Hydrodynamic properties of adenosine R_i receptors solubilized from rat cerebral-cortical membranes

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Adenosine R_i receptors and inhibitory guanine-nucleotide-regulatory components were solubilized from rat cerebral-cortical membranes with sodium cholate. (–)- N^6 -Phenylisopropyl[2,8- 3 H]adenosine ($[^3$ H]PIA) binds with high affinity to the soluble receptors, which retain the pharmacological specificity of adenosine R_i receptors observed in membranes. The binding is regulated by bivalent cations and guanine nucleotides. Bivalent cations increase $[^3$ H]PIA binding by increasing both the affinity and the apparent number of receptors. Guanine nucleotides decrease agonist binding by increasing the dissociation of the ligand–receptor complex. Adenosine agonists stabilize the high-affinity form of the soluble receptor. The hydrodynamic properties of the adenosine receptor were determined with cholate extracts of membranes that were treated with $[^3$ H]PIA. Sucrose-gradient-centrifugation analysis indicates that the receptor has a sedimentation coefficient of 7.7 S. The receptor is eluted from Sepharose 6B columns with an apparent Stokes radius of 7.2 nm. Labelling of either sucrose-gradient or gel-filtration-column fractions with pertussis toxin and $[^{32}$ P]-NAD $^+$ reveals that both the 41 000- and 39 000- M_r substrates overlap with the receptor activity. These studies suggest that the high-affinity adenosine-receptor-binding activity in the cholate extract represents a stable R_i –N complex.

INTRODUCTION

Binding to adenosine R_i (or A_1) receptors is regulated by both guanine nucleotides and bivalent cations [1,2]. Bivalent cations promote the formation of high-affinity binding states of the receptor for agonists, whereas guanine nucleotides elicit low-affinity binding states of the receptor for agonists. The inhibitory GTP-regulatory component N_i (also called G_i) mediates the effects of GTP on binding [3]. However, the site of action of bivalent cations in regulating inhibitory receptor binding is not clear [2,4]. Mg^{2+} is believed to play a crucial role in hormonal inhibition of adenylate cyclase [2,5], and one site of Mg^{2+} action may be N_i , since purified N_i has been shown to require Mg^{2+} for its activation by guanine nucleotides [6].

The molecular details of the interactions between N_i and adenosine R_i receptors in membranes are poorly understood. Such information requires the availability of active receptor molecules in highly purified form, as well as an efficient reconstitution assay. A key step in this direction is the solubilization of receptors with retention of their regulatory properties. A few such investigations have been reported on adenosine R_i receptors, yet little is known about the subunit composition of the receptor-binding activity, or whether agonists stabilize the soluble receptor [7–10]. Gavish *et al.* [7] reported that cholate solubilized R_i receptors from bovine brain membranes. The GTP regulation of agonist binding to the soluble receptor was retained, although the influence of bivalent cations was lost [7]. These results suggested that soluble receptors interacted with an N component in detergent solution and that Mg^{2+} did not act via N_i . More recently,

Klotz *et al.* [10] suggested that bivalent cations could enhance soluble adenosine-receptor-binding activity by two mechanisms: by protecting receptors from inactivation and by a regulatory enhancement of binding activity. Preliminary studies have appeared on the hydrodynamic properties of the adenosine receptor [8,9,11]. However, the very large sedimentation coefficients that were reported for the receptor-binding activity (approx. 14 S) suggested that aggregates may have been formed [8,11].

The present work set out to determine: (i) whether high-affinity adenosine R_i receptors could be solubilized by cholate with retention of their regulation by both bivalent cations and guanine nucleotides, (ii) whether agonist occupancy would stabilize the soluble receptor, (iii) the hydrodynamic properties of the soluble receptor, and (iv) whether the soluble receptor represented an R_i –N complex.

