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Species Differences in A_1 Adenosine Receptor/G Protein Coupling: Identification of a Membrane Protein that Stabilizes the Association of the Receptor/G Protein Complex

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SUMMARY

Reconstitution experiments with purified components reproduce the basic characteristics of receptor/G protein coupling, i.e., GTP-sensitive high affinity agonist binding and receptor-promoted GTP binding. However, the interaction of agonists with the A_1 adenosine receptor in rat and bovine but not human brain membranes deviates from the ternary complex model since the agonist/receptor/G protein complex cannot be dissociated by high concentrations (≥100 μM) of the hydrolysis-resistant analogue GTPγS. The reason for this phenomenon referred to as a "tight coupling mode" has remained enigmatic. We show that it is attributable to a distinct membrane protein, which we labeled the coupling cofactor. Extraction of the protein from rat brain membranes with the detergent 3[3-(cholamidopropyl)diamethylammonio]-1-propanamium increased the potency of GTPγS by 1000-fold. After extraction, the potency was comparable to that in human brain membrane. Detergent extracts from rat brain membranes were used to resolve the component from solubilized receptors and G protein α and βγ subunits by sequential DEAE-Sephacel chromatography and Superose gel filtration (molecular weight of ~150 kDa in 3[3-(cholamidopropyl)diamethylammonio]-1-propanamium). Coupling cofactor restored guanine nucleotide refractoriness in a concentration-dependent manner to both detergent-extracted rat brain membranes and, albeit with lower affinity, human brain membranes. However, in human brain extracts, cofactor activity was detectable on reconstitution with rat acceptor membranes, indicating an intrinsic difference between rat and human receptors in their ability to interact with the cofactor. With high amounts of coupling cofactor present, GTPγS no longer decreased but rather increased agonist affinity. Readdition of partially purified coupling cofactor to acceptor membranes reduced the rate of A_1 adenosine receptor-mediated G protein turnover. These observations show that the component identified traps the ternary agonist/receptor/G protein complex in a stable conformation, impedes signaling of the A_1 adenosine receptor, and thereby regulates the level of signal amplification.

The purine nucleoside adenosine exerts various actions in the brain, including modulation of neurotransmitter release, sedation, anticonvulsant activity, and a protective effect against ischemic damage. In the cerebral tissue, adenosine elicits effects on neuronal, glial, vascular, and blood cells, all of which express adenosine receptors on the cell surface. Among the adenosine receptor subtypes identified (A_1, A_{2a}, A_{2b}, and A_3), the A_1 adenosine receptor represents a primary signaling target for adenosine in brain cortex, where it is present in abundant levels. The A_1 receptor is differentiated on the basis of its pharmacological binding profile of agonist and antagonist ligands as well as by the mechanism of signal transduction in response to adenosine receptor agonists. Signaling of the A_1 receptor is mediated by pertussis toxin-sensitive G proteins of the G_i and G_o class (G proteins mediating inhibition of adenylyl cyclase and regulation of neuronal calcium channels, respectively). cDNAs encoding for the cerebral A_1 receptor protein have been cloned from rat, bovine, and human brain (1–5). The nucleic acid sequence transcribed from these genes uniformly predicts proteins composed of 326 amino acid residues that are highly homologous (≥90%).

Despite the eminent similarity among the three A_1 receptor species at the structural level, some differences were revealed through functional characterization of the purified receptor protein. One distinct feature was discovered through examination of the ligand binding profile. Thus, the bovine A_1 receptor has an affinity for agonist and antagonist ligands that is 10-fold higher than that of rat and human

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ABBREVIATIONS: GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; HPIA, N^6-(4-hydroxyphenylisopropyl)adenosine; IHPIA, N^6-3-(iodo-4-hydroxyphenylisopropyl)adenosine; CPA, N^6-cyclopentyladenosine; XAC, xanthine amine congener; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; HEPES, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid; CHAPS, 3[3-(cholamidopropyl)diamethylammonio]-propanesulfonate; BSA, bovine serum albumin; DTT, dithiothreitol.

receptors; in the bovine receptor, the typical A_1 receptor rank order of potency (−)-N^6(-*R*-phenylisopropyl)adenosine > NECA > (+)-N^6(-*S*-phenylisopropyl)adenosine is partially altered in that it has a specifically reduced binding affinity for the 5′-substituted adenosine analogues compared with rat and human receptors (3). This binding profile of the bovine A_1 receptor is specified by the fifth membrane spanning domain of the receptor protein (6).

We previously examined the interaction between human and bovine brain A_1 adenosine receptor and G proteins and found that both receptors interact preferentially with G_i than with G_o but display different α_i subtype selectivity (7–9). The human A_1 adenosine receptor activated each of the $G_{i\alpha}$ subtypes with similar potencies, whereas the bovine brain A_1 receptor showed selectivity for $G_{i\alpha 3}$ over $G_{i\alpha 1}$ and $G_{i\alpha 2}$. A particular coupling preference may be specified by the primary peptide sequence and the species differences found therein. In addition to the differences in the pharmacological profile and the G protein selectivity, the mode of receptor/G protein coupling was found to be dissimilar in native brain membranes. In human brain membranes, the A_1 adenosine receptor is highly susceptible to modulation by guanine nucleotides (9) and membrane solubilization destabilizes receptor/G protein interaction (10). In contrast, in rodent and bovine brain membranes, up to 90% of the high affinity agonist binding (reflecting the ternary complex of agonist/A_1 adenosine receptor/G protein) is insensitive to the allosteric modulation by guanine nucleotides (11–13). On solubilization, the receptor/G protein complex remains associated, but the potency of guanine nucleotides to dissociate the ternary complex is markedly increased (13). Both features, i.e., stability of the receptor/G protein complex in detergent solution and resistance to guanine nucleotides, have been considered to reflect a common cause, although the mechanistic basis for this resistance to GTP, which has been referred to as a "tight coupling mode," has remained enigmatic. In addition, this phenomemon is not unique to the rodent and bovine A_1 adenosine receptors; related findings have been obtained with the various species of the A_{2a} adenosine receptor and with bovine muscarinic receptors (14–16).

In the present study, we examined brain cortical A_1 adenosine receptors of various species and found that this tight coupling mode is determined by a membrane component that can be resolved from receptor and G protein. Specifically, when rat and bovine brain membranes were extracted by detergent solubilization, an inherent constraint was removed from the receptor/G protein interaction. A component was recovered in the soluble extract that, on reconstitution, restores resistance to guanine nucleotides.

Experimental Procedures

Materials. [^{3}H]DPCPX, [^{35}S]GTPγS, and [^{125}I] were purchased from NEN (Boston, MA). [^{125}I]HPIA was synthesized and purified according to Linden (17). Guanine nucleotides, *R*- and *S*-(phenylisopropyl)adenosine, and adenosine deaminase were obtained from Boehringer Mannheim (Mannheim, Germany). XAC and DPCPX were obtained from RBI (Natick, MA), and purified IgG, thymidine, theophylline, and CPA were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin was purchased from Serva (Heidelberg). The sources of the materials required for G protein purification have been reported previously (18, 19).

Membrane preparation and solubilization. Brains were removed from male Sprague-Dawley rats immediately after decapitation and were transferred to ice-cold preparation buffer (10 mM HEPES·NaOH, pH 7.2, 1 mM $MgCl_2$, and 250 mM sucrose). Preparation of plasma membranes from rat brain cortex was carried out as described previously (13). The final pellet was washed and resuspended in HME (20 mM HEPES·NaOH, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA) at a protein concentration of ~5 mg/ml. Bovine brain membranes were prepared as described previously (16). The source and preparation of human cortical brain membranes were reported by Jockers *et al.* (9). Solubilization of rodent brain membranes was performed according to Ströher *et al.* (13). In brief, the membrane suspension was centrifuged for 20 min at 37,000 × *g* and resuspended at a protein/detergent ratio (w/w) of 4:1 in solubilization buffer (10 mM CHAPS in HME and 20% glycerol, w/v). The membranes were stirred on ice for 60 min and centrifuged at 100,000 × g for 60 min. The pellet was washed once with, resuspended in HME at ~5 mg/ml, and subsequently used as detergent-extracted acceptor membranes. The volume of the supernatant was reduced to 1/20 through concentration over a PM 30 membrane in an Amicon ultrafiltration cell (Amicon, Beverly, MA). Aliquots at a protein concentration of ~10 mg/ml were frozen in liquid nitrogen and stored at −80°.

