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 [³H]PSB-298 [(8-{4-[2-(2-hydroxyethylamino)-2-oxoethoxy]phenyl}-1-propylxanthine] in radioligand binding studies. [³H]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 [³H]PSB-298 is a selective radioligand for adenosine A$_{2B}$ binding sites in this cell line.

Key words adenosine A$_{2B}$ receptor · A$_{2B}$ antagonist radioligand · CHO cells · HEK-293 cells · INS-1 cells · NG108-15 cells · [³H]PSB-298 · sodium shift assays

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-200 2-chloro-N$^6$-(3-iodobenzyl)-9-[5-(methylcarbamoyl)-β-D-ribofuranosyl]adenine
DMF dimethylformamide
DMSO dimethyl sulfoxide
DPCPX 1,3-diisopropyl-8-cyclopentylxanthine
DPX 1,3-diethyl-8-phenylxanthine
GPCR G protein-coupled receptor
HEK-293 human embryonic kidney
KRH Krebs-Ringer-HEPES buffer
[¹²⁵I]ABOPX [¹²⁵I]3-(4-amino-3-iodobenzyl)-8-phenyl-(4-oxyacetic acid)-1-propylxanthine
[¹²⁵I]MSX-2 [¹²⁵I]3-(3-hydroxypropyl)-7-methyl-8-(m-methoxystyryl)-1-propargylxanthine
MRE2029-19 F20 N-benzo[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-1H-pyrazol-3-yl)acetamide
MRS1754 N-(4-cyanophenyl)2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide
NECA 5’-N-ethylcarboxamidoadenosine
mouse neuroblastoma x rat glioma hybrid cell line
OSIP-339391 N-(2-{(2-phenyl-6-[4-(3-phenylpropyl)-piperazine-1-carbonyl]-7H-pyrrolo[2,3-d]pyrimidin-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
$[^3]H$PSB-11 $[^3]H$8-ethyl-4-methyl-2-phenyl-(8R)-4,5,7,8-tetrahydro-1H-imidazo[2,1-i]purin-5-one
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, A1, A2A, A2B and A3. Our knowledge of adenosine A2B 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 A2B 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 A2B receptors was available; for radioligand binding studies at adenosine A2B receptors, the adenosine A1 receptor selective antagonist $[^3]H$DPCPX ($[^3]H$1,3-dipropyl-8-cyclopentylxanthine) or the adenosine A2A receptor selective antagonist $[^3]H$ZM241385 ($[^3]H$4-(2-((7-amino-2-(furyl)-1,2,4-triazolo[2,3-a]-1,3,5-triazin-5-yl)amino)ethyl)phenol, see Figure 1) were frequently used,
but both radioligands are actually much more potent at other adenosine receptor subtypes than at the adenosine A$_{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-iodobenzy1-8-phenyl-(4-oxacyclic 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 cell [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-[3-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydropurin-8-yl)-1-methyl-1H-pyrrozol-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-OSSIP339391 (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 highly lipophilic and similarly bulky as the two other adenosine A$_{2B}$ receptor selective radioligands (Figure 1). Thus, $^{[3]}$H-OSSIP339391 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-OSSIP339391 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, $^{[3]}$HPSB-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 $^{[3]}$HPSB-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 $^{[3]}$HPSB-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 Toerics; $^{[3]}$HMSX-2 ($^{[3]}$H-(3-hydroxypropyl)-7-methyl-8-(m-methoxystyryl)-1-propargylxanthine) (84 C/mmol) [14]. $^{[3]}$HPSB-11 ($^{[3]}$H-8-ethyl-4-methyl-2-phenyl-(8R)-4,5,7,8-tetrahydro-1H-imidazo[2,1-i]-purin-5-one (53 C/mmol) [15] and $^{[3]}$HPSB-298 (124 Ci/mmol) were custom labeled by Amersham from precursors that were synthesized in our laboratory as described. $^{[2,8-3]}$H$^6$-cyclopentyladenosine ($^{[2,8-3]}$H$^6$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 $^{[3]}$HPSB-298

Tritiated $^{[3]}$HPSB-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 (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 (PSB-297) using tritium gas (custom labeling).