EXPERIMENTAL

Materials

(–)- N^6 -Phenylisopropyl[2,8- 3 H]adenosine ($[^3$ H]PIA, sp. radioactivity 49.9 Ci/mmol) and $[^{32}$ P]NAD $^+$ (sp. radioactivity 35 Ci/mmol) were obtained from New England Nuclear. *Bordetella pertussis* toxin was purchased from List Biochemicals (Campbell, CA, U.S.A.). Sepharose 6B was from Pharmacia. Adenosine deaminase (bovine intestine), sodium cholate, β -galactosidase (*Escherichia coli*) and malate dehydrogenase (pig heart) were from Sigma. Catalase (bovine liver) and cytochrome

Abbreviations used: R_i , inhibitory adenosine receptor; N_i , inhibitory GTP-regulatory component; PIA, (–)- N^6 -phenylisopropyladenosine; IBMX, 3-isobutyl-1-methylxanthine.

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c (horse heart) were from Boehringer Mannheim. Other chemicals were from either Sigma or Fisher.

Solubilization

Rat (Sprague-Dawley) cerebral-cortical membranes were prepared as previously described [12]. Solubilization conditions were chosen that had previously been demonstrated to retain N_i regulation of adenylate cyclase activity [12]. Cortical membranes were prepared in buffer 1, containing: 50 mM-Tris/HCl, pH 7.4, 1 mM-dithiothreitol, 0.1 mM-diethylenetriaminepenta-acetic acid, 0.1 mM-phenylmethanesulphonyl fluoride, 0.1 mM-benzamidine, 1 mg of aprotinin/l, 1 mg of leupeptin/l and 1 mg of pepstatin A/l. Membranes (5–6 mg/ml) were solubilized with 30 mM-sodium cholate by stirring for 20 min in an ice bath. The cholate extract was separated by centrifugation for 60 min at 130 000 *g* in a Beckman SW-60 rotor at 4 °C. The cholate extract was frozen in liquid nitrogen. Binding activity in these extracts was stable for at least 1 year.

Adenosine deaminase is required to metabolize endogenous adenosine so that specific binding of [3 H]PIA can be detected in brain preparations [13]. The effects of adenosine deaminase and adenosine were investigated in the present study to determine if soluble receptors were stabilized by endogenous adenosine. Pretreatment of membranes with 12 units of adenosine deaminase/ml for 30 min at 24 °C before and during solubilization decreased the recovery of binding activity in the cholate extract by 70 % ($B_{\max}$: control, 512 fmol/mg; + adenosine deaminase, 160 fmol/mg). Pretreatment of membranes with 100 nM-adenosine, as above, slightly increased the recovery of binding activity in the cholate extract (12 % above control). These results indicated that adenosine stabilized the high-affinity form of the soluble receptor, and that adenosine was present as a contaminant under normal solubilization conditions.

Receptor-binding assays

Soluble preparations (30–50 μ g/assay; final volume 100 μ l) were incubated for 3 h at 24 °C with 4 nM-[3 H]-PIA, 30 mM-Tris/HCl, pH 7.4, 3 mM-MgCl₂, 5 mM-phosphocreatine, 25 units of creatine kinase/ml and 1 unit of adenosine deaminase/ml. The assays were terminated by incubation for 5 min in an ice bath with 100 μ l of 22 % (w/v) poly(ethylene glycol) and 20 μ l of either 5 % (w/v) bovine serum albumin or 5 % (w/v) γ -globulin. Then 4 ml of 30 mM-Tris/HCl (pH 7.4)/3 mM-MgCl₂/8 % poly(ethylene glycol) was added to each sample, which was vortex-mixed, followed by rapid filtration and two 4 ml rinses with the same solution through Whatman GF/C filters [7,14]. Radioactivity bound to the filter paper was determined by liquid-scintillation counting. Non-specific binding was determined in the presence of 0.1 mM-PIA. The final concentration of cholate in the binding assay was 3 mM. Concentrations of cholate above 10 mM in the assay decreased specific binding and disrupted Mg²⁺ regulation of binding. This requirement for dilution may indicate that reconstitution of receptors and N components was occurring.

Binding parameters (K_d and $B_{\max}$ values) were determined by computer analysis of the untransformed data with the non-linear curve-fitting program LIGAND [15]. Values are summarized as means $\pm$ S.E.M., and *n* is the number of separate experiments.

