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Purine receptors and guinea-pig trachea: evidence for a direct action of ATP

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1 Adenosine (3–300 μM) and ATP (1–300 μM) each induced concentration-dependent relaxation of guinea-pig isolated tracheal preparations that had been precontracted with methacholine.

2 Ectonucleotidase enzymes on the trachea dephosphorylated ATP to form adenosine which was then further metabolised to inosine and hypoxanthine.

3 Dipyridamole (2 μM) inhibited the metabolism of adenosine but did not inhibit the dephosphorylation of ATP. Nevertheless dipyridamole potentiated the effects of both adenosine and ATP in relaxing tracheal smooth muscle.

4 Although adenosine 5'-[β,γ-imido] triphosphate (AMP-PNP), an analogue of ATP, was resistant to catabolism by ectonucleotidases, it was more potent at inducing relaxation than either ATP or adenosine, and was also potentiated by dipyridamole (2 μM).

5 Relaxations induced by ATP and by AMP-PNP were more rapid than those induced by adenosine.

6 We therefore conclude that the intact ATP molecule can itself induce relaxation of the guinea-pig trachea, without first having to be metabolised to adenosine, and furthermore that dipyridamole does not act simply by inhibiting the degradation (or uptake) of adenosine.

Introduction

Adenosine and the adenine nucleotides (AMP, ADP and ATP) each induce relaxation of guinea-pig isolated tracheal smooth muscle (Coleman, 1976; Farmer & Farrar, 1976). It has been suggested that only adenosine relaxes the tissue directly (Coleman, 1976; Christie & Satchell, 1980), via the A_2 purinoceptor (Brown & Collis, 1982), and that the nucleotides themselves produce relaxation only after being metabolised to adenosine (Christie & Satchell, 1980; Satchell, 1985).

It is assumed that ATP, ADP and AMP are rapidly dephosphorylated by pyrophosphohydrolase enzymes (ectonucleotidases) on the surface of the tissue (Satchell, 1982; 1985). Although ectonucleotidases do exist on other types of smooth muscle, such as guinea-pig taenia coli and urinary bladder (Welford *et al.*, 1986; 1987), their existence on the guinea-pig trachea has not yet been demonstrated directly, although it has been inferred (Coleman, 1976; Farmer & Farrar, 1976; Christie & Satchell, 1980; Jones *et al.*, 1980). Dipyridamole potentiates the effect of adenosine and of the adenine nucleotides, purportedly by blocking the uptake of adenosine into the tissue (Coleman, 1976; Farmer & Farrar, 1976; Christie & Satchell, 1980; Jones *et al.*, 1980); these authors concluded that the nucleotides are rapidly dephosphorylated to adenosine and that catabolism to adenosine is essential for their activity on this tissue.

In the present paper we have made a study of the enzymic dephosphorylation of ATP by ectonucleotidases, the degradation of adenosine, and the effects thereon of dipyridamole, in the isolated trachea of the guinea-pig. Furthermore, using adenosine 5'-[β,γ-imido] triphosphate (AMP-PNP) which resists degradation by ectonucleotidases on other tissues (Welford *et al.*, 1986; 1987), we have considered the possibility that the intact ATP molecule may itself induce relaxation without prior dephosphorylation to adenosine.

Methods

Mechanical studies

Albino guinea-pigs (200–500 g) of either sex were killed by a blow to the head and exsanguinated. The trachea was dissected out, freed of connective tissue, and cut transversely into several segments, each comprising 2–3 rings of cartilage. These segments

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were then mounted, by means of two stainless steel hooks inserted through the lumen (after Hooker *et al.*, 1977), within a 10 ml tissue bath containing Krebs Henseleit solution of the following composition (mM): NaCl 113, $NaHCO_3$ 25, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2 and glucose 5.7, gassed with 95% O_2 and 5% CO_2 and maintained at 37°C. Each preparation was initially mounted under 1 g tension, and mechanical activity was recorded isometrically with a Grass FT03C transducer and displayed on a Grass 79D polygraph. The preparations were equilibrated for 60 min before the addition of any drugs and the Krebs solution in the bath was replaced every 15 min.

