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Xanthine derivatives as antagonists at A_1 and A_2 adenosine receptors

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Summary. A variety of alkylxanthines has been comparatively examined as antagonists of A_1 adenosine receptors in rat fat cells, rat and bovine cerebral cortex and of A_2 adenosine receptors in human platelets. With few exceptions all xanthine derivatives with 7-position substituents such as diprophylline, proxyfylline, pentoxifylline and etofylline were less potent antagonists than xanthine itself which had K_i-values of 170 μmol/l (A_1) and 93 μmol/l (A_2). Theophylline, caffeine and 3-isobutyl-1-methylxanthine were more potent than xanthine but nearly equipotent antagonists at both receptor subtypes. 8-Phenyl substituents considerably increased the antagonist potency at A_1 and A_2 receptors. 1,3-Diethyl-8-phenylxanthine was the most potent A_2 antagonist (K_i 0.2 μmol/l) in human platelets. At A_1 receptors 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX) was the most potent antagonist in all three tissues with K_i-values from 0.3 to 8.6 nmol/l. Several 8-phenylxanthine derivatives were remarkably selective antagonists at A_1 receptors. 8-Phenyltheophylline was approximately 700 times more potent as antagonist at A_1 receptors (bovine brain) than at A_2 receptors (human platelets), and PACPX was even 1,600 times more potent as A_1 adenosine receptor antagonist. These compounds offer a possibility for a subtype-selective blockade of adenosine receptors.

Key words: Adenosine receptors – Adenylate cyclase – Theophylline – Alkylxanthines

Introduction

The discovery of adenosine receptors resulted in the proposal of a new mechanism of action for theophylline and related alkylxanthines. For many years these compounds were believed to act via an inhibition of cyclic nucleotide phosphodiesterases, but now it has been recognized that theophylline and other xanthine derivatives have additional effects on adenosine receptors and are more potent as adenosine antagonists than as inhibitors of phosphodiesterases (for review see Fredholm 1980; Daly 1982).

Based on adenylate cyclase studies and radioligand binding data adenosine receptors are subdivided into A_1 (R_i) receptors, which mediate an inhibition of adenylate cyclase activity, and A_2 (R_a) receptors, which stimulate adenylate cyclase activity (van Calker et al. 1978; Londos et al. 1980). Xanthine derivatives are antagonists at both receptor subtypes. The A_1 adenosine receptor-mediated inhibition of adenylate cyclase is competitively antagonized by theophylline, caffeine and 3-isobutyl-1-methylxanthine in isolated rat fat cells and rat brain (Londos et al. 1978; Cooper et al. 1980). These xanthine derivatives also compete for the binding of radioligand agonists and antagonists in membrane preparations of bovine brain, rat brain and rat fat cells (Bruns et al. 1980; Schwabe and Trost 1980; Williams and Risley 1980; Trost and Schwabe 1981). Adenosine receptor binding studies have been employed to design highly potent adenosine receptor antagonists (Bruns et al. 1983). Furthermore, it has been demonstrated that A_2 receptor-mediated activation of adenylate cyclase is competitively blocked by theophylline and caffeine in human platelets and mouse neuroblastoma cells (Haslam and Rosson 1975; Blume et al. 1975). In addition, a great number of alkylxanthines and related compounds reverse the adenosine-induced stimulation of cyclic AMP formation in human fibroblasts (Bruns 1981).

Recently, xanthine derivatives and mesoionic xanthine analogues have been comparatively investigated in A_1 and A_2 adenosine receptor systems, but markedly selective antagonists for one of both receptor subtypes were not obtained (Fredholm and Persson 1982; Glennon et al. 1984). We have studied a number of recently developed xanthine derivatives in subtype-selective cell systems and report here that several 8-phenylxanthine derivatives are selective antagonists at the inhibitory A_1 adenosine receptors. Part of the results have been presented at the Spring meeting of the Deutsche Pharmakologische Gesellschaft 1985 (Schwabe 1985).

Methods and materials

Preparation of human platelet membranes. Platelet membranes were prepared as described by Tsai and Lefkowitz (1979). The protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Preparation of rat fat cell membranes. Isolated rat fat cells were prepared according to the method of Rodbell (1964). Plasma membranes were prepared as described by McKeel and Jarett (1970). After the last centrifugation step the membranes were resuspended in 50 mmol/l Tris-HCl, pH 7.4, frozen in liquid nitrogen and stored at −80° C.

Preparation of brain membranes. Membranes from rat and bovine cerebral cortex were prepared according to the

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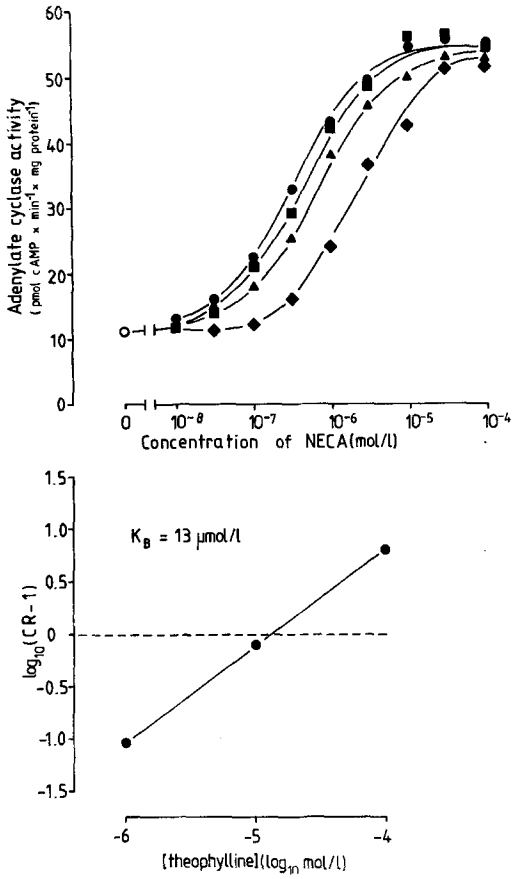

Fig. 1. Stimulation of adenylate cyclase activity of human platelet membranes by NECA in the absence (●——●) and presence of theophylline (1 µmol/l ■——■, 10 µmol/l ▲——▲, 100 µmol/l ◆——◆). Adenylate cyclase activity was determined at 37° C for 10 min. *Lower panel*: Schild plot of the same data with the concentration ratio (CR) of the EC_{50}-values for NECA in presence and absence of theophylline versus the theophylline concentration (slope 0.90, $r = 0.998$). The EC_{50}-values for NECA were 0.33 µmol/l in the absence and 0.36, 0.59 and 2.4 µmol/l in the presence of 1, 10 and 100 µmol/l theophylline, respectively. Mean values of three experiments done in duplicate are shown