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$_5$O$_5$ (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).
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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 9.54 ± 0.13 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 A2B receptor (Bmax = 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 A2B 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 cm² culture flasks (5 × 10⁶ cells/20 ml) or 24-well culture plates (1.5 × 10⁷ cells/well) in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum, 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% CO₂/95% air.

Binding studies with [³H]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 6 h before being counted in a liquid scintillation counter. The data were analyzed using Graphpad Prism (San Diego, Calif., USA), version 3.0. IC₅₀ 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 [³H]PSB-298 or 1 nM [²,⁸-³H]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 [³H]PSB-298) or 120 min (for [²,⁸-³H]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.

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% polyethyleneimine 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. IC₅₀ 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.

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Binding studies with the adenosine A₂A receptor selective antagonist radioligand [³H]MSX-2 in NG108-15

Figure 2 Preparation of [³H]PSB-298 from the propargyl precursor PSB-297 by catalytic hydrogenation (* denotes position of radiolabel)
Results

Preparation and physicochemical characterization of $[^3\text{H}]$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 $[^3\text{H}]$-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. $[^3\text{H}]$PSB-298 was prepared by catalytic hydrogenation of the corresponding 1-propargyl derivative PSB-297 using tritium gas (see Figure 2).

$[^3\text{H}]$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 $[^3\text{H}]$PSB-298. A typical electrophero-
gram of $[^3]$H]PSB-298 in the presence of its precursor PSB-297, used as internal standard, is shown in Figure 3. If $[^3]$H]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 $[^3]$H]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 ($B_{\text{max}}$ value of 1.6 pmol per milligram protein) were used to evaluate the new antagonist radioligand. Kinetics studies were performed using 1 nM of $[^3]$H]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 $[^3]$H]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 $B_{\text{max}}$ value of 3,511 ± 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 $[^3]$H]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 $[^3]$H]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 $[^3]$H]PSB-298, whereas the presence of NaCl led to a significant increase in the specific binding of $[^3]$H]PSB-298 in a concentration-dependent manner (Figure 7). For example, in the presence

![Figure 6 Saturation experiment of $[^3]$H]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 (▲).](image-url)

![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 $[^3]$H]PSB-298. Non-specific binding was determined with 1 mM NECA. The data are means of four independent experiments ± SEM.](image-url)
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).

Ki 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 Ki 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.

Table 1 Ki values for the displacement of [3H]PSB-298 binding to recombinant human adenosine A2B receptors expressed in HEK-293 cell membranes.

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

a [7]; radioligand: [125I]-ABOPX.
b [9]; radioligand: [3H]MR3 1754.
c [6]; radioligand: [3H]ZM241385.
d [12]; radioligand: [3H]OSIP339391.
e [22]; radioligand: [125I]-ABOPX.
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 A2B 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 A2A as well as adenosine A2B receptors on the basis of functional assays [20]. With the new adenosine A2B receptor radioligand, we were now able to directly determine and quantify the adenosine A2B 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 A2A 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 A2A 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 A2B receptor expression using [3H]PSB-298. We used this rodent cell line because of further experiments concerning the influence of adenosine 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 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.
Cl-IB-MECA showed a displacement of $[^3\text{H}]$PSB-298 binding. The $\text{A}_1$-selective agonist CHA displaced the radioligand only at rather high concentrations (100 $\mu$M) (Figure 11).

