Br. J. Pharmac. (1985), **85**, 291–296

PACPX – a substituted xanthine – antagonizes both the A_1 and A_2 subclasses of the P_1-purinoceptor: antagonism of the A_2 subclass is competitive but antagonism of the A_1 subclass is not

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1 1,3-Dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX) was examined for its ability to antagonize adenosine acting on the A_1 and A_2 subclasses of the P_1-purinoceptor. A_1-purinoceptors were studied in the isolated, driven left atria of the guinea-pig, and A_2-purinoceptors in the isolated, carbachol-contracted taenia coli of the guinea-pig.

2 PACPX antagonized the actions of adenosine in both types of preparation and was a more potent antagonist than 8-phenyltheophylline.

3 The antagonism at the A_2-purinoceptor was competitive with a pA_2 of 5.95.

4 The antagonism at the A_1-purinoceptor was not competitive, although antagonism at the A_1-purinoceptor was greater than that at the A_2-purinoceptor, based on a comparison of pD_2 values.

5 The manner of antagonism of PACPX on the A_1-purinoceptors of the heart was different from that found for the A_1-receptors in bovine brain, implying that there is a fundamental difference between these central and peripheral A_1 subclasses of P_1-purinoceptor.

Introduction

Methylxanthines in general are known to act as P_1-purinoceptor antagonists in a variety of tissues (see Burnstock, 1978, Daly *et al.*, 1981; Berne *et al.*, 1983). Theophylline antagonizes responses to adenosine in the guinea-pig left atrium (Burnstock & Meghji, 1981) and also in the guinea-pig ileum (Okwuasaba *et al.*, 1977). Some derivatives of theophylline have been shown to be more potent antagonists of adenosine activity. For example, 8-phenyltheophylline (8-PT) is more potent than theophylline in antagonizing adenosine-induced accumulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in the guinea-pig cerebral cortex (Smellie *et al.*, 1979) and in human fibroblasts (Bruns, 1981). In peripheral tissues, 8-PT has been shown to be more potent than theophylline in antagonizing: (a) the inotropic response to adenosine in the guinea-pig left atrium; (b) the presynaptic inhibition of cholinergic neurotransmission by adenosine in the guinea-pig ileum; and (c) the adenosine induced relaxations of the histamine-contracted rabbit basilar artery (Griffith *et al.*, 1981).

Recently, a more highly substituted xanthine, 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX), has been found to antagonize binding of the P_1-purinoceptor agonist N^6-[^{3}H]cyclohexyladenosine (CHA) to bovine brain membranes at the A_1 subtype of the purinoceptor with a potency 70,000 times that of theophylline and 7 times that of 8-PT (Bruns *et al.*, 1983).

In the guinea-pig left atrium, the P_1-purinoceptors appear to be of the A_1 subtype (Collis, 1983) and in the guinea-pig taenia coli they appear to be of the A_2-subtype (Burnstock *et al.*, 1984). In central systems the A_1-purinoceptor and A_2-purinoceptor have, respectively, a high and low affinity for adenosine (Van Calker *et al.*, 1979); similarly in peripheral systems adenosine appears to have a greater potency on A_1-receptors as in both the guinea-pig left atrium (Collis, 1983) and adrenergic nerve terminals in the rabbit portal vein (Brown & Collis, 1983), than on the A_2-purinoceptors, as in the trachea (Brown & Collis, 1982) or guinea-pig taenia coli (Burnstock *et al.*, 1984), based on comparison of pD_2 values.

The purpose of the present study was to determine whether or not PACPX was a potent antagonist of peripheral P_1-purinoceptors and whether or not it

showed a specificity for the A_1 or A_2 P_1-purinoceptor subtypes.

Methods

Guinea-pigs of either sex (300–600 g) were killed by a sharp blow to the back of the head. The heart was excised and the left atrium dissected free in cold Krebs solution of the following composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.4, $NaHCO_3$ 16.3, $MgSO_4$ 0.6, $CaCl_2$ 2.5 and glucose 7.7 and included dipyridamole (0.5 μM), an adenosine uptake inhibitor. The left atrium was mounted on a punctate electrode and then transferred to a 10 ml organ bath. A load of 0.5 g was applied and a cathodal pulse (5 ms, 2.5 Hz, twice threshold voltage) was delivered through the punctate electrode to stimulate the atrial muscle.