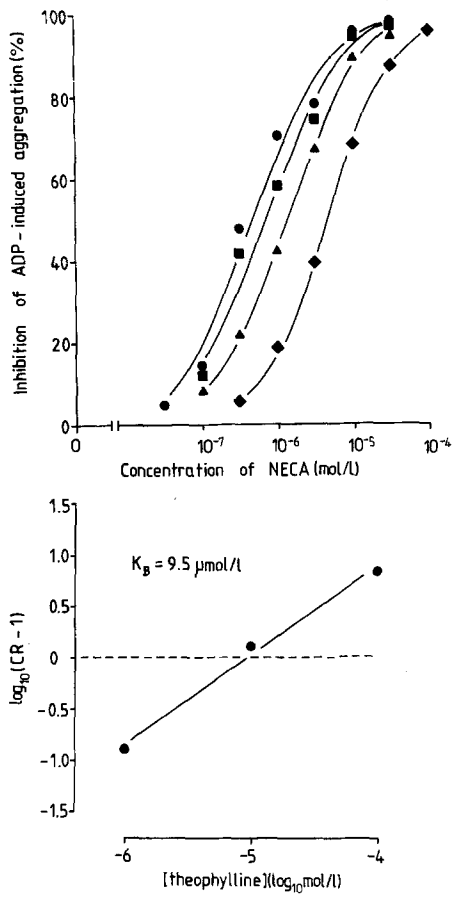

Fig. 2. Inhibition of ADP-induced aggregation of human platelet by NECA in the absence (●——●) and presence of theophylline (1 µmol/l ■——■, 10 µmol/l ▲——▲, 100 µmol/l ◆——◆). Platelet-rich plasma was incubated with theophylline and NECA for 3 min 37° C before the addition of 5 µmol/l ADP. *Lower panel*: Schild plot of the same data with the concentration ratio (CR) of the IC_{50}-values for NECA in presence and absence of theophylline versus the theophylline concentration (slope 0.92, $r = 0.999$). The IC_{50}-values for NECA were 0.54 µmol/l in the absence and 0.61, 1.2 and 4.5 µmol/l in the presence of 1, 10 and 100 µmol/l theophylline, respectively. Mean values of three experiments are shown

method described by Whittaker (1969). Male Sprague-Dawley rats (150 – 250 g) were killed by cervical dislocation, and the forebrains were quickley removed and immediately placed in ice-cold 0.32 mol/l sucrose. Bovine brain was obtained from a local slaugtherhouse and was placed in ice-cold 0.32 mol/l sucrose within 30 min of slaughter. Cerebral cortex was dissected and the tissue was homogenized at 0° C in a glass-Teflon homogenizer in 10 volumes of 0.32 mol/l sucrose (clearing 0.2 mm, at 500 rpm for 30 s). The homogenate was centrifuged at $1{,}000 \times g$ for 10 min and the supernatant again centrifuged at $100{,}000 \times g$ for 30 min to give the combined P_2 and P_3 fractions. The pellets were resuspended in 10 ml of water, centrifuged at $100{,}000 \times g$ for 30 min and washed once with 50 mmol/l Tris-HCl, pH 7.4, using the same centrifugation step. Finally, the membranes were resuspended in 50 mmol/l Tris-HCl, pH 7.4, at a concentration of 6 – 10 mg of protein per ml, frozen in liquid nitrogen, and stored at −80° C. Protein was measured according to the method of Lowry et al. (1951).

Platelet aggregation. Human platelet-rich plasma was separated from citrated venous blood by centrifugation at $260 \times g$ for 20 min at room temperature. For measurement of platelet aggregation, incubation mixtures consisting of 210 µl platelet-rich plasma (platelet count about 300,000/µl) and additions of 5′-N-ethylcarboxamidoadenosine (NECA) and xanthine derivatives in 0.154 mmol/l NaCl were stirred in a final volume of 250 µl at 37° C in an aggregometer (Elvi 840, Elvi Milano, Italy). After preincubation for 3 min platelet aggregation was initiated by addition of 5 µmol/l ADP and recorded for 5 min.

Adenylate cyclase assay. Adenylate cyclase activity of rat fat cell membranes and human platelet membranes was determined as described by Jakobs et al. (1976). The assay medium contained 1 mmol/l $MgCl_2$, 1 µmol/l GTP, 0.1 mmol/l cyclic AMP, 0.5 mmol/l Ro 20-1724, 0.2 mmol/l ethyleneglycol-bis-(β-aminoethylether)-N,N′-tetraacetic acid (EGTA), 5 mmol creatine phosphate as Tris-salt, 0.4 mg/ml

Table 1. Effects of xanthine derivatives on human platelets

Xanthine derivative	Adenylate cyclase K_B, μmol/l	Aggregation K_B, μmol/l	[^{3}H]NECA binding K_i, μmol/l
7-(2,3-Dihydroxypropyl)-theophylline (Diprophylline)	4,600	no antag.	7,500
Hypoxanthine	3,800	no antag.	> 10,000
1-(2-Hydroxypropyl)-theobromine (Protheobromine)	1,800	no antag.	8,400
7-(2-Hydroxypropyl)-theophylline (Proxyfylline)	850	no antag.	7,300
3,7-Dimethylxanthine (Theobromine)	590	no antag.	> 10,800
7-(2-Diethylaminoethyl)-theophylline (Etamiphylline)	520	no antag.	10,000
1-(5-Oxo-hexyl)theobromine (Pentoxifylline)	510	no antag.	2,900
7-(2-Hydroxyethyl)-theophylline (Etofylline)	180	no antag.	7,600
3-Propylxanthine (Enprofylline)	130	no antag.	160
Xanthine	93	no antag.	1,800
1,3,7-Trimethylxanthine (Caffeine)	43	21	5,000
1,7-Dimethylxanthine (Paraxanthine)	14	63	2,500
1,3-Dimethylxanthine (Theophylline)	13	9.5	380
3-Isobutyl-1-methylxanthine (IBMX)	7.2	4.2	47
8-Phenyltheophylline	4.1	3.4	> 100
8-(4-Acetoxyphenyl)theophylline	1.8	2.3	> 100
8-(4-Hydroxyphenyl)theophylline	0.51	0.63	> 100
1,3-Dipropyl-8-(4-hydroxyphenyl)xanthine	0.51	0.60	> 100
1,3-Dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX)	0.47	0.44	> 100
1,3-Diethyl-8-phenylxanthine (DPX)	0.21	0.27	> 100