Next, we investigated the selectivity of the $\text{A}_{2\text{B}}$ antagonists PSB-1115 and PSB-53. We tried to displace the $\text{A}_1$ selective radioligand $[^3\text{H}]$CHA with increasing concentrations of the adenosine $\text{A}_{2\text{B}}$ 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 $\text{A}_{2\text{B}}$ antagonists PSB-1115 and PSB-53 on $[^3\text{H}]$PSB-298 binding. Since the radioligand $[^3\text{H}]$PSB-298 may interact with $\text{A}_1$ binding sites in addition to $\text{A}_{2\text{B}}$ binding sites, the experiments were performed in the presence of the $\text{A}_1$-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 $\mu$M. Both $\text{A}_{2\text{B}}$ 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 $\text{A}_{2\text{B}}$ receptors on the protein level, we synthesized a new radioligand, $[^3\text{H}]$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 $[^3\text{H}]$PSB-298 saturation and kinetics studies in HEK-293 cells recombinantly expressing the human adenosine $\text{A}_{2\text{B}}$ 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, $[^3\text{H}]$PSB-298 showed sufficiently high affinity and appropriate kinetic and physicochemical properties to be useful as an adenosine $\text{A}_{2\text{B}}$ receptor radioligand. This $K_D$ value corresponded well with the $K_i$ value of 58 nM for the unlabeled PSB-298 determined with $[^3\text{H}]$PSB-298. It was higher than the originally determined $K_i$ value that had been obtained in binding studies using the adenosine $\text{A}_{2\text{A}}$ receptor selective radioligand $[^3\text{H}]$ZM241385 [16]. $[^3\text{H}]$ZM241385 has been useful for the labeling of adenosine $\text{A}_{2\text{A}}$ 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 $\text{A}_{2\text{B}}$ 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 $[^3\text{H}]$PSB-298 revealed $K_i$ values that were well in accordance with literature data [6, 7, 9, 13, 24] (Table 1) proving that $[^3\text{H}]$PSB-298 is a valuable tool for the identification of potential ligands for the adenosine $\text{A}_{2\text{B}}$ receptor.

With a $K_i$ value of 5 $\mu$M at human adenosine $\text{A}_{2\text{A}}$ receptors $[^3\text{H}]$PSB-298 is highly selective for adenosine $\text{A}_{2\text{B}}$ over $\text{A}_{2\text{A}}$ receptors [16]. Selectivity versus the human adenosine $\text{A}_3$ receptor is somewhat lower ($K_i$ $A_3$ 422 nM, [16]), but if used at a concentration of 1 nM, adenosine $\text{A}_3$ receptors should not be labeled. However, PSB-298 is not selective versus the adenosine $\text{A}_1$ receptor at which it is almost as potent as at adenosine $\text{A}_{2\text{B}}$ receptors ($K_i$ 68 nM, [16]). Thus, the new radioligand $[^3\text{H}]$PSB-298 may not be superior to recently developed adenosine $\text{A}_{2\text{B}}$ receptor radioligands [9, 10, 13] with respect to adenosine $\text{A}_{2\text{B}}$ 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 $\text{A}_{2\text{B}}$ receptor density of 1.6 pmol per milligram of protein using 50 $\mu$g 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 $\text{A}_{2\text{B}}$ radioligands described so far, $[^3\text{H}]$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 $[^3\text{H}]$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]. $[^3\text{H}]$ MRE-2092-F20 showed 30–45% non-specific binding at its $K_D$ value (1.65 nM) [12].
In contrast to other recently developed adenosine A2B receptor antagonist radioligands, [3H]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 [3H]MRS1754) or adding bovine serum albumin (as required for [3H]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 A1 receptors [28] and adenosine A2A 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 A2A receptors, using the antagonist radioligand [3H]-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 A2B receptor. Sodium ions increased in a concentration-dependent manner the specific binding of the radioligand [3H]PSB-298 to human recombinant adenosine A2B 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 [3H]PSB binding of up to 594 ± 116%. An EC50 value for 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 A2A receptors, GTP also did not appear to exhibit a significant effect on the conformation of adenosine A2B Receptors. This has been explained with an unusually tight coupling of the adenosine A2A receptor with G proteins [32, 33]. The same may be true for the adenosine A2B receptor.

