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Differences in the Order of Potency for Agonists But Not Antagonists at Human and Rat Adenosine A_{2A} Receptors*

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ABSTRACT. To examine possible species differences in pharmacology, rat adenosine A_{2A} receptors were studied in PC12 (pheochromocytoma) cells, and human receptors in Chinese hamster ovary (CHO) cells transfected with the cloned human A_{2A} receptor cDNA. Using [^{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) as radioligand, the estimated B_{max} (maximal binding) was 538 and 2085 fmol/mg in CHO and PC12 cells, respectively. The K_d (dissociation constant) values for [^{3}H]-SCH 58261 were 1.05 and 5.6 nM in the two cell types, respectively. The order of potency of antagonists and most agonists was the same in both cell types, but 2-phenylaminoadenosine and 2-chloroadenosine were relatively less potent in PC12 cells than in CHO cells. In the functional assay, using cyclic AMP accumulation, all agonists tested were more potent in CHO than in PC12 cells, but this could not be readily explained by differences in adenylyl cyclase or in the expression of G proteins. As in the case of binding, the relative agonist potencies were similar for most compounds, but 2-phenylaminoadenosine and 2-chloroadenosine were more potent at human A_{2A} receptors in CHO cells than predicted from the data obtained on rat A_{2A} receptors in PC12 cells. Antagonists were approximately equipotent in the two cells. These results show that, despite only small differences in amino acid sequences and no difference in antagonist pharmacology, the relative order of potency of receptor agonists can differ between species homologues of the adenosine A_{2A} receptor. BIOCHEM PHARMACOL **57**;1:65–75, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. adenosine receptors; PC12 cells; species differences; cyclic AMP; cloned receptor; receptor binding

Adenosine is a modulator of many cellular functions. It exerts its effects via a family of G protein-coupled receptors of which four members have been cloned from several species [2, 3]. The presence of two different adenosine receptors, A_1 and A_2, was first suggested by opposing actions on adenylyl cyclase and differences in relative agonist potency [4, 5]. The existence of two subtypes of the A_2 receptor, which when stimulated both increased cAMP¶ formation, was postulated largely on the basis of pharmacological evidence [6, 7]. This contention has been borne out by cloning. One of eight "orphan" receptors cloned from a dog thyroid cDNA library, RDC8, was identified as the canine counterpart of the A_{2A} receptor [8]. Based on this information, the rat and human A_{2A} receptors were cloned [9, 10], and subsequently the related A_{2B} receptor was also cloned from rat [11] and man [12].

There is considerable interest in the therapeutic potential of agonists and antagonists acting at A_{2A} receptors [13]. It is therefore important to determine to what extent functional data obtained in rats can be extrapolated to man. Rat and human A_{2A} receptors are structurally very similar. It is, however, known that there are significant differences in pharmacology between species homologues of A_1 adenosine receptors despite only minor differences in amino acid composition [14], but thus far the information regarding species differences for A_{2A} receptors is limited. We there-

* A preliminary account of some of these results has been given [1].

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¶ *Abbreviations:* PC12, pheochromocytoma cells; CHO, Chinese hamster ovary cells; cAMP, adenosine 3′,5′-cyclic monophosphate; NECA, 5′-*N*-ethylcarboxamidoadenosine; CV-1674, 2-(4-methoxyphenyl)-adenosine; CV-1808, 2-phenylaminoadenosine, CGS 21680, 2-[*p*-(2-carbonylethyl)-phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine; R-PIA, N^6-(R-phenylisopropyl)adenosine; CHA, N^6-cyclohexyladenosine; SCH 58261, 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; APNEA, N^6-2(4-aminophenyl)ethyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; CGS 15943, 9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo[1,5]quinazolin-5-imine monomethanesulfonate; CSC, 8-(3-chlorostyryl)caffeine; metrifudil, N^6-(2-methylphenyl)methyladenosine; HE-NECA, 2-hex-1-ynyl-5′-*N*-ethylcarboxamidoadenosine; KF 17837S, (1,3-dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine; G protein, GTP binding protein; G.418, Geneticin; hA_{2A}, human adenosine A_{2A}; rA_{2A}, rat adenosine A_{2A}; PCR, polymerase chain reaction; B_{max}, maximal binding; and SSC, 150 mM sodium chloride, 15 mM sodium citrate, pH 7.

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fore examined the potency of some prototypic adenosine receptor agonists and antagonists on rat and human receptors. The cloned human A_{2A} receptors were expressed in CHO cells. To examine rat A_{2A} receptors we used PC12 cells. These cells derive from rat chromaffin tissue [15] and have been shown to possess an adenosine-stimulated adenylyl cyclase [16, 17]. We used binding assays with the antagonist radioligand [^{3}H]-SCH 58261. In addition, cAMP accumulation in intact cells was used as a functional assay. Our results indicate a considerable similarity in antagonist potency, but some important differences in agonist potency between species.

MATERIALS AND METHODS

Chemicals

Rolipram (4-(3-cyclopentyloxymetoxyphenyl)-2-pyrrolidone) was a gift from Schering AG. cAMP, 2-chloroadenosine, NECA, paraxanthine, theophylline, caffeine, forskolin, and dipyridamole were purchased from Sigma. CV-1674 and CV-1808 were gifts from Takeda Chemical Industries Ltd. CGS 21680 and its [^{3}H]-labelled form were gifts from the CIBA-GEIGY Corporation, or were obtained from Research Biochemicals Inc. R-PIA and CHA were from Calbiochem Corporation. Adenosine deaminase was purchased from Boehringer Mannheim. Both the unlabelled and [^{3}H]-labelled forms of SCH 58261 were gifts from Dr. Ennio Ongini, Schering-Plough Research Institute. APNEA, DPCPX, CGS 15943, CSC, and nitrobenzylthioinosine were from RBI. Metrifudil was a gift from Dr. Lars Knutsen, Novo Nordisk. HE-NECA was a gift from Prof. Gloria Cristalli, Dept. of Chemistry, Camerino, Italy. KF 17837S was a gift from Dr. Fumio Suzuki, Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co. Oligonucleotide primers were synthesised by Promega, Scandinavian Diagnostic Services. Antibodies against G proteins were from Santa Cruz Biotechnology.

Cloning of the Human A_{2A} Receptor

The cDNA library of a human B-cell lymphoblast cell line (Gm03299, NIGMS Human Genetic Mutant Cell Repository), containing 7.5×10^6 recombinants, was screened [18] using a mixture based on a 29-mer oligonucleotide with 128-fold degeneracy to transmembrane region VI [19]. The oligonucleotide 5′-A(T/G)G(A/T)AG(A/T)AGGGCAGCCAGCAGA(C/G)(C/G)(G/A)(T/C)GAA-3′ was end-labelled with ^{32}P using [γ-^{32}P]-dATP (5000 Ci/mmol; Amersham) and T4 polynucleotide kinase. Hybridisation was carried out in 3 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), pH 7.0, at 60° and washing in 1 × SSC at 60°. A 2.5-kb hybridising BamH1 fragment separated by 0.8% agarose gel electrophoresis in 1 × Tris borate EDTA buffer was used to identify a positive clone (designated Lymb42-14). The 2.5-kb fragment was cut into smaller fragments that were subcloned into M13 and sequenced with the Sanger dideoxynucleotide chain termination method. The sequence agreed with the sequence no. X68486 submitted to GeneBank.