Protein purification. The G protein βγ-dimer was chromatographically resolved from the $G_{o,i}$ oligomer according to Casey *et al.* (18). Myristoylated recombinant $G_{i\alpha\text{-}1}$ (the α subunit of G_i, of which three subtypes exist, $G_{i\alpha\text{-}1}$, $G_{i\alpha\text{-}2}$, and $G_{i\alpha\text{-}3}$) was purified as described (19). The amount of G protein α subunits used in individual experiments was determined by [^{35}S]GTPγS binding in a medium containing 50 mM HEPES·NaOH, pH 8, 1 mM EDTA, 10 mM $MgSO_4$, 0.1% Lubrol PX, and 1 μM [^{35}S]GTPγS (specific activity, ~20 cpm/nmol). The incubation (2 hr at 30°) was terminated by filtration over nitrocellulose BA85 filters. The concentration of G protein βγ subunit used was calculated from the protein concentration assuming a molecular weight of 45 kDa.

Gel filtration of the soluble rat brain extract was performed on a Superose 12 HR10/30 column with the use of a Pharmacia FPLC at a flow rate of 0.5 ml/min (Pharmacia, Uppsala, Sweden). The resin was equilibrated with 4 bed volumes of elution buffer (10 mM CHAPS, 200 mM NaCl, and HME in 10% glycerol, w/v). The column was calibrated with purified IgG (molecular weight of ~150 kDa), BSA (molecular weight of ~67 kDa), and lysozyme (molecular weight of ~17 kDa). The elution volume for thymidine (molecular weight of 240 kDa) indicated the total bed volume (V_t). Approximately 2.5 mg of either prepurified or crude soluble protein was loaded onto the column and eluted with running buffer. The eluate was collected in 0.5-ml portions. The fractions were pooled as indicated and concentrated using Centricons fitted with a PM30 or PM10 polycarbonate membrane (Amicon). Aliquots of the pooled fractions were frozen in liquid nitrogen at a protein concentration of ~4–7 mg/ml and stored at −80°. If soluble protein had been pretreated with GTPγS (10 μM) for G protein subunit dissociation, the concentration of Mg^{2+} was increased to 10 mM in the elution buffer.

Ion exchange chromatography was carried out with a DEAE-Sephacel (Pharmacia) column equilibrated with 10 mM CHAPS and HME in 10% glycerol. Crude solubilized rat brain extract (~10 mg soluble protein) was applied to a 2.5-ml bed volume; the column was subsequently washed with 15 ml of equilibration buffer at a flow rate of 0.7 ml/min. The protein was eluted batchwise with ~15 ml buffer containing increasing concentrations of NaCl (50, 150, and 300 mM). The protein peaks indicated by UV absorption (280 nm) were collected, and the volume of protein solution reduced to 1/20 of the fraction volume by concentration in PM30 Centricons. The protein recovery in the 50, 150, and 300 mM eluate was 5%, 25%, and 25%, respectively. Aliquots were frozen in liquid nitrogen at a protein concentration of ~5 mg/ml and stored at −80°. The column was regenerated with 15 ml 1 M NaCl in equilibration buffer followed by removal of NaCl. Larger amounts of solubilized extract (50–70 mg

protein) were chromatographed using a 15-ml DEAE-Sephacel bed volume.

Hydroxylapatite chromatography was performed on a HTP-Econo 5-ml column (Bio-Rad, Hercules, CA) with the use of a Pharmacia FPLC at a flow rate of 0.75 ml/min. The resin was equilibrated in 10 mM CHAPS, 10 mM potassium phosphate, pH 6.8, and 1 mM $MgCl_2$. DEAE-Sephacel eluate containing coupling cofactor activity (~20 mg protein) was diluted 1:4 in equilibration buffer and loaded onto the column. On washing the column gradient elution was performed by increasing the potassium phosphate concentration from 10 mM, pH 6.8, to 400 mM, pH 7.2, as delineated in Fig. 4. The eluate was collected in 2.25-ml portions and concentrated over PM30 membranes. Aliquots were frozen in liquid nitrogen and kept at −80°.

Radioligand binding experiments. Binding assays were carried out in a volume of 40–100 μl containing 20 mM HEPES·NaOH, pH 7.5, 0.5 mM EDTA, 5 mM $MgCl_2$, 1–2 nM [^{125}I]HPIA, 0.2 units/ml adenosine deaminase, and brain membranes (10–25 μg protein), solubilized receptors (20 μg protein), or a combination of concentrated solubilized material with brain membranes. Also, CHAPS, glycerol, and NaCl were present at the concentrations indicated in the figure legends. The effect of soluble protein, i.e., of purified G protein or of detergent-solubilized proteins, on IHPIA binding was evaluated after preincubation of soluble proteins with detergent-extracted acceptor rat brain membranes or human brain membranes at a detergent concentration of 6 mM CHAPS (15 min at 22°). Before initiating the binding reaction, we diluted the mixture in HME, resulting in a final detergent concentration of 1 mM CHAPS. In control experiments, an ultrafiltrate obtained while concentrating crude or chromatographed soluble material was substituted for the retentate and was processed in the same way. The ultrafiltrate did not contain significant amounts of protein. The binding assay was carried out at 25° for 90 min and was terminated by filtration over glass-fiber filters, which were thoroughly washed using a cell harvester (Skatron, Lier, Norway). Filters were soaked in 0.3% PEI if binding was performed on soluble receptors or on membranes in the presence of soluble protein. Nonspecific binding was determined with the addition of 5 μM CPA or 1 μM XAC and amounted to 5% of total binding in the K_D concentration range. The radioligand concentration varied from 0.5 to 10 nM in IHPIA saturation binding experiments.

[^{3}H]DPCPX binding was performed in a final volume of 50 μl containing 0.5–20 nM [^{3}H]DPCPX, 20 mM HEPES·NaOH, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA, and 0.2 units/ml adenosine deaminase. The reaction was initiated by the addition of native or detergent-extracted brain membranes (~40 μg). After incubation for 60 min at 30°, the incubation was terminated by filtration through glass-fiber filters using a cell harvester. Nonspecific binding was determined in the presence of 1 μM XAC and amounted to 15% in the K_D range. Binding to soluble A_1 adenosine receptors eluted from the DEAE-Sephacel column was performed in the presence of 20 nM [^{3}H]DPCPX; the carryover resulted in NaCl concentrations of up to 150 mM, which, however, did not affect binding of the antagonist to the A_1 adenosine receptor (not shown; see Ref. 12). Bound ligand and free ligand were separated by filtration over glass-fiber filters treated with polyethylenimine.

