British Journal of Pharmacology (1997) 122, 1202–1208

Species differences in brain adenosine A_1 receptor pharmacology revealed by use of xanthine and pyrazolopyridine based antagonists

Takuya Maemoto, *Keith Finlayson, *Henry J. Olverman, **Atsushi Akahane, ***Roger W. Horton & [1]Steven P. Butcher

Fujisawa Institute of Neuroscience, *Department of Pharmacology, University of Edinburgh, Edinburgh EH8 9JZ; **New Drug Research Laboratories, Fujisawa Pharmaceutical Co. Ltd., Osaka 532, Japan and ***Department of Pharmacology, St. George's Hospital Medical School, London SW17 0RE

1 The pharmacological profile of adenosine A_1 receptors in human, guinea-pig, rat and mouse brain membranes was characterized in a radioligand binding assay by use of the receptor selective antagonist, [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]-DPCPX).

2 The affinity of [^{3}H]-DPCPX binding sites in rat cortical and hippocampal membranes was similar. Binding site affinity was higher in rat cortical membranes than in membranes prepared from guinea-pig cortex and hippocampus, mouse cortex and human cortex. pK_D values (M) were 9.55, 9.44, 8.85, 8.94, 8.67, 9.39 and 8.67, respectively. The binding site density (B_{max}) was lower in rat cortical membranes than in guinea-pig or human cortical membranes.

3 The rank order of potency of seven adenosine receptor agonists was identical in each species. With the exception of 5′-N-ethylcarboxamidoadenosine (NECA), agonist affinity was 3.5–26.2 fold higher in rat cortical membranes than in human and guinea-pig brain membranes; affinity in rat and mouse brain membranes was similar. While NECA exhibited 9.3 fold higher affinity in rat compared to human cortical membranes, affinity in other species was comparable. The stable GTP analogue, Gpp(NH)p (100 μM) reduced 2-chloro-N^6-cyclopentyladenosine (CCPA) affinity 7–13.9 fold, whereas the affinity of DPCPX was unaffected.

4 The affinity of six xanthine-based adenosine receptor antagonists was 2.2–15.9 fold higher in rat cortical membranes compared with human or guinea-pig membranes. The rank order of potency was species-independent. In contrast, three pyrazolopyridine derivatives, (**R**)-1-[(E)-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl) acryloyl]-2-piperidine ethanol (FK453), (**R**)-1-[(E)-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl) acryloyl]-piperidin-2-yl acetic acid (FK352) and 6-oxo-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl)-1(6H)-pyridazinebutyric acid (FK838) exhibited similar affinity in human, guinea-pig, rat and mouse brain membranes. pK_i values (M) for [^{3}H]-DPCPX binding sites in human cortical membranes were 9.31, 7.52 and 7.92, respectively.

5 Drug affinity for adenosine A_{2A} receptors was determined in a [^{3}H]-2-[4-(2-carboxyethyl)phenethylamino]-5′-N-ethylcarboxamidoadenosine ([^{3}H]-CGS 21680) binding assay in rat striatal membranes. The pyrazolopyridine derivatives, FK453, FK838 and FK352 exhibited pK_i values (M) of 5.90, 5.92 and 4.31, respectively, compared with pK_i values of 9.31, 8.18 and 7.57 determined in the [^{3}H]-DPCPX binding assay in rat cortical membranes. These novel pyrazolopyridine derivatives therefore represent high affinity, adenosine A_1 receptor selective drugs that, in contrast to xanthine based antagonists, exhibit similar affinity for [^{3}H]-DPCPX binding sites in human, rat, mouse and guinea-pig brain membranes.

Keywords: Adenosine receptors; [^{3}H]-DPCPX; [^{3}H]-CGS 21680; radioligand binding; FK453; brain

Introduction

Although adenosine receptors were originally subdivided into two classes according to functional data (van Calker *et al*., 1979), recent pharmacological and molecular cloning studies suggest the existence of at least four subclasses, termed A_1, A_{2A}, A_{2B} and A_3 receptors (Fredholm *et al*., 1994; Palmer & Stiles, 1995). The adenosine A_1 receptor has been particularly well characterized in both pharmacological and functional studies (Linden, 1991; Fredholm *et al*., 1994; Palmer & Stiles, 1995). Receptor selective agonists, such as N^6-cyclopentyladenosine (CPA) and 2-chloro-N^6-cyclopentyladenosine (CPA) (Williams *et al*., 1986; Klotz *et al*., 1989), and xanthine-based antagonists are available (Schwabe *et al*., 1985; Bruns *et al*., 1987; Lohse *et al*., 1987); [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]-DPCPX) is presently the ligand of choice for use in radioligand binding assays (Bruns *et al*., 1987; Lohse *et al*., 1987).

Marked species differences in the affinity of adenosine receptor agonists and antagonists have been described (Murphy & Snyder, 1982; Ferkany *et al*., 1986; Ukena *et al*., 1986; Klotz *et al*., 1991; Nonaka *et al*., 1996). Molecular cloning studies of the adenosine A_1 receptor from dog, human, rat, cow, and guinea-pig suggest that these pharmacological discrepancies may be related to the primary amino acid sequence of the receptor (Libert *et al*., 1991; 1992; Mahan *et al*., 1991; Reppert *et al*., 1991; Townsend-Nicholson & Shine, 1992; Tucker *et al*., 1992; Olah *et al*., 1992; Meng *et al*., 1994). Differences in G protein coupling with adenosine A_1 receptors purified from rat, human, and bovine brain (Jockers *et al*., 1994; Nanoff *et al*., 1995), and immunological differences between the human and rat receptor (Nakata, 1993), have also been demonstrated. In the present study a comparison was obtained of the pharmacological profile of three novel pyrazolopyridine derivatives, FK453 (Terai *et al*., 1995), FK352 and FK838 (Figure 1), and reference adenosine receptor agonists and antagonists, in a

[1] Author for correspondence at: Fujisawa Institute of Neuroscience, Dept. of Pharmacology, University of Edinburgh, 1 George Square, Edinburgh EH8 9JZ

[^{3}H]-DPCPX binding assay by use of brain membranes prepared from human, rat, mouse and guinea-pig. Drug selectivity for adenosine A_1, compared with A_{2A}, receptors was also assessed by use of a [^{3}H]-CGS 21680 binding assay with rat striatal membranes.