The use of [3H]PSB-298 allowed the direct evidence of human adenosine A2B 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 [3H]DPCPX or [3H]ZM241385 to label the adenosine A2B 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 Hampsrecht [34], which is frequently used as a model for neuronal cells, expressed two different adenosine receptor subtypes, A2A and A2B [20]. Ohkubo et al. used reverse transcription polymerase chain reaction to try to detect the mRNA for adenosine A2A and A2B receptors with subtype-specific primers. With this method, however, they only detected adenosine A2A but not A2B message in NG108-15 cells [35]. In radioligand binding studies using the new antagonist [3H]PSB-298 we have been able, for the first time, to detect adenosine A2B receptors in this rodent hybrid cell line on the protein level. We could show the predominance of the adenosine A2B receptor in this cell line. Adenosine A2A receptors were not detectable under the conditions of radioligand binding studies using the adenosine A2A receptor antagonist radioligand [3H]MSX-2 [14]. These experiments suggest that the expression level of adenosine A2B receptors is much higher than that of adenosine A2A receptors in NG108-15 cells. The radioligand binding studies revealed a KD value of 226 nM for [3H]PSB-298 at the rodent adenosine A2B receptors, showing an affinity that is approximately four-times lower than the affinity to human adenosine A2B receptors. Such a difference in the affinity for rodent and human receptors has previously been observed for a series
These studies showed that, although [3H]PSB-298 has a somewhat lower affinity for the rodent adenosine A\textsubscript{2B} receptors than for the human adenosine A\textsubscript{2B} receptor subtype, it can still be used as an adenosine A\textsubscript{2B} receptor radioligand for the labeling of rodent adenosine A\textsubscript{2B} receptors.

Thus, we used the radioligand for characterizing adenosine A\textsubscript{2B} receptors expressed on the rat pancreatic beta-cell line INS-1. Adenosine A\textsubscript{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\textsubscript{1} receptors in insulin release [36] and the involvement of adenosine A\textsubscript{2} receptors with respect to glucagon release [37] have been elucidated. Adenosine A\textsubscript{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\textsubscript{2B} receptor antagonists PSB-1115 and PSB-53, which are planned to be used in animal experiments to study their anti-diabetic effects, selectively bind to adenosine A\textsubscript{2B} receptors on the rat pancreatic beta-cell line INS-1 using [3H]PSB-298 as an adenosine A\textsubscript{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 [3H]PSB-298 labeled exclusively adenosine A\textsubscript{2B} receptor binding sites, but not A\textsubscript{1}, A\textsubscript{2A} or A\textsubscript{3} receptors in INS-1 cells. Thus, the radioligand appears to be selective for A\textsubscript{2B} receptors in these cells; however, at present, it is not known whether the cells express enough adenosine A\textsubscript{2A} and A\textsubscript{3} subtype receptors to reveal full selectivity. The IC\textsubscript{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\textsubscript{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\textsubscript{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 [3H]-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\textsubscript{1} receptor [40]. When we used CHA as an A\textsubscript{1} receptor agonist in the experiments, to avoid a possible interaction of the radioligand with A\textsubscript{1} binding sites, we obtained the same results. Under our conditions [3H]PSB-298 is a new and highly selective radiolabel for adenosine A\textsubscript{2B} receptor binding sites and is a valuable tool for adenosine receptor research. Since the adenosine A\textsubscript{2B} antagonists tested are able to antagonize NECA-induced inhibition of insulin secretion (data not shown), the data obtained on adenosine A\textsubscript{2B} receptor binding sites in INS-1 cells are likely to be physiologically and pharmacologically relevant. The hitherto described characteristics of these adenosine A\textsubscript{2B} receptor antagonistic compounds are not found in our experiments, since the concentrations to be used are higher than the EC\textsubscript{50} values found in other cells. The concentration of CHA half-maximally inhibiting [3H]CHA binding in INS-1 cells being in the range of 1 \textmu M is much higher than the previously observed K\textsubscript{i} value for CHA at adenosine A\textsubscript{1} receptors.

Conclusions

Recombinant (hA\textsubscript{2B}-HEK-293, hA\textsubscript{2B}-CHO cell membranes) and native (NG108-15 cell membranes, INS-1 whole cells) human and rodent adenosine A\textsubscript{2B} receptors were successfully characterized in radioligand binding studies using [3H]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\textsubscript{2B} receptors.

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