GTPγS binding to native or detergent-extracted acceptor membranes (~10 μg protein) was carried out in the presence of 1 μM DPCPX or 10 μM CPA in a final volume of 50 μl containing 20mM HEPES·NaOH, pH 7.5, 0.5 mM EDTA, 0.1 mM GDP, 1.5 mM $MgCl_2$, and 150 mM NaCl. These conditions were found to suppress basal GTPγS association and provide for optimal quantification of the receptor-induced increment. After a 10-min preincubation period at 25°, the reaction was initiated by the addition of [^{35}S]GTPγS (5 nM) at a specific activity of ~50 nCi/fmol. The reaction proceeded at 25° for the indicated periods and was terminated by the addition of ice-cold stop solution (10 mM Tris·HCl, pH 8.0, 20 mM $MgCl_2$, 150 mM NaCl, and 0.1 mM GTP). Bound radioligand and free radioligand were separated by filtration over glass-fiber filters. For GTPγS binding in the presence of soluble proteins, acceptor membranes were preincubated with 5 μl soluble protein at a detergent concentration of 6 mM CHAPS (15 min at 22°). The mixed sample was diluted to give the composition described and subjected to GTPγS binding. To quantify the amount of G protein α subunits in soluble rat brain extract, aliquots were diluted 10-fold into the assay medium containing 50 mM HEPES·NaOH, pH 8.0, 1 mM EDTA, 1 mM DTT, 10 mM $MgSO_4$, and 2 μM GTPγS at a specific activity of 45 pCi/pmol. The incubation was carried out for 60 min at 30° and terminated by filtration over nitrocellulose BA85 filters. After [^{35}S]GTPγS binding, filters were rinsed with 15 ml stop solution without GTP.

Pertussis toxin-mediated ADP-ribosylation of rat brain extract. Pertussis toxin treatment was performed according to Carty (20). DEAE-Sephacel prepurified rat brain extract was treated with pertussis toxin at a concentration of 5 μg/ml. Pertussis toxin was preactivated by incubating with 100 mM DTT, 5 mM CHAPS, 0.8 M urea, and 50 mM phosphate buffer, pH 8.0, for 20 min at 32° in a volume of 10 μl. Pertussis toxin was diluted 20-fold into the final assay volume of 0.2 ml consisting of 75 mM Tris, pH 8.0, 10 mM thymidine, 1 mM $MgCl_2$, 45 mM NaCl, 3 mM CHAPS, 0.1 mM NAD, 5 mM DTT, 0.1 mM EDTA, 0.01 mM GDP, 550 μg/ml DMPC, and 0.25 mg of soluble protein. The reaction proceeded for 30 min at 25° and was terminated by sequential dilution and concentration (final dilution, 250-fold) in ice-cold solubilization buffer. As a control, rat brain extract was incubated in the same medium in the absence of NAD. From a final volume of 0.1 ml, aliquots (4 μl) were reconstituted with acceptor membranes and assayed by IHPIA binding. Pretreatment with pertussis toxin inactivated ≥75% of α subunits in the $G_{i/o}$ class. This estimate was obtained as follows: After the cycles of concentration and dilution, a 10-μl aliquot from the first reaction was radiolabeled with [^{32}P]NAD (specific activity, 5.4 nCi/pmol) through a second exposure to pertussis toxin. This reaction was terminated by the addition of Laemmli-sample buffer containing 40 mM DTT. After polyacrylamide gel electrophoresis, radioactive bands corresponding to G_α subunits were identified by autoradiography, and the incorporation of [α^{32}P]ADP was quantified by liquid scintillation counting.

Miscellaneous procedures. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using the Laemmli-buffer system; the sample buffer contained 40 mM DTT. Electrophoretic transfer to nitrocellulose for immunoblots was performed as described by Towbin *et al.* (21). Immunostaining was carried out with a second antibody conjugated to horseradish peroxidase using the Amersham ECL reagents (Amersham, Madison, WI). The antisera 11 (anti-$G_{\alpha\text{-common}}$) and 7 (anti-$G_{\beta\text{-common}}$) were raised against the peptides used to generate the original antisera A569 and K521 (16). The protein concentration was determined by dye binding using the BioRad Coomassie-blue kit (Hercules, CA) or the sodium bicinchonate reagent provided by Pierce (Rockford, IL); BSA was the standard. Data were subjected to nonlinear, least-squares curve fitting using the appropriate equations (e.g., rectangular hyperbola, Hill equation), and parameter estimates were derived. If not indicated otherwise, a representative experiment is shown. Each experiment was reproduced at least twice.

Results

Species differences in A_1 adenosine receptor/G protein coupling. Guanine nucleotides dissociate the high affinity ternary complex of agonist/receptor/G protein and shift the interaction between receptor and agonist to the low affinity binding state. In human brain membranes, GTPγS modulated the entire agonist-liganded receptor population (Fig. 1A). In contrast in rat and bovine brain, high concentrations of GTPγS were required to inhibit [^{125}I]HPIA binding, and only a limited proportion of A_1 adenosine receptors was susceptible (Fig. 1A). Thus, the concentration of GTPγS required for half-maximal inhibition of [^{125}I]HPIA binding

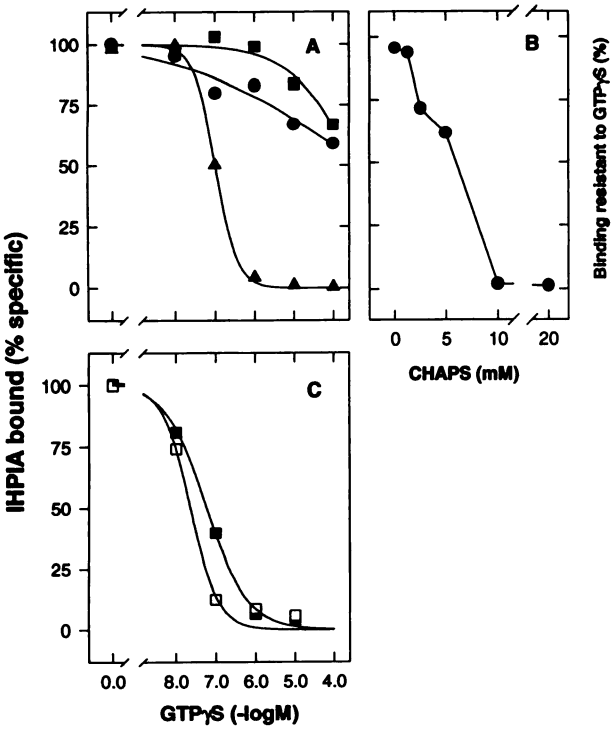

Fig. 1. Species-specific differences in guanine nucleotide modulation of the cerebral A_1 adenosine receptor. A, Membranes (~20 μg protein) prepared from rat (■, □), bovine (●), and human (▲) brain cortex were incubated with 1 nM [^{125}I]HPIA in the presence of increasing concentrations of GTPγS. The assay volume was 50 μl composed of 20 mM HEPES·NaOH, pH 7.5, 5 mM $MgCl_2$, and 1 mM EDTA; the incubation was carried out at 25° for 90 min. Bound and free ligands were separated by vacuum filtration. Nonspecific binding was evaluated in the presence of 1 μM XAC. Specific binding amounted to 508, 218, and 205 fmol/mg in bovine, rat, and human membranes, respectively, and was set 100%. Data were fitted to the Hill equation. B, The detergent CHAPS enhances guanine nucleotide modulation of A_1 adenosine receptor agonist binding. Rat brain membranes (10 μg) were diluted in HME plus CHAPS to give the indicated detergent concentration. Binding of [^{125}I]HPIA (1 nM) was performed in the absence or presence of 3 μM GTPγS; shown is the specific binding in the presence of GTPγS expressed as percentage of binding in the absence of guanine nucleotides. This value was taken as an estimate of the binding resistant to GTPγS. C, Rat brain membranes were solubilized with 10 mM CHAPS as described in Experimental Procedures. [^{125}I]HPIA binding (1 nM) was performed on detergent-extracted membranes (■) and on the soluble supernatant (□) using 19 μg of solubilized protein. Specific binding was 79 and 151 fmol/mg in the soluble preparation and detergent-extracted membranes, respectively. The soluble preparation was diluted to yield a final CHAPS concentration of 1 mM in the assay.

(IC_{50}) varied by three orders of magnitude (320 ± 70, 0.64 ± 0.10, and 0.10 ± 0.02 μM in rat, bovine, and human brain membranes, respectively).

