Solubilization of stable adenosine A1 receptors from rat brain

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Despite numerous reports of solubilization of adenosine A1 receptors, little progress has been made in isolating or purifying the receptor, owing to the extreme lability of the preparations. The present solubilization strategies recognized the possible role of endogenous adenosine to produce adenosine-receptor-N-protein complexes, which are intrinsically unstable, and instead attempted to use caffeine to solubilize free adenosine receptors, which might be more stable. Endogenous adenosine was removed from membranes by using adenosine deaminase along with GTP to accelerate the release of receptor-bound adenosine. The receptors were then occupied with caffeine and solubilized with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (CHAPS) in the presence of glycerol. These soluble preparations exhibited the characteristics of free adenosine receptors. They bound the A1-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (CPDPX) with high affinity to a single class of binding sites, which were insensitive to GTP. The binding activity was extremely stable, with a half-life of about 5 days at 4 °C; there was little change in either receptor number or affinity during 3 days at 4 °C. This methodology should greatly facilitate the characterization, isolation and purification of the adenosine A1 receptor.

INTRODUCTION

Adenosine receptors are widely distributed in the nervous system and in peripheral tissues. Two adenosine-receptor subclasses, which display distinctive pharmacological profiles, are currently recognized: A1 and A2, which mediate the inhibition and stimulation, respectively, of adenylate cyclase [1]. Progress on the purification of adenosine A1 receptors has been hampered by the fact that the receptors tend to be occupied during detergent solubilization owing to the presence in many tissues of endogenous adenosine, which has a relatively high affinity for A1 receptors. As has been well described for other receptors (and also for adenosine A1 receptors) that are coupled to either the stimulation or the inhibition of adenylate cyclase, binding of agonists promotes the stabilization of R-N complexes [2,3]. For adenosine receptors, this situation, coupled with the high concentrations of endogenous adenosine, results in the exclusive extraction by detergents of adenosine-receptor-N-protein complexes. In every study that has been reported describing solubilized adenosine-receptor preparations, agonists bind with high affinity [2,4–8] and guanine nucleotides regulate the agonist binding [2,4,5,7,8]. Extensive pretreatment of the membranes with adenosine deaminase before solubilization results in a substantial loss of the receptors that are subsequently detected, as monitored by agonist binding [2]. The reason that this situation poses a problem in the purification of adenosine receptors is that R-N complexes are intrinsically highly unstable, having rarely been demonstrated for any receptor system to withstand fractionation procedures. Against this background, the present studies attempted to extract an antagonist-stabilized form of the receptor, which might thereby be more stable and more amenable to subsequent purification procedures. The studies were

greatly facilitated by the development of an A1-selective high-affinity antagonist, CPDPX [9,10]. In addition, extensive measures were taken to occupy the receptors with the antagonist caffeine, rather than the endogenous adenosine, before and during solubilization. Additional measures were taken to optimize the stability of the preparation. The result of these steps is that an antagonist-stabilized preparation is described that is stable, with a half-life of about 5 days at 4 °C.

EXPERIMENTAL

Preparation of brain membranes

Male Sprague-Dawley rats were anaesthetized with CO₂ and killed by decapitation. The brains were quickly dissected, immediately frozen by immersion in liquid N₂, and stored at –80 °C. For the preparation of membranes the frozen brain tissue was crushed and then homogenized in a glass homogenizer with 20 ml of 50 mM-Tris/HCl (pH 7.4)/1 mM-dithiothreitol (buffer 1) containing 0.3 M-sucrose and proteinase inhibitors/g of frozen tissue. All buffers used for membrane preparation and solubilization contained the following proteinase inhibitors; 0.1 mM-diethylenetriaminepenta-acetic acid, 0.1 mM-phenylmethanesulphonyl fluoride, 0.1 mM-benzamide, 1 µg of aprotinin/ml, 1 µg of leupeptin/ml and 1 µg of pepstatin A/ml. The homogenate was centrifuged at 600 g for 10 min and the pellet discarded. The supernatant was centrifuged at 12000 g for 10 min, and the pellet was resuspended in buffer 1 containing 0.5 M-NaCl. After 10 min on ice, the membranes were pelleted at 12000 g for 10 min and washed with buffer 1. The final pellet was resuspended to ~10 mg of protein/ml in buffer 1, frozen and stored in liquid N₂. Membranes prepared from whole brain contained very similar