Transfection of A_{2A} Receptor into CHO Cells

Chinese hamster ovary cells (CHO-K1 cells; CCL61, American Type Culture Collection) were transfected with plasmid-DNA for stable expression using the calcium phosphate precipitation method [20]. The cells were cotransfected with the pcD_{neo} vector to allow for selection with the neomycin analogue G.418 (500 μg/mL), which was added 71 hr after the transfection. A monoclonal line expressing Lymb42-14 was obtained by limiting dilution. Expression of corresponding message RNA was determined with Northern blot hybridisation using an internal ^{32}P-labelled 48-mer oligonucleotide probe. Nontransfected cells were used as controls in both instances.

Culture of CHO Cells

CHO cells transfected with the human A_{2A} receptor cDNA were grown adherent and maintained in α-minimum essential medium without nucleosides, containing 10% foetal bovine serum, penicillin (50 U/mL), streptomycin (50 μg/mL), L-glutamine (2 nM) and G-418 (GIBCO; 0.5 mg/mL) at 37° in 5% CO_2/95% air. Cells were subcultured three times weekly at a ratio of 1:5. Before recultivation the cells were washed twice with PBS, trypsinized, and resuspended in new medium and counted. Viability was more than 95%, as assessed by the exclusion of trypan blue.

Culture of PC12 Cells

PC12 cells (clone 1) [21] were cultured in Dulbecco's modified Eagle's medium with glucose (4.5 g/mL) but without sodium pyruvate, supplemented with 10% heat-inactivated horse serum, 5% foetal bovine serum, 2 mM of L-glutamine and penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37° in a humidified 5% CO_2/95% air incubator. The cells were recultivated 2–3 times per week.

[^{3}H]-CGS 21680 Binding

PC12 or CHO cells were washed twice in PBS and homogenised in 50 mM Tris–HCl pH 7.4 containing 7.5 mM $MgCl_2$ and 5 mM EDTA. The supernatant after centrifugation at 1000 *g* was further centrifuged at 30,000 *g* for 60 min. The resulting membrane pellet was resuspended in 50 mM Tris–HCl pH 7.4 with 2 mM $MgCl_2$, and frozen in aliquots at −80° until used. The binding assay was carried out in 500 μL of 50 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$, and 5 U/mL of adenosine deaminase using 200 μg of membrane protein per assay for 4 hr at room temperature. The adenosine A_1 receptor antagonist DPCPX was included at a final concentration of 30 mM, because it is known that [^{3}H]-CGS 21680 can bind to sites that are not identical to adenosine A_{2A} receptors and that DPCPX can

potently displace binding at these sites [22, 23]. Saturation curves were determined using 0.5 to 200 nM [^{3}H]-CGS 21680. Nonspecific binding was determined using 100 μM of R-PIA.

[^{3}H]-SCH 58261 Binding

Membranes for radioligand assay were prepared in the same way as for [^{3}H]-CGS 21680 binding. Thawed membranes were resuspended in Tris–HCl to a final protein concentration of 1 mg/mL. Adenosine deaminase (2 units/mL) was added for 1 hr at 37° before radioligand assay to remove endogenous adenosine. Assays were carried out in duplicate and in a final volume of 300 μL containing mM [^{3}H]-SCH 58261, 50 mM Tris–HCl, pH 7.4 and CHO cell membrane suspension (0.1 mg protein/assay). In saturation studies, membranes were incubated with ten different concentrations of [^{3}H]-SCH 58261 ranging from 0.0625–32 nM. In competition experiments, at least nine different concentrations of some adenosine receptor agonists and antagonists were used. Nonspecific binding was determined in the presence of 100 μM NECA. After 30-min incubation at room temperature, samples were filtered through Whatman GF/B filters using a semiautomatic cell harvester (Skatron A/S). Radioactivity was determined in an LKB 1209 RackBeta liquid scintillation counter with 3 mL of ReadySafe (LKB/Pharmacia) scintillation liquid. Protein concentration was determined by the method of Bradford [24], with BSA used as standard.

Measurement of cAMP Accumulation

The comparison between CHO and PC12 clone 1 cells was carried out using adherent cells. The cells were sown out in 12-well plates (200,000 cells per well) and were allowed to grow for 36–48 hr. Cells were then washed twice with HEPES-buffered (20 mM) α-minimum essential medium (CHO) or Dubecco's modified Eagle's medium (PC12) (pH 7.4). The cells were incubated at 37° for 10 min in 0.9 mL of HEPES-buffered medium. The agonist to be tested was added in 0.1 mL of medium and the cells were incubated for a further ten min. To determine pA_2 values for a set of adenosine antagonists we incubated the cells with eight different concentrations of an adenosine agonist (NECA) in the presence of 3–4 different concentrations of the antagonist to measure the dose ratio. The reactions were terminated by the addition of perchloric acid to a final concentration of 0.4 M. After 1 hr at 4°, the acidified cell suspensions were transferred to tubes and neutralised with 4 M KOH/1 M Tris–HCl.

In initial experiments, it was found that the addition of a potent inhibitor of cAMP phosphodiesterase, rolipram (30 μM; 4-(3-cyclopentyloxymetoxyphenyl)-2-pyrrolidone), did not significantly affect the cAMP accumulation in either cell type. The experiments were therefore carried out in its absence. The cAMP content in the samples was determined using a competitive radioligand-binding assay [25]. Briefly, sample (cell culture supernatant) or cAMP standard (0–8.0 pmol) was incubated with [^{3}H]-cAMP and cAMP binding protein in 96-well microtitre plates at 4° for 150 min. Free and bound [^{3}H]-cAMP were separated by filtration over Whatman GF/B filters using a semiautomatic cell harvester (Skatron A/S). Each filter was rinsed with 3 mL of 50 mM Tris–HCl pH 7.4. With a filter punch (Skatron A/S) the filters were punched out into scintillation vials and the radioactivity measured in an LKB/Pharmacia scintillation counter with 3 mL of ReadySafe (LKB/Pharmacia) scintillation fluid.

PCR Analysis of A_{2A} and A_{2B} Receptor Message RNA

Total RNA was isolated from CHO and PC12 clone 1 cells using the Ultraspec™ RNA Isolation system (BIOTECH BULLETIN NO:27, 1992) and resuspended in diethylpyrocarbonate-treated water. The RNA was further diluted in diethylprocarbonate-treated water to a final concentration of 0.4–0.5 μg/μL. cDNA was synthesised by reverse transcription of 1 μg of total RNA. The DNA amplification was performed by PCR in a PTC-100™ (Programmable Thermal Controller) (MJ Research). The PCR reaction mixture contained 5 μL of 10 × PCR buffer (Promega, Scandinavian Diagnostic Services), 1 μL of dNTP (10 mM mix) (Pharmacia-LKB), 8 μL of $MgCl_2$ (25 mM) (Promega), 21 μL of H_2O, 0.25 μL of Taq DNA Polymerase (5000 U/mL) (Promega), 1.25 μL of each primer (5 μM), and 12.5 μL of cDNA. The timing was: 1-min denaturation at 95°, 1.5-min annealing at 55°, and 2-min elongation at 72°, for 35 cycles. After the 35 cycles, a 7.5-min elongation at 72° was carried out. The PCR product was separated on a 1.4% agarose gel (BRL) and stained with ethidium bromide.