Hydrodynamic studies

The hydrodynamic properties of the adenosine R_i receptor were determined with cholate extracts that were prebound with [3 H]PIA. In these experiments, membranes were incubated with 2–4 nM-[3 H]PIA in buffer 1 containing 3 mM-MgCl₂ for 2 h at 24 °C. The membranes were then centrifuged at 12 000 *g* for 20 min at 4 °C. The supernatant was discarded, and the membranes were washed once and sedimented as above. The pellet was resuspended in buffer 1 and solubilized as described above. Treatment of these cholate extracts with GTP abolished all the specifically bound [3 H]PIA, indicating that the radioactivity was associated specifically with adenosine receptors that interacted with N proteins. Before centrifugation, these preparations were filtered over a 10 cm column of Sephadex G-50 to separate the free from the bound radioligand [8,14]. After centrifugation for 7 h at 480 000 *g*, approx. 60 % of the radioactivity loaded was recovered from the 7 S region of the gradient.

Linear 2.5–10 % (w/v) sucrose gradients (4.4 ml) were prepared in buffer 1 containing 2 mM-MgCl₂ and 24 mM-cholate. Cholate extracts (0.25 ml) containing marker enzymes were layered on the gradients, then centrifuged for 6 h at 480 000 *g* (59 000 rev/min) in a Beckman SW-60 rotor at 4 °C. Fractions (125 or 150 μ l) were collected with an ISCO model 185 gradient fractionator connected to a Pharmacia F100 fraction collector. Sedimentation of the marker enzymes was determined by using assay procedures described in the Worthington Enzyme Manual [16]. The *s* values of the marker enzymes were as follows: β -galactosidase, 15.9 S; catalase, 11.3 S; lactate dehydrogenase, 6.95 S; malate dehydrogenase, 4.3 S; cytochrome *c*, 1.71 S [17].

Sephacrose 6B chromatography was performed by applying 0.75–1.0 ml of cholate extract, which was prebound with [3 H]PIA, containing the marker enzymes to a column (0.9 cm $\times$ 55 cm) equilibrated with buffer 1 containing 2 mM-MgCl₂ and 24 mM-cholate. Fractions (1 ml) were eluted with a flow rate of 6–10 ml/h. The Stokes radius of the receptor was interpolated from a plot of the Stokes radii of the marker enzymes versus their elution volume. Elution of marker enzymes was determined under assay conditions described previously [16]. The Stokes radii of the marker enzymes were as follows: β -galactosidase, 6.84 nm; catalase, 5.21 nm; malate dehydrogenase, 3.69 nm; cytochrome *c*, 1.87 nm [17]. Approx. 60 % of the radioactivity loaded on the column was eluted with an apparent Stokes radius of 7 nm.

Toxin labelling

Localization of the pertussis-toxin substrates in either gradient or column fractions (30 μ l samples) was analysed by SDS/polyacrylamide-gel electrophoresis after treatment with the following mixture (100 μ l final volume): 200 ng of activated pertussis toxin/ml, 10 μ M-NAD⁺ (10 Ci/mmol), 10 mM-Hepes, pH 7.4, 2.5 mM-MgCl₂, 0.3 mM-EDTA, 0.1 mM-ATP, and 0.1 mM-GDP. Electrophoresis was performed in the Laemmli [18] system with 11 % acrylamide gels. The M_r values of the pertussis-toxin substrates were determined by comparing their mobilities relative to the following Sigma standards: bovine serum albumin, 66 800; ovalbumin, 45 000; glyceraldehyde-3-phosphate dehydrogenase, 36 000; carbonic anhydrase, 29 000; trypsinogen, 24 000; trypsin inhibitor, 20 100; lactalbumin, 14 000. The distribution of

^{32}P -labelled proteins was determined by autoradiography of dried gels with Kodak XRP5 film exposed for 24 h at -70°C .

