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Comparative pharmacology of human adenosine receptor subtypes – characterization of stably transfected receptors in CHO cells

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Abstract Four adenosine receptor subtypes of the family of G protein-coupled receptors, designated A_1, A_{2A}, A_{2B} and A_3 are currently known. In this study all human subtypes were stably transfected into Chinese hamster ovary (CHO) cells in order to be able to study their pharmacological profile in an identical cellular background utilizing radioligand binding studies (A_1, A_{2A}, A_3) or adenylyl cyclase activity assays (A_{2B}). The A_1 subtype showed the typical pharmacological profile with 2-chloro-N^6-cyclopentyladenosine (CCPA) as the agonist with the highest affinity and a marked stereoselectivity for the N^6-phenylisopropyladenosine (PIA) diastereomers. In competition with antagonist radioligand biphasic curves were observed for agonists. In the presence of GTP all receptors were converted to a single low affinity state indicating functional coupling to endogenous G proteins. For A_{2A} adenosine receptors CGS 21680 (2-[p-(2-carboxyethyl)phenylethylamino]-5′-N-ethylcarboxamidoadenosine) and N-ethylcarboxamidoadenosine (NECA) were found to be the most potent agonists followed by R- and S-PIA with minor stereoselectivity. The relative potencies of agonists for the A_{2B} adenosine receptor could only be tested by measurement of receptor-stimulated adenylyl cyclase activity. NECA was the most potent agonist with an EC_{50}-value of 2.3 μM whereas all other compounds tested were active at concentrations in the high micromolar range. Inhibition of NECA-stimulated adenylyl cyclase identified xanthine amino congener (XAC; 8-[4-[[[[(2-aminoethyl)amino]-carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine) as the most potent antagonist at this receptor subtype. The A_3 receptor was characterized utilizing the nonselective agonist [^{3}H]NECA. The N^6-benzyl substituted derivatives of adenosine-5′-N-methyluronamide (MECA) turned out to be the most potent agonists. The notion of xanthine-insensitivity of the A_3 receptor should be dropped at least for the human receptor as xanthines with submicromolar affinity were found. Overall, the pharmacological characteristics of the human receptors are similar to other species with some species-specific characteristics.

In this study we present for the first time the comparative pharmacology of all known human adenosine receptor subtypes. The CHO cells with stably transfected adenosine receptors provide an identical cellular background for such a pharmacological characterization. These cells are valuable systems for further characterization of specific receptor subtypes and for the development of new ligands.

Key words Adenosine receptors · G protein-coupled receptors · Stable transfection · Signal transduction · A_1, A_{2A}, A_{2B}, A_3, ligand

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Introduction

Adenosine modulates a great variety of physiological functions via different subtypes of G protein-coupled receptors (Engler and Gruber 1992). Originally, A_1 and A_2 adenosine receptors were the only subtypes that were distinguished on the basis of their effector coupling and pharmacological profile. Both receptor subtypes modulate cAMP formation such that the A_1 subtype mediates an inhibition of adenylyl cyclase whereas A_2 receptors stimulate cyclase activity (van Calker et al. 1979; Londos et al. 1980). However, many lines of evidence pointed to the existence of additional subtypes and recent cloning of A_{2B} and A_3 receptors (Pierce et al. 1992; Salvatore et al. 1993) confirmed the existence of a total of at least four adenosine receptor subtypes (Fredholm et al. 1994). The A_{2A} and A_{2B} subtypes are both positively coupled to adenylyl

cyclase (Pierce et al. 1992), and A_3 receptors inhibit cAMP formation (Salvatore et al. 1993) and also stimulate phospholipase C (Ramkumar et al. 1993). This further subdivision renewed the interest in the therapeutic potential of adenosine receptors and the respective signaling mechanisms (Jacobson et al. 1995; Müller and Stein 1996).

For further elucidation of the therapeutic potential of individual receptor subtypes and the development of pharmacological tools model systems with human receptor subtypes are indispensable. In recent years recombinant expression of receptors in heterologous cell systems has become a valuable method to provide such models (Kenakin 1996). Expression of single receptor subtypes allows for the investigation of signal transduction in subtype-selective models where conflicting effects via multiple receptor subtypes do not occur. In addition, transfection of cells with receptor genes typically allows for high expression level (≥ 1 pmol receptor/mg membrane protein) which helps to overcome problems in the characterization of e.g. A_3 adenosine receptors which are physiologically expressed at low levels (Ji et al. 1994). In some instances ligand affinity may not only be dependent on the receptor but also on the cellular model used (Kenakin 1996). Therefore, it would be advantageous to compare receptor subtypes in an identical cellular background.

In this study, we present Chinese hamster ovary (CHO) cell lines stably transfected with each of the four human adenosine receptor subtypes. The adenosine receptors expressed by these cell clones are characterized with selected agonists and antagonists that are readily available from commercial sources. These adenosine receptor models will also aid in the development of selective high-affinity ligands and radioligands that are currently not available for all receptor subtypes.

Methods

Cloning of the human adenosine receptors. The cDNAs of the human A_1, A_{2B} and A_3 adenosine receptors were obtained by polymerase chain reactions (PCR) using oligonucleotides matching the very ends of the coding sequences as deposited in the GenBank plus attached restriction sites for BamHI or EcoRI at the 5′-end and XbaI at the 3′-end. PCR was done on randomly transcribed cDNA from human liver and brain, the ends of the PCR products were cut at the respective restriction sites and then ligated into the corresponding sites of the expression vector pcDNA3 (Invitrogen, Leek, The Netherlands).

