Characterization of human and rodent native and recombinant adenosine A_{2B} receptors by radioligand binding studies

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Abstract Adenosine A_{2B} receptors of native human and rodent cell lines were investigated using [^{3}H]PSB-298 [(8-{4-[2-(2-hydroxyethylamino)-2-oxoethoxy]phenyl}-1-propylxanthine] in radioligand binding studies. [^{3}H]PSB-298 showed saturable and reversible binding. It exhibited a K_D value of 60 ± 1 nM and limited capacity ($B_{max} = 3.511$ fmol per milligram protein) at recombinant human adenosine A_{2B} receptors expressed in human embryonic kidney cells (HEK-293). The addition of sodium chloride (100 mM) led to a threefold increase in the number of binding sites recognized by the radioligand. The curve of the agonist 5′-N-ethylcarboxamidoadenosine (NECA) was shifted to the right in the presence of NaCl, while the curve of the antagonist PSB-298 was shifted to the left, indicating that PSB-298 may be an inverse agonist at A_{2B} receptors. Adenosine A_{2B} receptors were shown to be the major adenosine A_2 receptor subtype on the mouse neuroblastoma x rat glioma hybrid cell line NG108-15 cells. Binding studies at rat INS-1 cells (insulin secreting cell line) demonstrated that [^{3}H]PSB-298 is a selective radioligand for adenosine A_{2B} binding sites in this cell line.

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Abbreviations

CGS-15943	9-chloro-2-(2-furyl)[1,2,4]-triazolo[1,5c]-chinazoline-5-amine
CGS-21680	(2-p-[2-carboxyethyl]phenethylamino)-5′-N-ethylcarboxamidoadenosine
CHA	N^6-cyclohexyladenosine
CHO	Chinese hamster ovary
CI-IB-MECA	2-chloro-N^6-(3-iodobenzyl)-9-[5-(methylcarbamoyl)-β-D-ribofuranosyl]adenine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
DPX	1,3-diethyl-8-phenylxanthine
GPCR	G protein-coupled receptor
HEK-293	human embryonic kidney
KRH buffer	Krebs–Ringer–HEPES buffer
[^{125}I]ABOPX	[^{125}I]3-(4-amino-3-iodobenzyl)-8-phenyl-(4-oxyacetic acid)-1-propylxanthine
INS-1	rat insulinoma cell line
LOD	limit of detection
LOQ	limit of quantitation
MRE2029-F20	N-benzo[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydropurin-8-yl)-1-methyl-1H-pyrazol-3-yloxy]acetamide
MRS1754	N-(4-cyanophenyl)2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)-phenoxy]acetamide
[^{3}H]MSX-2	[^{3}H]3-(3-hydroxypropyl)-7-methyl-8-(m-methoxystyryl)-1-propargylxanthine
NECA	5′-N-ethylcarboxamidoadenosine

NG-108-15	mouse neuroblastoma x rat glioma hybrid cell line
OSIP-339391	N-(2-{2-phenyl-6-[4-(3-phenylpropyl)-piper-azine-1-carbonyl]-7H-pyrrolo[2,3-d]pyrimi-din-4-ylamino}ethyl)acetamide
PSB-53	(4-(1-butyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)-benzoic acid
PSB-297	(8-{4-[2-(2-hydroxyethylamino)-2-oxo-ethoxy]phenyl}-1-propargylxanthine
PSB-298	(8-{4-[2-(2-hydroxyethylamino)-2-oxo-ethoxy]phenyl}-1-propylxanthine
[³H]PSB-11	[³H]8-ethyl-4-methyl-2-phenyl-(8R)-4,5,7,8-tetrahydro-1H-imidazo[2,1-i]-purin-5-one
PSB-1115	1-propyl-8-p-sulfophenylxanthine
ZM241385	(4-(2-((7-amino-2-(furyl)-1,2,4-triazolo[2,3-a]-1,3,5-triazin-5-yl)amino)ethyl)phenol

Introduction

Adenosine receptors are subdivided into four different subtypes, A_1, A_{2A}, A_{2B} and A_3. Our knowledge of adenosine A_{2B} receptors lags behind that of the other adenosine receptor subtypes because a lack of selective pharmacological tools has hindered research in this area [1]. The adenosine A_{2B} receptor subtype has recently attracted considerable interest as a novel potential therapeutic target, e.g., for anti-asthmatic, anti-diabetic, anti-diarrheal, anti-Alzheimer, analgesic and anti-inflammatory drugs [2–5]. Radioligand binding assays are widely used for the screening of new potential receptor ligands; they provide data of the direct interaction between ligand and receptor protein and, therefore, are ideally suitable for structure–activity relationship analysis and molecular modeling studies. In contrast, functional studies are only an indirect indicator for the formation of a receptor–ligand complex. Radioligands are also useful for the detection and quantification of receptor proteins in recombinant or native cell lines or tissues. When we started this project, no selective radioligand for adenosine A_{2B} receptors was available; for radioligand binding studies at adenosine A_{2B} receptors, the adenosine A_1 receptor selective antagonist [³H]DPCPX ([³H]1,3-dipropyl-8-cyclopentylxanthine) or the adenosine A_{2A} receptor selective antagonist [³H]ZM241385 ([³H](4-(2-((7-amino-2-(furyl)-1,2,4-triazolo[2,3-a]-1,3,5-triazin-5-yl)amino)ethyl)phenol, see Figure 1) were frequently used,

Figure 1 Structures of antagonist radioligands used for adenosine A_{2B} receptor binding assays
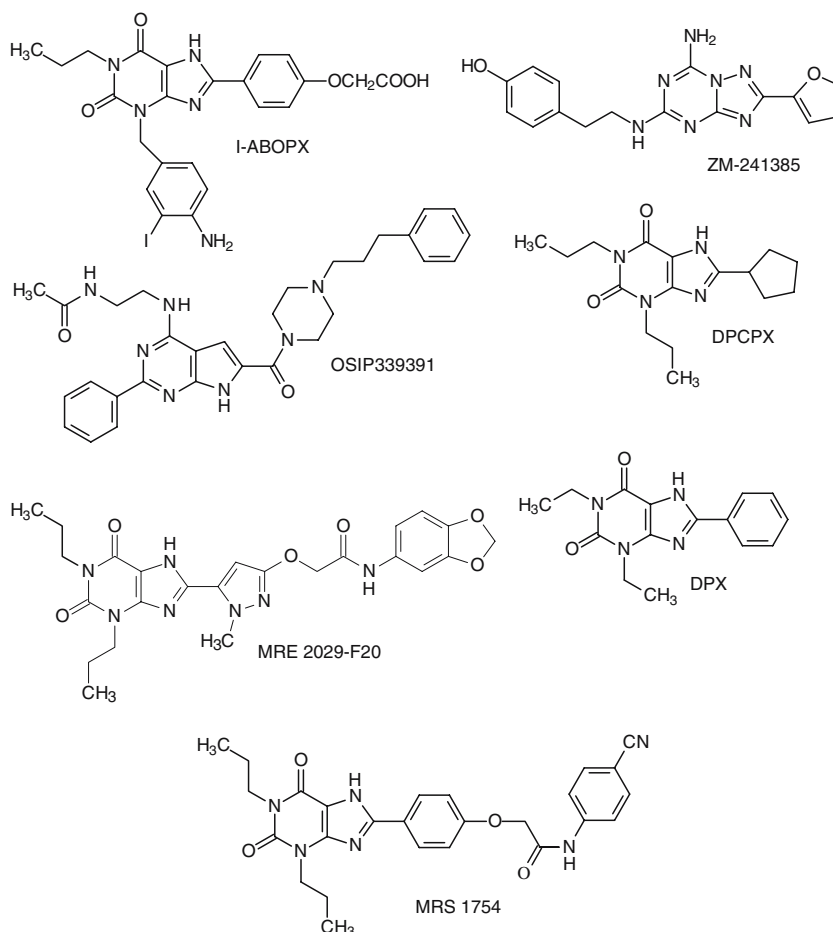
but both radioligands are actually much more potent at other adenosine receptor subtypes than at the adenosine A_{2B} receptor [6]. Further non-selective radioligands for the labeling of adenosine A_{2B} receptors have been used, including [^{125}I]ABOPX ([^{125}I]3-amino-4-iodobenzyl-8-phenyl-(4-oxyacetic acid)-1-propylxanthine) [7] and [^{3}H]-DPX (1,3-diethyl-8-phenylxanthine) [8]. Jacobson et al. developed [^{3}H]MRS1754 (N-(4-cyanophenyl)2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)-phenoxy]acetamide), the first potent and selective adenosine A_{2B} receptor antagonist radioligand (Figure 1) [9]. [^{3}H]-MRS1754, however, is a very hydrophobic compound showing only low solubility in water. Thus, the solution of the radioligand added to the test tubes has to contain 50% dimethylsulfoxide (DMSO) in order to allow constant concentrations of the dissolved radioligand, and the final DMSO concentration in the assays was 4.5% [9]. [^{3}H]-MRS1754 exhibited a relatively high level of non-specific binding of approximately 30% at a concentration of 0.7 nM (K_D = 1.13 nM) in Chinese hamster ovary (CHO) cells expressing a very high level of >10 pmol of receptors per milligram of protein [9]. Recently, Baraldi et al. developed another potent and selective adenosine A_{2B} receptor antagonist radioligand, [^{3}H]MRE2029-F20 (N-benzo[1,3]-dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahy-dropurin-8-yl)-1-methyl-1H-pyrazol-3-yloxy]-acetamide) for human adenosine A_{2B} receptors [10–12]. The compound was potent (K_D 1.65 nM) and showed ca. 40-fold selectivity versus human adenosine A_1 receptors. So far it has been used to detect only human, not rodent, A_{2B} receptors. Very recently, another adenosine A_{2B} receptor radioligand was described, [^{3}H]OSIP339391 (N-(2-{2-phenyl-6-[4-(3-phenylpropyl)-piperazine-1-carbonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-ylamino}ethyl)acetamide with a K_D value of 0.17 nM [13]). The deazapurine derivative is also as highly lipophilic and similarly bulky as the two other adenosine A_{2B} receptor selective radioligands (Figure 1). Thus, [^{3}H]OSIP339391 can be used only in the presence of 0.5% of bovine serum albumin to prevent its adsorption to laboratory ware, e.g., vials, incubation plates and pipette tips [13]. In addition, [^{3}H]OSIP339391 may label a second, low affinity, binding site in HEK-293 cells transfected with the human adenosine A_{2B} receptor, since the saturation assay showed a non-linear Rosenthal plot. It has also only been investigated at human adenosine receptor subtypes.