For the inhibition of NECA-induced changes of adenylate cyclase and platelet aggregation K_B-values were calculated from Schild plots using 3 concentrations of the antagonist as shown in Figs. 1 and 2 for theophylline. Values for slope and correlation coefficients were similar to those shown for theophylline. Mean values of duplicate determinations are shown. [^{3}H]NECA (10 nmol/l) binding was 540 ± 40 fmol/mg protein (means ± SEM), and the K_i-values were calculated from IC_{50}-values according to Cheng and Prusoff (1973) using the high affinity K_D of [^{3}H]NECA (0.16 μmol/l) taken from Hüttemann et al. (1984). Each K_i-value is the geometric mean of 3 experiments using 7–9 concentrations of the competitor

creatine kinase, 2 mg/ml bovine serum albumin and 50 mmol/l Tris-HCl, pH 7.4, in a total volume of 100 μl. In the case of rat fat cell membranes, adenosine deaminase (1 μg/ml) and 150 mmol/l NaCl were included in the assay (Londos et al. 1978, 1980). The concentration of [α-^{32}P]ATP was 0.1 mmol/l (0.75 μCi/tube) for rat fat cells and 0.1 mmol/l (0.5 μCi/tube) for human platelets. Incubations were initiated by the addition of fat cell membranes (approximately 15 μg protein/tube) or human platelet membranes (approximately 20–30 μg protein/tube) to reaction mixtures that had been preincubated for 5 min at 37° C and were conducted for 10 min at 37° C. Reactions were stopped by the addition of 0.4 ml of 125 mmol/l zinc acetate. Under these conditions cyclic AMP formation was linear as a function of time for at least 12 min. Cyclic AMP was purified by coprecipitation of other 5′-nucleotides with $ZnCO_3$ and by chromatography on neutral alumina. $ZnCO_3$ was formed by the addition of 0.5 ml of 144 mmol/l Na_2CO_3. After centrifugation for 5 min at 12,000 × *g*, 0.8 ml of the supernatant was applied to neutral alumina columns (approximately 1.2 g) equilibrated with 0.1 mol/l Tris-HCl, pH 7.4, and was followed by two 2 ml portions of the same buffer. The effluent was collected, and cyclic [^{32}P]AMP was determined by measuring Cerenkov radiation in a liquid scintillation counter. Cyclic AMP recovery was more than 98% as determined in recovery experiments with tritiated cyclic AMP.

Radioligand binding. The binding of 5′-N-ethylcarboxamido[^{3}H]adenosine ([^{3}H]NECA) to human platelet membranes was performed as described by Hüttemann et al. (1984). The standard assay contained 50 mmol/l Tris-HCl, pH 7.4, 10 nmol/l [^{3}H]NECA and 60–80 μg platelet membrane protein in a final volume of 250 μl. The incubation was carried out for 60 min at 0° C and was terminated by rapid filtration of a 200 μl aliquot through a Whatman GF/B glass fiber filter (25 mm diameter). The filter was immediately washed with two 3 ml portions of the ice-cold incubation buffer. After addition of 4 ml Triton-based scintillation fluid (Quickszint 402, Zinsser Analytic GmbH, Frankfurt, FRG) radioactivity on the filters was determined in a Tracor Analytic liquid scintillation counter (Mark III) with a counting efficiency of 55%. Specific binding was defined as the amount of the radioligand bound in the absence of competing ligand minus the amount bound in the presence of 0.3 mmol/l NECA.

The measurement of N^6-R-(−)-phenylisopropyl[^{3}H]-adenosine ([^{3}H]PIA) binding to brain membranes was carried out as described previously (Lohse et al. 1984). Binding of [^{3}H]PIA to membranes (100 μg of protein per tube) was carried out in 50 mmol/l Tris-HCl buffer, pH 7.4, in a total volume of 250 μl in the presence of 0.08 U/ml of adenosine deaminase to remove endogenous adenosine; [^{3}H]PIA was present in a final concentration of 1 nmol/l. Other substances were added as indicated. Incubation was carried out at 37° C for 90 min for rat brain membranes and 120 min for bovine brain membranes and was terminated by rapid filtration of an aliquot of 200 μl through a Whatman GF/B glass fiber filter. The subsequent washing and counting procedures were as described above. Nonspecific binding was determined in the presence of 10 μmol/l N^6-R-(−)-phenylisopropyladenosine (R-PIA).

Materials. 5′-N-Ethylcarboxamido[^{3}H]adenosine ([^{3}H]-NECA; 27 Ci/mmol) was purchased from Amersham Buchler (Braunschweig, FRG). N^6-R-(−)Phenylisopropyl[^{3}H]-adenosine ([^{3}H]PIA; 49.9 Ci/mmol) and carrier-free ^{32}P

Table 2. Effect of xanthine derivatives on NECA-induced inhibition of platelet aggregation

Xanthine derivative	Inhibition of platelet aggregation (%)	Increase in potency of NECA	
		IC_{50}, μmol/l	left shift
Control		0.58	
Diprophylline, 1 mmol/l	2	0.18	3.2
Hypoxanthine, 1 mmol/l	5	0.15	3.9
Protheobromine, 1 mmol/l	12	0.15	3.9
Proxyfylline, 1 mmol/l	9	0.30	1.9
Theobromine, 1 mmol/l	15	0.08	7.3
Etamiphylline, 1 mmol/l	4	0.26	2.2
Pentoxifylline, 1 mmol/l	7	0.16	3.7
Etofylline, 1 mmol/l	7	0.29	2.0
Xanthine, 1 mmol/l	3	0.48	1.2
Enprofylline, 0.1 mmol/l	7	0.10	5.8

