0026-895X/92/050765-08$03.00/0

MOLECULAR PHARMACOLOGY, 42:765–772

Indirect Effect of Guanine Nucleotides on Antagonist Binding to A_1 Adenosine Receptors: Occupation of Cryptic Binding Sites by Endogenous Vesicular Adenosine

M. RENEE PRATER, HEIDI TAYLOR, RAVI MUNSHI, and JOEL LINDEN

Departments of Internal Medicine (Cardiology) and Physiology, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

Received June 1, 1992; Accepted August 10, 1992

SUMMARY

Guanine nucleotides such as guanosine 5′-(3-*O*-thio)triphosphate (GTPγS) have been found to increase the binding of antagonists to adenosine A_1 receptors. This response can be attributed either to a direct effect of GTP on receptors to increase antagonist affinity or to an indirect effect to decrease the affinity of receptors for a pool of endogenous adenosine that cannot be readily removed from membranes. In this study, adenosine content was measured in preparations of membranes and 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS)-solubilized receptors by a sensitive radioimmunoassay. In both preparations, pools of adenosine (2.5–10 pmol/mg of protein) were detected that were resistant to deamination by added adenosine deaminase (0.5–3 units/ml) unless membrane lipids were first dissolved in acetone. Electron microscopic examination of crude CHAPS-solubilized receptors revealed the existence of small vesicles (<1 μm in diameter). Furthermore, most "solubilized" receptors were retained by a 0.1-μm filter. The effects of GTPγS were evaluated on the binding of an antagonist, 3-(4-amino-3-^{125}I-phenethyl)-1-propyl-8-cyclopentylxanthine (^{125}I-BW-A844U), to A_1 receptors of bovine brain membranes, receptors solubilized in CHAPS (crude solubilized), or receptors partially co-purified with G proteins by agonist affinity chromatography (partially purified). GTPγS (10 μM) increased antagonist binding to membranes (20–50%) and crude CHAPS-solubilized receptors (>200%) but increased binding to partially purified receptors by only 10–15%. GTPγS decreased agonist (^{125}I-N^6-aminobenzyladenosine) binding and increased antagonist B_{max}, but did not significantly decrease (5%) the dissociation rate of the antagonist. Omission of Mg^{2+} mimicked the effects of GTPγS on agonist and antagonist binding and increased both the association and dissociation rates of ^{125}I-BW-A844U. These data suggest that a Mg^{2+}-dependent GTPγS-induced increase in antagonist binding to membranes and solubilized receptors is primarily due to unmasking of cryptic binding sites occupied by contaminating vesicular adenosine. These findings are consistent with the observation that adenosine receptor antagonists have been found to have little or no inverse agonist physiological effects in well oxygenated tissues.

GTP (and GTP analogs such as GTPγS) decrease the affinity of A_1 receptors for agonists by uncoupling receptors from G proteins (1–9). GTP also has sometimes been found to increase the binding of antagonists to A_1 receptors on brain membranes by as much as 2-fold (1, 4–7, 10). Effects of guanine nucleotides on antagonist binding have been interpreted as supporting the existence of "negative efficacy," i.e., the concept that antagonists bind preferentially to uncoupled receptors. A significant physiological corollary to negative efficacy is that antagonists will elicit "inverse agonist" responses in tissues, i.e., trigger responses opposite to agonists, even if no agonist is present. An alternative hypothesis is that GTPγS increases antagonist binding due to endogenous adenosine trapped inside membrane vesicles. By decreasing the affinity of receptors for trapped adenosine, the guanine nucleotides may indirectly increase the binding of antagonists. Klotz *et al.* (10) noted that guanine nucleotides increase the binding of the antagonist [^{3}H]DPCPX to bovine brain A_1 receptors solubilized in CHAPS. This was taken as evidence of a direct effect of GTP on the binding of antagonists to A_1 receptors. However, "solubilized" receptors, defined as receptors remaining in a 100,000 × *g* supernatant after centrifugation of detergent-treated membranes for 1 hr, appear slightly turbid and are not necessarily free of small vesicles. In this study we undertook four lines of experimentation to test the notion that both membranes and solubilized receptors contain a pool of trapped vesicular adenosine that is primarily responsible for the effect of GTPγS to increase

This work was supported by National Institutes of Health Grant RO1-HL-37942.

ABBREVIATIONS: GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; CHAPS, 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate; G proteins, guanine nucleotide-binding proteins; ABA, N^6-aminobenzyladenosine; BW-A844U, 3-aminophenyethyl-1-propyl-8-cyclopentylxanthine; PIA, N^6-phenylisopropyladenosine; RIA, radioimmunoassay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

antagonist binding, i.e., 1) RIA to define a cryptic pool of adenosine inaccessible to the enzyme adenosine deaminase, 2) electron microscopy to visualize small vesicles in CHAPS-solubilized receptors, 3) evaluation of the effects of GTPγS on antagonist binding to A_1 receptors of membranes, crude solubilized receptors, and partially purified receptor-G protein complexes, and 4) evaluation of the effects of GTPγS on the kinetics of antagonist binding. The results of all of these approaches are consistent with the notion that effects of GTPγS on antagonist binding to adenosine A_1 receptors are due primarily to cryptic vesicular pools of endogenous adenosine that exist in membranes and CHAPS-solubilized receptors.

Materials and Methods

RIA of adenosine in membranes and soluble receptors. Bovine brain membranes or CHAPS-solubilized receptors were incubated with adenosine deaminase (0.1–3 units/ml) for 30 min at 21°. Aliquots (250 μl) were added to 750 μl of ice-cold acetone to dissolve membranes lipids and extract adenosine. Acetone extracts were centrifuged at 10,000 × *g* for 5 min, and the supernatants were dried under argon. After the residues were resuspended in 50 μl of water, nucleotides, which cross-react weakly with antiadenosine antisera, were removed by precipitation upon sequential addition of 25 μl each of 0.3 M $ZnSO_4$ and $Ba(OH)_2$, followed by centrifugation. Adenosine remaining in the supernatant was benzylated and quantitated by RIA as previously described (11), except that the volume of benzylbromide/dimethylacetamide was reduced to 25 μl. In some experiments adenosine deaminase was added after the zinc/barium precipitation step to establish that immunoreactive adenosine could be deaminated to nearly undetectable levels after acetone extraction and zinc/barium treatment.

