Apparent affinity of 1,3-dipropyl-8-cyclopentylxanthine for adenosine A₁ and A₂ receptors in isolated tissues from guinea-pigs

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1 The classification of adenosine receptor subtypes (A₁ and A₂) in intact tissues has been based on the order of agonist potency. In this study the apparent affinity of 1,3-dipropyl-8-cyclopentylxanthine (CPX), an antagonist which has been reported to be A₁ selective, and the non-selective antagonist 1,3-dimethyl-8-phenylxanthine (8PT) has been evaluated on isolated tissues from the guinea-pig.

2 The isolated tissues used were atria (bradycardic response, proposed A₁ sub-type), aorta and trachea (relaxant response, proposed A₂ sub-type).

3 Both the xanthines antagonized responses to adenosine in the three tissues but had little or no effect on responses to carbachol (atria), sodium nitrite (aorta) or isoprenaline (trachea).

4 pA₂ values for 8PT were similar on the three tissues (6.3–6.7), however, the pA₂ value for CPX on the atria (7.9–8.4) was greater than that on the aorta (6.6) or trachea (6.6).

5 These results support the suggestion that the adenosine receptors which mediate bradycardia in the atrium are of the A₁ sub-type and that those which mediate relaxation in the aorta and trachea are of the A₂ type.

Introduction

It has been proposed that adenosine receptors can be subdivided into A₁ and A₂ sub-types (Van Calcar *et al.*, 1979). This subdivision is based on the relative potency or binding affinity of a series of adenosine analogues in a variety of biochemical and isolated tissue preparations (Collis, 1985). However, there have been few reports of antagonists which show selective affinity for the proposed subtypes of adenosine receptors, particularly in intact tissues (Collis *et al.*, 1987). Recently, 1,3-dipropyl-8-cyclopentylxanthine (CPX) has been reported to exhibit selective A₁ receptor affinity in rat brain membranes (Bruns *et al.*, 1987) and in membranes from adipocytes and platelets (Martinson *et al.*, 1987). It has also been reported to antagonize the bradycardic effect of an adenosine analogue in the rat isolated heart without effects on coronary flow (Haleen *et al.*, 1987). In the present study we have evaluated the apparent affinity of this xanthine in guinea-pig tissues which are

thought to possess either A₁ (atria, Collis, 1983; Collis & Saville, 1984) or A₂ receptors (aorta and trachea, Brown & Collis, 1982; Collis & Brown, 1983) based on the relative potency of agonists. For comparison, 1,3-dimethyl-8-phenyl xanthine (8PT) was also evaluated since it is thought to be non-selective for adenosine receptor sub-types (Collis *et al.*, 1985).

Methods

Rings of isolated thoracic aorta and trachea or atrial pairs from guinea-pigs were mounted in organ baths containing modified Krebs solution at 37°C (composition mM: NaCl 118.4, NaHCO₃ 25.0, glucose 5.5, KCl 4.7, CaCl₂ 2.5, KHPO₄ 1.2, MgSO₄ 1.2, CaNa₂ EDTA 0.03) gassed with 95% O₂:5% CO₂. Isometric tension development was recorded with UFI strain gauges and 'Lectromed' polygraphs.

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Figure 1 The effect of 1,3-dipropyl-8-cyclopentyl-xanthine (CPX) on responses of guinea-pig isolated tissues to adenosine. (a) Negative chronotropic responses of isolated atria: (○) control ($n = 12$); (△) 10 nM CPX ($n = 3$); (▲) 100 nM CPX ($n = 4$) and (●) 1 μM CPX ($n = 5$). (b) Relaxant responses of isolated aorta: (○) control ($n = 10$); (●) 1 μM CPX ($n = 6$) and (□) 5 μM CPX ($n = 4$). (c) Relaxant responses of isolated trachea: (○) control ($n = 11$); (●) 1 μM CPX ($n = 4$); (□) 5 μM CPX ($n = 7$). Responses are expressed as a % of the maximum obtained on the first concentration response curve.

The nucleoside transport inhibitor dipyridamole (10 μM) and the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (10 μM) were routinely present in the Krebs solution when adenosine was used as the agonist. When 2-chloroadenosine was used as the agonist, dipyridamole (10 μM) was present in the Krebs solution. Indomethacin (5 μM) was added to the Krebs solution when tracheal preparations were used to reduce spontaneous activity and prevent contractile effects of adenosine (Advenier *et al.*, 1982; Farmer *et al.*, 1987).

