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Measurement of Guanine Nucleotide-Binding Protein Activation by A_1 Adenosine Receptor Agonists in Bovine Brain Membranes: Stimulation of Guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate Binding

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SUMMARY

Signal transduction by A_1 adenosine receptors was investigated by measuring the modulation by adenosine agonists of guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTP[S]) binding to guanine nucleotide-binding proteins (G proteins). The extent of stimulation of [^{35}S]GTP[S] binding was dependent on the presence of high concentrations of Mg^{2+} (1–10 mM), GDP (10 μM), and NaCl (100 mM). Under optimal conditions, the agonist (*R*)-N^6-phenylisopropyladenosine [(*R*)-PIA] stimulated binding of [^{35}S]GTP[S] to G proteins approximately 2.3-fold. All adenosine receptor agonists tested stimulated the binding of [^{35}S]GTP[S] with a rank order of potency typical of A_1 adenosine receptors in bovine tissues, (*R*)-PIA > 2-chloro-N^6-cyclopentyladenosine > (*S*)-PIA > 5′-*N*-ethylcarboxamidoadenosine > 2-[4-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine. The EC_{50} values for G protein activation correlated with the K_i value of agonists for inhibition of radioligand binding to the high affinity state of the A_1 adenosine receptor. The inclusion of 100 mM NaCl as well as increasing GDP concentrations led to a parallel increase of K_i values and EC_{50} values. In addition, both compounds induced a shift of A_1 adenosine receptors from the high affinity state for agonists to the low affinity state.

The A_1 adenosine receptor regulates the activity of several effector systems, e.g., adenylate cyclase (1, 2), potassium channels (3, 4), and phospholipase C (5), via a regulatory G protein. In brain, the interactions between this receptor and its G protein have been investigated mainly by radioligand binding studies (6–10). Functional studies of adenylate cyclase regulation by A_1 adenosine receptors are impeded by a very low signal to noise ratio in brain (2, 11, 12). Modulation of G protein activity by A_1 adenosine receptors has been demonstrated by stimulation of a low-K_m GTPase (13) in intact membranes and by stimulation of [^{35}S]GTP[S] binding to purified G proteins by affinity-purified A_1 adenosine receptors (14).

In this study the activation of G proteins by adenosine receptor agonists in bovine cerebral cortex has been investigated by measurement of [^{35}S]GTP[S] binding to G proteins, which reflects the GDP-GTP exchange reaction stimulated by receptor agonists. Stimulation of [^{35}S]GTP[S] binding to G proteins and adenosine receptor radioligand binding experiments have been performed under identical assay conditions. The effects of NaCl and GDP on G protein activation and receptor binding were compared.

Experimental Procedures

Materials

[^{35}S]GTP[S] (1000–1500 Ci/mmol) and [^{3}H]DPCPX (120 Ci/mmol) were obtained from New England Nuclear (Dreieich, Germany). Adenosine deaminase (from calf intestine; 200 units/mg), CHAPS, GDP, GDP[S], (*R*)-PIA, (*S*)-PIA, and unlabeled GTP[S] were purchased from Boehringer (Mannheim, Germany). Bovine serum albumin, dithiothreitol, and NEM were from Sigma (Deisenhofen, Germany). CCPA, CGS 21,680, and NECA were from Research Biochemicals Inc. (Cologne, Germany). All other chemicals were obtained from standard sources and were of the highest purity commercially available.

Preparation of Bovine Cortical Membranes

Fresh bovine brain was obtained from a local slaughterhouse. The pia mater and arachnoidea were removed, and the cortical layer was dissected from the white matter. The tissue was homogenized with a motor-driven glass-Teflon pestle in 0.32 M sucrose (9 ml of sucrose/g of tissue). All centrifugation procedures were performed at 4°. The crude homogenate was centrifuged for 10 min at 1000 × *g*. The supernatant was recentrifuged at 100,000 × *g* (37,000 rpm in a Beckmann Ti 60 rotor). The supernatant was discarded, and the pellet was washed twice in water. The final pellet was resuspended in 50 mM Tris·HCl,

ABBREVIATIONS: G protein, guanine nucleotide-binding protein; CCPA, 2-chloro-N^6-cyclopentyladenosine; CGS 21,680, 2-[4-(2-carboxyethyl)-phenethylamino]-5′-*N*-ethylcarboxamidoadenosine; DPCPX, 1,3-dipropyl-8-cyclopentyladenosine; GDP[S], guanosine-5′-*O*-(2-thio)diphosphate; GTP[S], guanosine-5′-*O*-(3-thio)triphosphate; NECA, 5′-*N*-ethylcarboxamidoadenosine; NEM, *N*-ethylmaleimide; PIA, N^6-phenylisopropyladenosine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

pH 7.4, at a protein concentration of 6–10 mg/ml, frozen in liquid nitrogen, and stored at −70°.

Preparation of Rat Brain Membranes

Rat brain membranes were prepared from whole brains according to the protocol described for bovine brain membranes.

Protein Determination

Protein content was measured according to the method described by Peterson (15), using bovine serum albumin as standard.

Binding of [^{35}S]GTP[S] to Membranes

Method I. Unless indicated otherwise, the incubation mixture for measuring [^{35}S]GTP[S] binding contained, in a total volume of 100 μl, 50 mM Tris·HCl, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 10 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 0.2 units/ml adenosine deaminase, 0.3–0.5 nM [^{35}S]GTP[S] (about 50,000 cpm), and 0.5% bovine serum albumin. The incubation was started by addition of the membrane suspension (usually 7.5 μg of membrane protein; for time course experiments, 2 μg/tube; for saturation experiments, 0.25 μg/tube) and was carried out in triplicate for 45 min at 25°, unless stated differently. Incubations were terminated by rapid filtration of the samples through glass fiber filters (Whatman GF/B), which had been previously soaked in 50 mM Tris·HCl, 5 mM $MgCl_2$, pH 7.4, followed by two 4-ml washes with the same buffer. No differences were detectable when nitrocellulose filters were used instead of glass fiber filters (data not shown), indicating that both filter types were similarly effective in retaining the membrane protein. Because nitrocellulose filters yielded higher filter blank values (incubations in the absence of membranes), glass fiber filters were preferred. Nonspecific binding of the radioligand was determined in the presence of 10 μM unlabeled GTP[S] and amounted to 0.2–0.5% of total [^{35}S]GTP[S]. Nonspecific binding was subtracted from total bound radioactivity. The concentration of free Mg^{2+} was calculated as described by Bartfai (16).

Method II. In some experiments, [^{35}S]GTP[S] binding and [^{3}H]DPCPX binding assays were carried out under identical conditions. In this case, incubation time for [^{35}S]GTP[S] binding was 150 min. Incubations contained 2 μg of membrane protein. At this protein concentration, [^{35}S]GTP[S] binding displayed a linear time course up to 180 min in the absence and in the presence of 1 μM (*R*)-PIA.