The cDNA of the human A_{2A} receptor was isolated from a human B-cell lymphoblast cDNA library (GM03299, NIGMS Human Genetic Mutant Cell Repository, Camden, N.J., USA), which was screened at 60° C in 3 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) pH 7.0 (Bonner et al. 1987) with an end-labeled 29-mer oligonucleotide with 128-fold degeneracy to transmembrane region VI (Libert et al. 1989): 5′-A(T/G)G(A/T)AAGA(T/A)GGGCAGCCAGCAGA(C/G)(C/G)(G/A)(T/C)GAA-3′. A 2.5-kb hybridizing BamH1 fragment was used to identify a positive clone (designated Lymb42-14).

All cDNAs were verified by sequencing and comparison with the respective GenBank entries. They were found to correspond to the published sequences for the A_1 (Libert et al. 1992), A_{2A} (Salvatore et al. 1992), A_{2B} (Pierce et al. 1992) and A_3 (Salvatore et al. 1993) receptors.

Stable transfection of cells. Chinese hamster ovary cells (CHO-K1 cells; CCL61, American Type Culture Collection, Rockville, Md., USA) were transfected with plasmid-DNA for stable expression using the calcium phosphate precipitation method (Chen and Okayama 1987) as described recently for the A_1 adenosine receptor (Freund et al. 1994). Positive clones were selected with 450 µg/ml of the neomycin analogue G418, and single clonal lines were isolated by limiting dilution. Expression of the receptors was verified by ligand binding for the A_1 and A_3 receptors (see below) or in the case of the A_2 receptors in adenylyl cyclase assays (see below) and in Northern blots.

Cell culture. The cells were grown adherently and maintained in Dulbecco's Modified Eagles Medium with nutrient mixture F12 (DMEM/F12) without nucleosides, containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM) and Geneticin (G418, 0.2 mg/ml; A_{2B}, 0.5 mg/ml) at 37° C in 5% CO_2/95% air. Cells were split 2 or 3 times weekly at a ratio between 1 : 5 and 1 : 20. For binding assays the culture medium was removed, cells were washed with PBS and frozen in the dishes until preparation of membranes. The cells utilized for cAMP determinations had a viability > 95%, as assessed by the exclusion of trypan blue.

Membrane preparation. Crude membranes for radioligand binding experiments were prepared by thawing frozen cells followed by scraping them off the petri dishes in ice-cold hypotonic buffer (5 mM Tris/HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized on ice (Ultra-Turrax, 2 × 15 s at full speed) and the homogenate was spun for 10 min (4° C) at 1,000 g. The supernatant was then centrifuged for 30 min at 100,000 g. The membrane pellet was resuspended in 50 mM Tris/HCl buffer pH 7.4 (for A_3 adenosine receptors: 50 mM Tris/HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 8.25), frozen in liquid nitrogen at a protein concentration of 1–3 mg/ml and stored at –80° C.

For the measurement of adenylyl cyclase activity a slightly modified protocol with only one centrifugation step was used. Fresh cells were homogenized and the homogenate was sedimented for 30 min at 54,000 g. The resulting pellet was resuspended in 50 mM Tris/HCl pH 7.4 and used for the adenylyl cyclase assay immediately.

Radioligand binding. Dissociation constants of radioligands (K_D-values) were measured in saturation binding experiments. Dissociation constants of unlabeled compounds (K_i-values) were determined in radioligand competition experiments. All binding data were calculated by non-linear curve fitting with the program SCTFIT (De Lean et al. 1982).

Binding experiments at A_1 adenosine receptors were based on the binding assay as described previously (Lohse et al. 1987). This protocol was adapted to a microplate formate utilizing a 96-well microplate filtration system (Millipore MultiScreen MAFC). For saturation binding increasing concentrations of the radioligands [^{3}H]DPCPX (8-cyclopentyl-1,3-dipropylxanthine) or [^{3}H]CCPA (2-chloro-N^6-cyclopentyladenosine) were incubated in a total volume of 200 µl containing 0.2 U/ml adenosine deaminase and 20 µg of membrane protein in 50 mM Tris/HCl pH 7.4.

In competition experiments the wells contained 1 nM [^{3}H]-DPCPX and the compound to be tested at different concentrations. Samples were incubated for 3 h at 25° C, filtered through the built-in filter at the bottom of the wells and washed three times with 200 µl of ice-cold binding buffer. After addition of 20 µl of scintillator to the dried filter plates samples were counted in a Wallac MicroBeta counter.

The conditions for A_{2A} adenosine receptor binding were essentially the same as for A_1 receptor binding. In competition experiments [^{3}H]NECA (N-ethylcarboxamidoadenosine) at 30 nM was used as radioligand. Samples with a protein concentration of 50–80 µg were incubated for 3 h at 25° C and filtered individually as described for conventional radioligand binding.

A_3 adenosine receptor binding was performed by incubation in 96-well microplates as described above for A_1 receptors. The non-

selective agonist [^{3}H]NECA was utilized as radioligand. The incubation conditions were adopted from Ji et al. (1994). Samples were incubated for 3 h at 25° C with a binding buffer containing 50 mM Tris/HCl, 1 mM EDTA, 10 mM $MgCl_2$, pH 8.25. Nonspecific binding was determined in the presence of 1 mM theophylline ([^{3}H]CCPA) or 100 μM R-PIA (all other radioligands).

