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Apparent affinity of some 8-phenyl-substituted xanthines at adenosine receptors in guinea-pig aorta and atria

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1 Some 8-phenyl-substituted, 1,3 dipropyl xanthines have previously been demonstrated to have a 20–400 fold greater affinity for A_1 binding sites in rat CNS membranes than for A_2 adenosine receptors in intact CNS cells from guinea-pigs. In the present study these compounds (1,3, dipropyl-8-phenylxanthine: DPPX; 1,3 dipropyl-8-(2 amino-4-chlorophenyl) xanthine: PACPX; 8-(4-(2-aminoethyl)amino) carbonyl methyl oxyphenyl)-1,3-dipropylxanthine: XAC; and D-Lys-XAC) together with two that have not been reported to exhibit A_1-receptor selectivity (8-(*p*-sulphophenyl)theophylline: 8-PST; 8-(4-carboxy methyl oxyphenyl)-1,3-dipropylxanthine: XCC) have been evaluated as antagonists of the effects of 2-chloroadenosine in two isolated cardiovascular tissues.

2 The isolated tissues used were guinea-pig atria (bradycardic response) and aorta (relaxation), which are thought to possess A_1 and A_2 adenosine receptors, respectively.

3 All the xanthines antagonized responses evoked by 2-chloroadenosine in both tissues but did not affect responses evoked by acetylcholine (atria) or sodium nitrite (aorta).

4 The xanthines, 8-PST, XAC, D-Lys XAC, XCC and DPPX appeared to be competitive antagonists of the effects of 2-chloroadenosine, as Schild plot slopes did not differ significantly from unity. The 1,3-dipropyl substituted compounds had pA_2 values from 6.5 to 7.4 and were more potent than the 1,3 dimethyl substituted 8-PST (pA_2 4.9 to 5).

5 For individual xanthines, there was no difference between pA_2 values obtained in the atria and in the aorta.

6 Responses evoked by 2-chloroadenosine in atria and aorta were antagonized to a similar degree by PACPX (1 μM). The agonist dose-ratio evoked by 10 μM PACPX was no greater than that evoked by 1 μM of the xanthine in both tissues. This was probably a consequence of the limited aqueous solubility of PACPX.

7 These results fail to demonstrate A_1 receptor selectivity for DPPX, XAC, D-Lys XAC or PACPX in tissues from the guinea-pig. The A_1 selectivity previously found for these compounds may therefore be due to their high affinity for binding sites in rat CNS cell membranes.

Introduction

It has been proposed that adenosine receptors can be subdivided into two types which have been designated A_1 and A_2 (Van Calker *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, the observation of differing agonist potency in various tissues is not proof of distinct populations of receptors, since potency can be influenced by tissue factors which may be unrelated to the receptor type (Kenakin, 1984). The discovery of selective antagonists for the putative A_1 and A_2 adenosine receptors is therefore of major importance in establishing their separate existence (Collis, 1985).

Recently, some 8-phenyl-substituted xanthines have been proposed to have A_1 receptor selectivity in CNS tissue (Daly *et al.*, 1986; Jacobson *et al.*, 1985b; 1986). In these studies, the affinity of the xanthines for the A_1

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site was assessed by the inhibition of the binding of the ligand [^{3}H]-N^6-cyclohexyladenosine to rat cerebral cortical membranes. Affinity for the A_2 receptor was assessed by the antagonism of adenosine 3': 5'-cyclic monophosphate (cyclic AMP) accumulation elicited by 2-chloroadenosine in guinea-pig cerebral cortical slices. Based on these two assay systems, 1,3 dipropyl-8-(2 amino-4-chlorophenyl) xanthine (PACPX) was 400 fold more selective for the A_1 receptor, whilst other compounds exhibited 20–180 fold A_1 selectivity (Table 1). In the present study, we have evaluated the apparent affinity of some of these selective xanthines in two isolated tissue preparations, using 2-chloroadenosine as the agonist. Two xanthines which were not selective in the biochemical studies cited above, 8-(*p*-sulphophenyl) theophylline (8-PST) and 8-(4-carboxymethyloxyphenyl)-1,3-dipropylxanthine (XCC), were also evaluated for comparative purposes. The isolated preparations used were guinea-pig atria and aorta which possess adenosine receptors that appear to be similar to the A_1 and the A_2 subtype, respectively (Collis & Brown, 1983; Collis & Saville, 1984).

