British Journal of Pharmacology (1996) 117, 1381-1386

Binding of the radioligand [^{3}H]-SCH 58261, a new non-xanthine A_{2A} adenosine receptor antagonist, to rat striatal membranes

[1]Cristina Zocchi, Ennio Ongini, Silvia Ferrara, *Pier Giovanni Baraldi & Silvio Dionisotti

Schering-Plough Research Institute, San Raffaele Science Park, via Olgettina 58, I-20132, Milan, and *Department of Pharmaceutical Sciences, University of Ferrara, 1-44100 Ferrara, Italy

1 The present study describes the binding to rat striatal A_{2A} adenosine receptors of the new potent and selective antagonist radioligand, [^{3}H]-5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo [1,5-*c*] pyrimidine, [^{3}H]-SCH 58261.

2 [^{3}H]-SCH 58261 specific binding to rat striatal membranes (>90%) was saturable, reversible and dependent upon protein concentration. Saturation experiments revealed that [^{3}H]-SCH 58261 labelled a single class of recognition sites with high affinity (K_d = 0.70 nM) and limited capacity (apparent B_{max} = 971 fmol mg^{-1} of protein). The presence of 100 μM GTP in the incubation mixture did not modify [^{3}H]-SCH 58261 binding parameters.

3 Competition experiments showed that [^{3}H]-SCH 58261 binding is consistent with the labelling of A_{2A} striatal receptors. Adenosine receptor agonists competed with the binding of 0.2 nM [^{3}H]-SCH 58261 with the following order of potency: 2-hexynyl-5'-N-ethyl carboxamidoadenosine (2HE-NECA) > 5'-N-ethylcarboxamidoadenosine (NECA) > 2-[4-(2-carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamido-adenosine (CGS 21680) > 2-phenylaminoadenosine (CV 1808) > **R**-N^6-phenylisopropyladenosine (**R**-PIA) > N^6-cyclohexyladenosine (CHA) = 2-chloro-N^6-cyclopentyladenosine (CCPA) > **S**-N^6-phenylisopropyladenosine (**S**-PIA).

4 Adenosine antagonists inhibited [^{3}H]-SCH 58261 binding with the following order: 5-amino-9-chloro-2-(2-furyl)-[1,2,4]-triazolo[1,5-*c*] quinazoline (CGS 15943) > 5-amino-8-(4-fluorobenzyl)-2-(2-furyl)-pyrazolo [4,3-*e*]-1,2,4-triazolo [1,5-*c*] pyrimidine (8FB-PTP) = SCH 58261 > xanthine amine congener (XAC) = (*E*,18%-Z,82%)7-methyl-8-(3,4-dimethoxystyryl)-1,3-dipropylxanthine (KF 17837S) > 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) ≥ 8-phenyltheophylline (8-PT).

5 The K_i values for adenosine antagonists were similar to those labelled with the A_{2A} agonist [^{3}H]-CGS 21680. Affinities of agonists were generally lower. The A_1-selective agonist, **R**-PIA, was found to be about 9 fold more potent than its stereoisomer, **S**-PIA, thus showing the stereoselectivity of [^{3}H]-SCH 58261 binding. Except for 8-PT, the adenosine agonists and antagonists examined inhibited [^{3}H]-SCH 58261 binding with Hill coefficients not significantly different from unity.

6 The present results indicate that [^{3}H]-SCH 58261 is the first non-xanthine adenosine antagonist radioligand which directly labels A_{2A} striatal receptors. High receptor affinity, good selectivity and very low non-specific binding make [^{3}H]-SCH 58261 an excellent probe for studying the A_{2A} adenosine receptor subtype in mammalian brain.

Keywords: Adenosine receptors; A_{2A} receptor antagonists; non-xanthine adenosine receptor antagonists; SCH 58261; CGS 21680

Introduction

Adenosine modulates a variety of physiological functions in both central nervous system and peripheral tissues by interacting with different cell surface receptors coupled to adenylate cyclase in an inhibitory or stimulatory manner (Van Calker *et al.*, 1979; Londos *et al.*, 1980). On the basis of biochemical studies and molecular cloning, these receptors have been classified into A_1, A_{2A}, A_{2B} and A_3 subtypes (Fredholm *et al.*, 1994). In the past, the lack of selective ligands hampered the pharmacological characterization of A_{2A} adenosine receptors. By use of different strategies to block the interaction with A_1 receptors, the tritium-labelled form of the non-selective agonist [^{3}H]-5'-N-ethylcarboxamidoadenosine ([^{3}H]-NECA) has been used in binding studies on A_{2A} adenosine receptors in rat striatal membranes (Yeung & Green, 1984; Bruns *et al.*, 1986). Over the last few years, the 2-substituted analogue of NECA, the agonist (2-[4-(2-carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680), which shows high affinity (K_i = 14 nM) and selectivity (A_1/A_{2A} ratio of about 180 fold) for rat striatal A_{2A} adenosine receptors, has become the radioligand of choice to investigate this receptor subtype in binding studies (Jarvis *et al.*, 1989). Therefore, all binding data on A_{2A} receptors are now obtained with this radiolabelled agonist.