Preparation of bovine brain membranes and solubilized receptors. Bovine cortices purchased from Pel-Freez were disrupted in a Brinkmann homogenizer at setting 2.5 for 20 sec in 4 volumes of ice-cold buffer A (10 mM HEPES, 10 mM EDTA, 0.1 mM benzamide, 0.1 mM phenylmethylsulfonylfluoride, pH 7.4), filtered through four layers of gauze, and centrifuged at 20,000 × *g* for 30 min. The pellet was washed twice in 5 volumes of HE buffer (10 mM HEPES, 0.1 mM benzamidine, 1 mM EDTA, pH 7.4) and resuspended in 3.5 volumes of E buffer. For solubilization, 0.5 volumes of 10% CHAPS was added and membranes were stirred on ice for 20 min before centrifugation at 140,000 × *g* for 90 min. The supernatant was frozen at −20° after addition of 10 mM $MgCl_2$ and 10% glycerol and readjustment of the pH to 7.4. For electron microscopy this solubilization protocol was modified such that membranes were resuspended in 10 volumes of 1% CHAPS and centrifuged at 100,000 × *g* for 1 hr.

Electron microscopy. CHAPS-solubilized receptors were mixed 1:1 with warm agar in isotonic saline. Receptors in the solidified agar were fixed in 2% glutaraldehyde, rinsed, and postfixed in osmium tetroxide. After incubation for 2 hr in 3% uranyl acetate, samples were dehydrated, infiltrated with Polybed 812 resin (Polysciences, Inc.), sectioned, stained with saturated uranyl acetate and lead citrate, and viewed at magnifications of 4,000 to 31,500.

Affinity chromatography. Adenosine A_1 receptor-G protein complexes were co-purified 1000–2000-fold as described (12). Adenosine deaminase (1 unit/ml) and 0.1 M NaCl were added to solubilized receptors (150 ml), which were then applied to a 3-ml ABA-agarose column at a rate of 50 ml/hr and washed with buffer (10 mM HEPES, 10% glycerol, 0.1% CHAPS, 0.01% asolectin, 0.1 M NaCl, 10 mM $MgCl_2$, pH 7.4) for 15 hr at 21°. Receptor-G protein complexes were eluted in 40 ml of the same buffer supplemented with 10 μM GTP. The guanine nucleotide was removed by gel filtration before radioligand binding assays.

Radioligand binding assays. Equilibrium binding was measured in 100 μl, using 50 μl of 20–40-fold diluted membranes, crude soluble receptors, or partially purified soluble receptors with 2.5 units/ml adenosine deaminase, with or without 5 mM $MgCl_2$, with or without 10 μM GTPγS, and with 50 μl of agonist (^{125}I-ABA) or antagonist (^{125}I-BW-A844U) radioligand. Preliminary dose-response curves indicated that 10 μM GTPγS was sufficient to produce maximal effects on both agonist and antagonist binding. Paired assays (with or without GTPγS) were terminated by filtration after a 3-hr incubation at ambient temperature (19–22°). Nonspecific binding was measured in the presence of 1 μM DPCPX. Association and dissociation kinetics were measured during incubation in a water bath maintained at 30 ± 0.5°. This was to ensure that precisely the same temperature was used in evaluating kinetic constants in experiments run on different days. Dissociation kinetics were measured beginning with the addition of 1 μM DPCPX. Data were fit to the equation:

$$B = B_{eq}(\exp(-k_{-1} \cdot t)) + NS$$

where B_{eq} is specific binding at equilibrium (3 hr), NS is nonspecific binding, and k_{-1} is the dissociation rate constant. Association data were fit simultaneously to two equations:

$$B = B_{eq}(1 - \exp(k_{-1} - k_1 \cdot L) \cdot t) + NS$$

$$L_t = L + B$$

where L_t is total radioligand, L is free radioligand, and k_1 is the association rate constant. Parameters were fit to these equations using Marquardt's nonlinear least squares method (13).

Results

Cryptic pools of adenosine in membranes and crude soluble receptors. Adenosine was measured in membranes and crude solubilized receptors by a sensitive RIA. As shown in Fig. 1A, the size of a residual adenosine pool in membranes incubated with 1 unit/ml adenosine deaminase was linearly

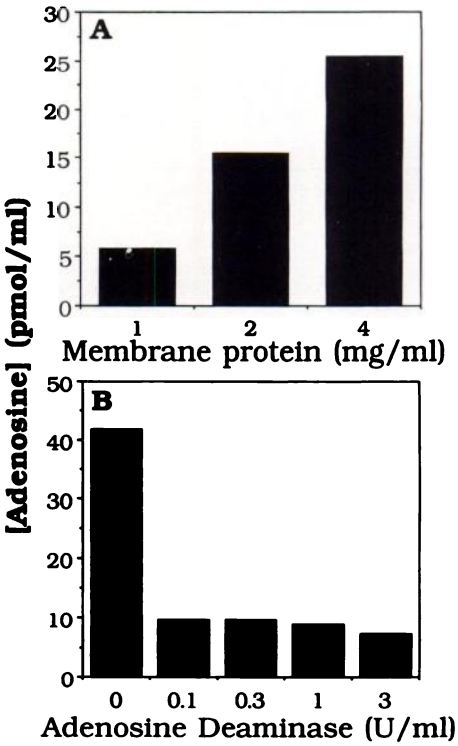

Fig. 1. Effects of adenosine deaminase on adenosine concentrations in bovine brain membranes. Membranes were incubated for 30 min with 1 unit/ml adenosine deaminase and various concentrations of membrane protein (A) or 1 mg/ml membrane protein and various concentrations of adenosine deaminase (B). Residual adenosine was detected by RIA.