We have synthesized a new adenosine A_{2B} receptor antagonist ligand, [^{3}H]PSB-298, a polar compound with relatively high water solubility, which exhibits a moderate degree of non-specific binding and has been useful for the testing of potential adenosine A_{2B} receptor agonists and antagonists in radioligand binding studies. Membranes from HEK-293 cells expressing human adenosine A_{2B}

receptors in a high density were used to evaluate the new antagonist radioligand and to test its suitability for radioligand binding studies at adenosine A_{2B} receptors. Subsequently, three different cell lines were characterized using [^{3}H]PSB-298 as an antagonist radioligand for adenosine A_{2B} receptors: a frequently used recombinant CHO cell line expressing the human adenosine A_{2B} receptor, a native rodent mouse neuroblastoma x rat glioma hybrid cell line (NG-108-15) and the rat insulinoma cell line INS-1. Using [^{3}H]PSB-298 we have been able for the first time to characterize the latter cell lines with respect to adenosine A_{2B} receptor expression in radioligand binding studies.

Materials and methods

Chemicals

Tris was obtained from Acros Organics (Leverkusen, Germany), DMSO was from Fluka (Switzerland), HCl was from Merck, HAT supplement from Gibco, [^{3}H]-ZM241385 (17 Ci/mmol) was from Tocris; [^{3}H]MSX-2 ([^{3}H]3-(3-hydroxypropyl)-7-methyl-8-(m-methoxystyryl)-1-propargylxanthine) (84 Ci/mmol) [14], [^{3}H]PSB-11 [^{3}H]-8-ethyl-4-methyl-2-phenyl-(8R)-4,5,7,8-tetrahydro-1H-imidazo[2,1-i]-purin-5-one (53 Ci/mmol) [15] and [^{3}H]-PSB-298 (124 Ci/mmol) were custom labeled by Amersham from precursors that were synthesized in our laboratory as described. [2,8-^{3}H]N^6-cyclopentyladenosine ([2,8-^{3}H]CHA) (10–20 Ci/mmol) was from Moravek Biochemicals, Inc., Cal., USA; all other chemical reagents, cell culture materials and adenosine receptor ligands were obtained from Sigma.

Synthesis of [^{3}H]PSB-298

Tritiated [^{3}H]PSB-298 was prepared by catalytic hydrogenation of the corresponding 1-propargyl derivative (8-{4-[2-(2-hydroxyethylamino)-2-oxoethoxy]-phenyl}-1-propargylxanthine (PSB-297) using tritium gas (custom labeling). Unlabeled PSB-298 was synthesized as described [16]. PSB-297 was prepared analogously starting from 6-amino-3-propargyluracil [17]. The product PSB-297 was purified by column chromatography on silica gel 60 using dichloromethane:methanol [9:1 (v/v)] as the mobile phase.

Analytical data for PSB-297: mp, 295–297°C (decomposition); MS, m/z (%) = 383.1 (M$^+$; 100); 365.1 (59); 282.1 (79); 253.1 (19); 85.1 (25). HRMS: found, 383.1221; calculated, 383.1226; $C_{18}H_{17}N_5O_5$ (383.12).

^{1}H-NMR: 3.04 (t, 1H, J = 2.2 Hz, C3′H); 3.21 (quart, 2H, J = 6.0 Hz, CH_2); 3.43 (t, 2H, J = 6.1 Hz, CH_2); 4.55 (s, 2H, OCH_2); 4.58 (d, 2H, J = 2.2 Hz, C1′H_2); 4.68 (s, 1H, OH); 7.07 (d, 2H, J = 8.8 Hz, aryl CH); 8.01 (s, NH amide); 8.03 (d, 2H, J = 8.8 Hz, aryl CH); 12.03 (s, 1H, N3-H); 13.56 (s, 1H, N7-H).

^{13}C-NMR: 29.33 (C1′H$_2$); 41.37 (CH$_2$NH); 59.80 (HOCH$_2$); 67.21 (OCH$_2$); 72.02 (C3′H); 80.93 (C2′); 110.02 (C5); 115.00 (C3″, C5″); 125.20 (C1″); 127.46 (C2″, C6″); 149.75 (C4); 151.34 (C8); 151.61 (C2); 155.42 (C6); 158.32 (C4″); 167.69 (amide CO).

Purity confirmation by capillary electrophoresis: phosphate buffer containing 100 mM sodium dodecyl sulfate (pH 7.4); voltage 10 kV; retention time 9.2 min; purity 100%; ultraviolet (UV) detection 318 nm; UV [methanol: DMSO = 9:1 (v/v)] λ_{max1} = 246 nm, λ_{max2} = 318 nm; solubility: soluble in dimethylformamide (DMF), DMSO, hot methanol, hot methanol with water (80:20), slightly soluble in cold methanol, cold ethanol and cold water. R_f [silica gel plates Merck F254, dichloromethane:methanol = 9:1 (v/v)] 0.30 (compare R_f value of PSB-298 under the same conditions: 0.32).

The radiolabeling was performed by Nycomed Amersham, Buckinghamshire, UK through Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany. The specific activity of [3H]PSB-298 was 124 Ci (4.59 TBq)/ mmol. UV spectroscopy (λ_{max} = 318 nm, ε = 35,780) and capillary electrophoresis with UV detection was used for the quantification of [3H]PSB-298 and for purity determination (see below).

Purity determination of [3H]PSB-298
by capillary electrophoresis

Capillary electrophoresis was performed on a P/PACE System 5500 (Beckman Coulter Instruments, Fullerton, Calif., USA) equipped with a photodiode array detection system. The instrument was controlled by P/ACE station software (Beckman instruments). The runs were performed under the following conditions: T = 25°C, λ = 321 nm, V = 10 kV, in 150 mM Tris-HCl buffer containing 100 mM sodium dodecyl sulfate, pH 9.1. The electrophoretic separations were carried out using an eCAP fused silica capillary [75.0 μm internal diameter (I.D.) × 375 μm outside diameter (O.D.), 37.0 cm length (30 cm to the detector)]. The capillary was washed with 0.1 N aqueous NaOH solution for 2 min, followed by deionized water for 1 min, and 150 mM Tris-HCl buffer containing 100 mM sodium dodecyl sulfate, pH 9.1, for 1 min before each injection. Injections were made by applying a slight pressure (0.5 p.s.i.) for 5 s delivering approximately 5 nl of the sample solution.

