Biochemical Pharmacology, Vol. 45, No. 4, pp. 847–851, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

ADENOSINE RECEPTOR-BLOCKING XANTHINES AS INHIBITORS OF PHOSPHODIESTERASE ISOZYMES

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(*Received* 4 *May* 1992; *accepted* 1 *December* 1992)

Abstract—The pharmacological actions of methylxanthines such as theophylline and caffeine may be due to blockade of adenosine receptors and/or inhibition of phosphodiesterase (PDE) activities. In the last years, potent xanthines have been developed that display some selectivity for A_1 and A_2 adenosine receptors. Little is known about the PDE inhibitory potency of these xanthines. The aim of the present study was to determine the potencies of A_1 and A_2 receptor selective xanthines as inhibitors of several PDE isozymes, the PDE I–V subtypes. The IC_{50} values of 8-phenyl- and 8-cycloalkyl-1,3-dialkylxanthines for inhibiton of PDE isozymes from different sources are up to 10,000-fold higher than their antagonistic potencies at adenosine receptors. However, the A_1 receptor selective antagonists 1,3-diethyl-8-phenylxanthine and 1,3-dipropyl-8-cyclopentylxanthine are comparatively potent inhibitors of PDE IV activity with IC_{50} values in the 10 μM range and are, therefore, nearly as potent as the PDE IV selective inhibitor, rolipram. The A_2 receptor selective 1,3-dipropyl-7-methylxanthine is about 10–300-fold more potent as an adenosine receptor antagonist than as a PDE inhibitor. The results indicate that some of these novel xanthines can be used as selective adenosine receptor antagonists without interference due to inhibitory effects on PDEs.

Caffeine is the most widely used behavioral stimulant, and theophylline is extensively used for treatment of diseases such as bronchial asthma and obstructive airways disease. The molecular mechanisms by which theophylline, caffeine and related compounds exert their effects have not been definitively identified. The early proposal that xanthines act by inhibiting the activity of cyclic nucleotide phosphodiesterases (PDE‡) and consequently increasing cellular cyclic AMP content, has gradually fallen out of favor. The demise of the PDE hypothesis relates mainly to the relatively poor potency of theophylline as a PDE inhibitor versus its potency as a bronchodilator [1]. Major advances have recently been made in the knowledge of cyclic nucleotide phosphodiesterases with the discovery of different isozymes. Five families, each composed of several isoforms and having differing tissue distribution, have been described [2, 3]. Isozyme selective inhibitors have been identified [2, 3].

Since the discovery of adenosine receptors, it is felt that many pharmacological actions of theophylline and caffeine are primarily due to blockade of these receptors [4]. The relevance of this hypothesis may be examined using more potent and selective adenosine antagonists without PDE inhibitory capacity. Presently, two major classes of adenosine receptors are recognised, with the A_1 receptor class inhibitory to adenylate cyclase and the A_2 receptor class stimulatory to adenylate cyclase [5]. Recent structure–activity data suggest that subtypes of A_1 and A_2 receptors may exist [6]. Theophylline and caffeine are more potent as adenosine receptor antagonists than as inhibitors of PDE, but are virtually non-selective for A_1 and A_2 receptors [5]. In recent years, xanthine derivatives have been developed that show selectivity for the adenosine receptor subtypes. 1,3-Di-alkylxanthines with 8-aryl substituents display remarkable selectivity for A_1 receptors [7, 8]. Some caffeine analogs show selectivity for A_2 adenosine receptors [9, 10]. In contrast to the classical xanthines such as theophylline and caffeine, information about the PDE-inhibitory potency of these newly developed xanthines is very limited.

In the present study, we investigated the potency of some novel adenosine receptor-blocking xanthines as inhibitors of the different PDE isozymes. Part of these results have been presented at the SEP-SEPCR Meeting 1990 [11].

MATERIALS AND METHODS

Preparation of PDE isozymes. Separation of isozymes was carried out by a method similar to that described by Reeves *et al.* [12], as recently published [13]. All subsequent procedures were carried out at 4°. Four grams of rat cardiac ventricle tissue were minced with scissors in 10 vol. of buffer A containing 20 mM bis-Tris, 5 mM mercaptoethanol, 2 mM EDTA, 2 mM benzamidine and 50 mM sodium acetate, pH 6.5. For homogenization, 50 μM phenylmethyl-sulfonyl-fluoride, 10 μM pepstatin, 10 μM leupeptin, 0.1 mg/mL soybean trypsin inhibitor and 0.015% (v/v) genapol X-080 were added to buffer

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‡ Abbreviations PDE, phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine; 8-CPI, 8-cyclopentyltheophylline; 1,3-DPX, 1,3-diethyl-8-phenylxanthine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; PACPX, 8-(2-amino-4-chlorophenyl)-1,3-dipropylxanthine; XAC, 8-[4-(2-aminoethylaminocarboxylmethyloxy)phenyl]-1,3-dipropylxanthine, xanthine amine congener.