The longitudinal muscle of the caecum (taenia coli) together with its underlying myenteric plexus was dissected free and placed in cold Krebs solution (composition as above) which included the adenosine uptake inhibitor dipyridamole (0.2 μM). Lengths of about 1.5 cm were attached by silk thread to a rigid support and were transferred to 10 ml organ baths. A tension of 1.0 g was applied.

Organ baths and reservoirs of Krebs solution were maintained at 36 ± 0.5°C and were continually gassed with 95% O_2 plus 5% CO_2. All preparations were allowed to equilibrate for 1 h. Mechanical activity was measured isometrically with a Dynamometer UF1 or Grass FTOC3 force displacement transducer output to a Grass polygraph.

Concentration-response curves

For the atria, concentration-response curves were constructed by cumulative addition of adenosine producing bath concentrations from 0.1 μM to 1000 μM. Responses were allowed to plateau before the concentration was increased and were measured as percentages of basal levels. PACPX (0.1 μM to 10 μM) was added to the bath and allowed to equilibrate for 10–15 min before a cumulative adenosine concentration-response was performed.

For the taenia, carbachol (50 nM) was added to the organ bath in order to produce a standardized tone, thereby allowing quantification of the relaxant response to adenosine. When the contraction due to carbachol had plateaued adenosine (1 μM to 300 μM) was added to the organ bath and was washed out after a maximum response had been obtained. Ten minutes was allowed to elapse before the next addition of carbachol and adenosine. PACPX (0.22 μM to 4.64 μM) was added to the organ bath and allowed to equilibrate for 10 min before carbachol and then adenosine were added. In the presence of PACPX (10 μM), the nature of response to carbachol was altered in that it took a long time to attain a maximal contraction and that the concentration of carbachol needed to be doubled (100 nM) to induce the standard level of tone; hence results with PACPX (10 μM) have not been included in analysis of experiments on taenia coli.

Specificity of PACPX

In the guinea-pig left atrium PACPX (10 μM) was tested against carbachol which, like adenosine, is a negative inotrope. Concentrations of carbachol (0.1–1.0 μM) were applied to the organ bath cumulatively in order to determine its EC_{50} from responses in the 20–80% maximum inotropy range, before and after incubation with PACPX. The mean of three evaluations of the pD_2 was taken from each atrium in the presence and absence of PACPX.

In the carbachol-contracted guinea-pig taenia coli, PACPX (4.64 μM) was tested against the P_2-purinoceptor agonist adenosine 5′-triphosphate (ATP), the adrenoceptor agonist noradrenaline and also NADP, which like ATP and noradrenaline causes a relaxation mediated via a Ca^{2+}-dependent K^+-channel (Burnstock & Hoyle, 1985). Partial concentration-response curves were performed in the 20–80% maximum response range in order that the EC_{50} could be evaluated.

Analysis of results

Responses to adenosine in the atria were expressed as a percentage of the maximum response obtained, which was approximately equivalent to a 90% decrease in the force of contraction. Concentration-response curves were constructed by calculating the mean log (concentration of adenosine) ± standard error (s.e.) which produced a given response (Waud, 1975).

For the taenia coli the maximum response was 100% relaxation and concentration-response curves were constructed as for atria.

Slopes of concentration-response curves were calculated from the regression of the response on the log (concentration of adenosine). Mean slopes ± s.e. were used for testing parallelism in the presence and absence of PACPX.

The pA_2 for the antagonism of adenosine by PACPX, with its 95% confidence limits, was calculated from a Schild plot (Arunlakshana & Schild, 1959) which was constructed by the calculated regression of log (dose-ratio − 1) on log (concentration of PACPX) for each preparation where the dose-ratio was taken as (concentration of adenosine in presence of PACPX)/(concentration of adenosine in absence of PACPX). Schild plot regression lines were calculated from the 50% maximal level of response.

The Schild plot regressions were analysed for linearity and the slope being 1.

Statistical analyses of variances were carried out using Student's t tests (paired or unpaired, as appropriate). The 5% level was taken as being significant.