In binding studies, however, the use of antagonist radioligands is preferred because they have certain advantages over agonists. For example, complication of high and low receptor affinity states can be avoided (Stiles & Jacobson, 1987). Among the adenosine antagonists available, the non-xanthine heterocycle 5-amino-9-chloro-2-(2-furyl)-[1,2,4]-triazolo[1,5-*c*]quinazoline (CGS 15943), has affinity in the low nanomolar range for A_{2A} adenosine receptors but little or no A_{2A} *vs* A_1 selectivity (Williams *et al.*, 1987). In fact, [^{3}H]-CGS 15943 has been used only to characterize A_1 adenosine receptors in rat cortex (Jarvis *et al.*, 1990). In investigating the A_{2A} receptor subtype, the [^{3}H]-xanthine amine congener ([^{3}H]-XAC) and [^{3}H]-N-[2-dimethylamino) ethyl]-*N*-methyl-4-(2,3,6,7 tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)-benzenesulphonamide ([^{3}H]-PD 115,199) have been used in binding studies. These non-selective adenosine antagonists label rat (Bruns *et al.*, 1987) and human (Ji *et al.*, 1992) A_{2A} striatal

[1] Author for correspondence.

receptors, but the presence of an A_1 antagonist is needed to eliminate their interaction with this receptor subtype. Only recently, important progress has been made with the development of selective A_{2A} antagonists having an interesting pharmacological profile. A series of 8-styrylxanthine derivatives, including (*E*,18%-Z,82%) 7-methyl-8-(3,4-dimethoxystyryl)-1,3-dipropylxanthine (KF 17837S) (Nonaka *et al.*, 1993) and 8-(3-chlorostyryl)caffeine (CSC) (Jacobson *et al.*, 1993), have been reported to have high affinity and selectivity for this receptor subtype. [^{3}H]-KF 17837S has also been found to label A_{2A} receptors directly in rat striatum. Binding occurs at concentrations in the low nanomolar range (K_d = 7.1 nM), but non-specific binding (about 30–40%) still appears high (Nonaka *et al.*, 1994).

With further optimization of the chemical structure of the prototype CGS 15943 we have found some interesting compounds (Gatta *et al.*, 1993; Dionisotti *et al.*, 1994; Baraldi *et al.*, 1994). In particular, the 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH 58261) is a new potent and selective non-xanthine A_{2A} antagonist (Baraldi *et al.*, 1994). In binding studies on rat and bovine brain tissues, SCH 58261 showed A_{2A}-receptor affinity in the low nanomolar range (K_i values of 2.3 and 2.0 nM, respectively) associated with good selectivity (A_1/A_{2A} ratio of 50 or 100 fold, respectively) (Zocchi *et al.*, 1995). The compound did not interact with a variety of other receptors including the A_{2B} and A_3 adenosine receptor subtypes (Zocchi *et al.*, 1996). Moreover, binding and functional assays indicated that SCH 58261 is a competitive A_{2A} antagonist (Zocchi *et al.*, 1995).

On the basis of this pharmacological profile and because it would be highly desirable to have antagonist radioligands for binding studies on A_{2A} receptors, the tritium-labelled form of SCH 58261 was prepared (Figure 1). In the present study, we describe the characterization of the binding of [^{3}H]-SCH 58261 to rat A_{2A} striatal receptors.

Methods

Membrane preparation

Male Sprague-Dawley rats (Charles-River, Calco, Como, Italy), weighing 250–350 g, were killed by decapitation and brain striatum was rapidly removed. Striatal tissues were homogenized with a Polytron PTA 10 probe (setting 5, 30 s) in 25 vol of 50 mM Tris-HCl buffer, pH 7.4. The homogenate was centrifuged at 48000 *g* for 10 min at 4°C and resuspended in Tris-HCl containing 2 units ml^{-1} adenosine deaminase. After 30 min incubation at 37°C, membranes were centrifuged and the pellet was stored at −70°C.

Radioligand binding assay

Binding of [^{3}H]-SCH 58261 to rat brain striatal membranes was performed by a modification of the method previously described for [^{3}H]-CGS 21680 (Jarvis *et al.*, 1989). Assays were carried out in duplicate and with a final volume of 0.5 ml in test tubes containing 0.2 nM [^{3}H]-SCH 58261, 50 mM Tris-HCl buffer, pH 7.4, and striatal membranes (0.1 mg protein/assay).

In saturation studies, striatal membranes were incubated with 11 different concentrations of [^{3}H]-SCH 58261 ranging from 0.0625 to 64 nM. In competition studies, at least 7 different concentrations of several adenosine agonists and antagonists were used. Non-specific binding was determined in the presence of 50 μM NECA. After 30 min incubation at 25°C, samples were filtered through Whatman GF/B filters using a Brandel cell harvester (Gaithersburg, MD, U.S.A). Radioactivity was determined in an LS-60001C Beckman liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA, U.S.A.), at an efficiency of 50 to 60%. Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin used as standard.

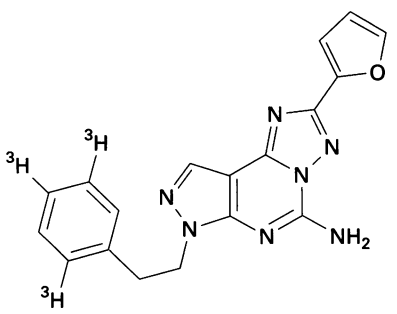

Figure 1 Chemical structure of [^{3}H]-5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine ([^{3}H]-SCH 58261).

Statistical analysis

Binding parameters were estimated by the computerized non-linear fitting programme LIGAND (Munson & Rodbard, 1980). Data are expressed as geometric mean, with 95% or 99% (Hill coefficients) confidence limits in parentheses.

