0026-895X/89/030412-08$02.00/0

MOLECULAR PHARMACOLOGY, 36:412-419

Density Gradient Profiles of A_1 Adenosine Receptors Labeled by Agonist and Antagonist Radioligands Before and After Detergent Solubilization

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Received December 1, 1988; Accepted May 24, 1989

SUMMARY

A_1 adenosine receptors in bovine cerebral cortex have been solubilized and subjected to sedimentation analysis using sucrose density gradient centrifugation. Because the receptors bound both agonists and antagonists with high affinity after solubilization, receptors labeled with an agonist or an antagonist radioligand could be studied before solubilization, after solubilization but before sucrose gradient centrifugation, or after sucrose gradient centrifugation. In each instance the agonist radioligand ^{125}I-*N*^{6}-*p*-aminobenzyladenosine (^{125}I-ABA)-labeled receptor migrated as a single symmetrical peak that was located in the same area of the gradient. In contrast, the location of the receptor labeled with the antagonist [^{3}H]xanthine amine congener ([^{3}H]XAC) varied in the different types of samples. When membranes were incubated with radioligands before solubilization, the peak of antagonist-labeled receptor was symmetrical and was located at a lower density than the peak of agonist-labeled receptor. In addition, receptors incubated with antagonist before solubilization migrated with an apparent lower density than receptors labeled with antagonist either after solubilization or after density gradient centrifugation. Treatments with agents that alter receptor/G protein interactions also resulted in a shift of antagonist-labeled receptors to lower density. These results suggest that the receptors that migrate to the lower density fractions of the gradients are free receptors, whereas those that migrate to the higher density fractions are coupled to a G protein. It is hypothesized that a large proportion of A_1 receptors exist in the membrane coupled to a G protein and that this is the species labeled by the agonist radioligand ^{125}I-ABA. It is, furthermore, hypothesized that the antagonist radioligand [^{3}H]XAC preferentially binds to the free uncoupled A_1 receptors. The finding that receptors incubated with antagonist before solubilization migrate at a lower density than receptors labeled with antagonist after solubilization and sucrose gradient centrifugation could result from (a) a preferential stabilization of the free receptor by the antagonist, (b) an instability of antagonist/receptor/G protein complexes during solubilization that results in the production of antagonist/receptor complexes, or (c) an antagonist-induced dissociation of receptor/G protein complexes. The authors favor the latter possibility.

Numerous receptors are known to couple to their effectors via G or N proteins (reviewed in Ref. 1). The first system that was thoroughly studied and that has subsequently served as a conceptual model in thinking about other systems was the β-adrenergic receptor/stimulatory G protein (G_s)/adenylate cyclase system. Extensive studies with this system led to a model in which occupation of the free receptor by an agonist causes a coupling of the receptor to G_s. This in turn facilitates the exchange of GTP for bound GDP, the dissociation of the heterotrimer G protein into its α and β-γ subunits, and the subsequent activation of the catalytic subunit of adenylate cyclase by the activated α subunit (reviewed in Refs. 1 and 2). According to this model, antagonists bind to free receptors and receptor/G protein complexes with equal high affinity, whereas agonists only form high affinity complexes consisting of the agonist, the receptor, and the G protein liganded with GDP (2). The latter complex is destabilized in the presence of GTP, which causes the dissociation of the receptor/G protein complex. Physical evidence for an agonist-induced coupling of the receptor to G_s was provided by gel exclusion chromatographic studies of Limbird and Lefkowitz (3), in which they showed that (a) antagonist but not agonist radioligands will form high affinity complexes with solubilized β receptors, (b) the apparent molecular weight of receptors solubilized in the presence of an agonist radioligand is greater than that of receptors solubilized in the presence of an antagonist radioligand, and (c) the apparent molecular weight of β receptors labeled with an antagonist radioligand is the same when the receptors are labeled before or after solubilization. Subsequent studies using high pressure

These studies were supported by a grant from the National Science Foundation (BNS-8719594).
[1] Recipient of a Postdoctoral Fellowship from the Chicago Heart Association.

ABBREVIATIONS: G protein, guanine nucleotide-binding protein; Gpp(NH)p, 5′-guanylylimidodiphosphate; XAC, xanthine amine congener; ABA, *N*^{6}-*p*-aminobenzyladenosine; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; NEM, *N*-ethylmaleimide.

gel exclusion chromatography or sucrose gradient centrifugation gave similar results with α_2 adrenergic (4, 5) and D_2 dopaminergic receptors (6).