Methods

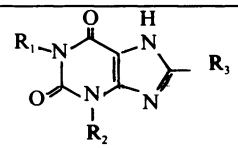
Rings of guinea-pig thoracic aorta or guinea-pig atrial pairs were mounted in organ baths containing oxygenated Krebs solution (composition mM: NaCl 118.4, $NaHCO_3$ 25.0, glucose 5.5, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaNa_2$ EDTA 0.03) at 37°C (Collis *et al.*, 1985). Isometric tension development was recorded using UFI strain gauges and Devices polygraphs. The nucleoside transport inhibitor, dipyridamole (10 μM) was present in the Krebs solution, since the agonist, 2-chloroadenosine, has been shown recently to be a substrate for the transporter (Collis & Brown, 1983; Jarvis *et al.*, 1985). Atenolol (1 μM) was present in the Krebs solutions for the atrial preparations. The tissues were placed under a resting tension of 1 g and allowed to equilibrate for 1 h.

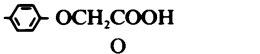
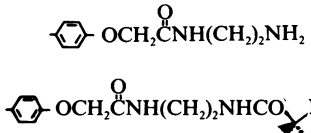
Submaximal contractions of the aortic rings were evoked by addition of 2 μM noradrenaline. Then 2-chloroadenosine was added cumulatively to the bath contents and the evoked relaxation measured. The atrial pairs were allowed to beat spontaneously before 2-chloroadenosine was added cumulatively to the bath contents and the decrease in rate measured. Concentration-response curves were also generated to the relaxant effects of sodium nitrite (aorta) and to the bradycardic effect of acetylcholine (atria) as examples of agonists that act independently of purinoceptors.

After generation of the first agonist concentration-response curve, the tissues were incubated with one of the xanthines. The incubation period required to reach equilibrium for each antagonist in either tissue was determined by examining the dextral shift of the 2-chloroadenosine concentration-response curve at 30 min intervals after the addition of the antagonist. When a vehicle was used to dissolve a xanthine, the control concentration-response curve was performed

Table 1 Structures of 8-phenyl substituted xanthines

General structure:



Compound (abbreviation)	R_1	R_2	R_3	A1:A2 *selectivity*[1]
8-(*p*-sulphophenyl)theophylline (8-PST)	CH_3	CH_3		1.4
1,3 dipropyl-8-phenyl xanthine (DPPX)	C_3H_7	C_3H_7		23
1,3 dipropyl-8-(2 amino-4-chlorophenyl) xanthine (PACPX)	C_3H_7	C_3H_7		400
8-(4-carboxymethyloxyphenyl)-1,3-dipropyl xanthine (XCC)	C_3H_7	C_3H_7		0.6
8-(4-(2-aminoethyl)amino)carbonylmethyloxyphenyl)-1,3-dipropyl xanthine (XAC)	C_3H_7	C_3H_7		41
D-Lys-XAC	C_3H_7	C_3H_7		183

[1] Based on IC_{50} values in rat cerebral cortex and guinea-pig cerebral cortex from Daly *et al.* (1985) and Jacobson *et al.* (1985b, 1986).

in the presence of the vehicle.

The magnitude of the xanthine-induced rightward shift of the agonist concentration-response curve was assessed by the dose-ratio at the 50% maximal level. Dose-ratios over a range of antagonist concentrations were analysed as described by Arunlakshana & Schild (1959) and pA_2 values calculated by linear regression.

Data are expressed as the mean ± s.e.mean. Significant differences ($P < 0.05$) were calculated by Student's t test.