Effect of detergent extraction on receptor/G protein coupling in brain membranes. Detergent solubilization of plasma membranes is generally known to disrupt receptor/G protein coupling. Adenosine receptors appear to be an exception; during solubilization, the rodent A_1 and A_2 adenosine receptors remain associated with the G protein, and the complex becomes more susceptible to guanine nucleotide modulation (12, 13, 15). In rat brain membranes, addition of the zwitterionic detergent CHAPS to the binding reaction enhanced the potency of GTPγS to inhibit formation of the high affinity binding state (Fig. 1B), whereas the binding kinetics of [^{125}I]HPIA were unaffected. The resulting concentration-response curve for CHAPS was very steep, and the effect rapidly attained a maximum once the concentration of CHAPS surpassed the critical micellar concentration (~4–8 mM; Fig. 1B). However, this increase in GTPγS sensitivity did not require the presence of detergent at a concentration exceeding the critical micellar concentration in the binding reaction (Fig. 1C). Rat brain membranes were solubilized with 10 mM CHAPS, followed by centrifugation. [^{125}I]HPIA binding was performed on detergent-extracted membranes recovered from the pellet (Fig. 1C) and to the soluble supernatant (Fig. 1C), which was diluted to give a final assay concentration of 1 mM CHAPS. High affinity [^{125}I]HPIA binding to the solubilized A_1 adenosine receptor/G protein complex as well as to the receptors present in the extracted membranes was now highly susceptible to the effect of guanine nucleotides. The GTPγS concentration range was similar to that required to uncouple the human brain A_1 adenosine receptor (IC_{50} = 23 ± 6 and 12 ± 3 nM in solubilized and CHAPS-extracted rat brain membranes, respectively). Similar observations were made with bovine and porcine brain membranes (not shown).

The assay used, i.e., destabilization of high affinity agonist binding by GTPγS, does not allow determination of whether detergent treatment promotes signal transfer from the receptor to the G protein or reduces the catalytic efficiency of the receptor. This was assessed by determining the rate of agonist-stimulated [^{35}S]GTPγS binding in native and detergent-extracted membranes (Fig. 2A). [^{35}S]GTPγS binding was assayed under conditions that suppress the spontaneous guanine nucleotide exchange to G protein α subunits (i.e., in the presence of 100 μM GDP, 150 mM NaCl, and 1.5 mM $MgCl_2$), yielding an optimal signal-to-noise ratio. These conditions allowed for the detection of a receptor-induced increment in GTPγS binding over basal (Fig. 2A, *inset*). After detergent extraction (Fig. 2A), the rate of agonist-mediated G protein activation in rat brain membranes increased markedly compared with native controls (Fig. 2A); from the linear part of the GTPγS association curves in three experiments, the molar turnover number was estimated to increase ~2–4-fold.

Detergent extraction removed ~50% (see below) of the receptors from the membrane. To account for this loss, agonist-induced [^{35}S]GTPγS binding was related to the number of A_1 receptors determined in parallel from [^{125}I]HPIA saturation isotherms (not shown). However, it is difficult to correct for the loss of G proteins; from the changes in basal GTPγS binding, which were confirmed in immunoblots using an α-common antiserum, we estimate that ~80% of $G_{i\alpha}/G_{o\alpha}$ was extracted from the membrane through treatment with detergent (data not shown). Thus, although the concentration of reaction partners had been severely reduced, the catalytic efficiency of the receptor clearly increased after solubilization. This increase in the molar turnover number provides compelling evidence that detergent extraction removes a membrane-bound constraint imposed on receptor/G protein coupling. It was reasonable to speculate that the constraining element may be the same component that modifies reciprocal receptor modulation by guanine nucleotides.

Binding of xanthine antagonists to the A_1 adenosine receptor is inversely regulated by G proteins (7, 13, 24) as they bind preferentially to the G protein-free receptor. The receptor density determined in saturation binding experiments

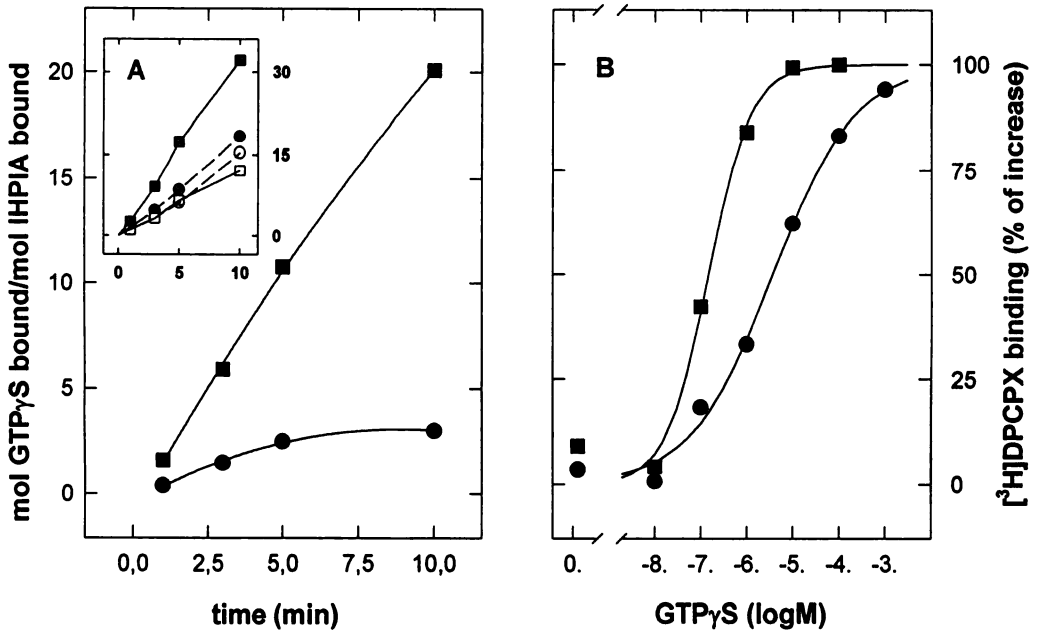

Fig. 2. Effect of solubilization on agonist-induced [^{35}S]GTPγS binding (A) and GTPγS-dependent regulation of antagonist binding (B) in rat brain membranes. A, [^{35}S]GTPγS binding was carried out at 30° in the presence of 1 μM DPCPX or of 10 μM CPA in a final volume of 50 μl containing 10 μg native (●) or 20 μg detergent-extracted brain membranes (■) for the incubation periods indicated. Assay conditions were chosen to suppress the basal GTPγS association rate (see Experimental Procedures). An aliquot of the membranes used was subjected to [^{125}I]HPIA saturation binding to determine the concentration of A_1 adenosine receptors (see Fig. 8B). *Inset*, GTPγS binding in the presence of agonist (*shaded symbols*) and binding in the presence of antagonist (*open symbols*); this value was normalized for the total number of agonist high affinity binding sites present. The difference between binding in the presence of agonist and antagonist (*filled symbols*) yields the molar turnover number of the receptor. B, The effect of GTPγS on antagonist binding was assayed in a final volume of 50 μl containing 3–6 nM [^{3}H]DPCPX, ~25 μg native (●) or ~50 μg CHAPS-extracted rat brain membranes (■) as described in Experimental Procedures. The incubation was carried out for 60 min at 30° in the absence and presence of GTPγS at the indicated concentrations. Nonspecific binding determined in the presence of 1 μM XAC amounted to 20% of total binding and was not affected by the addition of GTPγS. In the absence of GTPγS, specific binding amounted to ~200 and ~500 fmol/mg in CHAPS-extracted and native membranes, respectively; the GTPγS-induced maximal increment in DPCPX binding is quantified and given in the text.