A_1 adenosine receptors in the central nervous system are known to couple to both the inhibition of adenylate cyclase (7, 8) and the activation of a K^+ channel (9). Unlike the receptor systems discussed above, A_1 receptor agonist radioligands bind to A_1 receptors solubilized from rat cerebral cortex with high affinity (10, 11). This appears to be due to the solubilization of receptor/G protein complexes, because the high affinity binding is destabilized by the addition of GTP or GTP analogs (10, 11) and the sedimentation of the solubilized receptors on sucrose gradients is the same whether an agonist radioligand is bound before or after the receptor is solubilized (10). Stiles (10) postulated that adenosine receptors are "intimately associated" with a G protein even in the absence of an agonist. Yeung *et al.* (11) reported that treatment of rat cerebral cortical membranes with adenosine deaminase (12 units/ml, 30 min at 24°) before and during solubilization with sodium cholate decreases the recovery of agonist radioligand binding by 70%. These authors suggest that the adenosine receptor is not different from other receptors that have been studied and that the recovery of agonist binding after solubilization is due to the presence of the endogenous agonist adenosine, i.e., that endogenous adenosine induces the formation of adenosine receptor/G protein complexes. However, these studies do not differentiate between the ability of adenosine to induce the formation of receptor/G protein complexes, and the subsequent stabilization of this complex, and the ability to stabilize precoupled receptor/G protein complexes. Obviously, these conflicting alternate hypotheses have fundamental implications as to the mechanisms by which adenosine receptor agonists act at A_1 receptors to produce their responses. We have reinvestigated this question by performing experiments with bovine cerebral cortical membranes treated with high concentrations of adenosine deaminase, with or without GTP, before the receptors were solubilized and subjected to sucrose gradient centrifugation. Our results are consistent with the hypothesis that adenosine stabilizes A_1 adenosine receptors but that most of the receptors are coupled to a G protein(s) in the absence of an agonist. Additional results are interpreted to suggest that antagonists preferentially bind to free receptors and thus cause an "uncoupling" of unliganded receptor/G protein complexes.

Experimental Procedures

Preparation of bovine cerebral cortical membranes and solubilization of receptors. Brains from freshly killed calves were obtained at a local abattoir. To prepare membranes, 10–15 g of cerebral cortex were homogenized (Polytron; Brinkmann Instruments) in 4 volumes of ice-cold buffer A (10%, w/v, sucrose, 20 mM Tris·HCl, pH 7.0 at 25°, 1 mM EDTA, 100 μM phenylmethylsulfonyl fluoride) passed through gauze, and centrifuged (400 × *g*, 10 min at 4°). The supernatant was saved and the pellet was resuspended in buffer A and recentrifuged, after which the two supernatants were pooled and recentrifuged (6500 × *g*, 20 min). The resulting pellets were resuspended in buffer B (20 mM Tris·HCl, pH 7.5 at 25°, 1 mM EDTA) and, after 30 min on ice, were recentrifuged (6500 × *g*, 20 min). The pellets were resuspended in buffer B and aliquots were stored in liquid nitrogen for later study. In a few of the initial experiments, the membranes were immediately resuspended in solubilization buffer (10) (20 mM Tris·HCl, pH 7.5 at 25°, 10 mM $MgSO_4$, 5 mM EDTA, 100 mM NaCl, 1% digitonin), to give a protein:detergent ratio of about 2:1, and were centrifuged at 100,000 × *g* for 45 min (type 40 rotor, Beckman Instruments, Fullerton, CA). The supernatants were discarded and the pellets were reextracted with solubilization buffer (protein:detergent ratio of about 1:2), using an ultrasonic probe (five 5-sec bursts at maximum intensity; model W140; Heat Systems-Ultrasonics, Inc., Plainview, NY) and recentrifuged at 100,000 × *g* for 60 min. The second extract, which was considerably enriched in A_1 receptors compared with the first, was stored in aliquots in liquid nitrogen.

Radioligand binding. Two radioligands have been studied; ^{125}I-ABA is an agonist with high affinity for A_1 adenosine receptors (12), and [^{3}H]XAC is an antagonist with high affinity for the same receptors (13–15). Samples containing 20 mM Tris·HCl, pH 7.5 at 25°, 10 mM $MgSO_4$, 1 mM EDTA, 0.2 units/ml adenosine deaminase, radioligand, and solubilized receptor preparation so that the final digitonin was ≤0.2% were incubated at 37° until equilibrium (90 min for ^{125}I-ABA, 30 min for [^{3}H]XAC), at which time bound and free radioligand were separated by filtration through polyethylenimine-soaked GF/B glass fiber filters (16). (Initially, we also used a 90-min incubation for [^{3}H]XAC but changed to a 30-min incubation when it appeared that the specific binding slowly declines after this time.) Nonspecific binding was defined as that observed in the presence of 5 mM theophylline.

Preparation of samples for sucrose gradient centrifugation. Three types of samples, which we designate as membrane labeled, soluble labeled, and postgradient labeled, have been studied. Membrane-labeled samples were samples in which the ligand was bound to the receptors before solubilization. These samples were prepared by (a) incubation of membranes (2–5 mg of protein) in a medium containing 20 mM Tris·HCl, pH 7.5 at 25°, 10 mM $MgSO_4$, 1 mM EDTA, 5 units/ml adenosine deaminase, a mixture of protease inhibitors (17), and a near saturating concentration of radioligand (0.7–1 nM for ^{125}I-ABA, 1.5–2 nM for [^{3}H]XAC, for 75 min at 37°; (b) centrifugation at 100,000 × *g* for 30 min; (c) solubilization of the pellet in 1 ml of solubilization buffer with the aid of 5-sec bursts of the sonicator; (d) centrifugation at 100,000 × *g* for 60 min; and (e) concentration of the supernatants with Centricon 30 concentrators (Amicon).

Soluble-labeled samples were those samples that were incubated with the radioligands after solubilization but before the solubilized preparations were run on the sucrose gradients. Soluble preparations prepared from membranes simultaneously with the preparation of membrane-labeled samples and frozen solubilized preparations were studied. "Freshly prepared" solubilized preparations were made by incubation of membranes with protease inhibitors and 5 units/ml adenosine deaminase for 30 min at 37°, followed by centrifugation and solubilization as discussed above for the preparation of membrane-labeled samples. Both types of preparations were incubated with radioligand as discussed above for membrane-labeled samples, using a volume so that the digitonin was diluted to 0.2%. At the end of the incubation, the samples were cooled and concentrated using Centricon 30 concentrators. Samples prepared from frozen solubilized and "freshly solubilized" preparations gave similar results in the density gradient experiments.