RESULTS

High-affinity binding of $[^3\text{H}]\text{PIA}$ was detected in 30 mM-cholate extracts prepared in the absence of added adenosine. This concentration of cholate extracted about 70% of the total membrane protein and 60–70% of the initial binding sites. Higher concentrations of cholate (50 mM) did not increase the apparent yield of receptor. The agonist, $[^3\text{H}]\text{PIA}$, bound to the soluble receptors slowly at 24°C (Fig. 1a). Specific binding reached equilibrium in about 1 h in the absence of added bivalent cations. In the presence of 3 mM- MgCl_2 , the amount of specific binding was increased, and equilibrium was reached in 3 h. Equilibrium binding was maintained for at least 2 h in either the absence or presence of Mg^{2+} (Fig. 1a). Fig. 1(b) shows that the rate of $[^3\text{H}]\text{PIA}$ dissociation is slow in either the absence or the presence of Mg^{2+} . However, dissociation occurred very rapidly in the presence of 0.1 mM-GTP, such that, of the $[^3\text{H}]\text{PIA}$ bound, either all (without Mg^{2+}) or more than 50% (with Mg^{2+}) had dissociated within 1 min. Thus, as observed in membranes [1,2,19], soluble adenosine R_1 receptors were regulated by both Mg^{2+} and GTP.

The kinetic studies described above indicated that Mg^{2+} increased $[^3\text{H}]\text{PIA}$ binding and stabilized the agonist-receptor complex. To examine further the effects of Mg^{2+} , the steady-state characteristics of $[^3\text{H}]\text{PIA}$ binding to the soluble receptors were compared in the absence of added Mg^{2+} or in the presence of 3 mM- MgCl_2 (Fig. 2). Binding of $[^3\text{H}]\text{PIA}$ was saturable in the

absence or presence of Mg^{2+} . LIGAND [15] analysis indicated that the data (with or without Mg^{2+}) could be described by a model assuming the presence of a single class of receptors. In the absence of added Mg^{2+} , the K_d was 8.0 ± 1.8 nM and the B_{max} was 322 ± 13 fmol/mg ($n = 5$). Mg^{2+} increased the binding by increasing both the affinity ($K_d = 2.9 \pm 0.8$ nM; $n = 5$) and the total number of receptors detected ($B_{\text{max}} = 512 \pm 10$ fmol/mg; $n = 5$). Similar results were observed in cerebral-cortical membranes, where Mg^{2+} increased the formation of the high-affinity state of the adenosine receptor [11,19]. Other bivalent cations also increased binding to soluble receptors with the following efficacies: $\text{Ca}^{2+} > \text{Mg}^{2+} > \text{Mn}^{2+}$ (Fig. 3). Concentrations of MnCl_2 above 0.25 mM decreased $[^3\text{H}]\text{PIA}$ binding, possibly owing to the formation of insoluble detergent-receptor complexes [22].

In order to characterize further the properties of the soluble receptor, the inhibition of $[^3\text{H}]\text{PIA}$ binding by both adenosine-receptor agonists and antagonists was examined in the absence of added Mg^{2+} or in the presence of 3 mM- MgCl_2 (Fig. 4). The potency of the antagonist 3-isobutyl-1-methylxanthine (IBMX) at displacing binding was 2–10-fold lower in the presence of Mg^{2+} . This effect of Mg^{2+} was shared by Ca^{2+} (results not shown). The following order of potency was observed for a series of adenosine-receptor ligands in competing for $[^3\text{H}]\text{PIA}$ binding to the soluble receptor (with or without Mg^{2+}): $\text{PIA} > N^6$ -cyclohexyladenosine $> 5'$ - N -ethylcarboxamidoadenosine $> \text{IBMX}$ (Fig. 5). This order of potency is characteristic of adenosine R_1 receptors [20].