Adenylyl cyclase activity. Due to the lack of a suitable radioligand the affinity of antagonists and the relative potency of agonists at A_{2B} adenosine receptors was determined in adenylyl cyclase experiments. The procedure was carried out as described previously (Klotz et al. 1985) with minor modifications. Membranes were incubated with about 150,000 cpm of [α-^{32}P]ATP for 20 min in the incubation mixture as described (Klotz et al. 1985) without EGTA and NaCl. For agonists the EC_{50}-values for the stimulation of adenylyl cyclase were calculated with the Hill equation. Hill coefficients in all experiments were near unity. IC_{50}-values for concentration-dependent inhibition of NECA-stimulated adenylyl cyclase caused by antagonists was calculated accordingly. Dissociation constants (K_i) for antagonist were then calculated with the Cheng and Prusoff equation (Cheng and Prusoff 1973).

cAMP accumulation. For the measurement of A_{2B} receptor-mediated accumulation of cAMP cells were grown as confluent monolayers in 24 well cluster dishes. The cells were washed twice with 2 ml medium containing 20 mM HEPES pH 7.4 prewarmed to 37° C. The cells were then preincubated in 0.4 ml fresh medium containing the cAMP phosphodiesterase inhibitor rolipram (30 μM) for 15 min. Drugs were added in a volume of 0.1 ml medium and incubations were continued for 15 min. Experiments were carried out either with fixed concentrations of the antagonist and increasing concentrations of the agonist NECA (0.03–300 μM) or with a fixed concentration (0.3 μM NECA that gave a close to half-maximal response) and increasing concentrations of the antagonists.

The cAMP content in the samples was determined using a competitive radioligand-binding assay (Nordstedt and Fredholm 1990). Briefly, cell culture supernatants or cAMP standards (0–8 pmol) were incubated with [^{3}H]cAMP and cAMP binding protein in 96-well microtiter plates at 4° C for 150 min. Free and bound [^{3}H]cAMP were separated by filtration over Whatman GF/B filters using a semi-automatic cell harvester (Skatron A/S, Lier, Norway). Each filter was rinsed with 3 ml of 50 mM Tris-HCl pH 7.4. With a filter punch (Skatron A/S, Lier, Norway) the filters were punched out into scintillation vials and counted in a LKB/Pharmacia scintillation counter with 3 ml ReadySafe (LKB/Pharmacia) scintillation fluid.

Material. [^{3}H]CCPA, [^{3}H]DPCPX, [^{3}H]NECA, [^{3}H]CGS21680 (2-[p-(2-carboxyethyl)phenylethylamino]-5′-N-ethylcarboxamidoadenosine), [^{3}H]XAC and [α-^{32}P]ATP were from Du Pont NEN, Dreieich, Germany and [γ-^{32}P]-dATP (5000 Ci mmol^{-1}) was from Amersham, Braunschweig, Germany. DPCPX and CCPA were synthesized as described by Lohse et al. (1987, 1988 b). All other unlabeled adenosine receptor agonists and antagonists were from RBI, Natick, Mass., USA. The 96-well microplate filtration system (MultiScreen MAFC) was obtained from Millipore, Eschborn, Germany. Cell culture media and fetal calf serum were purchased from PanSystems, Aidenbach, Germany. Penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine and G418 were from Gibco-Life Technologies, Eggenstein, Germany. All other materials were from sources as described earlier (Klotz et al. 1985; Lohse et al. 1987).

Results

Untransfected CHO cells appear to be lacking endogenous adenosine receptors according to two criteria: First, A_1 and A_3 receptor binding was undistinguishable from nonspecific binding in both transfected and control cells, and, second, no adenosine receptor-mediated stimulation of adenylyl cyclase was observed in control cells whereas in cells transfected with A_{2A} or A_{2B} receptors 25–50% and 70–120% of AlF_3-stimulated cyclase activity were reached, respectively (not shown). The receptor-mediated stimulation of adenylyl cyclase after transfection of the cells demonstrates that CHO cells possess the appropriate G protein to couple A_{2A} or A_{2B} receptors to the endogenously expressed adenylyl cyclase. They also express the appropriate G protein that mediates inhibition of adenylyl cyclase (Freund et al. 1994). Thus, CHO cells may serve as a suitable model system for the expression of different adenosine receptor subtypes.

Receptor expression

CHO cells were transfected with each of the four adenosine receptors and several stably expressing clones were obtained in each case. The saturation binding data shown in Table 1 reveal that for A_1 and A_3 receptors expression levels of 0.8–1.1 pmol/mg membrane protein were reached. This is in the same order of magnitude as A_1 receptor densities, e.g. in brain where typically the highest physiological adenosine receptor concentrations are found (Lohse et al. 1987; Klotz et al. 1989). However, no tissue with such high expression levels is known for A_3 receptors.

A_{2A} adenosine receptors were expressed at much lower levels compared to the inhibitory subtypes (Table 1). This appears to be a receptor-specific trait because transfection of HEK 293 cells with A_{2A} receptor resulted in similarly low receptor expression (not shown). For A_{2B} adenosine receptors no radioligand is available and, thus, absolute receptor levels cannot be determined. The functional response to A_{2B} receptor stimulation, however, is dramatic with a receptor-stimulated adenylyl cyclase activity in the same order of magnitude as reached with receptor-independent AlF_3 stimulation (see above).

Although receptors were stably transfected, expression levels typically dropped when the cells were maintained in culture continuously for extended periods of time resulting in variation of B_{max}-values (Table 1). Therefore, one batch of cells should not be passaged for more than 4–6 weeks.