Before starting each experiment, the stability of the tissue was ascertained by administering methacholine (0.5 μM; EC_{50}) until a consistent response was obtained. Against such a contraction to methacholine, a cumulative concentration-response curve was obtained to adenosine, ATP or AMP-PNP. Test tissues were then washed, allowed to recover, and equilibrated for 30 min with dipyridamole (2 μM; a similar concentration to that used by Coleman, 1976; Farmer & Farrar, 1976; Christie & Satchell, 1980; Clark *et al.*, 1980; Jones *et al.*, 1980). The purine concentration-response curve was then reconstructed. In parallel experiments, vehicle (10 μl ethanol) replaced the dipyridamole solution added to the tissue bath; the reproducibility of the concentration-response curves for adenosine, ATP and AMP-PNP was thereby assessed. The relaxations produced by each of the compounds were measured as percentage inhibition of the contraction induced by methacholine (0.5 μM).

Studies of the catabolism of adenosine and adenine nucleotides

Tracheal segments prepared as described above were equilibrated for at least 60 min in Krebs Henseleit solution containing methacholine (0.5 μM) so that it was of the same composition as that used in the mechanical studies.

Each segment was then incubated at 37°C with methacholine-containing Krebs solution (1.8 ml) containing either adenosine, ATP, AMP-PNP or adenosine 5'-[β,γ-methylene] triphosphate (AMP-PCP) (each 100 μM, concentrations having been verified spectrophotometrically). When the effects of dipyridamole were being studied, the tissue was equilibrated with dipyridamole (2 μM) for 30 min before the addition of adenosine or the nucleotide being studied.

Aliquots (70 μl) of the incubation medium were removed at intervals and stored frozen for later analysis by high pressure liquid chromatography (h.p.l.c.). A Waters C_{18} Microbondapak reverse phase h.p.l.c. column was eluted with 0.05 M $NH_4H_2PO_4$ (pH 6.0) containing a gradient of 0–30% v/v methanol; this produced a high-resolution separation of each of the degradation products.

Separated catabolites were detected by ultraviolet absorption at 254 nm. Concentrations were estimated by measuring the heights of the corresponding peaks displayed on a chart recorder, and comparing the peak height to that of a standard peak representing a 100 μM concentration. Peak height had previously been shown to be linearly related ($r = 0.9999$) to concentration.

Controls were performed, as described in Welford *et al.* (1986), to assess (i) non-enzymic degradation of adenosine, ATP, AMP-PNP and AMP-PCP occurring during the 120 min incubation, (ii) the possible release of nucleosides, nucleotides or of enzymes from the tissue, and (iii) evaporation from the solution.

Drugs

Methacholine chloride, dipyridamole, adenosine, ATP and AMP-PCP were obtained from Sigma, Switzerland. AMP-PNP was purchased from Fluka AG, Switzerland.

Methacholine chloride was dissolved in distilled water as a 1 mM stock solution, 5 μl aliquots of which were added to the tissue bath to give a final concentration of 0.5 μM. Dipyridamole was dissolved in ethanol as a 2 mM stock solution, 10 μl of which were added to the tissue bath to give a final concentration of 2 μM.

Adenosine and the adenine nucleotides were each dissolved in distilled water as 1 mM and 10 mM stock solutions; concentrations were verified with a Beckman Model 24 spectrophotometer, measuring ultraviolet absorbance at 259 nm.

Due to the limited solubility of adenosine, aliquots of up to 300 μl of the stock solutions were introduced to the organ bath to give final concentrations of up to 300 μM. The addition to the organ bath of equivalent volumes of distilled water alone had no effect upon the tone of the muscle preparations.

Statistical analysis

Statistical calculations were performed using an IBM AT Computer with RS/1 software. The significance of differences was assessed by Student's t test, a probability of $P < 0.05$ allowing rejection of the null hypothesis. EC_{40} values were calculated by linear regression analysis of the steep portion of the concentration-response curve.

