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Characterization of human A_{2A} adenosine receptors with the antagonist radioligand [^{3}H]-SCH 58261

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1 We have characterized the binding 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, to human cloned A_{2A} adenosine receptors.

2 In Chinese hamster ovary (CHO) cells transfected with the human cloned A_{2A} receptor, [^{3}H]-SCH 58261 specific binding (about 70%) was rapid, saturable, reversible and proportional to protein concentration. The kinetic K_D value was 0.75 nM. Saturation experiments showed that [^{3}H]-SCH 58261 labelled a single class of recognition sites with high affinity ($K_D = 2.3$ nM) and limited capacity (apparent $B_{max} = 526$ fmol mg^{-1} protein).

3 Competition experiments revealed that binding of 0.5 nM [^{3}H]-SCH 58261 was displaced by adenosine receptor agonists with the following order of potency: 2-hexynyl-5′-N-ethylcarboxamidoadenosine (2HE-NECA) > 5′-N-ethylcarboxamidoadenosine (NECA) = 2-phenylaminoadenosine (CV 1808) > 2-[4-(2-carboxyethyl)-phenethylamino]-5′-N-ethylcarboxamidoadenosine (CGS 21680) > **R**-N^6-phenylisopropyladenosine (**R**-PIA) ≥ N^6-cyclohexyladenosine (CHA) > **S**-N^6-phenylisopropyladenosine (**S**-PIA).

4 Adenosine receptor 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) > 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) > theophylline.

5 Affinity values and rank order of potency of both receptor agonists and antagonists were similar to those previously obtained in human platelet and rat striatal membranes, except for CV 1808 which was more potent than CGS 21680. SCH 58261 was a competitive antagonist at inhibiting NECA-induced adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) accumulation in CHO cells transfected with human A_{2A} receptors. Good agreement was found between binding and functional data.

6 Thus, the new antagonist radioligand is preferable to the receptor agonist radioligand [^{3}H]-CGS 21680 hitherto used to examine the pharmacology of human cloned A_{2A} adenosine receptors.

Keywords: Adenosine receptors; human A_{2A} receptors; A_{2A} receptor antagonists; non-xanthine adenosine receptor antagonists; [^{3}H]-SCH 58261

Introduction

Adenosine interacts with the four G protein-coupled receptors, A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al*., 1994), which have been cloned from several animal species including rat, dog, mouse and man (Jacobson, 1995). Adenosine A_{2A} receptors are important in the regulation of a variety of biological functions, such as vascular conductance, blood platelet aggregation, polymorphonuclear leukocyte activation and motor behaviour (Ongini & Fredholm, 1996). There are several selective receptor agonists acting at A_{2A} receptors (Hutchison *et al*., 1989; van der Ploeg *et al*., 1996). Of these, 2-[4-(2-carboxyethyl)-phenethylamino]-5′-N-ethyl-carboxamidoadenosine (CGS 21680) has proven especially valuable (Hutchison *et al*., 1989). This compound is also a useful agonist radioligand (Jarvis *et al*., 1989). However, as discussed previously (Stiles & Jacobson, 1987), agonists at G protein-coupled receptors are not ideal radioligands. Indeed, like other G protein-linked receptor agonist radioligands, [^{3}H]-CGS 21680 binding to A_{2A} receptors is influenced by several factors including the state of the G proteins (Johansson *et al*., 1992). Moreover, in addition to the known limitation of the agonists when used as radioligands, [^{3}H]-CGS 21680 binds also to non-A_{2A} receptor binding sites. For example, it is now clear that in brain cortex and hippocampus [^{3}H]-CGS 21680 labels sites different from A_{2A} receptors (Johansson *et al*., 1993; Cunha *et al*., 1996; Lindström *et al*., 1996). Moreover, [^{3}H]-CGS 21680 labels the adenotin site in human platelets (Varani *et al*., 1994). Thus, the availability of antagonist radioligands, having high A_{2A} receptor affinity, would be a step forward for the characterization of this adenosine receptor subtype.

Only recently, important progress has been made with the development of selective A_{2A} adenosine receptor radioligands, such as the 8-styrylxanthine derivative, 7-[^{3}H]-methyl-8-(3,4-dimethoxystyryl)-1,3-dipropylxanthine ([^{3}H]-KF 17837S) (Nonaka *et al*., 1994), and the non-xanthine heterocyclic compounds, [^{125}I]-4 (2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-*a*}{1,3,5}triazin-5yl-amino]ethyl)phenol ([^{125}I]-ZM 241-385) (Palmer *et al*., 1995) and [^{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) (Zocchi *et al*., 1996). These radioligands have been shown to bind to the A_{2A} adenosine receptor with high affinity in membranes from different sources, such as rat striatum (Nonaka *et al*., 1994; Zocchi *et al*., 1996), bovine striatum (Palmer *et al*., 1995), porcine coronary arteries (Belardinelli *et al*., 1996), Chinese hamster ovary (CHO) cells expressing recombinant canine and rat A_{2A} receptors (Palmer *et al*., 1995) and human platelets (Dionisotti *et al*., 1996).