Methods

Human brain tissue

Human brain tissue was obtained at autopsy from 5 male patients with no history of psychiatric or neurological disease. The age of the subjects was 55.0 ± 2.5 years (mean ± s.e.mean), and the *post mortem* delay was 34.6 ± 9.6 h (mean ± s.e.mean). Parietal cortex (Brodman area 7) was dissected from fresh tissue, pooled (total weight 9.6 g), and frozen at −80°C until use.

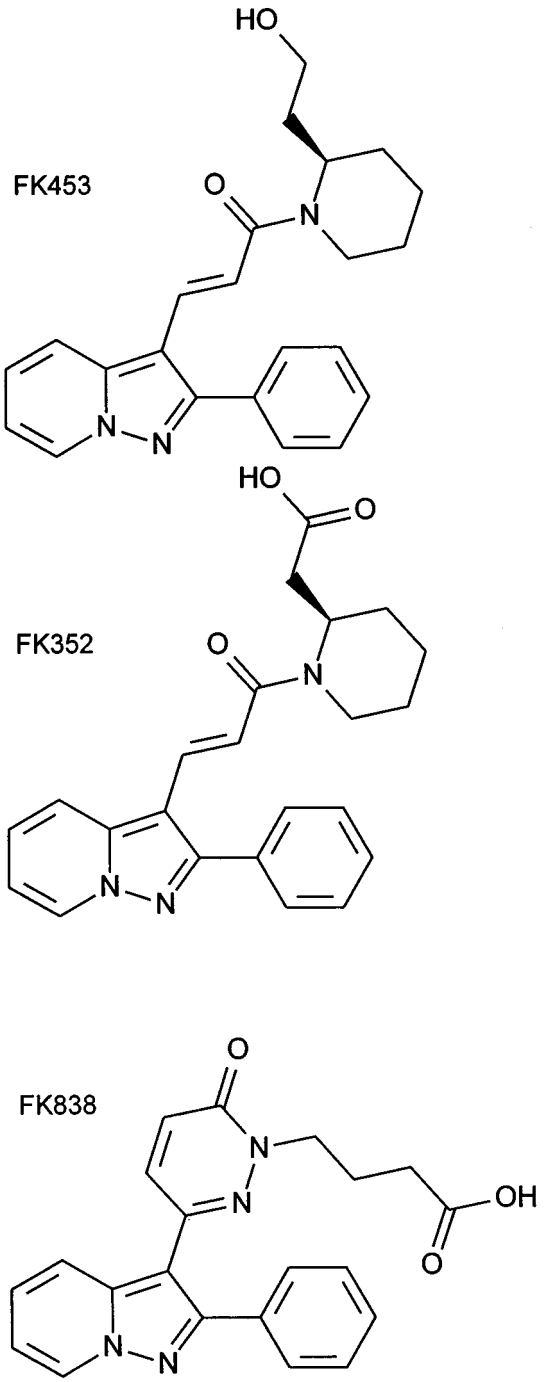

Figure 1 Chemical structures of the pyrazolopyridine derivatives, FK453, FK352 and FK838.

Membrane preparation

Male Sprague-Dawley rats (200–400 g; Charles-River), male Dunkin Hartley guinea-pigs (500–1000 g; Darley-Oak-Farm) and male HPG n/n mice (bred in-house) were killed by cervical dislocation. The brains were removed and immediately placed in ice-cold saline, before the cortex, striatum and hippocampus were dissected as appropriate. Animal and human tissues were homogenized in 15 volumes (vol) of 0.32 M sucrose with a glass/Teflon homogenizer, the homogenate centrifuged at 1,000 *g* for 10 min, and the resulting supernatant was centrifuged at 17,000 *g* for 20 min. The synaptosomal/mitochondrial P_2 pellet was lyzed in 30 vol of ice-cold water for 30 min, and centrifuged at 48,000 *g* for 10 min. The membrane pellet was resuspended in 30 vol of 50 mM Tris-HCl buffer (pH 7.4), centrifuged at 48,000 *g* for 10 min, resuspended in 5 vol of 50 mM Tris-HCl buffer (pH 7.4) and stored at −20°C. Frozen membranes were thawed on the day of use and resuspended in 30 vol of 50 mM Tris-HCl buffer (pH 7.4). The suspension was centrifuged at 48,000 *g* for 10 min, and the pellet resuspended in 200 vol of 50 mM Tris-HCl buffer (pH 7.4) and kept on ice before use in the binding assay; protein content was determined as described previously (Bradford, 1976).

[^{3}H]-DPCPX binding assay

[^{3}H]-DPCPX binding was assessed by use of a modification of previous methods (Bruns *et al*., 1987; Lohse *et al*., 1987). DMSO 10 μl or test drug was incubated with 100 μl of adenosine deaminase (ADA; 0.1 iu ml^{-1} final assay concentration), 290 μl of 50 mM Tris-HCl buffer (pH 7.4), 100 μl of [^{3}H]-DPCPX (NEN; 98.1 Ci mmol^{-1}) and 500 μl of membrane suspension. The final assay concentration of [^{3}H]-DPCPX was 0.1 nM for rat and mouse membranes, 0.2 nM for guinea-pig membranes and 0.7 nM for human membranes. Non-specific binding was determined in the presence of 10 μM **R**(-)N^6-(2-phenylisopropyl)adenosine (**R**-PIA). ADA was included to remove endogenous adenosine at a concentration that did not influence [^{3}H]-DPCPX binding (data not shown). Test compounds were prepared by serial dilution in DMSO; the final assay concentration of 1% DMSO did not affect [^{3}H]-DPCPX binding (data not shown). The incubation times were 20 min, 120 min and 60 min at 25°C for rat, mouse, human and guinea-pig membranes respectively; preliminary experiments confirmed that binding was at equilibrium under these conditions (data not shown). The binding assay was terminated by filtration onto glass filters (GF/B, Whatman) by use of a Brandel Cell Harvester, followed by three washes (3 ml) with 50 mM Tris-HCl buffer (pH 7.4). Filter disks were transferred to scintillation vials, 100 μl of formic acid was added, followed 10 min later by 4 ml Emulsifier SAFE scintillation fluid. Vials were left overnight before radioactivity was determined in a Packard 2500TR liquid scintillation analyser with automatic quench correction.