Stock solutions of the unlabeled compounds 8-[4-(2-hydroxyethylamino)-2-oxoethoxy)phenyl-1-propylxanthine (PSB-298) and 8-[4-(2-hydroxyethylamino)-2-oxoethoxy)-phenyl-1-propargylxanthine (PSB-297) were prepared in DMSO at a concentration of 1.0 mg/ml and were then diluted with a mixture of DMSO and deionized water (1:1)

to obtain a range of different concentrations. The radioactive compound [3H]PSB-298 was dissolved in DMSO at a concentration of 4.4 mg/ml and then diluted 1:1 with deionized water. For the determination of linearity and the limit of detection and quantitation, solutions of PSB-298 and PSB-297 were run in concentrations of 0.002 mg/ml, 0.005 mg/ml, 0.01 mg/ml, 0.02 mg/ml, 0.06 mg/ml, 0.08 mg/ml and 0.1 mg/ml. A high degree of linearity was found in the range from their limit of quantitation (LOQ; the lowest measured concentration within the linear range) to the highest measured concentration of 0.1 mg/ml. The regressive linearity coefficients for PSB-298 and PSB-297 were 0.996 and 0.993, respectively. The LOQs for PSB-298 and PSB-297 were 0.04 ± 0.001 mg/ml (SEM, n = 3) and 0.05 ± 0.002 mg/ml (SEM, n = 3), respectively. The limit of detection (LOD) was defined as a signal-to-noise ratio of 3. The LOD value for PSB-298 and PSB-297 was 0.002 mg/ml. Each sample was measured 3–4 times.

The concentration of the solution of [3H]PSB-298 in DMSO was determined by capillary electrophoresis. Peaks were identified by two means: (1) by comparing the migration times of the observed peak with that of the non-radioactive PSB-298 eluted under the same conditions, and (2) by spiking the radioactive sample with non-radioactive PSB-298 standard. Under the same conditions the peak for the precursor compound PSB-297 appeared at 6.31 ± 0.07 min and that for PSB-298 (radioactive and non-radioactive) at 9.54 ± 0.13 min. The solution of the radioactive compound gave only a single peak; no peak of the starting compound was found, indicating high purity (>95%). Therefore, quantitative determination could be performed in the presence of PSB-297 (0.1 mg/ml) as an internal standard.

Receptor-radioligand binding studies

HEK-293 cell membranes recombinantly expressing the human adenosine A$_{2B}$ receptor (B$_{max}$ = 1.6 pmol per milligram protein detected with [3H]DPCPX) were purchased from Perkin Elmer Life Sciences, Boston, USA. CHO cells recombinantly expressing the human adenosine A$_{2B}$ receptor were a gift from Dr. K.-N. Klotz and were grown as described [18]. Membrane preparations were obtained as described [18]. NG108-15 cells were provided by Dr. Brüss, Pharmacological Institute, University of Bonn and were grown as described [19, 20]. Membranes for radioligand binding experiments were prepared by thawing frozen cells followed by scraping them off the dishes in ice-cold Tris-HCl buffer (50 mM Tris, pH 7.4). The cell suspension was homogenized on ice and spun down for 10 min at 1,000 g at 4°C. After centrifugation of the supernatant for 30 min at 20,000 r.p.m., the membrane

pellets were re-suspended in Tris-HCl buffer (50 mM Tris, pH 7.4) and centrifuged once more under the same conditions. The protein concentration was determined by the method of Lowry et al. using bovine serum albumin as a standard reference [21]. The membranes were frozen at −80°C at a protein concentration of 1–3 mg/ml until they were used in the binding assays. INS-1 cells generously provided by Dr. C.B. Wollheim (Geneva, Switzerland) were grown in monolayer cultures (75 cm^2 culture flasks (5 × 10^6 cells/20 ml) or 24-well culture plates (1.5 × 10^5 cells/well) in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum, 10 mM HEPES, 2 mM glutamine, 1 mM pyruvate, 50 μM mercaptoethanol, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin and cultured at 37°C in humidified 5% CO_2/95% air.

Binding studies with [^{3}H]PSB-298 were performed in Tris-HCl buffer (50 mM Tris, pH 7.4) containing adenosine deaminase (1 U/ml) in a final volume of 200 μl. Stock solutions of test compounds were made in DMSO; the final DMSO concentrations did not exceed 1%. Non-specific binding was determined in the presence of 1 mM 5′-N-ethylcarboxamidoadenosine (NECA). After 60 min of incubation at room temperature (25°C) to allow equilibrium to be reached, bound and free radioactivity were separated by filtering the assay mixture through GF/B glass-fiber filters using a Brandel cell harvester (Brandel, Gaithersburg, Md., USA). The filters were washed twice with 1 ml ice-cold Tris buffer and the punched-out filters were immediately transferred to mini-vials and incubated with 2.5 ml Ultima Gold scintillation cocktail (Canberra Packard) for 9 h before being counted in a liquid scintillation counter Tricarb 2100TR (Canberra Packard) with an efficiency of 65%. Two-to-four independent experiments were performed. Saturation studies at the recombinant adenosine A_{2B} receptors of HEK-293 cell membranes were performed in duplicate using 50 μg of protein and were conducted over a concentration range of 0.25 nM to 100–200 nM using 10–12 different concentrations of [^{3}H]PSB-298 ($n = 3$). For saturation experiments at CHO and NG108-15 cell membranes 100 μg of protein per assay were incubated in duplicate with 10–12 different concentrations of the radioligand in a concentration range of 0.25–200 nM ($n = 2$) and 0.3–300 nM ($n = 4$), respectively. Incubation conditions were as described above. For kinetics studies and competition assays, 50 μg of recombinant protein and 1.0 nM [^{3}H]PSB-298 were used. Association kinetics analysis was conducted over a time range of 180 min with 14 different time points; dissociation analysis was performed after 180 min of pre-incubation by the addition of 1 mM NECA (final concentration) to initiate dissociation. Data reported are means ± SEM of experiments performed in duplicate.

Binding studies with the adenosine A_{2A} receptor selective antagonist radioligand [^{3}H]MSX-2 in NG108-15

cells were performed in Tris-HCl buffer (50 mM Tris, pH 7.4) containing adenosine deaminase (1 U/ml) in a final volume of 1,000 μl and 100 μg of protein per vial. Non-specific binding was determined in the presence of 50 μM NECA. After 30 min of incubation at room temperature the assay mixture was filtered through a GF/B glass-fiber filter that had been pretreated for 1 h with a 0.3% polyethylenimine solution using a Brandel cell harvester. The punched-out filters were transferred to mini-vials and incubated with 2.5 ml of Ultima Gold scintillation cocktail (Canberra Packard) for 6 h before being counted in a liquid scintillation counter. The data were analyzed using Graphpad Prism (San Diego, Calif., USA), version 3.0. IC_{50} values were determined by fitting data to a sigmoidal curve with variable slope. We used the equation built into the program and chose standard weighting.