Binding of [^{3}H]DPCPX to Membranes

Incubations were performed under equilibrium conditions in a total volume of 2 ml and contained, unless stated differently, 50 mM Tris·HCl, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 10 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 0.2 units/ml adenosine deaminase, 0.1 nM [^{3}H]DPCPX, and 0.5% bovine serum albumin. Reactions were started by addition of membranes (40 μg/tube) and were performed in duplicate for 150 min at 25°. Nonspecific binding was determined in the presence of 100 μM (*R*)-PIA. Reactions were terminated by filtration of the samples through GF/B glass fiber filters, which had been soaked in 50 mM Tris·HCl, 5 mM $MgCl_2$, 0.02% CHAPS, pH 7.4, followed by two 4-ml washes with the same buffer.

Treatment of Membranes with NEM

Membranes at a protein concentration of 1 mg/ml were incubated for 15 min on ice with NEM at the concentrations indicated. At the end of the reaction period, membranes were centrifuged at 100,000 × *g* (37,000 rpm in a Beckmann Ti 60 rotor) for 30 min at 4°. The incubation was repeated with fresh solutions of NEM, followed by three washes with Tris buffer. Finally, the membranes were resuspended in this buffer for measurement of protein content and [^{35}S]GTP[S] binding.

Data Analysis

EC_{50} values for agonists were calculated from dose-response curves with the commercially available program GraphPad (ISI Scientific Software, Philadelphia, PA). B_{max} and K_d values, as well as K_H and K_L values (K_i values for the high and low affinity states of the receptor for agonists, respectively), were analyzed with the nonlinear curve-fitting program SCTFIT (17). Affinity and EC_{50} values from at least three independent experiments are expressed as geometric means, with 95% confidence limits, and B_{max} values are expressed as arithmetic means ± standard errors.

Results

High affinity binding sites for [^{35}S]GTP[S] were characterized by saturation experiments. Membrane protein (0.25 μg) was incubated for 150 min with increasing concentrations of the radioligand in the presence of 100 mM NaCl and in the absence of other guanine nucleotides. Nonlinear curve fitting revealed a K_d value of 1.41 nM (95% confidence limits, 1.12–1.77 nM) and a binding capacity of 90.0 ± 5.3 pmol/mg of protein (Fig. 1). Although the number of concentrations presented in Fig. 1 is too small for a two-site analysis of the saturation experiments, it seems possible that high and low affinity components are present in the membranes. However, additional data are necessary to clarify this point and the possible cause of the two components.

In the absence of GDP and NaCl, the A_1-selective agonist (*R*)-PIA did not stimulate binding of [^{35}S]GTP[S] to membranes (Fig. 2). [^{35}S]GTP[S] binding reached maximal levels after 90–120 min. The addition of increasing concentrations of unlabeled GDP markedly slowed the association of the radioligand. In the absence of NaCl, addition of 10 μM GDP was necessary to detect differences between agonist-stimulated and basal values. NaCl (100 mM) had an effect similar to that of GDP on the time course of [^{35}S]GTP[S] binding (Fig. 2). In the absence of GDP and in the presence of 0.1 μM concentrations of this nucleotide, no agonist effects were detectable. However, with 1 and 10 μM GDP (*R*)-PIA clearly stimulated binding of [^{35}S]GTP[S] to membranes. Both basal values and agonist-stimulated values for [^{35}S]GTP[S] binding showed a linear time course up to 180 min in the presence of 10 μM GDP (both in the absence and in the presence of 100 mM NaCl) and in incubations containing 1 μM GDP (only in the presence of 100 mM NaCl).

The absolute amount of membrane-bound [^{35}S]GTP[S] was diminished in the presence of NaCl and by GDP (Fig. 2). However, the relative stimulation by (*R*)-PIA was increased in

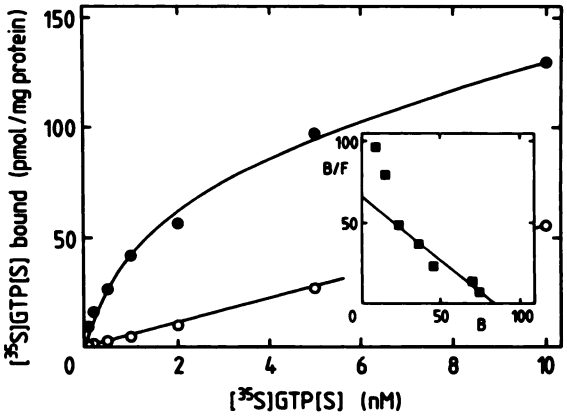

Fig. 1. Saturation of G proteins with [^{35}S]GTP[S]. Membrane protein (0.25 μg) was incubated for 150 min with increasing concentrations of the radioligand (0.1–10 nM) in the absence of other nucleotides and in the presence of 100 mM NaCl, in a total volume of 500 μl. One representative experiment of three independent experiments is shown. *Inset*, Scatchard plot of the data. ●, Total binding; ○, nonspecific binding in the presence of 10 μM GTP[S]. Nonlinear curve fitting revealed a K_d value of 1.41 (95% confidence limits, 1.12–1.77) nM and a maximum binding capacity (B_{max}) of 90.0 ± 5.3 pmol/mg of protein.

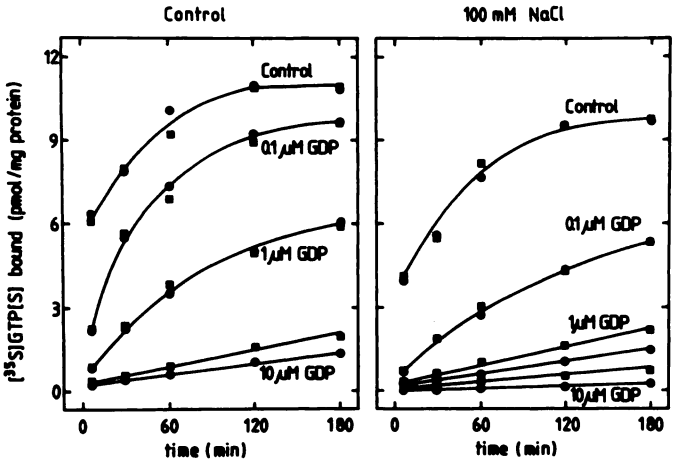

Fig. 2. Time course of [^{35}S]GTP[S] binding to bovine brain cortical membranes in the absence or presence of 0.1, 1, or 10 μM GDP. ●, Basal; ■, with 1 μM (*R*)-PIA. Incubations were performed for varying time intervals at 25° in the absence or presence of 100 mM NaCl, as described in Experimental Procedures.