[^{3}H]-CGS 21680 binding assay

[^{3}H]-CGS 21680 binding was determined by use of identical conditions to the [^{3}H]-DPCPX binding assay except that 500 μl of rat striatal membrane suspension was added to 290 μl of 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$ (final assay concentration), 100 μl of [^{3}H]-CGS 21680 (NEN; 40.5 Ci mmol^{-1}; 2 nM final assay concentration), 100 μl of ADA (0.1 iu ml^{-1}; final assay concentration) and 10 μl of DMSO or test drug. Addition of ADA and DMSO (1% final assay concentration) did not affect [^{3}H]-CGS 21680 binding (data not shown). The incubation was continued for 60 min at 25°C before termination by filtration with a Brandel Cell

Harvester. Filter disks were washed and radioactivity determined as described previously. Non-specific binding was determined in the presence of 10 μM NECA.

Data analysis

Data were analysed by use of an iterative, non-linear least square curve fitting programme (SigmaPlot; Jandel, U.S.A.) to the logistic expression; $Y = M.\ IC_{50}{}^{P}/(I^{P} + IC_{50}{}^{P}) + B$, where P is the Hill coefficient and Y is bound ligand in the presence of inhibitor concentration, I; M and B are specific binding in the absence of inhibitor and non-specific binding, respectively. Estimates of M and B were within 10% of experimentally determined values. If the inhibitor was the unlabelled form of the radioligand, K_D and B_{max} were calculated by use of the equations; $K_D = IC_{50}$-[ligand] and $B_{max} = (M\ .\ IC_{50})$/[ligand]. For other test compounds, K_i values were calculated from the equation $K_i = IC_{50}/(1 + ([ligand]/K_D))$. Statistical comparisons were performed by use of commercially available software (JMP 3.2; SAS Institute Inc.). Before statistical analysis by ANOVA, box plots were inspected to ensure a normal symmetrical distribution of data, and the homogeneity of variance was confirmed to be within acceptable limits. Thereafter, ANOVA was used to demonstrate a significant interaction between the receptor affinity of each drug and the tissue source (data not shown), with further *post hoc* analysis by use of Dunnett's method.

Materials

CCPA, CPA, **R**-PIA, 2-[4-(2-carboxyethyl)phenylethylamino]-5′-N-ethylcarboxamido-adenosine (CGS 21680), 5′-N-ethylcarboxamidoadenosine (NECA), DPCPX, 2-chloroadenosine (CADO), N^6-cyclohexyladenosine (CHA), 1,3-diethyl-8-phenylxanthine (DPX), 8-cyclopentyl-1,3-dimethylxanthine (CPT), 8-phenyltheophylline (8-PT) were purchased from Research Biochemicals International (Natick, U.S.A.). 8-(Noradamantan-3-yl)-1,3-dipropylxanthine (KW 3902; Nonaka *et al*., 1996), KFM-19 (Linden, 1991), (**R**)-8-(1-phenylpropyl)-1,3-dipropylxanthine (MDL 102234; Dudley *et al*., 1992), (**R**)-1-[(E)-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl)acryloyl]-2-pipendine ethanol (FK453), (**R**)-1-[(E)-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl)acryloyl]pipendin-2-yl acetic acid (FK352) and 6-oxo-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl)-](6H0-pyridazinebutyric acid (FK838) were synthesized by Fujisawa Pharmaceutical Co. Ltd. (Japan). Adenosine deaminase Type III (ADA), 5′-guanylimidodiphosphate (Gpp(NH)p) and other chemicals were from Sigma (Poole, U.K.).

Results

Species differences in [^{3}H]-DPCPX binding site affinity (pK_D) and density (B_{max})

Competition experiments with membranes prepared from human, rat, mouse and guinea-pig brain tissue revealed marked species differences in [^{3}H]-DPCPX binding site affinity. pK_D values (M) ranged from 9.55 in rat cortical membranes to 8.67 in human cortical membranes (Figure 2; Table 1). Affinity in rat cortical membranes was significantly higher than in mouse, guinea-pig or human brain membranes ($P < 0.001$). The rank order of affinity was rat cortex $\cong$ rat hippocampus $\geqslant$ mouse cortex > guinea-pig hippocampus $\cong$ guinea-pig cortex > human cortex. [^{3}H]-DPCPX binding site affinity in cortical and hippocampal membranes prepared from the same species was similar (Table 1). Binding site density (B_{max}) was lower in rat cortical membranes than in guinea-pig and human brain membranes ($P < 0.05$; Table 1). The affinity of DPCPX was unaltered in the presence of Gpp(NH)p, a stable GTP analogue (100 μM; Table 2). Hill coefficients in the presence and absence of Gpp(NH)p were not significantly different from unity (data not shown).

Affinity of adenosine receptor antagonists for brain [^{3}H]-DPCPX binding sites

Although species differences in the affinity of adenosine receptor agonists were apparent, the rank order of potency was CCPA $\cong$ CPA $\geqslant$ **R**-PIA $\geqslant$ CHA > NECA $\cong$ CADO > > CGS 21680 in each membrane preparation (Table 2). With the ex-

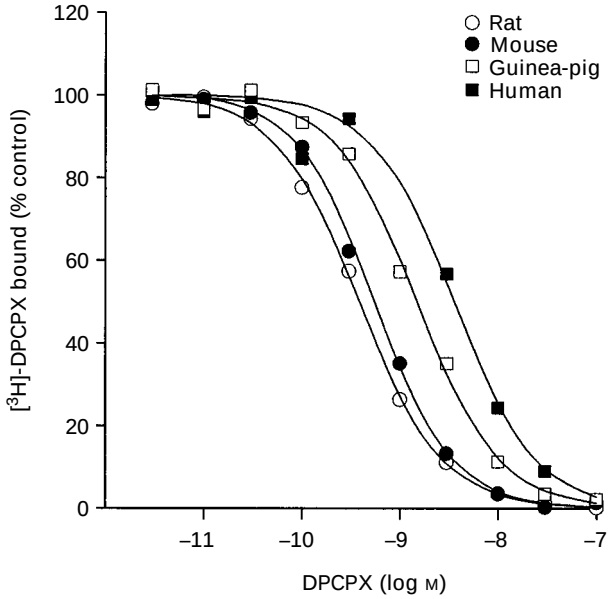

Figure 2 Inhibition of [^{3}H]-DPCPX binding to human, rat, mouse and guinea-pig cortical membranes by DPCPX. P_2 membranes were incubated with [^{3}H]-DPCPX (0.1 nM, rat and mouse; 0.2 nM, guinea-pig; 0.7 nM, human) in 50 mM Tris-HCl buffer (pH 7.4) containing unlabelled DPCPX and 0.1 iu ml^{-1} ADA. Binding was terminated after varying incubation times (20 min, rat and mouse; 60 min, guinea-pig; 120 min, human) by filtration with a Brandel Cell Harvester. Data shown are representative competition curves from a single experiment; pK_D and B_{max} values were determined from at least three independent experiments.