amounted to ~1250 and ~550 fmol/mg in native and extracted brain membranes, respectively. The addition of GTPγS increased binding of [^{3}H]DPCPX to rat brain membranes. The effect was a 2-fold increase in affinity and was indistinguishable between native and detergent-extracted membranes. The K_D (mean value and 95% confidence intervals) was 3.9 nM (2.4–5.5) and 1.9 nM (1.0–3.7) in the absence and presence of 100 μM GTPγS, respectively, with no alteration in binding maximum (data not shown). GTPγS concentration-response curves were performed at a radioligand concentration of [^{3}H]DPCPX in the K_D concentration range (Fig. 2B) and revealed a difference between native and detergent-extracted membranes. The concentration requirement for GTPγS shifted to the left by 1 log-unit, and the slope of the curve was markedly steeper. Parameter estimates were derived from fitting the data to the Hill equation. EC_{50} values were 2.3 ± 1.7 and 0.27 ± 0.17 μM in native and CHAPS-extracted rat brain membranes, respectively (three experiments). There was no alteration in the maximal GTPγS-induced increment in DPCPX binding [46% (18–118) in native versus 35% (16–78) in detergent-extracted brain membranes]. Thus, detergent extraction promoted guanine nucleotide sensitivity of antagonist binding in a fashion analogous to the effect on agonist binding. However, the consequences of detergent extraction were more pronounced if assessed by agonist/ than by antagonist/receptor interaction.

We have shown previously that proteolytic degradation enhanced the sensitivity of the membrane-bound A_{2a} adenosine receptor to modulation by guanine nucleotides (14). This was not the case for the A_1 receptor subtype. Both native and detergent-extracted brain membranes were subjected to trypsin digestion (for 30 min at 25°; range of trypsin concentration, 0.1–10 μg/100 μg membrane protein) followed by washing and membrane centrifugation, resulting in a loss in the number of agonist binding sites. In native membranes, receptors were uniformly resistant to GTPγS before and after limited trypsin digestion; the remainder of the receptor population in detergent-extracted membranes, however, became increasingly refractory with increasing degree of trypsin digestion (data not shown). A difference in the effect of partial proteolysis is not surprising since the receptor subtypes differ in primary structure; the A_{2a} receptor has an extended carboxyl terminus, whereas the A_1 receptor is fairly small (34 kDa), representing almost the "core version" of a G protein-coupled receptor. A similarity between the two receptor subtypes is that the A_{2a} adenosine receptor also becomes more sensitive to guanine nucleotide modulation on detergent extraction of striatal membranes (not shown).

Identification of a membrane-associated protein that regulates receptor/G protein coupling. In the soluble detergent extract prepared from rat brain membranes, an active principle was detected which, on readdition to detergent-extracted acceptor membranes, restored guanine nucleotide refractoriness to the A_1 adenosine receptor. The activity was not recovered from rat brain cytosolic fractions and was therefore assigned to a membrane-bound component. Its as-

sociation with the membrane was not resolved by treatment with chelating agents (EDTA/EGTA at 2 mM). On detergent extraction, the activity was completely abolished by heating or trypsin digestion but was not removed through repetitious diluting and concentrating over a porous polycarbonate membrane (molecular cutoff of 30 kDa; data not shown). We therefore assumed that the activity that conveys GTP refractoriness is attributable to a distinct protein component, and we subjected the detergent-solubilized brain extract to gel filtration (Fig. 3). Protein fractions were combined, and the proteins were concentrated 10-fold; the pooled fractions were added to detergent-extracted acceptor membranes and were assayed by [^{125}I]HPIA binding. Their ability to reconstitute guanine nucleotide refractoriness to [^{125}I]HPIA binding was determined in the absence and presence of 3 μM GTPγS (Fig. 3). The activity restoring guanine nucleotide resistance eluted with fractions 15–21 and 22–28, the latter pool containing the activity peak. The maximum of agonist binding, which presumably corresponds to the solubilized A_1 adenosine receptor/G protein complex, was recovered in fractions 15–21; an additional component, which suppresses [^{125}I]HPIA binding to the membrane-bound A_1 adenosine receptor, was distributed to pool 29–40. The nature of this factor was not further investigated.

When the soluble protein recovered in fractions 22–28 was added to detergent-extracted rat brain membranes (Fig. 3A) or to native human brain membranes (Fig. 3B), the concentration-response curve of GTPγS shifted to the right by 1 order of magnitude. Reconstitution of acceptor membranes with the soluble protein transferred agonist binding activity to the membrane. The magnitude of the shift and the shape of the curve, which is similar with or without the addition of soluble protein, suggest that guanine nucleotide refractoriness was restored not only to the receptor/G protein complex suspended in the fraction volume but also to the A_1 adenosine receptor in the acceptor membranes.

Resolution of the coupling cofactor from solubilized receptor and G proteins. The findings of the present study suggest that a component was extracted from and reconstituted with brain membranes, which modifies the guanine nucleotide sensitivity of the A_1 adenosine receptor/G protein complex. In previous experiments, the interaction between recombinant human A_1 adenosine receptors and purified recombinant G protein species was fully susceptible to the action of guanine nucleotides and indistinguishable from that of the human A_1 adenosine receptor in brain membranes (9). As mentioned, species differences exist in the amino acid sequence of A_1 adenosine receptors. There may be additional particularities on the level of post-translational modification (e.g., protein palmitoylation). It was therefore mandatory to obtain additional evidence that the putative coupling cofactor can be resolved from receptor and G protein. This should rule out further that the "tight" mode of receptor/G protein coupling might be an intrinsic feature of either receptor or G protein species involved.

Physical resolution from solubilized A_1 adenosine receptor was achieved by ion exchange chromatography (Table 1). Solubilized rat brain extract was applied to a DEAE-Sephacel column, and protein was eluted batchwise by increasing the concentration of NaCl. The fractions were assayed for reconstitution of guanine nucleotide-resistant [^{125}I]HPIA binding to detergent-extracted acceptor membranes. Significant resistance to guanine nucleotides was restored only by the fraction recovered at 150 mM NaCl (Table 1, *bottom*). [^{3}H]DPCPX binding as well as the major proportion of specific [^{125}I]HPIA binding activity eluted with 300 mM NaCl

