Hz.

Degradation studies

Studies on the degradation of the nucleotides were performed as described in detail elsewhere (Cusack & Hourani, 1984; Hourani *et al.*, 1985; Welford *et al.*, 1986). Briefly, muscle strips were incubated in the appropriate Krebs buffer (800 μl) as described above and containing a nucleotide (100 μM), and degradation of the nucleotide was followed by high performance liquid chromatography (h.p.l.c.) analysis of aliquots (70 μl) taken after various incubation times.

Drugs

AMP, ATP, AMP-PCP, methylenediphosphonic acid, carbonyldiimidazole, atropine and carbachol were obtained from Sigma Chemical Co., Poole. Guanethidine was obtained from Ciba-Geigy, Horsham.

2-Methylthioadenosine 5'-monophosphate (2-methylthio-AMP) was synthesized as described by Michal *et al.* (1969), and 9-β-L-ribofuranosyladenine 5'-monophosphate (L-AMP) was synthesized as described by Holý & Sǒrm (1971). Dichloromethylenediphosphonic acid was synthesized as described by Quimby *et al.* (1967) and difluoromethylenediphosphonic acid was synthesized as described by Blackburn *et al.* (1981a). Adenosine 5'-(β,γ-dichloromethylene)triphosphonate (AMP-$PCCl_2P$) and adenosine 5'-(β,γ-difluoromethylene)triphosphonate (AMP-PCF_2P) (Blackburn *et al.*, 1981b) were obtained by activation of AMP with carbonyldiimidazole followed by addition of dichloro- or difluoromethylenediphosphonic acid respectively. Similarly, 2-methylthioadenosine 5'-(β,γ-methylene) triphosphonate (2-methylthio-AMP-PCP), 2-methylthioadenosine 5' - (β,γ - dichloromethylene)triphosphonate (2 - methylthio-AMP-$PCCl_2P$) and 2-methylthioadenosine 5' - (β,γ - difluoromethylene)triphosphonate (2 - methylthio-AMP-PCF_2P) were obtained by activation of 2-methylthio-AMP followed by addition of the appropriate methylenediphosphonic acid. L-AMP-PCP was synthesized by activation of L-AMP with carbonyldiimidazole foll-

owed by addition of methylenediphosphonic acid as described by Cusack *et al.* (1983), and L-adenosine 5'-(β,γ-dichloromethylene)triphosphonate (L-AMP-$PCCl_2P$) and L-adenosine 5'-(β,γ-difluoromethylene)triphosphonate (L-AMP-PCF_2P) were similarly synthesized by reaction of activated L-AMP with dichloro- or difluoromethylenediphosphonic acid respectively.

Results

Pharmacological studies

AMP-PCP, 2-methylthio-AMP-PCP and their isopolar analogues each induced relaxation of the taenia coli in a dose-dependent manner (Figure 1). AMP-$PCCl_2P$ (EC_{50} 56 μM) and AMP-PCF_2P (EC_{50} 30 μM) were approximately 1.4 and 2.6 times as potent respectively as AMP-PCP (EC_{50} 78 μM) (Figure 1a), and 2-methylthio-AMP-$PCCl_2P$ (EC_{50} 7 μM) and 2-methylthio-AMP-PCF_2P (EC_{50} 0.6 μM) were approximately 5.7 and 67 times as potent respectively as 2-