Table 1 Comparison of the affinity (pK_D) and binding site density (B_{max}) of [^{3}H]-DPCPX binding sites in membranes prepared from the indicated species and brain regions

		Rat		*Mouse*	*Guinea-pig*		*Human*
		Cortex	*Hippocampus*	*Cortex*	*Cortex*	*Hippocampus*	*Cortex*
Control	pK_D	9.55 ± 0.04	9.44 ± 0.05	9.39 ± 0.06***	8.85 ± 0.09***	8.94 ± 0.10***	8.67 ± 0.28***
	B_{max}	2.38 ± 0.49	3.86 ± 1.23	3.41 ± 0.73	5.26 ± 1.68***	4.50 ± 1.75**	4.96 ± 4.22*
+100mM	pK_D	9.56 ± 0.07	9.56 ± 0.02	9.33 ± 0.09***	8.84 ± 0.09***	8.82 ± 0.17***	n.d.
Gpp(NH)p	B_{max}	2.45 ± 1.77	3.60 ± 0.96	3.54 ± 0.29	4.27 ± 0.69*	5.78 ± 2.86***	n.d.

pK_D (M) and B_{max} (pmol/mg^{-1} protein) values are means ± 95% confidence limits determined from at least three independent experiments. Gpp(NH)p was included in the assay buffer at 100μM where indicated. Statistical analyses compared data with values obtained in rat cortex; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Dunnett's method). ND = not determined

Table 2 Affinity (pK_i) of adenosine receptor agonists for [^{3}H]-DPCPX binding sites in membranes prepared from the indicated species and brain regions

	Rat		*Mouse*	*Guinea-pig*		*Human*
	Cortex	*Hippocampus*	*Cortex*	*Cortex*	*Hippocampus*	*Cortex*
CCPA	8.48±0.08	8.50±0.48	8.33±0.29	7.60±0.19***	7.78±0.23***	7.33±1.22***
+Gpp(NH)p	7.43±0.24	7.61±0.34	7.14±0.06	6.69±0.26***	6.71±0.19***	6.43±0.18***
CPA	8.40±0.16	8.55±0.47	8.48±0.58	7.54±0.21***	7.49±0.34***	7.22±0.43***
R-PIA	8.29±0.08	8.20±0.21	8.21±0.17	7.30±0.29***	7.43±0.33***	7.08±0.48***
CHA	8.17±0.15	8.23±0.40	8.19±0.35	7.18±0.24***	7.28±0.39***	6.75±0.30***
NECA	7.52±0.15	7.75±0.17	7.83±0.17	7.29±0.40	7.52±0.42	6.53±0.47***
CADO	7.45±0.11	7.42±0.66	7.34±0.94	6.94±0.23**	6.86±0.29**	6.51±2.16***
CGS21680	4.44±0.26	n.d.	4.85±0.30*	4.66±0.52	4.65±0.25	3.95±0.08*

pK_i values (M) are means±95% confidence limits determined from at least three independent experiments. Gpp(NH)p was included in the assay buffer at 100 μM where indicated. Statistical analyses compared data with values obtained in rat cortex; *$P<0.05$, **$P<0.01$, ***$P<0.001$ (Dunnett's method). ND = not determined.

ceptions of NECA and CGS 21680, adenosine based receptor agonists exhibited 3.5–26.2 fold higher affinity in rat cortical membranes than in brain membranes prepared from human or guinea-pig tissue ($P<0.001$; Table 2); agonist affinity in rat and mouse cortical membranes was similar. Regional differences between cortical and hippocampal membranes prepared from the same species were not noted (Table 2). In the case of CCPA, pK_i values (M) ranged from 8.50 in rat hippocampal membranes to 7.33 in human cortical membranes (Figure 3; Table 2). While NECA exhibited 9.3 fold higher affinity in rat compared to human cortical membranes ($P<0.001$), affinity in other species was comparable (Table 2). The receptor selectivity of the [^{3}H]-DPCPX binding assay was confirmed by the finding that CGS 21680, a specific adenosine A_{2A} agonist, exhibited negligible affinity with pK_i values (M) ranging from 4.44 in rat cortical membranes to 3.95 in human cortical membranes. With the exception of CGS 21680 (Hill coefficient = 1), Hill coefficients of 0.6–0.8 were noted in each membrane preparation, suggesting the presence of more than one agonist affinity state (data not shown). The effect of Gpp(NH)p on the affinity of CCPA for [^{3}H]-DPCPX binding sites was also determined. In contrast to DPCPX, addition of Gpp(NH)p (100 μM) reduced CCPA affinity 7–13.9 fold ($P<0.05$), with no obvious inter-species differences (Table 2).

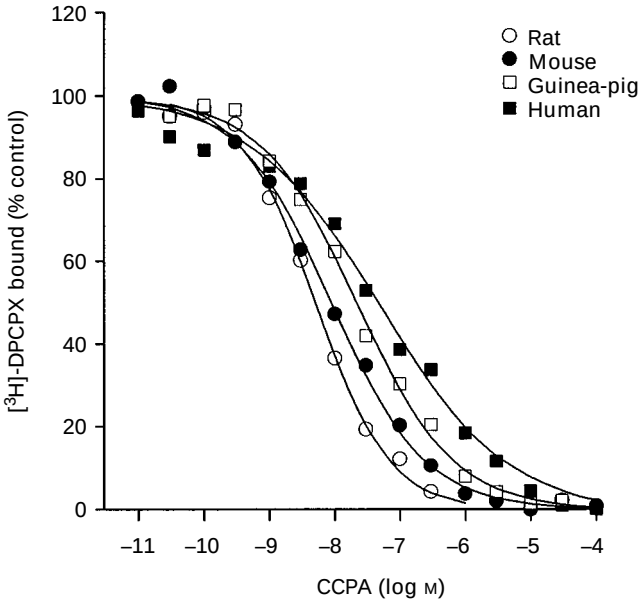

Figure 3 Inhibition of [^{3}H]-DPCPX binding to human, rat, mouse and guinea-pig cortical membranes by CCPA. P_2 membranes were incubated with [^{3}H]-DPCPX (0.1 nM, rat and mouse; 0.2 nM, guinea-pig; 0.7 nM, human) in 50 mM Tris-HCl buffer (pH 7.4) containing competing drug and 0.1 iu ml^{-1} ADA. Binding was terminated after varying incubation times (20 min, rat and mouse; 60 min, guinea-pig; 120 min, human) by filtration with a Brandel Cell Harvester. Data shown are representative competition curves from a single experiment; pK_i values were determined from at least three independent experiments.