A. The suspension was homogenized 3 × 15 sec in an ultra turrax, followed by 3 × 15 sec in a Potter–Elvejhem homogenizer at 1200 rpm. After centrifugation for 25 min at 25,000 *g*, the supernatant was filtered through a millipore filter (0.45 μm). The filtrate (about 40 mL) was applied to a column (17 × 1.6 cm) containing Q-sepharose "fast flow" (Pharmacia) pre-equilibrated with buffer A. The flow rate was 80 mL/hr throughout the chromatography. The column was washed with 90 mL buffer and was then eluted with a linear gradient of 500 mL 0.05–1.0 M sodium acetate. Fractions of 7.5 mL were collected. Sodium acetate concentration in the eluted fractions was measured by osmometry. The fractions were tested for PDE activity, and the peaks containing the different isozymes were identified. Fractions containing preferentially one isozyme were pooled and 2 mL portions were frozen at −40°. From this PDE preparation, the PDE stimulated by cyclic GMP (PDE II) and the cyclic GMP-inhibited isozyme (PDE III) were used in the following experiments.

Canine tracheae were taken from euthanized dogs. Fat and adhering tissue were removed from four frozen tracheae and the remaining tissue was washed in buffer A. It was then sliced with scissors and homogenized in 3 vol. of buffer A containing protease inhibitors (as above) for 3 × 60 sec in a Waring blender. The homogenate was centrifuged 10 min at 1500 *g*, and the supernatant was again homogenized 3 × 15 sec in an ultra turrax. After centrifugation at 25,000 *g* for 25 min, the resulting supernatant was filtered, and chromatography was carried out as described above. For further experiments, the rolipram-inhibitable PDE isozyme (PDE IV) from canine trachea was used.

Calmodulin-activated PDE from bovine brain with substrate specificity for cyclic GMP (PDE I_B) was obtained from Dr C. Gietzen, University Ulm, F.R.G. [13]. The procedure used for isolation was described by Gietzen *et al.* [14]. The cyclic GMP-specific PDE V was isolated from human platelets [13].

Assay of phosphodiesterase. PDE activity was determined in a standard reaction mixture containing 40 mM Tris, pH 8.0, 5 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin and 0.5 μM cyclic nucleotide/^{3}H-labeled cyclic nucleotide (about 50,000 cpm) in a total volume of 200 μL [15]. The reaction was initiated by addition of the enzyme solution (5–20 μg) and was carried out at 37° for 10 min. The reaction was stopped by addition of 50 μL 0.2 M HCl, followed by cooling on ice for 10 min. *Crotalus atrox* snake venom (50 μL) (1 mg/mL in 0.2 M Tris, pH 8.0) was added. After 10 min incubation at 37°, the reaction was stopped on ice. An 0.2 mL aliquot of the assay volume was applied to small columns (Econo column, Biorad) containing 1 mL QAE-$A_2$5-sephadex, followed by 2 mL of 30 mM ammonium formate, pH 6.0. The effluent was collected directly in scintillation vials.

For cyclic GMP-selective PDE, 0.5 μM cyclic GMP/[^{3}H]cyclic GMP was used as substrate. For the assay of cyclic GMP-stimulated PDE isozyme hydrolysing cyclic AMP (PDE II), 1 μM cyclic GMP was added to the reaction mixture containing [^{3}H]-cyclic AMP as substrate. Calmodulin-dependent PDE was assayed in the presence of 10 nM calmodulin (M_r 16,700) and with 0.5 μM cyclic GMP/[^{3}H]cyclic GMP. Unstimulated activity (control value) was determined in the presence of 1 mM EGTA.

Drug addition and statistics. All drugs were diluted in 50% dimethyl sulfoxide/H_2O. They were added to the assays at 100-fold higher concentrations in the appropriate volume. The final dimethyl sulfoxide concentration of 0.5% did not influence PDE assays. IC_{50} values were calculated from concentration inhibition curves by non-linear curve fitting using the program GraphPad (Institute for Scientific Research).