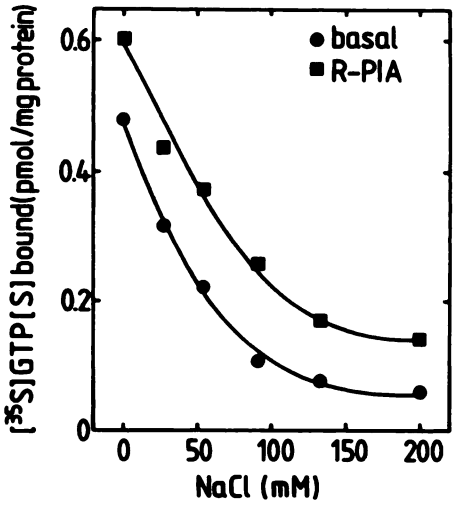

Fig. 3. Influence of NaCl on [^{35}S]GTP[S] binding to bovine cortical membranes not stimulated (●) or stimulated (■) with 1 μM (*R*)-PIA. Incubations were carried out at 25° for 45 min in the presence of increasing concentrations of NaCl and 10 μM GDP, with the additions described in Experimental Procedures.

the presence of NaCl and 10 μM GDP, compared with the values obtained in the absence of NaCl and in the presence of 1 μM GDP. To obtain a reasonable ratio between basal and agonist-stimulated data, additional incubations with adenosine receptor agonists were performed in the presence of NaCl and 10 μM GDP for 45 min.

The influence of NaCl on the binding of [^{35}S]GTP[S] was investigated in the absence and presence of 1 μM (*R*)-PIA. Fig. 3 shows the modulation of agonist-stimulated binding by NaCl. Basal values for [^{35}S]GTP[S] binding were decreased more than agonist-stimulated values. Additional incubations were carried out in the presence of 100 mM NaCl. At this concentration, the unstimulated binding of [^{35}S]GTP[S] amounted to approximately 40% of the values obtained at maximal stimulation by adenosine receptor agonists.

Stimulation of [^{35}S]GTP[S] binding above basal levels by the A_1-selective agonist (*R*)-PIA showed an absolute requirement for the presence of GDP (Fig. 4). GDP decreased both nonstimulated and agonist-stimulated binding of this radioligand. In the absence of this nucleotide and with 0.01 μM GDP, highest levels of [^{35}S]GTP[S] binding were obtained; however, no stimulation by (*R*)-PIA was evident. At higher concentrations of GDP, basal levels were decreased more than agonist-stimulated values, giving rise to clearly measureable agonist effects.

The requirement for a second, unlabeled, nucleotide was investigated in more detail by comparison of the effects of GDP, GDP[S], GTP, GTP[S], and GMP. We measured binding of [^{35}S]GTP[S] in the absence or presence of 1 μM (*R*)-PIA in the absence or presence of 0.01–100 μM concentrations of these nucleotides (Table 1). All nucleotides inhibited binding of [^{35}S]GTP[S] in nonstimulated and in agonist-stimulated incubations. GTP[S] was the most potent nucleotide, whereas inhibition by GMP was very weak. Agonist stimulation of G protein activity was observed only in the presence of GDP, GDP[S], and GTP. GDP was the most efficient compound in enhancing the ratio between agonist-stimulated and basal levels of [^{35}S]GTP[S] binding, as revealed by the fold stimulation in the presence of 1 μM (*R*)-PIA (Table 1). Additional experiments were performed to determine whether the stimulation of [^{35}S]GTP[S] binding in the presence of GTP was statistically significant. The fold stimulation and statistical significance versus control incubations in the absence of guanine nucleotides were obtained in the presence of increasing GTP concentrations (six experiments) and were as follows: 0.1 μM GTP, 1.14-fold stimulation ($p < 0.001$); 1 μM GTP, 1.73-fold stimulation ($p < 0.0001$); 10 μM GTP, 1.81-fold stimulation ($p < 0.0001$); 100 μM GTP, 1.24-fold stimulation ($p < 0.05$). The corresponding adenine nucleotides could not substitute for GDP, GDP[S], or GTP in assays of agonist-induced stimulation of [^{35}S]GTP[S] binding (data not shown).

In the presence of 10 μM GDP and 100 mM NaCl, binding of [^{35}S]GTP[S] was linear with respect to protein concentrations ranging from 1 to 20 μg/tube (data not shown).

Binding of [^{35}S]GTP[S] to bovine brain membranes required Mg^{2+} ions. The actions of Mg^{2+} appeared to be biphasic. Binding of [^{35}S]GTP[S] to G proteins occurred at concentrations above 0.1 μM free Mg^{2+}. A second, even more pronounced, increase in radioligand binding in the absence of agonists occurred at concentrations above 30 μM Mg^{2+} and proceeded steadily up to 10 mM Mg^{2+} (Fig. 5). Agonist-stimulated binding of [^{35}S]GTP[S] was also absolutely dependent on the presence

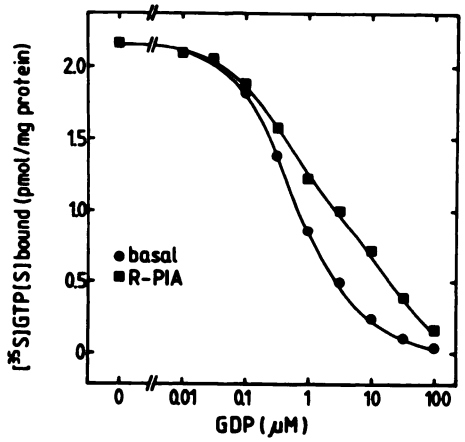

Fig. 4. Influence of GDP on [^{35}S]GTP[S] binding to bovine cortical membranes in the absence (●) or presence (■) of 1 μM (*R*)-PIA. Incubations were performed at 25° for 45 min in the presence of 100 mM NaCl, with the additions described in Experimental Procedures.

TABLE 1

Influence of five different guanine nucleotides on [^{35}S]GTP[S] binding in the absence or presence of 1 μM (*R*)-PIA

Membranes were incubated for 45 min with increasing concentrations of nucleotides and the additions described in Experimental Procedures. Levels of [^{35}S]GTP[S] binding are given as pmol of radioligand bound/mg of protein. Mean values from two experiments performed in triplicate are shown.