Chemicals

5-Amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH 58261) was synthesized as described elsewhere (Baraldi *et al.*, 1994). [^{3}H]-SCH 58261 (specific activity 68.6 Ci mM^{-1}; radiochemical purity 99%) was obtained using the precursor 5-amino-7-[2-(2',4',5'-tribromo)-phenylethyl]-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo-[1,5-*c*]-pyrimidine. Then, the radiolabelled form was prepared by NEN-Dupont (Boston, MA, U.S.A.) through reduction with tritium gas. (*E*,18%-*Z*,82%)7-methyl-8-(3,4-dimethoxystyryl)-1,3-dipropylxanthine (KF 17837S) and 5-amino-8-(4-fluorobenzyl)-2-(2-furyl)-pyrazolo [4,3-*e*]-1,2,4-triazolo [1,5-*c*] pyrimidine (8FB-PTP) were synthesized by Dr F. Gatta (Laboratory of Medicinal Chemistry, Italian Institute of Health, Rome, Italy) according to methods described elsewhere (Shimada *et al.*, 1992; Gatta *et al.*, 1993). 5-Amino-9-chloro-2-(2-furyl)-1,2,4-triazolo[1,5-*c*]quinazoline (CGS 15943) was kindly supplied by Ciba-Geigy (Summit, NJ, U.S.A.). 2-Hexynyl-5'-N-ethylcarboxamidoadenosine (2HE-NECA) was synthesized by Dr G. Cristalli (Department of Chemical Sciences, University of Camerino, Camerino, Italy) (Cristalli *et al.*, 1992). 2-[4-(2-Carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680), 5'-N-ethylcarboxamidoadenosine (NECA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 2-chloro-N^6-cyclopentyladenosine (CCPA), 2-phenylaminoadenosine (CV-1808), N^6-cyclohexyladenosine (CHA), **R**-N^6-phenylisopropyladenosine (**R**-PIA), **S**-N^6-phenylisopropyladenosine (**S**-PIA), xanthine amine congener (XAC) and 8-phenyltheopyhylline (8-PT) were from Research Biochemicals Inc. (Natick, MA, U.S.A.). Guanosine triphosphate was from Sigma Chemical Co. (St Louis, MO, U.S.A.). Adenosine deaminase was supplied by Boehringer Mannheim GmbH (Mannheim, Germany). All other reagents were from commercial sources.

Results

When incubated for 30 min at 25°C, at a concentration as low as 0.2 nM the radioligand [^{3}H]-SCH 58261 bound to rat striatal membranes with specific binding of 92 (90–94)% of total binding. Specific [^{3}H]-SCH 58261 binding was found to increase linearly with respect to protein concentration over the range of 50–300 μg of protein/assay (data not shown). The presence of 10 mM $MgCl_2$ in the assay mixture did not modify the percentage of specific binding (89%).

Kinetic studies ($n=4$) showed that [^{3}H]-SCH 58261 binding reached equilibrium after approximately 5 min and was stable for at least 4 h. [^{3}H]-SCH 58261 binding was rapidly reversed by the addition of 50 μM NECA (Figure 2). Computer analysis demonstrated that both association and dissociation data fit a one-component model significantly better than a two-component model ($P<0.05$) with the following rate constants: $K_{obs}=0.85$ (0.70–1.04) min^{-1} and $K_{-1}=0.62$ (0.55–0.69) min^{-1} from a $t_{1/2}=1.12$ (1.00–1.26) min. These values gave a kinetic dissociation constant (K_d) of 0.54 nM.

Saturation experiments ($n=4$) showed that [^{3}H]-SCH 58261 bound to a single class of receptors in rat striatal membranes, with a K_d value of 0.70 (0.64–0.76) nM and an apparent B_{max} value of 971 (859–1099) fmol mg^{-1} of protein. Computer analysis revealed that a one-component model described the data significantly better than a two-component model ($P<0.05$). A representative saturation isotherm and Scatchard plot are shown in Figure 3. The presence of 100 μM GTP in the incubation mixture ($n=3$) did not modify [^{3}H]-SCH 58261 binding parameters, K_d and B_{max} values being 0.85 (0.66–1.11) nM and 960 (920–1002) fmol mg^{-1} of protein, respectively.

In competition studies ($n=4-8$) we have evaluated selected adenosine agonists and antagonists. Adenosine agonists inhibited [^{3}H]-SCH 58261 binding with the following order of potency: 2HE-NECA > NECA > CGS 21680 > CV 1808 > R-PIA > CHA = CCPA > S-PIA (Table 1, Figure 4). The potent and selective A_{2A} agonist, 2HE-NECA, was the most effective compound with affinity in the low nanomolar range ($K_i=3.1$ nM). The A_1-selective agonist, **R**-PIA, was found to be about 9 fold more potent than its stereoisomer, S-PIA, thus showing the stereoselectivity of [^{3}H]-SCH 58261 binding. The ability of several xanthine and non-xanthine adenosine receptor antagonists in inhibiting [^{3}H]-SCH 58261 binding was also examined. The order of potency was: CGS 15943 > 8FB-PTP = SCH 58261 > XAC = KF 17837S > DPCPX ≥ 8-PT (Table 1, Figure 5). CGS 15943 was the most potent compound in inhibiting [^{3}H]-SCH 58261 binding with a K_i value of 0.38 nM. Except for 8-PT, both adenosine agonists and antagonists inhibited [^{3}H]-SCH 58261 binding with Hill coefficients not significantly different from unity (Table 1).

Inhibition curves of selected adenosine agonists and antagonists were not modified by the presence of 100 μM GTP (Table 2).

