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Solubilisation and immunoprecipitation of rat striatal adenosine A_{2A} receptors

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Abstract

In the present study, we have sought to solubilise adenosine A_{2A} receptors from rat striatal membranes using a variety of different detergents. Of the detergents tested, 1% CHAPS (3-[(3-deoxycholic acid (cholamidopropyl) dimethylammonio]-1-propanesulfonate) yielded optimal conditions for solubilisation (in the presence of 3 mg/ml protein, 44% of receptor was solubilised, 50% of total protein was solubilised). An antipeptide antibody was raised against a 15 amino-acid sequence within the predicted third intracellular loop region of the human and rat adenosine A_{2A} receptor. The antibody was coupled to protein A immobilised on sepharose CL-4B and used to immunoprecipitate adenosine A_{2A} receptors from solubilised rat striatal preparations. Radioligand-binding studies were performed using the selective adenosine A_2 antagonist [^{3}H]ZM 241385 (4-(2-[7-amino-2-(2-fury1)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol). Using [^{3}H]ZM 241385, the pharmacology of immunoprecipitated adenosine A_{2A} receptors was composed to striatal membrane bound adenosine A_{2A} receptors and detergent solubilised adenosine A_{2A} receptors. [^{3}H]ZM 241385 labelled a single saturable binding site with high affinity in all three preparations (membrane bound K_d 2.7 nM $\pm$ 1.0; solubilised K_d 1.9 nM $\pm$ 0.3; immunoprecipitated K_d 2.2 nM $\pm$ 0.7). Additionally, all three assays confirmed a rank order of potency for displacers consistent with adenosine A_{2A} receptor pharmacology: ZM 241385 > KW 6002 ((*E*)-8-[2-(3,4-dimethoxyphenyl)ethynyl]-1-3-diethyl-3,7-dihydro-7-methyl-1-purine 2,6 dione) > CGS 21680, (2-(4-(2 carboxyethyl)phenylethylamino)-5′-*N*-ethylcarboxamidoadenosine) > DPCPX (8-cyclopentyl-1,3-dipropylxanthine). We conclude that we have solubilised and immunoprecipitated adenosine A_{2A} receptors from rat striatum and that their pharmacology is consistent with native striatal adenosine A_{2A} receptors.

Keywords: Adenosine A_{2A} receptor; Solubilisation; CHAPS; [^{3}H]ZM 241385; Immunoprecipitation; Striatum

1. Introduction

Adenosine receptors were initially classified as A_1 and A_2 subtypes due to their opposing actions on adenylyl cyclase and their different agonist-binding profiles (Londos et al., 1980). Based on the ability of adenosine to stimulate adenylyl cyclase in brain slices, Daly et al. (1983) further subdivided the adenosine A_2 receptor into two subtypes: A_{2A}, having a high affinity for its endogenous ligand (adenosine) and A_{2B} displaying a considerably lower affinity (0.1–1.0 and > 10 μM, respectively). The presence of these three subtypes of adenosine receptor and further subtype, the adenosine A_3 receptor, has been confirmed by molecular cloning (Fredholm et al., 1994). Recent interest in the adenosine A_{2A} receptor has been stimulated by its proposed role in cerebral ischemia and neurological disorders. In the central nervous system, it is found in the highest density in the striatum where it has been proposed as a novel therapeutic target for anti-Parkinsonian drugs (Richardson et al., 1997).

Information regarding the structure, function and regulation of the adenosine A_{2A} receptors has been hampered by the lack of a potent selective radiolabel. Previous studies have used [^{3}H]NECA (5′ethylcarboxamido-adenosine), a low-affinity agonist, which has since been found to bind to proteins other than the adenosine A_{2A} receptor (Schutz et al., 1982). More recently, studies using another agonist [^{3}H]CGS 21680 (2-(4-(2 carboxyethyl)phenylethylamino)-5′-*N*-ethylcarboxamidoadenosine) have been performed. Though it has an affinity for the adenosine A_{2A} receptor, recent studies have implied that it binds to sites other than classical adenosine A_{2A} receptors (Johansson et al., 1993; Cunha et al., 1996; Lindstrom et al., 1996). It is

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not known for certain whether it is binding to another adenosine receptor, which has not yet been described, or to an atypical adenosine A_{2A} receptor.