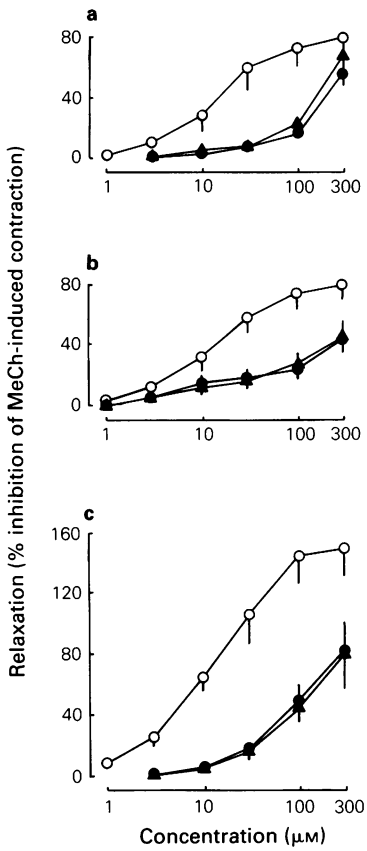

Figure 1 Relaxation of methacholine-contracted guinea-pig trachea induced by the purines adenosine (a), ATP (b) and AMP-PNP (c) in the absence and in the presence of dipyridamole. Purine without dipyridamole (●); purine with dipyridamole (2 μM (○)); purine with dipyridamole vehicle control (▲) (see Methods). Each point is the mean of at least 6 determinations using tissues from at least 4 animals. Vertical bars denote s.e.

Results

Mechanical studies

Adenosine (10–300 μM; Figure 1a), ATP (1–300 μM; Figure 1b) and AMP-PNP (3–300 μM; Figure 1c), in the absence of dipyridamole, each induced relaxation of the precontracted guinea-pig tracheal muscle in a concentration-dependent manner. Relaxations to ATP (Figure 2b) and AMP-PNP (Figure 2c), typically reaching a plateau within 3 min, were more rapid than those to adenosine (Figure 2a) which took at least 10 min to reach a plateau.

In the absence of dipyridamole, none of the purine compounds used produced a 100% relaxation

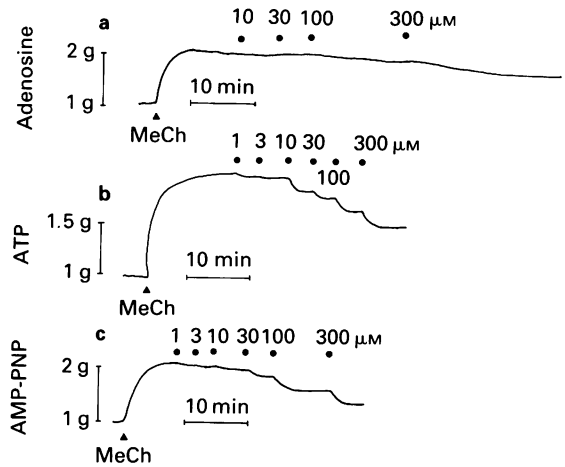

Figure 2 Guinea-pig isolated trachea: representative concentration-response curves to adenosine (a), ATP (b), and AMP-PNP (c), following precontraction with methacholine (MeCh; 0.5 μM).

(Figure 1). Because adenosine did not induce a 50% relaxation with the highest concentration usable (300 μM; Figure 1), EC_{40} values (i.e. an estimated 40% relaxation) were calcualted as a measure of potency of each of the purines (after Coleman, 1976). AMP-PNP, which had mean $EC_{40} = 77.6\,\mu M$ ($n = 6$; mean $-\log EC_{40} \pm$ s.e.mean $= 4.11 \pm 0.10$) (Figure 1c) was more potent than either ATP, which had mean $EC_{40} = 234\,\mu M$ ($n = 7$; $-\log EC_{40} = 3.63 \pm 0.13$) (Figure 1b), or adenosine which had mean $EC_{40} = 195.0\,\mu M$ ($n = 7$; $-\log EC_{40} = 3.71 \pm 0.03$) (Figure 1a).