For the binding experiments at INS-1 cells half confluent cells grown in 24-well culture plates were washed with Krebs–Ringer–Hepes (KRH) buffer and thereafter incubated at 22°C with 20 nM [^{3}H]PSB-298 or 1 nM [2,8-^{3}H]CHA with or without increasing concentrations of unlabeled compounds, as indicated. The final incubation volume was 200 μl. To determine the non-specific binding, the incubation was performed in the presence of 1 mM unlabeled NECA. The incubation was terminated after 80 min (for [^{3}H]PSB-298) or 120 min (for [^{3}H]CHA) by cooling (4°C) and washing twice with ice-cold KRH buffer. After the buffer had been removed the pellet was lysed with 50 μl of 0.5% sodium dodecyl sulfate and counted in a scintillation counter (TRI CARB 300 CD, Packard, Frankfurt, Germany) with Quickscint 212, Zinsser Analytic (Frankfurt, Germany). In these experiments at INS-1 cells no adenosine deaminase was added.
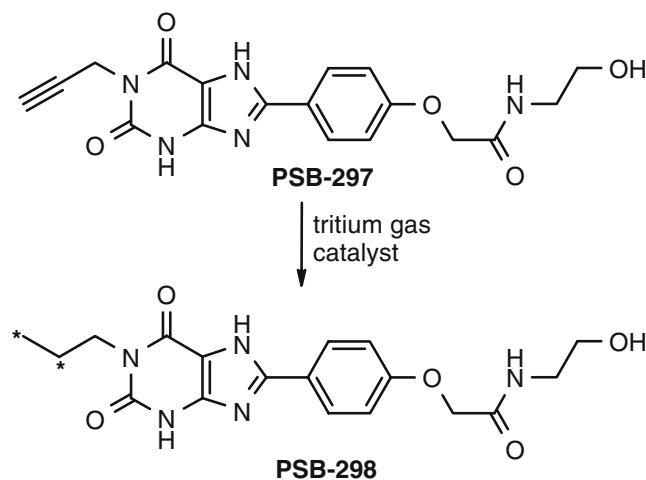

Figure 2 Preparation of [^{3}H]PSB-298 from the propargyl precursor PSB-297 by catalytic hydrogenation (* denotes position of radiolabel)
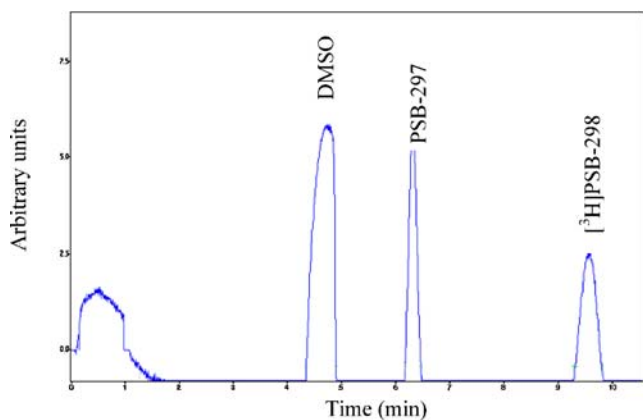
Figure 3 Electropherogram of [³H]PSB-298 in the presence of PSB-297 as an internal standard. The separation conditions were 150 mM Tris-HCl, 100 mM sodium dodecyl sulfate, pH 9.1, fused silica capillary, 37 cm length (30 cm to the detector), 75 µM I.D.; 10 kV; 25°C; detection at 321 nm; pressure injection (0.5 p.s.i., 5 s)
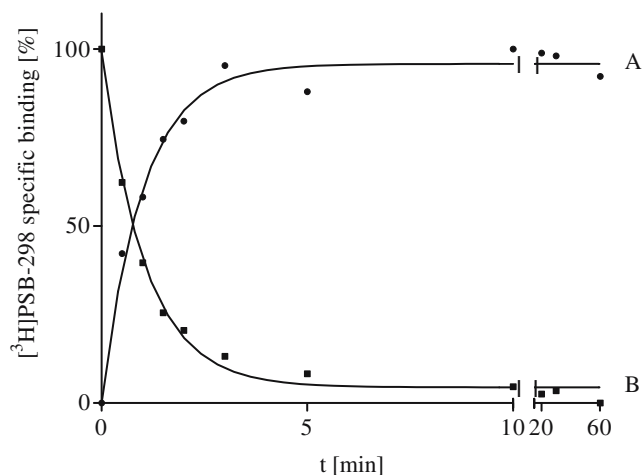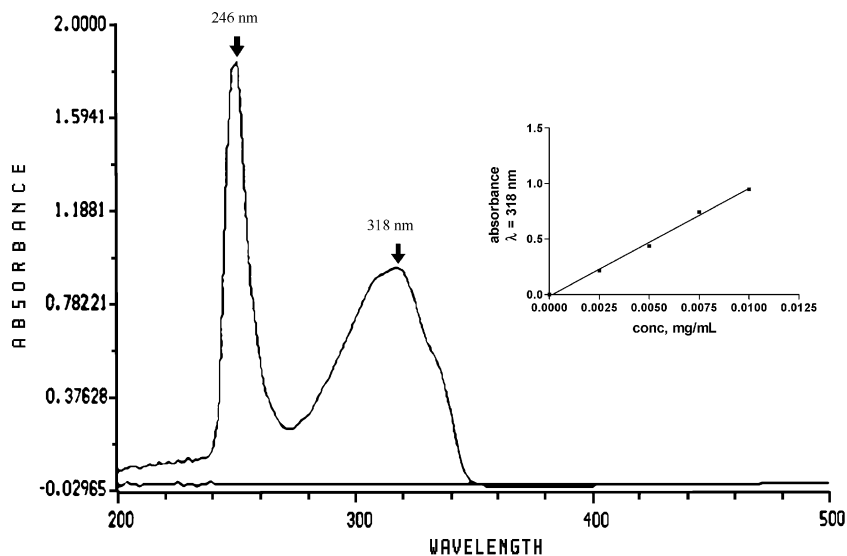

Figure 5 Kinetics of [³H]PSB-298 binding (1 nM) to membranes recombinantly expressing the human adenosine A_{2B} receptor at 25°C. *A* Association curve, *B* dissociation curve. Dissociation was initiated by the addition of 1 mM NECA (final concentration) after 180 min of pre-incubation. Experiments revealed a kinetic K_D value of 25 nM. Data points represent means from a typical experiment performed in duplicates

Results

Preparation and physicochemical characterization of [³H]PSB-298

In a series of 1,8-disubstituted xanthine derivatives, which had been optimized for high adenosine A_{2B} receptor affinity and selectivity, (8-{4-[2-(2-hydroxyethylamino)-2-oxoethoxy]phenyl}-1-propylxanthine (PSB-298, Figure 2) was identified as one of the most potent and most selective adenosine A_{2B} receptor antagonists (K_i = 1.2 nM vs [³H]-ZM241385 at recombinant human adenosine A_{2B} receptors, [16]). In addition, the compound is rather polar due to its hydroxyethylamide structure and might therefore exhibit only a moderate degree of non-specific binding. For these reasons we decided to synthesize PSB-298 in tritium-

labeled form and investigate its usefulness as an antagonist radioligand for adenosine A_{2B} receptor binding studies. [³H]PSB-298 was prepared by catalytic hydrogenation of the corresponding 1-propargyl derivative PSB-297 using tritium gas (see Figure 2).

[³H]PSB-298 was obtained with a high specific activity of 124 Ci/mmol (4.59 TBq/mmol) and stored as a solution in DMSO at a concentration of 4.4 mg/ml. Since the compound was insoluble in solvents commonly used for high performance liquid chromatography (HPLC) analysis, micellar electrokinetic capillary chromatography combined with UV detection was used for the purity determination and quantification of [³H]PSB-298. A typical electrophero-

Figure 4 UV spectrum of PSB-298 (at a concentration of 0.01 mg/ml)

Figure 6 Saturation experiment of [³H]PSB-298 binding to human adenosine A$_{2B}$ receptors recombinantly expressed in HEK-293 cells. The Scatchard–Rosenthal plot of the saturation binding experiment visualizes the presence of one single class of binding sites. Total binding (■), specific binding (●), non-specific binding (▲)
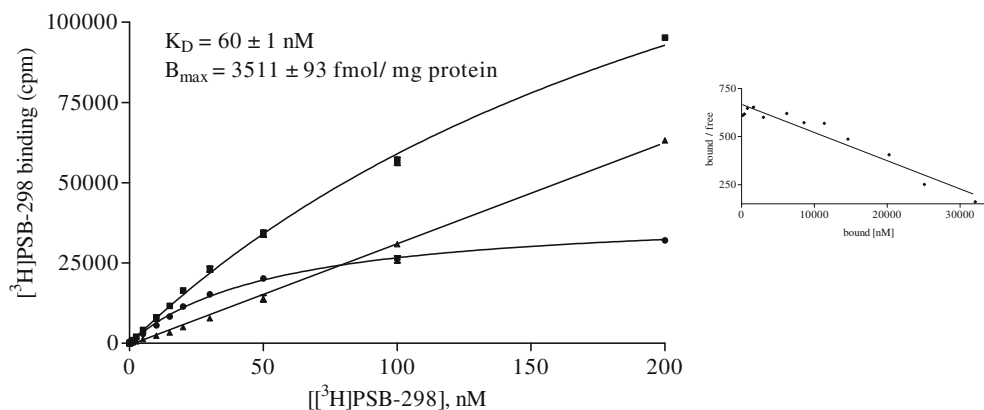
gram of [³H]PSB-298 in the presence of its precursor PSB-297, used as internal standard, is shown in Figure 3.