To fully characterise the adenosine A_{2A} receptor protein at a molecular level, it is necessary to isolate the receptor from its membrane environment and purify the protein. As a first step towards purification of the adenosine A_{2A} receptor, we have solubilised the receptor with CHAPS (3-[3-deoxycholic acid (cholamidopropyl) dimethylammonio]-1-propanesulfonate) and have used anti-adenosine A_{2A} receptor antibodies to immunoprecipitate the receptors. A potent and selective antagonist radiolabel has now become available, [^{3}H]ZM 241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol) (Poucher et al., 1995). In this present study, [^{3}H]ZM 241385 has been used to assay adenosine A_{2A} receptors in striatal membranes, solubilised preparations and immunoprecipitated preparations.

2. Materials and methods

2.1. Materials

[^{3}H]ZM 241385 (17 Ci/mmol) was purchased from Tocris (St. Albans, UK). ZM 241385, CGS 21680 and DPCPX (8-cyclopentyl-1,3-dipropylxathine) were all purchased from RBI (Poole, UK). Protease inhibitors were from Boehringer-Mannheim (Lewis, UK). Digitonin, Triton X-100, Tween 20, deoxycholic acid (DOC), CHAPS, glucopyranoside, thioglucanopyranoside, zwittergent 3–14 were purchased from Calbiochem (CA, USA). All other chemicals used were purchased from Sigma.

2.2. Designing the antibody

A 15 amino-acid sequence was selected from the third intracellular loop of the human adenosine A_{2A} receptor: KQMESQPLPGERARS. A BLAST search, using the Swissprot database, was performed to asses the suitability of this sequence in making a selective A_{2A} antibody. The peptide was synthesised and conjugated with keyhole limpet heamocyanin by Genosys Biotechnologies. The antipeptide antiserum was generated in rabbits also by Genosys Biotechnologies. Six immunisations with the antigen were carried out over a period of three months, and antibody titre was checked by western blotting until an adequate signal was seen.

2.3. Preparation of striatal membranes

The method used for the preparation of membrane bound and solubilised adenosine A_{2A} receptors is based on the method by Ji and Jacobson (1993). Freshly dissected rat striata were homogenised in 20 volumes of 50 mM Tris–HCl buffer (pH 7.4) containing 5 mM EDTA in the presence of protease inhibitors (Boehringer, Complete) using an Ultra-Turrax homogeniser. The homogenate was centrifuged at $37{,}000 \times g$ for 20 min. The resultant pellet was resuspended (20 mg tissue/ml) in the above buffer, also containing 3 IU/ml adenosine deaminase (ADA, EC 3.5.4.4), and incubated at 30 °C for 30 min to remove any endogenous adenosine. The homogenate was then centrifuged at $37{,}000 \times g$ for 20 min and the pellet was resuspended (200 mg wet weight/ml) in the above buffer using an Ultra-Turrax homogeniser and stored at −80 °C for up to 1 month.

2.4. Preparation of solubilised receptor

One gram (tissue wet weight) of membranes was thawed and suspended in a total volume of 20 ml of 50 mM Tris–HCl (pH 7.4) containing 5 mM EDTA and centrifuged at $37{,}000 \times g$ for 10 min at 4 °C. The resultant pellet was resuspended in 7 ml of solubilisation buffer containing 10 mM Tris–HCl (pH 7.4) with 1 mM EDTA, 3 IU/ml adenosine deaminase and 1% detergent (digitonin, Triton X-100, Tween 20, deoxycholate, CHAPS, glucopyranoside, thioglucanopyranoside, zwittergent 3–14). CHAPS extraction yielded the most favourable results and was used in subsequent experiments unless noted. The detergent membrane suspension was incubated at 4 °C for 30 min and gently mixed by passing the suspension through a 1 ml 25 gauge 5/8 inch syringe. The suspension was then centrifuged at $100{,}000 \times g$ for 50 min at 4 °C. The supernatant containing the solubilised fraction was carefully removed.

