1,3,8- and 1,3,7-substituted xanthines: relative potency as adenosine receptor antagonists at the frog neuromuscular junction

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1 The ability of 1,3,8-substituted xanthines (1,3-dipropyl-8-(4-(2-aminoethyl)amino)carbonylmethoxyphenyl)xanthine (XAC), 1,3-dipropyl-8-(4-carboxymethoxyphenyl)xanthine (XCC), 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 1,3-diethyl-8-phenylxanthine (DPX) and 8-phenyltheophylline (8-PT)), of 1,3,7-substituted xanthines (1-propargyl-3,7-dimethylxanthine (PGDMX) and caffeine), and of a 3-substituted xanthine (enprofylline) to antagonize the inhibitory effect of 2-chloroadenosine (CADO) on the amplitude of nerve-evoked twitches was investigated in innervated sartorius muscles of the frog.

2 All the 1,3,8-substituted xanthines, in concentrations virtually devoid of effect on neuromuscular transmission, shifted to the right, in a near parallel manner the log concentration-response curve for CADO. Linear Schild plots with slopes near to unity at concentration-ratios less than 14 were obtained for XAC, XCC, DPCPX, DPX and 8-PT.

3 The order of potency of the 1,3,8-substituted xanthines as antagonists of the effect of CADO was XAC ($K_i = 23$ nM) $\geq$ DPCPX (35 nM) $>$ 8-PT (200 nM) $\geq$ DPX (295 nM) $>$ XCC (1905 nM) $\geq$ PACPX (2291 nM). No correlation was found between the potency of these xanthines as antagonists of the adenosine receptor at the frog neuromuscular junction and their reported potency as antagonists of the A_1 - or A_2 -adenosine receptors.

4 The 1,3,7-substituted xanthines, PGDMX and caffeine, in concentrations virtually devoid of effect on neuromuscular transmission, also caused parallel shifts to the right of the log concentration-response curves for CADO, but were less potent than the 1,3,8-substituted xanthines. PGDMX was more than 20 times more potent than caffeine.

5 Enprofylline in concentrations up to 100 μ M did not antagonize the inhibitory effect of CADO on neuromuscular transmission.

6 It is concluded that the antagonist profile of the adenosine receptor mediating inhibition of transmission at the frog neuromuscular junction is different from the antagonist profile of the A_1 - and A_2 -adenosine receptors.

Introduction

Two subtypes of xanthine-sensitive adenosine receptors have been postulated on biochemical grounds: A_1 (R_1) operating inhibition and A_2 (R_2) operating stimulation of adenylate cyclase (Van Calcar *et al.*, 1979; Londos *et al.*, 1980). These two subtypes of adenosine receptor have different pharmacological profiles for agonists. At A_1 -adenosine receptors the order of agonist potency is L-N⁶-phenylisopropyladenosine (L-PIA), N⁶-cyclohexyladenosine (CHA) $>$ 2-chloroadenosine (CADO) $>$ 5'-N-ethylcarboxamide adenosine (NECA). At A_2 -adenosine

receptors the agonist profile is reversed, i.e. NECA $>$ CADO $>$ L-PIA, CHA (e.g. Daly *et al.*, 1981).

The adenosine receptor mediating the adenosine-induced presynaptic inhibition of neuromuscular transmission in the innervated frog sartorius muscle has an agonist profile with L-PIA, CHA and NECA being of similar potency and more potent than CADO (Ribeiro & Sebastião, 1985). The observation that this agonist profile is similar to that described for the adenosine receptors mediating a decrease in

neurotransmitter release in most of the peripheral and central synapses, but is different from the agonist profile of both A_1 - and A_2 -adenosine receptors, prompted the hypothesis that the adenosine receptor-mediated presynaptic inhibition of synaptic transmission by adenosine belongs to a third (A_3) subtype of xanthine-sensitive adenosine receptors (Ribeiro & Sebastião, 1986). Agonist potency can, however, be influenced by factors unrelated to receptor type (see e.g. Kenakin, 1987), which complicates receptor classification on the basis of different agonist profiles.