Postgradient-labeled samples were those samples that were labeled with radioligand after solubilization and sucrose gradient centrifugation. Freshly solubilized and frozen solubilized preparations were studied. In some experiments, the solubilized preparation was incubated with protease inhibitors and 5 units/ml adenosine deaminase at 37° for 30 min, cooled, diluted 5-fold with dilution buffer (20 mM Tris, pH 7.4 at 25°, 10 mM $MgSO_4$, 1 mM EDTA), and concentrated using a Centricon concentrator before sucrose gradient centrifugation. In another series of experiments, membranes were incubated for 30 min at 37° with or without 5 units/ml adenosine deaminase and 0.1 mM GTP, pelleted, incubated a second 30 min at 37° with or without adenosine deaminase (GTP omitted), pelleted and solubilized using the two-step solubilization procedure. Binding experiments using ^{125}I-ABA and [^{3}H]XAC were performed immediately to quantitate the receptors in the different preparations. These preparations were layered directly on the gradients, with the omission of the Centricon concentrator step. This

allowed a direct quantitation of the recovery of receptor after sucrose gradient centrifugation, unaffected by variable recoveries on the Centricon concentrators.

Sucrose gradient centrifugation. In order to follow the fate of agonist- and antagonist-labeled receptors in the same gradient, concentrated samples (~40 μl) labeled with ^{125}I-ABA or [^{3}H]XAC (membrane-labeled or soluble-labeled samples) were mixed together in a relative proportion of 10–20:1 starting material ([^{3}H]XAC:^{125}I-ABA) to compensate for the different specific activities of the radioligands. The "mixed" samples were diluted to 500 μl with dilution buffer containing 0.1% digitonin. Concentrated samples (~40 μl) for postgradient labeling were diluted with 450 μl of the same digitonin-containing dilution buffer. Each sample was layered on top of a 12.5-ml 5–20% sucrose gradient in 20 mM Tris, 10 mM $MgSO_4$, 1 mM EDTA, 0.1% digitonin, and centrifuged at 40,000 rpm for 15 hr (SW40 rotor). Each gradient was fractionated into 10-drop fractions using a Buchler fractionator and a fraction collector. Postgradient-labeled samples were prepared by incubating appropriate aliquots of the unlabeled gradient samples with 0.7–1 nM ^{125}I-ABA or 1.5–3 nM [^{3}H]XAC. Bound radioactivity was determined by filtration over polyethylenimine-soaked GF/B filter paper as described above. Samples containing ^{125}I-ABA were counted in a γ counter (75% efficiency); samples containing [^{3}H]XAC were counted in a scintillation counter (45% efficiency). Samples containing both radioligands were counted in a γ counter before scintillant was added, and total radioactivity was subsequently counted in a scintillation counter. In these instances, the relative difference in the counting efficiency for counting ^{125}I-ABA in the γ counter (75%) versus using the [^{3}H] window of the scintillation counter (60%) was used to calculate the radioactivity attributable to [^{3}H]XAC. Identical results were obtained using a correction obtained by allowing the ^{125}I-ABA to decay 1 half-life and recounting the samples. All values were corrected to give dpm. Nonspecific binding for both ^{125}I-ABA and [^{3}H]XAC in the fractionated samples of membrane-labeled and soluble-labeled samples was assessed by labeling aliquots of the same preparations in the presence of 5 mM theophylline. Nonspecific binding was constant throughout the gradients and accounted for about 5% of the radioactivity in the peak fractions. The values given for membrane-labeled and soluble-labeled samples are, consequently, total radioactivity. The nonspecific component in postgradient-labeled samples was similarly estimated and found to account for ~15% of the peak activity for ^{125}I-ABA and ~25% of the peak activity for [^{3}H]XAC. Because this component was also relatively constant throughout the gradients and was closely approximated by the radioactivity in fraction numbers 40 and above, the latter were averaged and subtracted from that in all fractions to give the estimates of the specific binding in the postgradient-labeled samples.

Materials. ^{125}I-ABA was synthesized and purified as previously described (17). A theoretical specific activity of 2200 Ci/mmol was assumed. [^{3}H]XAC (specific activity, 130 Ci/mmol) was from DuPont-NEN. Digitonin was from Gallard-Schlesinger Industries, Inc. All other chemicals were from Sigma Chemical Company. Centricon 30 concentrators were from Amicon.

Results

Agonist and antagonist radioligand binding to soluble A_1 receptors. Although CHAPS effectively solubilizes A_1 receptors in bovine brain membranes (18–20),[2] sucrose gradient centrifugation of CHAPS-solubilized preparations did not yield well defined peaks of receptor binding activity (data not shown). Because Stiles (10) had previously reported experiments on rat brain A_1 receptors solubilized with digitonin, we investigated the use of digitonin for these studies. Preliminary experiments with various procedures led to the protocol outlined in Experimental Procedures. In the initial set of experiments, the K_d and B_{max} values for five such preparations were 0.40 ± 0.08 nM (mean ± SE) and 475 ± 64 fmol/mg of protein for the antagonist [^{3}H]XAC and 0.10 ± 0.01 nM and 222 ± 31 fmol/mg of protein for the agonist ^{125}I-ABA. The B_{max} for [^{3}H]XAC was probably underestimated in these experiments, because these incubations were all for 90 min (see Experimental Procedures).