Materials. ^{3}H-Labeled cyclic nucleotides were obtained from New England Nuclear (Dreieich, F.R.G.). Q-sepharose "fast flow" and QAE sephadex were obtained from Pharmacia (Freiburg, F.R.G.). 4 - (3' - Cyclopentyloxy - 4' - methoxyphenyl) - 2 - pyrrolidone (rolipram, Zk 62711) was a gift from Schering A.G. (Berlin, F.R.G.); 4,5-dihydro-6-(4-(1*H*-imidazol-1-yl)-2-thienyl)-5-methyl-3(2*H*)-pyridazinone (motapizone) from Nattermann (Köln, F.R.G.); 1,6-dihydro-2-methyl-6-oxo-(3,4-bipyridine)-5-carbonitril (milrinone) from Sterling Winthrop (Rensselaer, NY, U.S.A.); 2-*O*-propoxyphenyl-8-azapurine-6-one (zaprinast) from Rhone-Poulenc (Dagenham, U.K.); the xanthine derivatives were obtained from RBI (Research Biochemicals Incorporated, Natick, MA, U.S.A.). All other chemicals and supplies were from standard commercial sources.

RESULTS

As recently published [13], the following PDE isozymes were used to determine the inhibitory potency of xanthine derivatives:

(1) PDE I_B was isolated from bovine brain; this enzyme is calmodulin-sensitive and displays sensitivity for cyclic GMP (K_m 7 μM);

(2) PDE II was taken from rat cardiac ventricle and is stimulated by cyclic GMP (K_m cyclic AMP 14 μM);

(3) PDE III was obtained from rat cardiac ventricle (K_m cyclic AMP 0.7 μM, K_m cyclic GMP 0.5 μM) and is inhibited by cyclic GMP;

(4) PDE IV was isolated from canine trachea and shows specificity for cyclic AMP (K_m cyclic AMP 2.0 μM; K_m cyclic GMP 7.4 μM);

(5) PDE V was taken from human platelets and is cyclic GMP-specific (K_m cyclic GMP 3.4 μM; K_m cyclic AMP >20 μM).

For each compound tested, concentration–response curves were conducted to define IC_{50} values. The substrate concentration of 0.5 μM cyclic AMP and cyclic GMP was kept constant in all assays. The results are summarized in Table 1. For comparison, the adenosine receptor-blocking potencies of the xanthines in two well-defined A_1 and A_2 receptor models, respectively, are also provided in Table 1 [8, 16].

Theophylline inhibited PDE I–V activities with IC_{50} values in the range of 155–630 μM. In contrast, its affinity for A_1 and A_2 adenosine receptors is in the 10 μM range. Enprofylline (3-propylxanthine) has been shown to be an effective bronchodilator,

Table 1. Effects of xanthines on PDE isozymes and adenosine receptors

Xanthine	PDE Ib	PDE II	PDE III	PDE IV	PDE V	A_1	A_2
1,3-Dimethylxanthine (theophylline)	3.55 ± 0.36	3.57 ± 0.06	3.42 ± 0.30	3.81 ± 0.06	3.20 ± 0.27	4.89	4.86
3-Propylxanthine (enprofylline)	3.57 ± 0.32	3.58 ± 0.07	3.97 ± 0.32	4.02 ± 0.09	3.23 ± 0.13	4.40	3.89
IBMX	5.05 ± 0.26	5.20 ± 0.21	4.98 ± 0.12	5.04 ± 0.10	5.01 ± 0.06	5.21	5.38
1,3-Dimethyl-8-cyclopentylxanthine	3.52 ± 0.09	3.76 ± 0.05	<4	4.60 ± 0.18	4.16 ± 0.14	6.29	6.85
1,3-DPX	3.31 ± 0.31	3.75 ± 0.06	<4	5.69 ± 0.17	4.24 ± 0.16	7.22	6.68
1,3-Dipropyl-8-*p*-sulfophenylxanthine	3.20 ± 0.19	4.56 ± 0.03	<4	4.56 ± 0.07	4.70 ± 0.27	6.85	5.72
DPCPX	<3	3.07 ± 0.15	<4	5.03 ± 0.18	4.21 ± 0.22	8.92	6.86
PACPX	<3	3.56 ± 0.19	<4	<4	3.53 ± 0.30	8.17	6.33
XAC	4.08 ± 0.12	4.61 ± 0.21	<4	4.75 ± 0.22	3.90 ± 0.34	8.92	7.60
1,3,7-Trimethylxanthine (caffeine)	3.32 ± 0.15	3.15 ± 0.21	<4	<4	3.16 ± 0.19	4.36	4.37
1,3-Dipropyl-7-methylxanthine	3.09 ± 0.11	3.81 ± 0.08	<4	4.53 ± 0.13	4.19 ± 0.11	5.47	5.55
Motapizone	3.40 ± 0.36	3.21 ± 0.25	6.12 ± 0.18	4.27 ± 0.21	4.10 ± 0.28		
Milrinone	3.57 ± 0.32	3.58 ± 0.07	6.14 ± 0.23	4.74 ± 0.11	3.84 ± 0.13		
Rolipram (Zk 62,711)	3.09 ± 0.12	3.90 ± 0.40	3.80 ± 0.12	5.82 ± 0.31	3.20 ± 0.14		
Zaprinast	3.95 ± 0.33	3.81 ± 0.13	—	4.00 ± 0.26	5.82 ± 0.34		