Nucleotide	[^{35}S]GTP[S] binding		Stimulation
	Basal	1 μM (*R*)-PIA	
μM	pmol/mg		fold
None	6.490	6.339	0.98
GDP			
0.01	5.367	5.228	0.97
0.1	2.424	2.562	1.06
1	0.689	1.108	1.61
10	0.187	0.499	2.40
100	0.081	0.173	2.14
GDP[S]			
0.01	5.927	5.093	0.97
0.1	3.651	3.852	1.06
1	1.489	1.780	1.20
10	0.416	0.727	1.74
100	0.112	0.212	1.89
GTP			
0.01	4.939	5.093	1.03
0.1	1.729	1.960	1.13
1	0.376	0.565	1.50
10	0.086	0.131	1.52
100	0.058	0.066	1.14
GTP[S]			
0.01	1.855	1.882	1.02
0.1	0.321	0.333	1.04
1	0.095	0.098	1.03
10	0.061	0.062	1.02
100	0.062	0.055	0.89
GMP			
0.01	6.394	6.336	0.99
0.1	6.338	6.304	1.00
1	6.233	6.144	0.99
10	5.940	5.836	0.98
100	3.524	3.254	0.92

of free Mg^{2+} ions. To obtain reasonable ratios between basal and (*R*)-PIA-stimulated binding of [^{35}S]GTP[S], Mg^{2+} concentrations of 100 μM or higher were required (Fig. 5). The stimulation obtained with 1 μM (*R*)-PIA was 2.58-fold at 100 μM Mg^{2+}, 3.03-fold at 300 μM Mg^{2+}, 2.74-fold at 1 mM Mg^{2+}, 2.41-fold at 3 mM Mg^{2+}, and 2.06-fold at 10 mM Mg^{2+}.

Treatment of the membranes with the sulfhydryl-alkylating agent NEM abolished agonist stimulation of [^{35}S]GTP[S] binding to G proteins (Fig. 6). An IC_{50} value of 6 μM was determined for NEM.

For a preliminary characterization of the receptor-G protein complexes activated by (*R*)-PIA, we investigated the time course of stimulation of [^{35}S]GTP[S] binding (15–180 min) by various adenosine receptor agonists. We compared the effects of equal concentrations (1 μM) of CCPA, (*R*)-PIA, NECA, and CGS 21,680 (Fig. 7). The degrees of stimulation effected by (*R*)-PIA and CCPA were identical. In contrast, in the presence of 1 μM CGS 21,680 no stimulation of radioligand binding was evident. NECA induced an intermediate level of stimulation.

To pharmacologically characterize in more detail the receptor-stimulated binding of [^{35}S]GTP[S] with respect to adenosine receptor subtypes, dose-response curves were established for five different adenosine receptor agonists. Table 2 lists the EC_{50} values for (*R*)-PIA, CCPA, (*S*)-PIA, NECA, and CGS 21,680 with bovine brain and rat brain membranes. All agonists except CGS 21,680 stimulated binding of [^{35}S]GTP[S] to a

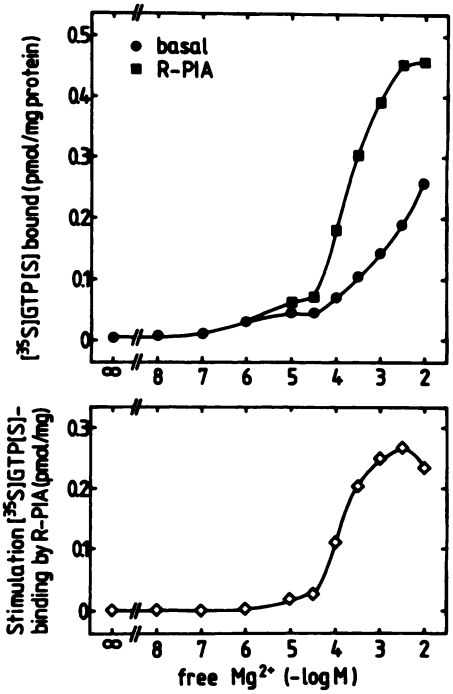

Fig. 5. Mg^{2+} requirement of control and (*R*)-PIA-stimulated binding of [^{35}S]GTP[S] to membranes from bovine brain cortex. Binding of the radioligand was measured after incubation for 45 min at 25° in the presence of 10 μM GDP and 100 mM NaCl. *Upper*, absolute values in the absence (●) or presence (■) of 1 μM (*R*)-PIA; *lower*, difference between stimulated and nonstimulated incubations (◇).

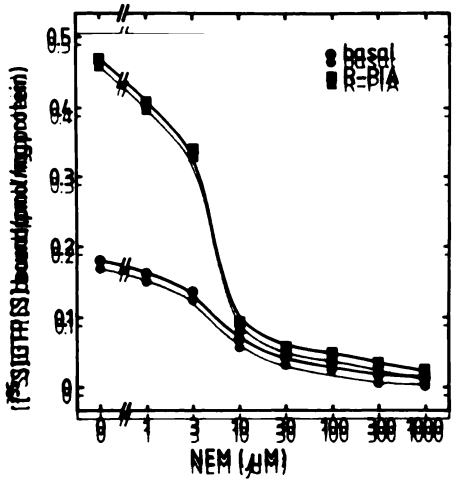

Fig. 6. Stimulation by the agonist (*R*)-PIA of [^{35}S]GTP[S] binding to control membranes and to membranes pretreated with increasing concentrations of NEM. NEM pretreatment was performed twice for 15 min on ice, followed by three washes with 50 mM Tris·HCl, pH 7.4, at 100,000 × *g* for 30 min at 4°. Membranes were resuspended in Tris buffer and incubated in the absence (●) or presence (■) of 1 μM (*R*)-PIA for measurement of [^{35}S]GTP[S] binding, as described in Experimental Procedures.

similar degree (Fig. 8). Stimulation by (*R*)-PIA, CCPA, (*S*)-PIA, and NECA amounted to approximately 110–130% above unstimulated controls. The rank order of potency with bovine brain membranes was (*R*)-PIA ≥ CCPA ≥ (*S*)-PIA ≥ NECA ≥ CGS 21,680. Stimulation by CGS 21,680 was very weak and

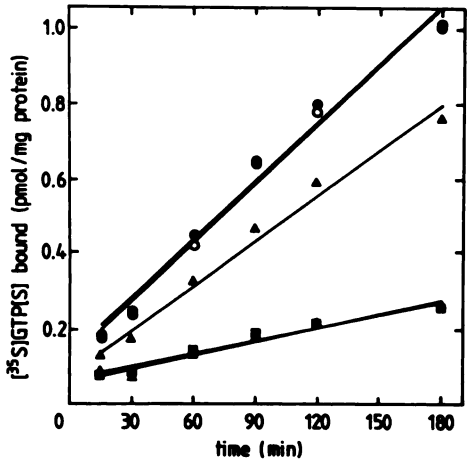

Fig. 7. Time course of [^{35}S]GTP[S] binding to bovine brain membranes (2 μg/tube) in the absence of agonists (■) or in the presence of 1 μM CCPA (○), 1 μM (*R*)-PIA (●), 1 μM NECA (▲), or 1 μM CGS 21,680 (△). Incubations were performed for different time intervals at 25°, as described in Experimental Procedures.

reached approximately 30% above base-line values at a 100 μM concentration of this agonist.