Sucrose density centrifugation profiles of agonist and antagonist radioligand binding in membrane-labeled, soluble-labeled, and postgradient-labeled samples. In order to assess the sedimentation characteristics of receptors that were labeled with agonist or antagonist under various conditions, we performed three experiments comparing the density gradient profiles of agonist- and antagonist-labeled receptors under three different conditions. Labeling was performed either in the membranes (Fig. 1A), after solubilization (Fig. 1B), or

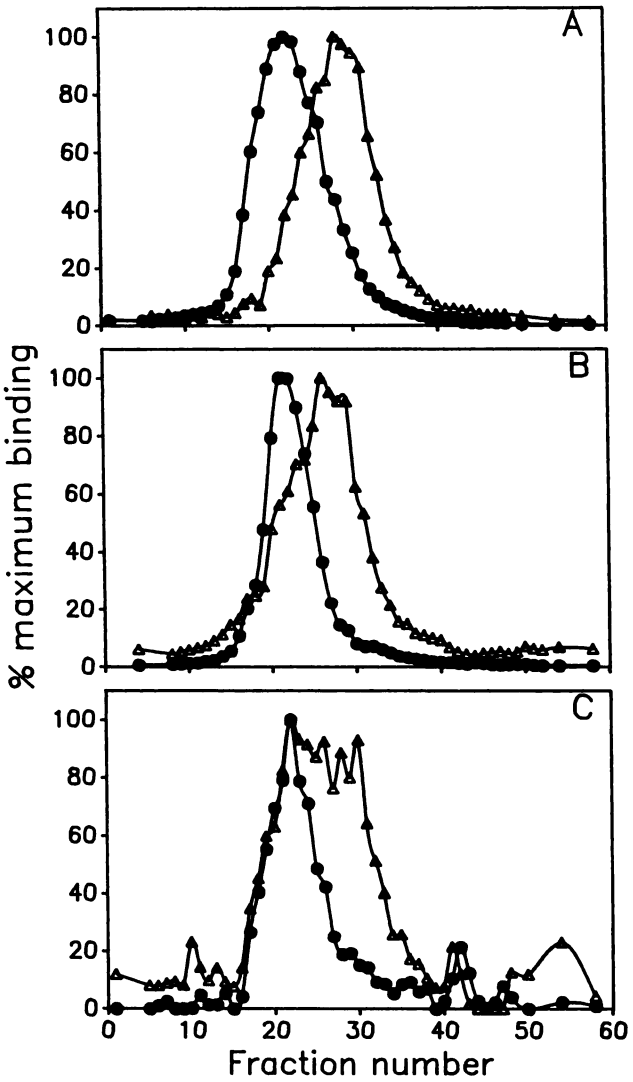

Fig. 1. Sucrose density gradient profiles of membrane-labeled (A), soluble-labeled (B), and postgradient-labeled A_1 receptors (C). ●, ^{125}I-ABA; △, [^{3}H]XAC. In this and the succeeding figures, the *left side* of the figure is the *bottom* of the gradient. The peak radioactivities for [^{3}H]XAC in A, B, and C were 3560, 3500, and 1710 dpm, respectively. Those for ^{125}I-ABA were 6035, 6036, and 7103 dpm, respectively. In all three instances, data for the two radioligands are from single gradients. Fifty- and 150-μl aliquots of the fractions of the same gradient were used for postgradient labeling with ^{125}I-ABA and [^{3}H]XAC, respectively.

[2] Unpublished results.

after density gradient centrifugation (Fig. 1C). All parts of these experiments were simultaneously performed on the same membrane preparation.

Several observations were made. First, in all three experiments the peak of agonist (^{125}I-ABA) binding in the membrane-labeled samples (Fig. 1A) was at a significantly higher density than that of the antagonist ([^{3}H]XAC). Second, the peak of agonist binding was observed at similar densities in preparations labeled in membranes (Fig. 1A), in solubilized extracts (Fig. 1B), or after sucrose gradient centrifugation (Fig. 1C).

In contrast, the density of receptors labeled by the antagonist varied with the different labeling conditions. After density gradient centrifugation, the receptors that could be detected with the antagonist were partially (Fig. 1C) or entirely (see below) located in heavier density fractions, as compared with receptors that were labeled with the antagonist before solubilization (Fig. 1A). The location of the receptors labeled with antagonist after solubilization but before sucrose gradient centrifugation also differed from that of receptors labeled by the antagonist before solubilization, in that variable amounts of the antagonist-labeled receptors were in the light and heavy fractions. In the experiment shown in Fig. 1, some of the soluble receptors labeled by the antagonist were in the heavier fractions while the main peak was in lighter fractions (Fig. 1B).