The tissues were placed under a resting tension of 1 g and allowed to equilibrate for 1 h. Sub-maximal

contractions (close to 50% maximal) of aortic and tracheal rings were evoked by 3 μM phenylephrine or 0.1 μM carbachol, respectively. Adenosine was then added cumulatively to the bath contents and the evoked relaxation measured. The atrial pairs were allowed to beat spontaneously and the decrease in rate evoked by adenosine or 2-chloroadenosine was measured. Concentration-response curves were also generated to carbachol (atria) to sodium nitrite (aorta) and to isoprenaline (trachea, in presence of 10 μM phentolamine) to check for effects of the xanthines which were not related to adenosine receptors. After generation of the first agonist concentration-response curve, the tissues were incubated with one of the xanthines and the agonist concentration-response curve was repeated. Preliminary experiments established the incubation period needed for equilibrium to be reached with the xanthines (30 min). The effect of the vehicle for CPX (dimethylsulphoxide (DMSO), final concentration 0.005%) on concentration-response curves to adenosine and 2-chloroadenosine was also determined. The magnitude of the xanthine-induced rightward shift of the agonist concentration-response curve was assessed at the 50% maximal level, (in trachea where CPX caused an increase in maximal response to adenosine the concentration-ratio was measured at an equal level of relaxation which was 50% of the maximal response on the control concentration-response curve).

Concentration-ratios over a range of xanthine concentrations were analysed as described by Arunlakshana & Schild (1959) using regression analysis of the pooled data to generate pA_2 values. At least three replicates were performed for each concentration of antagonist. The maximum concentration of each antagonist used was dictated by its aqueous solubility (5 μM for CPX, 10 μM for 8PT).

Data are expressed as mean $\pm$ s.e.mean or mean with 95% confidence limits in the case of pA_2 values. Significant differences ($P < 0.05$) were calculated by Student's *t* test.

Compounds used

Adenosine (Sigma), adenosine deaminase (EC 3.5.4.4., Sigma), carbachol (Sigma), 2-chloroadenosine (Sigma), 1,3 dipropyl-8-cyclopentyl xanthine (CPX, synthesized by Dr R. James, ICI), dipyridamole (Persantin, Boehringer Ingelheim), erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA, Burroughs Wellcome), phentolamine mesylate (Ciba), (–)-phenylephrine hydrochloride (Sigma), 8-phenyltheophylline (Sigma), (±)-isoprenaline hydrochloride (Sigma), indomethacin (Sigma), sodium nitrite (Analar). CPX was dissolved in 1% aqueous

DMSO at 1 mM, 8PT in 80% v/v methanol containing 0.2 M NaOH at 10 mM.

Results

Both xanthines antagonized responses to adenosine, causing dextral displacements of the agonist concentration-response curves. The effect of a range of concentrations of CPX on responses in the atria, aorta and trachea are shown in Figure 1. CPX was a more potent antagonist in the atria (e.g. concentration-ratio at $1 \mu\text{M}$ = 116) than in the aorta and trachea (e.g. concentration-ratios at $1 \mu\text{M}$ = 6 and 4 respectively). In the trachea, but not in the aorta, CPX (1 and $5 \mu\text{M}$) enhanced the maximum relaxation evoked by adenosine. 8PT appeared to be an equi-effective antagonist in the three tissues (e.g. concentration-ratios at $10 \mu\text{M}$ = 19, 27, 28 in atria, aorta and trachea respectively).

The Schild plot slopes obtained with CPX and 8PT in the three tissues did not differ significantly from unity over the antagonist concentration-ranges that could be used. The pA_2 values for 8PT were similar in all three tissues (Table 1, Figure 2), however CPX exhibited a higher pA_2 on atria compared with aorta and trachea (Table 1, Figure 2).

At the highest concentrations used the xanthines did not alter responses of atria to carbachol or of aortae to sodium nitrite ($n > 4$), however CPX $5 \mu\text{M}$ caused a small but significant potentiation of responses to isoprenaline on the trachea (control $-\log EC_{50} = 8.04 \pm 0.05$, in presence of CPX = 8.27 ± 0.03 , $P < 0.05$). The vehicle for CPX had no significant effect on concentration-response curves to adenosine or 2-chloroadenosine ($n > 4$).

In the absence of exogenous adenosine, neither CPX nor 8PT altered the amplitude of the contractile responses evoked by phenylephrine or carbachol in the aorta and trachea respectively. Both antagonists increased the rate of beating of isolated atria,

Figure 2 Schild plots of the interaction of (a) 1,3-dipropyl-8-cyclopentylxanthine and of (b) 8-phenyltheophylline with adenosine evoked responses in guinea-pig isolated atria (O), aorta (●), and trachea (□); $n = 3$ or more for each concentration of antagonist.

e.g. CPX increased rate from 151.8 ± 6.8 beats min^{-1} to 208.9 ± 4.9 beats min^{-1} ($n = 15$, $P < 0.001$). Since this increase in rate could be due to antagonism of the negative chronotropic action of endogenous adenosine, further experiments were performed to investigate the effects of adenosine deaminase (1 U ml^{-1}) (EHNA was omitted). In these experiments 2-chloroadenosine was used as the agonist, as it is resistant to deamination.