2.5. Immunoprecipitation

Protein A Sepharose beads were suspended in water and centrifuged at $1000 \times g$ to remove any preservatives. Beads (1 mg) swelled to 4 ml in water. Beads were resuspended in 2 × volume with water; 2.5 ml of beads (25 μl packed beads/well) were removed and recentrifuged. Antiserum of 1.25 ml (25 μl antiserum/well) was added unless otherwise stated, and the mixture was allowed to incubate for at least 1 h at room temperature with constant rotation. The protein A-antiserum mixture was centrifuged at $1000 \times g$ and washed three times with 10 volumes of Tris Buffered Saline (TBS) to remove any unbound antisera. Solubilised receptor preparation (3 ml) was diluted to 6 ml with solubilisation buffer and added to the beads. The receptor-bead mixture was incubated overnight at 4 °C with constant rotation. The mixture was centrifuged at $1000 \times g$ and washed three times with 10 volumes of 0.5% CHAPS in TBS to remove any unbound receptors. The beads were then resuspended in 50 mM Tris, 10 mM $MgCl_2$, 0.1 IU/ml adenosine deaminase and 0.05% CHAPS.

Table 1
Solubilisation of rat striatal membranes was performed, as described in Methods, using various detergents, all at a final concentration of 1%: digitonin, Triton X-100, Tween 20, deoxycholate, glucanopyranoside, CHAPS, thioglucanopyranoside, zwittergent 3–14

Detergent	% Total protein solubilised		% Total protein insoluble	
	Mean	S.E.M.	Mean	S.E.M.
Deoxycholate	66.1	12.0	43.9	11.2
Zwittergent 3–14	66.6	6.7	33.4	6.7
Thioglucopyranoside	65.3	4.0	34.7	4.0
Triton X-100	47.7	9.2	78.6	8.4
CHAPS	47.1	3.4	55.0	1.6
Glucanopyranoside	34.7	2.5	65.3	2.5
Digitonin	24.3	4.2	75.7	4.2
Tween 20	20.2	4.5	90.0	2.3

A 50 μl/150 μl sample of solubilised membranes was assayed for protein concentration (Bradford method, Bradford, 1979), a 50 μl sample for the detergent buffer was also taken and assayed to remove interference values. The data shown are a mean of three individual experiments.

2.6. Radioligand-binding studies

Radioligand-binding assays were carried out in a total volume of either 250 or 500 μl, containing [^{3}H]ZM 241385 (17 Ci/mmol), receptor preparation and displacing compounds. ZM 241385 (10 μM) was used to define non specific binding. All assays were incubated for 1 h at 25 °C. The reaction was terminated by rapid filtration through Whatman GF/B filters, which had been presoaked in 0.1% polyethylenimine and washed five times with ice cold 50 mM Tris–HCl pH 7.4. Filters were allowed to stand for 4 h in scintillation fluid before being counted on a Beckman LS6500. All curves were fitted using Graphpad Prism. K_i's were calculated according to the Cheng–Prusoff equation. (Cheng and Prusoff, 1973). Protein levels were determined using Biorad colorimetric reagent with bovine serum albumin as standard (Bradford, 1979).

3. Results

3.1. Optimisation of detergent solubilisation

Initially, a selection of eight detergents, deoxycholate (anionic), CHAPS and zwittergent 3–14 detergent (zwitterionic), glucanopyranoside, thioglucopyranoside, Triton X-100, digitonin and Tween-20 (nonionic) with different physiochemical properties were investigated for their ability to solubilise proteins from rat striatal tissue as described in the methods. After ultracentrifugation, both the pellet containing the insoluble fraction and the supernatant containing the soluble fraction were assayed for protein content. Detergents present in the buffers can cause interference with protein assays; hence, each buffer was also assayed through the protein assay. The absorbance value for each buffer was, therefore, subtracted from the value for each receptor preparation. Table 1 shows the amount of protein solubilised by each detergent and the amount of protein remaining unsolubilised in the pellet after ultracentrifugation. Deoxycholate, the anionic detergent, solubilised 66% of total protein from the rat striatum with the zwitterionic detergents solubilising between 45% and 66% and the non ionic detergents solubilising from 20% to 65% of total striatal protein.