Drugs and compounds used

Acetylcholine chloride (Sigma), 8-(4-((2 aminoethyl)amino) carbonylmethyl oxyphenyl)-1,3-dipropylxanthine (XAC), D-Lys-XAC, atenolol (Tenormin, ICI), 2-chloroadenosine (Sigma), 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX, Research Biochemicals), 1,3-dipropyl-8-phenylxanthine (DPPX), 8-(4-carboxymethyloxyphenyl)-1,3-dipropylxanthine (XCC), dipyridamole (Persantin, Boehringer Ingleheim), noradrenaline bitartrate (Sigma), L-N^6-phenylisopropyl adenosine (L-PIA, L-N_6-[R-phenylisopropyl]adenosine, Boehringer Mannheim), 8-phenyltheophylline (Calbiochem), sodium nitrite (Analar), 8-(*p*-sulphophenyl) theophylline (8-PST, Research Biochemicals) were used.

The preparation and properties of XAC, XCC and D-Lys-XAC have been described previously by (Jacobson *et al.*, 1985b; 1986).

Compounds were dissolved at 10 mM in distilled water, except for XAC, D-Lys-XAC and XCC (10 mM in DMSO), DPPX and 8-phenyltheophylline (10 mM in 80% methanol, 20% M NaOH, v/v) and PACPX (1 mM in M NaOH). A 1 h incubation period was judged to be sufficient to reach equilibrium for the majority of the xanthines with the exception of 8-PST (30 min, both tissues) and D-Lys-XAC (1.5 h, aorta).

Results

All of the xanthines tested were found to antagonize responses evoked by 2-chloroadenosine, causing a shift of the concentration-response curve to the right of control with no effect on the maximum response (see Table 2 for concentrations). At the highest concentrations used, none of the xanthines had any significant effect on negative chronotropic responses evoked by acetylcholine in the atria or on relaxant responses to sodium nitrite in the aorta ($n > 6$).

The xanthines 8-PST, XAC, D-Lys XAC, XCC and DPPX appeared to be competitive antagonists over the concentration range used, as the Schild plot slopes did not differ significantly from unity (Table 2). 8-PST was the least potent antagonist (pA_2 4.94–5.05). The 1,3-dipropyl substituted compounds had pA_2 values which ranged from 6.5–7.4. There was no significant difference between pA_2 values in the two tissues for individual xanthines.

PACPX (0.3 μM) shifted the 2-chloroadenosine concentration-response curve in the atrium to the right with no change in the maximum (log dose-ratio = 0.35 ± 0.1, $n = 6$). The concentration-response curve shifts evoked by 1 and 10 μM PACPX were not significantly different from each other (log dose-ratios = 0.92 ± 0.12, $n = 7$ and 1.1 ± 0.13, $n = 7$, respectively, Figure 1). Because of this phenomenon it was not possible to calculate a pA_2 value for this antagonist. The unusual behaviour of PACPX was investigated further by examining its effects on the negative chronotropic responses evoked by the A_1 selective agonist, L-PIA. When L-PIA was used as the agonist, the log dose-ratios evoked by 1 and 10 μM PACPX were also not significantly different (0.84 ± 0.06, $n = 8$ and 0.89 ± 0.09, $n = 9$, respectively).

The interaction of PACPX with the non-selective adenosine receptor antagonist 8-phenyltheophylline (8-PT; Collis *et al.*, 1985) was determined to ascertain whether the two xanthines acted at a common site. A control concentration-response curve was generated to 2-chloroadenosine and was repeated after exposure for 1 h to either PACPX (10 μM) or 8-PT (10 μM). Finally, a third concentration-response curve was generated after exposure for a further hour to both PACPX (10 μM) and 8-PT (10 μM). The log dose-ratios evoked by PACPX or 8-PT alone were 0.85 ± 0.11 ($n = 7$) and 1.38 ± 0.06 ($n = 7$), respectively. The combination of the two xanthines evoked a log dose-ratio of 1.49 ± 0.08 ($n = 14$, Figure 2). Thus, the sum of the dose-ratio for 8-PT (24) and PACPX (7) was equal to the dose-ratio for the combination of the antagonists − 1 (31), which is consistent with an action at a common receptor (Paton & Rang, 1965).