If [³H]PSB-298 was injected alone, only a single compound peak was observed; no impurities could be detected (not shown), indicating a radiochemical purity of >95%. The UV spectrum of PSB-298 is shown in Figure 4. The absorption maximum at ca. 320 nm was used for quantitative determination of the compound.

Characterization of [³H]PSB-298 at recombinant human adenosine A$_{2B}$ receptors

Commercially available membranes from HEK-293 cells expressing the human adenosine A$_{2B}$ receptor in a high density (B$_{max}$ value of 1.6 pmol per milligram protein) were used to evaluate the new antagonist radioligand. Kinetics studies were performed using 1 nM of [³H]PSB-298. Both association and dissociation appeared to be monophasic (Figure 5). The equilibrium was reached after fewer than 5 min, and, after the addition of 1 mM NECA, the binding could rapidly be reversed. A kinetic K$_D$ value of 25 nM was calculated from the kinetic association and dissociation constants. The equilibrium binding was stable for at least 180 min (not shown).

Saturation experiments demonstrated that [³H]PSB-298 bound to a single class of binding sites in the HEK-293 cell membranes recombinantly expressing the human adenosine A$_{2B}$ receptor with a K$_D$ value of 60 ± 1 nM and an apparent B$_{max}$ value of $3{,}511 \pm 93$ fmol per milligram protein (Figure 6). A two-site model did not give an improved fit over a one-site model. Control experiments with native HEK-293 cells, which are known to endogenously express a very low density of human adenosine A$_{2B}$ receptors did not show any detectable specific binding of [³H]PSB-298 (data not shown).

In competition assays a radioligand concentration of 1 nM and 50 μg of recombinant protein per tube in Tris buffer pH 7.4 was employed. The use of a buffer of pH 6.4, as described for [³H]MRS-1754 [9], gave essentially identical results. Pretreatment of the glass fiber filters with polyethyleneimine was not required – it did not have any effect on the amount of non-specific binding, which amounted to 20–30% of total binding. Polypropylene and glass tubes gave the same results. The presence of MgCl$_2$ (10 mM), or EDTA (1 mM), or GTP (1 mM), respectively, had no effect on the specific binding of 1 nM [³H]PSB-298, whereas the presence of NaCl led to a significant increase in the specific binding of [³H]PSB-298 in a concentration-dependent manner (Figure 7). For example, in the presence

Figure 7 Effects of different concentrations of sodium chloride on radioligand binding to recombinant human adenosine A$_{2B}$ receptors expressed in HEK-293 cell membranes. Membranes (50 μg) were incubated for 60 min at 25°C with 1 nM [³H]PSB-298. Non-specific binding was determined with 1 mM NECA. The data are means of four independent experiments ± SEM
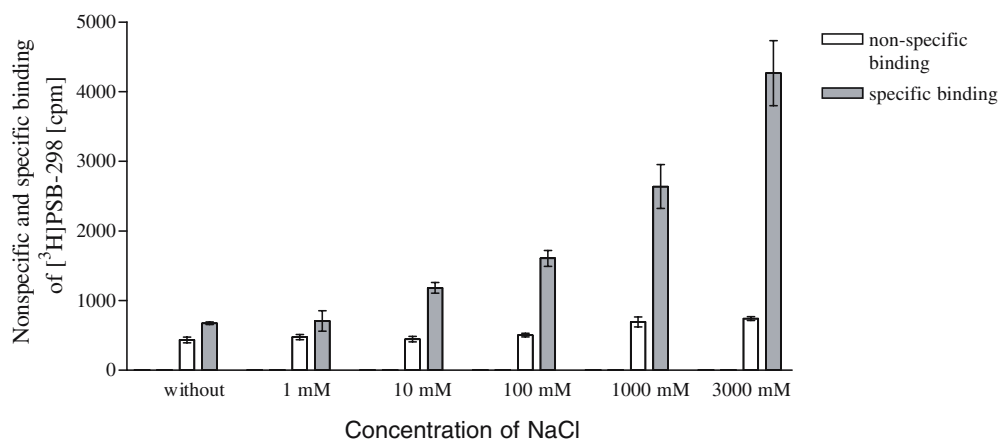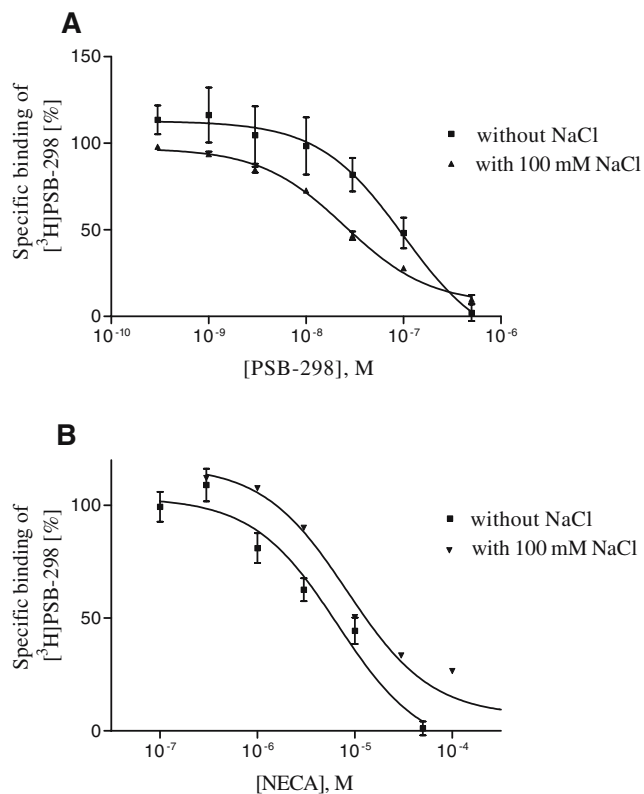
Figure 8 Effect of sodium chloride (100 mM) on the binding of the antagonist PSB-298 and the agonist NECA, respectively, versus the radioligand [³H]PSB-298. Sodium shift experiments were performed at human A$_{2B}$ adenosine receptors expressed in HEK-293 cell membranes with 1 nM [³H]PSB-298 and 50 μg of protein
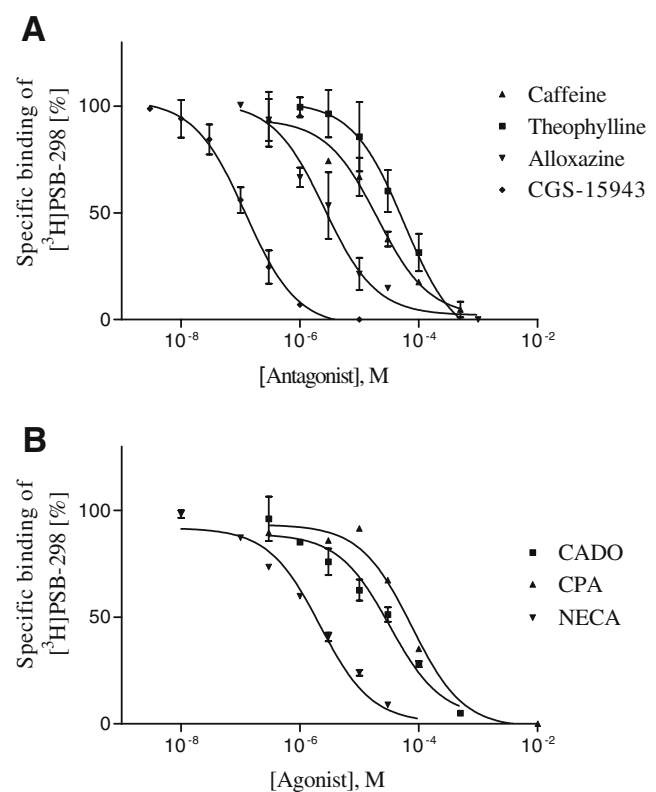

Figure 9 Competition experiments of the antagonists caffeine, theophylline, alloxazine and CGS-15943 (a) and the agonists CADO, CPA and NECA (b) versus 1 nM [³H]PSB-298 performed at human recombinant adenosine A$_{2B}$ receptors expressed in HEK-293 cells

Table 1 K$_i$ values for the displacement of [³H]PSB-298 binding to recombinant human adenosine A$_{2B}$ receptors expressed in HEK-293 cell membranes.