Effect of treatment of membranes with adenosine deaminase plus GTP on the recovery of adenosine receptors in solubilized preparations and on the density gradient profiles of the solubilized receptors. In the experiments just described, membranes and/or solubilized receptor preparations were treated with high concentrations of adenosine deaminase to metabolize contaminating adenosine, which could affect the location of the adenosine receptors in the sucrose gradients. However, it is impossible to prove that this, or any other treatment, removes all of the endogenous adenosine and it could be argued that adenosine may bind to the bovine adenosine receptors with very high affinity, which protects it from being metabolized by adenosine deaminase. Because guanine nucleotides destabilize high affinity agonist binding to A_1 adenosine receptors (8, 10–12, 18), we performed another series of experiments in which we treated membranes with 5 units/ml adenosine deaminase plus 0.1 mM GTP for 30 min at 37°, pelleted the membranes, and incubated the membranes for an additional 30 min at 37° with 5 units/ml adenosine deaminase before the receptors were solubilized using the two-step procedure. Membranes incubated in the absence of adenosine deaminase and GTP served as control. Scatchard plots for the binding of [^{3}H]XAC and ^{125}I-ABA to each preparation were constructed so that the effect of the pretreatment on receptor solubilization could be quantified; recovery of the receptor after sucrose gradient centrifugation was also estimated. The kinetic parameters determined in four such experiments are summarized in Table 1. The recovery of receptor in solubilized preparations was decreased 25–35% in the adenosine deaminase/GTP-treated preparations. Identical results were obtained in preparations similarly treated with adenosine deaminase in the absence of GTP (data not shown). The effect of adenosine deaminase was absent when the adenosine deaminase preparation was treated with the adenosine deaminase inhibitor deoxycoformycin, thus verifying that the effect was, in fact, due to adenosine deaminase and not a contaminant of the adenosine deaminase preparation (data not shown). Fig. 2.

TABLE 1

Effect of the treatment of membranes with adenosine deaminase plus GTP on the adenosine receptors in subsequently solubilized preparations that are detectable with agonist and antagonist radioligands

Values shown are means ± standard errors from four experiments.

	[^{3}H]XAC		^{125}I-ABA	
	K_d	B_{max}	K_d	B_{max}
	nM	fmol/mg	nM	fmol/mg
Control	1.45 ± 0.25	836 ± 47	0.08 ± 0.01	316 ± 49
Treated	1.15 ± 0.29	622 ± 22	0.09 ± 0.01	205 ± 47

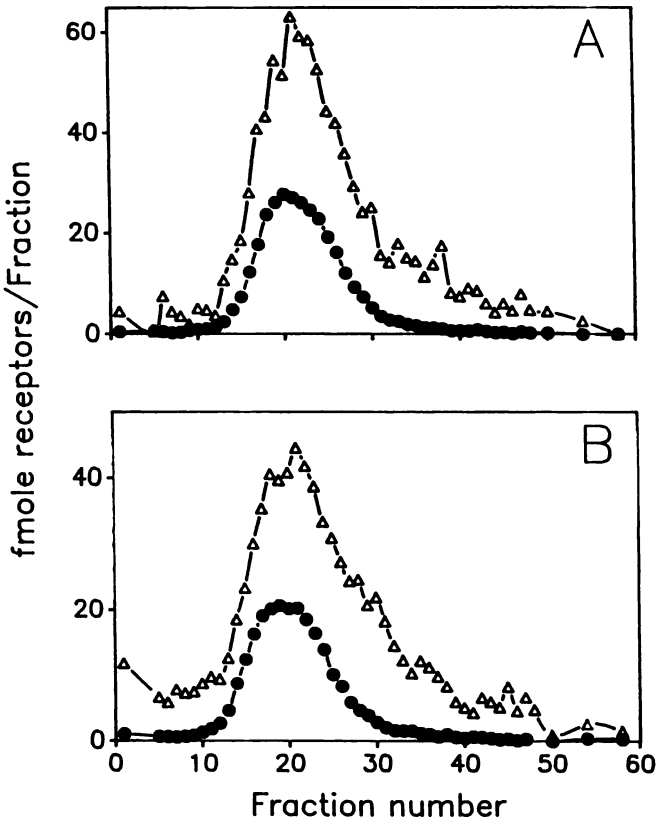

Fig. 2. Effect of treatment of membranes with adenosine deaminase and GTP on sucrose gradient profiles of receptors detected by [^{3}H]XAC (△) and ^{125}I-ABA (●). Membranes were incubated in the absence (A) or presence (B) of adenosine deaminase and GTP, pelleted, and extracted by the two-step method described in Experimental Procedures. After sucrose gradient centrifugation, the gradients were fractionated and the receptors in aliquots of each fraction were determined using 1.0 nM ^{125}I-ABA and 2.5 nM [^{3}H]XAC. The quantities shown are the total amounts theoretically present in each fraction, calculated from the ligand concentrations used and the K_d values of the ligands determined in the same experiment.

shows the sucrose gradient profiles obtained from postgradient labeling with [^{3}H]XAC and ^{125}I-ABA. Postgradient labeling was performed on aliquots of the same gradients and was corrected to 100% labeling, using the ligand concentrations used and the K_d values determined in the same experiment. This allowed a direct comparison of the numbers of receptors capable of binding the agonist and antagonist radioligands and the recovery of the receptors in the gradients after centrifugation. An estimate of recovery was calculated by summing the receptors in all fractions that contained more than 35% of that in the peak fraction. These values, expressed as percentages of the amount of receptor theoretically applied to the gradients,

did not differ for ^{125}I-ABA and [^{3}H]XAC and averaged 47 ± 5%. (Inspection of Fig. 2 shows that this estimate of recovery was conservative and, no doubt, considerably underestimated the true recovery). Two important points should be noted. (a) Although treatment with adenosine deaminase resulted in a modest decrease in the amount of receptors detectable in the soluble preparations, the density gradient profiles of both agonist- and antagonist-detectable receptors were unaffected. (b) A high percentage of the receptors were in the heavier fractions. This was true for both agonist- and antagonist-detectable receptors.