The human cloned A_{2A} adenosine receptor (Furlong *et al*., 1992; Salvatore *et al*., 1992) shows about 80–90% sequence identity with the receptor from other species such as rat, mouse, guinea-pig or dog (Jacobson, 1995). In the present

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study, we have characterized binding properties of the new potent and selective antagonist radioligand, [^{3}H]-SCH 58261, to the human A_{2A} adenosine receptor stably transfected in CHO cells. We have also examined the ability of SCH 58261 to block the agonist-induced increase of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in CHO cells as an index of functional antagonism.

Methods

Cell cultures and membrane preparation

Chinese hamster ovary (CHO) cells (American Type Culture Collection, Rockville, MD, U.S.A) stably expressing the human A_{2A} adenosine receptor were a gift from Drs Christer Owman and Christer Nilsson (Wallenberg Neuroscience Center, Lund, Sweden). The cloning and initial characterization is described elsewhere (Kull *et al*., 1995; Arslan *et al*., unpublished observations). The sequence of the cloned and expressed A_{2A} receptor is identical, in the coding region, to that previously obtained (Furlong *et al*., 1992; Salvatore *et al*., 1992). The cells were grown adherent and maintained in α-minimum essential medium (α-MEM) without nucleosides, containing 10% foetal calf serum, 2 mM L-glutamine, 50 u ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 500 μg ml^{-1} Geneticin at 37°C in 5% CO_2/95% air. Cells were subcultured 3 times weekly at a ratio of 1:5. Before recultivation, the cells were washed twice with phosphate buffered saline, trypsinized, resuspended and counted. Viability was more than 95% as assessed by the exclusion of trypan blue. Cells, obtained at passage 6–15, were washed twice with ice-cold phosphate buffered saline (PBS) and centrifuged at 200 × *g* for 5 min. The pellet was resuspended into 10 ml of 50 mM Tris-HCl buffer, pH 7.4, sonicated (4 times 10 s) and centrifuged at 1,000 × *g* for 10 min at 4°C. The resulting supernatant was recentrifuged at 30,000 × *g* for 60 min at 4°C and the pellet obtained was resuspended in Tris-HCl. Aliquots were rapidly frozen and stored at −70°C.

Radioligand binding assay

Thawed membranes were resuspended in the same buffer to a final protein concentration of 2 mg ml^{-1}. Adenosine deaminase (2 u ml^{-1}) was added for 30 min at 37°C, before radioligand binding assays, to remove endogenous adenosine.

Binding of [^{3}H]-SCH 58261 to human A_{2A} adenosine receptors was performed as previously described in rat striatal membranes (Zocchi *et al*., 1996). Assays were carried out in duplicate and in a final volume of 0.5 ml, containing 0.5 nM [^{3}H]-SCH 58261, 50 mM Tris-HCl buffer, pH 7.4 and CHO membrane suspension (0.1 mg protein/assay).

In saturation studies 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 some reference adenosine agonists and antagonists were used. Non-specific binding was determined in the presence of 100 μM 5′-N-ethylcarboxamidoadenosine (NECA). After 30 min incubation at 25°C, samples were filtered through Whatman GF/B filters with a Brandel cell harvester (Gaithersburg, MD, U.S.A.). Radioactivity was determined in an LS-6000IC 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.

Cyclic AMP assay

Cells were distributed in 12-well plates (200,000 cells per well) and were allowed to grow for 36–48 h. Cells were washed twice with HEPES-buffered (20 mM) α-MEM medium (pH 7.4) and incubated at 37°C for 10 min in 810 μl of HEPES buffered medium. The adenosine receptor antagonist to be tested was added in 90 μl of medium and cells were preincubated for 15 min. Then, NECA was added in 100 μl of medium and cells were incubated for a further 10 min. The reaction was stopped by addition of perchloric acid to a final concentration of 0.4 M. After 1 h at 4°C, the acidified cell suspension was transferred and neutralized with 4 M KOH/1 M Tris-HCl. In preliminary experiments, it was found that the addition of a potent inhibitor of cyclic AMP phosphodiesterase, rolipram (30 μM), did not significantly affect cyclic AMP accumulation. Therefore, the experiments were carried out in its absence. The cyclic AMP level was determined by use of a competitive radioligand binding assay (Nordstedt & Fredholm, 1990). Briefly, sample (cell culture supernatant) or cyclic AMP standard (0–8.0 pmol) was incubated with [^{3}H]-cyclic AMP and cyclic AMP binding protein in 96-well microtitre plates at 4°C for 150 min. The incubate was harvested by filtration over Whatman GF/B filters with a semi-automatic cell harvester (Skatron A/S, Norway). Each filter was rinsed with 3 ml of 50 mM Tris-HCl, pH 7.4. Then, the filters were punched out into scintillation vials and counted in an LKB 1209 RackBeta Liquid Scintillation Counter with 3 ml ReadySafe (LKB/Pharmacia) scintillation liquid. To determine pA_2 values for a series of adenosine receptor antagonists, cells were incubated with 8 different concentrations of the agonist NECA in the presence of 3–4 different concentrations of the antagonist.