Table 1 Saturation analysis of adenosine receptor subtypes with various radioligands

Ligand	Receptor	K_D (nM)	95% Confidence limit	B_{max} (fmol/mg protein)	± SD	*n*[a]
[^{3}H]DPCPX	A_1	3.86	3.53–4.21	1001	418	3
[^{3}H]CCPA	A_1	0.61	0.52–0.71	1069	569	5
[^{3}H]NECA	A_{2A}	20.1	11.6–34.8	247	104	3
[^{3}H]NECA	A_3	6.18	3.47–11.0	807	613	3

[a] Number of independent experiments done in triplicate

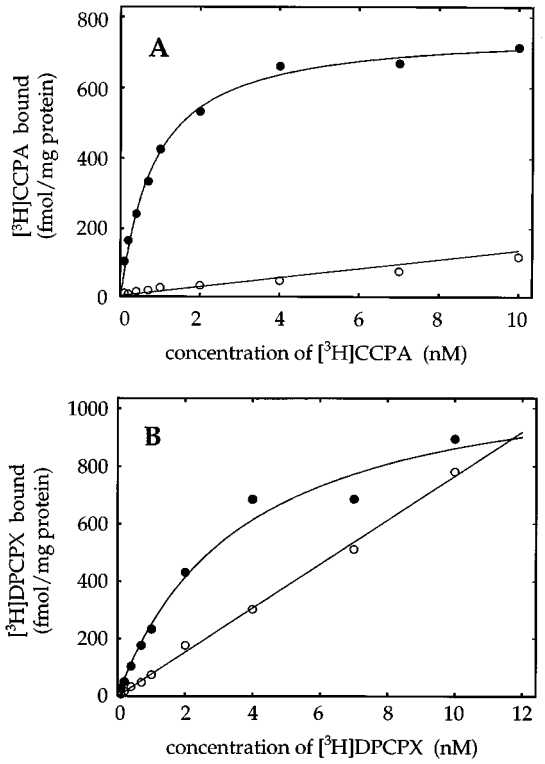

Fig. 1A, B [^{3}H]CCPA and [^{3}H]DPCPX saturation binding to A_1 adenosine receptors. The results of representative saturation experiments with agonist (**A**) and antagonist (**B**) radioligands are shown as specific (●) and nonspecific binding (○). The K_D-values are 0.78 and 3.6 nM and the corresponding B_{max}-values were 764 and 1177 fmol/mg membrane protein for [^{3}H]CCPA and [^{3}H]DPCPX, respectively. The binding experiments were carried out in the same membrane preparation showing that the antagonist labeled 1.5-fold more receptors compared to the agonist

A_1 adenosine receptor

Saturation analysis of the human A_1 adenosine receptor in stably transfected CHO cells gave K_D-values of 0.6 nM for the agonist [^{3}H]CCPA and 3.9 nM for the antagonist [^{3}H]DPCPX (Fig. 1, Table 1). In saturation experiments at A_1 receptors in the same membrane preparation about 40–60% higher B_{max}-values were detected with the antagonist [^{3}H]DPCPX compared to the agonist [^{3}H]CCPA (Fig. 1). The fact that high-affinity binding with [^{3}H]CCPA was detected suggests that at least a fraction of the transfected receptors was coupled to endogenous G protein. In agreement with this notion high and low affinity states for agonists were identified (Table 2).

In Fig. 2 the GTP sensitivity of the high-affinity state for agonists is shown. In the absence of GTP, competition of CCPA with the antagonist [^{3}H]DPCPX resulted in a biphasic curve. The presence of GTP caused a shift of the competition curve to the right and the resulting curve was monophasic, suggesting that the receptors were indeed functionally coupled to G proteins.

The K_i-values in Table 2 show the typical characteristics of an A_1 adenosine receptor with CCPA being an agonist with a subnanomolar affinity followed by CPA (N^6-cyclopentyladenosine) and R-PIA (R-N^6-phenylisopropyladenosine). The characteristic stereoselectivity for the PIA diastereomers was also found for the human receptor expressed in CHO cells. The A_{2A}-selective agonist CGS 21680 is about 350-fold less potent than CCPA. Interestingly, AB-MECA (N^6-4-aminobenzyladenosine-5′-N-methyluronamide) which has been introduced has an A_3-selective agonist (Gallo-Rodriguez et al. 1994 a) was indeed a rather weak ligand at A_1 receptors. However, introduction of an iodo substituent into the N^6-benzyl moiety increased the affinity not only for human A_3 but in an equally dramatic fashion for A_1 receptors.

The most potent antagonists were DPCPX and the non-xanthine compound CGS 15943 (9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine, Tables 1 and 2). For [^{3}H]DPCPX a K_D-value of 3.9 nM was determined and for CGS 15943 a K_i-value of 3.5 nM was found in radioligand competition. The xanthine derivatives DPCPX and XAC (xanthine amine congener, 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine) are about 10–30 times less potent at the human receptor compared to the rat A_1 receptor whereas no such species difference was found for theophylline (Klotz et al. 1991).

A_{2A} adenosine receptor

Saturation analysis with [^{3}H]NECA gave a K_D-value of 20 nM for the human A_{2A} adenosine receptor (Fig. 3, Table 1). A similar affinity was found for the human A_{2A} receptor in platelets (Lohse et al. 1988 a), however, in both human cell types this ligand was somewhat less potent than at the receptor in rat brain (Bruns et al. 1986). In initial saturation experiments [^{3}H]CGS 21680 (K_D 32 nM, not shown) was also used as a radioligand. However, because of the somewhat higher affinity of [^{3}H]NECA this ligand gave slightly better results and was routinely used. The pharmacological agonist profile for the transfected receptors was compatible with an A_{2A} receptor. The stereoselectivity for the PIA diastereomers was about ninefold and, therefore, much less pronounced than at the A_1 receptor (Table 3). Adenosine derivatives with both N^6- and 5′-substitutions (AB-MECA; IB-MECA, N^6-3-iodobenzyladenosine-5′-N-methyluronamide; IAB-MECA, N^6-4-amino-3-iodobenzyladenosine-5′-N-methyluronamide) were much less potent than NECA and CGS 21680 (Table 3).