Xanthine derivatives with some antagonist selectivity for A_1 - and A_2 -adenosine receptors have recently been developed (e.g. Daly *et al.*, 1987). In the present work we have studied the relative potency of some of the substituted xanthines as antagonists of the adenosine receptor that mediates the presynaptic inhibition of neuromuscular transmission in the innervated sartorius muscle preparation of the frog. Several 1,3,8- and 1,3,7-substituted xanthines were used. CADO was used as the agonist because this adenosine analogue is a full agonist of the adenosine receptor in the frog innervated sartorius preparation and is easily washed out (Ribeiro & Sebastião, 1985).

A brief account of some of the results has already been published (Sebastião & Ribeiro, 1988a).

Methods

The experiments were carried out at room temperature (22–25°C) on innervated sartorius muscles of the frog (*Rana ridibunda*) (see e.g. Kharkevich, 1986). The preparations were set up in a 25 ml organ bath through which the solutions flowed continuously with the aid of a roller pump. The solutions were changed by transferring the inlet tube of the pump from one flask to another. The flow rate was 25 ml min⁻¹ during the first 2 min after changing the solutions and 5 ml min⁻¹ until the next changeover of solutions. Rectangular pulses of 0.1 ms duration and supramaximal voltage were applied to the nerve once every 5 s. The twitch responses to nerve stimulation were recorded isometrically at a resting tension of 50 mN with a Sanborn transducer and displayed on a Hewlett-Packard recorder. The bathing solution (pH 7.0) contained (mM): NaCl 117, KCl 2.5, CaCl₂ 1.8, MgCl₂ 1.2, NaH₂PO₄ 1, Na₂HPO₄ 1. The average nerve-evoked twitch amplitude in the control bathing solution for the experiments described in this paper was 22 ± 1.3 mN.

Concentration-response curves and Schild regressions

In each experiment the cumulative concentration-response curves to the adenosine receptor agonist,

2-chloroadenosine (CADO), were obtained first in the absence of the antagonist. CADO was then washed out and the preparation equilibrated with the antagonist for at least 40 min. A second cumulative concentration-response curve for CADO, now in the presence of the antagonist, was then performed. After a washing out period of 60–90 min, a third cumulative concentration-response curve for CADO again in the absence of the antagonist was finally obtained. The usual procedure was to average the responses in the same experiment to the same concentration of CADO in the absence of antagonist, to obtain the control responses to CADO. Whenever the control effect of CADO after washing out the antagonist was smaller than the effect of the same concentration of CADO before exposure to the antagonist, we considered that the action of the antagonist was not fully reversible; in these cases the post-control concentration-response curve for CADO was not included in the calculations. This was frequently observed in the experiments using the highest concentration (100 nM) of 1,3-dipropyl-8-(4-(2-aminoethyl)amino) carbonylmethoxyphenyl)-xanthine (XAC), but not in the experiments using other xanthines.

The concentration of CADO producing 35% decrease in the amplitude of nerve-evoked twitches was calculated in each experiment by regression analysis of the linear part of the log concentration-response curves for CADO. The concentration of CADO causing 35% effect is near its EC₅₀ value since the maximal effect of CADO (determined from the double reciprocal plot of the averaged concentration-response curves for CADO, $n = 51$) was 65% decrease in twitch amplitude. The ratios between equi-active concentrations (CR) of CADO (causing 35% effect) in the presence and in the absence of the antagonist were used in the Schild equation (Arunlakshana & Schild, 1959)

$$\log(\text{CR} - 1) = n \log[B] - \log K_i$$

where $[B]$ is the molar concentration of antagonist and K_i the equilibrium dissociation constant of the antagonist for the receptor. pA_2 values were determined on the abscissae intercept of the regression of $\log(\text{CR} - 1)$ upon $\log[B]$. Where the regression was linear and the slope (n) approximated to unity, the K_i value was taken as the negative antilog of the pA_2 value.

Drugs

Drugs used were: caffeine, 2-chloroadenosine (CADO) (Sigma); 1,3-diethyl-8-phenylxanthine (DPX), 1,3-dipropyl-8-(2-amino-4-chlorophenyl)-xanthine (PACPX), 1,3-dipropyl-8-cyclopentylxan-

Figure 1 Structures of the 1,3,8-substituted xanthines. The structure of xanthine is illustrated at the top. 8-PT: 8-phenyltheophylline; DPX: 1,3-diethyl-8-phenylxanthine; PACPX: 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine; DPCPX: 1,3-dipropyl-8-cyclopentylxanthine; XAC: 1,3-dipropyl-8-(4-((2-aminoethyl)amino)carbonylmethoxyphenyl)xanthine; XCC: 1,3-dipropyl-8-(4-carboxymethoxyphenyl)xanthine.