Oligonucleotide primers for A_{2A} receptors corresponded to amino acids NLQNVT [5′-CGAATTCAACCTGCAG AACGTCACC-3′, sense] and amino acids IAIDR [5′-TC GAATTCGCGGTC(G/A)ATGGCGAT(A/G)-3′, antisense], i.e. to a sequence between the first and the beginning of the second cytoplasmic loop of the receptor. Those for A_{2B} receptors corresponded to amino acids QTPTNYF [5′-CAGAC(G/C)CCCACCAACTACTT-3′, sense] and amino acids AVLFIKI [5′-GCCACCA(G/T)GAAGAT (C/T)TT(A/G)ATG-3′, antisense], i.e. to a sequence between the first and the beginning of the third cytoplasmic loop.

Immunoblots for G Proteins

Cell membranes were prepared in the same way as those for binding assays. Proteins were run on a 12% polyacrylamide, denaturing gel, blotted onto a membrane (Immobilon, Millipore) and nonspecific binding blocked by an overnight incubation in Tris-buffered saline Tris–HCl, pH 8, 150 mM NaCl) containing Tween-20 (0.5%) and 5% dry milk. Membranes were incubated with primary antibodies for 1 hr, rinsed several times in Tris-buffered saline, incubated

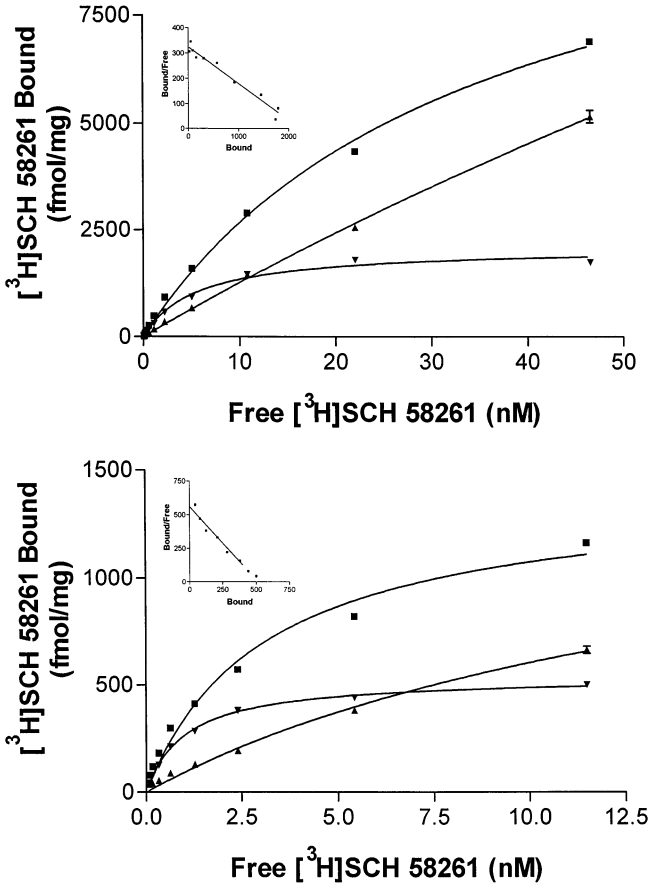

FIG. 1. Saturation isotherm for [^{3}H]-SCH 58261 binding to A_{2A} receptors in PC12 cell membranes (upper panel) and membranes from CHO cells transfected with the human A_{2A} receptor cDNA (lower panel). Membranes were incubated with increasing concentrations of radioligand as described in the experimental section. The insets show the Scatchard plots of the data. Using [^{3}H]-SCH 58261 nonlinear curve fitting gave a K_d of 5.6 nM in PC12 cells and 1.05 in transfected CHO cells, with B_{max} values of 2085 fmol/mg and 538 fmol/mg, respectively. (■) Total binding, (▲) nonspecific binding, and (▼) specific binding. Mean of 2–4 separate experiments.

with a secondary antibody (horseradish peroxidase-conjugated anti-rabbit) and developed by an enhanced chemiluminescence system (Amersham, UK). The data were quantitated using densitometry (MCID system, Imaging Research).

Data Analysis

Binding data were analysed using the Ligand program or the GraphPad InPlot program. The latter was also used to analyse individual dose-response curves. For statistical comparisons 95% confidence intervals were used.

RESULTS

Receptor Binding

When these studies were initiated, the only radioligand available for adenosine A_{2A} receptors was the agonist [^{3}H]-CGS 21680. Initial experiments with this radioligand showed saturable binding to both cell types that was best fitted to a one-site model of binding. The estimated K_d (dissociation constant) values were 31.9 (22–42) nM (mean and 95% confidence interval) with a calculated B_{max} of 352 (313–391) fmol/mg in membranes from PC12 cells and 53.3 (33–74) mM with a calculated B_{max} of 234 (198–270) fmol/mg in membranes from CHO cells transfected with human A_{2A} receptor cDNA. However, in the latter cell type, low affinity and a rather high nonspecific binding precluded meaningful binding data for competing agonists and antagonists.

Saturable binding of [^{3}H]-SCH 58261 could be detected in both cell types (Fig. 1). Nonlinear curve fitting of the data to a one-site model yielded estimated K_d (dissociation constant) values of 1.05 (0.96–1.14) mM in CHO cells and 5.6 (4.5–6.7) nM in PC12 cells. The calculated B_{max} was 538 (524–552) fmol/mg protein in CHO cells and 2085 (1953–2217) fmol/mg protein in PC12 cells. These values are similar to those reported earlier [26, 27]. Thus, [^{3}H]-SCH 58261 binds to a larger number of sites than [^{3}H]-CGS 21680 and with a markedly higher affinity.

[^{3}H]-SCH 58261 binding to membranes from both cell types was displaced by a series of adenosine analogues (Fig.

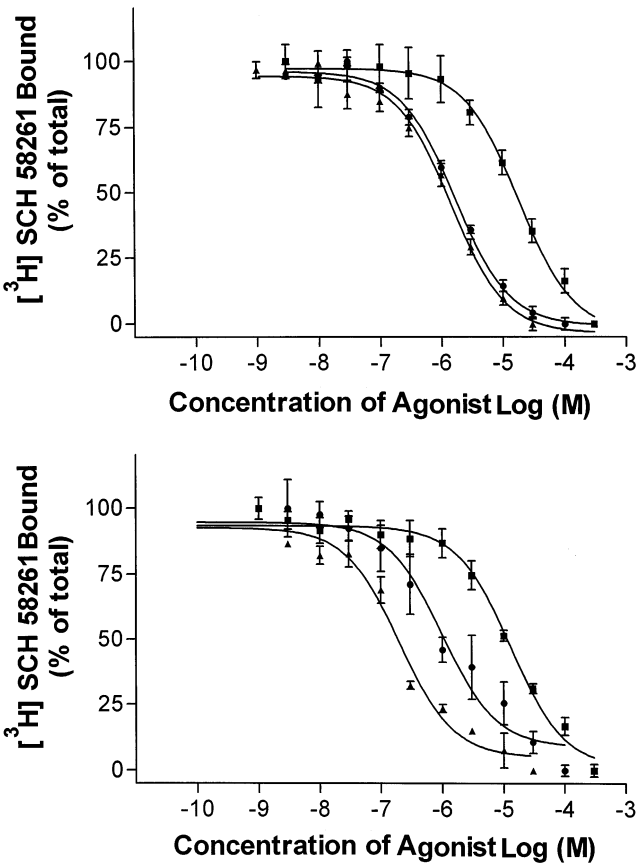

FIG. 2. Displacement of bound [^{3}H]-SCH 58261 (1 nM) in PC12 cell membranes (upper panel) and transfected CHO cell membranes (lower panel) by selected receptor agonists. CV-1808 (▲), 2-chloroadenosine (●), and APNEA (■). Mean ± SEM of 3–5 duplicate determinations.