Compound	K$_i$ ± SEM [μM]	Literature K$_i$ ± SEM [μM]
Agonists		
CADO	33.9 ± 2.7	25.5 ± 6.2[a]
CPA	87.0 ± 0.5	20.6 ± 7.50[b]; 34.4 ± 11.1[a]; 21.1 ± 4.30[c]; no inhibition at 5 μM[d]
NECA	1.85 ± 0.15	0.570 ± 0.170[b]; 0.33 ± 0.06[a]; 0.361 ± 0.123[c]; 0.489 ± 0.109[d]
Antagonists		
Caffeine	20.5 ± 2.2	10.4 ± 1.8[e]
Theophylline	74.0 ± 16.0	15.2 ± 4.10[b]; 9.07 ± 1.49[e]; 15.2 ± 8.8[c]; 17% inhibition at 10 μM[d]
Alloxazine	1.34 ± 0.7	2.04 ± 0.57[b]; 0.462 ± 0.078[c]
CGS-15943	0.13 ± 0.01	0.0342 ± 0.001[b]; 0.0164 ± 0.0036[c]

[a] [7]; radioligand: [¹²⁵I]I-ABOPX.
[b] [9]; radioligand: [³H]MRS 1754.
[c] [6]; radioligand: [³H]ZM241385.
[d] [12]; radioligand: [³H]OSIP339391.
[e] [22]; radioligand: [¹²⁵I]I-ABOPX.

of 1 M NaCl the specific binding 1 nM [³H]PSB-298 was more than doubled.

Competition experiments of the agonist NECA indicated that the addition of 100 mM NaCl led to a rightward shift, while the curve of the antagonist PSB-298 was shifted to the left (Figure 8). In contrast, the addition of GTP (1 mM) had no significant effect on the competition curve of NECA (data not shown).

K$_i$ values of standard adenosine receptor agonists and antagonists were determined in radioligand binding assays versus 1 nM [³H]PSB-298; they were in accordance with the K$_i$ values described in the literature (Table 1, Figure 9). NECA was more potent than 2-chloroadenosine and N⁶-cyclopentyladenosine. The rank order of antagonists was CGS15943 (9-chloro-2-(2-furyl)[1,2,4]-triazolo[1,5c]chinazoline-5-amine) >alloxazine >caffeine >theophylline.

In addition, another recombinant cell line (CHO) expressing the human adenosine A$_{2B}$ receptor was investigated. The frequently used cell line was previously characterized in adenylate cyclase assays due to its positive coupling to adenylate cyclase via G$_s$ protein [18]. Saturation experiments with [³H]PSB-298 confirmed the presence of adenosine A$_{2B}$ receptors in the recombinant CHO cell line; a K$_D$ value of 62 ± 8 nM and a B$_{max}$ value of 561 ± 120 fmol

per milligram protein was determined (data not shown). Experiments with different amounts of protein showed a direct proportional relationship of the protein amount and the specific binding of [³H]PSB-298 (data not shown).

Based on these results, the new radioligand [³H]PSB-298 was subsequently used for characterizing two cell lines believed to be natively expressing adenosine A_{2B} receptors.

Characterization of NG108-15 cells with [³H]PSB-298

The mouse neuroblastoma x rat glioma hybrid cell line had previously been proposed to express adenosine A_{2A} as well as adenosine A_{2B} receptors on the basis of functional assays [20]. With the new adenosine A_{2B} receptor radioligand, we were now able to directly determine and quantify the adenosine A_{2B} receptor protein in the cell membranes. Saturation experiments with [³H]PSB-298 at membranes of NG108-15 cells revealed a single class of binding sites with a K_D value of 226 ± 51 nM and a B_{max} value of 453 ± 199 fmol per milligram protein (Figure 10).

The adenosine A_{2A} receptor selective antagonist radioligand [³H]MSX-2 [14] showed no specific binding at NG108-15 cell membranes; there was no significant difference between total and non-specific binding (data not shown). Thus, adenosine A_{2A} receptors were not detectable under the applied conditions of radioligand binding studies.

Inhibition experiments using [³H]PSB-298 at the rat pancreatic beta cell line INS-1

The rat pancreatic beta cell line INS-1 was investigated with respect to adenosine A_{2B} receptor expression using [³H]PSB-298. We used this rodent cell line because of further experiments concerning the influence of adenosine
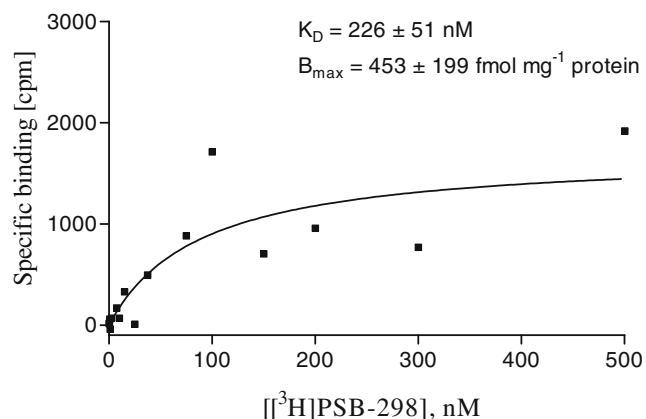

Figure 11 Inhibition of [³H]PSB-298 binding to INS-1 cells by CHA, CGS-21680 and Cl-IB-MECA. INS-1 cells were incubated for 80 min at 22°C in 200 μl KRH buffer containing 5.6 mM glucose, 20 nM [³H] PSB-298, and increasing concentrations of indicated compounds. Results are expressed as percent of maximum specifically bound radioactivity (non-specific binding in the presence of 1 mM unlabeled NECA was subtracted). Each value represents the mean ± SEM of five separate experiments

receptor antagonists on insulin secretion *in vitro* and *in vivo*. This is because our experiments have to be seen in the context of diabetes, as is outlined later in more detail. We tested the selectivity of the radioligand by trying to displace [³H]PSB-298 binding by rising concentrations of an adenosine A_1, A_{2A} or A_3 receptor agonist (CHA, CGS21680 and CI-IB-MECA, respectively). After an increase of binding at low concentrations neither the A_{2A}-selective agonist CGS21680 nor the A_3-selective agonist

Figure 10 Saturation of [³H]PSB-298 binding to native adenosine A_{2B} receptors in NG108-15 cells. The curve is representative of a single experiment from four independent experiments performed in duplicate
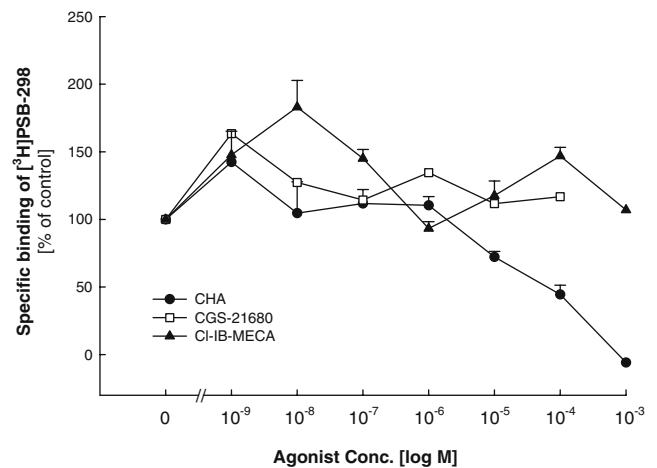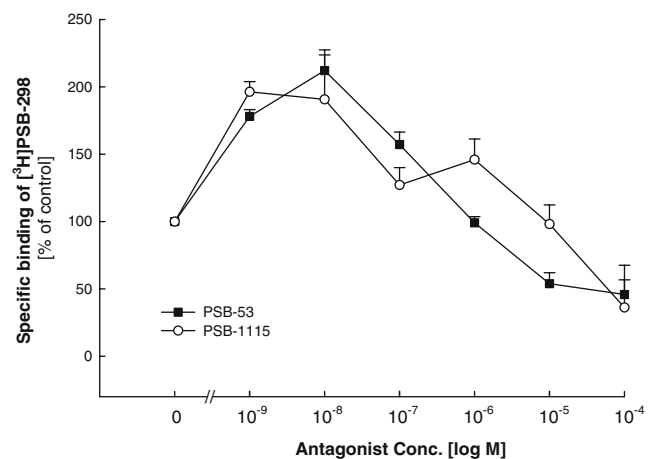