thine (DPCPX), enprofylline, 8-phenyltheophylline (8-PT) (R.B.I.); 1,3-dipropyl-8-(4-((2-aminoethyl)amino) carbonylmethoxyphenyl)xanthine (XAC), 1,3-dipropyl-8-(4-carboxymethoxyphenyl)xanthine (XCC) and 1-propargyl-3,7-dimethylxanthine (PGDMX) were kindly supplied by Drs J.W. Daly and K.A. Jacobson (National Institute of Health,

Bethesda, U.S.A.). 8-PT and DPX were made up into 10 mM and 50 mM stock solutions, respectively, in 80% methanol/20% M NaOH (v/v); PACPX and DPCPX were made up into 10 mM and 5 mM stock solutions, respectively, in 99% dimethylsulphoxide (DMSO)/1% M NaOH (v/v); XAC and XCC were made up into 80 μ M and 2.5 mM stock solutions,

Figure 2 Action of the adenosine receptor antagonist 1,3-dipropyl-8-((2-aminoethyl)amino)-carbonylmethylphenyl)xanthine (XAC) on the inhibitory effect of 2-chloroadenosine (CADO) on twitch responses evoked by nerve stimulation of frog sartorius muscles. (A) Concentration-response curves for the inhibitory effect of CADO. (a) Antagonism by XAC (25 nM) ($n = 3$); (b) antagonism by XAC (50 nM) ($n = 4$); (c) antagonism by XAC (100 nM) ($n = 4$). Each panel represents pooled data from the same experiments. The ordinates are percentage decreases in twitch amplitude caused by CADO in the absence (●) and in the presence (▲, ■, ▼) of XAC. Zero% is the twitch amplitude in the control bathing solution and 100% represents a complete inhibition of the nerve-evoked twitches. The vertical bars represent s.e.mean and are shown when they exceed the symbols in size; * $P < 0.05$ (paired Student's t test as compared with the effect of CADO alone in the same experiments). When testing the effect of CADO in the absence of XAC, the solvent of XAC (DMSO) was present in the same concentration (0.03–0.125% v/v) as that present in the XAC solutions applied to the preparations. Average twitch amplitude in the control bathing solution: 20.5 ± 2.1 mN. (B) shows pen-recorder traces of nerve-evoked twitch responses obtained in one experiment using XAC (100 nM). (a) Effect of CADO in the absence of XAC; responses recorded in control bathing solution (i) before applying CADO, and after 10 min in (ii) 0.3 μ M, (iii) 1 μ M and (iv) 3 μ M CADO. (b) Effect of CADO in the presence of XAC; responses recorded (i) 55 min after applying XAC (100 nM), and after 10 min in (ii) 0.3 μ M, (iii) 1 μ M and (iv) 3 μ M CADO + XAC (100 nM).

respectively, in DMSO. In each experiment all the solutions without the antagonist contained the same amount of solvent as the solutions with the antagonist.

Statistics

Data are expressed as mean $\pm$ s.e.mean, and n represents the number of experiments. The significance of the differences between the means was calculated by paired or unpaired Student's t test. P values of

0.05 or less were considered to represent significant differences.

Results

1,3,8-substituted xanthine derivatives

The structures of the 1,3,8-substituted xanthines used in the present work are shown in Figure 1. These

include xanthines with substitutions at the 1- and 3-positions by methyl, ethyl or propyl groups, the 8-position being substituted either by aryl or cycloalkyl groups.

Figure 2 shows the effect of the adenosine receptor agonist, 2-chloroadenosine (CADO, 0.1–30 μM) on nerve-evoked twitch responses of the frog sartorius, in the absence and in the presence of 1,3-dipropyl-8-(4 - ((2 - aminoethyl)amino) - carbonylmethoxy - phenyl)xanthine (XAC, 25–100 nM). CADO decreased, in a concentration-dependent manner, the amplitude of the nerve-evoked twitches, its maximal effect being almost obtained with 3–10 μM of this adenosine analogue. The comparisons between the concentration-response curves for CADO in the absence and in the presence of antagonist were made using data from the same experiments because there is considerable variation between the quantitative effects of the same concentration of CADO in different experiments. XAC (25–100 nM) used alone was virtually devoid of effect on nerve-evoked twitches, but shifted to the right the concentration-response curve for CADO (0.1–30 μM), this shift being greater the higher the concentration of XAC. The maximal effect of CADO was not significantly modified by XAC (25–100 nM) (Figure 2). The pA_2 value for XAC calculated from the data shown in Figure 2 was 7.63 ($n = 11$) and the slope of the regression line of the Schild plot was 1.05, giving a dissociation constant (K_i) for XAC of 23 nM.