In addition to GTP, analogues of GTP also decreased $[^3\text{H}]\text{PIA}$ binding in the cholate extract (Fig. 6). The order of potency was guanosine 5'-[γ -thio]triphosphate $>$

Fig. 1. Kinetics of $[^3\text{H}]\text{PIA}$ binding to cholate extracts of rat cerebral-cortical membranes

(a) Binding of $[^3\text{H}]\text{PIA}$ to cholate extracts (30–60 $\mu\text{g}/\text{assay}$) was determined as described in the Experimental section in the absence of added Mg^{2+} (■), or in the presence of 3 mM- MgCl_2 (●). Non-specific binding was determined with 0.1 mM-PIA (▲). (b) Dissociation of $[^3\text{H}]\text{PIA}$ binding was measured after 3 h of incubation in the absence (■, □) or presence of 3 mM- MgCl_2 (●, ○). Dissociation was initiated by the addition of either 0.1 mM-PIA (●, ■) or 0.1 mM-GTP (○, □). The reaction was stopped at the individual time points. Separation of bound from free ligand was performed after poly(ethylene glycol) precipitation as described in the Experimental section.

Fig. 2. Effect of Mg^{2+} on $[^3\text{H}]\text{PIA}$ binding in the cholate extract

Binding was measured for 2 h with 3.32 nM- $[^3\text{H}]\text{PIA}$ in the absence of added Mg^{2+} (■) or in the presence of 3 mM- MgCl_2 (●). Non-radioactive PIA was added such that the concentrations of total PIA ranged between 3.32 and 63.3 nM. Specific binding was 94% of the total. The data have been transformed into a Scatchard plot.

guanosine 5'-[β,γ -imido]triphosphate > GTP > 9 β -D-arabinofuranosylguanine 5'-triphosphate. Similar potencies for GTP analogues were obtained in the presence of Mg^{2+} (results not shown). GMP was essentially ineffective at decreasing binding (Fig. 6).

To evaluate the hydrodynamic properties of soluble R_1 adenosine receptors, sucrose-gradient centrifugation and gel filtration were performed on cholate extracts prebound with $[^3\text{H}]\text{PIA}$. The sedimentation of this $[^3\text{H}]\text{PIA}$ -labelled receptor is shown in Fig. 7(a); the receptor displayed a sedimentation coefficient of $7.7 \pm 0.3 \text{ S}$ ($n = 9$).

The receptor that was solubilized in the absence of $[^3\text{H}]\text{PIA}$ could also be detected after sucrose-gradient centrifugation, by using direct binding assays with 50 μl samples of each gradient fraction (results not shown). This binding activity also displayed an apparent sedimentation coefficient of about 7 S after 3 h of centrifugation (cf. Fig. 7), and GTP decreased binding of $[^3\text{H}]\text{PIA}$ in these fractions to non-specific values, indicating that $\text{N}_1\text{-R}_1$ interactions were maintained. However, this form of the receptor was unstable: recovery of binding activity was only 10% after 6 h of centrifugation. Since adenosine stabilized high-affinity binding in the cholate extract (see the Experimental section), the sedimentation characteristics of the receptor were determined with soluble preparations that were prebound with either $[^3\text{H}]\text{PIA}$ or adenosine. The adenosine-treated cholate extracts were centrifuged through gradients, as

Fig. 3. Effects of bivalent cations on specific $[^3\text{H}]\text{PIA}$ binding to the cholate extract

Binding was measured with 3 nM- $[^3\text{H}]\text{PIA}$ as described in the Experimental section. Incubations contained different concentrations of CaCl_2 (■), MnCl_2 (○) or MgCl_2 (●).

Fig. 4. Effects of bivalent cations on the competition of the agonist PIA and antagonist IBMX for $[^3\text{H}]\text{PIA}$ binding in cholate extracts.