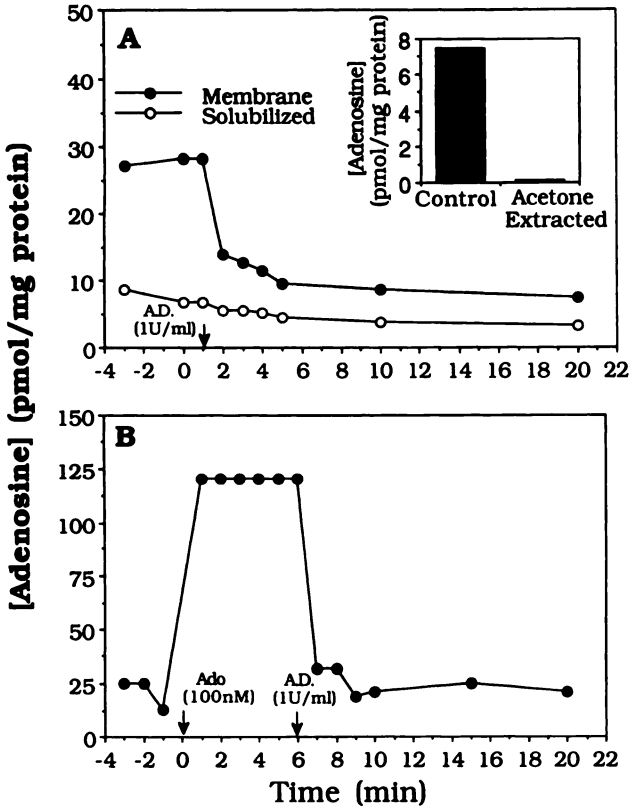

Fig. 2. Kinetics of changes in the adenosine content of membranes and CHAPS-solubilized bovine brain after the addition of adenosine deaminase or adenosine. A, Residual adenosine was assayed at various times before and after the addition of adenosine deaminase (*A.D.*). *Inset*, virtually complete removal of residual adenosine after disruption of membrane lipids and extraction of adenosine with acetone. B, Residual membrane adenosine was assayed at various times before and after the sequential addition of adenosine (*Ado*) and adenosine deaminase.

related to membrane concentration. When normalized to protein level, residual adenosine found in bovine brain membranes ranged from 6 to 10 pmol/mg of protein. If this pool is inaccessible to adenosine deaminase we reasoned that increasing the enzyme concentration would not eliminate this cryptic adenosine. Such a result is shown in Fig. 1B. Varying the concentration of adenosine deaminase between 0.1 and 3 units/ml had very little effect on the size of the cryptic pool. Fig. 2A shows the time course of adenosine deaminase action and demonstrates that a cryptic adenosine pool exists in CHAPS-solubilized receptors as well as in membranes. The cryptic adenosine was apparently released by dissolving membrane lipids with acetone, because the addition of adenosine deaminase to the acetone extract resulted in virtually complete removal of immunoreactivity (Fig. 2A, *inset*). This indicates that adenosine immunoreactivity can be reduced to a low level (<0.15 nM) by deamination with adenosine deaminase and that residual immunoreactivity in membranes is not due to an immuno-cross-reactive adenyl purine that cannot be deaminated. Membranes do not contain any inhibitor of adenosine deaminase, inasmuch as the enzyme could rapidly and completely deaminate exogenously added adenosine (Fig. 2B).

Small vesicles in CHAPS-solubilized receptors. The existence of cryptic pools of adenosine resistant to deamination could be accounted for by the presence of vesicles that exclude the enzyme. In order to determine whether such vesicles exist in CHAPS-solubilized receptors, solubilized preparations were embedded in agar and processed for electron microscopy. Fig. 3 shows microscopic evidence of what appear to be small vesicles, 0.1–0.5 μm in diameter. Such receptors are defined as "soluble" because they are found in the supernatant after centrifugation at 100,000 × *g* for 1 hr. Because it is possible that the apparent vesicles are fixation artifacts, we sought additional evidence in support of the existence of vesicles. CHAPS-"solubilized" preparations were passed through an Amicon type H1MPO1-43 hollow-fiber filter with a pore size of 0.1 μm. In three such experiments, only 20–30% of receptors (^{125}I-BW-A844U binding sites) passed through the pores. These data suggest that most receptors in CHAPS-solubilized preparations are not free in solution but are associated with large structures such as vesicles.

Effects of GTPγS on radioligand binding to A_1 adenosine receptors. Figs. 4 and 5 illustrate typical effects of 10 μM GTPγS on agonist and antagonist binding, respectively, to A_1 receptors on bovine brain membranes. Membranes were pretreated with 10 mM EDTA (see Materials and Methods) to strip tightly bound divalent cations. Under these circumstances, high affinity agonist binding was completely dependent on the addition of a divalent cation. In the presence of Mg^{2+}, the agonist ^{125}I-ABA binds with high affinity to A_1 receptor-G protein complexes in bovine membranes. The effect of GTPγS was to convert 80–85% of receptors to a low affinity, probably uncoupled, state (Fig. 4). In the presence of GTPγS, Scatchard plots of agonist binding appeared curvilinear, indicative of binding to coupled and uncoupled receptors, but due to the low level of binding two-site binding parameters could not be precisely determined. Single-site ^{125}I-ABA binding parameters summarized from four experiments in the absence and presence of GTPγS, respectively, are as follows: B_{max}, 354 ± 11 and 99 ± 7 fmol/mg protein; apparent K_d, 0.12 ± 0.02 and 0.33 ± 0.04 nM. It is possible that a small pool of residual high affinity agonist binding sites observed in the presence of GTPγS is due

TABLE 1

Kinetic parameters of ^{125}I-BW-A844U binding to bovine brain membranes

Bovine brain membranes were incubated at 30 ± 0.5° in buffer containing 2.5 units/ml adenosine deaminase and 1 mM EDTA, without and with 5 mM Mg^{2+} and 10 μM GTPγS. Results are from four or five experiments.

	+Mg^{2+}		−Mg^{2+}	
	−GTPγS	+GTPγS	−GTPγS	+GTPγS
B_{max}[a]	1.0 (control)	1.28 ± 0.13[b]	1.21 ± 0.11[b]	1.23 ± 0.12[b]
K_{-1} (min^{-1})	0.098 ± 0.008	0.093 ± 0.005	0.14 ± 0.006[b]	0.14 ± 0.004[b]
K_1 (nM^{-1} min^{-1})	0.60 ± 0.03	0.67 ± 0.13	1.03 ± 0.17[b]	1.03 ± 0.17[b]
K_{-1}/K_1 (nM)	0.16	0.13	0.14	0.14

[a] In each experiment the four conditions indicated were assayed on the same day. B_{max} was normalized to control (+Mg^{2+} and −GTPγS).
[b] Different from control, $p < 0.05$ by the paired Student's *t* test.