Affinity of xanthine-based adenosine receptor antagonists for brain [^{3}H]-DPCPX binding sites

The affinity of xanthine-based adenosine receptor antagonists for [^{3}H]-DPCPX binding sites was species-dependent, despite a common rank order of potency of KW 3902 > DPCPX > KFM-19 > CPT ≅ MDL 102234 > DPX ≥ 8-PT (Tables 1 and 3). Regional differences between cortical and hippocampal membranes prepared from rat and guinea-pig were not noted (Table 3). Antagonist Hill coefficients were close to unity in all species, indicating the presence of a single antagonist affinity state (data not shown). The affinity of CPT, DPX and 8-PT was 4.8–15.9 fold higher in rat cortical membranes than in human or guinea-pig brain membranes ($P<0.001$); affinity in rat and mouse membranes was similar (Table 3). The affinity of KW 3902, KFM-19 and MDL 102234 was 2.2–8.3 fold higher in rat cortical membranes compared with human and guinea-pig brain membranes ($P<0.05$), whereas pK_i values in rat and mouse membranes were similar (Table 3).

Affinity of pyrazolopyridine derivatives for brain [^{3}H]-DPCPX binding sites

In contrast to data obtained with xanthine-based adenosine receptor antagonists, three novel pyrazolopyridine derivatives, FK453, FK838 and FK352 did not exhibit species differences in affinity for [^{3}H]-DPCPX binding sites (Table 3). For example, pK_i values (M) for FK453 and FK352 in rat cortical membranes were 9.31 and 7.57, respectively, compared with 9.31 and 7.52, respectively, in human cortical membranes (Figure 4; Table 3). The affinity of FK838 was two fold higher in rat cortical membranes than in human cortical membranes (Table 3), whereas pK_i values in rat hippocampal and human cortical membranes were similar. Hill coefficients of close to unity were noted in each species (data not shown).

Pharmacological selectivity studied in a [^{3}H]-CGS 21680 binding assay

Adenosine receptor selectivity was evaluated by use of a [^{3}H]-CGS 21680 binding assay with rat striatal membranes (Jarvis *et al*., 1989; Table 4). The pK_D of rat striatal [^{3}H]-CGS 21680 binding sites was 8.44, compared with a pK_i value in the [^{3}H]-DPCPX binding assay of 4.44, confirming the receptor selectivity of the assay (Tables 2 and 4). On the basis of pK_i values for [^{3}H]-DPCPX and [^{3}H]-CGS 21680 binding sites in rat cortical and striatal membranes, respectively (Tables 2 and 4),

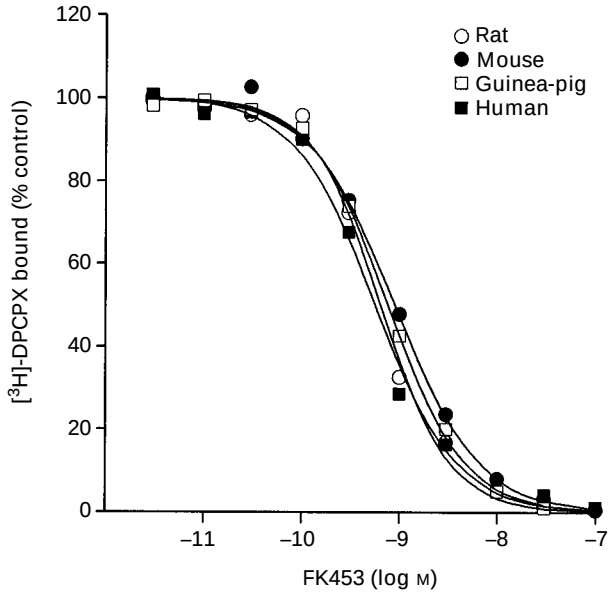

Figure 4 Inhibition of [^{3}H]-DPCPX binding to human, rat, mouse and guinea-pig cortical membranes by FK453. P_2 membranes were incubated with [^{3}H]-DPCPX (0.1 nM, rat and mouse; 0.2 nM, guinea-pig; 0.7 nM, human) in 50 mM Tris-HCl buffer (pH 7.4) containing competing drug and 0.1 iu ml^{-1} ADA. Binding was terminated after varying incubation times (20 min, rat and mouse; 60 min, guinea-pig; 120 min, human) by filtration with a Brandel Cell Harvester. Data shown are representative competition curves from a single experiment; pK_i values were determined from at least three independent experiments.

the adenosine receptor agonists CCPA, CPA, **R**-PIA, CHA, CADO and NECA showed 93, 46, 13, 43, 0.55 and 0.13 fold selectivity for adenosine A_1 compared with adenosine A_{2A} receptors. The xanthine based adenosine receptor antagonists, DPCPX, CPT, KFM-19, KW 3902, MDL 102234 exhibited 546, 129, 1464, 1352 and 100 fold selectivity for the rat cortical adenosine A_1 receptor (Tables 3 and 4). Similarly, the pyrazolopyridine derivatives, FK453, FK352 and FK838 showed 2514, 1855 and 181 fold selectivity for the rat cortical adenosine A_1 receptor (Tables 3 and 4).

Discussion

The novel pyrazolopyridine derivatives, FK453, FK352 and FK838 exhibit high affinity for [^{3}H]-DPCPX binding sites in brain membranes prepared from human, rat, mouse and guinea-pig. The adenosine A_1:A_{2A} receptor selectivity of these compounds was comparable to xanthine based antagonists; data concerning drug affinity for the adenosine A_3 receptor is not presently available. These data compliment functional studies demonstrating that FK453, FK352 and FK838 act as competitive adenosine A_1 receptor antagonists (Terai *et al*., 1995; Ito *et al*., unpublished observations). In contrast to xanthine based antagonists, the pyrazolopyridine derivatives exhibited similar affinity for [^{3}H]-DPCPX binding sites in each species tested. The use of well established radioligands (Bruns *et al*., 1987; Lohse *et al*., 1987; Jarvis *et al*., 1989), and the systematic nature of the present study, ensured the consistency and reliability of this finding.