Radioligand-binding using [^{3}H]ZM 241385 was performed on each of the soluble and insoluble preparations to determine the proportion of specific functional adenosine A_{2A} receptor solubilised (fmol/mg protein). Table 2 compares the effects of the various detergents on [^{3}H]ZM 241385 binding in both the soluble and insoluble fractions, ranked in the following order of descending specific binding (solubilised): CHAPS > digitonin > Tween 20 > zwittergent 3–14 > glucanopyranoside > Triton X-100 > thioglucanopyranoside > deoxycholate. Though deoxycholate had resulted in a very high percentage of protein solubilised, it resulted in the lowest level of receptor solubilisation as demonstrated by specific binding (27.6

Table 2
Solubilisation of rat striatal membranes was performed using various detergents, all at a final concentration of 1%: digitonin, Triton X-100, Tween 20, deoxycholate, glucanopyranoside, CHAPS, thioglucanopyranoside, zwittergent 3–14

Detergent	Soluble preparation (fmol/mg protein)		Insoluble preparation (fmol/mg protein)	
	Mean	S.E.M.	Mean	S.E.M.
CHAPS	1776.3	533.2	2118.7	549.7
Digitonin	857.7	111.6	4067.8	225.9
Zwittergent 3–14	358.2	207.8	128.2	60.1
Glucanopyranoside	350.2	142.5	894.9	516.8
Triton X-100	88.1	56.4	339.5	4.5
Thioglucanopyranoside	61.8	25.5	104.3	28.5
Deoxycholate	27.6	8.7	349.3	38.6

Solubilised receptor preparations were incubated for 1 h at 25 °C, as described in Methods, with 5 nM [^{3}H]ZM 241385 and either buffer or 10 μM ZM 241385 representing total and nonspecific binding, respectively. Protein concentration determination was performed using the Bradford assay (Bradford, 1979). The data shown are a mean of three individual experiments.

Table 3
Solubilised receptor preparations were prepared under various conditions, varying protein:detergent ratio and in the presence of Mg^{2+}

Condition % CHAPS, ×mg/ml protein	Soluble specific binding (fmol/mg protein)		Insoluble specific binding (fmol/mg protein)	
	Mean	S.E.M.	Mean	S.E.M.
1% CHAPS, 3 mg/ml	2488.0	371.2	1993.4	96.0
1% CHAPS, 3 mg/ml + Mg^{2+}	1188.1	46.0	3071.2	342.8
0.5% CHAPS, 1.5 mg/ml	1171.7	90.4	2605.8	820.2
0.5% CHAPS, 0.75 mg/ml	1106.7	86.1	1987.6	430.1
0.1% CHAPS, 0.75 mg/ml	980.0	252.8	1054.6	138.7
1% CHAPS, 1.5 mg/ml	904.1	281.1	2029.3	676.7
1% CHAPS, 0.75 mg/ml	864.0	24.3	866.0	33.2

They were then assayed by radioligand binding using [^{3}H]ZM 241385 where they were incubated for 1 h at 25 °C, as described in Methods, with 5 nM [^{3}H]ZM 241385 and either buffer or 10 μM ZM 241385 representing total and nonspecific binding, respectively. Protein concentration determination was performed using the Bradford assay (Bradford, 1979). The data shown are a mean of three individual experiments.

fmol/mg protein). However, CHAPS solubilisation resulted in a high protein yield (47%) whilst retaining a high degree of specific binding (1776.3 fmol/mg protein). Therefore, from these results, the detergent of choice, CHAPS, was selected for assay optimisation.