Statistical analysis

Binding parameters were estimated by the computerized nonlinear fitting programme LIGAND (Munson & Rodbard, 1980). In the functional assay, individual dose-response curves were analysed with GraphPAD Prism software (GraphPAD, San Diego, CA, U.S.A.). Data are expressed as geometric mean with 95% confidence limits in parentheses, except for Hill coefficients (99%).

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 previously (Baraldi *et al*., 1994). [^{3}H]-SCH 58261 (specific activity 68.6 Ci mM^{-1}; radiochemical purity 99%) was obtained by use of 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 (Baraldi *et al*., 1996). 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) was synthesized by Dr Franco Gatta (Laboratory of Medicinal Chemistry, Istituto Superiore di Sanita', Rome, Italy) according to the method described previously (Shimada *et al*., 1992). 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 Gloria Cristalli (Department of Chemical Sciences, University of Camerino, Italy) (Cristalli *et al*., 1992). 2-[4-(2-Carboxyethyl)-phenethyl-amino]-5′-N-ethylcarboxamido-adenosine, (CGS 21680), 5′-N-ethylcarboxamidoadenosine (NECA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 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 theophylline, were from Research Biochemicals Inc. (Natick, MA, U.S.A.). Guanosine 5′-triphosphate and adenosine 3′:5′-cyclic monophosphate (cyclic AMP) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Adenosine deaminase was supplied by Boehringer Mannheim GmbH (Mannheim, F.R.G.). 4-(3-Cyclopentyloxymethoxyphenyl)-2-pyrrolidone (rolipram) was a gift from Schering AG, Berling (F.R.G.). All other reagents were from commercial sources.

Results

Radioligand binding assay

Since adenosine was found to be present in the binding assays with CHO cell membranes (Cohen *et al*., 1996), a pretreatment with adenosine deaminase (2 u ml^{-1}) was included. Specific binding of [^{3}H]-SCH 58261 to membranes from CHO cells expressing the human A_{2A} adenosine receptor was found to be rapid, saturable, reversible and dependent upon protein concentration. When incubated for 30 min, 25°C, pH 7.4, at a concentration of 0.5 nM the radioligand showed a specific binding of 66 (61–72)%. Specific [^{3}H]-SCH 58261 binding was found to increase linearly with respect to protein concentration over the range of 25–250 μg of protein/assay. The presence of 10 mM $MgCl_2$ or 100 μM GTP in the assay mixture did not modify the specific binding (data not shown).

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 100 μM NECA (Figure 1). Association and dissociation were fitted to a one-component model significantly better than to a two-component model ($P<0.05$). The rate constants were: $K_{obs}=0.78$ (0.39–1.53) min^{-1} and $K_{-1}=0.46$ (0.25–0.87) min^{-1} from a $t_{1/2}=1.5$ min (0.8–2.8). With these values, a kinetic dissociation constant (K_D) of 0.75 (0.64–0.88) nM was calculated.

Saturation experiments ($n=3$) revealed that [^{3}H]-SCH 58261 bound to a single class of receptors in membranes from CHO cells expressing human A_{2A} adenosine receptor, with a K_D value of 2.3 (1.7–3.2) nM and an apparent B_{max} value of 526 (476–581) fmol mg^{-1} protein. Computer analysis indicated that a one-component model described the data significantly better than a two-component model ($P<0.05$). Figure 2 shows a representative saturation isotherm and Scatchard plot.

In competition studies ($n=3$–6) representative adenosine receptor agonists and antagonists were evaluated (Figure 3). Agonists inhibited [^{3}H]-SCH 58261 binding with the following order of potency: 2HE-NECA > NECA = CV 1808 > CGS 21680 > **R**-PIA ≥ CHA > **S**-PIA (Table 1; Figure 3). The A_1 selective agonist, **R**-PIA, was found to be about 15 fold more potent than **S**-PIA, thus showing the stereoselectivity of [^{3}H]-SCH 58261 binding. In order to evaluate whether Mg^{2+} affects agonist binding, inhibition competition curves of the A_{2A} selective agonist CGS 21680 were constructed in the presence of $MgCl_2$. K_i and Hill coefficient values were 221 (156–311) nM and 0.92 (0.82–1.02) in the absence of Mg^{2+}, and 328 (191–565) nM and 0.88 (0.79–1.00) in the presence of Mg^{2+}. Thus, there was no significant changes of K_i values or Hill coefficients.

