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Biochemical Pharmacology, Vol. 36, No. 22, pp. 4024–4027, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

Biochemical characterization of the antagonist actions of the xanthines, PACPX (1,3-dipropyl-8(2-amino-4-chloro)phenylxanthine) and 8-PT (8-phenyltheophylline) at adenosine A_1 and A_2 receptors in rat brain tissue

(*Received* 29 *November* 1986; *accepted* 18 *May* 1987)

Caffeine, the most widely used psychoactive agent consumed today [1, 2], produces its therapeutic effects by antagonizing the interaction of endogenous adenosine with specific cell surface recognition sites or receptors [1–3]. These may be subdivided into A_1 and A_2 subtypes on the basis of their pharmacological profiles [4] and subserve different functions. Agonists selective for the cardiac A_1 receptor can attenuate arrhythmic abnormalities such as supraventricular tachycardia [5], whereas A_2 selective agonists are potent coronary vasodilators [6]. Antagonists of A_1 and A_2 receptors are also useful as therapeutic agents; theophylline and its ethylenediamine-salt, aminophylline, have been used as anti-asthmatic [7] and cardiotonic agents [8] respectively. However, the use of these agents is limited by their low potency together with unwanted side effects such as phosphodiesterase inhibition [3, 9, 10].

Xanthines substituted in the 1, 3 and 8-positions of the molecule have increased adenosine antagonist activity with a corresponding loss of phosphodiesterase inhibitory activity [9–12]. One of the most potent of these analogs is PACPX (1,3 - dipropyl - 8(2 - amino - 4 - chloro)phenylxanthine), which is the most active A_1 adenosine antagonist known in bovine brain tissue [10]. In contrast to theophylline which has been reported to be a competitive adenosine antagonist, PACPX has been reported to have non-competitive properties [13], a fact that may be attributable to the phenyl substitutent. Examination of the adenosine antagonist activity of PACPX in the driven guinea pig left atria and in the carbachol-contracted guinea pig *Taenia coli* led to the conclusion that PACPX was a non-competitive antagonist at A_1 receptors and a competitive antagonist at A_2 receptors, at which it was less potent than A_1 receptors [13]. In brain tissue, however, the xanthine had been reported to be a competitive A_1 antagonist [10]. The discrepancy between the brain and atrial properties of PACPX was taken as evidence of differences in the A_1 receptors in the two tissues [13].

In the present study, the effects of PACPX and 8-phenyltheophylline (8-PT) on A_1 and A_2 receptor properties were assessed in rat brain tissue using radioligand binding techniques.

Methods

Binding to rat brain A_1 receptors was measured using [^{3}H]cyclohexyladenosine (CHA; sp. act. 25 Ci/mmol) as previously described [14]. For A_2 binding, [^{3}H]5′-*N*-ethylcarboxamidoadenosine (NECA; sp. act. 20 Ci/mmol) was used as a ligand in striatal tissue using 50 nM cyclopentyladenosine (CPA) to block the A_1 component of [^{3}H]NECA binding [15, 16].

For saturation analysis, [^{3}H]CHA was run at ten to twelve concentrations over the range of 0.01 to 20 nM and [^{3}H]NECA over the range 0.1 to 80 nM. Non-specific binding was determined for both ligands in the presence of 10 μM 2-chloroadenosine (2-CADO). Incubations were performed with adenosine deaminase pretreated tissues for 120 or 300 min at 23° as indicated, and receptor–ligand complexes were isolated by vacuum filtration. Data were analyzed using either the Lundon-1 or RS-1 [17] curve fitting programs. Radioligands were obtained from DuPont New England Nuclear, Boston, MA, and 8-PT and PACPX from Research Biochemicals, Natick, MA.

To assess the effects of PACPX on the kinetics of [^{3}H]NECA binding in the presence of CPA, ligand association and dissociation curves were generated in the absence and presence of PACPX. A 5 μM concentration of 2-CADO was used to initiate the dissociation experiments.

In previous experiments [16], IC_{50} values of 10 and 400–

700 nM for PACPX and 1 and 2 μM for 8-PT were determined at rat A_1 and A_2 receptors respectively. These concentrations, therefore, were used to assess the nature of the interactions of these two 8-phenylxanthines with [^{3}H]CHA and [^{3}H]NECA binding sites in rat cortex and striatum.