The xanthine derivative XAC turned out to be the most potent antagonist at the A_{2A} adenosine receptor with a K_i-value of 1.0 nM followed by the non-xanthine CGS 15943 (K_i 4.2 nM). Although theophylline did not reveal a marked subtype selectivity the K_i-value of 1.7 μM for A_{2A} receptors was the lowest detected for any adenosine receptor (Table 3).

Table 2 Competition of agonists and antagonists for [^{3}H]DPCPX binding at A_1 receptors

Agonist[a]	K_H (n*M*)	95% Confidence limit	K_L (n*M*)	95% Confidence limit	*n*[b]
CCPA	0.83	0.55–1.25	246	172–351	5
CPA	2.25	1.51–3.36	354	308–406	4
R-PIA	2.04	1.00–4.20	294	250–347	4
S-PIA	75.0	41.8–134	11,300	7,960–16,300	3
NECA	13.6	6.41–28.7	1,210	850–1,730	4
CGS 21680	289	232–361	102,000	37,300–280,000	3
AB-MECA	1,510	1,250–1,830	141,000	95,800–208,000	3
IB-MECA	3.73	1.80–7.71	2,306	2,080–2,557	4
IAB-MECA	8.50	4.75–15.2	3,743	1,503–9,320	3
Agonist + 100 μ*M* GTP			K_D (n*M*)	95% Confidence limit	*n*[b]
CCPA			209	136–320	5
R-PIA			458	285–735	3
Antagonist	K_D (n*M*)	95% Confidence limit	*n*[b]		
XAC	29.1	24.4 – 34.7	3		
CGS 15943	3.48	1.65– 7.32	4		
Theophylline	6,770	4,070 –11,300	3		

[a] Chemical names of compounds used:
AB–MECA N^6-4-aminobenzyladenosine-5′-N-methyluronamide
CCPA 2-chloro-N^6-cyclopentyladenosine
CGS 15943 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine
CGS 21680 2-[p-(2-carboxyethyl)phenylethylamino]-5′-N-ethyl-carboxamido-adenosine
CPA N^6-cyclopentyladenosine
IAB-MECA N^6-4-amino-3-iodobenzyladenosine-5′-N-methyl-uronamide
IB-MECA N^6-3-iodobenzyladenosine-5′-N-methyluronamide
NECA 5′-N-ethylcarboxamidoadenosine
PIA N^6-phenylisopropyladenosine
XAC 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy] phenyl]-1,3-dipropyl-xanthine

[b] Number of independent experiments done in duplicate

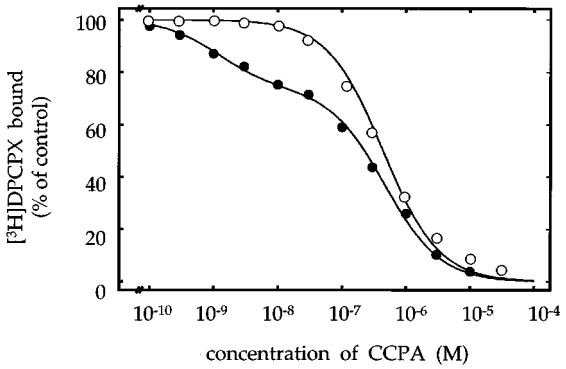

Fig. 2 GTP shift of agonist competition curves for antagonist radioligand binding. The curves show specific binding from a representative competition experiment of the agonist CCPA with the antagonist radioligand [^{3}H]DPCPX in the presence (○) and absence (●) of 100 μM GTP. Without GTP two affinity states were detected with K_H 0.93 nM and K_L 373 nM. GTP shifted the curves to the right and only one affinity state with a K_D of 347 nM was found which is in close agreement with K_L

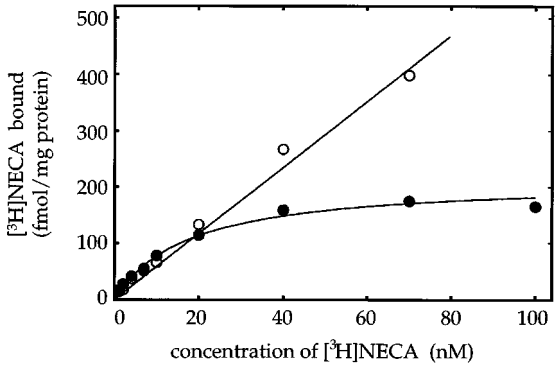

Fig. 3 [^{3}H]NECA saturation binding to A_{2A} adenosine receptors. Specific (●) and nonspecific (○) binding of [^{3}H]NECA from a representative experiment is shown (K_D 17.7 nM, B_{max} 214 fmol/mg protein). At a K_D concentration the nonspecific binding of [^{3}H]NECA amounted to about 50% of total binding

A_{2B} adenosine receptor

Due to the lack of potent ligands and radioligands for this receptor subtype, activation of adenylyl cyclase in membranes and accumulation of cAMP in cells was used to characterize A_{2B} receptors. NECA caused an about sevenfold increase of adenylyl cyclase activity and was the most potent agonist with an EC_{50}-value of 2.4 μM (Fig. 4, Table 4). All other agonists tested showed surprisingly little variation in EC_{50}-values which ranged from about 10 μM for IB-MECA and R-PIA to 90 μM for CGS 21680 (Table 4). In both assays similar maximal effects were observed for all agonists tested.