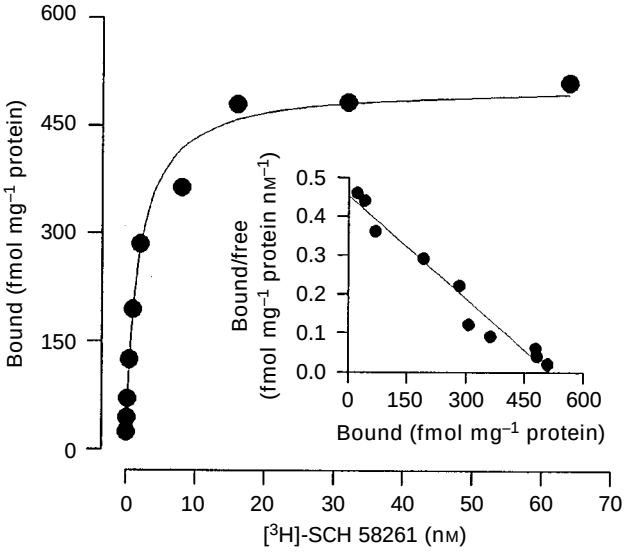

Figure 2 Saturation isotherm and Scatchard plot (inset) of [^{3}H]-SCH 58261 binding to membranes from CHO cells stably transfected with the human A_{2A} adenosine receptor. Curves are representative of a single experiment. Computer analysis revealed that data fit a one-component model significantly better than a two-component model ($P<0.05$). Binding parameters are as follows: $K_D=2.3$ (1.7–3.2) nM and B_{max} 526 (476–581) fmol mg^{-1} protein. Values indicate the geometric mean with 95% confidence limits in parentheses ($n=3$).

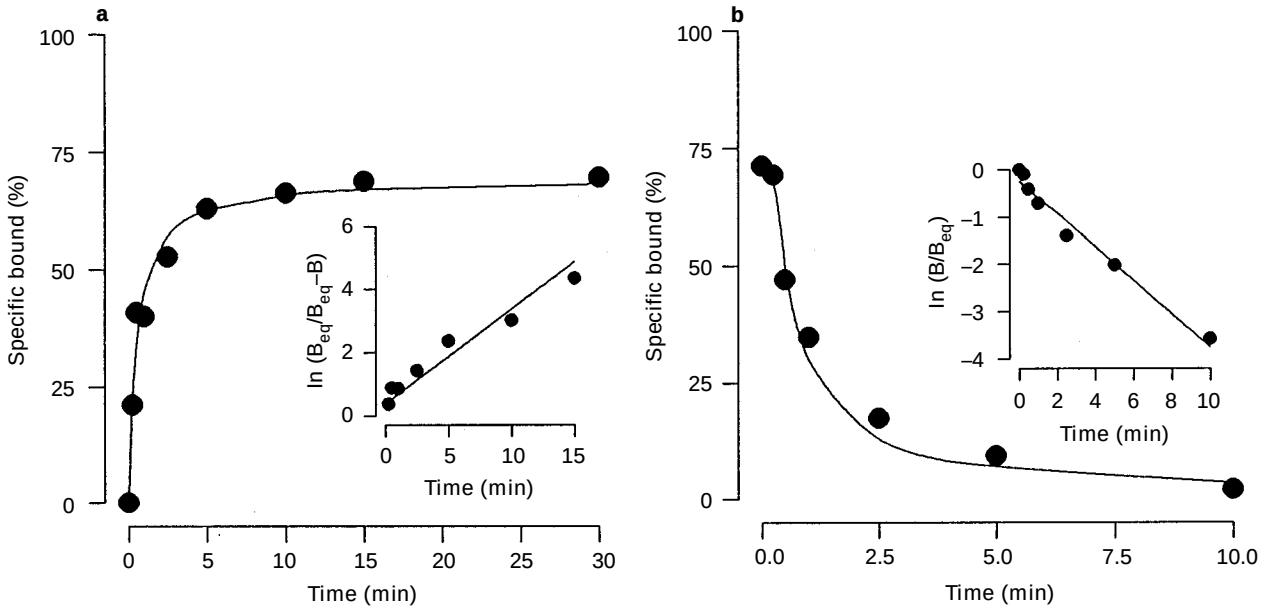

Figure 1 Kinetics of 0.5 nM [^{3}H]-SCH 58261 binding to membranes from CHO cells stably transfected with the human A_{2A} adenosine receptor, with association (a) and dissociation (b) curves representative of a single experiment. Dissociation was produced by the addition of 100 μM NECA. Insets: first-order 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 indicated that 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: $K_{obs}=0.78$ (0.39–1.53) min^{-1} and $K_{-1}=0.46$ (0.25–0.87) min^{-1} from a $t_{1/2}=1.5$ min (0.8–2.8). Values indicate the geometric mean with 95% confidence limits in parentheses ($n=4$).