Platelet-rich plasma was preincubated with the xanthine derivatives and NECA for 3 min at 37° C and aggregation was initiated by addition of 5 μmol/l ADP. IC_{50}-values for NECA were determined as described in Fig. 2. The left shift of the concentration-response curve for NECA was obtained from the ratio of the IC_{50}-values in the absence (control) and the presence of the xanthine derivatives. Values are the means of duplicate determinations

were purchased from New England Nuclear (Dreieich, FRG). [α-^{32}P]ATP was synthetized according to the method of Walseth and Johnson (1979). Other compounds used in this study were: bovine serum albumin, fraction V powder (Serva Feinbiochemica, Heidelberg, FRG); crude bacterial collagenase (Worthington Biochemical Corporation, Freehold, NJ, USA); adenosine, cyclic AMP, ATP, adenosine deaminase from calf intestine (200 U/mg), GTP, creatine kinase (Boehringer Mannheim, FRG); neutral alumina WN-3, creatine phosphate as Tris-salt, hypoxanthine, xanthine, 1,7-dimethylxanthine, theophylline, 3-isobutyl-1-methylxanthine (Sigma Chemie, Taufkirchen, FRG); caffeine (Merck, Darmstadt, FRG); 1,3-diethyl-8-phenylxanthine (New England Nuclear, Dreieich, FRG); 8-phenyltheophylline (Calbiochem, Frankfurt, FRG); etamiphylline, protheobromine (Degussa Pharma Gruppe, Asta-Werke, Bielefeld, FRG); enprofylline (Draco, Lund, Sweden); theobromine (Gödecke, Freiburg, FRG), pentoxifylline (Albert-Roussel Pharma GmbH, Wiesbaden, FRG); etofylline (Thiemann, Waltrop, FRG); diprophylline (Sagitta-Werk, Feldkirchen-Westerham, FRG). 5′-N-Ethylcarboxamidoadenosine (NECA) was kindly provided by Prof. Klemm (Byk Gulden Lomberg Chemische Fabrik, Konstanz, FRG); N^6-R(−)-phenylisopropyladenosine was a gift of Dr. K. Stegmeier (Boehringer Mannheim, Mannheim, FRG); 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) was provided by Dr. W. E. Scott (Hoffmann La Roche, Nutley, NJ, USA). 1,3-Dipropyl-8-(4-hydroxyphenyl)xanthine was kindly donated by Dr. John Daly (Laboratory of Bioorganic Chemistry, NIH, Bethesda, Maryland, USA), 8-(4-acetoxyphenyl)theophylline and 8-(4-hydroxyphenyl)theophylline by Prof. H. C. Erbler (Sanol-Schwarz, Monheim, FRG) and 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine by Prof. H. Osswald (Gödecke, Freiburg, FRG). All other chemicals were of analytical grade or best commercially available.

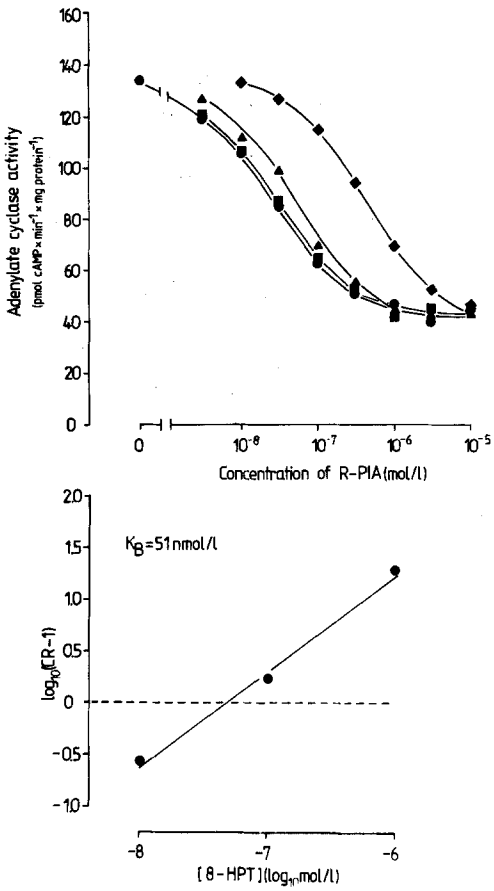

Fig. 3. Inhibition of adenylate cyclase activity of rat fat cell membranes by R-PIA in the absence (●——●) and presence of 8-(4-hydroxyphenyl)theophylline (10 nmol/l ■——■, 100 nmol/l ▲——▲, 1,000 nmol/l ◆——◆). Adenylate cyclase activity was determined at 37° C for 10 min. *Lower panel*: Schild plot of the same data with the concentration ratio (CR) of the IC_{50}-values for R-PIA in the presence and the absence of 8-(4-hydroxyphenyl)theophylline versus the concentration of the antagonist (slope 0.94, $r = 0.998$). The IC_{50}-values for R-PIA were 19 nmol/l in the absence and 24, 52 and 380 nmol/l in the presence of 10, 100 and 1,000 nmol/l 8-(4-hydroxyphenyl)theophylline, respectively. Mean values of triplicate determinations are shown

Results

Adenylate cyclase activity of human platelet membranes

5′-N-Ethylcarboxamidoadenosine (NECA) was used to evaluate the A_2 adenosine receptor antagonism by xanthine derivatives in human platelets because this adenosine analogue has been shown to be a potent A_2 receptor agonist for stimulation of adenylate cyclase and inhibition of platelet aggregation (Cusack and Hourani 1981).

Figure 1 shows the concentration-response curve for the effect of NECA on human platelet adenylate cyclase. NECA stimulated adenylate cyclase activity approximately 6-fold over basal values with an EC_{50} of 0.33 μmol/l (95% confidence limits 0.22 − 0.48). Theophylline alone did not affect basal enzyme activity, but produced a parallel right-shift of the concentration-response curve without a change of slope or maximal effect. The EC_{50} of NECA in the presence of 10 and 100 μmol/l theophylline was 0.59 and 2.4 μmol/l, respectively. The dissociation constant K_B for theophylline calculated from the Schild plot of these data was 13 μmol/l