Drugs used

Adenosine (Sigma); PACPX was generously supplied as a gift from Warner-Lambert Company, U.S.A. PACPX was dissolved in 80% methanol/20% molar NaOH(v/v) to produce a stock solution of 10^{-2}M, and subsequently diluted with distilled water. Noradrenaline (Arterenol, Sigma) was dissolved in ascorbic acid (10^{-4}M) to produce a stock solution of 10^{-2}M, subsequently diluted with distilled water. Carbachol (carbamylcholine chloride) and β-nicotonamide adenine dinucleotidephosphate (NADP) were also obtained from Sigma.

Results

Guinea-pig atria

In the presence of dipyridamole (0.5 μM), adenosine (1–1000 μM) produced a concentration-dependent negative inotropy. Increasing the concentration of PACPX produced successive parallel rightward shifts in the adenosine concentration-response curves (Figure 1 and Table 1). However, increasing the concentration of PACPX beyond 2.15 μM did not induce a greater antagonism (Table 1). At all concentrations of PACPX the antagonism was fully surmountable by adenosine. The Schild plot regression was not statistically significant (Figure 3).

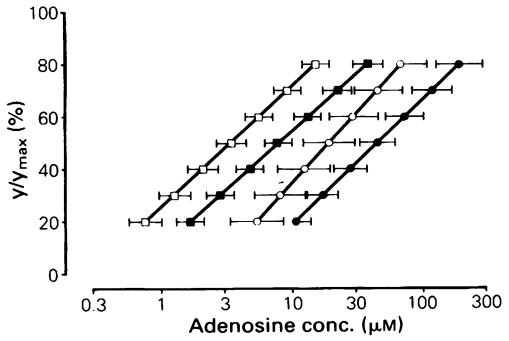

Figure 1 Guinea-pig driven left atrium. Antagonism of the negative inotropic response of adenosine by PACPX. Ordinate scale is (Y/Ymax) %, abscissa scale is adenosine concentration (μM) logarithmic scale. Adenosine alone (□, $n = 13$); + PACPX 0.22 μM (■, $n = 10$); + PACPX 1.0 μM (○, $n = 8$); + PACPX 4.64 μM (●, $n = 9$). Points represent means and horizontal lines show s.e. Curves for intermediate concentrations of PACPX have been omitted for clarity (i.e. PACPX at 0.1, 0.46, 2.15 and 10 μM). The curves for PACPX at 2.15, 4.64 and 10 μM all but overlie each other. Dipyridamole, 0.5 μM, was present throughout.

Guinea-pig taenia coli

Adenosine (1–300 μM) caused a concentration-dependent relaxation in the carbachol-contracted taenia coli in the presence of dipyridamole (0.2 μM). Increasing the concentration of PACPX produced a progressive rightward shift in the adenosine concentration-response curves (Figure 2 and Table 1). The shifts were all significantly parallel over the concentration range of 0.22 μM to 4.64 μM PACPX. The Schild plot constructed at the 50% level of response was linear ($r = 0.71$, $P < 0.001$) and its slope of 1.03 ± 0.41 (95% confidence limits) was not significantly different from 1 ($P < 0.01$) (Figure 3). The pA_2 value was determined to be 5.95 ± 0.158 (mean ± 95% confidence limits).

Specificity of PACPX

In the driven left atrium, PACPX (10 μM) did not antagonize the negative inotropic response to carbachol. In controls, the pD_2 for carbachol was 6.62 ± 0.080 and was virtually unchanged at 6.58 ± 0.091 after incubation with PACPX (mean ± s.e., $n = 4$).

In the carbachol-contracted taenia coli, PACPX (4.64 μM) did not antagonize the responses to ATP, NADP, or noradrenaline (Table 2).

Table 1 pD_2 values for adenosine in the guinea-pig driven left atrium and carbachol-contracted taenia coli in the absence and presence of PACPX and in the presence of dipyridamole

PACPX (μM)	pD_2(n)	
	Left atrium	*Taenia coli*
0	5.46 ± 0.115 (13)	5.27 ± 0.135 (11)
0.10	5.19 ± 0.108 (9)	-
0.22	5.10 ± 0.112 (10)	5.11 ± 0.162 (8)
0.46	4.81 ± 0.120 (10)	5.08 ± 0.168 (8)
1.00	4.71 ± 0.206 (8)	5.00 ± 0.137 (8)
2.15	4.44 ± 0.242 (10)	4.59 ± 0.156 (7)
4.64	4.34 ± 0.142 (9)	4.49 ± 0.138 (7)
10.00	4.39 ± 0.130 (10)	-

The values show mean ± s.e. of n number of observations.