TABLE 1. Potency of adenosine analogues as displacers of [^{3}H]-SCH 58261 binding in rat PC12 cells and CHO cells transfected with the human A_{2A} receptor

	PC12	CHO	
	K_d [^{3}H]-SCH 58261	K_d [^{3}H]-SCH 58261	
	1-site: 5.6 (4.49–6.74)	1-site: 1.05 (0.96–1.14)	Dose ratio
	K_i	K_i	PC12/CHO
Agonists			
HE-NECA	127 (6.5–247)	5.2 (4.3–6.3)*	24
NECA	160 (110–234)	66 (40–110)*	2.4
CGS 21680	298 (216–412)	221 (156–311)*	1.4
CV-1808	949 (589–1,530)	76 (62–93)*	12.5
R-PIA	2,301 (1,663–3,182)	684 (558–838)*	3.4
APNEA	12,850 (7,924–20,850)	4,718 (2,711–8,212)	2.7
2-chloroadenosine	879 (722–1,070)	164 (92–293)	5.4
CHA	5,859 (3,609–9,529)	1,386 (631–3,044)*	4.2
CV-1674	4,420 (3,624–5,390)	1,414 (908–2,203)	3.1
Metrifudil	1,813 (1,514–2,170)	3,448 (1,686–7,052)	0.5
Antagonists			
SCH 58261	3.5 (2.2–5.5)	1.1 (0.75–1.6)*	3.1
DPCPX	323 (236–445)	284 (178–452)*	1.1
Caffeine	8,182 (6,555–10,210)	2,396 (1,248–4,600)	3.4

K_d and K_i values were calculated from radioligand binding experiments as described. All results are given in nM. Mean and 95% confidence interval.
*Data from [27].

2, Table 1). All compounds induced a complete and concentration-dependent inhibition of specific radioligand binding. The displacement appeared monophasic for both agonists and antagonists. Comparison of one- and two-site binding models showed the one-site model to give the best fit for all compounds tested ($P < 0.05$ according to F-test in GraphPad Prism). The rank order of potency of a set of adenosine agonists (HE-NECA, NECA, CGS 21680, R-PIA, CHA, and APNEA) to displace the specific binding of [^{3}H]-SCH 58261 was similar for binding to membranes from the transfected CHO cells, and for binding to membranes from PC12 cells. However, for some agonist compounds there were notable differences. CV-1808 was equipotent with NECA and more potent than CGS 21680 on hA_{2A} receptors expressed in CHO cells, but significantly less potent on rA_{2A} receptors in PC12 cells. The latter finding is in good agreement with data on rA_{2A} receptors in striatum [28]. 2-Chloroadenosine was equipotent with CGS 21680 on hA_{2A} receptors. Metrifudil, finally, tended to be more potent on rA_{2A} receptors, where it was equipotent with R-PIA, than on hA_{2A} receptor, where it was significantly less potent than R-PIA (Table 1). In general, the results obtained for agonist potency in displacing [^{3}H]-SCH 58261 binding from rA_{2A} receptors in PC12 cells agreed with the reported values for rA_{2A} receptors in striatal membranes [28]. However, for unexplained reasons HE-NECA was clearly less potent in PC12 cells.

We also examined the ability of a series of adenosine receptor antagonists to displace the specific binding of [^{3}H]-SCH 58261 (Table 1). The rank order of potency in both PC12 and CHO cells was SCH 58261 $\gg$ DPCPX $\gg$ caffeine. All ligands were more potent in CHO cell membranes except for metrifudil.

cAMP Responses to Adenosine Analogues in Transfected CHO and PC12 Cells Inducing Activation of A_{2A} Receptors

Accumulation of cAMP in the two cell types was studied. It must be kept in mind that the A_{2A} receptor is not the only adenosine receptor able to afford such stimulation, and CHO cells transfected with human A_{2A} receptor message RNA also expressed A_{2B} receptor message RNA (Fig. 3). The A_{2B} receptors are also found in native CHO cells not transfected with A_{2A} receptor cDNA. However, neither NECA nor CGS 21680 could stimulate cAMP accumulation in CHO cells that had not been transfected with the A_{2A} receptor (Fig. 3b), indicating that the response even to the nonselective agonist NECA is almost entirely due to A_{2A} receptor stimulation. The increase in cAMP accumulation seen after stimulation with NECA (1 μM) was from 7.5 to 115.6 ± 4.0 pmol cAMP per well in transfected cells, but only from 5.3 to 7.1 ± 4.7 pmol cAMP per well in nontransfected cells. The very potent A_2 receptor agonist HE-NECA [29] (1 μM) was also ineffective in untransfected CHO cells (not shown).

It has been shown previously that PC12 cells possess not only A_{2A} but also A_{2B} receptors, but that the bulk of the response, especially that seen with lower concentrations of

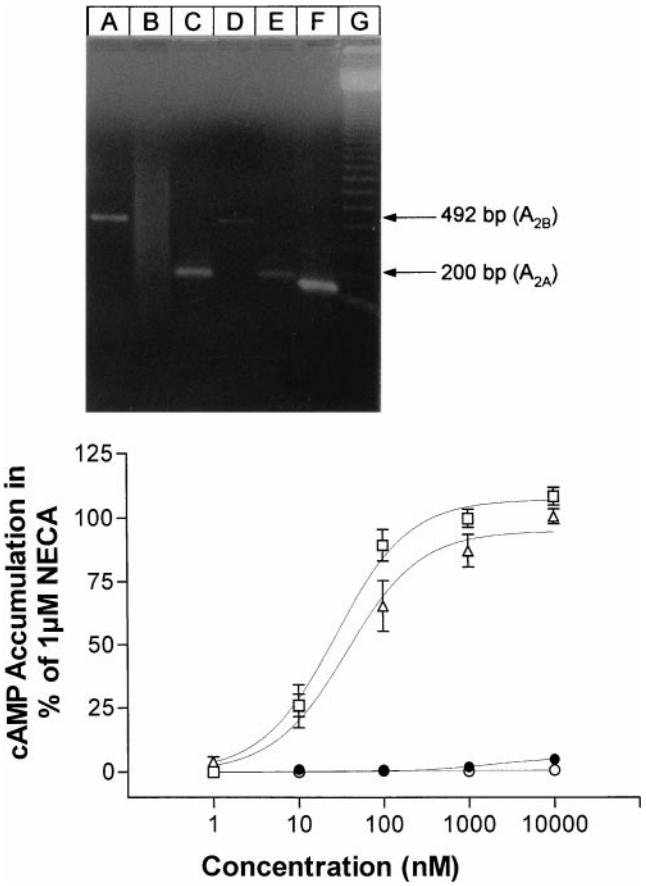

FIG. 3. Adenosine A_{2B} receptors present in PC12 cells and CHO cells (upper panel) do not contribute to the functional responses to agonists (lower panel). Upper panel, PCR analysis of cDNA obtained by reverse transcription of cellular RNA reflecting the expression of adenosine receptors in PC12 clone 1 cells, CHO cells transfected with human A_{2A} receptors, and untransfected CHO cells. Lane A: PC12 cells, A_{2B} primers. Lane B: Untransfected CHO cells, A_{2A} primers. Lane C: CHO cells, A_{2A} primers. Lane D: CHO cells, A_{2B} primers. Lane E: PC12 cells, A_{2A} primers. Lane F: Positive control. Lane G: Ladder (123 bp). Lower panel, Dose-response curves of cAMP accumulation in CHO cells transfected with human A_{2A} receptor cDNA and control cells. Results are presented as percent of the response to 1 μM NECA, which was always included as an internal control. (□) NECA with A_{2A} transfected CHO cells, (△) CGS 21680 with A_{2A} transfected CHO cells, (●) NECA with control cells, and (○) CGS 21680 with control cells. (The basal cAMP level was 5.3 pmol/well and stimulation with 1 μM NECA gave a cAMP level of 115.6 ± 4.0 pmol/well). Mean with SEM of 2 duplicate determinations.