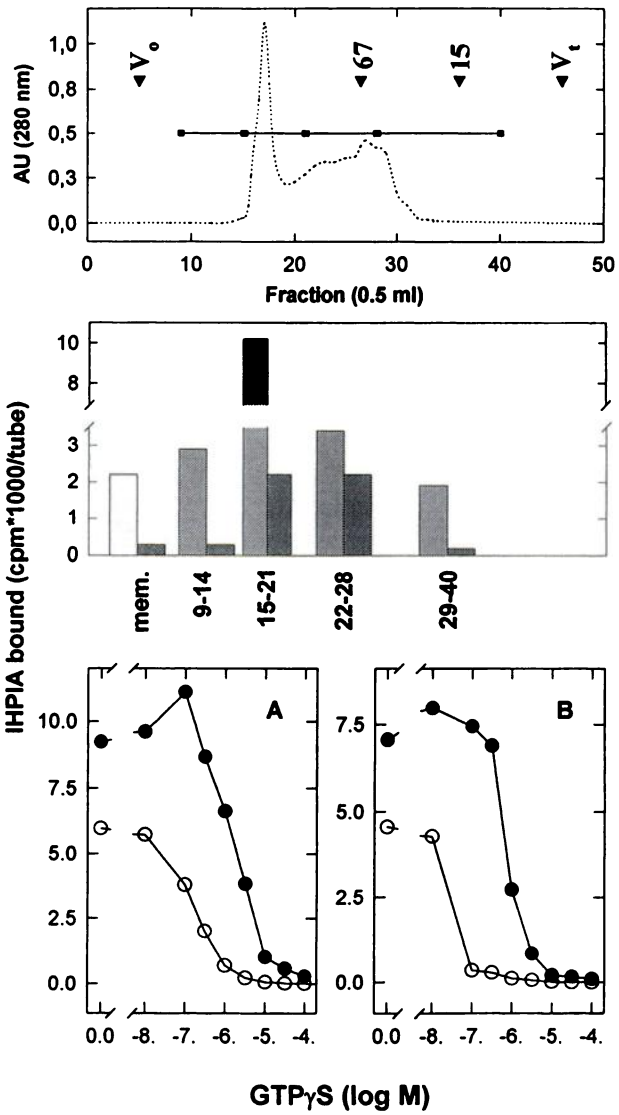

Fig. 3. A macromolecular component restores guanine nucleotide refractoriness to membrane-bound A_1 adenosine receptors. *Top*, A solubilized extract from rat brain cortical membranes (~5 mg) was applied to a Superose HR 10/30 gel filtration column and equilibrated in 10 mM CHAPS, 0.2 M NaCl, and HME in 10% glycerol. Given are the elution volumes for BSA (*67*), lysozyme (*15*), and thymidine (V_t). The protein was eluted in 0.5-ml fractions; *dashed line* represents the UV absorption trace (280 nm). Fractions were pooled as indicated by the *dots on the horizontal line*. *Middle*, The pooled fraction were concentrated 10-fold over PM30 membranes; fraction 29–40 was concentrated over a PM 10 membrane. Aliquots (5 μl) were combined with CHAPS-extracted acceptor membranes (~10 μg) and preincubated at a concentration of 6 mM CHAPS for 15 min at 22°. The binding reaction was initiated by dilution (1 mM final CHAPS) and addition of [^{125}I]HPIA (1 nM final) in the absence (*striped bars*) and presence (*cross-hatched bars*) of 3 μM GTPγS. *Numbers* indicate the number of the pooled fractions; *mem.* indicates [^{125}I]HPIA binding to acceptor membranes to which 5 μl ultrafiltrate had been added. *Bottom*, Inhibition by GTPγS of [^{125}I]HPIA binding to detergent-extracted rat brain membranes (A) or native human brain membranes (B). An aliquot (5 μl) of fraction 22–28 was reconstituted with CHAPS-extracted rat brain (~10 μg) or native human brain membranes (sim]10 μg).

TABLE 1

Separation of a detergent extract from rat brain membranes by DEAE chromatography: resolution of the coupling cofactor from A_1 receptor binding activity

Solubilized extract (~8 mg) was applied to a DEAE-Sephacel column (2-ml bed volume); protein was eluted batchwise with increasing concentrations of NaCl as indicated (50, 150, and 300 mM) and was concentrated over PM30 membranes. Binding of [^{3}H]DPCPX (20 nM) and of [^{35}S]GTPγS (2 μM; specific activity, 45 pCi/pmol) was determined in the DEAE-fractions as described in Experimental Procedures. For [^{125}I]HPIA (~2 nM) binding, 4-μl aliquots of the DEAE fractions were reconstituted with detergent-extracted brain membranes as described in the legend to Fig. 3. Binding experiments with the inclusion of ultrafiltrate did not reveal any significant effect of NaCl carried over on [^{3}H]DPCPX and IHPIA equilibrium binding as well as on the guanine nucleotide resistance of the A_1 receptor. Values are given as mean standard error of three experiments. IHPIA binding values given in the table are the difference between IHPIA binding to acceptor membranes in the presence of DEAE eluate and binding to acceptor membranes alone. Binding in the presence of 3 μM GTPγS is expressed as percentage of IHPIA bound specifically in the absence of GTPγS; given are geometric mean values with 95% confidence intervals.

	50 mM NaCl	150 mM NaCl	300 mM NaCl
Binding assay in solution			
DPCPX bound (fmol/fraction)	7.0 ± 5.3	61.4 ± 49.7	272.0 ± 137.1
GTPγS bound (pmol/fraction)	92.4 ± 47.1	172.3 ± 59.6	189.6 ± 167.3
Binding on reconstitution with acceptor membranes			
IHPIA bound (fmol/fraction)	0	158 ± 113	225 ± 131
Binding resistant to GTPγS (%)	4 (1–27)	64 (33–123)	14 (4–45)

TABLE 2

Pertussis toxin treatment of rat brain extract reduces the activity of the A_1 adenosine receptor/G protein coupling cofactor

[^{125}I]HPIA binding (~2 nM) was performed in a volume of 40 μl on detergent-extracted rat brain (35 μg) combined with solubilization buffer (Acceptor membranes) or with DEAE-Sephacel prepurified rat brain extract (10 μg) after pertussis toxin pretreatment in the presence (Pertussis toxin-treated extract) and absence (Sham-treated extract) of 0.1 mM NAD as outlined in "Experimental Procedures" and the legend to Fig. 3. Specific IHPIA binding is given as mean values ± standard deviation of two experiments. Binding in the presence of 3 μM GTPγS is expressed as percentage of IHPIA bound specifically in the absence of GTPγS; given are the geometric mean values with confidence intervals.

	Acceptor membranes	+ Pertussis toxin-treated extract	+ Sham-treated extract
IHPIA bound (fmol/mg)	147.0 ± 32.2	156.2 ± 28.3	159.3 ± 6.6
Binding resistant to GTPγS (%)	18 (14–21)	28 (18–43)	83 (56–123)

(Table 1). The latter, however, was more evenly distributed between fractions "150" and "300" than the solubilized A_1 receptors indicated by DPCPX binding. Because IHPIA binding was assayed after reconstitution of eluate with acceptor membranes, both soluble receptor and G protein, as well as soluble receptor/G protein complex, might have contributed to a rise in the number of agonist binding sites. Guanine nucleotide binding activity was recovered at 150 and 300 mM NaCl (Table 1). The [^{35}S]GTPγS binding assay was performed in the presence of 10 mM $MgCl_2$, which suppresses the guanine nucleotide exchange reaction of small GTP binding proteins but stimulates [^{35}S]GTPγS binding to the α subunits of heterotrimeric G proteins. Reducing the magnesium concentration to micromolar free concentrations favors binding to the small GTP binding proteins. Under these conditions, the peak of [^{35}S]GTPγS binding was found in the 150 mM fraction (not shown). Thus, the coupling cofactor was resolved from the major proportion of solubilized A_1 adenosine receptors but could not be entirely separated from the G protein α subunits (and small GTP binding proteins).

Pretreatment of the fraction eluting at 150 mM NaCl with pertussis toxin resulted in ADP-ribosylation of ≥75% of G protein α subunits and abolished coupling cofactor activity (Table 2). Likewise, if the DEAE fraction was preincubated with 10 μM GTPγS followed by gel filtration to remove unbound guanine nucleotide, coupling cofactor activity was undetectable (data not shown). The trivial explanation is that coupling cofactor activity resides in the G proteins *per se*. This is clearly not the case. Table 3 shows that neither the G protein heterotrimer nor the α- or βγ subunits alone promoted refractoriness of [^{125}I]HPIA binding to GTPγS; increasing the concentration of holo-G_i to 0.32 μM did not significantly alter GTPγS modulation of the A_1 adenosine receptor. G_{i1} as well as α_{i1}, however, interacted with the receptors; the addition of either the α-subunit or the heterodimer increased the amount of agonist high affinity binding.

Alternatively, G_{i1} was combined with soluble protein recovered after gel filtration (Table 3). A fraction was chosen that eluted in front of the coupling cofactor activity peak and whose addition to acceptor membranes restored little guanine nucleotide resistance (Table 3, *bottom*; Fig. 4, *middle*). Guanine nucleotide refractoriness of [^{125}I]HPIA binding was induced in extracted acceptor membranes if exogenous α_{i1}, βγ, and, most evidently, trimeric holo-G_{i1} had been combined with the same fraction (Table 3). Therefore, G proteins would promote guanine nucleotide resistance of receptor/G protein coupling but depended on the synergistic action of an ancillary component, the coupling cofactor.

