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Pyrazolopyridine derivatives act as competitive antagonists of brain adenosine A_1 receptors: [^{35}S]GTPγS binding studies

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Abstract

The effects of adenosine receptor ligands and three novel pyrazolopyridine derivatives on guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding to rat cerebral cortical membranes were examined. [^{35}S]GTPγS binding was stimulated in a concentration dependent manner by several adenosine receptor agonists. The adenosine A_{2a} receptor selective agonist, 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS 21680), was ineffective confirming specificity for adenosine A_1 receptor activation. 2-Chloro-N^6-cyclopentyladenosine (CCPA; 10^{-7} M)-stimulated [^{35}S]GTPγS binding was inhibited by xanthine and pyrazolopyridine based adenosine receptor antagonists. The concentration–response curve for CCPA-stimulated [^{35}S]GTPγS binding was shifted to the right with increasing concentrations of antagonist without significant changes in maximal response. Schild analyses determined pK_B values of 8.97, 8.88, 8.21, 8.16, 7.79 and 7.65 for 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), (*R*)-1-[(*E*)-3-(2-phenylpyrazolo[1,5*a*]pyridin-3-yl) acryloyl]-2-piperidine ethanol (FK453), 6-oxo-3-(2-phenylpyrazolo[1,5*a*]pyridin-3-yl)-1(6*H*)-pyridazinebutyric acid (FK838), 9-chloro-2-(2-furyl)[1,2,4]triazolo-[1,5*c*]quinazolin-5-amine (CGS 15943), 8-cyclopentyl-1,3-methylxanthine (CPT) and (*R*)-1-[(*E*)-3-(2-phenylpyrazolo[1,5*a*]pyridin-3-yl) acryloyl]-piperidin-2-yl acetic acid (FK352), respectively. Schild slopes were close to unity, confirming that these novel pyrazolopyridine derivatives act as competitive antagonists at rat brain adenosine A_1 receptors.

Keywords: Adenosine A_1 receptor; [^{35}S]GTPγS binding; Cortex, rat; FK453; Schild analysis

1. Introduction

Recent molecular cloning and pharmacological studies have resulted in the classification of the G-protein linked adenosine receptor family into four subclasses, termed A_1, A_{2a}, A_{2b} and A_3 receptors (Fredholm et al., 1994; Palmer and Stiles, 1995). However, the pharmacological profile of adenosine A_1 and A_2 receptors was originally characterised on the basis of inhibition and stimulation of adenylate cyclase respectively (Van Calker et al., 1979; Londos et al., 1980). Subsequent studies on native and recombinant adenosine A_1 receptors have confirmed this interaction, and have demonstrated functional coupling to other signal transduction pathways, including phospholipase C, K^+ and Ca^{2+} channels (Libert et al., 1992; Townsend-Nicholson and Shine, 1992; Mogul et al., 1993; Yawo and Chuhma, 1993; Mynlieff and Beam, 1994; Akbar et al., 1994; Alexander et al., 1994; Freund et al., 1994; Iredale et al., 1994; Pan et al., 1994, 1995). This promiscuity may result from the ability of adenosine A_1 receptors to interact with multiple members of the G-protein family (Munshi et al., 1991).

The diversity of effector systems potentially linked to the adenosine A_1 receptor suggests that pharmacological evaluation of functional activity by measurement of a single effector system (e.g., cAMP) may be inappropriate. Since agonist-induced exchange of GTP for GDP on G proteins represents a common activation step in all relevant signal transduction pathways, this provides a convenient assay of functional activity. Thus, an agonist-induced increase in guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding has been reported following adenosine A_1 receptor activation (Lorenzen et al., 1993; Sweeney