Results

At the A_1 receptor, binding of [^{3}H]CHA was best fit to a single site (Fig. 1a) as previously determined [14]. K_d values of 0.6 to 1.3 nM were determined in three separate assay series (Table 1) with corresponding apparent B_{max} values of 806 to 1200 fmol/mg protein. At a final concentration of 10 nM, PACPX incubated together with [^{3}H]CHA for 2 hr had no significant effect on ligand affinity at the receptor (K_d) but reduced the apparent receptor density (B_{max}) to 31% of control values (Table 1a; Fig. 1a). Binding of [^{3}H]NECA to rat striatal membranes over the concentration range studied was best fit to a single site with a non-saturating component (Fig. 1b). Previous reports using more data points [15, 16], however, have led to the description of two or more sites. K_d values of 7.6 to 9.2 nM were obtained under the experimental conditions used in the present study (Table 1) with corresponding B_{max} values for the higher affinity site determined by the curve fitting program of 940–999 fmol/mg protein. PACPX at a final concentration of 700 nM again had no significant effect on the dissociation constant for the A_2-selective ligand (Table 1a; Fig. 1b) but reduced the number of higher affinity binding sites to 38% of control values. Comparative data for 8-PT (Table 1b) showed that at the A_1-site, this xanthine analog, at a concentration of 1 μM increased the apparent dissociation constant for [^{3}H]CHA over five times that seen in control membranes without affecting the apparent B_{max}. At the A_2 site in striatal membranes, 2 μM 8-PT had no significant effect on the dissociation constant for [^{3}H]NECA at the higher affinity site but reduced the apparent B_{max} to 44% of control values. Neither xanthine affected non-specific binding (data not shown).

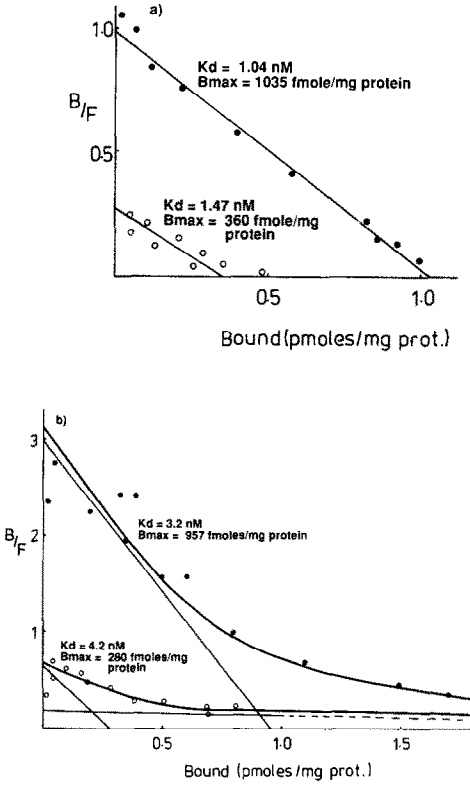

Fig. 1. (a) Transformed saturation data analysis of the effect of PACPX on [^{3}H]CHA (adenosine A_1) binding. Data are representative of a single experiment which is included in the data in Table 1. Results represent triplicate determinations. Data were fitted by the Lundon-1 curve fitting program [17] and best fit a single site model. Key: (●) control, and (○) in the presence of PACPX. (b) Transformed saturation data analysis of the effect of PACPX on [^{3}H]NECA (adenosine A_2) binding. See legend for panel (a) and Methods for details. Data best fit a single site model with a non-saturating component.

Because the physical characteristics of PACPX including its lipophilicity and pharmacokinetics [10] may raise criticism in regard to its use, the current assay conditions were extended to 8 hr to more readily allow for equilibrium binding conditions to be reached, and at the same time the effects of PACPX on the association/dissociation kinetics of [^{3}H]NECA binding were examined. Comparisons of total and non-specific binding at 2, 5 and 8 hr revealed no marked differences under control conditions (data not shown), and at 5 hr, 10 nM PACPX caused an increase in the K_d for [^{3}H]CHA (Table 1a) which, due to data variation, was not significant. A corresponding decrease in the apparent B_{max} to 71% of that seen in the absence of the xanthine was seen. At the A_2 receptor, a 5-hr incubation with 700 nM PACPX caused a 61% increase in the K_d of [^{3}H]NECA at the higher affinity site with a corresponding decrease in the apparent B_{max} for this site to 41% of control values (Table 1a).

In association experiments (Fig. 2; Table 2, a and b), inclusion of 700 nM PACPX had no effect on either the association or dissociation rate constants, although the total amount of specific binding was reduced by approximately 50%.

To further examine the effects of the xanthine on [^{3}H]NECA binding, PACPX was also added only at the time of dissociation (i.e. 700 nM PACPX + 5 μM 2-CADO were used to initiate dissociation) and, again, was found to have no effect on dissociation parameters or the half-life of dissociation (Table 2c).