Abbreviations used: CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate; CMC, critical micelle concentration; PIA, N⁶-(R)-1-methyl-2-phenylethyladenosine; CPDPX, 8-cyclopentyl-1,3-dipropylxanthine; CHA, N⁶-cyclohexyladenosine; R, receptor; N, GTP-regulatory protein.

amounts of adenosine A1 receptors compared with membranes prepared from only cerebral cortices. The membranes were analysed for protein by the method of Lowry *et al.* [11], modified to eliminate interference by lipid [12]. The phospholipid content of the membranes was determined by phosphate assay by the method of McClare [13]. The membranes were found to contain 0.78 μmol of phospholipid/mg of protein or, assuming an average M_r of 750 for phospholipid, to consist of 63% protein and 37% phospholipid (w/w).

Adenosine-receptor stabilization and solubilization

Unless stated otherwise, the adenosine receptors were stabilized before their solubilization by occupying their ligand-binding sites either with the natural agonist, adenosine, or with the antagonist, caffeine. For agonist stabilization, the membranes were treated with 10 μM -adenosine for 1 h at 24 °C. For antagonist stabilization, the membranes were treated for 1 h at 24 °C with 12 units of adenosine deaminase/ml, 100 μM -GTP and 10 mM-caffeine (1 unit of adenosine deaminase will deaminate 1.0 μmol of adenosine to inosine/min at pH 7.5 at 25 °C). The adenosine deaminase was added to eliminate most of the free endogenous adenosine, and the GTP was added to increase the dissociation of adenosine bound to the receptors [2] so that they could be occupied more efficiently by caffeine. Experiments performed with [^3H]adenosine, to validate the metabolism of adenosine to inosine (separated by t.l.c. [14]), indicated that this procedure decreased the adenosine content of the membranes to less than 10 fmol/mg of protein (results not shown). A concentration of 10 mM-caffeine gave a maximal yield of receptors. After treatment of the membranes, the agonist-stabilized and antagonist-stabilized receptors were both further stabilized by inclusion of 20% (v/v) glycerol. The membranes were chilled on ice for 5 min and then solubilized by addition of CHAPS. After incubation for 30 min on ice, the solubilization mixture was centrifuged at 100 000 g for 1 h at 4 °C. The supernatant containing the solubilized receptors was frozen and stored in liquid N_2 . Sodium cholate and CHAPS solubilized similar amounts of membrane protein; however, CHAPS gave a greater yield of receptors. The final concentration of CHAPS used for solubilization was 20–25 mM. A final concentration of membrane protein (6.5 mg/ml) was chosen so that there would be a considerable amount (~ 5 mM) of endogenous phospholipid present in the solubilization mixture to help stabilize the solubilized proteins. The range of CHAPS concentrations used was then chosen to give (after correcting for the CMC) an effective detergent/lipid molar ratio of 2–2.5, which has been shown to be effective for solubilizing functional membrane proteins [15,16].

Removal of stabilizing ligands

Before the binding properties of the solubilized receptors could be assayed, it was necessary to remove the stabilizing ligands adenosine and caffeine from the binding sites. These ligands were chosen because of their relatively low affinity and fast dissociation, which would make them easier to remove than agonists and antagonists that bind more tightly. This removal was readily achieved by centrifuging the solubilized preparations through small Sephadex G-50 columns [17]. Sephadex G-50 (fine grade) was equilibrated in 50 mM-Tris/HCl (pH 7.4)/2 mM-CHAPS and poured into 1 ml syringes or

pipette tips fitted with porous polyethylene frits to give bed volumes of ~ 1.5 ml. The columns were pre-spun (65 g , 2 min, 4 °C) in a swinging-bucket table-top centrifuge to remove excess buffer. Portions (150 μl) of solubilized preparations were applied to each column and centrifuged (65 g , 7 min, 4 °C). The eluate (150–200 μl) was immediately used for binding assays, after samples for protein determination were taken. The Sephadex G-50 columns served to remove GTP, as well as the proteinase inhibitors, dithiothreitol and glycerol, which might have interfered with the binding assays. The columns were equilibrated with 2 mM-CHAPS because this gave a better yield of receptors relative to columns with 20 mM-CHAPS or with no detergent.