Antagonist affinities were determined by inhibition of NECA-stimulated adenylyl cyclase activity in membrane preparations. XAC was the most potent antagonist (K_i 140 nM) followed by the non-xanthine CGS 15943 (Table 4). All xanthines showed little selectivity for the A_2 subtypes.

Table 3 Competition of agonists and antagonists for [^{3}H]NECA binding at A_{2A} receptors

Agonist	K_i (n*M*)	95% Confidence limit	*n*[a]
CCPA	2,270	1,950 –2,660	4
CPA	794	466 –1,360	4
R-PIA	859	479 –1,540	3
S-PIA	7,780	7,010 –8,640	3
CGS 21680	27.1	12.4 – 58.9	3
AB-MECA	3,570	2,710 –4.720	3
IB-MECA	2,520	1,870 –3,400	3
IAB-MECA	471	300 – 740	4
Antagonist			
DPCPX	129	64.6 – 259	4
XAC	1.00	0.58– 1.73	5
CGS 15943	4.18	2.64– 6.63	3
Theophylline	1,710	1,020 –2,900	4

[a] Number of independent experiments done in duplicate

A similar pharmacological profile was identified when cAMP accumulation was measured in whole-cell experiments. Although comparable EC_{50}-values for agonists were determined with the two methods a higher potency of the antagonists was always observed in the cAMP assay compared to adenylyl cyclase activation (Table 4). This difference occurred irrespective of whether fixed concentrations of antagonist were used to counteract increasing concentrations of an agonist (Fig. 4 B) or a single submaximal concentration of the agonist was antagonized with increasing concentrations of the antagonist (Fig. 4 C, Table 4). The difference in K_i estimated in the cyclase assay and the cAMP assay tended to increase with the potency of the antagonist.

The nanomolar affinity of XAC that was determined in the cAMP assay led us to attempt radioligand binding with [^{3}H]XAC. We were unable to detect specific binding in both membrane preparations and whole cells, probably owing to the very high nonspecific binding of [^{3}H]XAC which amounted to about 1 pmol/mg membrane protein at a concentration of 5 nM and 50 µg protein/200 µl.

A_3 adenosine receptor

Although once introduced as an A_2 receptor radioligand, [^{3}H]NECA exhibited remarkably high affinity for human A_3 adenosine receptors. The saturation analysis gave a K_D-value of 6 nM which is about threefold higher than at the A_{2A} receptor (Table 1, Fig. 5). This affinity was high enough to allow us the use of the microplate protocol for binding experiments. For the classical A_1/A_{2A} ligands, affinities between 21 nM for R-PIA and 85 nM for CGS 21680 were found. The derivatives of N^6-benzyladenosine-5′-N-methyluronamide that were recently introduced as A_3 ligands (Gallo-Rodriguez et al. 1994 b) were more potent than other agonists with IAB-MECA (K_i 0.6 nM)