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and Dolphin, 1995; Lorenzen et al., 1996), a finding replicated for other members of the G-protein receptor superfamily including β-adrenoceptors (Asano et al., 1984; May and Ross, 1988), muscarinic receptors (Hilf et al., 1989; Lazareno and Birdsall, 1993), α_2-adrenoceptors (Tian et al., 1994) and μ-opioid receptors (Traynor and Nahorski, 1995). The present study determined the effects of adenosine receptor agonists on [^{35}S]GTPγS binding to rat cortical membranes, and examined inhibition of 2-chloro-N^6-cyclopentyladenosine (CCPA)-stimulated [^{35}S]GTPγS binding by xanthine-based adenosine A_1 receptor antagonists and the novel pyrazolopyridine derivatives, (*R*)-1-[(*E*)-3-(2-phenylpyrazolo[1,5*a*]pyridin-3-yl) acryloyl]-2-piperidine ethanol (FK453), 6-oxo-3-(2-phenylpyrazolo[1,5*a*]pyridin-3-yl)-1(6*H*)-pyridazinebutyric acid (FK838) and (*R*)-1-[(*E*)-3-(2-phenylpyrazolo[1,5*a*]pyridin-3-yl) acryloyl]-piperidin-2-yl acetic acid (FK352), using Schild analysis.

2. Materials and methods

2.1. Membrane preparation

Cerebral cortices from male Sprague–Dawley rats (280–350 g; Charles River) were homogenised in 15 volumes (vol.) of 0.32 M sucrose. The homogenate was centrifuged at $1000 \times g$ for 10 min, and the supernatant recentrifuged at $17{,}000 \times g$ for 20 min. The resulting synaptosomal/mitochondrial P_2 pellet was lysed in 30 vol. of ice cold water for 30 min, and then centrifuged at $48{,}000 \times g$ for 10 min. The pellet was resuspended in 30 vol. of 50 mM Tris–HCl (pH 7.4), centrifuged at $48{,}000 \times g$ for 10 min, and the final pellet resuspended in 50 mM Tris–HCl (pH 7.4) at a protein concentration of 1.6 mg/ml for storage at −20°C until use; all procedures were performed at 4°C. Membrane protein was measured using bovine serum albumin (BSA) as the standard (Bradford, 1976).

2.2. [^{35}S]GTPγS binding assay

Membranes were incubated with 0.2 nM [^{35}S]GTPγS (1245 Ci/mmol; 50 fmol/tube; NEN) in 50 mM Tris–HCl (pH 7.4) buffer containing a final assay concentration of 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, 0.2 IU/ml adenosine deaminase (ADA), 0.5% BSA and test drug (Lorenzen et al., 1993). Non-specific binding was determined in the presence of 10^{-5} M unlabelled GTPγS. Test drugs were prepared by serial dilution in DMSO. The final assay concentration of 0.1% DMSO did not affect [^{35}S]GTPγS binding (data not shown). Assays were performed in duplicate in a final volume of 250 μl, and incubations of 45 min at 25°C were initiated by addition of membranes (7.5 μg in the presence of 10^{-5} M GDP, 0.5 μg in the absence of GDP). GDP (10^{-5} M) was routinely included in the assay buffer because agonist stimulation of [^{35}S]GTPγS binding was not observed in the absence of exogenous GDP (data not shown). Preliminary experiments confirmed that [^{35}S]GTPγS binding increased linearly for 60 min, and that ligand depletion did not occur in the presence of 10^{-5} M GDP; ligand depletion was only apparent when using >1 μg protein in the absence of exogenous GDP (data not shown). [^{35}S]GTPγS binding was not reversible either in the presence or absence of exogenous GDP; excess unlabelled GTPγS failed to displace previously bound ligand when added 60 min following initiation of the assay (data not shown). Assays were terminated by rapid filtration through glass fibre filters (Whatman GF/B; presoaked in 50 mM Tris–HCl buffer (pH 7.4)/5 mM $MgCl_2$) using a Brandel Cell Harvester. Filters were washed twice with 4 ml of 50 mM Tris–HCl buffer/5 mM $MgCl_2$ (pH 7.4), and filter disks were then transferred to scintillation vials and 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 using automatic quench correction.