In the aorta, PACPX (1 μM) displaced the 2-chloroadenosine concentration-response curve to the right (log dose-ratio 0.76 ± 0.09, $n = 4$). A similar rightward shift was evoked by 10 μM PACPX (log dose-ratio = 0.7 ± 0.1, $n = 16$). When the dose-ratio evoked by PACPX in the aorta was compared with that in the atria, there was no significant difference at 1 μM, and a small (2.5 fold) but significant ($P < 0.05$) difference at 10 μM (atria > aorta).

Discussion

The results of this study demonstrate that the high degree of A_1 adenosine receptor selectivity exhibited by some 8-phenyl-substituted xanthines when evaluated in a rat CNS binding system and a guinea-pig CNS cyclic nucleotide assay is not apparent in isolated tissue preparations from guinea-pigs that are thought to possess A_1 and A_2 adenosine receptors.

Table 2 Antagonist data for 8-phenyl-substituted xanthines in guinea-pig atria and aorta

Xanthine	*Tissue*	*Concentration range (μM)*	*Schild plot slope*	*pA₂*		n
8-PST	Atria	10–1000	− 1.10 ± 0.15	4.94	(4.10–6.0)*	12
	Aorta	10–100	− 1.18 ± 0.17	5.05	(4.53–5.71)	19
DPPX	Atria	0.3–10	− 0.97 ± 0.10	6.93	(6.43–7.53)	19
	Aorta	0.3–10	− 1.04 ± 0.11	6.71	(5.90–7.65)	48
XCC	Atria	0.1–10	− 1.12 ± 0.06	7.40	(7.00–7.85)	15
	Aorta	0.1–10	− 0.85 ± 0.11	7.17	(6.23–8.32)	20
XAC	Atria	0.1–10	− 1.03 ± 0.09	7.22	(6.68–7.87)	11
	Aorta	0.1–10	− 0.89 ± 0.10	7.00	(6.68–7.89)	23
D-Lys XAC	Atria	1–100	− 0.98 ± 0.07	6.72	(6.24–7.29)	21
	Aorta	1–50	− 0.81 ± 0.11	6.56	(5.72–7.67)	19

For key to abbreviations used see Table 1.
*Mean and 95% confidence limits.

Thus DPPX, XAC and D-Lys-XAC, which exhibit A_1 selectivity in the studies cited in Table 1, did not show any selective antagonism between adenosine receptors in the atria (A_1) and the aorta (A_2).

An explanation for the differences between the present results and some of those previously published on these xanthines may lie in the methods used to assess their affinity for the receptor. In Figure 3, pK_i values from binding studies in rat CNS and pK_b values, derived from antagonism of the cyclic AMP response in guinea-pig CNS tissue (Daly *et al.*, 1985; Jacobson *et al*; 1986), are compared with pA_2 values obtained on guinea-pig isolated tissues in this and in a previous study (Collis *et al.*, 1985), and in a recent study by Ukena *et al.* (1986) which utilised the cyclic AMP response of rat adipocyte membranes. There is reasonable agreement between the apparent affinity of individual xanthines at A_2 receptors in guinea-pig CNS tissue and in the guinea-pig aorta (Figure 3a). The apparent affinity of individual xanthines at A_1 receptors in the rat adipocyte and guinea-pig atrium are also similar (Figure 3b). The greatest discrepancy between these latter two tissues is exhibited by D-Lys-XAC. However, when pK_i values derived from the CNS A_1-receptor binding studies are compared with the apparent pK_b from the A_1 receptors in the rat adipocyte or pA_2 values in the guinea-pig atrium (Figure 3c and d), no consistent pattern is observed. For some of the xanthines, i.e. 8-PST, 8-PT and XCC, there is excellent agreement between the values