While several studies have noted species differences in the pharmacological profile of native adenosine A_1 receptors (Murphy & Snyder, 1982; Ukena *et al*., 1986; Ferkany *et al*.,

Table 3 Affinity (pK_i) of adenosine receptor antagonists for [^{3}H]-DPCPX binding sites in membranes prepared from the indicated species and brain regions

	Rat		*Mouse*	*Guinea-pig*		*Human*
	Cortex	*Hippocampus*	*Cortex*	*Cortex*	*Hippocampus*	*Cortex*
KW 3902	9.90±0.13	10.2±1.09	9.84±0.65	9.43±0.05***	9.49±0.41**	9.53±0.24**
KFM-19	8.87±0.10	8.80±0.19	8.67±0.09	8.04±0.22***	8.09±0.14***	7.93±0.10***
CPT	8.26±0.15	8.23±0.15	8.14±0.07	7.53±0.05***	7.55±0.04***	7.31±0.12***
MDL 102234	8.15±0.06	8.20±0.30	8.05±0.72	7.60±0.37***	7.59±0.20***	7.44±0.09***
DPX	7.50±0.0	7.40±0.09	7.37±0.06	6.51±0.07***	6.52±0.08***	6.36±0.45***
8-PT	7.35±0.07	7.32±0.16	7.28±0.16	6.44±0.05***	6.45±0.14***	6.15±0.37***
FK453	9.31±0.09	9.37±0.13	9.26±0.33	9.19±0.09	9.21±0.07	9.31±0.76
FK838	8.18±0.09	7.96±0.13	7.99±0.34	8.00±0.09	8.12±0.07	7.92±0.76*
FK352	7.57±0.09	7.55±0.09	7.50±0.42	7.65±0.08	7.72±0.07	7.52±0.43

pK_i values (M) are means±95% confidence limits determined from at least three independent experiments. Statistical analyses compared data with values obtained in rat cortex; *$P<0.05$, **$P<0.01$, ***$P<0.001$ (Dunnett's method).

Table 4 Affinity (pK_i) of adenosine receptor agonist and antagonists for [^{3}H]-CGS 21680 binding sites in rat striatal membranes

Agonist	pK_i	*Binding site selectivity (A_1:A_{2A})*	*Antagonist*	pK_i	*Binding site selectivity (A_1:A_{2A})*
CGS 21680	8.44±0.46	0.0014	DPCPX	6.82±0.05	546
NECA	8.38±0.15	0.13	KW 3902	6.74±0.10	1352
CADO	7.70±0.15	0.55	DPX	6.58±0.10	8.1
R-PIA	7.18±0.17	13	8-PT	6.45±0.06	7.8
CPA	6.83±0.55	46	MDL 102234	6.15±0.28	100
CHA	6.54±0.21	43	CPT	6.11±0.06	129
CCPA	6.53±0.25	93	FK838	5.92±0.03	181
			FK453	5.90±0.16	2514
			KFM-19	5.68±0.19	1464
			FK352	4.31±0.41	1855

pK_i values (M) are means±95% confidence limits determined from at least three independent experiments. Binding site selectivity determined from drug affinities in [^{3}H]-CGS 21680 and [^{3}H]-DPCPX binding assays is also shown (values>1 indicate selectivity for [^{3}H]-DPCPX binding sites).

1986; Klotz *et al*., 1991), this is the first detailed study to employ an antagonist radioligand. Xanthine-based adenosine receptor antagonists exhibited higher affinity for [^{3}H]-DPCPX binding sites in rat cortical membranes, compared with human and guinea-pig brain membranes. Although similar findings have been obtained with agonist radioligands, such as [^{3}H]-**R**-PIA (Ukena *et al*., 1986) or [^{3}H]-CHA (Murphy & Snyder, 1982; Ferkany *et al*., 1986), inter-species differences in antagonist affinity were more pronounced in the present study. The affinity of adenosine receptor agonists for [^{3}H]-DPCPX binding sites was also higher in rat than in human brain membranes, whereas equivalent K_i values were noted in studies with agonist radioligands (Ukena *et al*., 1986; Ferkany *et al*., 1986). Indeed, the affinities of NECA and CADO were shown to be higher in human than in rat brain membranes in one study with a [^{3}H]-CHA binding assay (Ferkany *et al*., 1986), although these data conflicted with earlier findings (Murphy *et al*., 1982). Discrepancies in agonist affinity between studies employing agonist and antagonist radioligands can be expected because of differential binding to high and low affinity agonist binding states. Thus, agonist radioligands preferentially bind to a high affinity state (Williams *et al*., 1986; Klotz *et al*., 1989), whereas [^{3}H]-DPCPX binds to both high and low affinity states, as confirmed by the low agonist Hill coefficients noted in this and earlier studies (Burns *et al*., 1987; Lohse *et al*., 1987). However, the conflicting data concerning inter-species affinity ratios between studies in which agonist and antagonist radioligands were used remain unexplained. The effect of Gpp(NH)p on the affinity of CCPA for rat cortical [^{3}H]-DPCPX binding sites also provides evidence for the existence of high and low affinity agonist binding states, as shown previously (Lohse *et al*., 1987). While the finding that this stable GTP analogue did not affect [^{3}H]-DPCPX affinity and binding site density would appear to conflict with previous data, this may be due to the experimental conditions employed. Consistent with the present data, Gpp(NH)p effects on antagonist binding kinetics were noted in studies which included magnesium in the assay buffer (Ströher *et al*., 1989; Prater *et al*., 1992), but not in the absence of this divalent cation (Lohse *et al*., 1987; Olah & Stiles, 1990; Prater *et al*., 1992).