Once the detergent had been selected, solubilisation of the receptors was then optimised by varying the protein:detergent ratio. Table 3 shows the results of the assay optimisation where the results are ranked in order of descending specific binding (fmol/mg protein). Solubilisation using 1% CHAPS and 3 mg/ml protein, a detergent:protein ratio of approximately 3:1 gave the highest level of specific [^{3}H]ZM 241385 binding (2488 fmol/mg protein). Mg^{2+} has previously been shown to increase the soluble yield of bovine adenosine A_{2A} receptors (Nanoff and Stiles, 1993). We, therefore, tried solubilising in the presence of Mg^{2+}. The presence of Mg^{2+} ions in this experiment did not show any benefit, it approximately halved the amount of specific binding (1188 fmol/mg protein). All the other conditions gave similar, but less favourable results, decreasing the amount of specific binding by twofold to fourfold. Thus, a detergent:protein ratio of 3:1 in the absence of Mg^{2+} ions provided optimal extraction of [^{3}H]ZM 241385 binding (31.9 fmol/25 μl beads). This preparation was used for subsequent immunoprecipitation and pharmacological experiments.

3.2. Immunoprecipitation of striatal membranes

Favourable immunoprecipitation conditions were explored by varying the ratio of Protein A beads to antisera and assaying the amount of receptor on the beads by radioligand binding. Assays were performed using 200 μl (packed) beads and antisera amounts 50, 100, 200, 400 and 500 μl, made up to a total volume of 1 ml in TBS (Tris buffered saline). Unbound antisera was removed from the protein A beads by three successive washes with 10× volume of TBS/CHAPS, 1 ml of the solubilised receptor

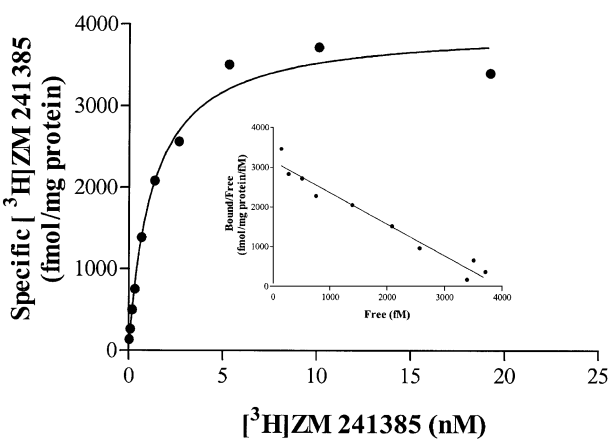

Fig. 1. Saturation-binding isotherm for [^{3}H]ZM 241385 binding to rat striatal membranes. Homogenates were incubated for 1 h at 25 °C, as described in Methods, with varying concentrations of [^{3}H]ZM 241385. Nonspecific binding was determined by the addition of 10 μM ZM 241385. The K_d was 2.7±1.0 nM with a B_{max} of 4.3±0.3 pmol/mg protein. The points shown are from a single representative experiment. Three independent experiments were carried out. The inset is a scatchard plot of the same points.

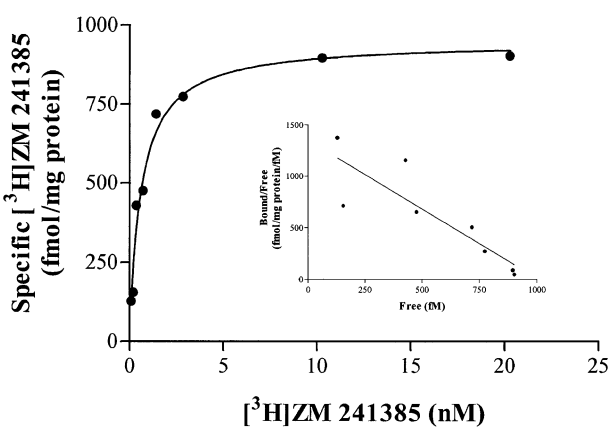

Fig. 2. Saturation isotherm for [^{3}H]ZM 241385 binding to CHAPS solubilised rat striatal membranes. 1% CHAPS-solubilised receptor preparation was incubated for 1 h at 25 °C, as described in Methods, with varying concentrations of [^{3}H]ZM 241385. Nonspecific binding was determined by the addition of 10 μM ZM 241385. The K_d was 1.9±0.3 nM, and the B_{max} was 1.3±0.08 pmol/mg protein. The points shown are from a single representative experiment. Three independent experiments were carried out. The inset is a scatchard plot of the same points.