the agonists, can be ascribed to A_{2A} receptor stimulation [17, 30]. This conclusion is further supported by the finding that the cAMP stimulation afforded by NECA can be antagonised in a purely competitive manner by the selective A_{2A} receptor antagonist KF 17387 (Fig. 4) and SCH 58261 (not shown). The fact that CGS 21680 and CV-1674, which are more than 1000-fold selective for A_{2A} vs. A_{2B} receptors [6, 31], appeared to be full agonists in both cells provides further evidence that the responses measured are due to A_{2A} receptor stimulation.

Agonist Potencies on Human and Rat A_{2A} Receptors

We next examined the effects of eleven adenosine receptor agonists on cAMP accumulation in CHO cells expressing human A_{2A} receptors and in PC12 cells expressing the rat counterpart. The results are summarised in Table 2. Although all agonists were more potent in CHO cells, the ratio between EC_{50} for the agonists in PC12 and CHO cells was remarkably different for the different agonists. For example, 2-chloroadenosine and adenosine were approximately 39 times less potent in PC12 than in CHO cells. This could not be accounted for by a more rapid uptake of adenosine and 2-chloroadenosine in PC12 cells than in CHO cells, as uptake inhibitors (nitrobenzylthioinosine; 3 μM or dipyridamole; 3 μM) only slightly (a shift to the left by less than 50%, which was not significant) altered the dose-response curves. Moreover, the effect of the uptake blockers was similar in PC12 and CHO cells (data not shown). The largest difference in agonist potency between the two cells was seen with CV-1808 and the smallest with NECA. CV-1808 was 121 times more potent in CHO than in PC12 cells, whereas NECA was only 1.9 times more potent in CHO than in PC12 cells. This difference could not be accounted for by CV-1808 being a partial agonist, since its E_{max} was as high as that of NECA in both PC12 cells and in CHO cells transfected with hA_{2A} receptors. Indeed, this was true for all agonists except CHA, CV-1674, and APNEA for which poor solubility prevented the generation of full concentration-response curves.

These differences can also be expressed in another way. Whereas the relative order of potency for most agonists was similar between cells, there were exceptions. Thus, on hA_{2A} receptors expressed in CHO cells, CV-1808 was at least as potent as NECA and CGS 21680, but was significantly less potent than both those compounds on cAMP generated by rA_{2A} receptors in PC12 cells (Table 2). 2-Chloroadenosine tended to be less potent than CHA on rA_{2A} receptors, but more potent on hA_{2A} receptors. The higher potency of the agonists in CHO cells might be due to differences in adenylyl cyclase or in the rate of cAMP breakdown. However, as seen in Fig. 5a, forskolin in concentrations above 1 μM raised cAMP accumulation in both cell types, but the magnitude of the response was higher in PC12 than in CHO cells. Similarly, there are only minor differences in the expression of $G_{\alpha s}$ and $G_{\alpha i}$ (Fig. 5b), suggesting that the higher absolute potency of the agonists in CHO compared to PC12 cells cannot be accounted for by dramatic differences in the amounts of G proteins.

Antagonist Potencies on Human and Rat A_{2A} Receptors

We examined eight different adenosine receptor antagonists for their ability to block the agonist-induced increase of cAMP in CHO cells transfected with the human A_{2A} receptor cDNA and in PC12 cells expressing the rat A_{2A} receptor. For each of the antagonists, we used three differ-