Concentration-dependent shifts to the right of the concentration-response curve for the inhibitory effect of CADO (0.1–30 μM) on nerve-evoked twitches were also observed with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 50–250 nM), 8-phenyltheophylline (8-PT, 0.25–2.5 μM), 1,3-diethyl-8-phenylxanthine (DPX, 0.5–2.5 μM) and 1,3-dipropyl-8-(4-carboxymethoxyphenyl)xanthine (XCC, 2.5–10 μM). The slope of the linear part of the log concentration-response curves for CADO as well as the maximal effect of CADO were not appreciably modified by these xanthines. In the concentrations used, the xanthines were devoid of effect on the nerve-evoked twitches, when applied to the preparations in the absence of CADO. In Figure 3 the Schild plots obtained for XAC, DPCPX, 8-PT, DPX and XCC are compared. As can be seen, linear Schild plots with slopes near unity at low concentration-ratios (less than 14) were obtained.

1,3-Dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX, 2.5 μM) shifted the log concentration-response curve for CADO (0.1–10 μM) to the right by a factor of 2.2 ± 0.3 ($n = 6$), without affecting in an appreciable manner the maximal effect of CADO. The averaged slopes of the linear part of the log concentration-response curves for CADO in the absence and in the presence of PACPX (2.5 μM) were

Figure 3 Schild plots for the antagonism by 1,3-dipropyl-8-(4-((2-aminoethyl)amino) carbonylmethoxyphenyl)xanthine (XAC, ●), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, ▲), 8-phenyltheophylline (8-PT, ◆), 1,3-diethyl-8-phenylxanthine (DPX, ▼) and 1,3-dipropyl-8-(4-carboxymethoxyphenyl)xanthine (XCC, ■) of the inhibitory effect of 2-chloroadenosine (CADO) on twitch responses evoked by nerve stimulation of frog sartorius muscles. Each point is the average of the concentration-ratio (CR) values obtained in 2–4 experiments. The regression lines were calculated by the method of the least squares using the individual CR values; the correlation coefficients ranged from 0.876 to 0.760 ($P < 0.05$ in all cases). CR values were calculated at the level of 35% decrease in twitch amplitude caused by CADO, which is near its EC_{50} value since the maximal effect of CADO (determined from the double reciprocal plot of the averaged concentration-response curves for CADO, $n = 51$) was 65% decrease in twitch amplitude.

not statistically different (27.6 ± 4.0 in the absence and 28.6 ± 4.3 in the presence of PACPX, $P > 0.05$). When 0.25 μM and 0.5 μM PACPX were used, no measurable modifications of the inhibitory effect of CADO (0.1–10 μM) on nerve-evoked twitch amplitude were observed ($n = 3$), and 1 μM PACPX shifted the concentration-response curve for CADO (0.1–10 μM) to the right by a factor of 1.5 (one experiment). To avoid the use of concentration-ratios below 2 to calculate pA_2 values, and since concentrations of PACPX higher than 2.5 μM could not be used due to its solubility limit in the bathing solution, the pA_2 value for PACPX was estimated using the concentration-ratios obtained with 2.5 μM PACPX in an abbreviated Schild analysis where a slope of unity was assumed for $\log(\text{CR} - 1)$ on $\log[\text{PACPX}]$. PACPX (0.25–2.5 μM) was virtually devoid of effect on nerve-evoked twitches when applied to the preparations in the absence of CADO.