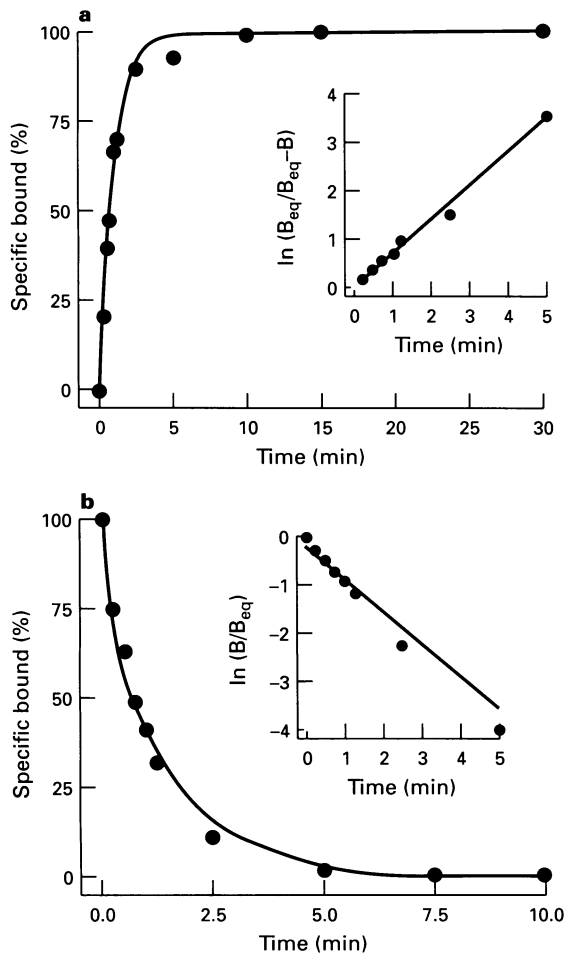

Figure 2 Kinetics of [^{3}H]-SCH 58261 (0.2 nM) binding to rat striatal membranes with association (a) and dissociation (b) curves representative of a single experiment. Dissociation was produced by the addition of 50 μM NECA. *Insets*, first-order plots of [^{3}H]-SCH 58261 binding. B_{eq}, amount of [^{3}H]-SCH 58261 bound to equilibrium; B, amount of [^{3}H]-SCH 58261 bound to each time. Computer analysis demonstrated that both association and dissociation data fit a one-component model significantly better than a two-component model ($P<0.05$). Association and dissociation rate constants were as follows: $K_{obs}=0.85$ (0.70–1.04) min^{-1} and $K_{-1}=0.62$ (0.55–0.69) min^{-1} from a $t_{1/2}=1.12$ (1.00–1.26) min. Values indicate the geometric mean with 95% confidence limits in parentheses ($n=4$).

Discussion

The present study demonstrates that the new radioligand, [^{3}H]-SCH 58261, binds specifically to rat striatal membranes with a pharmacological profile consistent with labelling of A_{2A} adenosine receptors. This new radioligand was found to label the A_{2A} receptor subtype directly with a high specific binding (≥90%) which was saturable, reversible and dependent upon protein concentration. The divalent cation Mg^{2+}, which is known to be important in promoting agonist binding to A_{2A} brain receptors (Johansson *et al.*, 1992), did not affect [^{3}H]-SCH 58261 specific binding. Likewise, the presence of Mg^{2+} did not influence specific binding of [^{3}H]-XAC to human striatal membranes (Ji *et al.*, 1992).

Association and dissociation of [^{3}H]-SCH 58261 binding to A_{2A} striatal receptors were very rapid and kinetic parameters

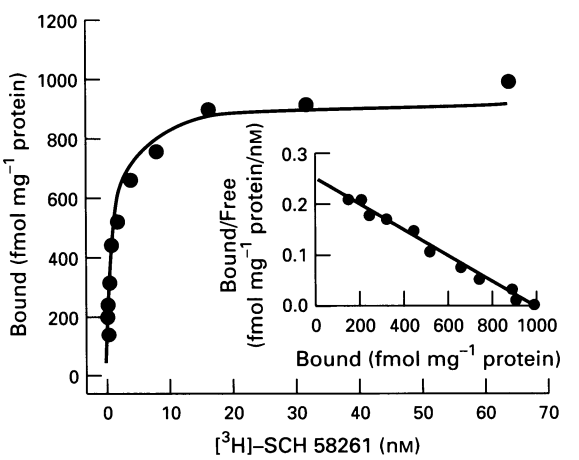

Figure 3 Saturation isotherm and Scatchard plot (*inset*) of [^{3}H]-SCH 58261 binding to rat striatal membranes. Curve is representative of a single experiment. Computer analysis revealed that a one-component model described the data significantly better than a two-component model ($P<0.05$). Binding parameters are as follows: $K_d=0.70$ (0.64–0.76) nM and B_{max} 971 (859–1099) fmol mg^{-1} of protein. Values indicate the geometric mean with 95% confidence limits in parentheses ($n=4$). (●) Specific binding.