PDE: pIC_{50} ± SD, N = 3; A_1/A_2: pK_i.
A_1: inhibition of radioligand binding to rat brain (from Ref. [16]).
A_2: antagonism of 5′-*N*-ethylcarboxamidoadenosine (NECA)-stimulated adenylate cyclase activity in human platelets (from Ref. [10]).

being about five times more potent than theophylline [17]. In contrast to the latter compound, enprofylline is a weaker adenosine receptor antagonist [17, 18]. The PDE inhibitory potency of enprofylline in the present experiments is similar to that of theophylline for PDE I, II and IV, and two or four times higher for PDE III and IV. The classical PDE inhibitor 3-isobutyl-1-methyl-xanthine (IBMX) had IC_{50} values in the 10 μM range and was, therefore, 10–100-fold more potent than theophylline. The potency of IBMX as PDE inhibitor is similar to its adenosine receptor blocking potency. None of these compounds showed considerable selectivity for PDE isozymes.

8-Cyclopentyltheophylline (8-CPT) displays an affinity to adenosine receptors in the submicromolar range and a remarkable A_1-selectivity in the rat [19]. This compound inhibited PDE IV from canine trachea with an IC_{50} of about 25 μM and PDE V with an IC_{50} of 69 μM, but was as weak an inhibitor of the other PDE isozymes as theophylline. With the exception of PDE IV, 8-CPT is about 100-fold more potent as an adenosine receptor antagonist than as a PDE inhibitor. 1,3-Diethyl-8-phenylxanthine (1,3-DPX) is a well-known adenosine receptor antagonist [20]. Its affinity for adenosine receptors is in the range of 60 nM (A_1) and 200 nM (A_2). As was the case with 8-CPT, DPX was a fairly potent inhibitor of PDE IV (IC_{50} 2.0 μM), but again was a weak inhibitor of the other PDE isozymes.

As shown by Daly *et al.* [21], polar substituents on the 8-aryl ring such as *p*-sulfo greatly increase water solubility, but also reduce potency and selectivity for A_1 adenosine receptors. The 8-*p*-sulfophenyl-1,3-dialkylxanthines have proven very useful adenosine antagonists in physiological experiments, since their lack of penetration into cells restricts their effects to extracellular sites, thereby eliminating the complication of inhibition of intracellular PDEs [22]. 1,3-Dipropyl-8-*p*-sulfophenylxanthine is a moderately potent adenosine receptor antagonist with some selectivity for A_1 receptors. Its inhibitory potency for PDE isozymes is at least 10-fold lower than its adenosine antagonistic potency.

1,3-Dipropyl-8-cyclopentylxanthine (DPCPX) is a very potent antagonist at adenosine receptors and shows a more than 100-fold selectivity for A_1 adenosine receptors [23]. The IC_{50} of this compound for inhibition of PDE activity (10 μM for PDE IV) is about 10,000-fold higher than the inhibition constant for antagonism at A_1 adenosine receptors (Table 1). 8-(2-Amino-4-chlorophenyl)-1,3-dipropylxanthine (PACPX) is another potent A_1 adenosine receptor antagonist, but has not proved very useful as a pharmacological tool [24]. Again, this xanthine is a very weak PDE inhibitor.

8-[4-(2-aminoethylaminocarboxylmethyloxy)-phenyl]-1,3-dipropylxanthine (xanthine amine congener, XAC) is a functionalized congener of xanthine drugs [25, 26] and in its tritiated form a useful antagonist radioligand for A_1 and A_2 adenosine receptors [27, 28]. XAC inhibited PDE isozymes with IC_{50} values between 17 and 126 μM. These values are several hundred-fold higher than the inhibition constants at adenosine receptors.