Figure 12 Inhibition of [³H]PSB-298 binding to INS-1 cells by PSB-53 and PSB-1115. INS-1 cells were incubated for 80 min at 22°C in 200 μl KRH buffer containing 5.6 mM glucose, 20 nM [³H] PSB-298, 1 mM CHA (adenosine A_1 receptor agonist) and increasing concentrations of indicated compounds (adenosine A_{2B} receptor antagonists). Results are expressed as percent of maximum specifically bound radioactivity (non-specific binding in the presence of 1 mM unlabeled NECA was subtracted). Each value represents the mean ± SEM of five separate experiments

Cl-IB-MECA showed a displacement of [3H]PSB-298 binding. The A_1-selective agonist CHA displaced the radioligand only at rather high concentrations (100 μM) (Figure 11).

Next, we investigated the selectivity of the A_{2B} antagonists PSB-1115 and PSB-53. We tried to displace the A_1 selective radioligand [3H]CHA with increasing concentrations of the adenosine A_{2B} receptor antagonists. None of them was effective in displacing; however unlabeled CHA was effective as expected (data not shown).

Figure 12 shows the effects of the A_{2B} antagonists PSB-1115 and PSB-53 on [3H]PSB-298 binding. Since the radioligand [3H]PSB-298 may interact with A_1 binding sites in addition to A_{2B} binding sites, the experiments were performed in the presence of the A_1-selective ligand N^6-cyclohexyladenosine (CHA). After an increase in binding at low concentrations there was a sigmoidal inhibition of binding in both cases. This displacement was complete at 100 μM. Both A_{2B} antagonists were similarly potent. The results were virtually identical when CHA was not present during the experiments (data not shown).

Discussion

As a pharmacological tool to investigate adenosine A_{2B} receptors on the protein level, we synthesized a new radioligand, [3H]PSB-298, by catalytic hydrogenation of a propargyl-substituted precursor. The radioligand showed high specific radioactivity of 124 Ci/mmol. For the characterization of the new antagonist radioligand [3H]PSB-298 saturation and kinetics studies in HEK-293 cells recombinantly expressing the human adenosine A_{2B} receptor in high density were performed. With a kinetic K_D value of 25 nM and a K_D value of 60 nM obtained in saturation assays, [3H]PSB-298 showed sufficiently high affinity and appropriate kinetic and physicochemical properties to be useful as an adenosine A_{2B} receptor radioligand. This K_D value corresponded well with the K_i value of 58 nM for the unlabeled PSB-298 determined with [3H]PSB-298. It was higher than the originally determined K_i value that had been obtained in binding studies using the adenosine A_{2A} receptor selective radioligand [3H]ZM241385 [16]. [3H]ZM241385 has been useful for the labeling of adenosine A_{2B} receptors in highly expressing artificial systems. The reason for the discrepant results are currently unknown; one explanation could be that both radioligands, which belong to different structural classes of adenosine receptor antagonists, bind to somewhat different receptor sites or conformations. Even though both compounds are antagonists at adenosine A_{2B} receptors, they might label different affinity states of the receptors. For example, antagonists may be "neutral", labeling all affinity states with the same affinity, or "inverse agonistic," binding preferably and with higher affinity to the low-affinity state for agonists than to the high affinity state for agonists [23]. Recently, it has become clear that multiple conformational states of G protein-coupled receptors (GPCRs) may exist that may all exhibit somewhat different affinities for different ligands [22]. The fact that the previously published data [16] were of a more preliminary character ($n = 2$) may also contribute to the discrepancy with the now thoroughly validated values.

Competition experiments with a series of adenosine receptor agonists and antagonists versus [3H]PSB-298 revealed K_i values that were well in accordance with literature data [6, 7, 9, 13, 24] (Table 1) proving that [3H]PSB-298 is a valuable tool for the identification of potential ligands for the adenosine A_{2B} receptor.

With a K_i value of 5 μM at human adenosine A_{2A} receptors [3H]PSB-298 is highly selective for adenosine A_{2B} over A_{2A} receptors [16]. Selectivity versus the human adenosine A_3 receptor is somewhat lower (K_i A_3 422 nM, [16]), but if used at a concentration of 1 nM, adenosine A_3 receptors should not be labeled. However, PSB-298 is not selective versus the adenosine A_1 receptor at which it is almost as potent as at adenosine A_{2B} receptors (K_i 68 nM, [16]). Thus, the new radioligand [3H]PSB-298 may not be superior to recently developed adenosine A_{2B} receptor radioligands [9, 10, 13] with respect to adenosine A_{2B} receptor selectivity. Nevertheless, it has other advantages, including high polarity and good water solubility, low molecular weight and moderate non-specific binding. The non-specific binding amounted to 20–30% of total binding at a radioligand concentration of 1 nM used in competition assays in membranes expressing an A_{2B} receptor density of 1.6 pmol per milligram of protein using 50 μg of protein in 200 ml TRIS buffer (50 mM, pH 7.4) containing no additives. A direct comparison with published values for non-specific binding of other radioligands is difficult, since non-specific binding is dependent on (1) the concentration of the radioligand, (2) the receptor density in the tissue, (3) the protein concentration used, and (4) the incubation conditions, buffer compositions, additives such as detergent or bovine serum albumin, and other factors. In comparison, the most polar of the other A_{2B} radioligands described so far, [3H]OSIP-339391, has been reported to exhibit 20% of non-specific binding at 1 nM concentration, but only in the presence of 0.5% bovine serum albumin and in tissues expressing an extremely high receptor density of 20 pmol per milligram of protein [13]. The non-specific binding of [3H]MRS-1754 at a concentration of 0.7 nM was reported to be less than 30% in membranes with a very high receptor density of 10.9 pmol per milligram of protein [9]. [3H]MRE-2029-F20 showed 30–45% non-specific binding at its K_D value (1.65 nM) [12].

In contrast to other recently developed adenosine A_{2B} receptor antagonist radioligands, [^{3}H]PSB-298 has no tendency to bind to plasticware, and it is therefore easy to handle without the necessity of preparing radioligand dilutions in DMSO, including high DMSO concentrations in the assays (as required for [^{3}H]MRS1754) or adding bovine serum albumin (as required for [^{3}H]OSIP339391).

Sodium ions are well known for modulating the affinity of agonists and antagonists for a number of GPCRs [25–27]. Frequently, they lead to a decrease in the affinity of agonists for the receptors and increase antagonist binding. Adenosine A_1 receptors [28] and adenosine A_{2A} receptors have been shown to be allosterically modulated by sodium ions, presumably by binding of the sodium to aspartate 55 in the second transmembranal helix of the receptor protein [29]. Gao et al. observed 2–27-fold rightward shifts of agonist affinities in competition experiments at adenosine A_{2A} receptors, using the antagonist radioligand [^{3}H]-ZM241385, upon addition of 1 M NaCl. Vogel et al. recently showed that different salts, including sodium chloride, are able to shift the equilibrium of metarhodopsin from the inactive (MI) to the active (MII) state and act, therefore, as allosteric modulators at this transmembrane protein. The authors postulated that this influence of salts on the conformation and stability may also apply to other transmembrane proteins and, particularly, to other G protein-coupled receptors [30].