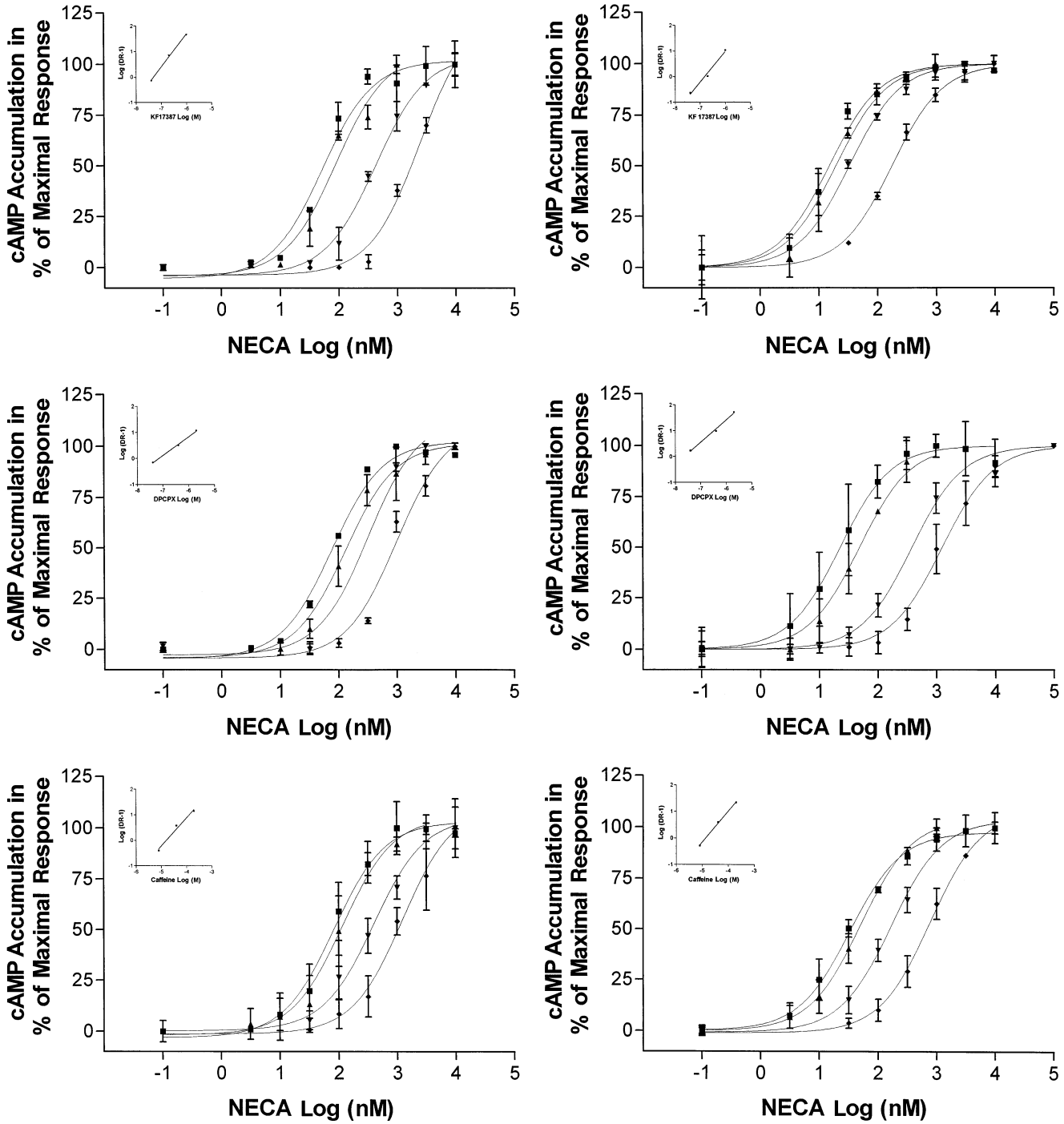

FIG. 4. Dose–response curves of NECA-induced cAMP accumulation in the absence and in the presence of some selected adenosine receptor antagonists in PC12 cells (left column) and in CHO cells transfected with human A_{2A} receptor cDNA (right column). Top two panels KF 17837S (■) 0 nM, (▲) 40 nM, (▼) 200 nM, (◆) 1 μM. Middle panels DPCPX (■) 0 nM, (▲) 40 nM, (▼) 400 nM, (◆) 2 μM. Bottom panels caffeine (■) 0 nM, (▲) 8 μM, (▼) 40 μM, (◆) 200 μM. Results are mean with SEM of 3 experiments carried out in duplicate and presented as percent of maximal response. In CHO cells, the basal level of cAMP was 6.6 ± 4.5 pmol/well, and the maximal response was 111.6 ± 0.2 pmol/well. In PC12 cells the basal cAMP level was 6.8 ± 3.2 pmol/well, and the maximal response was 120.5 ± 2.3 pmol/well. Insets show the Schild plots.

ent concentrations to antagonise the stimulatory effect of NECA. From these data, Schild plots were generally and only in one case (theophylline in PC12 cells) was the slope of the Schild plot different from 1. Typical examples are illustrated in Fig. 4 and the results are summarised in Table 3. There were no major species differences in potency for the antagonists tested and the rank order of potency was virtually the same in both cells: CGS 15943 > SCH 58261 > DPCPX > KF 17837S > CSC > paraxanthine = theophylline > caffeine.

TABLE 2. Potency of several adenosine analogues as agonists in PC12 clone 1 cells and transfected CHO cells

Drug	PC12 cells	CHO cells	Dose ratio PC12/CHO
HE-NECA	38.1 (18.9–76.6)	1.6 (1.0–2.6)	24
NECA	49.4 (20.4–119.5)	26.1 (8.3–81.5)	1.9
CGS 21680	128.7 (40.0–413.6)	36.8 (15.3–88.6)	3.5
R-PIA	414.5 (221.5–775.8)	62.1 (28.2–136.7)	6.7
CV-1808	812.1 (490.7–1344)	6.7 (1.35–33.26)	121
Metrifudil	1315 (564–3068)	136 (71.5–258.1)	9.7
CHA	1435 (778–2648)	318 (99.2–1023)	4.5
2-chloroadenosine	3988 (2531–6285)	103 (67.3–157)	39
CV-1674	6689 (3144–14230)	573 (256–1287)	11.7
APNEA	7926 (4203–14950)	623 (336–1156)	12.7
Adenosine	15300 (12430–18830)	363 (120–1100)	42.1

Results are given as EC_{50} values in nM: 95% confidence intervals within brackets.

DISCUSSION

A major conclusion of this study is that there appear to be some differences in agonist recognition between rat and human A_{2A} receptors despite only minor differences in amino acid composition and minimal differences in the potency of antagonists. All agonists tested, except metrifudil, were more potent at human A_{2A} receptors at displacing [^{3}H]-SCH 58261. They were also more potent in the functional assay. As discussed by Kenakin [32] such differences may reflect differences in G protein coupling and thus a potential artefact in recombinant systems. Many of the problems discussed by Kenakin [32] refer to systems where the recombinant receptor is overexpressed. However, this is not a concern in the present case. The number of receptors, as determined by antagonist binding, was in fact higher in the PC12 cells expressing the rat adenosine A_{2A} receptor endogenously than in CHO cells transfected with the human counterpart. It is also worth pointing out that the density of A_{2A} receptors in the transfected CHO cells if anything is lower than the number of receptors in naturally occurring cells in the brain. In membranes from the rat striatum that abundantly express native A_{2A} receptors, a B_{max} of close to 1000 fmol/mg protein was found [28]. It is known that these endogenous A_{2A} receptors are found only in neurones and not in glial cells and furthermore that only about 50% of the neurones express the receptor [13]. Thus, the number of receptors in these naturally A_{2A}-expressing cells is apparently at least as high as that found in the PC12 cells. Thus, there is no evidence that the transfected cells express an unnaturally high number of receptors.

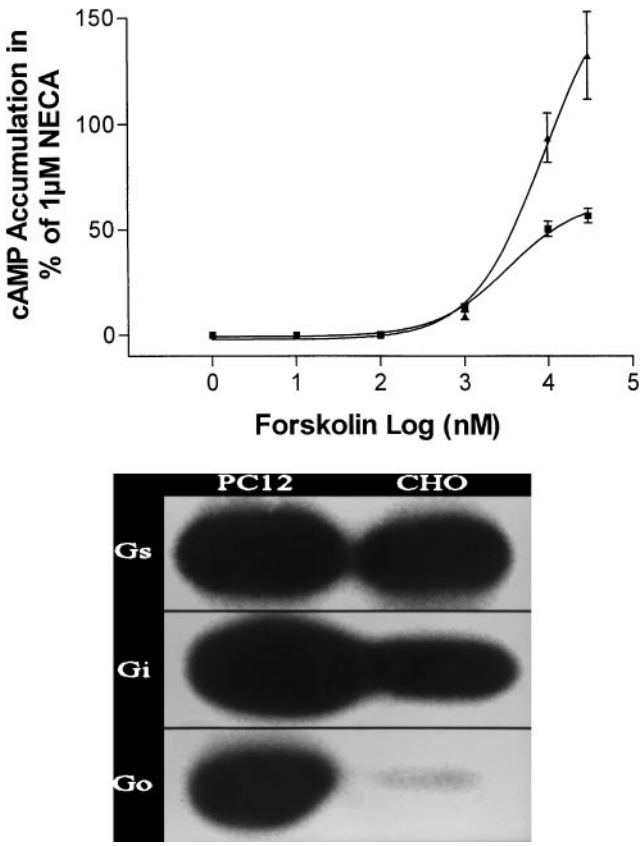

FIG. 5. Higher agonist potency of agonists in CHO cells than in PC12 cells cannot be ascribed to a generally increased responsiveness to adenylyl cyclase stimulation or to major differences in G proteins. Upper panel, Stimulatory effects of forskolin on cAMP accumulation in PC12 clone 1 (▲) and transfected CHO cells (■). Results are given as increases above control in per cent of the cAMP accumulation afforded by 1 µM of NECA and are presented as means ± SEM of 2 experiments each carried out in duplicate. Lower panel, Western blot showing the bands corresponding to $G_{\alpha s}$, $G_{\alpha i}$, and $G_{\alpha o}$ in CHO and PC12 cells.

Another possible concern is that the receptors in the transfected cells couple differently to G proteins. One way to detect such anomalous coupling is to determine differences in the relative binding of an agonist and an antagonist radioligand [32]. In both the cells examined here, the number of binding sites detected with the antagonist radioligand was higher than the number of binding sites detected with the agonist radioligand. The difference was approximately 2-fold in the CHO cells and almost 5-fold in the PC12 cells. This indicates that if anything the ratio between the number of receptors and the available G proteins coupling to the receptor is higher in the cells naturally expressing the receptor than in the transfected cells [32].