Table 3. Effects of xanthine derivatives on A_1 adenosine receptor systems

Xanthine derivative	Rat fat cell adenylate cyclase K_B, nmol/l	Rat brain [^{3}H]PIA binding K_i, nmol/l	Bovine brain [^{3}H]PIA binding K_i, nmol/l
1. Diprophylline	810,000	284,000	362,000
2. Protheobromine	770,000	411,000	745,000
3. Etamiphylline	690,000		514,000
4. Pentoxifylline	330,000	228,000	78,000
5. Proxyfylline	280,000	99,000	82,000
6. Theobromine	240,000	131,000	92,000
7. Etofylline	195,000	92,000	236,000
8. Xanthine	170,000	298,000	190,000
9. Caffeine	46,000	29,000	27,000
10. 1,7-Dimethylxanthine	33,000	33,000	31,000
11. Enprofylline	32,000	40,000	43,000
12. Theophylline	9,500	11,000	5,300
13. 3-Isobutyl-1-methylxanthine (IBMX)	7,600	3,200	2,500
14. 8-(4-Acetoxyphenyl)theophylline	310	238	23
15. 1,3-Diethyl-8-phenylxanthine (DPX)	170	61	4.1
16. 8-Phenyltheophylline	160	115	5.9
17. 1,3-Dipropyl-8-(4-hydroxyphenyl)xanthine	53	45	3.2
18. 8-(4-Hydroxyphenyl)theophylline	51	36	3.4
19. 1,3-Dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX)	8.6	6.8	0.3

Adenylate cyclase activity of rat fat cell membranes was determined in the presence of increasing concentration of R-PIA (1 nmol/l to 10 μmol/l, IC_{50} 19 nM) and the K_B-values for the reversal of R-PIA-induced inhibition by xanthine derivatives calculated from Schild plots are shown. The K_B-values are the means of duplicate determinations. K_i-values for the inhibition of [^{3}H]PIA binding to rat and bovine cerebral cortex membranes were calculated from IC_{50}-values of competition curves after transformation according to Cheng and Prusoff (1973). K_D-values for [^{3}H]PIA used for the calculation were determined from saturation experiments and were 1.4 nmol/l for rat brain and 0.13 nmol/l for bovine brain. The K_i-values are the means of 2 to 3 experiments done in duplicate with 6–10 different concentrations of the competitors

(95% confidence limits 10.7–16.1) as shown in the lower panel of Fig. 1. The slope of the line is near unity confirming a competitive antagonism between NECA and theophylline.

Platelet aggregation

We have further studied the effects of xanthine derivatives on the NECA-induced inhibition of platelet aggregation (Fig. 2). NECA inhibited the ADP-induced aggregation of human platelets with an IC_{50} of 0.54 μmol/l (95% confidence limits 0.41–0.70). At concentrations between 1 and 100 μmol/l theophylline alone had no effect on ADP-induced aggregation. The concentration-response curve of NECA was shifted to the right by theophylline resulting in an increase of the IC_{50} for NECA to 1.2 and 4.5 μmol/l at 10 and 100 μmol/l theophylline, respectively. The K_B for theophylline calculated from the Schild plot of these data was 9.5 μmol/l (95% confidence limits 4.8–19.1).

Both methods were used to evaluate the A_2 receptor antagonism of several xanthine derivatives. The K_B-values of antagonists for the inhibition of NECA effects on adenylate cyclase and platelet aggregation are listed in Table 1 and were calculated from Schild plots as described for theophylline. In addition, the K_i-values for inhibition of [^{3}H]NECA binding to human platelet membranes are shown. Xanthine itself had adenosine antagonistic properties as inhibitor of NECA-stimulated adenylate cyclase and the K_B-value was 93 μmol/l. Nearly all xanthine derivatives with 7-position substituents were less potent than xanthine. Among these compounds are several drugs which are used therapeutically as bronchodilators and vasodilators, such as diprophylline, proxyfylline, pentoxifylline and etofylline. Also enprofylline (3-propylxanthine) was less potent than xanthine.

All other compounds listed in Table 1 were more potent A_2 adenosine antagonists compared to xanthine. Thus, caffeine (1,3,7-trimethylxanthine) was 2 times more potent and theophylline (1,3-dimethylxanthine) and paraxanthine (1,7-dimethylxanthine) were 7 times more potent than xanthine, whereas theobromine (3,7-dimethylxanthine) was 6 times less potent than xanthine. If the two latter dimethyl derivatives are compared directly, there is a 45-fold difference in potency between the 1,7-dimethyl and the 3,7-dimethyl substituents. The 3-isobutyl substituent produced a twofold increase in antagonist activity compared to theophylline. A more pronounced increase in potency was seen with most of the 8-phenyl substituents. 8-Phenyltheophylline was 3 times more potent than theophylline. A 4-hydroxy group on the 8-phenyl ring had an additional effect on potency so that 8-(4-hydroxyphenyl)theophylline was 40 times more potent than theophylline. All other changes at the 1,3-positions by diethyl or dipropyl substituents as well as additional substituents on the 8-phenyl ring did not produce a marked additional increase in antagonist potency at A_2 adenosine receptors.

Somewhat different results were obtained in the study of platelet aggregation. Whereas all compounds which were more potent than xanthine in the cyclase studies exhibited a pharmacological profile nearly identical to that obtained in the adenylate cyclase studies, xanthine itself and all less potent derivatives did not antagonize the effect of NECA on platelet aggregation but potentiated the inhibitory action of NECA. As shown in Table 2, these compounds alone caused only marginal inhibition of aggregation between 2%

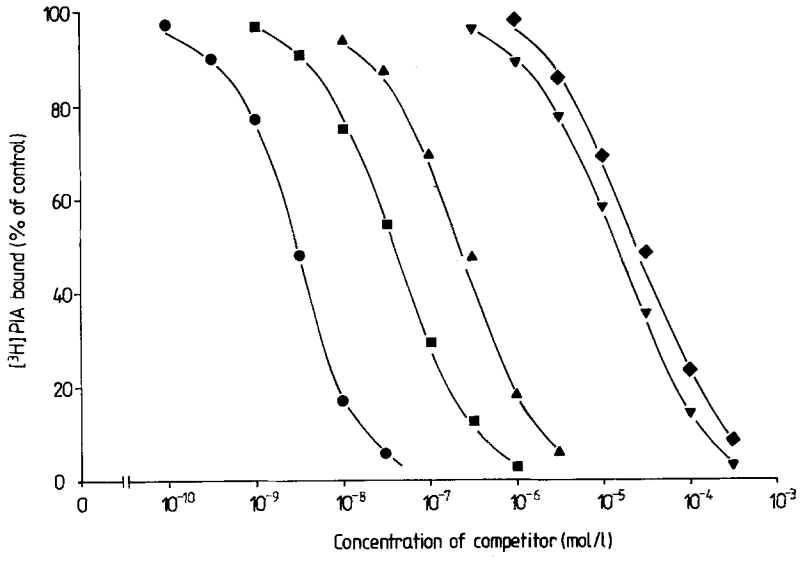

Fig. 4
Competition of xanthine derivatives for [^{3}H]PIA binding to bovine cerebral cortex membranes. Binding of 1 nmol/l [^{3}H]PIA was measured for 120 min at 37° C. Slope factors were 1.10 for PACPX (●——●), 0.91 for 8-phenyltheophylline (■——■), 0.97 for 8-(4-acetoxyphenyl)theophylline (▲——▲), 0.85 for 3-isobutyl-1-methylxanthine (▼——▼) and 0.86 for theophylline (◆——◆). Mean values of three experiments done in duplicate are shown