2.3. Data analysis

Data analysis was carried out using SigmaPlot (Jandel, USA). Agonist EC_{50} values were determined using a least

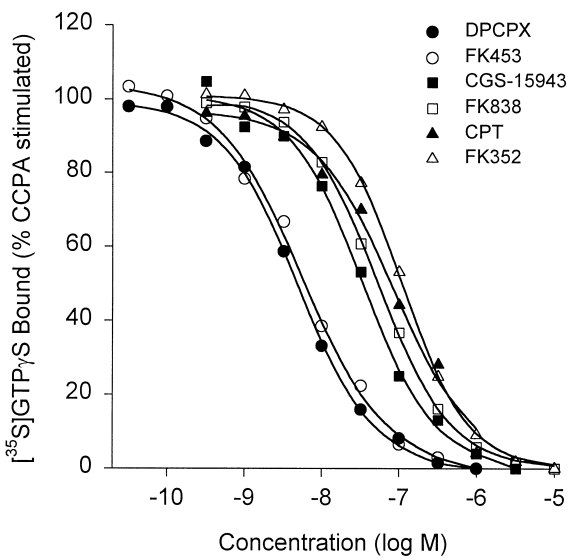

Fig. 1. Inhibition of CCPA-stimulated [^{35}S]GTPγS binding by adenosine receptor antagonists. Rat cortical P_2 membranes were incubated with [^{35}S]GTPγS and 10^{-7} M CCPA in assay buffer at 25°C for 45 min in the presence, and absence, of increasing concentrations of adenosine receptor antagonists. [^{35}S]GTPγS bound (% CCPA stimulated) is the mean of at least three independent experiments performed in duplicate. Basal [^{35}S]GTPγS binding, with no drug present (0.27 ± 0.07 pmol/mg protein; mean ± 95% confidence interval), and CCPA stimulated (0.54 ± 0.15 pmol/mg protein; mean ± 95% confidence interval), binding was determined in the presence of 0.1% DMSO. Basal binding for an antagonist was determined in the presence of the appropriate concentration of each drug (high antagonist concentrations inhibited basal binding by up to 10%).

Table 1
pEC_{50} values for adenosine receptor agonists

Agonist	[^{35}S]GTPγS binding (pEC_{50})	[^{3}H]DPCPX binding (pK_i)
CPA	7.99 ± 0.08	8.40
CCPA	7.79 ± 0.14	8.48
R-PIA	7.64 ± 0.23	8.29
CHA	7.40 ± 0.08	8.17
CADO	6.72 ± 0.17	7.45
NECA	6.66 ± 0.47	7.52
S-PIA	6.30 ± 0.22	6.68
CGS 21680	< 5	4.44

pEC_{50} values (M) were determined from concentration–response curves for agonist stimulation of [^{35}S]GTPγS binding to rat cerebral cortical membranes. Data are means ± 95% confidence intervals determined from at least three independent experiments. Agonist pK_i values determined in a [^{3}H]DPCPX binding assay are shown for comparison (Maemoto et al., 1997).

squares fit to the logistic equation $Y = MX^P/(X^P + EC_{50}^P)$ where Y is the increase in [^{35}S]GTPγS binding above basal levels noted in the presence of agonist concentration X; M is maximum stimulation of binding and P is the Hill coefficient. Antagonist IC_{50} values for inhibition of CCPA (10^{-7} M)-stimulated [^{35}S]GTPγS binding were determined using the equation $Y = MIC_{50}^P/(X^P + IC_{50}^P)$ where Y is the increase in [^{35}S]GTPγS binding above basal levels noted in the presence of antagonist concentration X; M is the calculated amount of binding in the absence of antagonist and P is the Hill coefficient. Calculated values of M were within 10% of the experimental value. To determine the dissociation constants (K_B) of antagonists, concentration–response curves for CCPA were obtained in the presence, and absence, of increasing concentrations of antagonists. Dose ratio values were calculated for each antagonist concentration, and data analysed using a Schild plot from which slope and K_B values were determined.

2.4. Materials

CCPA, DPCPX, CGS 21680, N^6-cyclopentyladenosine (CPA), $R(-)$-N^6-(2-phenyl-isopropyl)adenosine (*R*-PIA), N^6-cyclohexyladenosine (CHA), 2-chloroadenosine (CADO), 5′-*N*-ethylcarboxyamidoadenosine (NECA), $S(+)$-N^6-(2-phenyl-isopropyl)adenosine (*S*-PIA), 1,3-diethyl-8-phenylxanthine (DPX), 8-cyclopentyl-1,3-dimethylxanthine (CPT), 8-phenyltheophylline (8-PT) were purchased from Research Biochemicals, Natick, USA. FK453, FK352, FK838, 8-(noradamantan-3-yl)-1,3-dipropylxanthine (KW3902; Nonaka et al., 1996), KFM-19 (Linden, 1991), (*R*)-8-(1-phenylpropyl)-1,3-dipropylxanthine (MDL102234; Dudley et al., 1992) were synthesised by Fujisawa Pharmaceutical Company, Osaka, Japan. CGS 15943 was a generous gift from Ciba-Geigy. Unlabelled GTPγS, ADA, GDP and other chemicals were purchased from Sigma, Poole, UK.