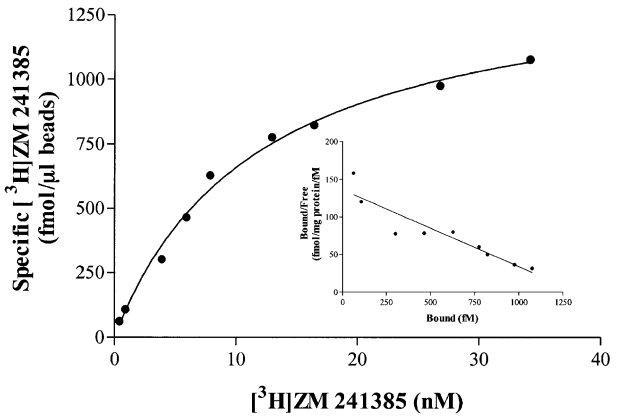

Fig. 3. Saturation isotherm for [^{3}H]ZM 241385 binding to immunoprecipated striatal receptors. Immunoprecipated receptors were incubated for 1 h at 25 °C with varying concentrations of [^{3}H]ZM 241385 as described in Methods. Nonspecific binding was determined by the addition of 10 μM ZM 241385. A K_d of 2.2 ± 0.7 nM and a B_{max} of 807.9 ± 269.3 fmol/10 μl beads were obtained. The points shown are from a single representative experiment. Three independent experiments were carried out. The inset is a scatchard plot of the same points.

preparation was then added to each set of beads. The rest of the assay was carried out as detailed in the methods section. The most favourable immunoprecipitation conditions were determined using the radioligand-binding signal and found to be where the sera:bead ratio is 1:1, i.e. 200 μl antisera 200 μl beads. This preparation was used for further pharmacological experiments.

3.3. Radioligand-binding studies

Scatchard analyses and competition curves were performed on all three receptor preparations. The specific binding of [^{3}H]ZM 241385 to rat striatal membranes is saturable, as shown by the saturation isotherm (Fig. 1). Scatchard analysis of the data indicates a single population of high-affinity binding sites with a dissociation constant (K_d) of 2.7 ± 1.0 nM and a maximum number of binding sites (B_{max}) of 4.3 ± 0.3 pmol/mg protein. Nonspecific binding represents 10.3% of the total binding at 1 nM [^{3}H]ZM 241385. There was a linear relationship between nonspecific binding and the concentration of [^{3}H]ZM 241385.

A saturation isotherm of solubilised receptors also reveals a single saturable high-affinity binding site (Fig. 2). Scatchard analysis of the data indicates a single population of high-affinity binding sites with dissociation constant (K_d) of 1.9 ± 0.3 nM, and a maximum number of binding sites (B_{max}) of 1.3 ± 0.08 pmol/mg protein. Nonspecific binding represents approximately 30.2% of the total binding at 1 nM [^{3}H]ZM 241385. There was a linear relationship between nonspecific binding and the concentration of [^{3}H]ZM 241385. This K_d value was found not to be statistically different from that in striatal membranes ($p < 0.05$, analysis of variance), suggesting that the binding site for [^{3}H]ZM 241385 is unaltered by solubilisation. The B_{max} values were significantly different with approximately only a quarter of the number of sites being labelled in the soluble preparation compared with the membrane bound preparation.

The specific binding of [^{3}H]ZM 241385 to immunoprecipitated striatal adenosine A_{2A} receptors is saturable, as shown by the representative saturation isotherm (Fig. 3). Scathard analysis of the data indicates a single population of high-affinity binding sites, in the concentration range studied with K_d of 2.2 ± 0.7 nM and a B_{max} of 807.9 ± 269.3 fmol/10 μl beads. For the immunoprecipitated receptors, it is not possible to measure the protein concentration as the receptors are in the presence of large amounts of protein A and of IgG, both of which would interfere with the protein assay. It is, therefore, not possible to express the amount of receptor binding as fmol per mg of protein and so cannot be compared directly to the membrane and soluble binding. The amount of receptor binding is normalised as per 10 ul of protein A beads so that we can compare consistency between immunoprecipitation experiments. Nonspecific binding represents 50% of the total binding at 1 nM [^{3}H]ZM 241385. There is a linear relationship between nonspecific binding and the concentration of [^{3}H]ZM 241385. There is a high level of nonspecific binding, which could be due to the properties of the polyclonal antibody or that of the beads. In the absence of