In the absence of adenosine deaminase, CPX had a pA_2 of 8.14 (7.7–8.7) against 2-chloroadenosine (Schild plot slope = 0.91, $n = 15$). In the presence of adenosine deaminase, the initial beating rate of the atria was 203.4 ± 8.1 beats min^{-1} , and this was not significantly enhanced by CPX (209.8 ± 7.3 beats min^{-1} , $n = 15$). In the presence of adenosine deaminase, the pA_2 value of CPX was increased to 8.41 (7.7–9.6, Schild plot slope = 0.95, $n = 15$); however, this increase was not statistically significant.

Table 1 Antagonist data for 1,3-dimethyl-8-phenylxanthine (8-PT) and 1,3-dipropyl-8-cyclopentylxanthine (CPX) in guinea-pig atria, aorta and trachea (adenosine as agonist)

Xanthine	Tissue	Concentration range (μM)	Schild plot slope	pA_2^*	n
8PT	Atria	1–10	0.84 ± 0.22	6.68(5.8–8.6)	14
	Aorta	1–10	0.82 ± 0.19	6.69(6.0–7.9)	11
	Trachea	1–10	1.20 ± 0.20	6.3 (5.6–7.2)	15
CPX	Atria	0.01–1	1.08 ± 0.12	7.91(7.2–8.8)	15
	Aorta	0.5–5	1.05 ± 0.24	6.63(6.4–7.16)	14
	Trachea	1–5	0.87 ± 0.08	6.55(6.3–6.9)	11

* 95% confidence intervals.

Discussion

The results of this study confirm those obtained with membrane preparations which have indicated that CPX is an A₁ selective adenosine receptor ligand. 8-PT, in contrast, did not show selectivity between the receptors in the three tissues, confirming previous suggestions that this compound is non-selective between adenosine receptor sub-types. The pA₂ values for 8PT reported in this paper are similar to those previously published (Collis *et al.*, 1985). The pA₂ values obtained for CPX in the aorta and trachea (6.55–6.6) are close to the $-\log K_i$ values reported in binding or adenylate cyclase based assays at A₂ receptors (6.47–7.16, Martinson *et al.*, 1987; Bruns *et al.*, 1987; Lohse *et al.*, 1987). It is difficult to reach a conclusion as to the nature of the antagonism evoked by CPX or 8PT in the aorta and trachea, because of the limited range of concentrations employed. However, higher concentrations could not be used because of the poor aqueous solubility of these xanthines. The enhancement of maximal responses to adenosine on the trachea may have been a consequence of a non-specific enhancement of relaxant responses in this tissue as evidenced by the effect of CPX on isoprenaline-induced relaxation.

Antagonism of adenosine-evoked responses by CPX in the guinea-pig atrium appears to be consistent with a competitive interaction over the concentration-range used. The pA₂ value for CPX at receptors in the atrium (7.9) is markedly less than the $-\log K_i$ values reported in cyclase based or binding studies with rat adipocytes or CNS membranes (8.9–9.3, Martinson *et al.*, 1987; Bruns *et al.*, 1987; Lohse *et al.*, 1987; Ukena *et al.*, 1986), and slightly less than that in binding data from guinea-pig tissues (8.4, Ukena *et al.*, 1986). One possible reason for the lower pA₂ values found in the guinea-pig atria could be that it is due to the increase in basal beating rate which the antagonist evokes. Kenakin & Beek (1987)

have shown that an increase in basal activity of atria caused by an adenosine antagonist can lead to an underestimation of antagonist affinity. Kenakin & Beek (1987) used pharmacological resultant analysis to compensate for the stimulatory effects of the antagonists that they used. We have used a different method, that of removing the effects of endogenous adenosine with a concentration of adenosine deaminase known to abolish responses to large doses of the purine in guinea-pig atria (Collis & Pettinger, 1982). When this procedure was used the pA₂ for CPX in the guinea-pig atria became similar to the $-\log K_i$ reported for guinea-pig CNS tissue (Ukena *et al.*, 1986).

The reason why the affinity of CPX for A₁ receptors is apparently less in guinea-pig tissue and membranes than in rat cell membrane preparations is unclear (present study and Ukena *et al.*, 1986; Martinson *et al.*, 1987; Bruns *et al.*, 1987; Lohse *et al.*, 1987). It has been shown that there are species differences in antagonist binding affinity at A₁ receptors in the mammalian brain and that the affinities are higher in membranes from the rat than from guinea-pig tissue (Ukena *et al.*, 1986; Ferkany *et al.*, 1986). A systematic study of antagonist affinity in isolated tissues from the two species is required to establish whether there is a consistent species-related difference. Preliminary studies have indicated, however, only small differences between the apparent affinities of some xanthines in rat and guinea-pig atria (Collis *et al.*, 1988).

In conclusion, the results of this study demonstrate that CPX is an adenosine receptor antagonist which exhibits a higher affinity for receptors in guinea-pig atria than those in the aorta and trachea of this species. The selectivity of this antagonist supports the hypothesis that there are two subtypes of adenosine receptors and that the A₁ type mediates the negative chronotropic effect of adenosine in the atrium whereas the A₂ subtype mediates the relaxant effect of the purine in the trachea and the aorta.

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