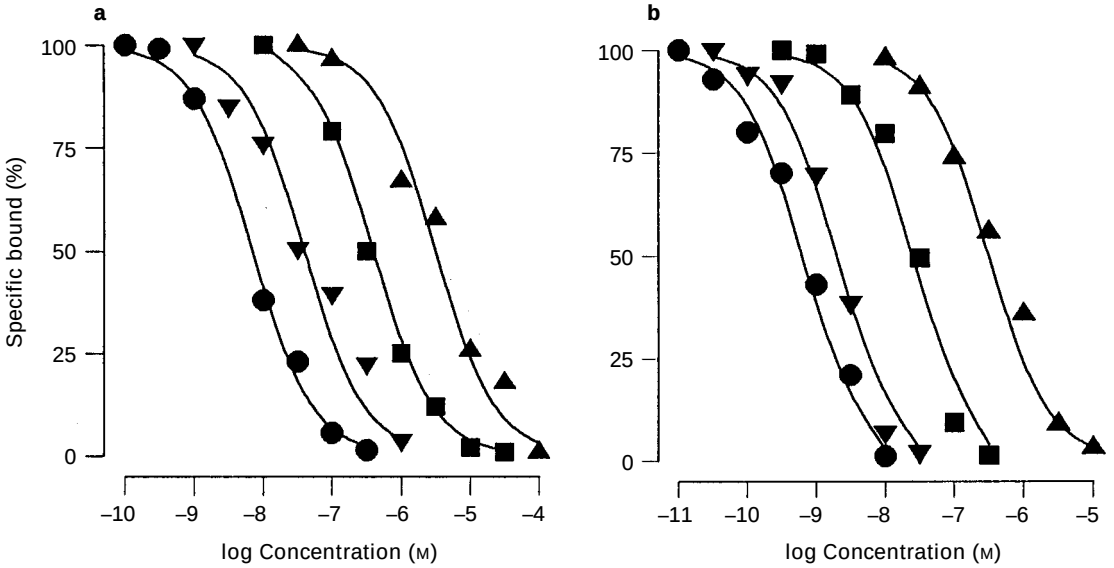

Figure 3 Competition curves of selected adenosine receptor agonists (a) or antagonists (b) in inhibiting 0.5 nM [^{3}H]-SCH 58261 binding to membranes from CHO cells stably transfected with the human A_{2A} adenosine receptor. (a) (●) 2HE-NECA, (▼) NECA, (■) CGS 21680, (▲) CHA. (b) (●) CGS 15943, (▼) SCH 58261, (■) KF 17837S, (▲) DPCPX. Curves are representative of a single experiment from a group of three to six independent experiments.

Table 1 Inhibition of [^{3}H]-SCH 58261 binding and functional activity of selected adenosine receptor agonists in CHO cells transfected with the human A_{2A} receptor

	[^{3}H]-SCH 58261		*Cyclic AMP assay*
	K_i (nM)	*Hill coefficient*	EC_{50} (nM)
2HE-NECA	5.2	0.91	1.7
	(4.3–6.3)	(0.71–1.15)	(1.0–2.6)
NECA	66	0.93	26
	(40–110)	(0.83–1.03)	(8–81)
CV 1808	76	0.98	6.8
	(62–93)	(0.96–1.00)	(1.4–33)
CGS 21680	221	0.92	37
	(156–311)	(0.82–1.02)	(15–89)
R-PIA	684	0.95	62
	(558–838)	(0.88–1.01)	(28–138)
CHA	1,386	0.93	316
	(631–3,044)	(0.84–1.02)	(100–977)
S-PIA	10,416	0.81	ND
	(6,821–15,904)	(0.64–1.01)	

Membrane preparations of CHO cells stably transfected with the human A_{2A} adenosine receptor were incubated with 0.5 nM [^{3}H]-SCH 58261 in the presence of at least 7 different concentrations of agonists. In the cyclic AMP assay, EC_{50} represents the concentration that produced a half-maximal stimulation of cyclic AMP production during 10 min incubation. Each value represents the geometric mean with 95% confidence limits in parentheses, except for Hill coefficients (99%), of 3–6 different experiments performed in duplicate. ND: not determined.

The ability of several xanthine and non-xanthine adenosine receptor antagonists inhibit [^{3}H]-SCH 58261 binding was also examined. The order of potency was: CGS 15943 > SCH 58261 > XAC > KF 17837S > DPCPX > theophylline (Table 2; Figure 3). CGS 15943 was the most potent compound in inhibiting [^{3}H]-SCH 58261 binding with a K_i value of 0.53 nM. Hill coefficients were not significantly different from unity (Tables 1 and 2).

Cyclic AMP assay

In order to confirm that SCH 58261 is a competitive antagonist at A_{2A} receptors expressed in CHO cells, we examined its ability to block the effects of NECA in these cells. We have previously observed that the cyclic AMP accumulation determined by NECA in CHO cells is entirely explained by stimulation of the transfected A_{2A} receptors (Kull *et al*., 1995; Arslan *et al*., unpublished observations). As shown in Figure 4, increasing concentrations of SCH 58261 caused a progressive rightward shift of the NECA dose-response curve. The estimated K_B value was 6.3 (3.2–10) nM.