A comparison between the different pA_2 values as well as the K_i values calculated for PACPX, XCC, DPX, 8-PT, DPCPX and XAC is shown in Table 1. It is evident that XAC and DPCPX were the most

Table 1 Potencies of the 1,3,8-substituted xanthine derivatives as antagonists of the inhibitory effect of 2-chloroadenosine on nerve-evoked twitch amplitude in the frog sartorius

	pA_2	Max. CR	Slope*	K_i (nM)	n
XAC	7.63 ± 0.08	5.39	1.05	23	11
DPCPX	7.46 ± 0.05	9.78	1.02	35	10
8-PT	$6.70 \pm 0.08^*$	13.17	0.94	200	13
DPX	6.53 ± 0.05	8.45	0.96	295	10
XCC	$5.72 \pm 0.10^{**}$	7.50	0.99	1905	7
PACPX ^b	5.64 ± 0.08	2.22	—	2291	6

Max. CR: averaged concentration-ratio obtained with the maximum concentration used. *Slope of the regression line from the Schild plot shown in Figure 3. ^bDue to solubility limits, pA_2 and K_i values were estimated using one concentration (2.5 μ M) of PACPX in an abbreviated Schild analysis, where a slope of unity was assumed for $\log(CR - 1)$ on $\log[PACPX]$. * $P < 0.05$ compared with DPCPX; ** $P < 0.05$ compared with DPX (unpaired Student's *t* test). For key to abbreviations used see legend to Figure 1.

potent antagonists, XCC and PACPX were the least potent, 8-PT and DPX having intermediate potency as antagonists of the inhibitory effect of CADO on nerve-evoked twitch responses in the frog sartorius.

1,3,7-substituted xanthine derivatives

The ability of 1-propargyl-3,7-dimethylxanthine (PGDMX, 20 μ M) to antagonize the inhibitory effect of CADO (0.1–3 μ M) on nerve-evoked twitches was investigated in two experiments. In this concentration PGDMX caused a small parallel shift to the right of the concentration-response curve for CADO (0.1–3 μ M), with an average concentration-ratio obtained in the two experiments of 1.9 ± 0.04 . Caffeine (1,3,7-trimethylxanthine) in concentrations from 50 μ M to 100 μ M was virtually devoid of antagonistic action against the inhibitory effect of CADO (0.1–10 μ M) on the amplitude of nerve-evoked twitches ($n = 3$). Used in a higher concentration (250 μ M) caffeine shifted to the right in a near parallel manner the concentration-response curve for CADO (0.1–10 μ M) by a factor of 1.7 ± 0.1 ($n = 3$). Neither PGDMX (20 μ M) nor caffeine (50–250 μ M) affected the nerve-evoked twitch responses when applied to the preparations in the absence of CADO.

Enprofylline

Enprofylline (3-propylxanthine) (50–100 μ M) did not antagonize in an appreciable manner the inhibitory effect of CADO (0.1–10 μ M) on nerve-evoked twitches ($n = 3$).

Discussion

The present results show that several 1,3,8- and 1,3,7-substituted xanthine derivatives, antagonize the

inhibitory effect of CADO on the amplitude of nerve-evoked twitch responses in the innervated sartorius muscle of the frog. The rank order of potency of the xanthines was $XAC \geq DPCPX > 8-PT \geq DPX > XCC \geq PACPX > PGDMX > \text{caffeine}$. The 3-substituted xanthine, enprofylline, was virtually devoid of antagonistic action, which conforms with the results obtained by others (cf. Persson *et al.*, 1986; but see Ukena *et al.*, 1985).

In concentrations similar to those used in the present work, CADO is devoid of effect on the amplitude of twitches evoked by direct stimulation of curarized frog sartorius muscles (Ribeiro & Sebastião, 1985) and decreases the amplitude and quantal content of the endplate potentials (e.p.ps) recorded from frog muscle fibres (Silinsky, 1980; Ribeiro & Sebastião, 1987). Thus, the inhibitory effect of CADO on nerve-evoked twitches is due to inhibition of neuromuscular transmission by decreasing transmitter release.

The xanthines XAC, DPCPX, 8-PT, DPX and XCC, appear to be competitive antagonists of the adenosine receptor stimulated by CADO at the frog neuromuscular junction, as can be concluded from the finding that the slopes of the Schild plots for these xanthines were near unity. Also, they appear to antagonize an homogeneous population of adenosine receptors since the Schild plots were linear at low concentration-ratios (cf. Kenakin, 1987). Schild plots for PACPX, PGDMX and caffeine were not done, but the findings that the concentration-response curves for CADO were shifted to the right in a near parallel manner and that the maximal effect of CADO was virtually unaffected by these xanthines, indicate that they also may behave as competitive antagonists.