In rat brain membranes, these agonists stimulated [^{35}S]GTP[S] binding to 110–120% above nonstimulated values. A different rank order of potency of the five agonists tested was found, with CCPA > (*R*)-PIA > NECA > (*S*)-PIA > CGS 21,680 (Table 2).

The EC_{50} values determined for stimulation of [^{35}S]GTP[S] binding correlated with the K_i values for the low affinity state for agonists of the adenosine A_1 receptor, rather than with the K_i values for the high affinity state (18). Therefore, we performed radioligand binding studies of the A_1 adenosine receptor using incubation conditions applied in the [^{35}S]GTP[S] binding assay. [^{3}H]DPCPX binding studies were performed under equilibrium conditions with an incubation time of 150 min. [^{35}S]GTP[S] binding assays were done for an equal period of time (method II, 2 μg of membrane protein/tube). Binding of this radioligand showed a linear time course up to 180 min both in the absence and in the presence of 1 μM (*R*)-PIA (Fig. 2).

Saturation of A_1 adenosine receptors with the highly A_1-selective antagonist [^{3}H]DPCPX revealed a K_d value of 100 (95% confidence limits, 85–117) pM and a maximum binding capacity (B_{max}) of 714 ± 48 fmol/mg of protein (three experiments; data not shown). Competition by unlabeled (*R*)-PIA for [^{3}H]DPCPX binding revealed a high affinity binding site with an affinity of 4.49 (2.06–9.82) nM and a low affinity binding site with an affinity of 32.4 (21.6–48.7) nM, with 46 ± 7% of the receptors in the high affinity state (four experiments; data not shown). When dose-response curves for stimulation of [^{35}S]GTP[S] binding were obtained under identical conditions, half-maximal stimulation was detected at 2.93 (2.21–3.89) nM (*R*)-PIA, which is in good agreement with the affinity of (*R*)-PIA for the high affinity state for agonists of the A_1 adenosine receptor.

However, the reported affinity of (*R*)-PIA for the high affinity state for agonists of the A_1 receptor in bovine brain membranes is in the subnanomolar range (9, 18). To clarify this discrepancy, the influences of NaCl and GDP were investigated in separate experiments. When the effects of NaCl were investigated, GDP (10 μM) was always present. In experiments designed to test the effects of GDP, NaCl was present at a concentration of 100 mM.

In saturation experiments of the A_1 adenosine receptor, we found that 100 mM NaCl increased the affinity of the receptor from a K_d of 282 (167–477) pM in the absence of NaCl to 127 (88–182) pM in the presence of 100 mM concentrations of this salt, with no significant change in B_{max} values (control, 689 ± 43 fmol/mg; 100 mM NaCl, 748 ± 75 fmol/mg). Competition curves for unlabeled (*R*)-PIA competition for [^{3}H]DPCPX binding in the absence and presence of 100 mM NaCl are depicted in Fig. 9. NaCl lowered the affinities of both the high and low affinity states of the receptor for (*R*)-PIA. The following affinity values were measured: absence of NaCl, K_H, 0.59 (0.45–0.77) nM; K_L, 9.78 (8.72–10.96) nM; presence of 100 mM NaCl, K_H, 1.15 (0.81–1.63) nM; K_L, 29.19 (22.53–37.82) nM. In the presence of NaCl, a greater fraction of the receptors were shifted to the low affinity state for agonists (61.3 ± 7.9% R_H in the absence versus 41.8 ± 3.6% R_H in the presence of 100 mM NaCl).

Dose-response curves for stimulation of [^{35}S]GTP[S] binding by (*R*)-PIA in the absence or presence of 100 mM NaCl reflected parallel changes, as observed in A_1 receptor binding studies (Fig. 10). In the presence of 100 mM NaCl, dose-response curves for (*R*)-PIA were shifted to the right, which is more clearly visible when the data are presented as a percentage of the maximal effect of (*R*)-PIA stimulation (Fig. 10). Half-maximal response was observed at 0.35 (0.21–0.58) nM (*R*)-PIA under control conditions and at 1.94 (1.32–2.87) nM (*R*)-PIA when 100 mM NaCl was present. In both cases, the EC_{50} values obtained for G protein activation correlated with the affinities of the high affinity state for agonists of the A_1 receptor.

A parallel approach was applied to test the influence of GDP on receptor binding and G protein activation. Saturation studies of the A_1 receptor in the absence of GDP and in the presence of 1, 10, and 100 μM concentrations of this nucleotide revealed that GDP induced an increase in the affinity for the antagonist [^{3}H]DPCPX and a small but significant increase in the maximum binding capacity. K_d and B_{max} values are summarized in

TABLE 2

Effect of adenosine analogs on [^{35}S]GTP[S] binding to G proteins in bovine and rat brain membranes

The EC_{50} values for stimulation of [^{35}S]GTP[S] binding were calculated from three to five independent experiments for each agonist. Values in parentheses are 95% confidence limits. n_H, Hill slope.

Agonist	Bovine brain		Rat brain	
	EC_{50}	n_H	EC_{50}	n_H
	nM		nM	
(*R*)-PIA	7.9 (3.4–18.4)	0.90	16.5 (12.7–21.4)	0.84
CCPA	25.4 (11.5–55.7)	1.05	12.7 (10.5–15.3)	0.80
(*S*)-PIA	161.9 (86.8–302.1)	1.00	1,231 (900–1,684)	0.80
NECA	1,458 (738–2,881)	0.78	87.2 (77.8–97.8)	0.78
CGS 21,680	>100,000		>100,000	

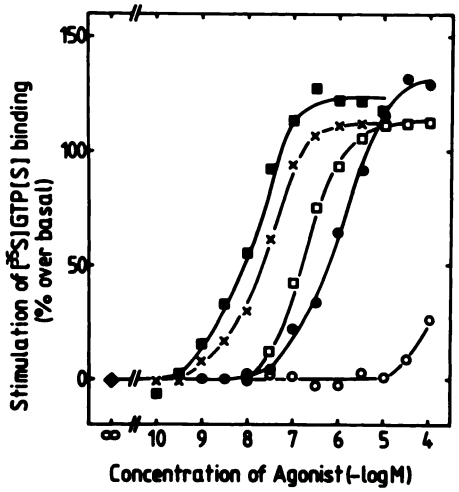

Fig. 8. Adenosine receptor-mediated stimulation of [^{35}S]GTP[S] binding to G proteins in bovine brain membranes induced by increasing concentrations of the agonists (*R*)-PIA (■), CCPA (×), (*S*)-PIA (□), NECA (●), and CGS 21,680 (○). Incubations were performed essentially as described in Experimental Procedures.