Dipyridamole (2 μM) itself had no effect upon tracheal tone, but it potentiated the purines in relaxing the trachea. In the presence of dipyridamole (2 μM), AMP-PNP had mean $EC_{40} = 4.8\,\mu M$ ($n = 6$; mean $-\log EC_{40} \pm$ s.e.mean $= 5.32 \pm 0.11$) (Figure 1c), ATP had mean $EC_{40} = 12.9\,\mu M$ ($n = 7$; $-\log EC_{40} = 4.89 \pm 0.14$) (Figure 1b) and adenosine had mean $EC_{40} = 17\,\mu M$ ($n = 7$; $-\log EC_{40} = 4.77 \pm 0.23$) (Figure 1a). AMP-PNP, ATP and adenosine were, respectively, 16, 18 and 11 times more potent in the presence of dipyridamole than in its absence. In time-matched control tissues, when vehicle alone (i.e. 10 μl ethanol) had been added to the tissue bath, concentration-response curves to adenosine, ATP and AMP-PNP were shown to be reproducible with no significant change ($P > 0.05$) in the potency of the purines (Figure 1).

Degradation studies

Adenosine (100 μM) was enzymically catabolised by the guinea-pig isolated trachea to yield inosine and

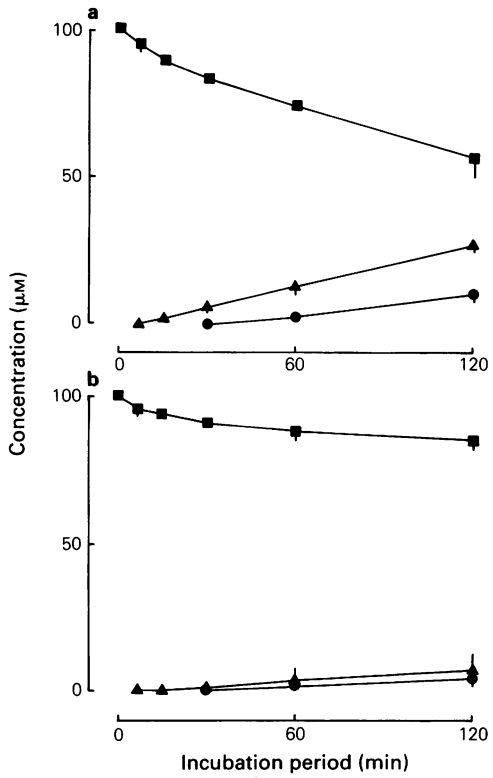

Figure 3 Metabolism of adenosine by the guinea-pig trachea in the absence (a) and in the presence (b) of dipyridamole (2 μM). Adenosine (■); hypoxanthine (●); inosine (▲). Each point is the mean of at least 3 determinations using tissues from 3 animals. Vertical bars denote s.d. where larger than symbols.

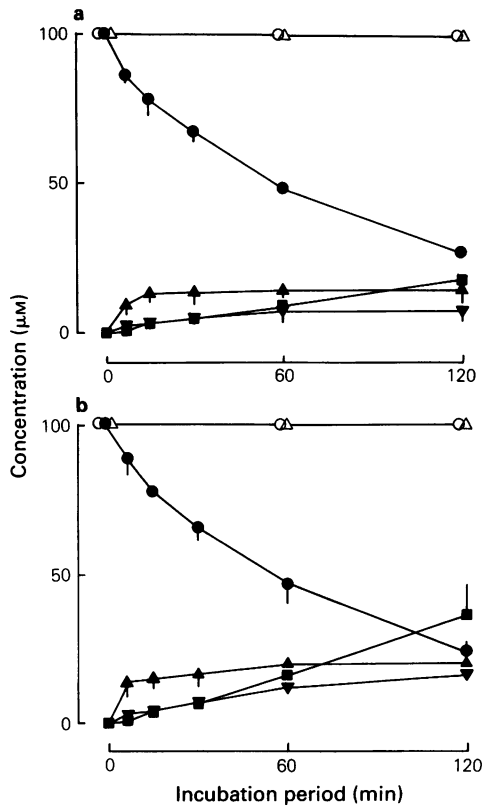

Figure 4 Metabolism of ATP by the guinea-pig trachea in the absence (a) and in the presence (b) of dipyridamole (2 μM). ATP (●); ADP (▲); AMP (▼); adenosine (■). Also shown is the lack of metabolism of AMP-PNP (○) or of AMP-PCP (△). Each point is the mean of at least 3 determinations using tissues from 3 animals. Vertical bars denote s.d. where larger than symbols.