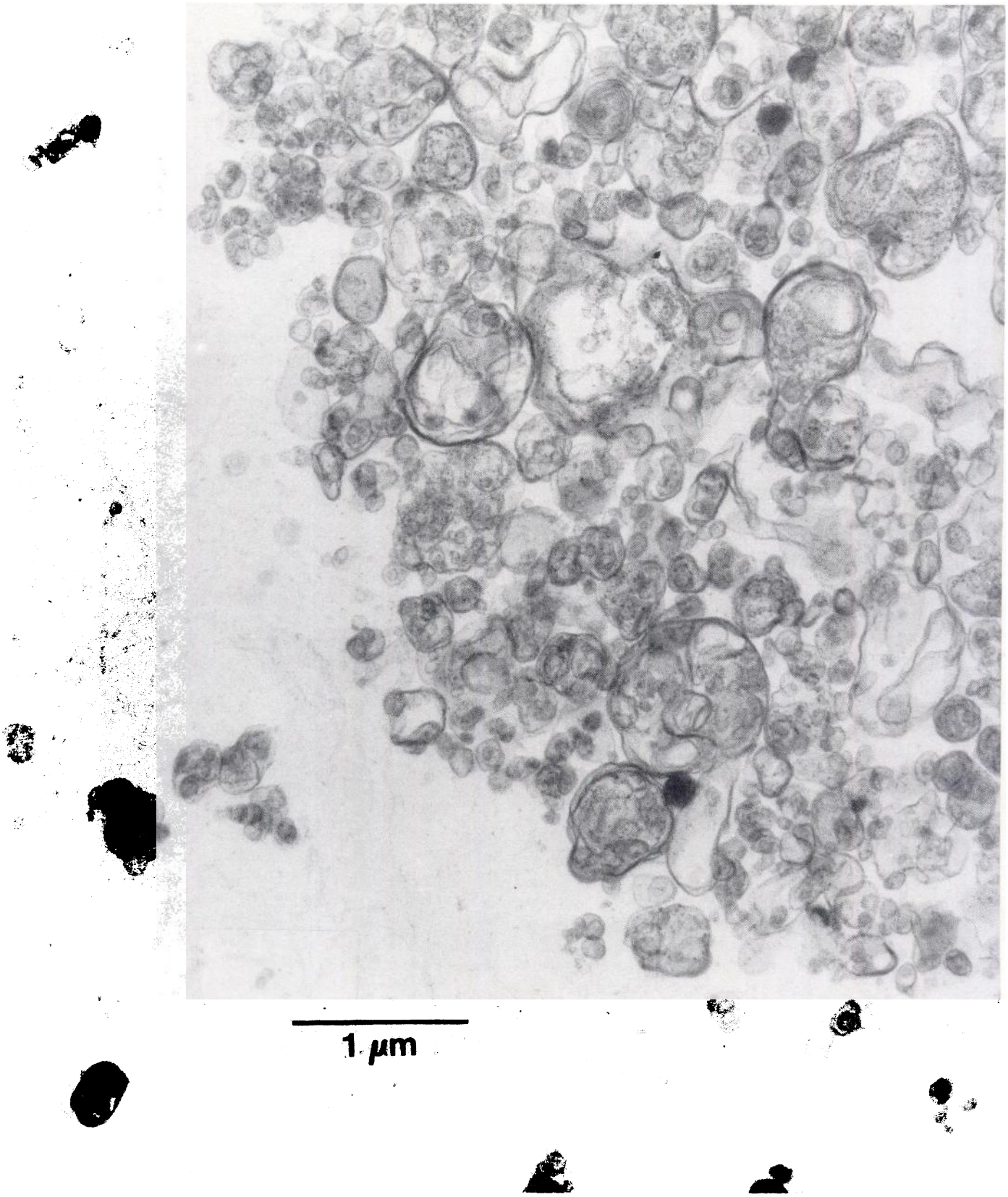

Fig. 3. Microscopic evidence of small vesicles in CHAPS-solubilized receptors. Solubilized receptors were prepared as described in Materials and Methods. The electron micrograph shown is typical of four experiments in which small vesicular structures were observed (magnification, 31,500).

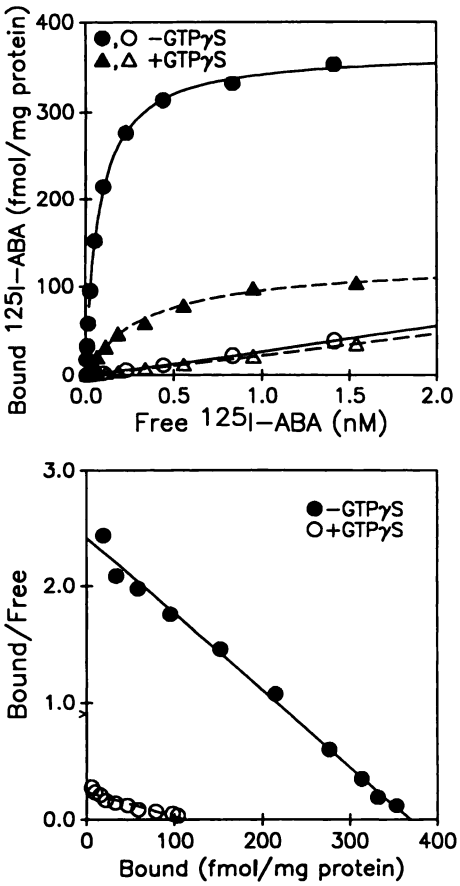

Fig. 4. Effect of GTPγS on the binding of ^{125}I-ABA to adenosine A_1 receptors of bovine brain membranes. Untransformed (*top*) and Scatchard-transformed (*bottom*) data from a single typical experiment are shown. Single-site binding parameters in the absence and presence of GTPγS, respectively, are as follows: B_{max}, 369 and 115 fmol/mg protien; K_d, 0.09 and 0.32 nM. In the *top graph*, *open symbols* depict nonspecific binding.

to the presence of some G proteins trapped within vesicles and hence inaccessible to the guanine nucleotide.