Caffeine (1,3,7-trimethylxanthine) is a non-selective adenosine receptor antagonist with IC_{50} values of about 40–50 μM. The IC_{50} values for inhibition of PDE isozymes are in the submillimolar range. 1,3-Dipropyl-7-methylxanthine shows a certain degree of selectivity for A_2 adenosine receptors in some tissues [7, 8]. With respect to its inhibitory potency for PDE isozymes, this caffeine analog is 10–300-fold more potent as an adenosine receptor antagonist.

For comparison, the IC_{50} values of some well-established PDE inhibitors are listed in Table 1. Motapizone and milrinone are new cardiotonic drugs which increase the force of contraction [29]. Both compounds are potent and selective inhibitors of

PDE III activities from rat cardiac ventricle. Rolipram has been implicated as a selective inhibitor of PDE IV activities [9]. The IC_{50} value of this compound for inhibition of PDE IV activities from canine trachea is about 100-fold lower than those for inhibition of other PDE isozymes. Zaprinast exerted a potent inhibitory effect on cyclic GMP specific PDE V (IC_{50} 1.5 μM), while having modest inhibitory effects on cyclic AMP hydrolysis by other PDE isozymes.

DISCUSSION

Antagonism of adenosine receptors has been proposed to be primarily responsible for many pharmacological actions of methylxanthines such as caffeine and theophylline. In the last few years, several xanthines have been developed that show a remarkable degree of selectivity for adenosine receptor subtypes [8–10, 23]. On the other hand, it has been claimed that at therapeutic plasma concentrations theophylline does not inhibit PDE activity appreciably and thus PDE inhibition cannot account for the clinical effects of theophylline [1, 17]. Nonetheless, theophylline does produce about 10–20% inhibition of PDE activity at concentrations that elicit bronchodilation *in vivo* [30]. Recently, there have been attempts to re-examine the proposal that PDE inhibition contributes to the therapeutic activity of xanthines [31]. However, there is little known of the activity of the novel adenosine receptor blocking xanthines as inhibitors of PDE isozyme activities.

In the present study, we used several well-defined PDE I–V isozymes to determine the PDE inhibitory potency of adenosine receptor blocking xanthines. All xanthine derivatives tested have been characterized extensively as adenosine antagonists in previous studies. The xanthines with 8-phenyl- and 8-cycloalkyl-substituents show selectivity for A_1 adenosine receptors and much less PDE inhibiting capacity. In the case of DPCPX, the IC_{50} for inhibition of PDE activity is roughly 10,000-fold higher than the inhibition constant at A_1 adenosine receptors. A remarkable adenosine antagonistic potency compared to PDE inhibitory potency was also found for the functionalized xanthine congener, XAC. Several of these xanthines, however, exhibited selectivity for PDE IV compared to the other PDE isozymes. Especially the xanthines DPX and DPCPX substituted with phenyl- or cyclopentyl groups in the 8-position exhibited IC_{50} values equal or near to rolipram (Table 1), which still appears to be the lead compound for selective PDE IV inhibition [2]. Similar low IC_{50} values for 8-aryl-substituted xanthines in the range of 10 μM for low K_m PDE from erythrocytes had been reported for 8-phenyltheophylline [32]. Analog data showed that 8-phenyltheophylline and certain 8-aryl analogs were weak inhibitors of PDEs in brain tissue [33, 34]. Similar results were reported for inhibition of PDEs from coronary artery [35]. All these data support the view that the PDE-inhibiting capacity is observed at 30–10,000-fold higher concentrations as compared to adenosine receptor antagonism.

The caffeine analog 1,3-dipropyl-7-methylxanthine had somewhat selective *in vivo* antagonistic effects on response due to activation of A_2 adenosine receptors [36]. Compared to its PDE inhibitory potency, this xanthine is about 10–300-fold more potent at adenosine receptors. However, as shown in rat brain, this compound inhibited Ca-independent PDEs with an IC_{50} of about 10 μM, which is similar to that for adenosine receptor antagonism [37].

In conclusion, the presented data supports the idea that some of the novel xanthines can be used as selective adenosine receptor antagonists without interference from inhibitory effects on PDEs. In order to analyse the mode of action involved in the pharmacological effects of theophylline and related compounds, these compounds may be particularly useful.

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