The presence of an 8-phenyl or 8-cycloalkyl group greatly increases the xanthine antagonistic potency at the adenosine receptor mediating inhibition of

transmission in the frog sartorius neuromuscular junction. This has also been shown to occur at A_1 (Bruns *et al.*, 1983) and A_2 (Bruns, 1981) adenosine receptors. Within the series of 8-substituted xanthines studied, 8-PT and DPX were of similar potency, indicating that the adenosine receptor at the frog sartorius neuromuscular junction does not discriminate between methyl or ethyl groups in the 1- and 3-positions of the xanthine molecule. XAC and DPCPX were about equipotent and more potent than 8-PT, whereas XCC and PACPX were less potent than 8-PT. It thus appears that the increase in the length of the 1,3-alkyl substituents from methyl to propyl groups can either increase or decrease the potency of the 1,3,8-substituted xanthines, the nature of the substituent in the 8-position being more important in determining the xanthine potency. This contrasts with that which was found at the A_1 -adenosine receptor where 1,3-dipropyl-8-substituted xanthines are more potent than 1,3-dimethyl-8-substituted xanthines (see e.g. Daly, 1985).

The potencies of the 1,3,8-substituted xanthine derivatives as antagonists of the A_1 -adenosine receptor have been determined by its ability to inhibit binding of [3 H]CHA or [3 H]-L-PIA to rat brain membranes (Ukena *et al.*, 1986a; Daly *et al.*, 1987; Lohse *et al.*, 1987) or by its ability to inhibit L-PIA-induced inhibition of adenylate cyclase activity in rat adipocytes (Ukena *et al.*, 1986b; Lohse *et al.*, 1987; Martinson *et al.*, 1987). The antagonistic profile found in brain membranes is DPCPX ($K_i = 0.3\text{--}1.2\text{ nM}$), XAC (1.2–3.5 nM), PACPX (2.5 nM) > XCC (58 nM), DPX (70 nM), 8-PT (76 nM), and in fat cells is DPCPX ($K_i = 0.45\text{--}0.47\text{ nM}$) > PACPX (3 nM), XAC (4.4–15 nM) > XCC (83 nM) > DPX (170 nM) > 8-PT (310 nM). The high potency of DPCPX observed in the present work could indicate that the adenosine receptor at the frog neuromuscular junction has an antagonist profile compatible with that of the A_1 -adenosine receptor, and the finding that DPCPX and XAC are of similar potency suggests that the adenosine receptor at the frog neuromuscular junction is closer to the adenosine receptor in brain membranes than in fat cells. However, no correlation was found between the potencies of these xanthines as antagonists of the A_1 -adenosine receptor in brain membranes or fat cells and as antagonists of the adenosine receptor at the frog sartorius neuromuscular junction (Figure 4a). This indicates that the antagonist profile of the adenosine receptor at the frog neuromuscular junction is different from the antagonist profile of the A_1 -adenosine receptor. Even excluding PACPX, which in some preparations behaves as a non-competitive antagonist of adenosine receptors (Burnstock & Hoyle, 1985; Collis *et al.*, 1987; Wil-

Figure 4 Scatter diagrams comparing the affinities of the 1,3,8-substituted xanthines for the adenosine receptor at the frog sartorius neuromuscular junction (NMJ) (expressed as the K_i values shown in Table 1) and for the A_1 -adenosine receptor (a) in rat brain membranes (●) or rat fat cells (○), and for the A_2 -adenosine receptor (b) in human platelets. The correlation coefficients in (a) were $r = 0.154$, $0.7 < P < 0.80$, for (●) and $r = -0.093$, $0.8 < P < 0.9$ for (○), and the correlation coefficient in (b) was 0.104 , $0.8 < P < 0.9$. Excluding PACPX, the correlation coefficients were $r = 0.502$, $0.1 < P < 0.2$, for (●), and $r = 0.125$, $0.7 < P < 0.8$, for (○), and the correlation coefficient in (b) was $r = 0.374$, $0.3 < P < 0.4$. Data which are not from the present study are from Daly *et al.* (1987), Lohse *et al.* (1987), Martinson *et al.* (1987), Schwabe *et al.* (1985) and Ukena *et al.* (1986a,b,c); when different K_i values for the same xanthine have been reported they are all represented (e.g. XAC (●, ○), DPCPX (●) in (a) and 8-PT in (b)) and used in the calculation of the correlation coefficients. For key to abbreviations used see legend to Figure 1. The broken line indicates a gradient of unity. Note the absence of correlation between the affinities of the xanthines for the adenosine receptor at the neuromuscular junction and for the A_1 - or the A_2 -adenosine receptors.

liams *et al.*, 1987), no correlation was observed between the potencies of the 1,3,8-substituted xanthines at the A_1 -adenosine receptor and at the adenosine receptor at the frog neuromuscular junction (see legend to Figure 4).