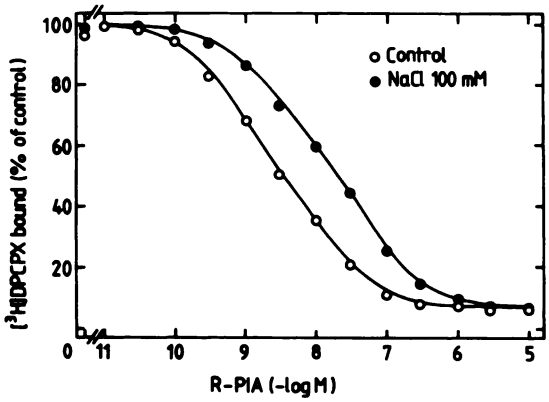

Fig. 9. Competition for [^{3}H]DPCPX binding to A_1 adenosine receptors in bovine brain membranes by unlabeled (*R*)-PIA in the absence (○) or presence (●) of 100 mM NaCl. *Curves* from one representative experiment of four independent experiments are shown. Data obtained by nonlinear curve fitting were as follows: absence of NaCl, K_H = 0.59 (0.45–0.77) nM, K_L = 9.78 (8.72–10.96) nM, 61.3 ± 7.9% R_H; presence of NaCl, K_H = 1.15 (0.81–1.63) nM, K_L = 29.19 (22.53–37.82) nM, 41.8 ± 3.6% R_H.

Table 3. In a fashion similar to that of NaCl, increasing concentrations of GDP shifted competition curves for (*R*)-PIA competition for [^{3}H]DPCPX binding to the left and increased the percentage of A_1 receptors in the low affinity state (Fig. 11; data in Table 4). However, in contrast to NaCl, the affinity of (*R*)-PIA for the low affinity state for agonists of the receptor remained unchanged (Table 4).

Stimulation of [^{35}S]GTP[S] binding by (*R*)-PIA proceeds only in the presence of GDP (Fig. 4). The influence of 1, 10, and 100 μM GDP on (*R*)-PIA dose-response curves is depicted in Fig. 12. Increasing concentrations of this nucleotide shifted the dose-response curves to the right. EC_{50} values of 0.25 (0.16–0.41) nM (1 μM GDP), 1.84 (1.10–3.04) nM (10 μM GDP), and 6.18 (4.49–8.49) nM (100 μM GDP) were calculated for (*R*)-PIA.

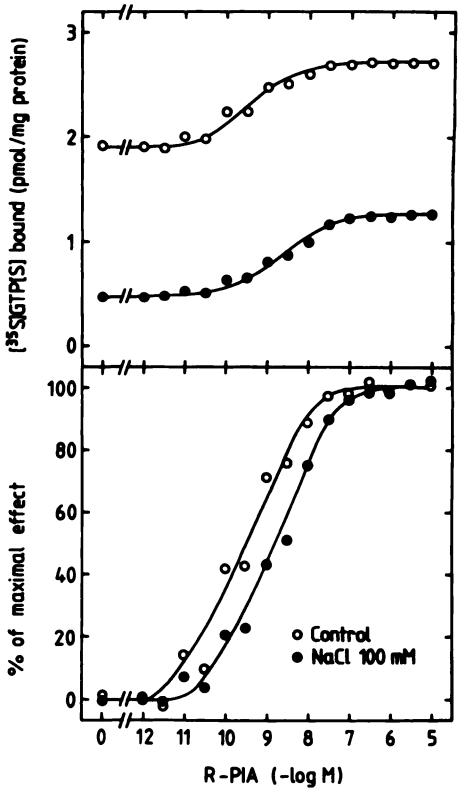

Fig. 10. Stimulation of [^{35}S]GTP[S] binding by (*R*)-PIA in the absence (○) or presence (●) of 100 mM NaCl. *Upper*, absolute values; *lower*, percentage of maximal effect. Incubations were performed for 150 min at 25° with 2 μg of membrane protein in the presence of 10 μM GDP, with the additions described in Experimental Procedures. In the absence of NaCl, an EC_{50} value of 0.35 (0.21–0.58) nM was determined. In the presence of 100 mM NaCl, the EC_{50} was shifted to 1.94 (1.32–2.87) nM (four experiments).

TABLE 3

Saturation of A_1 adenosine receptors in bovine brain membranes with the antagonist [^{3}H]DPCPX in the presence of increasing GDP concentrations

Membrane protein (40 μg) was incubated for 150 min with increasing concentrations of [^{3}H]DPCPX in the presence of 100 mM NaCl and without GDP (control) or with 1, 10, or 100 μM GDP. K_d and B_{max} values were calculated from three independent experiments. Values in parentheses are 95% confidence limits of K_d values. B_{max} values are given as arithmetic means ± standard errors. Significance of differences versus control (no GDP) was tested using the Student's *t* test for paired samples.

	K_d	*p*	B_{max}	*p*
	pM		fmol/mg	
Control	121 (114–127)		870 ± 42	
1 μM GDP	83 (79–87)	<0.001	998 ± 21	<0.05
10 μM GDP	78 (75–82)	<0.0005	1070 ± 14	<0.005
100 μM GDP	81 (76–85)	<0.0005	1025 ± 22	<0.005

Discussion

Agonist-stimulated binding of the poorly hydrolyzable GTP analog [^{35}S]GTP[S] to G proteins has been developed as a sensitive tool for investigation of the interaction of purified β-adrenergic receptors with G proteins after reconstitution (19, 20). This method has also been applied to study the coupling of purified A_1 adenosine receptors from bovine brain with purified G proteins (14, 21). Signal amplification, as determined by stimulation of [^{35}S]GTP[S] binding by other agonists, has been shown in native cardiac membranes (22) and subsequently in membranes from HL-60 cells (23). In the present study,

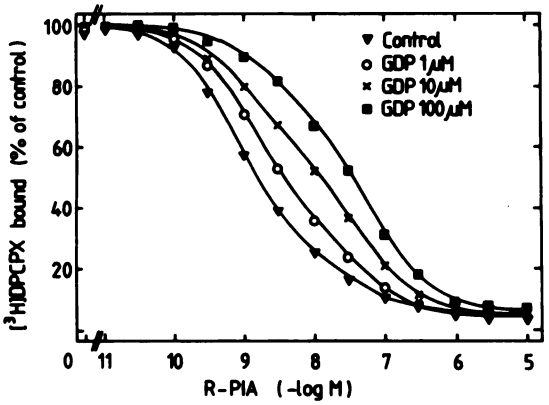

Fig. 11. Inhibition by unlabeled (*R*)-PIA of [^{3}H]DPCPX binding in the absence (▼) or presence of 1 (○), 10 (×), or 100 μM GDP (■). *Curves* from one of four independent experiments are shown. K_H and K_L values and the percentage of receptors in the high affinity state are shown in Table 4.