The antagonist ^{125}I-BW-A844U also binds with high affinity to bovine brain membranes. The effect of GTPγS on antagonist binding was to increase the B_{max} without changing the K_d (Fig. 5). We next examined the effects of GTPγS on association and dissociation kinetics of ^{125}I-BW-A844U in the absence and presence of Mg^{2+}, as illustrated in Fig. 6 and summarized in Table 1. On average, the addition of GTPγS in the presence of Mg^{2+} decreased the dissociation rate by 5% and increased the association rate by 12%, but these changes did not reach statistical significance. GTPγS did produce a variable but significant increase in the B_{max}. GTPγS had no effect on membranes in the absence of Mg^{2+} (and other divalent cations). The effect of GTPγS to increase B_{max} was mimicked by the omission of Mg^{2+} (Fig. 6; Table 1). It is notable that, although the omission of Mg^{2+} did not alter the K_d of ^{125}I-BW-A844U, the absence of divalent cations did substantially increase (by about 50%) both the association and dissociation rates constants of the antagonist (Table 1).

Effects of GTPγS on radioligand binding to crude and partially purified CHAPS-solubilized receptors. The effects of GTPγS on the binding of single concentrations of radioligands to membranes, crude soluble receptors, and partially purified receptors are shown in Fig. 7A. One surprising finding of this series of experiments was the relatively large increase in ^{125}I-BW-A844U binding in crude soluble receptors (2.5–3-fold versus <1.5-fold in membranes). By comparison with crude soluble receptors, GTPγS produced only a very modest 10–15% increase in antagonist radioligand binding to partially purified receptors. It could be argued that GTPγS produces only a small effect on antagonist binding to purified receptors because they are largely uncoupled from G proteins. However, as reported previously (12), agonist affinity chromatography results in the purification of receptor-G protein complexes that are well coupled. The guanine nucleotide substantially (60–80%) inhibited high affinity agonist binding to all three receptor preparations. This was further verified by the experiment shown in Fig. 7B, which shows competition by the agonist (*R*)-PIA for ^{125}I-BW-A844U binding to purified receptors. Based on the fraction of high affinity agonist binding, the purified receptors are >50% coupled.

Discussion

The results of this series of experiments indicate that there is a cryptic pool of adenosine present in bovine brain membranes that is inaccessible to adenosine deaminase. This pool of adenosine (6–10 pmol/mg of protein) can be metabolized by adenosine deaminase if lipids are first dissolved in acetone. We

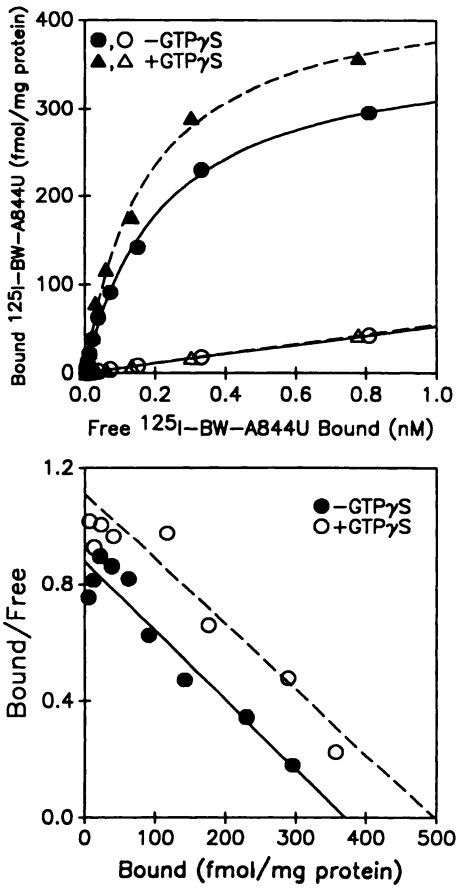

Fig. 5. Effect of GTPγS on the binding of ^{125}I-BW-A844U to adenosine A_1 receptors of bovine brain membranes. Untransformed (*top*) and Scatchard-transformed (*bottom*) data are shown. Binding parameters in the absence and presence of GTPγS, respectively, are as follows: B_{max}, 366 and 475 fmol/mg protein; K_d, 0.26 and 0.24 nM. In the *top graph*, *open symbols* depict nonspecific binding. Similar results were found in two additional experiments.

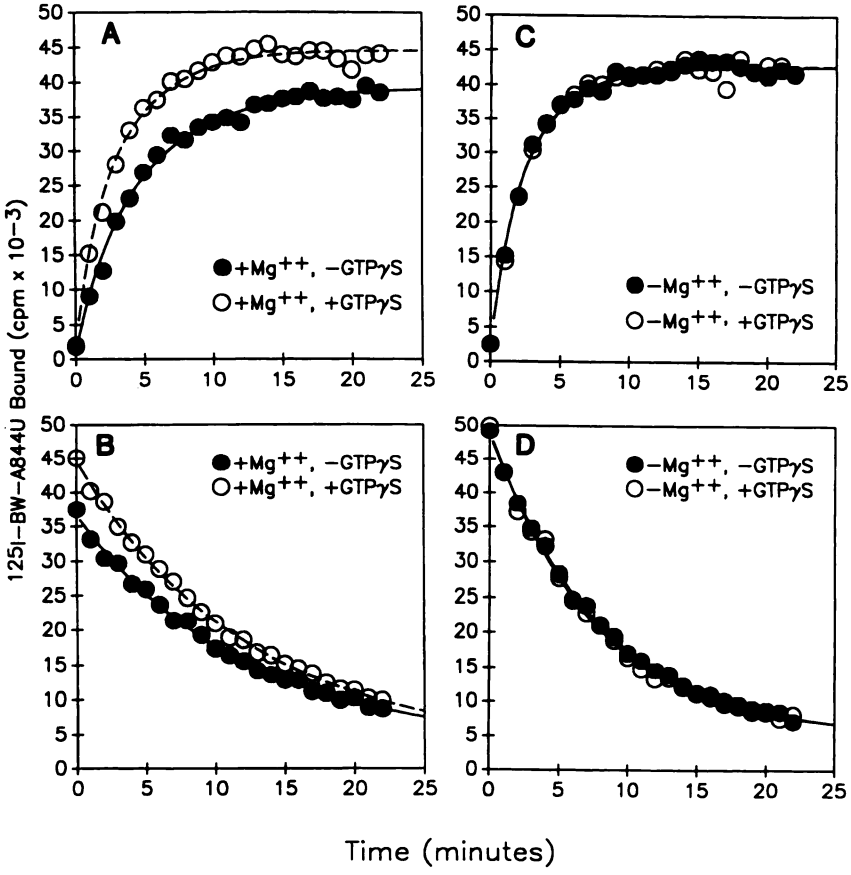

Fig. 6. Effects of GTPγS and Mg^{2+} on the kinetics of ^{125}I-BW-A844U binding to bovine brain membranes. A single typical experiment is shown. A and C, Association data, with nonspecific binding measured in the presence of 1 μM DPCPX (*time 0 point*). B and D, Dissociation data, initiated after binding reached equilibrium (3 hr). Nonspecific binding was entered as the *100-min point* (not graphed). The *lines* were fit by single-exponential functions, as described in Materials and Methods. Fitting the data to biexponential functions did not significantly improve the goodness of fits. Data from multiple experiments are summarized in Table 1.