Since the solubilized preparations contained 20–25 mM-CHAPS and this chromatography lowered the detergent concentration to 2 mM-CHAPS, which is below the CMC, this step could be considered a reconstitution procedure. We attempted to demonstrate acceptor binding under our solubilization conditions by using 20% (v/v) glycerol, 20 mM-CHAPS and 5 mM exogenous lipid, since the low concentration of protein in a binding assay (0.1–0.5 mg/ml) did not provide enough endogenous lipid to give an appropriate detergent/lipid ratio. Under these conditions, which mimicked the optimal solubilization conditions, specific adenosine-receptor binding activity was not detectable, even when the exogenous lipid was varied from pure phosphatidylcholine to a mixture of phospholipids similar to that found in native membranes. It appears that solubilized adenosine receptors must be reconstituted in order to bind ligands specifically. A review of the adenosine receptor solubilization literature [2,4–8] reveals that, in all such reports, binding assays were performed after dilution of the detergent below its CMC, so that, in effect, a detergent-dilution reconstitution procedure [18] was carried out. {One report [5] indicated that specific [^3H]CHA binding could be detected in 8.1 mM-CHAPS (i.e. above the assumed CMC of 5 mM) at about 20% of the value seen at 0.41 mM-CHAPS. This apparent discrepancy may be explained by the fact that CMC values are (i) difficult to measure (published values for the CMC of CHAPS vary from 8 to 12 mM [19,20]) and are (ii) influenced by factors such as the concentration of lipid and salts. Therefore the reported binding activity might have reflected a small population of receptors in a partially reconstituted milieu.}

Further evidence that reconstitution occurred during Sephadex G-50 chromatography was obtained from experiments in which solubilized preparations were supplemented with exogenous lipid before being applied to the columns. Addition of 10 mM-phosphatidylcholine or a mixture of phospholipids increased the yield of receptors after chromatography by 50–150% relative to unsupplemented controls. However, the preparations were not supplemented routinely, but relied on the endogenous lipid in the experiments presented.

Receptor-binding assays

After Sephadex G-50 chromatography, the eluted protein (15–40 μg /assay) was incubated for 2 h at 23 °C in a final volume of 0.1 ml with 1 mg of bovine serum albumin/ml, and 1 unit of adenosine deaminase/ml in 50 mM-Tris/HCl, pH 7.4, and the following ligands and additions. Agonist binding was carried out with 1 nM-[^3H]PIA (38 Ci/mmol); non-specific binding was deter-

mined in the presence of 10 μM -PIA (unlabelled). Antagonist binding was carried out with 1 nM-[^3H]CPDPX (119 Ci/mmol); non-specific binding was determined in the presence of 10 mM-caffeine. Binding was performed in either the absence or the presence of Mg^{2+} (2 mM) or GTP (10 μM). After incubation, the binding was terminated by rapid filtration through 0.3%-polyethyleneimine-treated Whatman GF/C glass-fibre filters [5]. The filters were quickly washed with 3 $\times$ 4 ml of ice-cold 50 mM-Tris/HCl, pH 7.4, and then dried. After gentle shaking overnight in scintillant, the radioactivity was measured in a liquid-scintillation counter. For antagonist saturation-binding experiments, a range of total CPDPX concentration of 0.2–6.0 nM was used in the presence of 0.2 nM-[^3H]CPDPX. These data were transformed into Scatchard plots by the program SCAFIT [21].

A concentration of 10 mM-caffeine was required to displace all specific CPDPX binding. The same amount of non-specific binding, which was $\sim 4\%$ of total binding, could be obtained with 100 μM -PIA. For assays of receptor stability over time, a single preparation was thawed, incubated at the indicated temperature, and at the indicated times samples were taken, passed through the Sephadex G-50 columns and immediately assayed for PIA or CPDPX binding.

Binding determinations were performed in duplicate. Variability between samples was always $< 5\%$. The data presented illustrate the results of typical experiments that had been performed at least three times.

RESULTS

Membranes, treated with 12 units of adenosine deaminase/ml, 100 μM -GTP and 10 mM-caffeine, were solubilized with 20 mM-CHAPS in the presence of 20% glycerol. These conditions had been found to maximize the yield of CPDPX-binding activity (see the Experimental section). The stability of the solubilized receptors was evaluated by incubation at 4° or 20 °C for up to 6 days and then assaying for PIA and CPDPX binding immediately after passage through Sephadex G-50 columns. The initial PIA-binding activity was low and decayed rapidly at either 4° or 20 °C (Fig. 1*a*). Binding of the antagonist CPDPX was much greater initially and more stable than that of the agonist PIA. Such stability might be expected of a preparation that was extracted with the antagonist caffeine. At 4 °C, this preparation displayed a half-life of approx. 5 days, although this binding was less stable at 20 °C, as is characteristic of solubilized preparations.