This hypothesis was substantiated by examination of the elution profile of receptors, G proteins, and coupling cofactor activity on a Superose gel filtration column subsequent to DEAE-chromatography (Fig. 4). The total amount of [^{125}I]HPIA binding (sum of binding activity in the column fraction and the acceptor membranes) was assayed after the addition of an aliquot of concentrated fractions to detergent-extracted acceptor membranes (Fig. 4, *top*). The [^{125}I]HPIA binding activity peak eluted with an apparent size of >150 kDa, which is consistent with the sum of the molecular weights of the G protein oligomer and the A_1 adenosine receptor bound to several detergent micelles. Resistance of [^{125}I]HPIA binding to 3 μM GTPγS was evaluated to track coupling cofactor activity and was expressed as percentage of [^{125}I]HPIA bound in the absence of GTPγS (Fig. 4, *bottom*). Coupling cofactor activity was partially resolved from receptor/G protein complex but was more clearly separated from the majority of free G protein α and β subunits (Fig. 4). However, activities overlapped; some fractions (23–26) comprised both coupling cofactor and free G protein subunits. If the fractions that were devoid of G proteins (15–22) were combined with exogenous G_{i1}, a significant extra amount of coupling cofactor activity was recruited. The synergistic effect of exogenously added G_i protein declined with fractions eluting later than fraction 22, i.e., those containing α and βγ. Thus, the G protein subunits were distinguished from the coupling cofactor, but this experiment provided further evidence that the cofactor activity relied on an abundance of G proteins capable of interacting with the receptor.

TABLE 3

The addition of rG_{i1} promotes the activity of the A_1 adenosine receptor/G protein coupling cofactor

IHPIA binding to detergent extracted rat brain membranes (Acceptor membrane; 25 μg protein) was determined in a final volume of 40 μl containing $rG_{i\alpha 1}$ (α_{i1}, 0.15 μM), βγ subunits (+βγ, 0.45 μM), or a combination of both (+αβγ) at a final concentration of 1 mM CHAPS (Control incubations); a second set of incubations was carried out in the presence of concentrated eluate (4 μl) corresponding to fractions 17/18 (compare with Fig. 4; +Coupling cofactor). Specific IHPIA binding is given as mean values ± standard error of three experiments. Binding in the presence of 3 μM GTPγS is expressed as percentage of IHPIA bound specifically in the absence of GTPγS; given are the geometric mean values with 95% confidence intervals.

	Acceptor membrane	+ α_{i1}	+ βγ	+ αβγ
Control				
IHPIA bound (fmol/mg)	102.5 ± 7.2	142.9 ± 11.4	117.0 ± 21.8	139.0 ± 13.4
Binding resistant to GTPγS (%)	7 (1–34)	17 (6–45)	12 (10–15)	17 (7–41)
+ Coupling cofactor				
IHPIA bound (fmol/mg)	125.9 ± 23.6	156.6 ± 11.0	139.5 ± 15.4	181.9 ± 36.9
Binding resistant to GTPγS (%)	18 (12–27)	66 (40–107)	31 (17–55)	79 (48–110)

Hydroxylapatite chromatography was used to confirm that the coupling cofactor is a physical entity distinct from G protein. A DEAE fraction containing coupling cofactor activity (fraction 150; see Table 1) was applied to an HTP column, and the protein was eluted by generating a potassium phosphate gradient as outlined in Fig. 5. Coupling cofactor activity was assayed after concentrating the fraction volume 10-fold and combining an aliquot with detergent-extracted rat brain membranes. Fig. 5 shows that only fractions 3 and 4 caused GTPγS refractoriness of A_1 agonist binding, whereas the peak of G protein α and β immunoreactivity was detected in fractions 4–6, resolved from the cofactor activity peak. Thus, fraction 3 conferred cofactor activity but was free of G protein subunits. The activity of coupling cofactor was enhanced in fraction 4, presumably through enrichment of the particular component or due to the concomitant presence of $G_{i/o}$. Hydroxylapatite chromatography and gel filtration of rat brain extract rule out that the activity of coupling cofactor resides on the G protein *per se* but strongly suggest that an additional protein component modifies receptor/G protein interaction.

Mechanism of action for the coupling cofactor. Coupling cofactor was enriched by the two-step, partial purification (corresponding to fraction 23/24; see Fig. 4). Increasing amounts of solubilized rat brain extract (Fig. 6A) or partially purified cofactor activity (Fig. 6B) were reconstituted with detergent-extracted acceptor membranes. Although the two-step purification performed resulted in an enrichment of the coupling cofactor activity (~5-fold; compare Fig. 6A with Fig. 6B), the degree of purification appears modest; however, we suspect that the reconstitution protocol used can be further optimized by taking advantage of the finding that the cofactor activity is supported by the readdition of G proteins. More important, the combination of the two chromatographic steps substantially depleted contaminating [^{125}I]HPIA binding activity (≥90%).

When human brain membranes were used as acceptor membranes, ~5 times the amount of purified soluble material was needed to achieve equal effects on guanine nucleotide resistance (compare Fig. 6B with Fig. 6C). The increase in GTPγS refractoriness truly reflected an interaction of the rat brain coupling cofactor with the human brain A_1 adenosine receptor and did not arise from contamination with rat receptor/G protein complex. If the rat brain fraction containing cofactor activity was added to heat-inactivated human acceptor membranes, specific [^{125}I]HPIA binding was entirely attributable to the addition of soluble protein (Fig. 6D). The binding increment was approximately equal to the amount of agonist binding transferred to noninactivated acceptor membranes but was not accompanied by any appreciable degree of guanine nucleotide refractoriness. This finding provides further evidence that the coupling cofactor is a separate moiety recognizing with lesser affinity the human than the rat A_1 adenosine receptor/G protein complex as a substrate for interaction. However, coupling cofactor is present in human brain membranes; the addition of a detergent extract from human brain restores GTPγS refractoriness to detergent-extracted rat membranes (Fig. 6A, *inset*). Because our access to human brain samples is limited, we have not pursued the characterization of coupling cofactor from human brain.

Detergent extraction not only enhances GTPγS-sensitivity of agonist binding but also increases the catalytic efficiency of the A_1 adenosine receptor. Fraction 22/23 (which contained coupling cofactor activity; see Fig. 4) was reconstituted with detergent-extracted acceptor membranes to assess the A_1 agonist-stimulated rate of [^{35}S]GTPγS binding (Fig. 7A). In the presence of coupling cofactor activity, the molar turnover number was reduced to a value similar to that obtained in native rat brain membranes (see Fig. 2A). Thus, the fraction containing coupling cofactor not only conferred resistance of agonist binding to GTPγS but also restored the constraint on receptor-catalyzed guanine nucleotide exchange, decreasing the catalytic efficiency of the A_1 adenosine receptor.

We noted in several experiments that in the presence of coupling cofactor, [^{125}I]HPIA binding was further enhanced by the addition of GTPγS (see Figs. 3A and Fig. 4, *middle*). The effect of GTPγS appeared paradoxical and was dependent on the concentration of soluble protein reconstituted with the acceptor membranes (Fig. 6, A and B). This finding provided a mechanistic clue to the action of the coupling cofactor as we assessed the effect of partially purified coupling cofactor (fraction 22/23) on the affinity of [^{125}I]HPIA in detergent-extracted membranes. Saturation isotherms were superimposable in detergent-extracted brain membranes before or after the addition of a fraction volume containing the coupling cofactor (Fig. 7B). In the presence of coupling cofactor, GTPγS did not decrease but rather increased [^{125}I]HPIA binding to the A_1 adenosine receptor due to a ~2-fold shift in binding affinity with no detectable effect on B_{max} (K_D = 3.5 ± 0.1 nM versus 1.8 ± 0.1 nM and B_{max} = 299 ± 5 and 294 ± 10 fmol/mg in the absence and presence of GTPγS, respectively).