Table 1 Affinity of selected adenosine receptor agonists and antagonists in inhibiting [^{3}H]-SCH 58261 binding to rat striatal membranes

	[^{3}H]-SCH 58261		*[^{3}H]-CGS 21680*
	K_i (nM)	*Hill coefficient*	K_i (nM)
Agonists			
2HE-NECA	3.1 (2.4–4.0)	0.93 (0.86–1.00)	2.2[a]
NECA	61 (48–77)	0.99 (0.98–1.00)	7.8[a]
CGS 21680	111 (83–148)	0.90 (0.73–1.11)	11[a]
CV-1808	332 (233–473)	0.97 (0.87–1.08)	62[a]
R-PIA	992 (834–1183)	0.95 (0.88–1.02)	164[a]
CHA	2840 (2367–3408)	1.07 (0.93–1.22)	685[b]
CCPA	3260 (2678–3968)	0.95 (0.80–1.12)	650[a]
S-PIA	8504 (8178–8843)	0.80 (0.62–1.04)	882[a]
Antagonists			
CGS 15943	0.38 (0.30–0.47)	0.90 (0.78–1.04)	1.2[c]
8FB-PTP	0.84 (0.61–1.2)	0.91 (0.79–1.05)	1.2[c]
SCH 58261	1.1 (0.84–1.3)	0.93 (0.83–1.05)	2.3[c]
XAC	9.0 (6.6–12.2)	0.87 (0.68–1.13)	50[b]
KF 17837S	9.4 (7.5–12.0)	1.01 (0.79–1.30)	7.9[d]
DPCPX	234 (124–445)	0.83 (0.66–1.03)	706[c]
8-PT	383 (263–557)	0.85 (0.75–0.97)**	550[b]

Rat striatal membranes were incubated with 0.2 nM [^{3}H]-SCH 58261 in the presence of at least 7 different concentrations of agonists or antagonists. Each value represents the geometric mean, with 95% or 99% (Hill coefficients) confidence limits in parentheses, of at least 4 different experiments performed in duplicate. **Hill coefficients significantly different from unity ($P<0.01$). [a]Dionisotti *et al.*, 1992; [b]Jarvis *et al.*, 1989; [c]Baraldi *et al.*, 1994; [d]Nonaka *et al.*, 1993. For abbreviations, see text.

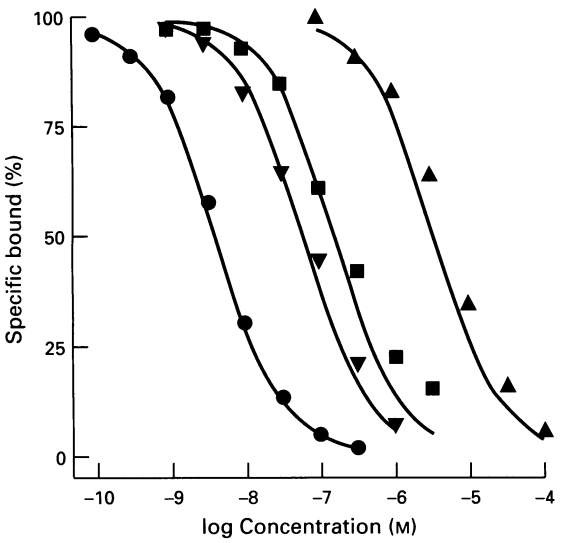

Figure 4 Competition curves of selected adenosine receptor agonists in inhibiting 0.2 nM [^{3}H]-SCH 58261 binding in rat striatal membranes. Curves are representative of a single experiment from a group of four to eight independent experiments: (●) 2HE-NECA; (▼) NECA; (■) CGS 21680; (▲) CHA.

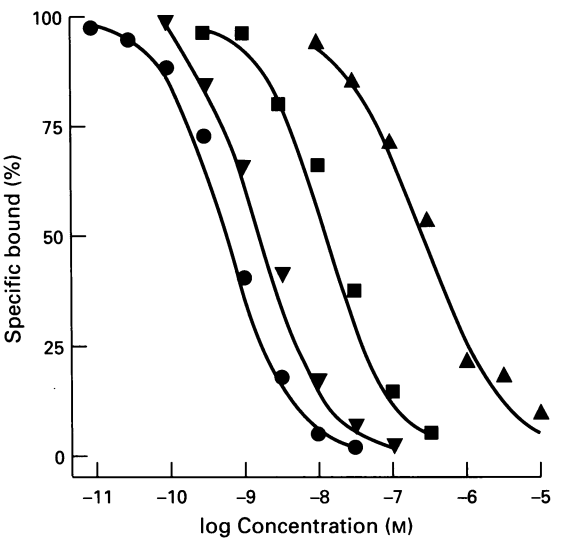

Figure 5 Competition curves of selected adenosine receptor antagonists in inhibiting 0.2 nM [^{3}H]-SCH 58261 binding in rat striatal membranes. Curves are representative of a single experiment from a group of four to eight independent experiments: (●) CGS 15943; (▼) SCH 58261; (■) KF 17837S; (▲) DPCPX.

were about 20 fold higher than those observed with [^{3}H]-CGS 21680 binding (Jarvis *et al.*, 1989). However, this is in agreement with previous studies in which adenosine antagonists have been used as radioligands at A_{2A} receptors (Bruns *et al.*, 1987; Nonaka *et al.*, 1994). These data clearly suggest that different mechanisms are involved in the binding of agonists or antagonists to A_{2A} adenosine receptors.