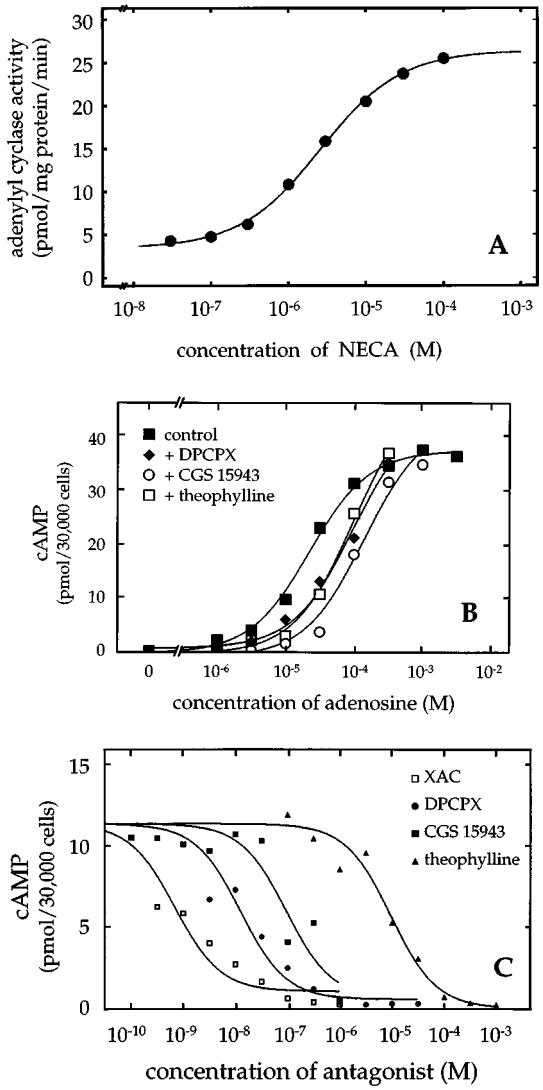

Fig. 4 Adenylyl cyclase activation via A_{2B} adenosine receptors. The curve in panel **A** shows concentration-dependent stimulation of adenylyl cyclase activity in a membrane preparation by NECA from a representative experiment. At maximal concentration NECA caused an approximately sevenfold stimulation over basal activity with an EC_{50}-value of 2.4 µM. Panel **B** shows the accumulation of cAMP in intact cells stimulated by increasing concentrations of the natural ligand adenosine in the absence (control) or presence of fixed concentrations of selected antagonists. Results are mean values of two to six determinations (each in duplicate) at *each point*. The EC_{50} for adenosine alone was 33.4 (25–44) µM (mean and 95% confidence interval); for adenosine in the presence of 0.5 µM DPCPX 96 (62–151) µM; in the presence of 0.3 µM CGS 15943 112 (74–169) µM; and in the presence of 30 µM theophylline 71 (54–94) µM. Assuming a slope of 1 in a Schild plot the following pseudo-pA_2 values could be calculated: DPCPX, 6.78; CGS 15943, 6.88; theophylline, 4.58. Panel **C** shows the effect of increasing concentrations of XAC, DPCPX, CGS 15943 and theophylline on the cAMP accumulation stimulated by 0.3 µM NECA – a concentration that produces a close to half maximal response. The *curves* show the result of a representative experiment (mean values of determinations in triplicate). The results from pooled data are given in Table 4

Table 4 Effect of agonists and antagonists on adenylyl cyclase activity and cAMP levels via A_{2B} receptors

Agonist[a]	Adenylyl cyclase activity			cAMP levels		
	EC_{50} (n*M*)	95% Confidence limit	*n*	EC_{50} (n*M*)	95% Confidence limit	*n*[a]
CCPA	18,800	12,700–27,900	4	42,700	37,900–48,000	3
CPA	18,600	10,300–33,400	3	34,700	29,800–40,300	6
R-PIA	11,200	9,200–13,500	3	16,600	14,500–19,000	3
S-PIA	21,700	15,300–30,800	4	109,600	79,400–151,500	3
NECA	2,360	1,860–3,000	4	692	586–817	9
CGS 21680	88,800	56,100–140,800	3	212,300	138,500–325,300	6
AB-MECA	51,500	41,100–64,500	3			
IB-MECA	11,000	10,500–11,500	3			
IAB-MECA	25,200	15,400–41,300	3			
Antagonist	K_i (n*M*)			K_i (n*M*)		
DPCPX	1,010	609–1,690	4	18.4	10.0–37.4	6
XAC	141	104–192	3	1.10	0.53–2.80	6
CGS 15943	912	605–1,370	3	65.8	34.4–126.2	6
Theophylline	40,100	36,500–44,000	3	6,670	5,110–8,700	9

[a] Number of independent experiments done in duplicate

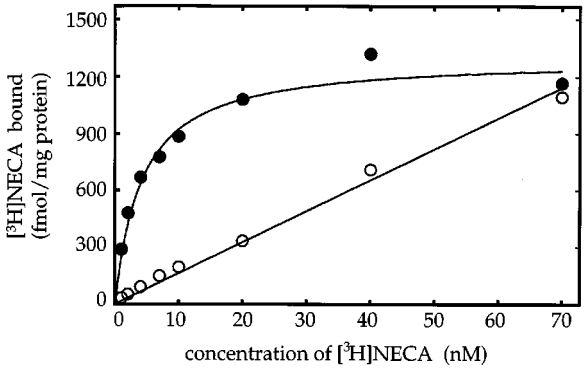

Fig. 5 [^{3}H]NECA saturation binding to A_3 adenosine receptors. Specific (●) and nonspecific (○) binding of [^{3}H]NECA from a representative experiment is shown (K_D 4.1 nM, B_{max} 1300 fmol/mg protein). Nonspecific binding of [^{3}H]NECA at a K_D concentration amounted to about 10% of total binding

Table 5 Competition of agonists and antagonists for [^{3}H]NECA binding at A_3 receptors

Agonist	K_i (n*M*)	95% Confidence limit	*n*[a]
CCPA	42.3	32.1–55.8	3
CPA	43.0	30.4–60.8	3
R-PIA	16.3	9.28–28.7	4
S-PIA	44.6	32.6–61.1	4
CGS 21680	67.1	50.0–89.9	3
AB-MECA	21.5	19.6–23.6	3
IB-MECA	1.20	0.96–1.49	3
IAB-MECA	0.64	0.58–0.70	4
Antagonist			
DPCPX	3,960	2,600–6,020	4
XAC	91.9	63.8–132	4
CGS 15943	50.8	42.8–60.3	3
Theophylline	86,400	73,600–101,300	3

[a] Number of independent experiments done in duplicate

being the most potent compound at this receptor subtype (Table 5).

The non-xanthine CGS 15943 was the most potent antagonist (K_i-value 50 nM). As shown in Table 5, XAC bound with a K_i-value of 92 nM. The affinity of theophylline (86 μM) was tenfold lower than at the A_1 receptor and 50-fold lower than at the A_{2A} subtype. However, DPCPX as the prototypical A_1-selective antagonist was 1000-fold less active at A_3 compared to A_1 receptors.

Discussion

With stable transfection of adenosine receptors into CHO cells we provide models for the investigation of the human receptor subtypes in an identical cellular background. We provide a comparative pharmacological characterization utilizing selected agonists and antagonists that are available from commercial sources. In general, the stably transfected receptors exhibit the typical pharmacology of the respective subtypes with some species-specific characteristics.