Perhaps the most interesting finding of the present study was that the affinity of xanthine based antagonists was species-dependent, whereas the affinity of pyrazolopyridine derivatives was similar in the four species examined. The molecular basis for species differences in the affinity of xanthine and pyrazolopyridine derivatives is intriguing, especially because the human, rat and guinea-pig cloned receptors exhibit approximately 90% sequence homology at the amino acid level (Mahan *et al*., 1991; Reppert *et al*., 1991; Libert *et al*., 1992; Townsend-Nicholson & Shine, 1992; Meng *et al*., 1994). However, this high degree of homology may not be of great importance because site directed mutagenisis studies on several members of the G protein linked receptor superfamily, including the 5-HT_{1B}, 5-HT_2, neurokinin and α_2-adrenoceptors, have demonstrated that a single amino acid can modulate ligand affinity and explain species differences in pharmacological profile (Oksenberg *et al*., 1992; Kao *et al*., 1992; Link *et al*., 1992; Jensen *et al*., 1994). In the case of the adenosine A_1, receptor, amino acid residues 270 and/or 277 in transmembrane domain 7 may be of critical importance (Tucker *et al*., 1994; Townsend-Nicholson & Scholfield, 1994). The former residue is involved in the binding of C^8 substituted xanthines and N^6 adenosine analogues, and the latter recognizes 5′-ribose derivatives. Interestingly, amino acid residue 270 may be largely responsible for pharmacological differences between canine and bovine adenosine A_1 receptors (Tucker *et al*., 1994). Since the isoleucine at this position of the rat receptor is replaced by a threonine in the human and guinea-pig receptor, this could also explain the inter-species differences in the binding site affinity of C^8 substituted xanthines and N^6 adenosine derivatives noted in this and earlier studies. While it is tempting to speculate that residue 270 is not crucial for binding of pyrazolopyridine derivatives, thereby explaining the maintenance of affinity across species, confirmation of this hypothesis will require further pharmacological studies at the molecular level. Such studies will also need to address the importance of other amino acid residues, including histidine residues 251 and 278 (Olah *et al*., 1992), as well as other regions of the receptor. For example, mutagenisis studies suggest that the second extracellular loop and transmembrane domains 1–4 may be involved in ligand recognition (Olah *et al*., 1994; Rivkess *et al*., 1995). In conclusion, the present study suggests that different structural determinants may be responsible for binding of xanthine and pyrazolopyridine derivatives to the adenosine A_1 receptor.

K.F. was supported by an MRC Fellowship.

References

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biol.*, **72,** 248–254.

BRUNS, R.F., FERGUS, J.H., BADGER, E.W., BRISTOL, J.A., SANTAY, L.A., HARTMAN, J.D., HAYS, S.J. & HUANG, C.C. (1987). Binding of the A_1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **335,** 59–63.

DUDLEY, M., RACKE, M., OGDEN, A.M., PEET, N., SECREST, R. & MCDERMOTT, R. (1992). MDL102,234: A selective adenosine A_1 receptor antagonist reflecting a new binding mode to the receptor. *Soc. Neurosci. Abs.*, **18,** 419.12.

FERKANY, J.W., VALENTINE, H.L., STONE, G.A. & WILLIAMS, M. (1986). Adenosine A_1 receptors in mammalian brain: Species differences in their interactions with agonists and antagonists. *Drug Devel. Res.*, **9,** 85–93.

FREDHOLM, B.B., ABBRACCHIO, M.A., BURNSTOCK, G., DALY, J.W., KENDALL HARDEN, T., JACOBSON, K.A., LEFF, P. & WILLIAMS, M. (1994). Nomenclature and classification of purinoreceptors. *Pharmacol. Rev.*, **46,** 143–156.

JARVIS, M.F., SCHULZ, R., HUTCHISON, A.J., DO, U.H., SILLS, M.A. & WILLIAMS, M. (1989). [^{3}H]CGS 21680, a selective A_2 adenosine receptor agonist directly labels A_2 receptors in rat brain. *J. Pharmacol. Exp. Ther.*, **251,** 888–893.

JENSEN, C.J., GERARD, N.P., SCHWARTZ, T.W. & GETHER, U. (1994). The species selectivity of chemically distinct tachykinin nonpeptide antagonists is dependent on common divergent residues of the rat and human neurokinin-1 receptor. *Mol. Pharmacol.*, **45,** 294–299.

JOCKERS, R., LINDER, M.E., HOHENENEGGER, M., NANOFF, C., BERTIN, B., STROSBERG, A.D., MARULLO, S. & FREISSMUTH, M. (1994). Species differences in the G protein selectivity of the human and bovine A_1-adenosine receptor. *J. Biol. Chem.*, **269,** 32077–32084.

KAO, H.-T., ADNAM, N., OLSEN, M.A., WEINSHANK, R.L., BRANCHEK, T.A. & HARTIG, P.R. (1992). Site-directed mutagenisis of a single residue changes the binding properties of the serotonin 5-HT_2 receptor from a human to a rat pharmacology. *FEBS Lett.*, **307,** 324–328.

KLOTZ, K.-N., LOHSE, M.J., SCHWABE, U., CRISTALLI, G., VITTORI, S. & GRIFANTINI, M. (1989). 2-Chloro-N^6-[^{3}H]cyclopentyladenosine ([^{3}H]CCPA) – a high affinity agonist radioligand for A_1 adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **340,** 679–683.

KLOTZ, K.-N., VOGT, H. & TAWFIK-SCHLIEPER, H. (1991). Comparison of A_1 adenosine receptors in brain from different species by radioligand binding and photoaffinity labeling. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **343,** 196–201.

LIBERT, F., SCHIFFMANN, S.N., LEFORT, A., PARMENTIER, M., GÉRARD, C., DUMONT, J.E., VANDERHARGHEN, J.-J. & VASSART, G. (1991). The orphan receptor cDNA RDC7 encodes an A_1 adenosine receptor. *EMBO J.*, **10,** 1677–1682.

LIBERT, F., VAN SANDE, J., LEFORT, A., CZERNILOFSKY, A., DUMONT, J.D., VASSART, G., ENSINGER, H.A. & MENDLA, K.D. (1992). Cloning and functional characterization of a human A_1 adenosine receptor. *Biochem. Biophys. Res. Commun.*, **187,** 919–926.

LINDEN, J. (1991). Structure and function of A_1 adenosine receptors. *FASEB J.*, **5,** 2668–2676.

LINK, R., DAUNT, D., BARSH, G., CHRUSCINSKI, A. & KOBILKA, B. (1992). Cloning of two mouse genes encoding α_2-adrenergic receptor subtypes and identification of a single amino acid in the mouse α_2-C10 homologue responsible for the interspecies variation in antagonist binding. *Mol. Pharmacol.*, **42,** 16–27.

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.