Discussion

In this study we provide the first binding characterization of the A_{2A} antagonist radioligand [^{3}H]-SCH 58261 to cloned human A_{2A} adenosine receptors. In membrane preparations of CHO cells stably transfected with this human adenosine receptor subtype, [^{3}H]-SCH 58261 bound a single class of recognition sites with nanomolar affinity (K_D = 2.3 nM) and limited capacity (B_{max} = 526 fmol mg^{-1} protein). Saturation studies revealed that [^{3}H]-SCH 58261 has a receptor affinity for human cloned A_{2A} receptors (K_D = 2.3 nM) comparable to that observed in rat (K_D = 0.7 nM), porcine striatum (K_D = 1.3 nM), and in peripheral tissues such as human platelets (K_D = 0.9 nM) and porcine coronary arteries (K_D = 2.2 nM) (Belardinelli *et al*., 1996; Dionisotti *et al*., 1996; Zocchi *et al*., 1996). Moreover, K_D

Table 2 Inhibition of [^{3}H]-SCH 58261 binding and functional activity of selected adenosine receptor antagonists in CHO cells transfected with the human A_{2A} receptor

	[^{3}H]-SCH 58261		*Cyclic AMP assay*
	K_i (nM)	*Hill coefficient*	K_B (nM)
CGS 15943	0.53 (0.37–0.75)	1.00 (0.80–1.24)	0.63 (0.40–1.0)
SCH 58261	1.1 (0.75–1.6)	0.94 (0.86–1.02)	6.3 (3.2–10)
XAC	3.6 (2.0–6.3)	0.93 (0.87–1.00)	ND
KF 17837S	19 (11–34)	0.91 (0.74–1.11)	125 (100–158)
DPCPX	284 (178–452)	0.97 (0.92–1.03)	20 (16–25)
Theophylline	4,970 (3,632–6,802)	0.97 (0.89–1.05)	7,940 (6,310–10,000)

Membrane preparations of CHO cells stably transfected with the human A_{2A} adenosine receptor were incubated with 0.5 nM [^{3}H]-SCH 58261 in the presence of at least 7 different concentrations of antagonists. Each value represents the geometric mean, with 95% confidence limits in parentheses, except for Hill coefficient (99%), of 3–6 different experiments performed in duplicate. Binding and functional experiments with KF 17837S were carried out in daylight conditions. ND: not determined.