Effect of NEM on sucrose gradient profiles of antagonist binding in membrane-labeled and soluble-labeled samples. It was clear from the type of experiment summarized in Fig. 1 that the peak of antagonist binding in membrane-labeled samples was in a lighter fraction and was better defined than that in the soluble-labeled or postgradient-labeled samples. This can be explained by postulating that (a) receptors in the higher density fractions are coupled to a G protein whereas those in lower density fractions are free uncoupled receptors; (b) in membranes, the antagonist [^{3}H]XAC binds to free receptors, which perturbs an equilibrium between free receptors and receptor/G protein complexes, thus causing the dissociation of the latter and resulting in an antagonist binding peak at lower density than agonist binding; but (c) for unknown reasons, this occurs to a lesser extent when preparations are exposed to [^{3}H]XAC after being solubilized with digitonin. If this is the case, then treatment of membranes with NEM, which is known to block high affinity agonist binding to A_1 receptors presumably by inactivating the relevant G protein (8), should decrease the density of antagonist binding in samples subsequently solubilized and then labeled. The results of such an experiment are shown in Fig. 3A; the peak of antagonist binding in NEM-pretreated samples migrated at a significantly lighter density than the peak of antagonist binding observed with the control preparation. The data summarized in Fig. 3B are from the same experiment and show that NEM treatment of soluble preparations before labeling with antagonist also shifts the antagonist peak to the lighter density reactions. Thus, perturbation of the preparations with NEM results in a decrease in the density of receptors that can be labeled with the antagonist, when the NEM treatment is performed with membranes (Fig. 3A) or with solubilized extracts (Fig. 3B). Furthermore, the results with the latter preparation indicate that the lack of a complete shift in the antagonist peak in soluble-labeled samples (Fig. 1B), compared with membrane-labeled samples (Fig. 1A), is not due to the inability to produce such a shift in the soluble preparation when the receptor/G protein complex is perturbed.

Effect of Gpp(NH)p on sucrose gradient profiles of antagonist binding in soluble-labeled and postgradient-labeled samples. The above results are consistent with the hypothesis that the labeling of membrane-bound receptors with an antagonist or perturbation with NEM results in the dissociation of receptor/G protein complexes and an increase in low density receptor. It is possible that the ability of the antagonist [^{3}H]XAC to dissociate receptor/G protein complexes in the membrane samples and, thus, label a lower density fraction in the sucrose gradients is dependent on a guanine nucleotide, most likely GDP, being liganded to the G protein. Thus, the inability of XAC to cause this shift in most soluble-labeled samples could be due to the loss of guanine nucleotide during

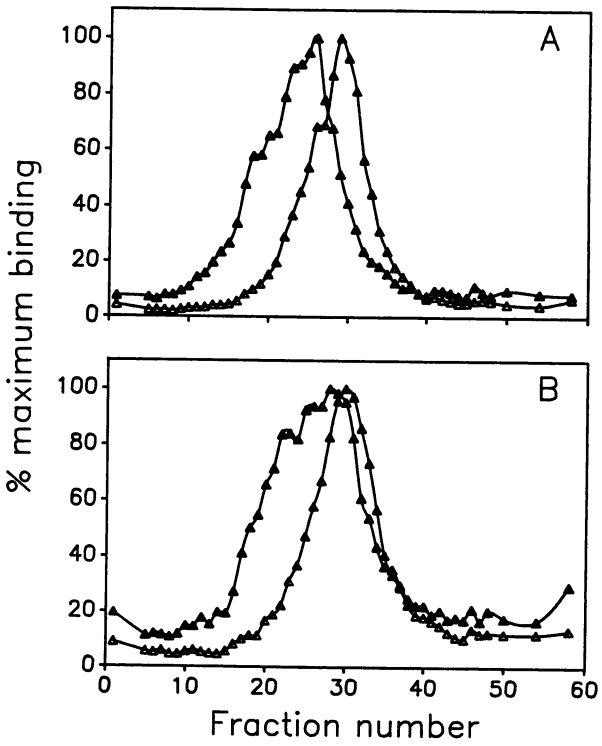

Fig. 3. Effect of NEM on sucrose density gradient profiles of antagonist ([^{3}H]XAC) binding. A, Membranes were incubated with adenosine deaminase (5 units/ml with or without 3 mM NEM for 15 min at 37° after which 4.5 mM dithiothreitol was added, the membranes were pelleted (100,000 × *g*, 30 min), and soluble preparations were made, labeled with [^{3}H]XAC, and processed as described in Experimental Procedures. The peak radioactivities for the control (▲) and NEM-treated (△) preparations were 2830 and 5430 dpm, respectively. B, A second solubilized preparation was made simultaneously, incubated with adenosine deaminase with or without NEM followed by the addition of dithiothreitol, labeled with [^{3}H]XAC, and processed. The peak radioactivities for the control (▲) and NEM-treated (△) preparations were 1260 and 2250 dpm, respectively.

or after the solubilization process. Fig. 4 summarizes sucrose gradient profiles from an experiment in which solubilized receptors were (a) labeled with [^{3}H]XAC in the absence or presence of Gpp(NH)p (100 μM) (Fig. 4A, soluble-labeled samples) or (b) incubated with or without Gpp(NH)p and run on the gradient before labeling with [^{3}H]XAC (Fig. 4B, postgradient-labeled samples). Fig. 4A shows that labeling of solubilized receptors with [^{3}H]XAC in the presence of Gpp(NH)p shifted the [^{3}H]XAC peak to the lower density region labeled by [^{3}H]XAC in NEM-treated samples (compare with Fig. 3) or in control [^{3}H]XAC membrane-labeled samples (Fig. 1A). Fig. 4B shows that the peak of [^{3}H]XAC binding to the postgradient-labeled, Gpp(NH)p-pretreated, samples was also shifted to a lower density compared with control but the shift to a lower density appeared to be less complete, compared with the samples labeled with [^{3}H]XAC in the presence of Gpp(NH)p (compare Fig. 4, A and B). This latter results suggests that the combination of antagonist plus guanine nucleotide (Fig. 4A) is more effective than antagonist alone (Fig. 1B) or guanine nucleotide alone (Fig. 4B) in promoting the formation of the lower density receptors. The experiment summarized in Fig. 4 is characteristic of three experiments with 100 μM Gpp(NH)p and/or GDP. Both nucleotides produced the same effects on the gradient profiles of [^{3}H]XAC binding and inhibited the