hypoxanthine (Figure 3a), which accumulated in the incubation medium. Only 56 ± 7% of the adenosine remained unchanged after 120 min. In the presence of dipyridamole (2 μM), the degradation of adenosine was significantly ($P < 0.05$) slower (Figure 3b); after 120 min, 85 ± 3% was unchanged.

ATP (100 μM) was rapidly degraded, via ADP and AMP, to adenosine and ultimately to hypoxanthine, via inosine (Figure 4a; inosine and hypoxanthine not shown). After 120 min incubation only 27 ± 0% of the ATP remained unchanged. The rate of dephosphorylation of ATP was unaffected ($P > 0.05$) by the presence of dipyridamole (2 μM) (Figure 4b). However, after the 120 min incubation more adenosine was present, as a catabolite, when dipyridamole was used (36 μM ± 11; Figure 4b) than when it was not (17 μM ± 4; Figure 4a), but this difference was not significant ($P > 0.05$).

No degradation of AMP-PNP (100 μM) or of AMP-PCP (100 μM) was detected, either in the absence (Figure 4a) or in the presence (Figure 4b) of dipyridamole (2 μM), even after 120 min incubation.

Non-enzymic degradation of adenine nucleotides was not detected, and there was no detectable release of degradative enzymes or purine compounds from the tissues after 120 min incubation, nor was there any evaporation from the solution.

Discussion

Adenosine and ATP each induced concentration-dependent relaxations of guinea-pig trachea which were potentiated in the presence of dipyridamole

(Figure 1). These results are in accordance with the findings of other workers (Coleman, 1976; Farmer & Farrar, 1976; Christie & Satchell, 1980; Clark *et al.*, 1980; Jones *et al.*, 1980).

Dipyridamole has been reported to inhibit the uptake of adenosine into tissues (Kolassa *et al.*, 1970). The potentiation of adenosine-induced relaxation of guinea-pig trachea by dipyridamole has often been attributed to this property (Coleman, 1976; Farmer & Farrar, 1976; Christie & Satchell, 1980; Jones *et al.*, 1980; Satchell, 1982), even though no such uptake mechanism has ever been demonstrated in this particular tissue. Our results show that the use of dipyridamole in the micromolar range inhibits the removal of adenosine, from the incubation medium, by the guinea-pig trachea (Figure 3). It is unclear from our results whether dipyridamole inhibited the extracellular enzymic degradation of adenosine (Bunag *et al.*, 1964) or whether the reduced catabolism was a consequence of the inhibition of adenosine uptake. It therefore appears that dipyridamole may indeed enhance the effect of adenosine by reducing its removal from the extracellular fluid either by inhibiting its uptake into cells or by inhibiting its extracellular catabolism.

Because ATP is also potentiated by dipyridamole, it has been assumed that the nucleotide is dephosphorylated, by ectonucleotidases, to adenosine and that such dephosphorylation is a prerequisite to ATP-induced relaxation (Christie & Satchell, 1980; Satchell, 1982; 1985). We have shown that ATP is indeed sequentially dephosphorylated to adenosine by ectonucleotidases on the guinea-pig trachea and that it is only further degraded to inosine and hypoxanthine in the absence of dipyridamole (Figure 4). However, the amount of adenosine produced as a catabolite of ATP in the presence of dipyridamole was not significantly greater than when dipyridamole was absent (Figure 4); this implies that dipyridamole may not after all be potentiating ATP via an inhibition of nucleoside uptake or degradation. Furthermore, relaxations to ATP were more rapid than those to adenosine (Figure 2), suggesting that ATP need not be dephosphorylated to adenosine prior to inducing relaxation. This result contrasts with the finding of Satchell (1982) that guinea-pig tracheal strips responded to ATP and adenosine with identical relaxations. When we studied the effects of ATP and adenosine on the same piece of tissue, the response to adenosine was consistently slower and of longer duration than that to ATP (as shown in Figure 2).