In order to assess the factors responsible for this stability, membranes were solubilized under a variety of conditions. The membranes were treated with adenosine or with adenosine deaminase, GTP and caffeine as described in the Experimental section. Membranes were also treated with adenosine deaminase and GTP without caffeine in order to vacate the receptors. The treated membranes were then supplemented with 20% glycerol (see the Experimental section) or with buffer alone. The membranes were solubilized with 25 mM-CHAPS and stored frozen in liquid N_2 . The solubilized preparations were thawed, and a portion was immediately passed through a Sephadex G-50 column and assayed for PIA and CPDPX binding. The remainder of the preparations were incubated at 4 °C for 2 days, passed through Sephadex columns and assayed for binding (Table 1).

Fig. 1. Time- and temperature-dependence of solubilized receptor inactivation

Rat brain membranes were treated with adenosine deaminase, GTP and caffeine as described in the Experimental section and were solubilized with 20 mM-CHAPS in the presence of 20% glycerol. The solubilized material was incubated at 4° or 20 °C for the indicated times and then centrifuged through Sephadex G-50 columns. The eluate was immediately assayed for binding of (a) PIA and (b) CPDPX under control conditions as described in the Experimental section. Note that MgCl_2 was not included in these incubations.

CPDPX binding was measured under control conditions, but, to maximize [^3H]PIA binding, the agonist binding was measured in the presence of Mg^{2+} to shift the receptors into the high-affinity form for agonists [2,8]. [Note that the PIA binding measured with Mg^{2+} is much greater than the PIA binding observed under control conditions, as in Fig. 1(a).] Although the precise locus for the action of Mg^{2+} has not been determined, it seems likely that the cation acts on the GTP-regulatory protein to shift receptors from a low- to a high-affinity form for agonists, perhaps as a consequence of promoting the hydrolysis of GTP that binds to the R-N complex, which would otherwise maintain the complex in a low-affinity state for agonists (cf. [22]).

Table 1 shows that both ligands and glycerol are important in solubilizing and stabilizing adenosine receptors. The highest yield of binding activity for the antagonist CPDPX was obtained with both glycerol and the

Table 1. Stabilization of solubilized receptors by ligands and glycerol

Brain membranes were treated with adenosine to occupy the receptors, or were treated with adenosine deaminase (ADA) and GTP to remove endogenous adenosine, or were treated with adenosine deaminase, GTP and caffeine to remove adenosine and occupy the receptors with caffeine. The treated membranes were solubilized with 25 mM-CHAPS in the presence or absence of 20 % glycerol. The solubilized material was stored in liquid N₂. Preparations were thawed and either immediately or, after 2 days at 4 °C, centrifuged through Sephadex G-50 columns. The eluates were assayed for CPDPX (1 nM) binding under control conditions and for PIA (1 nM) binding in the presence of Mg²⁺.

Membrane treatment	Stabilizing ligand	Binding	[Glycerol]. . .	Binding activity (fmol/mg)			
				Initial		After 2 days*	
				0	20 %	0	20 %
ADA, GTP, caffeine	Caffeine	CPDPX		416 ± 6	532 ± 14	278 ± 5 (67)	545 (102)
		PIA		361 ± 9	392 ± 25	160 ± 4 (44)	340 (87)
Adenosine	Adenosine	CPDPX		445 ± 3	485 ± 20	157 ± 5 (35)	492 (101)
		PIA		435 ± 9	500 ± 21	104 ± 8 (24)	467 (93)
ADA, GTP	None	CPDPX		108 ± 3	431 ± 23	33 ± 5 (31)	243 (56)
		PIA		74 ± 10	326 ± 15	24 ± 3 (32)	141 (43)

* The percentage of the initial binding activity that remains after 2 days is indicated in parentheses.