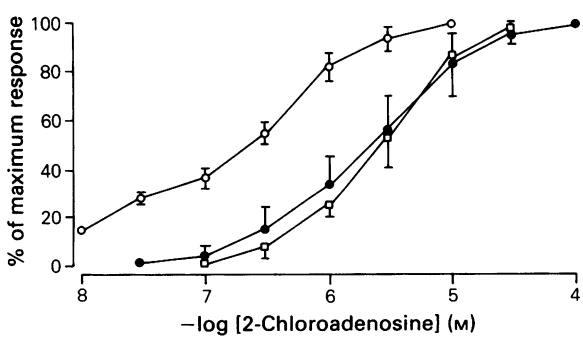


Figure 1 Effect of 1,3 dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX) on negative chronotropic responses to 2-chloroadenosine in guinea-pig atria. Each point represents the mean with vertical lines indicating s.e.mean; $n = 7$. Control (○), PACPX (1 μM) (●), PACPX, (10 μM) (□). The maximal effective concentration of 2-chloroadenosine caused cessation of mechanical activity.

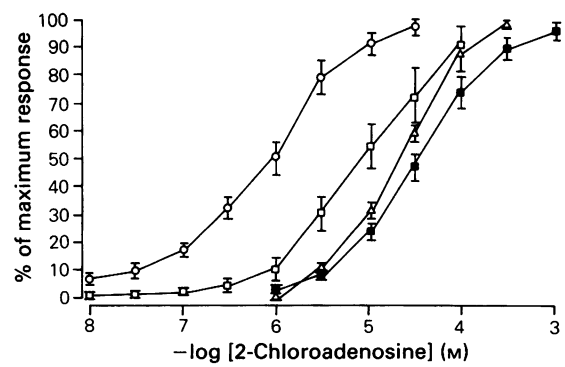


Figure 2 Effect of 1,3 dipropyl-8-(2-amino-4-chlorophenyl) xanthine (PACPX) and of 8-phenyltheophylline (8-PT) on negative chronotropic responses to 2-chloroadenosine in guinea-pig atria. Control (○) ($n = 14$), (□) PACPX (10 μM, $n = 7$), (△) 8-PT (10 μM, $n = 7$), (■) PACPX plus 8-PT (10 μM, $n = 14$). Each point represents the mean with vertical lines indicating s.e.mean.