AMP-PNP induced a rapid relaxation of the trachea (Figure 2), was more potent than either adenosine or ATP (Figure 1), and was potentiated 16 fold in the presence of dipyridamole. A similar analogue to AMP-PNP, AMP-PCP, which also possesses a hydrolysis-resistant linkage between the beta and gamma phosphate groups, has been shown to relax guinea-pig trachea (Christie & Satchell, 1980; Clark *et al.*, 1980; Satchell, 1982; Venugopalan *et al.*, 1986). Because the effect of AMP-PCP was potentiated by dipyridamole (Christie & Satchell, 1980; Clark *et al.*, 1980; Satchell, 1982), it was concluded that AMP-PCP must be degraded to adenosine, which then relaxed the tissue (Christie & Satchell, 1980; Satchell, 1982). This assumes the existence on the tissue of a pyrophosphohydrolase enzyme which would remove the two terminal phosphate groups, as a unit, to yield AMP which can be dephosphorylated to adenosine. However, we have shown that during a 120 min incubation, neither AMP-PCP nor AMP-PNP is degraded by enzymes on the guinea-pig trachea (Figure 4) and that therefore such a pyrophosphohydrolase does not occur on this tissue. As these two analogues induce relaxation, even though they are not catabolised to adenosine, prior degradation to adenosine is not a prerequisite for activity.

We have considered the possibility that ATP, AMP-PNP and AMP-PCP may be substrates *per se* for the adenosine uptake process and are thus potentiated by dipyridamole which inhibits the uptake of all of them. However, this explanation of our results is most unlikely since nucleotides do not usually cross intact cell membranes (Pearson, 1985) and this is demonstrated by the constancy (at 100 μM) of the concentrations of AMP-PNP and AMP-PCP during the 120 min incubation with the tissue in the absence of dipyridamole (Figure 4a). Thus neither AMP-PNP nor AMP-PCP is taken up into the tissue. Moreover, the removal of ATP from the incubation medium is unaffected by the presence of dipyridamole; from this it may be concluded that the progressive decrease in concentration of ATP during the incubation is effected by enzymic dephosphorylation alone, and not by an uptake mechanism inhibitable by dipyridamole.

Because dipyridamole markedly enhances the potency of AMP-PNP (Figure 1c) and AMP-PCP (Christie & Satchell, 1980; Satchell, 1982), even though no adenosine is produced as a catabolite of either of these analogues (Figure 4a), these results surely cast doubt on the suggestion that dipyridamole acts as a specific inhibitor of nucleoside uptake. Dipyridamole is also known to induce prostanoid synthesis (Blass *et al.*, 1980), inhibit prostanoid metabolism (Uotila *et al.*, 1981), competitively antagonize prostaglandin $F_{2\alpha}$-induced contraction of the guinea-pig trachea (Kamikawa & Shimo, 1979), and inhibit phosphodiesterase (Fredholm *et al.*, 1978). These phenomena were, however, observed with concentrations higher than that used in the present study. Nevertheless, much evidence argues

against the specificity of dipyridamole as an adenosine uptake inhibitor, and Jager (1976) has suggested that dipyridamole interferes with many mechanisms in smooth muscle cells.

In conclusion, we have shown that ATP is dephosphorylated by ectonucleotidases on the guinea-pig trachea to adenosine, and that the extracellular degradation of adenosine and/or its uptake is inhibited by dipyridamole (2 μM). The potentiation of adenosine-induced relaxation by dipyridamole (2 μM) may be attributable to this property, but the enhancement by dipyridamole of the effects of ATP, AMP-PNP and AMP-PCP cannot be reconciled with the inhibition of nucleoside uptake. ATP and AMP-PNP, an analogue of ATP which we have shown to be resistant to enzymic degradation by the tissue, each induce a more rapid relaxation than adenosine. It therefore appears that ATP, AMP-PNP, and indeed AMP-PCP, are themselves active on the trachea without the necessity of prior dephosphorylation to adenosine.

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