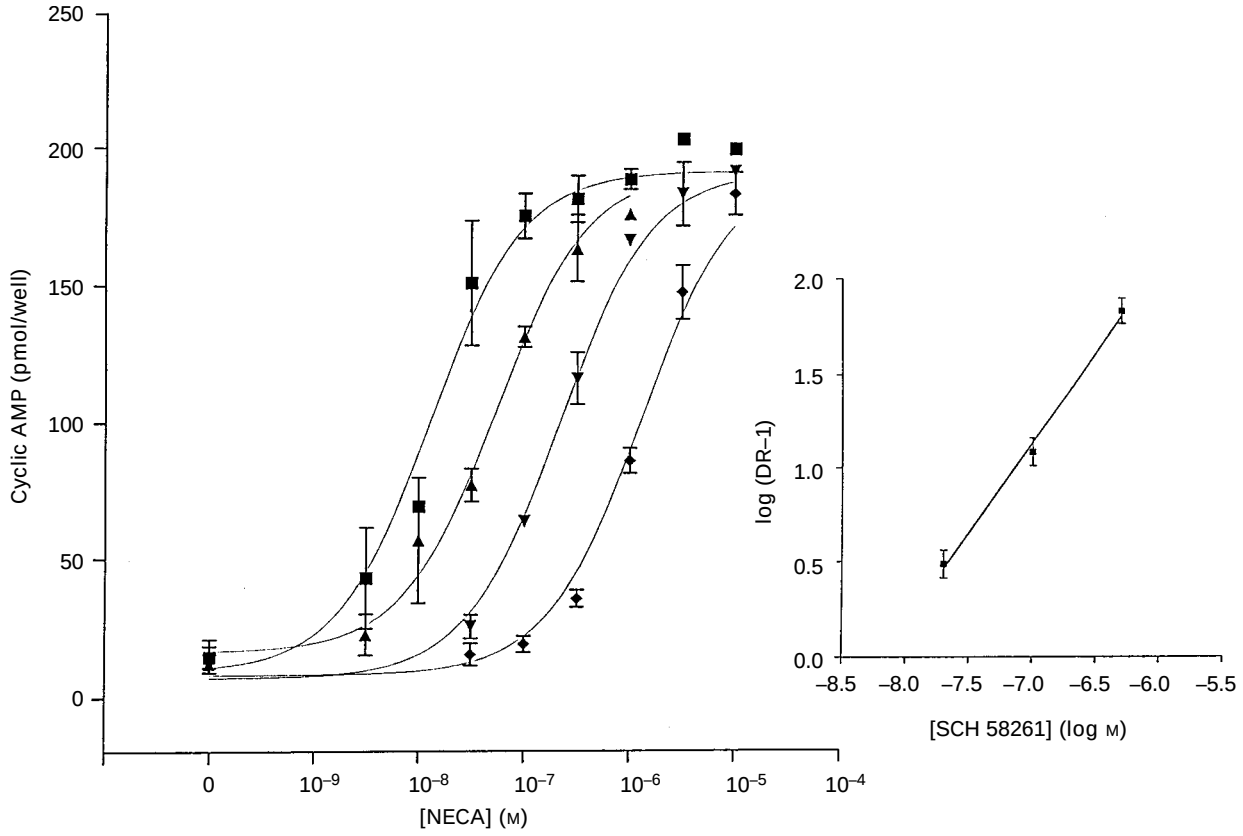

Figure 4 Dose-response curves of NECA-induced cyclic AMP accumulation in the absence (■) and in the presence of SCH 58261, 0.02 μM (▲), 0.1 μM (▼) and 0.5 μM (◆). Results are the geometric mean, with 95% confidence limits, of 3 experiments carried out in duplicate. Inset shows the Schild plot. A K_B value of 6.3 (3.2–10) nM was found.

values as evaluated in saturation and kinetic experiments were found to be in the same order of magnitude (2.3 and 0.75 nM, respectively). Interestingly, the B_{max} value was almost twice as high as that found when we used, instead, the agonist radioligand [^{3}H]-CGS 21680 in the same cell membrane preparation (Kull *et al.*, 1995; Arslan *et al.*, unpublished observations). This might be explained by considering that the agonist radioligand only binds to receptors present in the high-affinity state, *i.e.* associated with holotrimeric G proteins that have not bound any guanine nucleotide, whereas the antagonist radioligand binds to the receptor irrespective of its association with G proteins. The assumption is supplied by the fact that [^{3}H]-CGS 21680 binding is markedly affected by GTP (Parkinson & Fredholm, 1990; Johansson *et al.*, 1992). By contrast and in agreement with previous studies carried out with adenosine receptor antagonist radioligands (Nonaka *et al.*, 1994; Palmer *et al.*, 1995; Zocchi *et al.*, 1996), specific binding of [^{3}H]-SCH 58261 was not influenced by either Mg^{2+} or GTP.

Recently, it has been demonstrated that the agonist radioligands [^{3}H]-CGS 21680 and [^{125}I]-APE recognize two affinity states in rat transfected A_{2A} receptors, where the high affinity state corresponds to a G protein-coupled form of the A_{2A} receptor (Luthin *et al.*, 1995). However, all the binding studies carried out with antagonist radioligands recognize a single

Table 3 Affinity of selected adenosine receptor agonists and antagonists in inhibiting [^{3}H]-SCH 58261 binding to different tissues

		[^{3}H]-SCH 58261 K_i (nM)	
	Human A_{2A} in CHO cells	*Human platelets*[a]	*Rat striatum*[b]
Agonists			
2HE-NECA	5.2 (4.3–6.3)	17 (10–27)	3.1 (2.4–4.0)
NECA	66 (40–110)	30 (21–43)	61 (48–77)
CV 1808	76 (62–93)	ND	332 (233–473)
CGS 21680	221 (156–331)	753 (430–1,317)	111 (83–148)
R-PIA	684 (558–838)	1,614 (1,136–2,292)	992 (834–1,183)
CHA	1,386 (631–3,044)	7,829 (4,638–13,214)	2,840 (2,367–3,408)
S-PIA	10,416 (6,821–15,904)	7,833 (3,764–16,343)	8,504 (8,178–8,843)
Antagonists			
CGS 15943	0.53 (0.37–0.75)	0.22 (0.18–0.27)	0.38 (0.30–0.47)
SCH 58261	1.1 (0.75–1.6)	0.75 (0.40–1.4)	1.1 (0.84–1.3)
XAC	3.6 (2.0–6.3)	5.1 (3.7–7.0)	9.0 (6.6–12)
KF 17837S	19 (11–34)	48 (31–74)	9.4 (7.5–12)
DPCPX	284 (178–452)	501 (378–665)	234 (124–445)
Theophylline	4,970 (3,632–6,802)	ND	ND

Each value represents the geometric mean, with 95% confidence limits in parentheses. ND: not determined. [a]Dionisotti *et al.* (1996); [b]Zocchi *et al.* (1996). Binding experiments with KF 17837S were carried out in daylight conditions.

class of binding sites in several membrane preparations from different animal species (Nonaka *et al.*, 1994; Palmer *et al.*, 1995; Dionisotti *et al.*, 1996; Zocchi *et al.*, 1996). These data are consistent with the hypothesis that G-protein coupled receptors may exist in two affinity states (high and low) and receptor antagonists bind both states with the same affinity (Gilman, 1987).

In competition experiments, a series of both adenosine receptor agonists and antagonists displaced [^{3}H]-SCH 58261 with different affinity. Competition curves were best described by the one-component model (i.e. Hill coefficients not significantly different from unity), thus indicating the existence of a single coupling affinity state for the A_{2A} receptor (Tables 1 and 2).