Agonist affinities of the human A_1 adenosine receptor (Table 2) were very similar to the affinites of the prototypical rat receptor (Klotz et al. 1991). However, the human A_1 subtype exhibits an about tenfold lower affinity for the A_1 antagonist DPCPX (Table 1) compared to the rat brain receptor (Lohse et al. 1987). This confirms results of the initial cloning of the human A_1 receptor (Libert et al. 1992). XAC was less potent at the human receptor compared to rat. Thus, the pharmacological antagonist profile of the human receptor resembles most closely the A_1 receptor in guinea pig (Klotz et al. 1991).

The binding data for A_{2A} adenosine receptors reveal that none of the prototypical adenosine receptor agonists exhibits high affinity for the human subtype. Both NECA and CGS 21680, which are available as radioligands for

the A_{2A} subtype, have lower affinities at the human than the rat receptor. For [^{3}H]NECA a K_D-value of 20 nM was determined. The specific radioactivity of only about 20 Ci/mmol of this commercially available tritiated compound makes it a rather poor radioligand. Due to an even lower affinity [^{3}H]CGS 21680 is not a useful alternative. Recently, the new non-xanthine antagonist [^{3}H]SCH 58261 ([^{3}H]-5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4, 3-e]-1,2,4-triazolo[1, 5-c]pyrimidine) was introduced as a high-affinity radioligand at A_{2A} receptors with a K_D-value of 2.3 nM at the human subtype (Dionisotti et al. 1997). This compound shows fairly high nonspecific binding and is not yet commercially available. The data from agonist and antagonist competition experiments utilizing [^{3}H]SCH 58261 (Dionisotti et al. 1997) are in good agreement with our data from [^{3}H]NECA competition (Table 3).

The A_{2A} receptor was expressed at lower level compared to A_1 and A_3 receptors. Several strategies to increase receptor expression failed. Even in another cell type (HEK 293 cells) there appears to be a limit of 200–300 fmol receptors/mg membrane protein. One might speculate that A_{2A} adenosine receptors somehow regulate their own expression preventing higher expression yields.

Currently, no high-affinity agonists or antagonists are available for the A_{2B} adenosine receptor. Therefore, stimulation of adenylyl cyclase activity in membrane preparations and accumulation of cAMP in whole cells were utilized as functional assays to characterize the A_{2B} receptor. For agonists this strategy yields only relative potencies whereas inhibition of agonist-stimulated cyclase allows for the determination of absolute antagonist affinity. NECA was the most potent agonist and was confirmed to be a “universal” adenosine receptor agonist.

The pharmacological profile for antagonists identified XAC as the most potent compound at the human A_{2B} receptor. However, the two methods for determination of antagonist affinites used in this study resulted in discrepant results. The discrepancy was more pronounced with increasing affinity of the antagonists and was about 100-fold in the case of XAC compared to only sixfold for theophylline which was the weakest antagonist in our series. We do not have an explanation for this phenomenon which has been observed previously in studies with the A_{2B} receptor in NIH 3T3 fibroblasts (Brackett and Daly 1994). The fact that the potency difference between determination of adenylyl cyclase activity and measurement of cAMP levels appeared to increase with the potency of the antagonists could indicate that the effect is related to the kinetics of the drug-receptor-effector interactions, since more potent antagonists generally dissociate more slowly than less potent antagonists. For some of the antagonists we investigated in this study Alexander et al. (1996) reported similar potencies determined with cAMP accumulation experiments compared to our values from the same method.

For the nonselective agonist [^{3}H]NECA a surprisingly high affinity at A_3 adenosine receptors was detected. At the human receptor this ligand is about 20-fold more potent than at the rat receptor (Ji et al. 1994). The K_D-value of 6 nM (Table 1) was the highest determined at any subtype and, therefore, allowed us to use a binding assay in a 96-well microplate formate. The human A_3 subtype exhibits some distinct features compared to A_3 receptors from other species. In particular, the xanthine insensitivity that has been pointed out as characteristic in other species (Ji et al. 1994; Müller and Stein 1996) does not appear to hold true for the human A_3 subtype. For XAC a K_i-value of 90 nM was determined showing that it is over 1000-fold more potent than at the rat receptor (van Galen et al. 1994). Thus, corresponding to the agonist NECA, XAC turned out to be a universal antagonist for human adenosine receptors. The non-xanthine compound CGS 15943 was the most potent antagonist at the A_3 (Table 5) as well as at the A_1 subtype (Table 2).

The iodinated derivatives of N^6-benzyladenosine-5′-N-methyluronamide showed particularily high affinity for A_3 receptors. IAB-MECA was the most potent compound with a K_i-value of 0.6 nM. These agonists were also very active at A_1 receptors with the result of an only moderate A_3/A_1 selectivity of 13 for IAB-MECA.

Adenosine has been recognized as a regulatory metabolite in virtually all cells and tissues and, therefore, adenosine receptors were proposed as “targets for future drugs” already 15 years ago by Daly (1982). The confirmation of the existence of four different subtypes has stimulated new interest in adenosine receptors as therapeutic targets because they may serve as the basis for a more selective intervention. In this study we present model systems for the human adenosine receptor subtypes that were stably transfected into CHO cells. These models will help to discriminate and characterize effects mediated via specific receptor subtypes. In addition, we present for the first time the comparative pharmacology of the human adenosine receptors. The data underline that only for A_1 adenosine receptors a panel of high-affinity agonists and antagonists is available. Therefore, we have begun to utilize the transfected CHO cells for the development of new ligands for the human adenosine receptors (Klotz et al. 1997; Camaioni, Di Francesco, Volpini, Vittori, Klotz, Cristalli, submitted). Taken together, the receptor-selective cells developed in this study may prove to be valuable systems to advance our understanding of the diverse functions of adenosine in different tissues and cell types.

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