Table 2. Binding parameters of CPDPX to caffeine-stabilized and adenosine-stabilized receptors

The binding affinity (K_d) and receptor number (B_{max}) were determined from Scatchard plots by SCAFIT analysis. Rat brain membranes were treated with adenosine or with adenosine deaminase, GTP and caffeine as described in the Experimental section and were then solubilized with 25 mM-CHAPS in the presence of 20 % glycerol. Solubilized material was stored frozen in liquid N₂. Preparations were thawed and either immediately, or after 3 days at 4 °C, centrifuged through Sephadex G-50 columns, and the eluates were used in saturation binding experiments as described in the Experimental section. The data were transformed into Scatchard plots, which showed a single binding site. When assessed by two-tailed Student's *t* test, the following B_{max} values were significantly different from their appropriate controls; *significantly different ($P < 0.1$) from binding in the absence of Mg²⁺, ^bsignificantly different (^b $P < 0.25$ and ^c $P < 0.05$) from binding measured under the same assay conditions on day 0.

Receptors	Day	Treatment	K_d (nM)	B_{max} (pmol/mg)
Caffeine-stabilized	0	Control	0.549	0.911 ± 0.032
		GTP	0.545	0.938 ± 0.043
		Mg ²⁺	0.726	0.905 ± 0.046
	3	Control	0.616	0.935 ± 0.019
		GTP	0.593	0.593 ± 0.046
		Mg ²⁺	0.893	1.018 ± 0.062
Adenosine-stabilized	0	Control	0.907	1.168 ± 0.063
		GTP	0.884	1.241 ± 0.093
		Mg ²⁺	1.053	0.974 ± 0.045 ^a
	3	Control	0.758	0.729 ± 0.037 ^b
		GTP	0.592	0.737 ± 0.037 ^b
		Mg ²⁺	0.868	0.715 ± 0.042 ^c

antagonist caffeine. The highest yield of binding activity for the agonist PIA was solubilized with both glycerol and the agonist adenosine. Approx. 80 % of this yield could be achieved initially with any one stabilizing agent. However, in the absence of any stabilizing agents, the yield was only about 20 % of the maximum obtainable. Therefore, to maximize the yield of adenosine receptors, solubilization is optimally performed in the presence of both glycerol and stabilizing ligands: agonist or antagonist, as appropriate.

When the binding activity of these soluble preparations was assayed after 2 days at 4 °C, similar trends were

evident. Antagonist binding was most stable in the presence of glycerol and caffeine; agonist binding was most stable in the presence of glycerol and adenosine. With both glycerol and receptor ligands little loss of agonist- or antagonist-binding activity occurred after 2 days in solution. Any one stabilizing agent afforded some protection; caffeine alone was more effective than adenosine alone. In the absence of stabilizing agents, few receptors (approx. 5 %) survived after 2 days.

Saturation binding experiments were carried out with CPDPX to determine receptor number, affinity and stability on preparations that were solubilized in the

presence of glycerol and either caffeine or adenosine (Table 2). Measurements were repeated on the same preparations after incubation for 3 days at 4 °C (Table 2). The binding was carried out under control conditions, or in the presence of Mg^{2+} or GTP as described in the Experimental section. In all cases the binding of CPDPX could be attributed to a single class of binding sites (Table 2). When membranes were solubilized in the presence of glycerol and caffeine, the solubilized receptors bound CPDPX with a K_d of 0.55 nM, and the yield of receptors was 0.91 ± 0.03 pmol/mg (Table 2). The addition of GTP did not alter receptor affinity or number. The addition of Mg^{2+} decreased receptor affinity slightly. When receptors were solubilized in the presence of glycerol and adenosine, the yield was slightly higher (1.17 ± 0.06 pmol/mg), although the affinity was lower (K_d 0.91 nM). The receptor number and affinity were not affected significantly by GTP. Addition of Mg^{2+} decreased both the receptor number ($P < 0.1$) and affinity (Table 2).

After 3 days at 4 °C, the preparations were re-assayed to determine their stability. The caffeine-stabilized preparation displayed virtually the same parameters as measured initially, with only a very slight decrease in the affinity (Table 2). However, the adenosine-stabilized preparation had lost about 40% of the number of receptors, with a slight increase in their affinity. In addition, the number of receptors was no longer decreased by addition of Mg^{2+} (Table 2).

For caffeine-stabilized receptors, it appears that all of the binding was due to free adenosine receptors, since neither Mg^{2+} nor GTP affected receptor number. In contrast, PIA binding to R–N complexes is greatly increased by Mg^{2+} and almost eliminated by GTP [2]. With adenosine-stabilized receptors some R–N complexes must persist, since the overall affinity for CPDPX was somewhat lower, and Mg^{2+} decreased receptor number. The free receptors that are stabilized with caffeine are extremely stable in solution. However, the receptors stabilized with adenosine decay with time, probably owing to disruption of R–N complexes. In support of this possibility, after 3 days the binding begins to resemble that for free receptors, displaying a higher affinity for the antagonist CPDPX, and no effect of Mg^{2+} or GTP on receptor number. Therefore, even though adenosine allows slightly more receptor-binding activity to be solubilized initially, the receptors solubilized in the presence of caffeine are more stable and display more consistent binding properties with time.