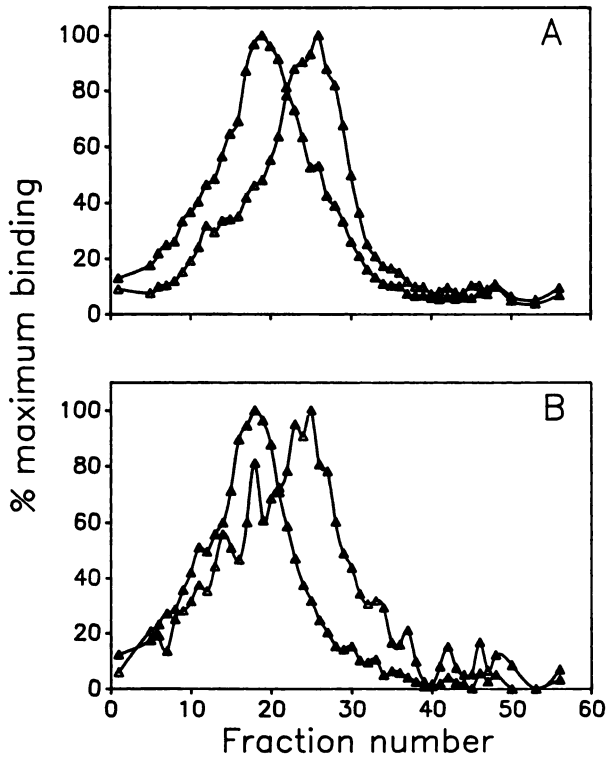

Fig. 4. Effect of Gpp(NH)p on sucrose density gradient profiles of antagonist ([^{3}H]XAC) binding. A, A solubilized preparation was incubated with [^{3}H]XAC with or without Gpp(NH)p (100 μM), for 75 min, under conditions used to study ligand binding and processed as described in Experimental Procedures. The peak radioactivities in the absence (▲) and the presence (△) of Gpp(NH)p were 5740 and 3970 dpm, respectively. B, Aliquots of the same soluble preparation were incubated under the same conditions with or without Gpp(NH)p but in the absence of [^{3}H]XAC and were processed. The entire gradient fractions were labeled with [^{3}H]XAC. The peak radioactivities for the control (▲) and the Gpp(NH)p-treated (△) preparations were 8075 and 3320 dpm, respectively.

binding of a saturating concentration of ^{125}I-ABA to the solubilized receptors over 80% (data not shown).

Discussion

The finding that adenosine receptors labeled with an agonist radioligand before solubilization have an apparent greater molecular size, i.e., are present in "heavier" fractions of sucrose density gradients, than receptors similarly labeled with an antagonist radioligand is the same as findings reported for other receptors that couple to G proteins (3–6). The findings that treatments of membranes with NEM or Gpp(NH)p before solubilization shift the location of receptors that can be labeled by the antagonist radioligand [^{3}H]XAC to "lighter" fractions of the gradients are consistent with the hypothesis that the adenosine receptors in the heavier fractions are coupled to a G protein(s) whereas those in the lighter fractions are not.

The behavior of the adenosine receptor subsequent to solubilization and sucrose gradient centrifugation as compared with the model system, the β-adrenergic receptor, is of great interest. The ability of the agonist radioligand ^{125}I-ABA to bind to soluble adenosine receptors clearly differs from the β-adrenergic receptor, which is unable to bind agonist ligands with high affinity after solubilization. The location of solubilized adenosine receptors in the heavier fractions of sucrose density gradients suggests that this is possible because adenosine receptor/G protein complexes are solubilized. Yeung *et al.* (11) reported that treatment of rat cerebral cortical membranes with adenosine deaminase before detergent solubilization decreases the concentration of receptors in the solubilized extract that are capable of binding (*R*)-[^{3}H]phenylisopropyladenosine by 70%. These workers argue that solubilized adenosine receptors retain their ability to bind agonists with high affinity because, in fact, adenosine/receptor/G protein complexes are solubilized. This would be possible if the endogenous adenosine that is bound to the solubilized receptors is able to dissociate from the receptors and agonist radioligand is able to bind to the receptors faster than the unliganded receptor/G protein complex dissociates. (This assumes that the detergent concentration is not allowed to fall below its critical micelle concentration so that reconstitution may occur.) An alternate explanation for the effect of adenosine deaminase would be that the receptor exists coupled to a G protein in the absence of an agonist but endogenous adenosine stabilizes the receptor/G protein complex, i.e., the adenosine/receptor/G protein complex is more stable than the unliganded receptor/G protein complex. We have confirmed that the treatment of membranes with adenosine deaminase decreases the concentration of receptors detectable after solubilization. However, the loss of receptor (~30%) was much less than that reported by Yeung *et al.* (11) (~70%), even though we used a combination of high concentrations of adenosine deaminase and GTP (Fig. 2). It should also be noted that the treatment decreased the recovery of receptors capable of binding agonist and antagonists roughly to the same proportion and that, in this series of experiments, almost all the receptors capable of binding the antagonist were in the same heavy fractions capable of binding agonist. These results suggest that contaminating adenosine does stabilize the A_1 receptors but that the dissociation constant for the binding of the adenosine receptor to its G protein is favorable so that a large portion, if not all, of the adenosine receptors in membranes are coupled to their G proteins in the absence of an agonist. With the numerous different experimental protocols used in the present experiments, a large proportion of the solubilized receptors appeared to remain coupled to a G protein, i.e., both agonist- and antagonist-detectable receptors were in the heavier fractions of the sucrose gradients (Figs. 2, 3, and 4). In some experiments (Fig. 1C), a larger proportion of the receptors appeared to uncouple from their G proteins. The reason for these variations in the results is not known but could be due to the different experimental protocols used or to differences in different batches of digitonin employed. It is possible that the variability in the amount of receptor in the lighter fractions that was detected with the antagonist radioligand is due to a relative instability of the free receptor after solubilization. Whatever the reason, a large proportion of the receptors were always present in the heavier fractions of the gradients.