Table 4. Relative potencies of xanthine derivatives at A_1 and A_2 adenosine receptors

Xanthine derivative	A_1 receptor selectivity		
	Rat fat cell (ratio I)	Rat brain (ratio II)	Bovine brain (ratio III)
Diprophylline	5.7	16.2	12.7
Protheobromine	2.3	4.4	2.4
Etamiphylline	0.8		1.0
Pentoxifylline	1.5	2.2	6.5
Proxyfylline	3.0	8.6	10.4
Theobromine	2.5	4.5	6.4
Etofylline	0.9	2.0	0.8
Xanthine	0.5	0.3	0.5
Caffeine	0.9	1.5	1.6
1,7-Dimethylxanthine	0.4	0.4	0.5
Enprofylline	4.0	3.3	3.0
Theophylline	1.4	1.2	2.5
3-Isobutyl-1-methylxanthine (IBMX)	0.9	2.3	2.9
8-(4-Acetoxyphenyl)theophylline	5.8	7.6	78
1,3-Diethyl-8-phenylxanthine (DPX)	1.2	3.4	51
8-Phenyltheophylline	26	36	700
1,3-Dipropyl-8-(4-hydroxyphenyl)xanthine	10	11	160
8-(4-Hydroxyphenyl)theophylline	10	14	150
1,3-Dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX)	55	69	1,600

The ratios express the A_1 receptor selectivity and were obtained by dividing the K_B-values for adenylate cyclase of human platelets by the K_B-values for adenylate cyclase of rat fat cells (ratio I), K_i-values of [^{3}H]PIA binding in rat brain (ratio II) and the K_i-values of [^{3}H]PIA binding in bovine brain listed in Tables 1 and 3

and 15%. However, they induced a left shift of the concentration-response curve for NECA and thereby produced a 2- to 7-fold decrease of the IC_{50}-values for NECA.

Inhibition of [^{3}H]NECA binding to human platelet membranes

Furthermore we have attempted to determine the A_2 receptor affinities of xanthine derivatives by measuring the inhibition of [^{3}N]NECA binding to human platelet membranes. As shown in Table 1, most of the K_i-values for [^{3}N]NECA binding are 10 to 100 times higher than the corresponding K_B-values from adenylate cyclase and platelet aggregation. The potent 8-phenylxanthine derivatives did not at all compete with [^{3}H]NECA binding at concentrations up to 100 μmol/l. Therefore, [^{3}H]NECA binding in platelet membranes is not a suitable model to evaluate structure-activity relationships of A_2 receptor antagonists.

Inhibition of adenylate cyclase of rat fat cell membranes

N^6-R-(−)-Phenylisopropyladenosine (R-PIA) was used to determine the potency of xanthine derivatives at A_1 receptors of isolated rat fat cells. R-PIA has been characterized

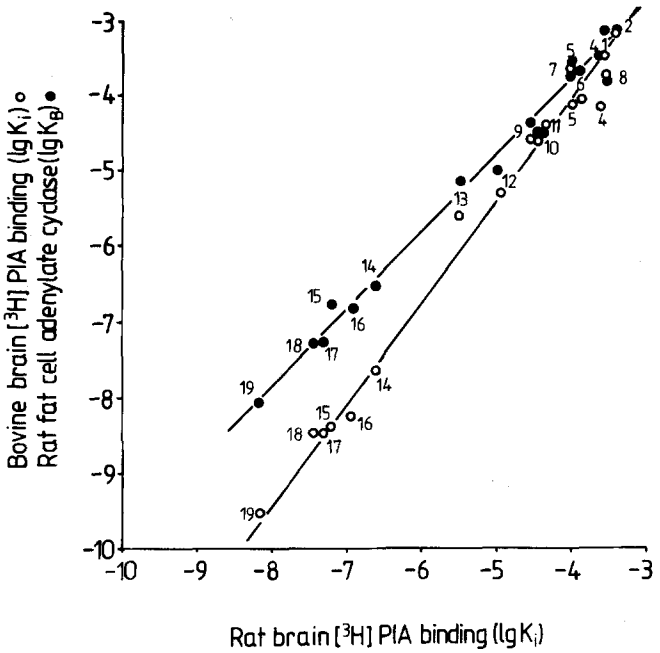

Fig. 5. Correlation of dissociation constants of xanthine derivatives for inhibition of [^{3}H]PIA binding to bovine brain (○——○, $r = 0.993$) and for the antagonism of R-PIA-induced inhibition of rat fat cell adenylate cyclase (●——●, $r = 0.993$) with the K_i-values of [^{3}H]PIA binding in rat brain. Data and numbers of the compounds are taken from Table 3

as a highly potent and selective A_1 receptor agonist for inhibition of adenylate cyclase, lipolysis and radioligand binding in fat cells (Londos et al. 1978, 1980; Trost and Schwabe 1981).

As shown in Fig. 3, basal activity of adenylate cyclase of rat fat cell membranes was inhibited by R-PIA with an IC_{50} of 19 nmol/l. The adenosine antagonist 8-(4-hydroxyphenyl)theophylline did not affect basal activity in the absence of R-PIA, but shifted the concentration-response curve of R-PIA to the right. The slope and the maximal effect were not changed. The IC_{50} of R-PIA in the presence of 10, 100 and 1,000 nmol/l 8-(4-hydroxyphenyl)theophylline was 24, 52 and 380 nmol/l, respectively. The K_B for 8-(4-hydroxyphenyl)theophylline calculated from the Schild plot of these data was 51 nmol/l.