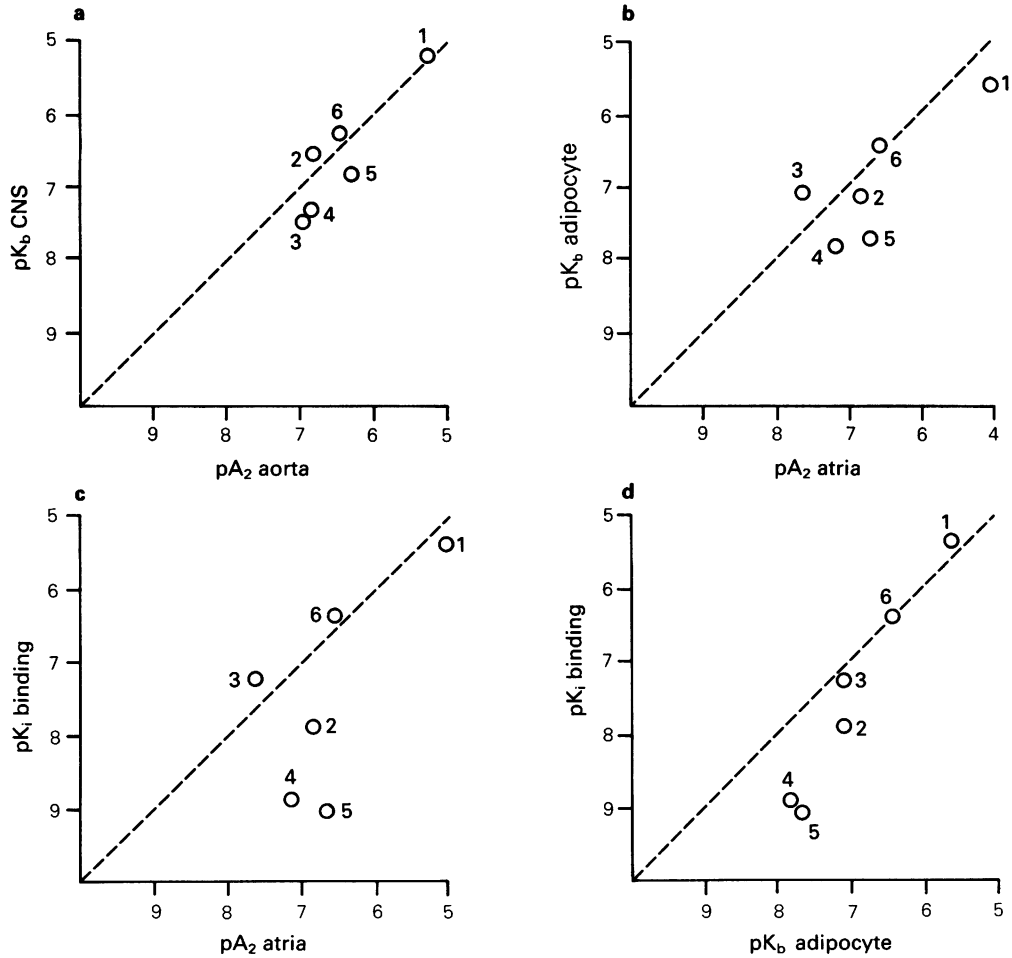


Figure 3 Scatter diagrams comparing pK_b, pA_2 and pK_i values for 8-phenyl-substituted xanthines in a variety of adenosine receptor systems. (a) Cyclic AMP response in guinea-pig CNS tissue (A_2) and relaxation of guinea-pig aorta (A_2); (b) cyclic AMP response in rat adipocytes (A_1) and rate in guinea-pig atria (A_1); (c) ligand binding in rat CNS (A_1) and rate in guinea-pig atria (A_1); (d) ligand binding in rat CNS (A_1) and cyclic AMP in rat adipocyte (A_1). The xanthines used are (1) 8-(*p*-sulphophenyl) theophylline (8-PST), (2) 1,3-dipropyl-8-phenylxanthine (DPPX), (3) 8-(4-carboxymethyloxyphenyl)-1,3-dipropyl xanthine (XCC), (4) 8-(4-((2 aminoethyl)amino)carbonylmethyloxyphenyl)-1,3-dipropyl xanthine (XAC), (5) D-Lys XAC, (6) 8-phenyltheophylline. Data are from the present study and those of Collis *et al.* (1985), Daly *et al.* (1985) Jacobson *et al.* (1985b, 1986) and Ukena *et al.* (1986). The broken line indicates a gradient of unity.

obtained in the different preparations. The pK_i values for DPPX, XAC and D-Lys-XAC are, however, higher than the pK_b values in the adipocyte or the pA_2 values in atria. The result of these high pK_i values is that these xanthines, which have been demonstrated to have A_1 selectivity, only exhibit this in a pronounced way when A_1 affinity is assessed in a ligand binding assay. Comparison of pA_2 values for these compounds in the present study using systems containing intact cells reveal no consistent selectivity. These observations are supported by the studies of Ukena *et al.* (1986), in which the A_1 selectivity of these compounds was less marked when they were compared in cell membranes possessing functional A_1 and A_2 adenosine receptors. A similar conclusion may also apply to PACPX. The pK_i for binding to A_1 receptors for PACPX was 8.6 (Daly *et al.*, 1985). A pA_2 of around 7 for PACPX seems likely from the results in the atria, and the

compound exhibits a similar potency on the aorta. However, the unusual characteristics of PACPX (see below) prevent an accurate assessment of its pA_2 value in isolated tissues.