The ability of 1,3,8-substituted xanthine derivatives to inhibit NECA-induced stimulation of adenylate cyclase in human platelets has been studied in order to characterize the antagonist profile of the A_2 -adenosine receptor (Schwabe *et al.*, 1985; Ukena *et al.*, 1986b; Lohse *et al.*, 1987). In these studies it was shown that XAC ($K_i = 24-25$ nM) > DPX (210 nM), DPCPX (390 nM), PACPX (470 nM) > 8-PT (1.9–4.1 μ M), XCC (2.4 μ M). The low potency of XCC observed in the present work, taken together with the finding that PGDMX was much more potent than caffeine (also a characteristic of the A_2 -adenosine receptor (Ukena *et al.*, 1986c)), could indicate that the adenosine receptor at the frog neuromuscular junction has an antagonist profile compatible with that of the A_2 -adenosine receptor. However, in disagreement with this idea is the absence of correlation between the potencies of the 1,3,8-substituted xanthines as antagonists of the A_2 -adenosine receptor at human platelets and as antagonists of the adenosine receptor at the frog neuromuscular junction (Figure 4b). Also, no correl-

ation was observed when PACPX was not included (see legend to Figure 4).

The adenosine receptor at the frog (Ribeiro & Sebastião, 1985) and rat (Sebastião & Ribeiro, 1988b) neuromuscular junctions has an agonist profile different from that of the A_1 - and A_2 -adenosine receptors and was recently named A_3 (Ribeiro & Sebastião, 1986). In the present work it was shown that the antagonist profile of the adenosine receptor at the frog sartorius neuromuscular junction is also different from the antagonist profile of the A_1 - and A_2 -adenosine receptors. Since differences might result from different experimental methods, i.e., binding vs cyclase vs functional studies, or from species differences (Ukena *et al.*, 1986a), i.e., rat vs human vs frog, characterization of the antagonist profile of the adenosine receptor mediating presynaptic inhibition of transmission in other preparations and species is needed to know whether the adenosine receptor antagonist profile found at the frog sartorius neuromuscular junction represents the antagonist profile of the A_3 -adenosine receptor.

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References

- ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.
- BRUNS, R.F. (1981). Adenosine antagonism by purines, pteridines and benzopteridines in human fibroblasts. *Biochem. Pharmacol.*, **30**, 325–333.
- BRUNS, R.F., DALY, J.W. & SNYDER, S.H. (1983). Adenosine receptor binding: structure-activity analysis generates extremely potent xanthine antagonists. *Proc. Natl. Acad. Sci., U.S.A.*, **80**, 2077–2080.
- BURNSTOCK, G. & HOYLE, C.H.V. (1985). PACPX – a substituted xanthine – antagonizes both the A_1 and A_2 subclasses of the P_1 -purinoceptor: antagonism of the A_2 subclass is competitive but antagonism of the A_1 subclass is not. *Br. J. Pharmacol.*, **85**, 291–296.
- COLLIS, M.G., JACOBSON, K.A. & TOMKINS, D.M. (1987). Apparent affinity of some 8-phenyl-substituted xanthines at adenosine receptors in guinea-pig aorta and atria. *Br. J. Pharmacol.*, **92**, 69–75.
- DALY, J.W. (1985). Adenosine receptors in the central nervous system: structure-activity relationships for agonists and antagonists. In *Purines: Pharmacology and Physiological Roles*, ed. Stone, T.W., pp. 5–15. London: Macmillan Press.
- DALY, J.W., BRUNS, R.F. & SNYDER, S.H. (1981). Adenosine receptors in the central nervous system: relationship to the central actions of methylxanthines. *Life Sci.*, **28**, 2083–2097.
- DALY, J.W., UKENA, D. & JACOBSON, K.A. (1987). Analogues of adenosine, theophylline, and caffeine: selective interactions with A_1 and A_2 adenosine receptors. In *Topics and Perspectives in Adenosine Research*, ed. Gerlach, E. & Becker, B.F., pp. 23–26. Berlin: Springer-Verlag.
- KENAKIN, T.P. (1987). *Pharmacologic Analysis of Drug-Receptor Interaction*. New York: Raven Press.
- KHARKEVICH, D.A. (1986). Methods for experimental evaluation of neuromuscular blocking agents. In *New Neuromuscular Blocking Agents*, ed. Kharkevich, D.A., pp. 279–299. Berlin: Springer-Verlag.
- LOHSE, M.J., KLOTZ, K.-N., LINDENBORN-FOTINOS, J., REDDINGTON, M., SCHWABE, U. & OLSSON, R.A. (1987). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) – a selective high affinity antagonist radioligand for A_1 adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **336**, 204–210.
- LONDOS, C., COOPER, D.M.F. & WOLFF, J. (1980). Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 2551–2554.
- MARTINSON, E.A., JOHNSON, R.A. & WELLS, J.N. (1987). Potent adenosine receptor antagonists that are selective for the A_1 receptor subtype. *Molec. Pharmacol.*, **31**, 247–252.
- PERSSON, C.G.A., ANDERSSON, K.-E. & KJELLIN, G. (1986). Effects of enprofylline and theophylline may show the role of adenosine. *Life Sci.*, **38**, 1057–1072.
- RIBEIRO, J.A. & SEBASTIÃO, A.M. (1985). On the type of receptor involved in the inhibitory action of adenosine at the neuromuscular junction. *Br. J. Pharmacol.*, **84**, 911–918.