In the present study we investigated the ability of sodium ions to modulate agonist and antagonist binding to the human adenosine A_{2B} receptor. Sodium ions increased in a concentration-dependent manner the specific binding of the radioligand [^{3}H]PSB-298 to human recombinant adenosine A_{2B} receptors (Figure 7). A physiological concentration of 100 mM NaCl increased specific binding of the radioligand by 245 ± 55%. A further increase in the NaCl concentration, up to 3 M NaCl, led to a further increase in specific [^{3}H]PSB binding of up to 594 ± 116%. An EC_{50} value for NaCl could not be determined, owing to its solubility limit. In contrast, 100 mM NaCl had not shown any effect on binding of the adenosine A_{2B} receptor antagonist radioligand [^{3}H]MRS1754 [9]. This may indicate that both radioligands bind to different receptor conformations.

In the presence of 100 mM NaCl, which corresponds to the extracellular physiological sodium concentration, the competition curve of the adenosine receptor agonist NECA was shifted to the right, whereas the competition curve for the adenosine A_{2B} receptor antagonist PSB-298 was shifted to the left (Figure 8). The Na^+ shift (the ligand affinity in the presence and absence of sodium ions) was previously shown to be correlated with the intrinsic activity of ligands at some GPCRs, including adenosine A_{2A} receptors [27, 29]. Our results show that Na^+-shift experiments may also be useful for the determination of the intrinsic activity of

ligands at human adenosine A_{2B} receptors. While agonist curves are shifted to the right in the presence of NaCl, the observed left shift for the curve of PSB-298 may indicate that it is an inverse agonist at adenosine A_{2B} receptors. NaCl shifts the equilibrium of receptor conformations to the inactive state to which agonists exhibit reduced affinity but inverse agonists exhibit increased affinity.

GTP usually results in an uncoupling of G protein-coupled receptors from their G proteins and a conformational change from a high-affinity conformation for agonists to a low-affinity conformation. In contrast, the affinity for antagonists may be increased in the presence of GTP [14, 31]. As previously observed for adenosine A_{2A} receptors, GTP also did not appear to exhibit a significant effect on the conformation of adenosine A_{2B} receptors. This has been explained with an unusually tight coupling of the adenosine A_{2A} receptor with G proteins [32, 33]. The same may be true for the adenosine A_{2B} receptor.

The use of [^{3}H]PSB-298 allowed the direct evidence of human adenosine A_{2B} receptors expressed in CHO cells that had been transfected and functionally characterized by Klotz et al. [18]. The receptor density was considerably lower than in the recombinant HEK-293 cells (561 fmol per milligram protein vs 3,511 fmol per milligram protein). This explains why previous attempts using [^{3}H]DPCPX or [^{3}H]ZM241385 to label the adenosine A_{2B} receptors in this recombinant cell line had failed (own unpublished observations).

Mundell and Kelly had shown in functional assays that the mouse neuroblastoma x rat glioma hybrid cell line NG108-15 developed by Hamprecht [34], which is frequently used as a model for neuronal cells, expressed two different adenosine receptor subtypes, A_{2A} and A_{2B} [20]. Ohkubo et al. used reverse transcription polymerase chain reaction to try to detect the mRNA for adenosine A_{2A} and A_{2B} receptors with subtype-specific primers. With this method, however, they only detected adenosine A_{2A} but not A_{2B} message in NG108-15 cells [35]. In radioligand binding studies using the new antagonist [^{3}H]PSB-298 we have been able, for the first time, to detect adenosine A_{2B} receptors in this rodent hybrid cell line on the protein level. We could show the predominance of the adenosine A_{2B} receptor in this cell line. Adenosine A_{2A} receptors were not detectable under the conditions of radioligand binding studies using the adenosine A_{2A} receptor antagonist radioligand [^{3}H]MSX-2 [14]. These experiments suggest that the expression level of adenosine A_{2B} receptors is much higher than that of adenosine A_{2A} receptors in NG108-15 cells. The radioligand binding studies revealed a K_D value of 226 nM for [^{3}H]PSB-298 at the rodent adenosine A_{2B} receptors, showing an affinity that is approximately four-times lower than the affinity to human adenosine A_{2B} receptors. Such a difference in the affinity for rodent and human receptors has previously been observed for a series

of 1-, 3-, 7- and 8-substituted xanthine derivatives [24]. These studies showed that, although [^{3}H]PSB-298 has a somewhat lower affinity for the rodent adenosine A_{2B} receptors than for the human adenosine A_{2B} receptor subtype, it can still be used as an adenosine A_{2B} receptor radioligand for the labeling of rodent adenosine A_{2B} receptors.

Thus, we used the radioligand for characterizing adenosine A_{2B} receptors expressed on the rat pancreatic beta-cell line INS-1. Adenosine A_{2B} receptor antagonists have been proposed as novel potential anti-diabetic drugs [1, 5]. Adenosine receptor subtypes have not been investigated in detail with respect to diabetes: the involvement of adenosine A_1 receptors in insulin release [36] and the involvement of adenosine A_2 receptors with respect to glucagon release [37] have been elucidated. Adenosine A_{2B} receptor antagonists are being developed for inhibiting hepatic glucose production [5], to improve glucose tolerance and to increase insulin sensitivity in skeletal muscle [38]. They may also provide new drugs for the treatment of proliferative and diabetic retinopathy [39]. Therefore, we wanted to investigate whether the adenosine A_{2B} receptor antagonists PSB-1115 and PSB-53, which are planned to be used in animal experiments to study their anti-diabetic effects, selectively bound to adenosine A_{2B} receptors on the rat pancreatic beta-cell line INS-1 using [^{3}H]PSB-298 as an adenosine A_{2B} receptor radioligand. In order to be close to physiological conditions the radioligand binding assays were performed at intact cells without the addition of adenosine deaminase to degrade physiological adenosine.

The data showed that [^{3}H]PSB-298 labeled exclusively adenosine A_{2B} receptor binding sites, but not A_1, A_{2A} or A_3 receptors in INS-1 cells. Thus, the radioligand appears to be selective for A_{2B} receptors in these cells; however, at present, it is not known whether the cells express enough adenosine A_{2A} and A_3 subtype receptors to reveal full selectivity. The IC_{50} values for PSB-53 and PSB-1115 were in the low micromolar range and were, therefore, higher than those determined in other binding assays where they showed K_i values of 20–50 nM [16]. The same high concentrations, however, were effective with respect to insulin secretion in the same cell line (INS-1 cells). Adenosine receptor agonist-induced inhibition of insulin secretion was antagonized by PSB-1115 and PSB-53 at micromolar rather than nanomolar concentrations (data not shown), in accordance with the radioligand binding data. As an agonist we used NECA, a non-degradable A_{2B} agonist, which is also a potent agonist at the other adenosine receptor subtypes.

A high increase in binding was obvious at low concentrations of either displacing compound versus [^{3}H]-PSB-298. The increase in binding at low concentrations of

the displacing compound was highly reproducible. One possible explanation for this behavior could be an allosteric effect, as it has already been described for the A_1 receptor [40]. When we used CHA as an A_1 receptor agonist in the experiments, to avoid a possible interaction of the radioligand with A_1 binding sites, we obtained the same results. Under our conditions [^{3}H]PSB-298 is a new and highly selective radiolabel for adenosine A_{2B} receptor binding sites and is a valuable tool for adenosine receptor research. Since the adenosine A_{2B} antagonists tested are able to antagonize NECA-induced inhibition of insulin secretion (data not shown), the data obtained on adenosine A_{2B} receptor binding sites in INS-1 cells are likely to be physiologically and pharmacologically relevant. The hitherto described characteristics of these adenosine A_{2B} receptor antagonistic compounds are not found in our experiments, since the concentrations to be used are higher than the EC_{50} values found in other cells. The concentration of CHA half-maximally inhibiting [^{3}H]CHA binding in INS-1 cells being in the range of 1 μM is much higher than the previously observed K_i value for CHA at adenosine A_1 receptors.

Conclusions

Recombinant (hA$_{2B}$-HEK-293, hA$_{2B}$-CHO cell membranes) and native (NG108-15 cell membranes, INS-1 whole cells) human and rodent adenosine A_{2B} receptors were successfully characterized in radioligand binding studies using [^{3}H]PSB-298 ((8-{4-[2-(2-hydroxyethylamino)-2-oxoethoxy]phenyl}-1-propylxanthine), a new antagonist radioligand. Modulation of radioligand binding by sodium chloride allows for the characterization of agonists and antagonists and, presumably, inverse agonists at adenosine A_{2B} receptors.

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