The same procedure was used to determine the A_1 receptor antagonism of several other xanthine derivatives. The K_B-values of antagonists for the R-PIA-induced inhibition of adenylate cyclase are listed in Table 3. Xanthine itself showed a relatively weak antagonistic effect as indicated by a K_B of 170 μmol/l. With the exception of caffeine and 1,7-dimethylxanthine all xanthine derivatives with substituents in the 7-position were less potent than xanthine. Theophylline and 3-isobutyl-1-methylxanthine (IBMX) were approximately 20 times more potent than xanthine. A marked increase in antagonist affinity was obtained with 8-phenylxanthine derivatives. 1,3-Diethyl-8-phenylxanthine (DPX) and 8-(4-acetoxyphenyl)theophylline were 30–40 times more potent than theophylline and the additional 4-hydroxy substituent on the 8-phenyl ring induced a further 3-fold increase of potency. The highest affinity was obtained with 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX) which gave a K_B of 8.6 nmol/l and thus was 1,000 times more potent than theophylline.

Inhibition of [^{3}H]PIA binding to brain membranes

Finally, the xanthine derivatives were characterized as A_1 adenosine receptor antagonists in binding studies with [^{3}H]PIA using membrane preparations of rat and bovine cerebral cortex. For several compounds competition curves obtained for bovine brain membranes are shown in Fig. 4. Again, PACPX was the most potent compound with a K_i of 0.30 nmol/l (95% confidence limits 0.23–0.39), followed by 8-phenyltheophylline, 8-(4-acetoxyphenyl)theophylline, 3-isobutyl-1-methylxanthine and theophylline. The competition curves were monophasic with Hill coefficients between 0.85 and 1.1, indicating the absence of cooperative interactions.

For these and several other xanthine derivatives IC_{50}-values were calculated from competition curves, transformed into K_i-values according to Cheng and Prusoff (1973) and listed in Table 3. The structure-activity profile of [^{3}H]PIA binding to rat brain is very similar to that obtained for adenylate cyclase activity in rat fat cells. This is also evident from the paired comparison of the dissociation constants of both systems shown in Fig. 5. The data obtained with bovine brain membranes agree well with those of the two other systems as far as the less potent xanthine derivatives are concerned. However, all 8-phenylxanthines are 10 to 20 times more potent inhibitors of [^{3}H]PIA binding to bovine brain than to rat brain and of R-PIA-mediated inhibition in rat fat cells. In bovine brain 8-phenyltheophylline is 1,000 times more potent than theophylline and PACPX has a K_i-value of 0.3 nmol/l. Despite these differences a good correlation ($r = 0.993$) between the K_i-values for inhibition of [^{3}H]PIA binding in bovine brain versus rat brain is obtained (Fig. 5), but the regression line is steeper due to the higher affinity of the 8-phenylxanthine derivatives to A_1 receptors of bovine brain.

Comparison of A_1 and A_2 adenosine receptors

The selectivity of xanthine derivatives as antagonists at the two subclasses of adenosine receptors can be derived from the potencies at A_1 and A_2 receptors. Ratios were calculated from the directly comparable functional parameters which are adenylate cyclase activity of human platelets and rat fat cells (ratio I). Furthermore, the K_B-values of platelet cyclase were related to the K_i-values for inhibition of [^{3}H]PIA binding in rat brain (ratio II) and in bovine brain (ratio III). The values are shown in Table 4.

For ratio I most of the compounds show values between 0.4 and 6, demonstrating that these xanthine derivatives are nearly equipotent as antagonists at A_1 and A_2 adenosine receptors. With the exception of 8-(4-acetoxyphenyl)theophylline and 1,3-diethyl-8-phenylxanthine, these compounds are relatively weak adenosine antagonists with K_B-values in the micromolar range. However, a substantial selectivity is observed with the last four compounds among which 8-phenyltheophylline is 26 times and PACPX is 55 times more potent at A_1 receptors than at A_2 receptors (Table 4). Very similar ratios result from correlations with the K_B-values of rat brain (ratio II). If the data of human platelet cyclase are correlated with those of the A_1 receptor system of bovine brain (ratio III) all 8-phenylxanthines exhibit a high degree of A_1 receptor selectivity. Particularly

high ratios are obtained for 8-phenyltheophylline (700) and PACPX (1,600).

Discussion

Methylxanthines and related xanthine derivatives have been studied as adenosine antagonists for more than two decades. After the proposal of the present adenosine receptor concept with two receptor subtypes, it was of interest to look for subtype-selective adenosine antagonists. The results of the present study show that several 8-phenylxanthines are remarkably selective for A_1 adenosine receptors. 8-Phenyltheophylline is approximately 700 times more potent as antagonist at A_1 than at A_2 adenosine receptors. The recently developed 8-phenylxanthine derivative, PACPX, is even 1,600 times more potent as A_1 adenosine antagonist.

These high selectivity ratios have been obtained with bovine brain membranes versus human platelets and display the optimal conditions among the three A_1 receptor systems assayed in the present study. In bovine brain 8-phenylxanthines are 10 to 20 times more potent than in rat brain or rat fat cells. If the data of the A_1 receptor systems of rat tissues are used for the correlation, the selectivity of 8-phenyltheophylline is still 20 to 40-fold and of PACPX 50 to 70-fold. Thus, our results consistently demonstrate an A_1 receptor selectivity in 3 different A_1 receptor systems as a characteristic property of several 8-phenylxanthine derivatives.

The enhanced affinity of 8-phenylxanthine derivatives for adenosine receptors has previously been observed for A_1 as well as for A_2 receptor systems. The prototype of this group of compounds, 8-phenyltheophylline, was first studied as adenosine antagonist in the A_2 receptor system of guinea pig cerebral cortex by Smellie et al. (1979). In this system, 8-phenyltheophylline (K_i 6 µmol/l) was 10 times more potent than theophylline in antagonizing the adenosine-induced accumulation of cyclic AMP. In a second A_2 receptor system, the human fibroblast, 8-phenyltheophylline (K_i 0.18 µmol/l) was even more potent than in guinea pig brain and, in addition, was 25 times more potent than theophylline (Bruns 1981). In the present study, the potency of 8-phenyltheophylline in antagonizing A_2 receptor-mediated responses of human platelets (K_i 4.1 and 3.6 µmol/l) is similar to that of guinea pig brain.