have shown previously that bovine brain membranes persistently produce adenosine, possibly from a pool of trapped nucleotide (14), and Schiemann *et al.* (15) detected an increase in antagonist radioligand binding to A_1 receptors of smooth muscle upon permeabilization of presumptive membrane vesicles with detergents. Parkinson and Fredholm (16) detected adenosine (25–50 pmol/mg of protein) and adenine nucleotides (300–1100 pmol/mg of protein) in tissue sections of rat striatum incubated with adenosine deaminase for 2 hr. The existence of vesicles containing adenosine may account for the ability of A_1 adenosine receptor antagonists to increase adenylyl cyclase activity in adipocyte membranes treated with adenosine deaminase (17, 18), a response the authors attributed to inactivation of G proteins by adenosine receptor antagonists, based on the probably incorrect premise that all endogenous adenosine is accessible to adenosine deaminase.

More surprising than the existence of a cryptic pool of adenosine in membrane vesicles was the detection of a similar pool in CHAPS-solubilized receptors. The existence of a cryptic pool of adenosine in the 100,000 × *g* × 1 hr supernatant of bovine brain membranes treated with CHAPS suggested that the supernatant might contain small buoyant vesicles that are not sedimented. In addition to the existence of an adenosine deaminase-resistant pool of adenosine present in the CHAPS-solubilized preparation, two other pieces of data support the existence of vesicles; the first is electron microscopic evidence that reveals what appear to be small (0.1–0.5-μm) vesicular structures in CHAPS-solubilized receptors after the receptors are embedded in agar, and the second is the finding that most solubilized receptors are found in the retentate after filtration through 0.1-μm pores, suggesting that receptors are associated with large structures. It is likely that the quantity and stability of the vesicular structures depend on a number of factors that were not systematically examined in this study, such as pH and concentrations of membrane protein, detergent, and divalent cations.

In this study we noted that GTPγS increases the binding of an antagonist radioligand, ^{125}I-BW-A844U, to A_1 receptors of membranes, to crude solubilized receptors, and to a small extent to partially purified solubilized receptors. Similar responses have been observed in several previous studies (1, 4, 6, 7, 10, 19). The magnitude of the increase in antagonist binding is quite variable and has been reported to result from an increase in antagonist affinity, an increase in the number of receptors, or both. Such variability in responses might be the result of differences in the number of vesicles formed and their orientation, their leakiness, or the amount of adenosine trapped within them or on species differences in the affinity of receptors for adenosine and various radioligands. We found no evidence that GTPγS increases the affinity of bovine A_1 receptors for antagonists, inasmuch as the guanine nucleotide increased the B_{max}, but did not change the K_d for ^{125}I-BW-A844U binding. Moreover, there was no discernible effect of GTPγS on the dissociation rate of prebound antagonist. An increase in affinity would predictably decrease the dissociation rate. Klotz *et al.* (10) argued that the effect of GTP on [^{3}H]DPCPX binding to adenosine A_1 receptors is not dependent on endogenous adenosine because washing reversed the effect of GTP, the effect was restored by readdition of the guanine nucleotide, and the effect also was observed in CHAPS-solubilized receptors. How-

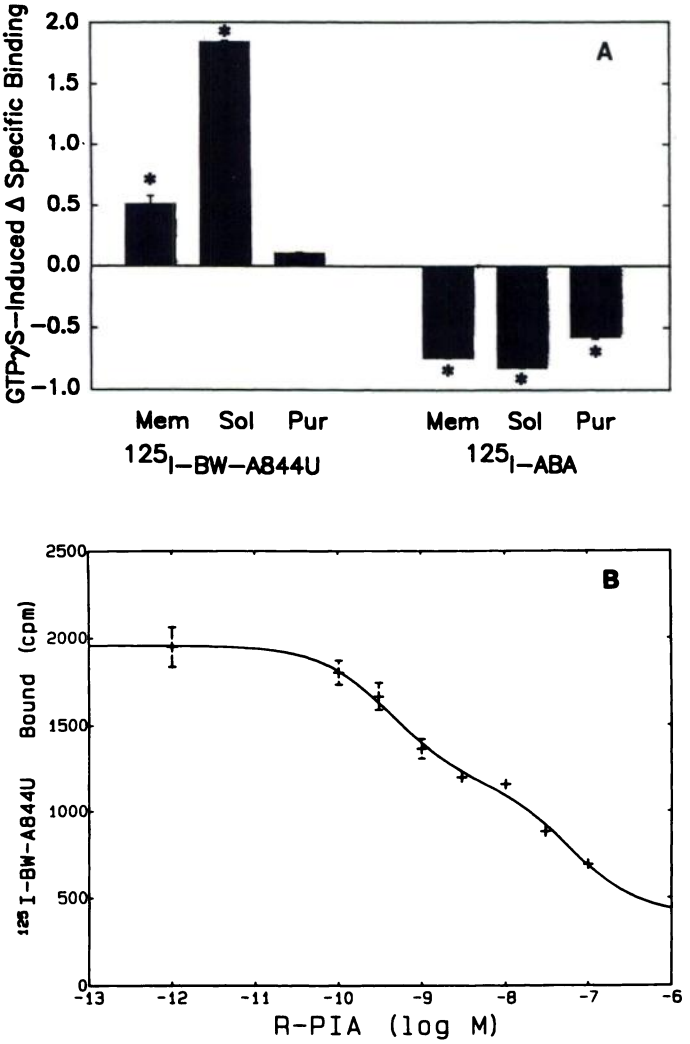

Fig. 7. Effects of GTPγS on equilibrium binding of ^{125}I-BW-A844U and ^{125}I-ABA to various adenosine A_1 receptor preparations. A, Single concentrations of radioligands were used in three or four experiments, each consisting of three to six replicates. The averaged ratio − 1 of specific binding measured in the absence and presence of 10 μM GTPγS is plotted. *Mem*, membranes; *Sol*, crude CHAPS-solubilized receptors; *Pur*, affinity-purified receptors. *, Significant effect of GTPγS ($p < 0.05$ by paired Student's *t* test). B, Competition by (*R*)-PIA for binding to purified receptors. K_h and K_l were calculated to be 0.29 and 37 nM, respectively. The fraction of receptors in the high affinity state is 51%. Similar results were obtained in a second experiment.