Table 4
Competition by log concentrations of four adenosine ligands for the specific binding of 2–5 nM [^{3}H]ZM 241385 to rat striatal, solubilised and immunoprecipitated A_{2A} receptors

Compound	Membrane bound K_i (nm)		Solubilised receptor K_i (nm)		Immunoprecipitated K_i (nm)	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
ZM 241385	1.3	0.6	0.89	0.3	3.08	1.2
KW 6002	11	2.5	5.4	2.3	13.9	6.2
CGS 21680	206	31	79	18	315	80
DPCPX	599	48	557	92	2805	1516

Assays were carried out as detailed in the Methods section in a total volume of 500 μl. IC_{50} concentrations were calculated using linear sigmoidal curve analysis using the graph pad prism program. K_i's (nM) were calculated using the Cheng–Prusoff equation above (Cheng and Prusoff, 1973). Data are mean and S.E.M. of three independent experiments.

polyclonal antisera, 1 nM [^{3}H]ZM 241385 binding to the beads represented 2–9% of the total binding.

The K_d values for all three of the preparations were compared using an analysis of variance test, which revealed that they were not statistically different from each other ($p < 0.05$). This suggests that the [^{3}H]ZM 241385 binding site remains unaltered by antibody binding.

In order to confirm the adenosine A_{2A} pharmacology of these three receptor preparations, we compared the relative potency of four adenosine ligands as competitors of [^{3}H]ZM 241385 binding in all three preparations using: ZM 241385, a potent and selective adenosine A_2 antagonist; CGS 21680, a selective adenosine A_{2A} agonist; KW 6002, a selective adenosine A_{2A} antagonist (Shimada et al., 1997); and DPCPX, a selective adenosine A_1 antagonist (Bruns et al., 1987). The K_i's (affinity constants), determined by the Cheng–Prusoff equation, show a good correlation between the three receptor preparations, and the rank order potency was conserved: ZM 241385 > KW 6002 > CGS 21680 > DPCPX. Table 4 shows a summary of the K_i and S.E.M. obtained for the compounds.

4. Discussion

This study investigated the adenosine A_{2A} receptor in rat striatal tissue using receptor solubilisation and immunoprecipitation. Eight detergents were investigated for their ability to solubilise proteins from rat striatal tissue. Although the anionic detergent deoxycholate solubilised the greatest amount of protein from the tissue preparations, it resulted in the lowest level of receptor solubilisation (specific binding of [^{3}H]ZM 241385). Solubilisation with some of the detergents, i.e. Triton X-100, deoxycholate, thioglucopyranoside and zwittergent 3–14, resulted in a loss of binding in both the soluble and insoluble fractions of the membrane preparation. This could be due to the harshness of the detergents, which may destroy the receptor, or it is possible that the detergents could interfere with the [^{3}H]ZM 241385 binding assay. Solubilisation with 1% CHAPS, a zwitterionic detergent, resulted in the highest degree of specific binding (1776.3 fmol/mg protein) and a 47% solubilisation of total protein and was, therefore, selected for further assay optimisation. Assay optimisation was investigated by varying the protein:detergent ratio. Solubilisation was also determined in the presence of Mg^{2+} ions. The presence of Mg^{2+} in this experiment did not show any benefit in contrast to the previous suggestion by Nanoff and Stiles (1993), who found that an absence of Mg^{2+} decreased the yield of solubilised bovine adenosine A_{2A} striatal receptors by 10%. Conversely, in our hands, Mg^{2+} approximately halved the amount of specific binding. Specific radioligand binding revealed that a protein:detergent ratio of 3:1 and the absence of Mg^{2+} ions gave the best results.