The effect of 8-phenylxanthines on A_1 adenosine receptors was first investigated in radioligand binding studies with several brain membrane preparations. Bruns et al. (1980) found that 1,3-diethyl-8-phenylxanthine (DPX) was the most potent A_1 receptor antagonist in guinea pig brain (IC_{50} 0.5 µmol/l) followed by 8-phenyltheophylline (IC_{50} 1 µmol/l), whereas 8-(4-sulfophenyl)theophylline (IC_{50} 20 µmol/l) was even less potent than theophylline. In two other species, 8-phenyltheophylline was considerably more potent as A_1 receptor antagonist than in guinea pig brain. In rat brain synaptosomal membranes an IC_{50} of 116 nmol/l (Williams and Risley 1980) and in bovine brain membranes an IC_{50} of 1.2 nmol/l (Bruns et al. 1983) were determined. These values agree closely with the results obtained with 8-phenyltheophylline as A_1 receptor antagonist in rat brain (K_i 115 nmol/l) and bovine brain (K_i 5.9 nmol/l) in the present study. 8-Phenyltheophylline has also been studied as adenosine antagonist in guinea pig atrium, rabbit basilar artery and guinea pig ileum, and K_B-values between 70 and 760 nmol/l were obtained (Griffith et al. 1981).

The structure-activity relationship of 8-phenylxanthine derivatives at A_1 receptors has been extensively analyzed by Bruns et al. (1983). These authors tested the effects of 43 xanthine derivatives in the sensitive A_1 receptor system of bovine brain and developed several compounds of extraordinary receptor affinity. The most potent adenosine antagonist was 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX) with a K_i-value of 0.022 nmol/l for A_1 receptors. In the present study, PACPX was less potent (K_i 0.3 nmol/l) in bovine brain but still the most potent and most selective antagonist for A_1 receptors. Bruns et al. (1983) did not assess the potency of the newly developed 8-phenylxanthines at an A_2 receptor system and, therefore, the relative selectivity for A_1 receptors cannot directly be evaluated. However, some of the compounds have previously been tested by the same author as A_2 receptor antagonists in human fibroblasts (Bruns 1981). If these two adenosine receptor systems are used for a correlation, a considerable selectivity can be calculated. 8-Phenyltheophylline is 150 times more potent at A_1 receptors of bovine brain than at A_2 receptors of human fibroblasts. A 270-fold selectivity for A_1 receptors is obtained for 8-(4-methylphenyl)theophylline. Our data yield an even higher selectivity for several 8-phenylxanthines at A_1 receptors, mainly because of the lower sensitivity of the A_2 receptors in human platelets compared to that of human fibroblasts.

In two comparative studies on A_1 and A_2 receptor subtypes a selectivity of 8-phenylxanthines for A_1 adenosine receptors has not been detected. Fredholm and Persson (1982) reported that the absolute and relative potencies of 10 xanthine derivatives were almost identical in two A_1 receptor systems (rat cerebral cortex membranes, rat fat cells) and an A_2 receptor system (rat hippocampal slices). 1,3-Diethyl-8-phenylxanthine and 8-phenyltheophylline were the most potent compounds with K_i-values of approximately 100 to 200 nmol/l. Only enprofylline was found to be 20 to 30 times more potent in the hippocampal system (A_2) than in the two other systems. In the present study enprofylline did not show selective antagonism at A_2 adenosine receptors. Glennon et al. (1984) tested 6 xanthines and 22 mesionic xanthine analogues on A_1 receptors (rat cerebral cortical membranes) and A_2 receptors (guinea pig cerebral cortical slices). Again, 8-phenyltheophylline was the most potent antagonist. However, a substantial selectivity was not obtained, as shown by IC_{50}-values of 800 nmol/l for the A_1 receptor and 1,700 nmol/l for the A_2 receptor system, whereas in the present study 8-phenyltheophylline (K_i 115 nmol/l) was more potent as A_1 receptor antagonist and less potent as A_2 antagonist in human platelets (K_i 4,100 nmol/l).

Antagonists with a substantial selectivity for A_2 adenosine receptors were were not detected in the present study. Xanthine and 1,7-dimethylxanthine were two times more potent as A_2 than as A_1 receptor antagonists, but both compounds had only weak antagonist activity. A similar observation was reported for several mesoionic benzothiazolopyrimidines which displayed IC_{50}-values between 30 and 40 µmol/l for A_1receptors and 15 and 20 µmol/l for A_2 receptors (Glennon et al. 1984). The only compound, for which a relatively high degree of selectivity for A_2 receptors has been reported, was 8-(4-sulfophenyl)theophylline (Bruns et al. 1980). This compound (K_i 1.2 µmol/l) was more potent as A_2 antagonist in human fibroblasts than theophylline and showed an 80-fold selectivity for A_2 receptors. However, this

observation was not confirmed by Fredholm and Persson (1982).

Many xanthine derivatives analyzed in the present paper have been previously examined as inhibitors of cyclic AMP phosphodiesterases. In general, xanthine derivatives are more potent as adenosine receptor antagonists than as phosphodiesterase inhibitors. Theophylline, for instance, has a K_i of 10 to 13 μmol/l as A_2 receptor antagonist in human platelets in the present study whereas the K_i for the inhibition of platelet phosphodiesterase was found to be 45 μmol/l (Asano et al. 1977). At A_1 receptors of rat fat cells we obtained a K_i of 9.5 μmol/l for theophylline compared to a K_i of 91 μmol/l for inhibition of phosphodiesterase in the same tissue (Schwabe et al. 1972). In rat brain theophylline is a very weak phosphodiesterase inhibitor (IC_{50} 500–1,000 μmol/l) and is already considered as a quite selective adenosine antagonist (Smellie et al. 1979). 3-Isobutyl-1-methylxanthine (IBMX) is usually regarded as a specific phosphodiesterase inhibitor with a relatively low K_i of 7.5 μmol/l (Smellie et al. 1979), but our present data show that A_1 and A_2 adenosine receptors (K_i 2.5 to 7.6 μmol/l) are antagonized in the same concentration range and a mixture of both effects must always be expected. 8-Phenyltheophylline and other 8-phenylxanthines are relatively weak phosphodiesterase inhibitors with IC_{50}-values ranging from 30 to more than 100 μmol/l (Smellie et al. 1979; Wu et al. 1982). Obviously, the 8-phenyl substituent does not substantially increase the potency as phosphodiesterase inhibitor but markedly enhances the affinity for A_1 and A_2 adenosine receptors. Therefore, 8-phenylxanthines can be used as selective adenosine receptor antagonists without substantial interference by inhibitory effects on phosphodiesterase. With their additional selectivity for the A_1 adenosine receptor subtype, several 8-phenylxanthines, such as 8-phenyltheophylline and PACPX, offer a first approach to study the pharmacological effects of a subtype-selective blockade of adenosine receptors.

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