ever, these data are consistent with the possibilities that washing of vesicles does not remove entrapped adenosine and that CHAPS-solubilized receptors contain small vesicles.

Another possible explanation for the GTP-induced increase in the B_{max} of various adenosine receptor antagonists suggested by some investigators (19, 20) is that antagonists bind selectively to uncoupled receptors. The strongest evidence against the notion that antagonists are incapable of binding to coupled receptors comes from radiation inactivation analyses, which indicate that GTP reduces the apparent target size of the [^{3}H]DPCPX binding site (21). These data suggest that antagonists can bind to both large coupled receptor-G protein complexes and small uncoupled receptors.

High affinity agonist binding to brain membranes is increased by divalent cations such as Mg^{2+}. Although this effect is small in membranes not pretreated with chelators (16), pretreatment of membranes with 10 mM EDTA (as in this study) induces a divalent cation requirement for high affinity agonist binding (4, 8). Because binding of endogenous adenosine also requires Mg^{2+} (or another divalent cation), omitting Mg^{2+} should have the same effect to increase the binding of antagonists as does adding GTPγS. This is what was observed (Table 1). Omitting Mg^{2+} differed in one respect from adding GTPγS, in that Mg^{2+} decreased both the association and dissociation rates of the antagonist ^{125}I-BW-A844U. Possibly this latter effect is due in part to stabilization of vesicles by Mg^{2+}, resulting in increased diffusional barriers. Another possibility is that Mg^{2+}, by stabilizing receptor-G protein complexes, produces conformational changes in the receptor that modify its accessibility to ligands. Of practical consideration is the conclusion that the addition of Mg^{2+} to radioligand binding assays will produce potential artifactual changes in the apparent B_{max} of radioligands if care is not taken to ensure that binding reaches equilibrium. Similar conclusions were reached by Parkinson and Fredholm (16) in their analysis of the effects of Mg^{2+} on [^{3}H]DPCPX binding to brain slices.

It is striking that the increase in antagonist binding induced by GTPγS was larger in crude solubilized receptors than in membranes (see Fig. 7). One possible explanation for this is that small vesicles that form during CHAPS solubilization may be predominantly inside out, i.e., with receptors internalized and G proteins facing outward. Consistent with this possibility is the observation that solubilized receptors have much greater sensitivity to guanine nucleotides than do receptors in membranes (9). We note also that reducing the protein concentration during solubilization attenuates the magnitude of the guanine nucleotide-induced increase in B_{max} (data not shown).

Recently, Freissmuth *et al.* (22) reported that GTP produced a large (40–50%) increase in the B_{max} of [^{3}H]-xanthine amine congener (antagonist) binding sites on purified A_1 receptors reconstituted with G proteins. Of significance in interpreting these data is that fact that receptors were eluted from affinity columns with 20 mM adenosine. It is unlikely that the gel permeation chromatography method used to remove this added adenosine reduced its concentration sufficiently ($>1 \times 10^6$-fold) to preclude residual contamination by the nucleoside in quantities sufficient to affect subsequent radioligand binding assays. In the present study, receptor-G protein complexes were eluted from affinity columns with GTP. This approach avoids the reintroduction of adenosine into the purified receptor-G protein preparation. Under these conditions only a small (10–15%) effect of GTPγS on antagonist binding to purified receptors is observed, despite that fact that such receptors are well coupled (50%). The absence of a significant effect of GTPγS on the dissociation rate of prebound antagonist (<6%; Table 1) suggests that even the small increase in antagonist binding that was observed in purified receptors may be the result of low level (nanomolar) residual contaminating nucleoside.

Neurotransmitters that exist in great abundance in terminal ganglia of tissues may, like purines, be entrapped in membrane vesicles during homogenization. In this regard it is notable that muscarinic antagonists have been reported to increase adenylyl cyclase activity in heart cell membranes (23), but addition of atropine alone to cultured heart cells has no effect on cellular cAMP (24). Entrapment of neurotransmitters in membrane vesicles may also have contributed to observations of a preference of several other classes of G protein-linked receptor an-

tagonists for uncoupled receptors, e.g., α_1- and α_2-adrenergic antagonists (25, 26).

In conclusion, we have detected cryptic, probably vesicular, pools of adenosine in membranes and even in CHAPS-solubilized receptor preparations. This endogenous adenosine is responsible for most and possibly all of the apparent increase in adenosine receptor antagonist binding sites observed upon the addition of guanine nucleotides or omission of Mg^{2+}. These results suggest that guanine nucleotides have little direct effect on antagonist binding that is not attributable to blockade of endogenous adenosine. This interpretation is consistent with the absence of a physiological inverse agonist response to adenosine receptor antagonists (e.g., absence of contractile effects in myocardium) (27) except during conditions when endogenous adenosine is elevated, as in hypoxia.

Acknowledgments

We thank Susan Ramos for her expert electron microscopy, Rose Saponja for suggesting microscopic examination of CHAPS-solubilized receptors, and Amy L. Tucker for her critical review of this manuscript.