Saturation studies revealed that [^{3}H]-SCH 58261 bound to a single class of receptors with a high affinity ($K_d = 0.70$ nM) similar to that determined by kinetic experiments ($K_d = 0.54$ nM) and also shown in competition studies using either [^{3}H]-SCH 58261 ($K_i = 1.1$ nM) or [^{3}H]-CGS 21680 ($K_i = 2.3$ nM) (Baraldi *et al.*, 1994). Thus, although significant levels of A_1 receptors are present in rat striatum (Yeung & Green, 1984; Bruns *et al.*, 1986; Kirk & Richardson, 1995) and SCH 58261 binds to the same rat cerebral receptor with a K_i value of 121 nM (Baraldi *et al.*, 1994), over the concentration-range used in saturation experiments it appears to have no significant interaction with this receptor subtype. The apparent B_{max} of [^{3}H]-SCH 58261 to rat striatal membranes (971 fmol mg^{-1} of protein) was approximately 2–3 fold greater than that typically obtained with [^{3}H]-CGS 21680 binding (Jarvis *et al.*, 1989) and it was close to that reported for the A_{2a}-selective antagonist [^{3}H]-KF 17837S (1300 fmol mg^{-1} of protein) (Nonaka *et al.*, 1994).

It is generally known that G protein-coupled receptors exist in low- and high-affinity states. While agonists preferentially recognize the high affinity state, antagonists can label both of them with the same affinity. The presence of guanine nucleo-

Table 2 Inhibition of [^{3}H]-SCH 58261 binding by adenosine receptor agonists and antagonists to rat striatal membranes in the absence and in the presence of 100 μM GTP

	[^{3}H]-SCH 58261	
Compound	K_i (nM)	*Hill coefficient*
2HE-NECA	3.1 (2.4–4.0)	0.93 (0.86–1.00)
2HE-NECA + GTP	4.0 (2.9–5.5)	0.96 (0.90–1.03)
NECA	61 (48–77)	0.99 (0.98–1.00)
NECA + GTP	71 (49–101)	0.93 (0.81–1.08)
CGS 21680	111 (83–148)	0.90 (0.73–1.11)
CGS 21680 + GTP	139 (89–218)	0.96 (0.79–1.15)
SCH 58261	1.1 (0.8–1.3)	0.93 (0.83–1.05)
SCH 58261 + GTP	1.1 (0.9–1.2)	0.95 (0.84–1.08)
CGS 15943	0.38 (0.30–0.47)	0.90 (0.78–1.04)
CGS 15943 + GTP	0.45 (0.41–0.49)	0.85 (0.72–1.01)

Each value represents the geometric mean, with 95% or 99% (Hill coefficients) confidence limits in parentheses, of at least 4 different experiments performed in duplicate. For abbreviations, see text.

tides, such as GTP or its analogues, determines dissociation of receptor-G protein complexes converting receptors from high- to a low-affinity state (Gilman, 1987). Unlike agonist radioligands, the binding of antagonist radioligands cannot be expected to be modulated by guanine nucleotides. However, since conflicting results have been reported in the literature concerning the influence of guanine nucleotides on A_1 antagonist binding (Ramkumar & Stiles, 1988; Klotz *et al.*, 1990), we assessed their effects on [^{3}H]-SCH 58261 binding parameters in saturation experiments. The presence of 100 μM GTP in the incubation mixture did not induce significant changes in either K_d or B_{max} values, thus excluding guanine nucleotide modulation of antagonist binding to rat A_{2A} striatal receptors. Surprisingly, similar results have been described in rabbit and bovine striatal membranes using the A_{2A}-selective agonist radioligands, [^{125}I]-PAPA-APEC (Nanoff *et al.*, 1991) and [^{3}H]-CGS 21680 (Nanoff & Stiles, 1993). The lack of guanine nucleotide modulation in the agonist binding has led to the suggestion that a tight association between the A_{2A} striatal receptor and the stimulatory G protein exists (Nanoff *et al.*, 1991; Nanoff & Stiles, 1993).

In competition experiments, the order of potency found for several adenosine agonists and antagonists in inhibiting [^{3}H]-SCH 58261 binding is in agreement with that observed using the agonist radioligand, [^{3}H]-CGS 21680 (Table 1) as well as the antagonist, [^{3}H]-KF 17837S (Nonaka *et al.*, 1994). The data are consistent with a selective interaction occurring at A_{2A} receptors. In agreement with previous studies (Bruns *et al.*, 1987; Nonaka *et al.*, 1994), the affinity of adenosine antagonists was similar or higher than that observed with [^{3}H]-CGS 21680 and computer analysis revealed that antagonist inhibition curves were best described by the one-component model (i.e. Hill coefficients not significantly different from unity). Conversely, adenosine agonists inhibited [^{3}H]-SCH 58261 binding with K_i values generally higher than those reported using agonist radioligands (Bruns *et al.*, 1986; Jarvis *et al.*, 1989). These changes of K_i values are usually found when binding studies are carried out using antagonist rather than agonist radioligands. This reduction of affinity observed in competition studies with the agonists could be explained by considering that the A_{2A} receptor exists in two G-protein coupling states. However, this hypothesis is not supported from data on agonist inhibition curves where Hill coefficients were found to be not significantly different from unity, thus excluding the involvement of multiple coupling affinity states. Contrasting results have been reported in the literature about the ability of A_{2A} antagonist radioligands to reveal both high and low affinity states (Bruns *et al.*, 1987; Ji *et al.*, 1992; Nonaka *et al.*, 1994). In agreement with our results, adenosine agonists displaced the antagonist radioligand [^{3}H]-PD 115,199 to A_{2A} striatal receptors with Hill coefficients close to unity (Bruns *et al.*, 1987). In contrast, they inhibited binding of both antagonists [^{3}H]-XAC (Ji *et al.*, 1992) and [^{3}H]-KF 17837S (Nonaka *et al.*, 1994) with shallow competition curves (i.e Hill coefficients less than unity) which are best described by a two-site model. However, in these two latter studies the addition of guanine nucleotides, which are expected to shift a biphasic to a simple inhibition curve (Gilman, 1987), had little or no influence. For example, in the presence of 100 μM GTP, Ji *et al.* (1992) did not observe significant changes in the potency of CGS 21680 in inhibiting [^{3}H]-XAC binding to A_{2A} receptors, whereas the guanine nucleotide increased only weakly (about 2–3 fold) the K_i value of the same agonist in [^{3}H]-KF 17837S binding, without changes of Hill coefficient. In the present study, the addition of 100 μM GTP modified neither the potency of selected A_{2A} agonists in competing with [^{3}H]-SCH 58261 binding nor Hill coefficients (Table 2). It remains to be investigated whether this slight or non-existent influence of guanine nucleotides on agonist inhibition curves constructed using A_{2A} antagonist radioligands can be ascribed to the atypical tight coupling previously mentioned or to vesicle formation in membrane suspensions which could limit the interaction of guanine nucleotides with the receptors on the inside of membrane vesicles (Schiemann *et al.*, 1990; Luthin *et al.*, 1995).