MAHAN, L.C., MCVITTIE, L.D., SMYK-RANDALL, E.M., NAKATA, H., FREDERICK, J., MONSMA, J., GERFEN, C.R. & SIBLEY, D.R. (1991). Cloning and expression of an A_1 adenosine receptor from rat brain. *Mol. Pharmacol.*, **40,** 1–7.

MENG, F., XIE, G.-X., CHALMERS, D., MORGAN, C., WATSON, S.J., Jr. & AKIL, H. (1994). Cloning and characterization of a pharmacologically distinct A_1 adenosine receptor from guinea pig brain. *Mol. Brain Res.*, **26,** 143–155.

MURPHY, K.M.M. & SNYDER, S. (1982). Heterogeneity of adenosine A_1 receptors in brain tissue. *Mol. Pharmacol.*, **22,** 250–257.

NAKATA, H. (1993). Development of an antiserum to rat brain A_1 adenosine receptor: application for immunological and structural comparison of A_1 adenosine receptors from various tissues and species. *Biochem. Biophys. Acta*, **1177,** 93–98.

NANOFF, C., MITTERAUER, T., ROKA, F., HOHENEGGER, M. & FREISSMUTH, M. (1995). Species differences in A_1 adenosine receptor/G protein coupling: Identification of a membrane protein that stabilises the association of the receptor/G protein complex. *Mol. Pharmacol.*, **48,** 806–817.

NONAKA, H., ICHIMURA,M., TAKEDA, M., KANDA, T., SHIMADA, J., SUZUKI, F. & KASE, H. (1996). KW 3902, a selective high affinity antagonist for adenosine A_1 receptors. *Br. J. Pharmacol.*, **117,** 1645–1652.

OLAH, M.E. & STILES, G.L. (1990). Agonists and antagonists recognize different but overlapping populations of A_1 adenosine receptors: Modulation of receptor number by $MgCl_2$, solubilisation and guanine nucleotides. *J. Neurochem.*, **55,** 1432–1438.

OLAH, M.E., REN, H., OSTROWSKI, J., JACOBSON, K.A. & STILES, G.L. (1992). Cloning, expression, and characterization of the unique bovine A_1 adenosine receptor: Studies on the ligand binding site by site-directed mutagenisis. *J. Biol. Chem.*, **267,** 10764–10770.

OLAH, M.E., JACOBSON, K.A. & STILES, G.L. (1994). Role of the second extracellular loop of adenosine receptors in agonist and antagonist binding: Analysis of chimeric A_1/A_3 adenosine receptors. *J. Biol. Chem.*, **269,** 24692–24698.

OKSENBERG, D., MARSTERS, S.A., O'DOWD, B.F., JIN, H., HAVLIK, S., PEROUTKA, S.J. & ASHKENAZI, A. (1992). A single amino-acid difference confers major pharmacological variation between human and rodent 5-HT_{1b} receptors. *Nature*, **360,** 161–163.

PALMER, T.M. & STILES, G.L. (1995). Neurotransmitter receptors VII: Adenosine receptors. *Neuropharmacology*, **34,** 683–694.

PRATER, M.E., TAYLOR, H., MUNSHI, R. & LINDEN, J. (1992). Indirect effect of guanine nucleotides on antagonist binding to A_1 adenosine receptors: Occupation of Cryptic binding sites by endogenous vesicular adenosine. *Mol. Pharmacol.*, **42,** 765–772.

REPPERT, S.M., WEAVER, D.R., STEHLE, J.H. & RIVKESS, S.A. (1991). Molecular cloning and Characterization of a rat A_1 adenosine receptor that is widely expressed in brain and spinal cord. *Mol. Endocrinol.*, **5,** 1037–1048.

RIVKESS, S.A., LASBURY, M.E. & BARBHAIYA, H. (1995). Identification of domains of the human A_1 adenosine receptor that are important for binding receptor subtype-selective ligands using chimeric A_1/A_{2a} adenosine receptors. *J. Biol. Chem.*, **270,** 20485–20490.

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.

STRÖHER, M., NANOFF, C. & SCHULTZ, W. (1989). Differences in the GTP-regulation of membrane-bound and solubilised A_1-adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **340,** 87–92.

TERAI, T., KITA, Y., KUSUNOKI, T., SHIMAZAKI, T., ANDO, T., HORIAI, H., AKAHANE, A., SHIOKAWA, Y. & YOSHIDA, K. (1995). A novel non-xanthine adenosine A_1 receptor antagonist. *Eur. J. Pharmacol.*, **279,** 217–225.

TOWNSEND-NICHOLSON, A. & SHINE, J. (1992). Molecular cloning and characterisation of a human brain A_1 adenosine receptor cDNA. *Mol. Brian Res.*, **16,** 365–370.

TOWNSEND-NICHOLSON, A. & SCHOLFIELD, P.R. (1994). A threonine residue in the seventh transmembrane domain of the human A_1 adenosine receptor mediates specific agonist binding. *J. Biol. Chem.*, **269,** 2373–2376.

TUCKER, A.L., LINDEN, J., ROBEVA, A.S., D'ANGELO, D.D. & LYNCH, K.R. (1992). Cloning and expression of a bovine adenosine A_1 receptor cDNA. *FEBS Lett.*, **297,** 107–111.

TUCKER, A.M., ROBEVA, A.S., TAYLOR, H.E., HOLETON, D., BOCKNER, M., LYNCH, K.R. & LINDEN, J. (1994). A_1 adenosine receptors: Two amino acids are responsible for species differences in ligand recognition. *J. Biol. Chem.*, **269,** 27900–27906.

UKENA, D., JACOBSON, K.A., PADGETT, W.L., AYALA, C., SHAMIM, M.T., KIRK, K.L., OLSSON, R.O. & DALY, J.W. (1986). Species differences in structure-activity relationships of adenosine agonists and xanthine antagonists at brain A_1 adenosine receptors. *FEBS Lett.*, **209,** 122–128.

VAN CALKER, D., MULLER, D. & HAMPRECHT, B. (1979). Adenosine regulates via two different types of receptors the accumulation of cyclic AMP in culutred brain cells. *J. Neurochem.*, **33,** 999–1005.

WILLIAMS, M., BRAUNWALDER, A. & ERIKSON, T.M. (1986). Evaluation of the binding of the A-1 selective adenosine radioligand, cyclopentyladenosine (CPA), to rat brain tissue. *Naunyn-Schiedeberg's Arch. Pharmacol.*, **332,** 179–183.

(Received February 26, 1997
Revised June 2, 1997
Accepted July 30, 1997)