3. Results

3.1. Effect of adenosine receptor agonists

Adenosine receptor agonists stimulated [^{35}S]GTPγS binding in a concentration dependent manner in the presence of 10^{-5} M GDP. With the exception of CGS 21680, the maximal stimulation of [^{35}S]GTPγS binding induced by all agonists tested was similar, averaging at about 100% above basal (see Fig. 1 for typical basal and stimulated values), indicating similar agonist efficacies. pEC_{50} values were determined from concentration–response curves (Table 1); the rank order of potency was CPA ≥ CCPA ≥ *R*-PIA > CHA > CADO ≥ NECA > *S*-PIA. CGS 21680, a selective adenosine A_{2a} receptor agonist (Jarvis et al.,

Table 2
pIC_{50} values for adenosine receptor antagonists

Antagonist	[^{35}S]GTPγS binding (pIC_{50})	Schild slope	[^{35}S]GTPγS binding (pK_B)	[^{3}H]DPCPX binding (pK_i)
DPCPX	8.54 ± 0.06	1.088	8.97 ± 0.64	9.55
FK453	8.34 ± 0.65	1.053	8.88 ± 0.37	9.31
KFM-19	7.82 ± 0.27			8.87
KW3902	7.62 ± 0.22			9.90
CGS 15943	7.56 ± 0.37	0.980	8.16 ± 0.24	8.82
FK838	7.34 ± 0.16	1.019	8.21 ± 0.27	8.18
CPT	7.13 ± 0.59	0.961	7.79 ± 0.84	8.26
FK352	7.01 ± 0.08	1.033	7.65 ± 0.38	7.57
MDL102234	6.44 ± 0.33			8.15
DPX	6.25 ± 0.26			7.50
8-PT	6.14 ± 0.91			7.35

pIC_{50} values (M) were determined from concentration–response curves for antagonist inhibition of CCPA (10^{-7} M)-stimulated [^{35}S]GTPγS binding to rat cerebral cortical membranes. In addition, concentration–response curves for CCPA-stimulated [^{35}S]GTPγS binding were obtained in the presence, and absence, of increasing concentrations of antagonist. Dose ratios were calculated, and data analysed using a Schild plot to determine pK_B values and Schild slope. Data are means ± 95% confidence intervals determined from at least three independent experiments. Antagonist pK_i values determined in a [^{3}H]DPCPX binding assay are shown for comparison (Maemoto et al., 1997).

1989), did not stimulate [^{35}S]GTPγS binding to rat cortical membranes (Table 1).

3.2. Effect of adenosine receptor antagonists

Stimulation of [^{35}S]GTPγS binding induced by a submaximal concentration (10^{-7} M) of the adenosine A_1 receptor selective agonist, CCPA (Klotz et al., 1989) was inhibited by both xanthine and pyrazolopyridine derivatives (Fig. 1). pIC_{50} values for all antagonists tested were determined (Table 2); the rank order of potency was DPCPX ≥ FK453 > KFM-19 > KW3902 ≥ CGS 15943 > FK838 > CPT ≥ FK352 > MDL102234 > DPX ≥ 8-PT. None of the antagonists stimulated [^{35}S]GTPγS binding at 1 μM (data not shown). However, antagonists at concentrations 10 to 100 fold in excess of their K_i values, inhibited basal [^{35}S]GTPγS binding to a maximum of about 10%. In order to determine pK_B values for receptor antagonists, concentration–response curves for CCPA were obtained in the presence, and absence, of increasing concentrations of three standard adenosine receptor antagonists, and three pyrazolopyridine derivatives. A representative experiment of CCPA-stimulated [^{35}S]GTPγS binding using FK453 as the antagonist is shown in Fig. 2, with the relevant Schild plot shown as an insert. The concentration–response curve for CCPA-stimulated [^{35}S]GTPγS binding was shifted to the right with increasing concentrations of FK453 without significant changes in the maximal response to CCPA. At 100 nM FK453 (K_i < 1 nM), up to 10% inhibition of basal [^{35}S]GTPγS binding was observed. Similar data were obtained with the other antagonists tested; DPCPX, FK838, CGS 15943, FK352 and CPT (data not shown). Schild slopes for all antagonists were close to unity allowing pK_B values to be calculated (Table 2); the rank order of potency was DPCPX ≥ FK453 > FK838 ≥ CGS 15943 > CPT ≥ FK352.