The adenosine A_{2A} receptor is coupled via G_s proteins to

TABLE 3. Potency of several adenosine receptor antagonists in CHO cells transfected with the human A_{2A} receptor cDNA and PC12 cells expressing the rat A_{2A} receptor

	PC12 cells		CHO cells		PC12/CHO Dose ratio
Antagonist	K_b	Slope	K_b	Slope	
CGS 15943	1.4 (0.15–6.7)	1.04 (0.86–1.22)	0.63 (0.4–1.0)	1.01 (0.87–1.16)	2.2
SCH 58261	5.8 (0.6–24.5)	1.10 (0.85–1.35)	6.63 (3.2–10)	0.96 (0.80–1.13)	0.9
DPCPX	98 (60–328)	0.85 (0.41–1.28)	20 (16–25)	0.91 (0.67–1.15)	4.9
KF 17837S	65 (4.7–229)	1.25 (0.81–1.69)	125 (100–158)	1.14 (0.98–1.31)	0.5
CSC	192 (30–490)	0.93 (0.42–1.44)	297 (159–467)	0.90 (0.76–1.05)	0.6
Paraxanthine	7778 (955–14791)	1.04 (0.49–1.6)	5384 (3802–6918)	0.99 (0.86–1.12)	1.4
Theophylline	4186 (2754–5623)	0.76 (0.62–0.90)	7940 (6310–9983)	1.26 (0.99–1.53)	0.5
Caffeine	18493 (15849–20893)	0.93 (0.81–1.05)	12331 (9772–14791)	1.07 (0.93–1.22)	1.5

Results are given as mean K_b values in nM (n = 3): 95% confidence intervals within brackets.

adenylyl cyclase. We found no major difference in the amount of $G_{s\alpha}$ using Western blot and there were no major differences in the maximal stimulation of cAMP formation induced by a concentration of forskolin that stimulates the enzyme directly. If the greater response in CHO cells were due to a much higher activity of adenylyl cyclase and/or a larger number of G_s proteins, one would expect a larger effect of forskolin on cAMP accumulation in these cells than in PC12 cells. However, the converse was found to be in this case. This experiment also provides evidence against the possibility that cAMP is broken down more rapidly in PC12 than in CHO cells. Thus, we have no ready explanation for the finding that all agonists tended to be more potent on the human than on the rat A_{2A} receptor according to both binding and functional assays. Thus, the possibility exists that there is a real difference between the two receptors in this regard, but the most parsimonious explanation is that there is a difference in the cellular background that we have not been able to identify.

A difference in cellular background cannot, however, explain the fact that there was not only a difference in absolute potency, but also a difference in relative potency for several agonists—but not for antagonists—between the rat and human A_{2A} receptor. These differences in the relative order of potency were observed both in binding experiments and the functional assay. Thus, the order of potency of agonists observed in binding experiments using [^{3}H]-SCH 58261 very nicely predicted the order of potency in the functional assay in the two cells expressing the rat and human A_{2A} receptors. In particular, CV-1808 was more potent than CGS 21680 on human receptors, whereas the opposite was true for the rat receptor. A 2-fold [33] or 4-fold [34] higher potency of CV-1808 as an agonist at the human neutrophil A_{2A} receptor has previously been described, whereas there are abundant data showing that CGS 21680 is more potent than CV-1808 on A_{2A} receptor-mediated responses in the rat [35]. It is difficult to ascribe such a difference in the order of potency that occurs both in a binding and in a functional assay to anything else than a difference in the receptor. Since there are differences for some, but not for all agonists, it is tempting to speculate that the differences are not localised to the main ligand recognition site, but rather to some adjacent or accessory site. Obviously, the precise structural basis for the functional differences that we have observed can only be examined using different methods from those used in the present study. Nonetheless, there are some suggestive hints in the literature.

It is generally accepted that agonists bind to sequences in the transmembrane helical domain in the adenosine receptors. In particular, hydrophilic side chains in the third and seventh domains have been strongly implicated [36]. There are a few differences between the rat and human sequences in the third, fifth, and seventh helical domains which might be responsible for the observed differences in agonist potency ratio. In a recent study on COS-7 cells transiently transfected with wild-type and mutated human A_{2A} receptors, it was found that 2-chloroadenosine binding was much more affected by several mutations in the third, fifth, and seventh helical domain than was CGS 21680 binding or NECA binding [37]. For example, mutation of the serine 281 to a threonine markedly *increased* the potency of 2-chloroadenosine [37]. A neighbouring threonine (279) in the human A_{2A} receptor is aligned with a serine (274) in the rat sequence. Perhaps this difference in amino acid sequence also results in differences in the potency of 2-chloroadenosine. Another important structural difference between rat and human A_{2A} receptors in the fifth transmembrane domain was also indicated [37]. Thus, changing a phenylalanine in the human sequence to a tryosine (in the rat sequence) had a much larger negative effect on 2-chloroadenosine binding than on CGS 21680 binding. This could indicate that there are differences between the rat and human receptors that are important for the functional state of the receptor, and that this affects some agonist ligands more than others. However, in a published computer model for the adenosine A_{2A} receptor [38], there are no amino acids that differ between the human and rat adenosine A_{2A} receptors within a 5.5 Å distance from the chosen docking ligand, 2-(cyclohexylmethylidinehydrazino)adenosine. Only one of the amino acids that differ between the sequences more of less points

into the postulated receptor pore, but this residue, isoleucine 80, is at the top of helix 3, where—according to the model—it would not interact with the ligand. The remaining residues that differ are probably involved in interhelical interactions, and may be important in the overall architecture of the receptor and particularly in the interchange between high- and low-affinity agonist states.

In conclusion, we have found that for rat A_{2A} receptors [^{3}H]-SCH 58261 binding predicts the functional agonist potency reasonably well. We also found some interesting differences in the order of potency for selected adenosine analogues as agonists at rat and human A_{2A} receptors. These differences with regard to agonist potencies should have consequences for drug development. Our results thus emphasise the importance of examining receptors from the appropriate species, and they also underline the importance of using functional assays as adjuncts to binding assays when examining agonists.