Binding assays were performed in the presence of increasing concentrations of either PIA (●, ○) or IBMX (■, □). Binding was measured in either the absence of added Mg^{2+} (○, □) or in the presence of 3 mM- MgCl_2 (●, ■).

described in the Experimental section, in the additional presence of 50 nM-adenosine. Direct binding of $[^3\text{H}]\text{PIA}$ was measured after preincubation of 50 μl portions of each fraction with 2 units of adenosine deaminase/ml for 15 min at 24 °C. The apparent sedimentation coefficient of the receptor detected by these means was also approx. 7 S (Fig. 7a). This latter binding could also be eliminated

Fig. 5. Competition for [3 H]PIA binding to the cholate extract by adenosine analogues

Binding was measured with 3 nM-[3 H]PIA in the absence of added bivalent cations. Incubations contained increasing concentrations of one of the following adenosine-receptor ligands: $\circ$, (–)PIA; $\bullet$, N^6 -cyclohexyladenosine; $\triangle$, 5'- N -ethylcarboxamide adenosine; $\blacktriangle$, IBMX.

Fig. 6. Effects of guanine nucleotides on [3 H]PIA binding to the cholate extract

The binding assay was performed with 4 nM-[3 H]PIA in the absence of added bivalent cations. Incubations contained increasing concentrations of one of the following guanine nucleotides: $\circ$, guanosine 5'-[γ -thio]triphosphate; $\bullet$, guanosine 5'-[β,γ -imido]triphosphate; $\blacksquare$, GTP; $\square$, 9- β -D-arabinofuranosylguanine 5'-triphosphate; $\blacktriangle$, GMP.

by including GTP, along with adenosine deaminase, during the preincubation (results not shown).

High-affinity binding of [3 H]PIA in sucrose-gradient fractions appeared to be dependent on the interaction of the R_1 adenosine receptor with N proteins. In order to determine the relationship of this functional N_1 activity with the multiple brain pertussis-toxin substrates [21], gradient fractions were treated with pertussis toxin and [32 P]NAD $^+$, followed by polyacrylamide-gel electrophoresis and autoradiography (Fig. 7b). Two pertussis-toxin substrates were detected, with different sedimentation coefficients: 39 000- M_r substrate, 3.5 S; 41 000- M_r substrate, 4.0 S. Although both pertussis-toxin substrates

appeared lighter than the binding activity, these substrates were present in the same fractions (10–16) as the receptor binding activity in amounts (approx. 3 pmol of N-protein, pertussis-toxin substrate) that exceeded the receptor concentration (approx. 0.2 pmol of receptor).

Cholate extracts that were prelabelled with [3 H]PIA were also analysed by Sepharose 6B chromatography. Fig. 8 shows the elution of radioactivity from the column. The major peak of radioactivity was eluted from the column with an apparent Stokes radius of 7.2 nm, as assessed by co-elution with several enzyme standards (Fig. 8, inset). In addition, radioactivity was eluted from the column in the void volume (fraction 20) and as free ligand (fraction 50). Pertussis-toxin labelling of column fractions revealed that both the 41 000- and the 39 000- M_r substrates were eluted from the column with apparent Stokes radii of 6 nm (Fig. 8). Thus, as observed with sucrose-gradient centrifugation, Sepharose 6B chromatography did not totally fractionate the adenosine receptor from the pertussis-toxin substrates. By using the hydrodynamic parameters of the adenosine R_1 receptor determined above, and assuming a partial specific volume of 0.75 ml/g, the M_r of the protein-detergent complex, and any associated phospholipid, was calculated to be 254 000 [17].

DISCUSSION

The present study demonstrates that cholate can solubilize functional R_1 adenosine receptors that are regulated by N components. The soluble receptor is regulated by either guanine nucleotides or bivalent cations in a manner similar to that observed in membranes [19]. In addition, the soluble receptor retains the specificity for receptor ligands that have been described for R_1 adenosine receptors in membranes [20]. The receptor is considered to be soluble for the following reasons: (i) it does not sediment after 60 min of centrifugation at 130 000 g; (ii) it passes through filters with 0.22 μ m pores without loss of activity; and (iii) it is observed between soluble protein markers after either gel filtration or sucrose-gradient centrifugation.