The findings that a large proportion of receptors detected by the antagonist [^{3}H]XAC in sucrose gradients are in the heavier fractions whereas the receptors labeled by [^{3}H]XAC before solubilization and density gradient centrifugation are in lighter fractions, along with our postulate that the adenosine receptors exist coupled to G proteins in the absence of an effector, would suggest that the antagonist [^{3}H]XAC induces an uncoupling of precoupled adenosine receptor/G protein complexes. This could occur if there is an equilibrium between free receptors, G

proteins, and receptor/G protein complexes and if the antagonist has a higher affinity for the free receptors. (This is not unlike the situation for β-adrenergic receptors, in which the receptors and G proteins are uncoupled in the absence of an effector, and agonists induce a coupling because the affinities of agonist/receptor complexes for the relevant G protein are greater than the affinity of the free receptor for the G protein; in this case, the affinities of agonists for the receptor/G protein complexes are greater than the affinities of the agonists for the free receptors. In the case at hand, if the affinity of the antagonist/receptor complex for the relevant G protein is less than the affinity of the free receptor for the G protein, then the affinity of the antagonist for the free receptor is greater than the affinity of the antagonist for the receptor/G protein complex.) If this is the case, and if the unliganded receptor/G protein complex exerts an effect on whatever effector system is involved, then the addition of a xanthine antagonist would have the potential to relieve this tonic effect. If the effect of the unliganded receptor/G protein complex were the same, albeit much less than that induced by an agonist, then the antagonist could exert a negative efficacy. The concept of antagonists with negative rather than zero efficacy has been discussed by Colquhoun (21). Different antagonists could differ in this regard. For example, an antagonist with equal affinities for the free receptor and the receptor/G protein complex would behave as a competitive antagonist, i.e., an antagonist with zero efficacy, whereas others could exert various negative effects depending on their relative preferences for free receptors and the receptor/G protein complexes (21). It should also be noted that such a mechanism would have an impact on the way agonists and antagonists interact. As pointed out by Wreggett and DeLean (22), a ternary complex model in which agonists prefer coupled receptors whereas antagonists prefer uncoupled receptors predicts that the interactions between the agonists and antagonists will appear "noncompetitive" in nature. Williams *et al.* (23) have reported that the adenosine receptor antagonist 1,3-dipropyl-8-(2-amino-4-chloro)phenylxanthine "noncompetitively" inhibits the binding of [^{3}H]N^6-cyclohexyladenosine to A_1 receptors in rat brain membranes. In addition, we have found "noncompetitive" interactions between [^{3}H]XAC and ^{125}I-ABA (and other adenosine receptor agonists and antagonists) in studies using bovine cerebral cortical membranes.[3]

A somewhat surprising finding was that the incubation of solubilized receptors with the antagonist did not produce a reproducible shift in the location of the receptor to the lower density area of the sucrose gradients. It could be argued that the shift of all of the receptors to the lighter fractions when the antagonist was bound before detergent solubilization was not due to an antagonist-induced dissociation of precoupled receptors but was rather due to inability of the antagonist/receptor/G protein complexes to withstand detergent solubilization and the subsequent dissociation of this complex to generate antagonist/receptor complexes. The difference between these possibilities would be a matter of degree, because in both cases the effect of the antagonist would be to destabilize receptor/G protein interactions, albeit to different extents. Because the solubilized receptor labeled by the antagonist in the presence of Gpp(NH)p was in the lighter fractions, as compared with that labeled by the antagonist alone (Fig. 4A), we prefer the hypothesis that the antagonist induces the dissociation of receptor/G protein complexes and propose that this process is modulated by the binding of a guanine nucleotide to the G protein. Obviously, further experiments must be done to more completely resolve this point.

In summary, we have presented evidence that membrane-bound A_1 adenosine receptors exist coupled to a G protein(s) in the absence of an agonist and have postulated that xanthine-type adenosine receptor antagonists perturb the equilibrium between coupled and free receptors, resulting in the uncoupling of the "precoupled" receptors. The implications of this hypothesis with regards to the pharmacological effects of these antagonists and the mechanisms by which adenosine receptor agonists and antagonists interact have been discussed.

Acknowledgments

We thank Christine Bobichon for her expert technical assistance.

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