In conclusion, the present study demonstrates that [^{3}H]-SCH 58261 is the first potent non-xanthine adenosine antagonist radioligand available for the characterization of A_{2A} striatal receptors. Compared to the other recently described A_{2A} antagonist, the xanthine [^{3}H]-KF 17837S (Nonaka *et al.*, 1994), the radioligand [^{3}H]-SCH 58261 shows about 10 fold higher affinity for A_{2A} striatal receptors (K_d value of 0.7 nM *vs* 7.1 nM) and significantly lower non-specific binding (less than 10% *vs* 30–40%). The overall pharmacological profile makes the radioligand [^{3}H]-SCH 58261 an excellent tool for studying the A_{2A} adenosine receptor in mammalian brain and therefore for elucidating the structure, function and regulation of this adenosine receptor subtype. Moreover, this new radioligand appears to have characteristics which will be useful for the investigation of A_{2A} receptors distributed in peripheral tissues, such as vascular smooth muscle, platelets and neutrophils, in which their presence has been clearly demonstrated (Collis & Hourani, 1993).

References

BARALDI, P.G., MANFREDINI, S., SIMONI, D., ZAPPATERRA, L., ZOCCHI, C., DIONISOTTI, S. & ONGINI, E. (1994). Synthesis of new pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*] pyrimidine and 1,2,3-triazolo[1,5-*c*] pyrimidine displaying potent and selective activity as A_{2A} adenosine receptor antagonists. *Bioorg. Med. Chem. Lett.*, **4**, 2539–2544.

BRUNS, R.F., FERGUS, J.H., BADGER, E.W., BRISTOL, J.A., SANTAY, L.A. & HAYS, S.J. (1987). PD 115,199: an antagonist ligand for adenosine A_2 receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **335**, 64–69.

BRUNS, R.F., LU, G.H. & PUGSLEY, T.A. (1986). Characterization of the A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. *Mol. Pharmacol.*, **29**, 331–346.

COLLIS, M.G. & HOURANI, S.M.O. (1993). Adenosine receptor subtypes. *Trends Pharmacol. Sci.*, **14**, 360–366.

CRISTALLI, G., ELEUTERI, A., VITTORI, S., VOLPINI, R., LOHSE, M.J. & KLOTZ, K.N. (1992). 2-Alkynyl derivatives of adenosine and adenosine-5′-ethyluronamide as selective agonists at A_2 adenosine receptors. *J. Med. Chem.*, **35**, 2363–2368.

DIONISOTTI, S., CONTI, A., SANDOLI, D., ZOCCHI, C., GATTA, F. & ONGINI, E. (1994). Effects of the new A_2 adenosine receptor antagonist 8FB-PTP, an 8 substituted pyrazolo-triazolo-pyrimidine, on *in vitro* functional models. *Br. J. Pharmacol.*, **112**, 659–665.

DIONISOTTI, S., ZOCCHI, C., VARANI, K., BOREA, P.A. & ONGINI, E. (1992). Effects of adenosine derivatives on human and rabbit platelet aggregation. Correlation of adenosine receptor affinities and antiaggregatory activity. *Naunyn-Schmied. Arch. Pharmacol.*, **346**, 673–676.

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

GATTA, F., DEL GIUDICE, M.R., BORIONI, A., BOREA, P.A., DIONISOTTI, S. & ONGINI, E. (1993). Synthesis of imidazo[1,2-*c*]pyrazolo[4,3-*e*]pyrimidines, pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidines and triazolo[5,1-*i*]purines: new potent A_2 adenosine receptor antagonists. *Eur. J. Med. Chem.*, **28**, 569–577.

GILMAN, A.G. (1987). G-proteins: transducer of receptor-generated signals. *Annu. Rev. Biochem.*, **56**, 615–649.

JACOBSON, K.A., GALLO-RODRIGUEZ, C., MELMAN, N., FISCHER, B., MAILLARD, M., VAN BERGEN, A., VAN GALEN, P.J.M. & KARTON, Y. (1993). Structure-activity relationships of 8-styrylxanthines as A_2-selective adenosine antagonists. *J. Med. Chem.*, **36**, 1333–1342.

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.

JARVIS, M.F., WILLIAMS, M., DO, U.H. & SILLS, M.A. (1990). Characterization of the binding of a novel nonxanthine adenosine antagonist radioligand, [^{3}H]CGS 15943, to multiple affinity states of the adenosine A_1 receptor in the rat cortex. *Mol. Pharmacol.*, **39**, 49–54.