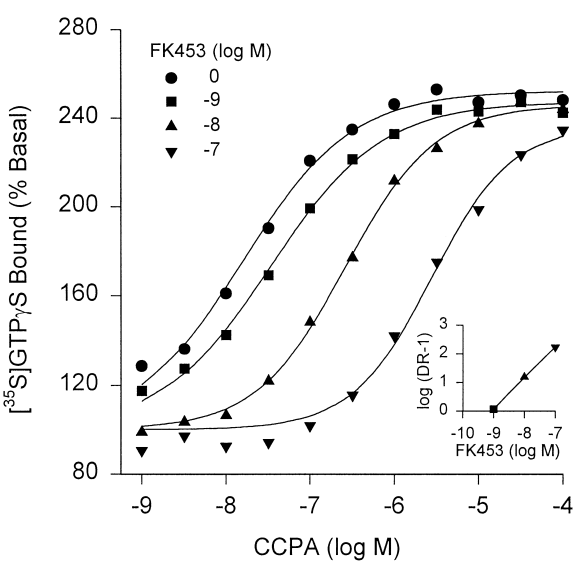

Fig. 2. Concentration response curves for CCPA-stimulated [^{35}S]GTPγS binding in the presence, and absence, of increasing concentrations of FK453. Rat cortical P_2 membranes were incubated with [^{35}S]GTPγS and increasing concentrations of CCPA in assay buffer at 25°C for 45 min in the absence, or presence, of three concentrations of FK453. Each data point represents the mean of three independent experiments performed in duplicate. The corresponding Schild plot for FK453 is shown as an insert.