A decrease of agonist affinity is generally observed when binding studies on A_{2A} adenosine receptors are carried out with an antagonist (Bruns *et al.*, 1987; Nonaka *et al.*, 1994; Zocchi *et al.*, 1996) rather than agonist radioligand (Bruns *et al.*, 1986; Jarvis *et al.*, 1989). It is well known that the binding of the agonist radioligand [^{3}H]-CGS 21680 is markedly enhanced by elevation of Mg^{2+} concentrations from 0.1 to 10 mM (Jarvis *et al.*, 1989; Johansson *et al.*, 1992; Parkinson & Fredholm, 1992). The effect of the divalent cation on agonist binding can be explained both by an increased coupling of the receptor to the holotrimeric G protein, which promotes high affinity binding (Parkinson & Fredholm, 1992), and by its direct effect on the receptor (Johansson *et al.*, 1992). We examined whether the absence of Mg^{2+} in the incubation mixture may contribute to the low affinity of agonists and found that the addition of 10 mM $MgCl_2$, altered neither the K_i value (220 and 328 nM with and without Mg^{2+}, respectively) nor the Hill coefficient of CGS 21680 binding, the compound being a reference A_{2A} selective agonist. The fact that the antagonist binding was not influenced by Mg^{2+} suggests that its binding is unaffected by coupling to G proteins. Moreover, the estimated Hill coefficient for agonists in displacing [^{3}H]-SCH 58261 binding was close to unity. This suggests that under these conditions a very small portion of the agonist binding is to the high affinity site.

In the present study, both adenosine agonists and antagonists displaced [^{3}H]-SCH 58261 with affinity and order of potency very close to those observed in both rat striatum and human platelets, with the same radioligand (Table 3) (Dionisotti *et al.*, 1996; Zocchi *et al.*, 1996). The Spearman test indicated that rank correlation coefficients were good as shown by their range from 0.89 to 0.99 ($P<0.01$). However, some adenosine receptor agonists were found to be more potent (from 3 to 5 fold) in inhibiting [^{3}H]-SCH 58261 binding to human cloned than platelet A_{2A} receptors. These differences could be explained by postulating that the platelet membrane receptor is less well coupled to G proteins than is the cloned A_{2A} receptor expressed in CHO cells. As for receptor antagonists, their potency to compete for [^{3}H]-SCH 58261 binding to the human cloned A_{2A} receptor was identical to that observed in human platelets (Table 3).

A reasonably good agreement was also found between binding results and functional data for adenosine receptor agonists and antagonists (Tables 1 and 2). Agonists were generally more potent as stimulators of cyclic AMP production in CHO cells expressing human A_{2A} receptors than predicted from binding data. This could be related to the fact that cells express a large number of receptors and that occupation of only a fraction of the receptors is sufficient to obtain a maximal activation of the subsequent steps in the signal transduction cascade. In the case of antagonists, SCH 58261 and KF 17837S appeared somewhat less (about 6 fold) and DPCPX somewhat more potent (about 15 fold) in functional assays than expected from binding data. We have no explanation for these differences at present, but it may be relevant that the functional assay was carried in intact cells at 37°C, whereas the binding assay used cell membranes incubated at room temperature.

As pointed out in the Introduction, there are some structural differences between rat and human A_{2A} receptors (Jacobson, 1995). In the past, Stone and coworkers (1988) showed that a considerable and significant interspecies variation exists in the K_i values of several xanthine adenosine receptor antagonists for the high affinity [^{3}H]-NECA binding site, whereas the rank order of potency of the same compounds was quite similar. In the present study, there are no apparent differences in the potency of receptor antagonists to displace [^{3}H]-SCH 58261 binding from rat striatal A_{2A} receptors or transfected human A_{2A} receptors (Table 3). However, there are some differences in the potency of receptor agonists. Specifically, the order of potency of CGS 21680 and CV 1808 is reversed. A similar difference in the order of potency was noted in a comparison of the potency of receptor agonists in a functional assay (Kull *et al.*, 1995). Moreover, a comparative study of A_{2A} receptor agonists in cells and tissues from different species (Gurden *et al.*, 1993) indicated that CGS 21680 is not a potent agonist at human A_{2A} receptors.

This finding adds to the above-mentioned argument that [^{3}H]-CGS 21680 has to be used with caution to study human A_{2A} receptors. An antagonist radioligand, such as [^{3}H]-SCH 58261 (and possibly [^{125}I]-ZM 241385), is much preferable. Its binding is apparently not affected by coupling to G proteins

and it shows a lower affinity to the non-A_{2A} receptor site to which CGS 21680 binds (Lindström *et al*., 1996). The results of this study also indicate that it is indeed important to characterize the human A_{2A} receptor since there are some species differences, at least in the case of receptor agonists. The cloned human A_{2A} receptors expressed in CHO cells may be a suitable system for such studies aiming to develop new pharmacological agents that act specifically at A_{2A} receptors.

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