appropriate conditions for A_1 adenosine receptor-induced stimulation of G proteins have been determined. So far, interaction of this receptor with G proteins in native brain membranes has been studied indirectly either by radioligand binding techniques (6–10) or by stimulation of second messenger systems, e.g., adenylate cyclase (2, 11, 12), by A_1 agonists. One of the main physiological actions of adenosine in the central nervous system, the inhibition of transmitter release, can be regulated by adenosine independently of cAMP levels (24). Therefore, it seems difficult to assess the physiological relevance of the modulation by adenosine of adenylate cyclase in brain. In addition, these studies have been hampered by a low signal to noise ratio. The central depressant effects of adenosine have been linked to the modulation of K^+ channels (3) and intracellular Ca^{2+} levels or Ca^{2+} sensitivity (25). Similarly to stimulation of the GTPase activity of G proteins (13), stimulation of [^{35}S]GTP[S] binding by agonists offers the advantage of studying receptor-G protein interactions independently of the effector system. Whereas agonist-stimulated binding of [^{35}S]GTP[S] reflects the GDP/GTP exchange that leads to G protein activation, cleavage of GTP represents the off-switch step for the G protein (26).

Stimulation of [^{35}S]GTP[S] binding by various adenosine receptor agonists revealed a pharmacological profile typical of A_1 adenosine receptors in bovine brain, (*R*)-PIA > CCPA > (*S*)-PIA > NECA > CGS 21,680 (Table 2). In bovine brain membranes, (*R*)-PIA has been shown to possess a higher affinity for A_1 adenosine receptors than does CCPA, which is more potent at rat brain membranes (18). Similarly, (*S*)-PIA is more potent in bovine brain than is NECA, whereas in rat brain the order of potency is reversed (18). The rank order of potency described by radioligand binding studies for rat brain membranes was confirmed in this study of G protein activation (Table 2). All agonists except CGS 21,680 stimulated G protein activity to a similar degree, indicating identical intrinsic activities.

In the tissues investigated in this study, we have found no evidence for stimulation of [^{35}S]GTP[S] binding by A_2 adenosine receptors. Hill slopes of dose-response curves were typical for one site of action of agonists (Table 2). In addition, we performed time course experiments in the presence of agonists with different degrees of selectivity for A_1 and A_2 adenosine receptors. Also, after shorter or longer periods of incubation, the A_{2a}-selective agonist CGS 21,680 did not stimulate G protein activation at a concentration that was in great excess of the concentration required to occupy A_{2a} receptors (Fig. 7). The EC_{50} values obtained for N^6-substituted agonists are not compatible with activation of A_{2b} receptors (27).

G protein activation by A_1 adenosine receptor agonists has been shown to be highly sensitive to the sulfhydryl-alkylating agent NEM (Fig. 6). This agent has been applied to inactivate the G proteins linked to A_1 adenosine receptors (7, 28). A_1 adenosine receptors in bovine brain interact with the G_{i1}, G_{i2}, and G_o subtypes of G proteins (21). Concentrations of NEM in the low micromolar range cause alkylation of the cysteine residue at the fourth position from the carboxyl terminus of the α subunit of G_i and G_o, which is also ADP-ribosylated by pertussis toxin. NEM pretreatment has been shown to abolish incorporation of [^{32}P]ADP-ribose into these G protein α subunits (29). Thus, our results are in agreement with previous findings that demonstrated that the G proteins linked to A_1 adenosine receptors are susceptible to alkylation of sulfhydryl groups by NEM and to ADP-ribosylation by pertussis toxin, which are directed at the same amino acid residue.

Three additions were absolutely necessary to detect A_1 adenosine receptor-induced G protein activation, i.e., the inclusion of Mg^{2+} ions, NaCl, and a second, unlabeled, guanine nucleotide. Binding of [^{35}S]GTP[S] was dependent on the presence of Mg^{2+} ions and occurred at concentrations of free Mg^{2+} above 0.1 μM. Agonist-stimulated binding of the radioligand required higher concentrations (Fig. 5). Stimulation was half-maximal and maximal at approximately 200 μM and 3 mM free Mg^{2+}, respectively. The biphasic action of this ion on basal and agonist-stimulated binding of [^{35}S]GTP[S] suggests two separate sites of action.

The addition of NaCl in the G protein activation assay led to diminished basal levels of [^{35}S]GTP[S] binding, whereas agonist-stimulated levels were influenced to a smaller degree (Figs. 3 and 10). The relative amount of agonist-mediated stimulation was thus increased from approximately 20% above

TABLE 4

Competition of (*R*)-PIA for [^{3}H]DPCPX binding to A_1 adenosine receptors in bovine brain membranes.

Experiments were performed in the presence of 100 mM NaCl and in the absence (control) or presence of 1, 10, or 100 μM GDP. K_H and K_L are the K_i values for the high and low affinity states of the receptor, respectively, and are given as geometric means with 95% confidence limits. R_H, percentage of receptors in the high affinity state (arithmetic means ± standard errors). Significance of differences was tested using the Student's *t* test for paired samples.

	K_H	*p*	K_L	*p*	R_H	*p*
	nM		nM		%	
Control	0.48 (0.43–0.53)		24.31 (19.31–30.60)		80.5 ± 2.7	
1 μM GDP	0.76 (0.36–1.63)	NS[a]	18.06 (11.48–28.41)	NS	69.1 ± 2.4	<0.05
10 μM GDP	0.97 (0.73–1.28)	<0.001	23.25 (18.02–30.00)	NS	49.3 ± 3.3	<0.0005
100 μM GDP	1.61 (0.98–2.67)	<0.001	32.75 (27.07–39.61)	NS	40.0 ± 4.5	<0.0005

[a] NS, not significant.