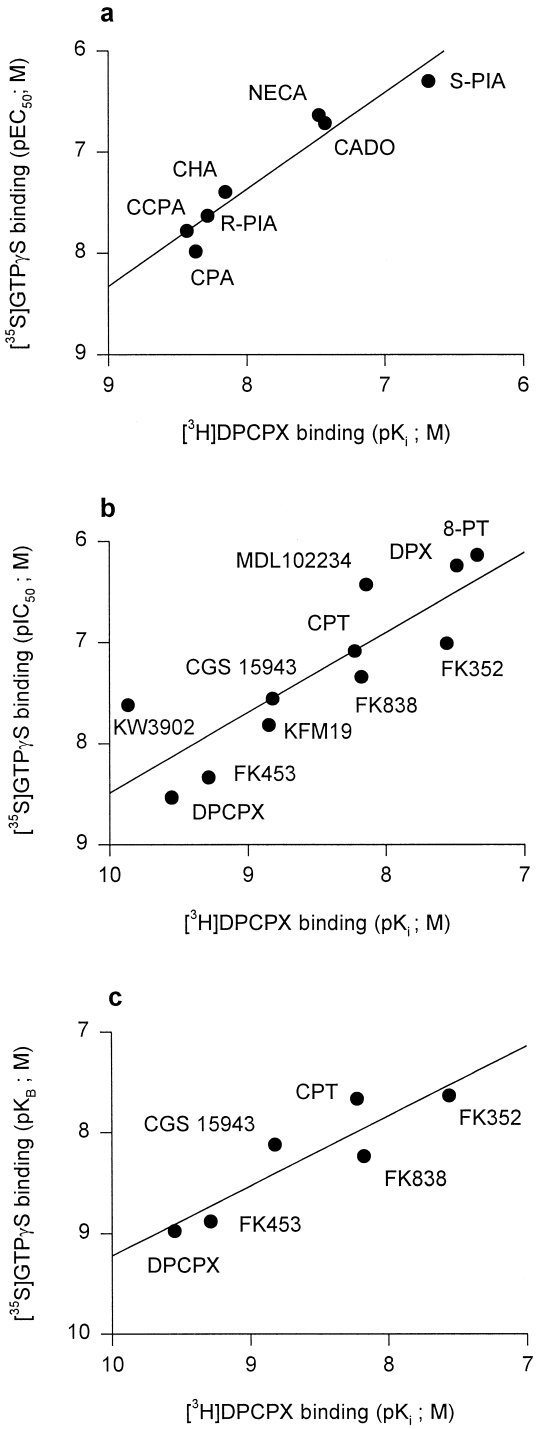

Fig. 3. Comparison of drug affinity for adenosine A_1 receptors in rat cortical P_2 membranes determined in [^{3}H]DPCPX and [^{35}S]GTPγS binding assays. pEC_{50} (a), pIC_{50} (b) and pK_B (c) values obtained in [^{35}S]GTPγS binding experiments were compared with pK_i values determined in [^{3}H]DPCPX binding studies (Maemoto et al., 1997).

3.3. Correlation of functional and receptor binding data

Pharmacological data determined using the [^{35}S]GTPγS functional binding assay were compared with radioligand binding data obtained in a similar cortical membrane preparation using [^{3}H]8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX), an adenosine A_1 receptor selective ligand (Maemoto et al., 1997). The rank order of potency for agonist stimulation of [^{35}S]GTPγS binding was identical to that obtained in [^{3}H]DPCPX binding studies (Fig. 3a); regression analysis confirmed a linear relationship with a correlation coefficient (r^2) of 0.96 ($F_{(1,5)} = 95.26$; $p < 0.001$). EC_{50} values in the [^{35}S]GTPγS binding assay were 2.5–7-fold higher than K_i values obtained using the [^{3}H]DPCPX binding assay (Table 1). A similar rank order of potency for receptor antagonists was also noted when pIC_{50} values determined in the [^{35}S]GTPγS binding assay were compared with pK_i values obtained using the [^{3}H]DPCPX binding assay (Fig. 3b). Regression analysis again confirmed a linear relationship with a correlation coefficient of 0.73 ($F_{(1,9)} = 24.42$; $p < 0.001$). Since the pIC_{50} value obtained in functional binding studies is dependent on the agonist concentration, comparison of pK_i values obtained in the [^{3}H]DPCPX binding assay with pK_B values determined from CCPA concentration–response curves in the presence of antagonists may be more appropriate (Fig. 3c). Regression analysis confirmed a linear relationship between these parameters with a correlation coefficient of 0.82 ($F_{(1,4)} = 17.96$; $p < 0.013$).

4. Discussion

While the functional activity of native adenosine A_1 receptors has been studied by measuring inhibition of forskolin-stimulated adenylate cyclase activity (Cooper et al., 1980; Ebersolt et al., 1983), drug effects in this assay using rat brain slices are generally small and unreliable (Ito, unpublished data). Furthermore, more recent data suggest that both native adenosine A_1 receptors, and recombinant receptors expressed in appropriate cell lines, can functionally couple to additional signal transduction pathways (Libert et al., 1992; Townsend-Nicholson and Shine, 1992; Akbar et al., 1994; Alexander et al., 1994; Freund et al., 1994; Iredale et al., 1994). The present study sought to determine the functional activity of a range of reference and novel compounds at rat brain adenosine A_1 receptors using a [^{35}S]GTPγS binding assay (Lorenzen et al., 1993, 1996). This functional assay monitors the exchange of GTP for GDP at the receptor associated G-protein, a common activation step following agonist stimulation of G-protein linked receptors irrespective of the downstream signal transduction pathway.