Discussion

The present data showing significant PACPX-elicited decreases in apparent receptor density (B_{max}) at both A_1 and A_2 sites without changes in K_d values are consistent with this xanthine being a non-competitive adenosine antagonist in rat brain tissue. While the A_1 data are in agreement with the studies performed in guinea pig atria [13], they contrast with that originally reported in bovine brain [10]. Similarly, the A_2 data contrast with those obtained in *T. coli* where PACPX has been reported as a competitive antagonist [13]. It should be noted, however, that, as mentioned above, the binding of [^{3}H]NECA in the presence of 50 nM CPA to block the A_1 component attributable to this non-selective agonist is complex [15, 16], and thus the PACPX interaction described may be somewhat oversimplified. Nonetheless, these data do clearly show a decrease in the B_{max} for this adenosine ligand under conditions selective for the A_2 receptor. Increasing the incubation time to 5 hr to ensure that PACPX is in equilibrium with the receptor showed a markedly weaker non-competitive interaction at the A_1 receptor with a mixed competitive (increased K_d)/non-competitive (decreased B_{max}) effect at the higher affinity A_2 receptor. 8-PT, another 8-phenyl substituted xanthine, was a clear competitive antagonist at the A_1 receptor (increased K_d, no change in B_{max}) with a non-competitive action (decreased B_{max}) at the A_2 receptor.

Further evaluation of these phenomena by measuring the effects of PACPX on the kinetics of A_2 receptor binding showed no effect on either association or dissociation rate parameters, the latter irrespective of whether the xanthine

Table 1. Effects of 8-phenylxanthines on radioligand binding to adenosine A_1 and A_2 receptors in rat brain membranes

		K_d (nM)	B_{max} (fmol/mg protein)
(a) PACPX			
[^{3}H]CHA			
2 hr	Control	1.03 ± 0.24	1020 ± 270
	+10 nM PACPX	1.34 ± 0.35 (130)	320 ± 130* (31)
5 hr	Control	0.60 ± 0.04	1201 ± 139
	+10 nM PACPX	1.12 ± 0.35 (187)	857 ± 214* (71)
[^{3}H]NECA			
2 hr	Control	7.8 ± 1.8	945 ± 287
	+700 nM PACPX	5.4 ± 3.8 (69)	339 ± 165* (38)
5 hr	Control	7.6 ± 1.7	940 ± 165
	+700 nM PACPX	12.3 ± 1.7* (161)	387 ± 40* (41)
(b) 8-PT			
[^{3}H]CHA	Control	4.81 ± 0.62	806 ± 21
	+1 μM 8-PT	26.6 ± 8.2* (553)	808 ± 157 (100)
[^{3}H]NECA	Control	9.2 ± 2.7	999 ± 228
	+2 μM 8-PT	11.9 ± 4.9 (129)	442 ± 143* (44)

Results are the mean ± SD for 3–7 separate observations. Specific binding data were analyzed by the curve fitting program, Lundon-1 [17]. Numbers in parentheses indicate percent of control (= 100). Data for [^{3}H]CHA were best fit to a single site; those for [^{3}H]NECA in the presence of 50 nM CPA were fit to a single site with a non-saturating component. The PACPX concentrations used represent approximate IC_{50} values [16].

* $P < 0.02$; Student's two-tailed t-test [18].

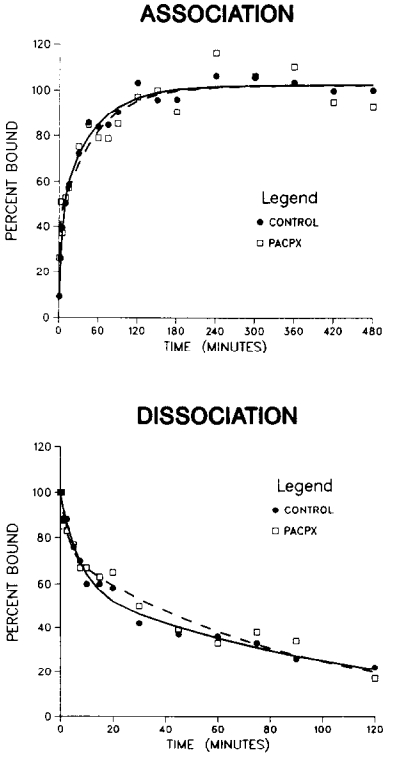

Fig. 2. Effects of 700 nM PACPX on the association and dissociation rate constants of [^{3}H]NECA binding. Association was initiated by the addition of 4 nM [^{3}H]NECA ± 700 nM PACPX to striatal membranes. Dissociation was initiated by the subsequent addition of 5 μM 2-CADO.

is present throughout the incubation period or is added at the time of dissociation. These data, like those obtained at the 2-hr period, are consistent with a non-competitive action and would indicate, again because of the complexity of using a non-selective ligand in the presence of a blocking agent, that the 61% increase in K_d at 5 hr in the presence of PACPX may be artifactual.