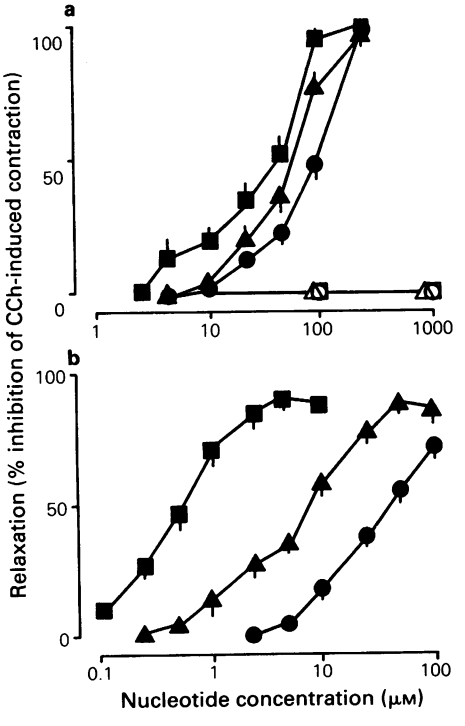

Figure 1 Relaxation of the carbachol (CCh)-contracted guinea-pig taenia coli by methylenephosphonate analogues of ATP. (a) AMP-PCP (●), AMP-$PCCl_2P$ (▲), AMP-PCF_2P (■), L-AMP-PCP (○), L-AMP-$PCCl_2P$ (△), L-AMP-PCF_2P (□); (b) 2-methylthio-AMP-PCP (●), 2-methylthio-AMP-$PCCl_2P$ (▲), 2-methylthio-AMP-PCF_2P (■). Each point is the mean of at least 10 determinations using tissues from at least 5 guinea-pigs. Vertical bars show the standard errors, where these are larger than the symbols.

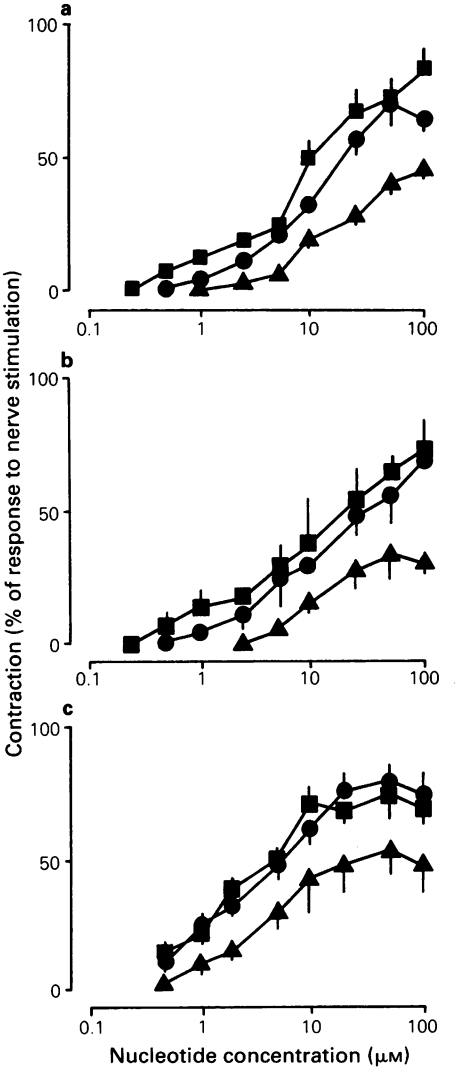

Figure 2 Contraction of the guinea-pig urinary bladder by methylenephosphonate analogues of ATP. (a) AMP-PCP (●), AMP-$PCCl_2P$ (▲), AMP-PCF_2P (■); (b) 2-methylthio-AMP-PCP (●), 2-methylthio-AMP-$PCCl_2P$ (▲), 2-methylthio-AMP-PCF_2P (■); (c) L-AMP-PCP (●), L-AMP-$PCCl_2P$ (▲), L-AMP-PCF_2P (■). Each point is the mean of at least 10 determinations using tissues from at least 5 guinea-pigs. Vertical bars show the standard errors, where these are larger than the symbols.

methylthio-AMP-PCP (EC_{50} 40 μM) (Figure 1b). L-AMP-$PCCl_2P$ and L-AMP-PCF_2P, like-AMP-PCP, were inactive at concentrations up to 100 μM (Figure 1a).