References

1. Yeung, S.-M. H., and R. D. Green. Agonist and antagonist affinities for inhibitory adenosine receptors are reciprocally affected by 5′-guanylylimidodiphosphate or *N*-ethylmaleimide. *J. Biol. Chem.* **258**:2334–2339 (1983).
2. Bruns, R. F., J. H. Fergus, E. W. Badger, J. A. Bristol, L. A. Santay, J. D. Hartman, S. J. Hays, and C. C. Huang. Binding of the A_1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **335**:59–63 (1987).
3. Patel, A., R. H. Craig, S. M. Daluge, and J. Linden. ^{125}I-BW-A844U, an antagonist radioligand with high affinity and selectivity for adenosine A_1 receptors, and ^{125}I-azido-BW-A844U, a photoaffinity label. *Mol. Pharmacol.* **33**:585–591 (1988).
4. Linden, J., C. Q. Earl, A. Patel, R. H. Craig, and S. M. Daluge. Agonist and antagonist radioligands and photoaffinity labels for the adenosine A_1 receptor, in *Topics and Perspectives in Adenosine Research* (E. Gerlach and B. F. Becker, eds.). Springer-Verlag, Berlin, 3–14 (1987).
5. Green, R. D. Reciprocal modulation of agonist and antagonist binding to inhibitory adenosine receptors by 5′-guanylylimidodiphosphate and monovalent cations. *J. Neurochem.* **4**:2472–2476 (1984).
6. Stiles, G. L. A_1 adenosine receptor-G protein coupling in bovine brain membranes: effects of guanine nucleotides, salt, and solubilization. *J. Neurochem.* **51**:1592–1598 (1988).
7. Fastbom, J., and B. B. Fredholm. Regional differences in the effect of guanine nucleotides on agonist and antagonist binding to adenosine A1-receptors in rat brain, as revealed by autoradiography. *Neuroscience* **34**:759–769 (1990).
8. Gavish, M., R. R. Goodman, and S. H. Snyder. Solubilized adenosine receptors in the brain: regulation by guanine nucleotides. *Science (Washington D. C.)* **215**:1633–1635 (1982).
9. Stroher, M., C. Nanoff, and W. Schutz. Differences in the GTP-regulation of membrane-bound and solubilized A_1-adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **340**:87–92 (1989).
10. Klotz, K.-N., R. Keil, F. J. Zimmer, and U. Schwabe. Guanine nucleotide effects on 8-cyclopentyl-1,3-[^{3}H]dipropylxanthine binding to membrane-bound and solubilized A_1 receptors of rat brain. *J. Neurochem.* **54**:1988–1994 (1990).
11. Linden, J., H. E. Taylor, M. D. Feldman, E. B. Woodward, C. R. Ayers, M. L. Ripley, S. Iflah, and A. Patel. The precise radioimmunoassay of adenosine: minimization of sample collection artifacts and immunocrossreactivity. *Anal. Biochem.* **201**:246–254 (1992).
12. Munshi, R., and J. Linden. Co-purification of A_1 adenosine receptors and guanine nucleotide-binding proteins from bovine brain. *J. Biol. Chem.* **264**:14853–14859 (1989).
13. Marquardt, D. M. An algorithm for least-squares estimation of nonlinear parameters. *J. Soc. Indust. Appl. Math.* **11**:431–441 (1963).
14. Linden, J. Adenosine deaminase to remove adenosine: how much is enough? *Trends Pharmacol. Sci.* **10**:260–262 (1989).
15. Schiemann, W. P., J. M. Walther, and I. L. O. Buxton. On the ability of endogenous adenosine to regulate purine nucleoside receptor binding of antagonists in smooth muscle membranes. *J. Pharmacol. Exp. Ther.* **255**:886–892 (1990).
16. Parkinson, F. E., and B. B. Fredholm. Magnesium-dependent enhancement of endogenous agonist binding to A_1 adenosine receptors: a complicating factor in quantitative autoradiography. *J. Neurochem.* **58**:941–950 (1992).
17. Parsons, W. J., V. Ramkumar, and G. L. Stiles. The new cardiotonic agent sulmazole is an A_1 adenosine receptor antagonist and functionally blocks the inhibitory regulator, G_i. *Mol. Pharmacol.* **33**:441–448 (1988).
18. Ramkumar, V., and G. L. Stiles. A novel site of action of a high affinity A_1 adenosine receptor antagonist. *Biochem. Biophys. Res. Commun.* **153**:939–944 (1988).
19. Leung, E., K. A. Jacobson, and R. D. Green. Analysis of agonist-antagonist interactions at A_1 adenosine receptors. *Mol. Pharmacol.* **38**:72–83 (1990).
20. Leung, E., and R. D. Green. Density gradient profiles of A_1 adenosine receptors labeled by agonist and antagonist radioligands before and after detergent solubilization. *Mol. Pharmacol.* **36**:412–419 (1989).
21. Reddington, M., K.-N. Klotz, M. J. Lohse, and B. Hietel. Radiation inactivation analysis of the A_1 adenosine receptor of rat brain: decrease in radiation inactivation size in the presence of guanine nucleotide. *FEBS Lett.* **252**:125–128 (1989).
22. Freissmuth, M., E. Selzer, and W. Schütz. Interactions of purified bovine brain A_1-adenosine receptors with G-proteins: reciprocal modulation of agonist and antagonist binding. *Biochem. J.* **275**:651–656 (1991).
23. Hilf, G., and K. H. Jakobs. Agonist-independent inhibition of G protein activation by muscarinic acetylcholine receptor antagonists in cardiac membranes. *Eur. J. Pharmacol.* **225**:245–252 (1992).
24. Linden, J. Enhanced cAMP accumulation after termination of cholinergic action in the heart. *FASEB J.* **1**:119–124 (1987).
25. Ernsberger, P., and D. C. U'Prichard. Modulation of agonist and antagonist interactions at kidney α1-adrenoceptors by nucleotides and metal ions. *Eur. J. Pharmacol.* **133**:165–176 (1987).
26. Asakura, M., T. Tsukamoto, J. Imafuku, M. Matsui, M. Ino, and K. Hasegawa. Quantitative analysis of rat brain α2-receptors discriminated by [^{3}H]clonidine and [^{3}H]rauwolscine. *Eur. J. Pharmacol.* **106**:141–147 (1984).
27. Clemo, H. F., A. Bourassa, J. Linden, and L. Belardinelli. Antagonism of the effects of adenosine and hypoxia on atrioventricular conduction time by two novel alkylxanthines: correlation with binding to adenosine A1 receptors. *J. Pharmacol. Exp. Ther.* **242**:478–484 (1987).

Send reprint requests to: Joel Linden, Box 158, Health Sciences Center, University of Virginia, Charlottesville VA, 22908.