Distinct species and tissue differences exist for adenosine receptors in terms of both their kinetic and pharmacological properties [16, 20], and their latter differences are especially pronounced for the xanthines, occurring at both A_1 and A_2 receptors. The present data, and their discrepancy from data reported in bovine brain [10] and peripheral preparations [13], may reflect such species and tissue differences in the interaction of xanthines with adenosine receptors.

Studies with CPA and the corresponding cyclopentyltheophylline derivative have led to the suggestion that xanthines bind "backwards" as compared to the purine agonists [15]. Despite considerable effort, little is known about the conformation of either adenosine receptor subtype [9], a probable reflection of a paucity of chemical structures dissimilar from the purines and xanthines [13]. PACPX is a non-competitive antagonist at both A_1 and A_2 receptors with a competitive component occurring at the A_2 receptor with increased incubation periods. 8-PT is a competitive antagonist at A_1 sites and a non-competitive antagonist at A_2 sites. These competition data indicate a complexity of xanthine interactions with adenosine receptors and may suggest that "backwards" binding, a phenomenon that would indicate multiple recognition domains for thse antagonists, may explain the observed data, although this suggestion requires further substantiation. It is of interest that the novel, non-xanthine adenosine antagonist, CGS 15943A, a triazoloquinazoline, like 8-PT, is a competitive antagonist at A_1 receptors and has a non-competitive profile at A_2 receptors [21]. The recent availability of potent and selective xanthine congeners in radiolabeled form [12] may provide an additional tool to probe the interactions of

Table 2. Association and dissociation kinetics for [^{3}H]NECA binding to A_2 receptors in the absence and presence of 700 nM PACPX

Association conditions	K_{obs} (min^{-1})	K_{+1} (min^{-1} nM^{-1})	Dissociation conditions	$T_{\frac{1}{2}}$ (min)	K_{-1} (min^{-1})
(a) [^{3}H]NECA	0.053 ± 0.007	0.0045	5 μM 2-CADO	20.2 ± 2.7	0.035 ± 0.004
(b) [^{3}H]NECA + 700 nM PACPX	0.047 ± 0.003	0.0035	5 μM 2-CADO	21.3 ± 2.9	0.033 ± 0.004
(c) [^{3}H]NECA			5 μM 2-CADO + 700 nM PACPX	21.2, 27.8	0.033, 0.025

Results are mean ± SD for 3–5 separate observations except in condition c where the results from two separate observations are given. Conditions a and b represent association and dissociation parameters from experiments in which 4 nM [^{3}H]NECA was incubated, in the presence and absence of 700 nM PACPX, with striatal membranes in the presence of 50 nM CPA to label A_2 receptors. Condition c represents the dissociation parameters of 4 nM [^{3}H]NECA when dissociation was initiated by the addition of 5 μM 2-CADO and 700 nM PACPX. Kinetic parameters were determined as described by Burt [19] where K_{+1} = association rate, K_{-1} = dissociation rate. K_{obs} = observed association rate, and $T_{\frac{1}{2}}$ = dissociation half-life.

xanthines with adenosine receptors and gain further information as to the steric requirements for high affinity binding and for the reasons related to the pronounced interspecies differences previously reported [16, 20]. That xanthine interactions may not always be described in terms of a simple competitive interaction is suggested by the finding [22] that chronic caffeine treatment can attenuate *in vitro* responses to the xanthine, as measured by the ability of the xanthine to increase transmitter release, but not those associated with adenosine agonist effects.

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Biochemical Pharmacology, Vol. 36, No. 22, pp. 4027–4032, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

Alpha$_1$-adrenergic receptor photoaffinity labeling in intact cells

(*Received* 14 *November* 1986; *accepted* 18 *May* 1987)

The molecular characterization of the α_1-adrenergic receptor has been the focus of intense research for the past several years [1]. Photoaffinity labeling and purification of the receptor from rat hepatic membranes suggest that the major polypeptide representing the hormone binding subunit has a molecular weight in the range of 75,000–80,000 daltons [2–5]. The α_1-adrenergic receptor in non-hepatic tissues appears to have a similar subunit molecular weight based on photoaffinity labeling of the receptor in membrane preparations [6–8]. To date, however, there have been no