Stability of the solubilised receptors was examined (data not shown) and found to decrease exponentially over 72 h, retaining 25% binding after 48 h. We then went on to investigate optimal conditions for immunoprecipitating adenosine A_{2A} receptors. Very little work has been done in this field due to the lack of an antibody with affinity for the rat adenosine A_{2A} receptors. Commercial antibodies (Calbiochem) have been designed from the published sequence for the canine adenosine A_{2A} receptor and have displayed cross-reactivity with the human adenosine A_{2A} receptor, but no cross-reactivity with the rat adenosine A_{2A} receptor. This custom-made antipeptide adenosine A_{2A} antibody, whose sequence was chosen from the third intracellular loop of the human adenosine A_{2A} receptor, has good homology with the rat adenosine A_{2A} receptor (93%), as revealed by a BLAST search using the SwissProt database. It displays a high degree of homology with mouse (93%), human (100%), dog (100%), rat (93%) and guinea pig (93%) sequences, potentially making it an excellent species tool for comparisons of the A_{2A} receptor. The most favourable conditions for immunoprecipitation of striatal adenosine A_{2A} receptors were investigated by radioligand binding using [^{3}H]ZM 241385. The highest yield of receptors was achieved using a sera:bead ratio of 1:1. Nonspecific binding, however, was quite high, 50% of total binding. Ratios of greater than 1:1 resulted in higher amounts of nonspecific binding and, therefore, lower specific binding. Further assay optimisation, in an attempt to reduce nonspecific binding by increasing assay volume, decreasing thickness of filter paper and absence of polyethylenimine, was not very successful (data not show), suggesting that the polyclonal antisera's properties are responsible for the high level of nonspecific binding.

Saturation studies performed on membrane bound, solubilised and immunoprecipitated striatal adenosine A_{2A} receptors revealed the same single population of high-affinity binding sites, indicated by the K_d values of 2.7 ± 1.0 nM, 1.9 ± 0.3 and 2.2 ± 0.7 nM, respectively. An analysis of variance test revealed no significant difference between the three receptor populations. The total number of receptors in each preparation varied; in the membrane-bound receptor preparation, the B_{max} was calculated to be 4.3 ± 0.3 pmol/mg protein, whereas in the soluble preparation, the B_{max} was 1.3 ± 0.08 pmol/mg protein. This decrease in binding sites in the solubilised preparation is probably due to the detergent destroying some of the binding sites or interference of the detergent with the binding assay. The pharmacology of the three preparations shown by displacement of four adenosine ligands—ZM 241385, CGS 21680, DPCPX and KW 6002—were similar and consistent with data reported in literature. The K_i values for ZM 241385 in all three preparations are similar to the K_d values of [^{3}H]ZM 241385 obtained in the saturation experiments. Additionally, the affinity for ZM 241385 for the adenosine A_{2A} receptor in all three preparations correlates with previous findings (Cunha et al., 1997) in rat striatal mem-

branes using [^{3}H]CGS 21680, where ZM 241385 was found to have a K_i of 0.4 nM. The K_i value for DPCPX in all three preparations (599, 557 and 2805 nM, respectively) agrees with the low-affinity value (570 nM) observed previously by Cunha et al. (1996). The K_i values for CGS-21680 displacing [^{3}H]ZM 241385 binding in all preparations are higher (i.e. less potent), than previously found by Cunha (1996), but agree with the antagonist studies using [^{3}H]SCH 58261 (111 nM) by Zocchi et al. (1996). This shift in potency is expected as we used an antagonist label, as opposed to [^{3}H]CGS 21680, an antagonist label; hence, all agonists will show a decreased potency since the agonist radiolabel labels both coupled and uncoupled receptor populations. Finally, the affinity of KW 6002 in all three of the receptor preparations agree with each other (11.0 nM membrane bound, 5.4 nM soluble and 13.9 nM immunoprecipitated), but show a slightly lower value than has been reported using [^{3}H]CGS 21680 (2.2 nM) (Shimada et al., 1997).

In summary, we have solubilised and immunoprecipitated adenosine A_{2A} receptors from rat striatum which retain ligand binding for [^{3}H]ZM 241385. We have confirmed the pharmacology of these two preparations as being the same as native rat striatal receptors. This is an important step towards the molecular characterisation and purification of native adenosine A_{2A} receptors.

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