High-affinity binding to the soluble adenosine receptor is stabilized by adenosine agonists. Treatment of membranes with 12 units of adenosine deaminase/ml before and during solubilization greatly decreased the receptor-binding activity detected in the cholate extract. This suggests that endogenous adenosine stabilizes the high-affinity form of the receptor under normal solubilization conditions. During the binding assay, the endogenous adenosine is metabolized by adenosine deaminase, which allows the binding of [3 H]PIA. Although the solubilization of adenosine receptors was reported previously, the effect of adenosine deaminase treatment was not evaluated [7–10]. Brief treatment (20 min) of cerebral-cortical membranes with adenosine deaminase is not sufficient to remove all the endogenous adenosine (results not shown). Since dissociation of adenosine ligands from the adenosine receptor is a slow process, a 4 h treatment with adenosine deaminase may be required to degrade all the adenosine in cerebral-cortical preparations. Indeed, in the previous studies [7–10], the adenosine R_1 receptor appeared to be unusual in that a high-affinity form of the receptor that was regulated by guanine nucleotides was extracted without the addition of stabilizing ligands. However, the present study demonstrates that the

Fig. 7. Sucrose-gradient centrifugation of the cholera extract

(a) Sucrose-gradient centrifugation of cholera extracts that were prelabelled with [3 H]PIA or adenosine before extraction. Membrane preparations were prelabelled with either 3 nM-[3 H]PIA or 50 nM-adenosine for 2 h at 24 °C. The membranes were centrifuged, washed with buffer 1, then solubilized with 30 mM-cholate at 4 °C. The free ligand was removed by Sephadex G-50 chromatography on a 10 cm column. A 0.25 ml portion (0.5 mg of protein; 18000 c.p.m.) plus the enzyme markers (cyt c, cytochrome c; MDH, malate dehydrogenase; LDH, lactate dehydrogenase; cat, catalase) was layered on a 2.5–10% linear sucrose gradient containing 24 mM-cholate and 2 mM-MgCl₂. The gradients were centrifuged for 6 h at 480000 g (59000 rev./min) in a Beckman SW-60 rotor at 4 °C. The amount of [3 H]PIA in each fraction was determined by liquid-scintillation counting, by using 40 μ l samples of each fraction (●). In preparations prelabelled with adenosine, the receptor was localized by measuring the direct binding of 5 nM-[3 H]PIA to 50 μ l samples of each fraction (▲). Gradient fractions were pretreated with 2 units of adenosine deaminase/ml at 24 °C for 15 min. Binding activity was measured by the poly(ethylene glycol)-precipitation method described in the Experimental section. The inset shows the plot of the sedimentation position of each marker enzyme versus its sedimentation coefficient. Fractions were treated with 200 ng of activated pertussis toxin/ml and 10 μ M-[32 P]NAD⁺ (sp. radioactivity 10 Ci/mmol) as described in the Experimental section. Incorporation of [32 P]ADP-ribose was determined by cutting the 39000–41000- M_r region from the gel, followed by liquid-scintillation counting (○). (b) Autoradiograph of a polyacrylamide gel from a similar experiment with different fraction sizes. The autoradiograph was made with Kodak XRP5 film exposed for 24 h at -70 °C. The M_r values ($\times 10^{-3}$) of protein standards are shown at the left of the autoradiograph. R indicates the fraction at which the peak of receptor binding activity was observed.

adenosine R₁ receptor is similar to some other inhibitory receptors coupled to adenylate cyclase, such as α_2 -adrenergic [23] and D₂-dopaminergic receptors [24], in that occupancy of the receptor with agonists before solubilization promotes the formation of a high-affinity state of the receptor. These results suggest that pre-treatment with agonists may be required for the purification of functionally active R₁ adenosine receptors.

Bivalent cations increase the affinity of agonists in several membrane receptor systems. The present study demonstrates that Mg²⁺ increases agonist binding to the soluble receptor by increasing both the affinity and the apparent number of receptors. In contrast, a previous

report did not detect the ability of bivalent cations to increase adenosine binding to soluble receptors [7]. A possible explanation for this discrepancy is that adenosine receptors are unstable in extracts prepared from membranes treated with 0.5 M-EDTA [10]. In addition to Mg²⁺, Ca²⁺ and Mn²⁺ were also capable of regulating binding of the soluble adenosine receptor. Similarly to the present results, bivalent cations were recently reported [10] to regulate soluble R₁ receptors and to stabilize the receptor from inactivation. Future studies should be directed at determining whether bivalent cations act via the N₁ or the R₁ receptor.