AMP-PCP, 2-methylthio-AMP-PCP, L-AMP-PCP and their isopolar analogues each induced contraction of the urinary bladder in a dose-dependent manner (Figure 2). AMP-$PCCl_2P$ was less effective than AMP-PCP at every concentration, while AMP-PCF_2P was equally or more effective than AMP-PCP (Figure 2a). Similarly, 2-methylthio-AMP-$PCCl_2P$ was less effective than, and 2-methylthio-AMP-PCF_2P equally or more effective than 2-methylthio-AMP-PCP (Figure 2b). Again L-AMP-$PCCl_2P$ was less effective than L-AMP-PCP, whereas L-AMP-PCF_2P was equipotent with AMP-PCP (Figure 2c).

Degradation studies

No degradation by ectonucleotidases of any of the analogues (100 μM) was detected in either tissue even after 60 min incubation, during which time ATP (100 μM) was completely dephosphorylated (results not shown).

Discussion

These results show that AMP-PCP and 2-methylthio-AMP-PCP and their isopolar analogues were active on both the taenia coli and the bladder, and that L-AMP-PCP and its isopolar analogues, while potent on the bladder, were inactive on the taenia coli.

The order of potency of the analogues depended on the tissue, and was independent of the nature of the purine or ribose moieties. In the taenia coli the order of potency was difluoromethylene > dichloromethylene > methylene, whereas in the bladder the order of potency was difluoromethylene > methylene > dichloromethylene. As none of these analogues was degraded by ectonucleotidases, these differences in potency were not complicated by any differences in their rates of dephosphorylation. This difference in the order of potency is therefore consistent with the suggestions that the P_2-purinoceptors on these two tissues are different (Burnstock & Kennedy, 1985; Hourani *et al.*, 1985).

The order or potency of the analogues on the taenia coli reflects the order of acidity reported by Blackburn *et al.* (1984), suggesting that the electronegativity of the non-hydrolysable linkage is important. AMP-$PCCl_2P$ was about 1.4 times, and AMP-PCF_2P about 2.6 times more potent than AMP-PCP and substitution at the 2-position of the purine ring by methylthio magnified these differences so that 2-methylthio-AMP-$PCCl_2P$ was about 5.7 times, and 2-methylthio-AMP-PCF_2P about 67 times more potent than 2-methylthio-AMP-PCP. Indeed, 2-methylthio-AMP-PCF_2P was about twice as potent as ATP itself ($EC_{50} \sim 1$ μM; Burnstock *et al.*, 1983), and as this analogue was not degraded it should be useful for studies on the P_{2Y}-purinoceptor. However, 2-methylthio-AMP-PCF_2P was still not as potent as 2-methylthio-ATP, which has been reported to be up to 200 times more potent than ATP (Satchell & Maguire, 1975; Burnstock *et al.*, 1983) and AMP-PCF_2P was still not as potent as ATP itself: this suggests that the electronegativity of the triphosphate is not the only factor determining pharmacological potency.

Unlike the taenia coli, in the bladder the order of potency of these analogues did not reflect their order of acidity, the dichloro analogues being the least active. This may be because some distortion of the triphosphate chain is necessary to accommodate the bulky chlorine groups, whereas the difluoro analogue is sterically more similar to ATP (Blackburn *et al.*, 1984). 2-Substitution of the purine ring did not generate more active analogues, and this is in accord with the earlier studies on the P_{2X}-purinoceptor (Burnstock *et al.*, 1983). The isopolar L-enantiomers, L-AMP-$PCCl_2P$ and L-AMP-PCF_2P were both active on the bladder, and as they were without activity on the taenia coli they are (like L-AMP-PCP itself) selective agonists for the P_{2X}-purinoceptor.

In conclusion, restoration of the electronegativity of the triphosphate chain did enhance the potency of non-hydrolysable methylene analogues on the taenia coli, although the isopolar methylene analogues were still not as potent as the equivalent analogues possessing the usual hydrolysable oxygen linkage. In the bladder, on the other hand, non-hydrolysable analogues are already more potent than ATP and restoration of the electronegativity of the triphosphate chain did not further enhance this potency.

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