Adenosine A_1 receptor agonists stimulated [^{35}S]GTPγS binding to rat cortical membranes, as reported previously (Lorenzen et al., 1993, 1996). The maximal level of stimulation was similar for all agonists except CGS 21680, suggesting that the six effective drugs act as full agonists. These data also indicate that receptor affinity and efficacy are independent variables as proposed previously (Lorenzen et al., 1996). While CCPA, CPA and CHA are recognised as adenosine A_1 receptor selective agonists, NECA and CADO are less selective also being agonists at adenosine A_{2a} receptors (Maemoto et al., 1997). Nevertheless, the rank order of potency for adenosine receptor agonist-stimulated [^{35}S]GTPγS binding was identical to that noted in [^{3}H]DPCPX binding experiments (Maemoto et al., 1997), and the pharmacological profile was characteristic of adenosine A_1 receptors (Bruns et al., 1987; Klotz et al., 1989). These data, combined with the finding that the adenosine A_{2a} receptor agonist, CGS 21680 did not stimulate [^{35}S]GTPγS binding in rat cortical membranes, confirm the selective involvement of adenosine A_1 receptors in this functional assay. The presence of rat cortical [^{3}H]CGS 21680 binding sites exhibiting a pharmacological profile similar to the striatal adenosine A_{2a} receptor has been reported (Kirk and Richardson, 1995), and these receptors might be expected to contribute to agonist-stimulated [^{35}S]GTPγS binding because the adenosine A_{2a} receptor is positively coupled to adenylate cyclase presumably via G_s. The present data suggest either that assay conditions do not detect this interaction or that the density of adenosine A_{2a} receptors in rat cortex is too low to provide a significant contribution to the signal.

It was noted that EC_{50} values determined in the [^{35}S]GTPγS binding assay were only 2.5–7-fold higher than the corresponding K_i from [^{3}H]DPCPX binding studies. While this may be due to the slightly different conditions used in the two assays (for example, sodium was present in the functional assay buffer), previous studies have noted a similar discrepancy using identical binding conditions (Lorenzen et al., 1996). From a theoretical point of view the close quantitative correlation between the receptor binding and functional data was not necessarily expected and indeed might be regarded as somewhat surprising. However, it was also noted that the Hill coefficients for the agonist stimulated [^{35}S]GTPγS binding curves were close to unity (data not shown), indicating perhaps little or no signal amplification and that the functional response was closely related to receptor occupancy. The K_i values for agonist inhibition of [^{3}H]DPCPX binding data are composite values, reflecting binding to both high and low agonist affinity states and therefore comparison of the EC_{50} values with the K_i for the low affinity state of the receptor may be more appropriate (for references see Maemoto et al., 1997).

Stimulation of [^{35}S]GTPγS binding by a submaximal concentration of the adenosine A_1 receptor agonist, CCPA (Klotz et al., 1989), was inhibited in a concentration dependent manner by a number of xanthine-based receptor antagonists. The rank order of potency was again similar to that observed in [^{3}H]DPCPX binding studies (Maemoto et

al., 1997), suggesting that blockade of adenosine A_1 receptors was responsible for this effect. Although none of the antagonists stimulated [^{35}S]GTPγS binding, at high concentrations they inhibited basal [^{35}S]GTPγS binding levels by up to 10%. While this could be interpreted as the antagonists displaying negative intrinsic activity, it is more likely to be due to inhibition of the activity of small amounts of endogenous adenosine remaining in the membrane preparation, even following extensive washing and incubation with ADA.

Interestingly, inhibition of CCPA-stimulated [^{35}S]GTPγSbinding was also noted using three novel pyrazolopyridine derivatives, FK453, FK352 and FK838. Antagonism of adenosine receptor mediated responses in isolated tissues by FK453 has been reported previously (Terai et al., 1995). In order to further characterise the antagonistic nature of xanthine and pyrazolopyridine based compounds, concentration–response curves for CCPA were obtained in the presence, and absence, of increasing concentrations of antagonist. Dose ratios were calculated and data were analysed further using Schild plots from which pK_B values and Schild slopes were determined. Schild slopes were close to unity for both xanthine-based antagonists and the pyrazolopyridine derivatives, indicative of competitive receptor antagonism. pK_B values correlated reasonably well with pK_i values determined in the [^{3}H]DPCPX binding assay using rat cortical membranes (Maemoto et al., 1997).

In summary, the present data demonstrate that [^{35}S]GTPγS binding provides a convenient pharmacological assay for the functional activity of adenosine A_1 receptors in rat cortical membranes, and suggest that the novel pyrazolopyridine derivatives, FK453, FK352 and FK838 act as competitive adenosine A_1 receptor antagonists.

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