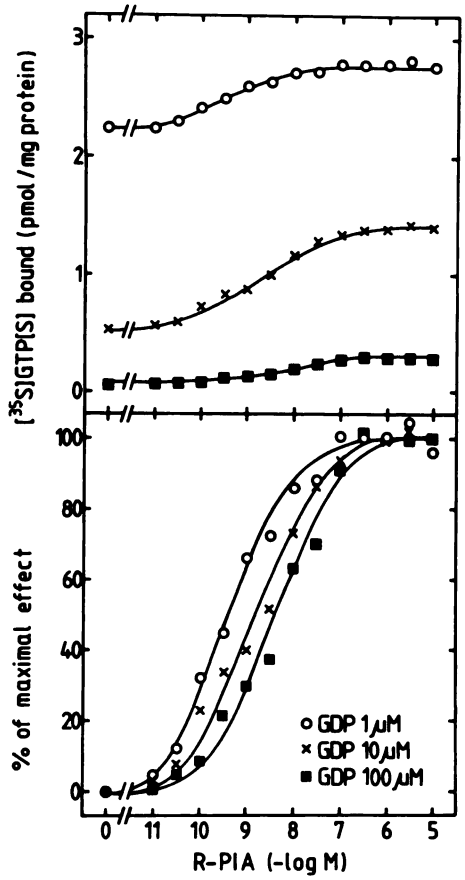

Fig. 12. Dose-response curves for stimulation of [^{35}S]GTP[S] binding by (*R*)-PIA obtained in the presence of 1 (○), 10 (×), and 100 µM (■) GDP. *Upper*, absolute values; *lower*, percentage of maximal effect. Membrane protein (2 µg) was incubated for 150 min at 25° in the presence of different GDP concentrations and in the presence of 100 mM NaCl, with the additions described in Experimental Procedures. Nonlinear curve-fitting gave the following EC_{50} values: 1 µM GDP, 0.25 (0.16–0.41) nM; 10 µM GDP, 1.84 (1.10–3.04) nM; 100 µM GDP, 6.18 (4.49–8.49) nM.

base-line levels in the absence of NaCl to approximately 130% in the presence of 100 mM concentrations of this salt. The observed effect of NaCl is in agreement with other studies of the modulation of G protein activity (13, 14, 21) or adenylate cyclase activation (2, 11, 12) by A_1 adenosine receptors in which NaCl was also included.

The molecular mechanism of action of NaCl was studied by performing [^{35}S]GTP[S] and A_1 adenosine receptor binding experiments under identical conditions. The shift of K_H and K_L values of the A_1 adenosine receptor for (*R*)-PIA to lower affinities is in agreement with the results of previous studies of the A_1 receptor (6, 9). Parallel observations have been reported for α_2 receptors (30, 31).

Although a greater portion of the A_1 receptors were in the low affinity state for agonists in the presence of 100 mM NaCl, there was obviously no receptor-G protein uncoupling, because the ability to stimulate [^{35}S]GTP[S] binding was retained. Dose-response curves were shifted to the right in the presence of NaCl. This effect of NaCl has also been described in other investigations of stimulation of low-K_m GTPases and [^{35}S]GTP[S] binding by muscarinic acetylcholine receptors (22, 32) and receptors for chemotactic peptides (23, 33). EC_{50} values for (*R*)-PIA were in good agreement with K_H values, indicating that G protein activation was induced by the high affinity state of the A_1 receptor. The low affinity state appeared functionally uncoupled from G proteins, because no stimulation of [^{35}S]GTP[S] binding was observed at agonist concentrations occupying this state. Because NaCl also lowered the affinity of the low affinity state for agonists, we may assume that NaCl acts directly on the receptor protein.

In addition, we found that NaCl slowed the association of [^{35}S]GTP[S] to bovine brain membranes. This effect was more pronounced in the presence of GDP (Fig. 2). Higashijima *et al.* (34) showed that NaCl had no major effect on the rate of association of [^{35}S]GTP[S] to purified G proteins but markedly inhibited the rate of dissociation of GDP bound to holotrimeric G proteins in the presence of Mg^{2+} ions. The marked slowing of [^{35}S]GTP[S] binding in the presence of NaCl might be interpreted as a slowing of the dissociation of GDP from G proteins, which in turn would lead to a slower association of [^{35}S]GTP[S].

Sodium chloride had a more pronounced inhibitory effect on basal than on agonist-stimulated binding of [^{35}S]GTP[S] (Fig. 3). Identical observations have also been reported in studies of G protein activation by muscarinic acetylcholine receptors (22, 32) and chemotactic peptide receptors (23, 33). This effect of NaCl is currently attributed to uncoupling of unoccupied receptors from their respective G proteins, which leads to a reduction of G protein activity in the absence of agonists. Activation of G proteins by unoccupied receptors has been shown in purified component studies, e.g., β-adrenergic receptors (35) and A_1 adenosine receptors (14). In light of these findings, the effect of NaCl reported in this study has to be interpreted as an inhibition of basal G protein activation by agonist-unoccupied receptors, which markedly enhances the signal to noise ratio when agonist activation of G protein-coupled receptors is studied. NaCl would have opposite effects on G protein-coupled agonist-free and agonist-occupied receptors; whereas activation of G proteins in the absence of agonists is inhibited, agonist activation proceeds in the presence of this salt (Figs. 3 and 10).

Similarly, the addition of GDP was necessary to obtain a reasonable ratio between unstimulated and agonist-stimulated values of [^{35}S]GTP[S] binding. In the absence of GDP, no stimulation of radioligand binding to G proteins by A_1 adenosine receptors was evident. This nucleotide lowered basal values more than agonist-stimulated values (Figs. 2, 4, and 12). However, the mechanism of action of GDP is not fully understood at present. Purified G proteins have been shown to contain GDP in their inactive state (26, 36). However, if this is also the case in membranes, addition of this nucleotide should have no influence on the levels of [^{35}S]GTP[S] binding. Therefore, additional experimental evidence is required to clarify the GDP content of the membranes, the GDP ligation state of G proteins in native membranes, and a possible contribution of nucleoside diphosphokinase to the actions of GDP.

In agreement with other studies (7, 37, 38), we found that the affinity for the antagonist was increased by the addition of the guanine nucleotide. In the studies cited, the poorly hydrolyzable guanylyl-5′-imidodiphosphate has been used as guanine nucleotide. Because GDP exerted effects identical to those of the metabolically stable GTP analog, we assume that the reported guanine nucleotide effects are not specific for guanine trinucleotides but are shared by guanine di- and trinucleoside phosphates. Indeed, Klotz *et al.* (10) have shown equal potencies of GTP and GDP in radioligand binding studies of the A_1 adenosine receptor.

The site of action of GDP is distinct from the site of action of NaCl, because GDP did not influence the affinity of the low

affinity state for agonists of the A_1 adenosine receptor (Table 4). In contrast, GDP lowered the affinity of the high affinity state for agonists, which was in good agreement with the higher EC_{50} values of (*R*)-PIA for stimulation of [^{35}S]GTP[S] binding (Fig. 12). Again, these effects of GDP are identical to the effects of guanylyl-5′-imidotriphosphate (9).

In summary, stimulation of [^{35}S]GTP[S] binding provides a simple and sensitive tool to study G protein activation by A_1 adenosine receptors in brain membranes independently of the second messenger system. This method simplifies the interpretation of the multiple affinity states observed in receptor binding studies in the presence of, for example, NaCl and guanine nucleotides, with respect to receptor-G protein coupling and G protein activation.

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