- RIBEIRO, J.A. & SEBASTIÃO, A.M. (1986). Adenosine receptors and calcium: basis for proposing a third (A_3) adenosine receptor. *Prog. Neurobiol.*, **26**, 179–209.
- RIBEIRO, J.A. & SEBASTIÃO, A.M. (1987). On the role, inactivation and origin of endogenous adenosine at the frog neuromuscular junction. *J. Physiol.*, **384**, 571–585.
- SCHWABE, U., UKENA, D. & LOHSE, M.J. (1985). Xanthine derivatives as antagonists at A_1 and A_2 adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **330**, 212–221.
- SEBASTIÃO, A.M. & RIBEIRO, J.A. (1988a). On the antagonist profile of the adenosine receptor in the frog neuromuscular junction. *Br. J. Pharmacol.*, **93**, 235P.
- SEBASTIÃO, A.M. & RIBEIRO, J.A. (1988b). On the adenosine receptor and adenosine inactivation at the rat diaphragm neuromuscular junction. *Br. J. Pharmacol.*, **94**, 109–120.
- SILINSKY, E.M. (1980). Evidence for specific adenosine receptors at cholinergic nerve endings. *Br. J. Pharmacol.*, **71**, 191–194.
- UKENA, D., SCHIRREN, C.G. & SCHWABE, U. (1985). Effects of enprofylline on A_1 and A_2 adenosine receptors. *Eur. J. Pharmacol.*, **117**, 25–33.
- UKENA, D., JACOBSON, K.A., PADGETT, W.L., AYALA, C., SHAMIM, M.T., KIRK, K.L., OLSSON, R.O. & DALY, J.W. (1986a). Species differences in structure-activity relationships of adenosine agonists and xanthine antagonists at brain A_1 adenosine receptors. *FEBS Letts*, **209**, 122–128.
- UKENA, D., DALY, J.W., KIRK, K.L. & JACOBSON, K.A. (1986b). Functionalized congeners of 1,3-dipropyl-8-phenylxanthine: potent antagonists for adenosine receptors that modulate membrane adenylate cyclase in pheochromocytoma cells, platelets and fat cells. *Life Sci.*, **38**, 797–807.
- UKENA, D., SHAMIM, M.T., PADGETT, W. & DALY, J.W. (1986c). Analogs of caffeine: antagonists with selectivity for A_2 adenosine receptors. *Life Sci.*, **39**, 743–750.
- VAN CALKER, D., MULLER, M. & HAMPRECHT, B. (1979). Adenosine regulates via two different types of receptors the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.*, **33**, 999–1005.
- WILLIAMS, M., JARVIS, M.F., SILLS, M.A., FERKANY, J.W. & BRAUNWALDER, A. (1987). 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. *Biochem. Pharmacol.*, **36**, 4024–4027.

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