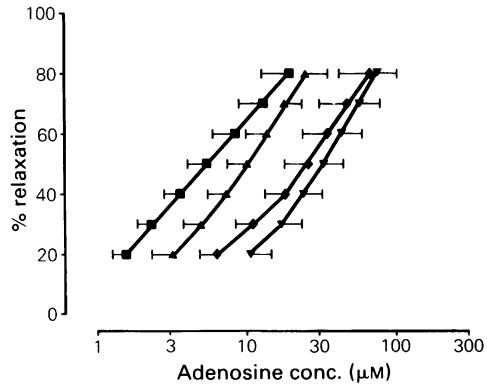

Figure 2 Guinea-pig carbachol-contracted taenia coli. Antagonism of adenosine by PACPX. Ordinate is % relaxation, abscissa is adenosine concentration on a logarithmic scale. Adenosine alone (■, $n = 11$); + PACPX 0.46 μM (▲, $n = 8$); + PACPX 1.0 μM (◆, $n = 8$); + PACPX 4.64 μM (▼, $n = 7$). Points represent mean and horizontal lines show s.e. Curves for intermediate concentrations of PACPX have been omitted for clarity (i.e. PACPX at 0.22 and 2.15 μM. Dipyridamole, 0.2 μM, was present throughout.

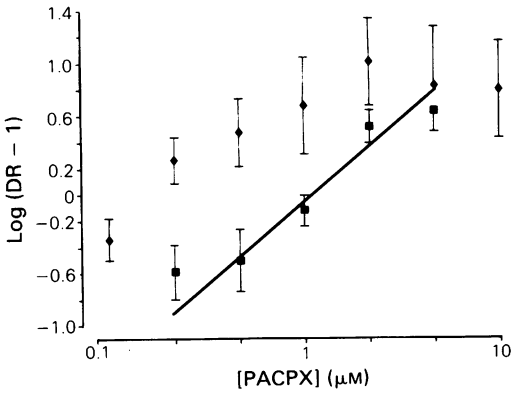

Figure 3 Schild plots for the antagonism by PACPX of the effects of adenosine in the guinea-pig driven left atrium and carbachol-contracted taenia coli. Ordinate is log (dose-ratio − 1), abscissa is concentration of PACPX on a logarithmic scale. Left atrium (◆), the regression was not statistically significant ($P < 0.40$). Taenia coli (■), solid line is the linear regression ($r = 0.71$ $P < 0.001$) which intersects the abscissa to yield a pA_2 of 5.95 ± 0.158 (95% confidence limits). For both atrium and taenia coli the points represent the mean of 7–10 observations and vertical lines show s.e.

Table 2 Guinea-pig carbachol-contracted taenia coli, pD_2 values for ATP, NADP and noradrenaline (NA) in the absence and presence of PACPX (4.64 μM)

	pD_2	
	Control	+ PACPX
ATP	6.38 ± 0.215	6.19 ± 0.204
NADP	5.40 ± 0.180	5.44 ± 0.140
NA	7.05 ± 0.105	7.02 ± 0.135

Results show mean ± s.e. ($n = 4$). All the pD_2 values in the presence of PACPX were not significantly different from the controls (Student's paired t test).

Discussion

The results show that PACPX antagonized the action of adenosine in both types of preparation used; although the A_2-purinoceptor of the taenia coli was antagonized competitively, the A_1-purinoceptor of the atrium was not.

The antagonism at the A_2-purinoceptor by PACPX in the taenia coli was competitive since it was surmountable and PACPX produced concentration-dependent parallel shifts in the adenosine concentration-response curves, from which the constructed Schild plot yielded a slope not significantly different from unity.

The antagonism of both A_1- and A_2-purinoceptors by PACPX appeared to be specific since the effect of carbachol, a cholinoceptor agonist, was not antagonized in the atrium and neither were the effects of ATP, NADP or noradrenaline antagonized in the taenia coli. Hence, PACPX did not antagonize inhibitory P_2-purinoceptors or adrenoceptors in the taenia coli or the inhibitory muscarinic receptors in the heart.