JI, X.D., STILES, G.L., VAN GALEN, P.J.M. & JACOBSON, K.A. (1992). Characterization of human striatal A_2-adenosine receptors using radioligand binding and photoaffinity labeling. *J. Recept. Res.*, **12**, 149–169.

JOHANSSON, B., PARKINSON, F.E. & FREDHOLM, B.B. (1992). Effects of mono- and divalent cations on the binding of the adenosine analogue CGS 21680 to adenosine A_2 receptors in rat striatum. *Biochem. Pharmacol.*, **44**, 2365–2370.

KIRK, I.P. & RICHARDSON, P.J. (1995). Further characterization of [^{3}H]-CGS 21680 binding sites in the rat striatum and cortex. *Br. J. Pharmacol.*, **114**, 537–543.

KLOTZ, K.N., KEIL, R., ZIMMER, F.J. & SCHWABE, U. (1990). Guanine nucleotide effects on 8-cyclopentyl-1,3-[^{3}H]dipropylxanthine binding to membrane-bound solubilized A_1 adenosine receptors of rat brain. *J. Neurochem.*, **54**, 1988–1994.

LONDOS, C., COOPER, D.M.F. & WOLFF, J. (1980). Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 2551–2554.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.I. & RANDALL, R.J. (1951). Protein measurement with Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

LUTHIN, D.R., OLSSON, R.A., THOMPSON, R.D., SAWMILLER, D.R. & LINDEN, J. (1995). Characterization of two affinity states of adenosine A_{2A} receptors with a new radioligand, 2-[2-(4-amino-3-[^{125}I]iodophenyl)ethylamino] adenosine. *Mol. Pharmacol.*, **47**, 307–313.

MUNSON, P.J. & RODBARD, P. (1980). LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.*, **107**, 220–239.

NANOFF, C., JACOBSON, K.A. & STILES, G.L. (1991). The A_2 adenosine receptor: guanine nucleotide modulation of agonist binding is enhanced by proteolysis. *Mol. Pharmacol.*, **39**, 130–135.

NANOFF, C. & STILES, G.L. (1993). Solubilization and characterization of the A_2-adenosine receptor. *J. Recept. Res.*, **13**, 961–973.

NONAKA, H., MORI, A., ICHIMURA, M., SHINDOU, T., KANAGAWA, K., SHIMADA, J. & KASE, H. (1994). Binding of [^{3}H]KF 17837S, a selective adenosine A_2 receptor antagonist, to rat brain membranes. *Mol. Pharmacol.*, **46**, 817–822.

NONAKA, Y., SHIMADA, J., NONAKA, H., KOIKE, N., AOKI, N., KOBAYASHI, H., KASE, H., YAMAGUCHI, K. & SUZUKI, F. (1993). Photoisomerization of a potent and selective adenosine A_2 antagonist, (*E*)-1,3-dipropyl-8-(3,4-dimethoxy-styryl)-7-methylxanthine. *J. Med. Chem.*, **36**, 3731–3733.

RAMKUMAR, V. & STILES, G.L. (1988). Reciprocal modulation of agonist and antagonist binding to A_1 adenosine receptors by guanine nucleotides is mediated *via* a pertussis toxin-sensitive G protein. *J. Pharmacol. Exp. Ther.*, **246**, 1194–1200.

SCHIEMANN, W.P., WALTHER, J.M. & BUXTON, I.L.O. (1990). On the ability of endogenous adenosine to regulate purine nucleoside receptor binding of antagonists in smooth muscle membranes. *J. Pharmacol. Exp. Ther.*, **255**, 886–892.

SHIMADA, J., SUZUKI, F., NONAKA, H., ISHII, A. & ICHIKAWA, S. (1992). (E) - 1,3 - dialkyl - 7 - methyl - 8 - (3,4,5-trimethoxystyryl)-xanthines: potent and selective adenosine A_2 antagonists. *J. Med. Chem.*, **35**, 2342–2345.

STILES, G.L. & JACOBSON, K.A. (1987).A new affinity, iodinated adenosine receptor antagonist as a radioligand/photoaffinity crosslinking probe. *Mol. Pharmacol.*, **32**, 184–188.

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., FRANCIS, J., GHAI, G., BRAUNWALDER, A., PSYCHOYOS, S., STONE, G.A. & CASH, W.D. (1987). Biochemical characterization of the triazoloquinazoline, CGS 15943, a novel non-xanthine adenosine antagonist. *J. Pharmacol. Exp. Ther.*, **241**, 415–420.

YEUNG, S.H. & GREEN, R.D. (1984). [^{3}H]5′-N-ethylcarboxamidoadenosine binds to both Ra and Ri adenosine receptors in rat striatum. *Naunyn-Schmied. Arch. Pharmacol.*, **325**, 218–225.

ZOCCHI, C., ONGINI, E., BARALDI, P.G. & DIONISOTTI, S. (1995). SCH 58261, a new potent and selective adenosine A_{2A} receptor antagonist. *Br. J. Pharmacol.*, **114**, 299P.

ZOCCHI, C., ONGINI, E., CONTI, A., MONOPOLI, A., NEGRETTI, A., BARALDI, P.G. & DIONISOTTI, S. (1996). The non-xanthine heterocyclic compound SCH58261 is a new potent and selective A_{2A} adenosine receptor antagonist. *J. Pharmacol. Exp. Ther.*, (in press).

(Received June 22, 1995
Revised October 26, 1995
Accepted November 22, 1995)