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References

1. Kull B, Arslan G, Owman C, Nilsson C and Fredholm BB, Differences in the potency of adenosine analogues at human and rat adenosine A_{2A} receptors. *Pharmacol Toxicol* **76**(Suppl II)**:** P1.26. 1995.
2. Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobsen KA, Leff P and Williams M, Nomenclature and classification of purinoceptors. *Pharmacol Rev* **46:** 143–156, 1994.
3. Palmer TM and Stiles GM, Adenosine receptors. *Neuropharmacology* **34:** 683–694, 1995.
4, Londos C, Cooper DMF and Wolff J, Subclasses of external adenosine receptors. *Proc Natl Acad Sci USA* **77:** 2551–2554, 1980.
5. van Calker D, Muller M and Hamprecht B, Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J Neurochem* **33:** 999–1005, 1979.
6. Bruns RF, Lu GH and Pugsley TA, Characterization of the A_2 adenosine receptor labelled by [^{3}H]-NECA in rat striatal membranes. *Mol Pharmocol* **29:** 331–346, 1986.
7. Bruns FR, Davis RE, Ninteman FW, Poschel BPH, Wiley JN and Heffner TG, Adenosine antagonists as pharmacological tools. In: *Adenosine and Adenine nucleotides. Physiology and Pharmacology* (Ed. Paton DM), pp. 39–49. Taylor & Francis, Basingstoke, 1988.
8. Maenhaut C, Van Sande J, Libert F, Abramowicz M, Parmentier M, Vanderhaegen JJ, Dumont JE, Vassart G and Schiffmann S, RDC8 codes for an adenosine A_2 receptor with physiological constitutive activity. *Biochem Biophys Res Commun* **173:** 1169–1178, 1990.
9. Fink JS, Weaver DR, Rivkees SA, Peterfreund R, Pollak R, Adler E and Reppert SM, Molecular cloning of a rat A_2 adenosine receptor: Selective co-expression with D_2 dopamine receptors in rat striatum. *Mol Brain Res* **14:** 186–195, 1992.
10. Furlong TJ, Pierce KD, Selbie LA and Shine J, Molecular characterization of a human brain adenosine A_2 receptor. *Brain Res Mol Brain Res* **15:** 62–66, 1992.
11. Stehle JH, Rivkees SA, Lee JJ, Weaver DR, Deeds JD and Reppert SM, Molecular cloning and expression of the cDNA for a novel A_2-adenosine receptor subtype. *Mol Endocrinol* **6:** 384–393, 1992.
12. Pierce KD, Furlong TJ, Selbie LA and Shine J, Molecular cloning and expression of an adenosine A_{2b} receptors from human brain. *Biochem Biophys Res Commun* **187:** 86–93, 1992.
13. Ongini E and Fredholm BB, Pharmacology of adenosine A_{2A} receptors. *Trends Pharmacol Sci* **17:** 364–372, 1996.
14. Tucker AL, Linden J, Robeva AS, D'Angelo DD and Lynch KR, Cloning and expression of a bovine adenosine A_1 receptor cDNA. *FEBS Lett* **297:** 107–111, 1992.
15. Greene LA, Nerve growth factor prevents death and stimulates neuronal differentiation of clonal PC12 pheochromocytoma cells in serum-free medium. *J Cell Biol* **78:** 747–755, 1978.
16. Guroff G, Dickens G, End D and Londos C, The action of adenosine analogues on PC 12 cells. *J Neurochem* **37:** 1431–1439, 1981.
17. Hide I, Padgett WL, Jacobson KA and Daly JW, A_{2A} adenosine receptors from rat striatum and rat pheochromocytoma PC12 cells: Characterization with radioligand binding and by activation of adenylate cyclase. *Mol Pharmacol* **41:** 352–359, 1992.
18. Bonner TI, Affolter HU, Young AC and Young III WS, A cDNA encoding the precursor of the rat neuropeptide, neurokinin B. *Mol Brain Res* **2:** 243–249, 1987.
19. Libert F, Parmentier M, Lefort A, Dinsart C, Van Sande J, Maenhaut C, Simons M-J, Dumont JE and Vassart G, Selective amplification and cloning of four new members of the G protein-coupled receptor family. *Science* **244:** 569–572, 1989.
20. Chen C and Okayama H, High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* **7:** 2745–2752, 1987.
21. Clementi E, Racchetti G, Zacchetti D, Panzeri MC and Meldolesi J, Differential expression of markers and activities in a group of PC12 nerve cell clones. *Eur J Neurosci* **4:** 944–953, 1992.
22. Cunha RA, Johansson B, Constantino MD, Sebastiao AM and Fredholm BB, Evidence for high-affinity binding sites for the adenosine A_{2A} receptor agonist [^{3}H]CGS 21680 in the rat hippocampus and cerebral cortex that are different from striatal A_{2A} receptors. *Naunyn-Schmiedeberg's Arch Pharmacol* **353:** 261–271, 1996.
23. Johansson B, Georgiev V, Parkinson FE and Fredholm BB, The binding of the adenosine A_2 selective agonist [^{3}H]CGS 21680 to rat cortex differs from its binding to rat striatum. *Eur J Pharmacol, Mol Pharmacol Sect* **247:** 103–110, 1993.
24. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–294, 1976.
25. Nordstedt C and Fredholm BB, A modification of a protein-binding method for rapid quantification of cAMP in cell-culture supernatants and body fluid. *Anal Biochem* **189:** 231–234, 1990.
26. Belardinelli L, Shryock JC, Ruble J, Monopoli A, Dionisotti S, Ongini E, Dennis DM and Baker SP, Binding of the novel nonxanthine A_{2A} adenosine receptor antagonist

[^{3}H]SCH58261 to coronary artery membranes. *Circ Res* **79:** 1153–1160, 1996.
27. Dionisotti S, Ongini E, Zocchi C, Kull B, Arslan G and Fredholm BB, Characterization of human A_{2A} adenosine receptors using the antagonist radioligand [^{3}H]-SCH 58261. *J Pharmacol* **121:** 353–360, 1997.
28. Zocchi C, Ongini E, Ferrara S, Baraldi PG and Dionisotti S, Binding of the radioligand [^{3}H]-SCH 58261, a new non-xanthine A_{2A} adenosine receptor antagonist, to rat striatal membranes. *Br J Pharmacol* **117:** 1381–1386, 1996.
29. Klotz K-N, Hessling J, Hegler J, Owman C, Kull B, Fredholm BB and Lohse MJ, Comparative pharmacology of human adenosine receptor subtypes—Characterization of stably transfected receptors in CHO cells. *Naunyn-Schmiedeberg's Arch Pharmacol* **357:** 1–9, 1998.
30. van der Ploeg I, Ahlberg S, Parkinson FE, Olsson RA and Fredholm BB, Functional characterization of adenosine A_2 receptors in Jurkat cells and PC12 cells using adenosine receptor agonists. *Naunyn-Schmiedeberg's Arch Pharmacol* **353:** 250–260, 1996.
31. Brackett LE and Daly JW, Functional characterization of the A_{2b} adenosine receptor in NIH 3T3 fibroblasts. *Biochem Pharmacol* **47:** 801–814, 1994.
32. Kenakin T, Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. *Trends Pharmacol Sci* **18:** 456–464, 1997.
33. Gurden MF, Coates J, Ellis F, Evans B, Foster M, Hornby E, Kennedy I, Martin DP, Strong P and Vardey CJ, Functional characterization of three adenosine receptor types. *Br J Pharmacol* **109:** 693–698, 1993.
34. Fredholm BB, Zhang Y and van der Ploeg I, Adenosine A_2 receptors mediate the inhibitory effect of adenosine on fMLP-stimulated respiratory burst in neutrophil leukocytes. *Naunyn-Schmiedeberg's Arch Pharmacol* **354:** 262–267, 1996.
35. Hutchison AJ, Webb RL, Oei HH, Ghai GR, Zimmerman MB and Williams M, CGS 21680C, an A_2 selective adenosine receptor agonist with preferential hypotensive activity. *J Pharmacol Exp Ther* **251:** 47–55, 1989.
36. Jiang Q, Van Rhee AM, Kim J, Yehle S, Wess J and Jacobson KA, Hydrophilic side chains in the third and seventh transmembranes helical domains of human A_{2A} adenosine receptors are required for ligand recognition. *Mol Pharmacol* **50:** 512–521, 1996.
37. Kim J, Wess J, van Rhee AM, Schoneberg T and Jacobson KA, Site-directed mutagenesis identifies residues involved in ligand recognition in the human A_{2a} adenosine receptor. *J Biol Chem* **270:** 13987–13997, 1995.
38. IJzerman AP, van der Wenden EM, van Galen PJM and Jacobson KA, Molecular modeling of adenosine receptors. The ligand binding site on the rat adenosine A_{2A} receptor. *Eur J Pharmacol, Mol Pharmacol Sect* **268:** 95–104, 1994.