Binding to the soluble adenosine receptor was also

Fig. 8. Sepharose 6B chromatography of the cholate extract

Sepharose 6B chromatography was performed by applying 0.75 ml of cholate extract, which was prebound with $[^3\text{H}]\text{PIA}$ (54 000 c.p.m.), containing the marker enzymes to a column (0.9 cm $\times$ 55 cm), equilibrated with buffer 1 containing 24 mM-cholate and 2 mM- MgCl_2 . Elution of 1 ml fractions was performed at a flow rate of 10 ml/h. The amount of $[^3\text{H}]\text{PIA}$ in 40 μl samples of each fraction was determined by liquid-scintillation counting (●). The column fractions were labelled with pertussis toxin as described in the Experimental section. Incorporation of $[^{32}\text{P}]\text{ADP-ribose}$ was determined by cutting the 39 000–41 000- M_r region from the gel, followed by liquid-scintillation counting (○). The inset shows the plot of the elution position of each marker enzyme versus its Stokes radius.

regulated by guanine nucleotides. Guanine nucleotides decrease binding in cerebral-cortical membranes by decreasing the affinity of adenosine receptors for agonists [1,2]. The specificity for guanine nucleotide analogues to decrease high-affinity binding in cholate extracts was similar to that observed in membranes. A similar order of potency has been observed for the N_1 -mediated inhibition of adenylate cyclase activity in cholate extracts [12]. In contrast with membranes, low-affinity binding could not be detected in the cholate extract; guanine nucleotides decreased high-affinity binding to non-specific values in either the absence or the presence of Mg^{2+} . These results suggest that $[^3\text{H}]\text{PIA}$ binds to R_1 receptors that interact with an N component. These R–N complexes may have formed either in membranes before solubilization, or in solution during the assay. Hydrodynamic studies on the R_1 receptor were performed in order to determine whether cholate solubilized a stable R_1 –N complex.

The ability of agonists to stabilize the high-affinity form of the soluble receptor allowed the determination of the hydrodynamic properties of the R_1 adenosine receptor. The receptor was labelled with $[^3\text{H}]\text{PIA}$ before cholate solubilization of the membranes, then subjected to either sucrose-gradient centrifugation or Sepharose 6B chromatography. The sedimentation coefficient of the R_1 adenosine receptor was previously reported to be 14 S in 0.1% digitonin solution [8]. In preliminary studies using low concentrations of cholate in the sucrose gradient (1 mM), we also observed that the soluble receptor sedimented as a very large particle of approx. 14–19 S which was sensitive to GTP [11]. In contrast, the present study demonstrates that, in gradients containing 24 mM-cholate, the receptor sediments as a discrete particle with a sedimentation coefficient of 7.7 S. It seems likely that soluble adenosine receptors form aggregates in gradients with low detergent concentrations. In the presence of 24 mM-cholate, the adenosine receptor

displayed the hydrodynamic properties of a particle with an M_r of about 250 000. The amount of detergent bound to this particle cannot be determined in cholate solution, since cholate has a partial specific volume similar to that of most proteins [17].

Two laboratories have reported that the adenosine receptor identified by photoaffinity labelling displayed an electrophoretic mobility corresponding to a protein of M_r about 38 000 [25,26]. Radiation inactivation studies demonstrated that high-affinity adenosine binding activity was inactivated as a target of M_r 63 000 [19]. Purified N_i displays a M_r of 108 000 in detergent solution, indicating that it binds significant amounts of detergent [6]. Whatever the precise size of the intact adenosine receptor subunit(s), the M_r determined in this study for the adenosine receptor probably relates to that of a functional R_i -N-detergent complex that may also contain endogenous phospholipids. Future studies should be directed at developing an assay for free soluble receptors that will allow the detection of active R_i adenosine receptors during purification.

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