DISCUSSION

The fractionation and purification of adenosine A1 receptors has been hampered by the lability of solubilized receptor preparations. The present study set out to establish reproducible conditions for extracting soluble receptors that would be stable. Consequently, the following strategies were adopted. Although our previous studies utilized sodium cholate [2], the less-denaturing detergent CHAPS provided higher yields of receptor. Analysis of the phospholipid content of brain membrane preparations was used to establish an effective detergent-to-lipid ratio for solubilization. The well-known stabilizing ability of glycols was exploited by including 20% glycerol. Attention was also paid to the fact that endogenous adenosine significantly enhances the ability to

solubilize adenosine receptors [2,7,8]. Since R–N complexes typically bind agonists with high affinity, and free receptors bind antagonist with high affinity, and since free receptors are usually more stable than R–N complexes [23], the antagonist ligand caffeine was chosen to stabilize free adenosine receptors. Caffeine was selected because its low affinity makes it easily removed (this property makes the choice of caffeine particularly useful in any projected affinity-chromatographic procedures). Endogenous adenosine was removed by using adenosine deaminase and GTP, so that all the receptors could be occupied with caffeine. The availability of the highly A1-receptor-specific high-affinity antagonist ligand CPDPX greatly facilitated the ability to measure free adenosine receptors.

When membranes were solubilized with CHAPS, at an appropriate detergent-to-lipid ratio, in the presence of glycerol and caffeine, soluble adenosine receptors were obtained that were extremely stable, with a half-life of about 5 days at 4 °C. Both glycerol and caffeine contributed to the observed stability, although either agent alone could confer considerable stability on the preparation. Thus a purification strategy need not necessarily include a specific receptor-binding ligand; the selection of an appropriate glycerol concentration and detergent:protein ratio may confer similar stabilization properties on the preparation. Solubilization with adenosine alone most closely resembles the solubilization conditions used by other investigators [2,4–8]. The high concentration of endogenous adenosine in effect stabilized the receptors as R–N complexes, which remained responsive to GTP. In the present study, exogenous adenosine provided a high yield of receptors from the initial solubilization. However, after 2 days, the agonist binding declined to 24% of its original activity. Such instability has impeded the characterization, fractionation and purification of adenosine receptors, despite numerous reports of A1-receptor solubilization. Such studies should be greatly facilitated by the present stabilization strategies employing the antagonist caffeine. Such a strategy (i.e. antagonist stabilization) was a key to the successful fractionation of muscarinic cholinergic receptors in high yield [23].

When CPDPX and PIA binding were compared in these preparations, antagonist-binding activity was always similar to or greater than the agonist-binding activity. In addition, antagonist-binding activity was always more stable than the agonist-binding activity. Both of these observations can be explained by assuming that (i) only high-affinity binding is measured in the assays, (ii) CPDPX binds with high affinity to all adenosine receptors and (iii) PIA binds with high affinity only to adenosine-receptor–N-protein complexes. Thus CPDPX binding estimates the total number of adenosine receptors, whereas PIA binding detects only a subset of the receptors that are coupled to N-proteins as R–N complexes. (It should be noted that both CPDPX and PIA are highly selective for A1 adenosine receptors [8,9]. Therefore the difference cannot be attributed to CPDPX binding to A1 and A2 receptors, whereas PIA binds only to A1 receptors.) The difference in stability may also be explained as follows: the loss of antagonist binding is due only to denaturation of the receptor; the loss of agonist binding is due not only to receptor denaturation, but also to the dissociation of R–N complexes. PIA binding measures the integrity of R–N complexes and thus

underestimates the total number of functional receptors. Since CPDPX binding more accurately reflects the state of the receptors, saturation-binding experiments were carried out with this compound to characterize the solubilized A1 receptors. These experiments confirmed that the caffeine-stabilized preparation was unaffected by GTP and most likely consisted of free receptors. This preparation and the stabilization strategies adopted in the present work should prove valuable in further characterization and fractionation of the adenosine A1 receptor.

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