If the apparent marked selectivity of certain xanthines for A_1 receptors is mainly a consequence of the binding assay used to assess affinity, then it may be that the binding site has different characteristics from A_1 receptors. This suggestion is supported by the high affinity of agonist ligands at the binding site (e.g. K_D for L-PIA = 2.4 nM; Daly *et al.*, 1986) when compared with EC_{50} values at functional adenosine receptors (e.g. EC_{50} for L-PIA on atria = 150 nM, on rat adipocytes = 26 nM, Collis *et al.*, 1985; Ukena *et al.*, 1986). The EC_{50} for an agonist would normally be lower than the K_D derived from binding studies because of the influence of receptor reserve in the former.

There are other possible explanations for the lack of marked A_1 selectivity of the xanthines examined in the present study on isolated tissues. One is that the adenosine receptors in the atria and the aorta are actually of the same type. This cannot be discounted until an antagonist is discovered with a pronounced selectivity for the receptor in either of these tissues.

The discrepancy between the binding data for some of the xanthines and the results obtained in the isolated atria could also be a consequence of an additional pharmacological action of the compounds in the latter preparation. In order to test this possibility, the effect of the xanthines on negative chronotropic responses to acetylcholine was also examined. The muscarinic agonist was chosen since it is known to act via the same regulatory protein and potassium ion channel as adenosine in atrial cells (Kurachi *et al.*, 1986). Since none of the xanthines altered the bradycardic action of acetylcholine, the possibility of an additional pharmacological action interfering with the results is regarded as unlikely.

The original binding and cyclic nucleotide studies of Daly *et al.* (1985) indicated that PACPX was 400 fold selective for A_1 adenosine receptors. In the isolated preparations used in this study, little evidence of A_1 selectivity was found. This xanthine exhibited unusual characteristics. In the atria; concentrations of 1 and 10 μM evoked similar rightward shifts of the dose-response curve to 2-chloroadenosine. This phenomenon did not appear to be a function of the relative A_1 to A_2 selectivity of the agonist used, as similar effects were seen when the A_1 selective agonist (L-PIA) was employed. These observations raised the possibility that PACPX is acting as an antagonist of the effect of adenosine receptor activation but not by a competitive interaction at the receptor site. The interaction of PACPX and the adenosine receptor antagonist 8-phenyltheophylline (8-PT) was determined in order to investigate this possibility. The dose-ratios evoked by PACPX and by 8-PT exhibited an additive relationship, which is consistent with an action of both xanthines at a common receptor site (Paton & Rang, 1965). The most feasible explanation of the results obtained with apparently increasing concentrations of PACPX is that the limited aqueous solubility of the compound prevents an increase in its antagonist effect. This suggestion is supported by the similar behaviour of the compound in aortic preparations. Burnstock & Hoyle (1985) have also observed that increasing the concentration of PACPX beyond 2 μM did not evoke a greater antagonism at adenosine receptors in the guinea-pig atrium or taenia coli.

The results of this study have not provided any additional support for the hypothesis that different adenosine receptor subtypes exist in the heart and blood vessels of the guinea-pig. It is interesting that one of the xanthines used in the present study (XAC) has been found to show a 16 fold selectivity against the bradycardic effects of 5-N-ethylcarboxamide adenosine when compared with the hypotensive effect of this analogue in the anaesthetized rat (Jacobson *et al.*, 1985a). This observation could imply the presence of two different adenosine receptor types in the heart and blood vessels of the rat with different susceptibilities to blockade by XAC (or it could be due to the tissue distribution of the xanthine). Indeed, marked species differences in the affinity of compounds for adenosine A_1 binding sites have been demonstrated (Ukena *et al.*, 1986). However, comparison of the data from the rat adipocyte with those from the guinea-pig atrium (Figure 3b) does not support this concept. The possibility of species differences in the affinity of xanthines for functional adenosine receptors merits further investigation.

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