In the left atrium, at the 50% response level, PACPX has an apparent pA_2 of 6.15 ± 0.41 which compares well with that for 8-PT (6.24) in the same preparation (Griffith *et al.*, 1981), i.e. PACPX is as potent as 8-PT.

The pA_2 for the antagonism of purinoceptors by 8-PT in the guinea-pig taenia coli has not been published, but 8-PT (10 μM) has been shown to have a relative antagonism of 6.9 on the EC_{50} of 2-chloroadenosine in the taenia coli (Burnstock *et al.*, 1984); the results here show that when the concentration of PACPX was 4.64 μM, the relative antagonism of the adenosine EC_{50} was 7.4. Since the action of 2-chloroadenosine is comparable to that of adenosine in the presence of dipyridamole (Muller & Paton, 1979), the inference can be drawn that PACPX is approximately twice as potent as 8-PT in antagonizing A_2-purinoceptors in the guinea-pig taenia coli.

The exact nature of PACPX antagonism of adenosine in the left atrium is unclear since, although induced shifts were parallel and the observed antagonism was surmountable, the degree of antagonism was limited at a concentration of PACPX of 2.15 μM at which the relative antagonism, based on pD_2 values, was 10.5. Since the antagonism was limited this indicates that PACPX is not competing with adenosine for a site of action at an A_1-purinoceptor.

That PACPX does not act in a competitive manner against the A_1-purinoceptors of the left atrium is at variance with the competitive antagonism against A_1-purinoceptors found in bovine brain (Bruns *et al.*, 1983). This may indicate a fundamental difference between central and peripheral A_1-purinoceptors, and it may well be a reflection of the fact that the classification of purinoceptor subtypes in central nervous tissues which subsequently led to subclassification of purinoceptors in peripheral tissues, has been based largely on biochemical assays and radioligand displacement studies (e.g. Bruns *et al.*, 1980), whereas peripheral classification has been mostly based on pharmacological studies.

At A_1-purinoceptors in cultured glial cells from mouse brain, the N^6-substituted adenosine analogue L-phenylisopropyladenosine (L-PIA) is more potent than adenosine (Van Calker *et al.*, 1979), and additionally at the A_1-purinoceptors of rat adipocytes and rat cerebral cortex adenosine is more potent than the C^5-substituted analogue N-ethylcarboxamidoadenosine (NECA) (Londos *et al.*, 1980; Cooper *et al.*, 1980). Such a rank order of agonist potency: L-PIA > adenosine > NECA is one of the criteria for identifying subclasses of adenosine receptors (Van Calker *et al.*, 1979; Londos *et al.*, 1980). Although all receptors subsequently claimed to be in the A_1-subclass show a high stereoselectivity between the pair of diastereomers L-PIA and D-PIA (for review see Burnstock & Buckley, 1984), profiles of agonism by adenosine analogues differ. For example, in the guinea-pig left atrium the order is NECA > L-PIA > adenosine (Collis, 1983); similarly at presynaptic noradrenergic terminals of the rat vas deferens and cholinergic terminals of the guinea-pig ileum the 5'-substituted adenosine analogue, N-cyclopropylcarbox-amidoadenosine (NCPCA) is more potent that L-PIA, and L-PIA is more potent than adenosine (Paton, 1981). The observation that PACPX acts competitively in one A_1-system (Bruns *et al.*, 1983) but not in another, reinforces the concept of heterogeneity amongst A_1-purinoceptors (Burnstock & Buckley, 1984).

The results presented here provide further evidence that subclasses of the P_1-purinoceptor exist in the periphery and that central A_1-purinoceptors differ from the peripheral A_1-purinoceptors of the guinea-pig atrium (which suggests that a different nomenclature should be adopted). Also, they show that PACPX is a specific and more potent antagonist than 8-PT on peripheral purinoceptors and demonstrate, for the first time, that an antagonist may be useful for identifying subclasses of P_1-purinoceptors.

C.H.V.H. is grateful to Merck Sharp and Dohme (UK) Ltd for a scholarship and to N.J. Buckley for helpful discussion. The authors thank Annie Evans and Mary Lewis for typing the manuscript and Dr Stephanie Clark for assistance in its preparation.

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(*Received November 29, 1984. Accepted December 24, 1984.*)