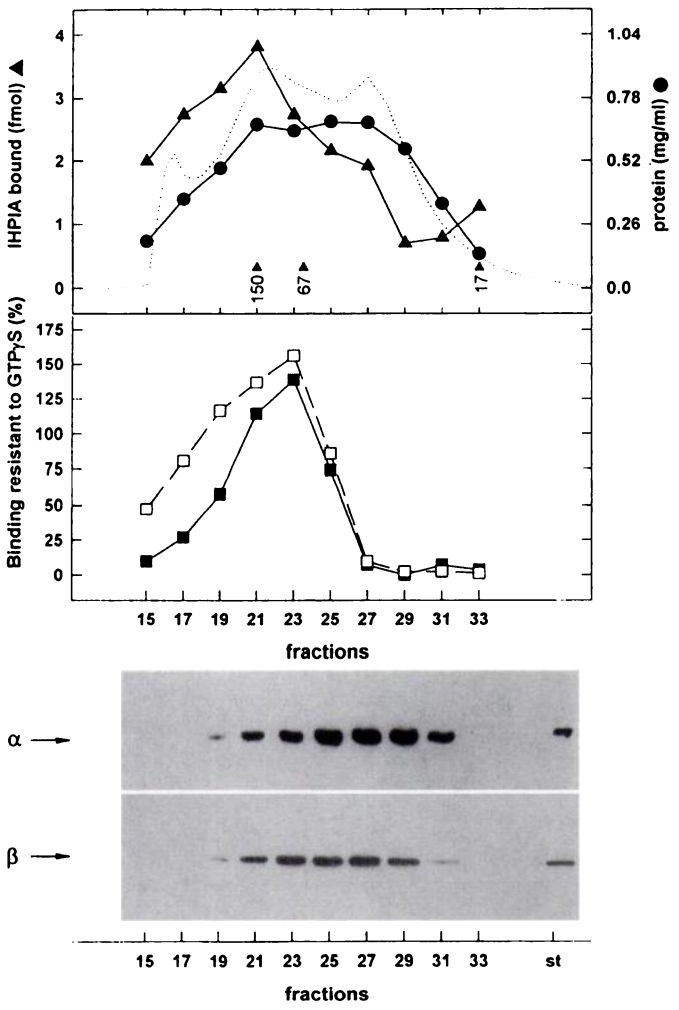

Fig. 4. Elution profile of a prepurified rat brain extract passed through a Superose HR 10/30 gel filtration column (resolution of the coupling cofactor from G proteins). Protein eluted from the DEAE column at 150 mM NaCl was concentrated, and ~3 mg was passed through a Superose column as described in Experimental Procedures. The eluate was collected in 0.5-ml fractions, and every two fractions were combined, starting with fraction 15. The volume was reduced to 0.12 ml by concentrating over PM30 (15–30) and PM10 (31–34) membranes. Before concentrating, an aliquot was reserved for protein determination and immunoblotting. *Top*, Protein concentration (●) and UV trace at 280 nm is given along the elution profile. The concentrated eluate was assayed by [^{125}I]IHPIA binding (2 nM) on addition of 5-μl aliquots to detergent-extracted membranes (see Fig. 3). *Numbers* indicate the elution volume of protein standards (IgG, BSA, lysozyme). *Middle*, Guanine nucleotide modulation of [^{125}I]IHPIA binding. On reconstitution of detergent-extracted membranes with soluble protein, the effect of 3 μM GTPγS on IHPIA binding was evaluated. Given is the ratio of guanine nucleotide-modulated IHPIA binding versus control binding, expressed in percentage (■). Also, the binding assay was carried out with the inclusion of purified holo-G_i as described in Table 3 (■). This experiment has been repeated twice with similar results. *Bottom*, For immunoblotting, 8 μl of eluate was electrophoretically separated on a 12% sodium dodecyl sulfate-polyacrylamide gel, and proteins were transferred to a nitrocellulose membrane. Membranes were probed with an antiserum raised against a peptide sequence conserved in G protein α subunits (*top band*) or an antiserum generated against a conserved β sequence (*bottom band*). Bands were visualized with a second antibody conjugated to horseradish peroxidase using the ECL reagents (Amersham). *Bands on the right* are recombinant α_o and a bovine brain β standard (*st*).

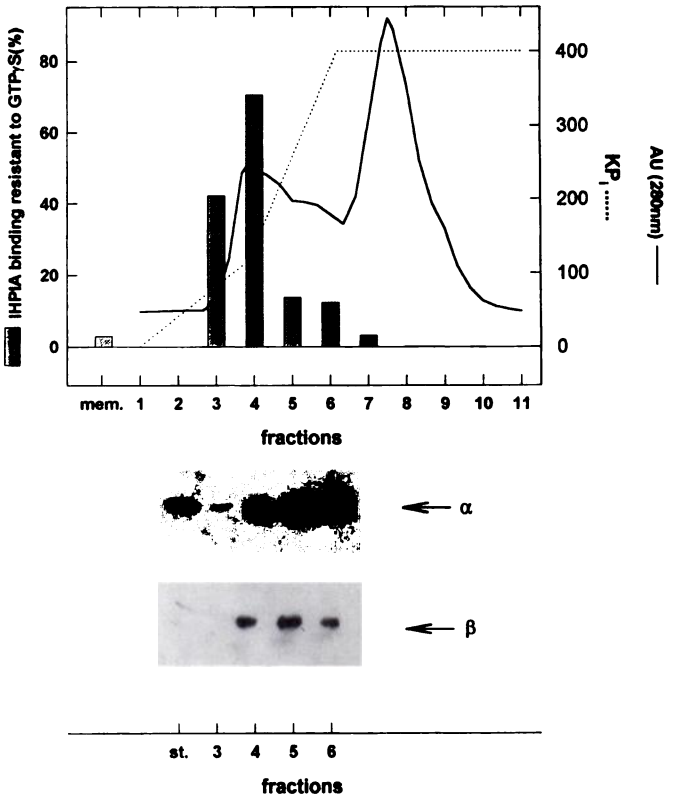

Fig. 5. Resolution of coupling cofactor activity from G protein subunits by hydroxylapatite chromatography. Protein eluted from DEAE-Sephacel column was diluted in equilibration buffer and applied to an HTP-Econo column as described in Experimental Procedures. After washing, the column protein was eluted by increasing the concentration of potassium phosphate to 0.4 M, generating a two-phase gradient as indicated. The eluate was collected in 2.25-ml fractions and concentrated over PM 30 membranes to ~0.2 ml. *Top*, Coupling cofactor activity was examined after reconstitution of a 4-μl aliquot with acceptor membranes; *bars* indicate the ratio of IHPIA bound specifically in the presence and the absence of 3 μM GTPγS (see Fig. 3). Specific IHPIA binding was between ~190 fmol/mg in acceptor membranes and ~320 fmol/mg membrane protein with the addition of fraction 6. *Bottom*, Immunoblotting was performed on a 2-μl aliquot of the indicated fractions after concentration as outlined in the legend to Fig. 4. *Left*, Standards (*st.*). These results are representative of two experiments.

Discussion

In the present study, we identified a membrane-bound component that imposes an inhibitory constraint on the A_1 adenosine receptor-dependent signaling. Previously, it has been reported that on solubilization of the functionally intact A_1 receptor/G protein complex from rodent brain membranes, receptor/G protein coupling became more susceptible to guanine nucleotide modulation (11–13). We show that relief from this constraint inherent to rodent and bovine membranes does not require separation of the receptor from the membrane environment. The component whose removal eliminates the constraint is a membrane-bound protein, and our results show that neither the A_1 adenosine receptor nor the G protein *per se* can account for its functional activity. We demonstrate that the component can be resolved from the bulk of the the receptor (DEAE chromatography) and is separated from G proteins through either gel filtration or hydoxylapatite chromatography. Although this component, which we refer to as the coupling cofactor, is physically dis-

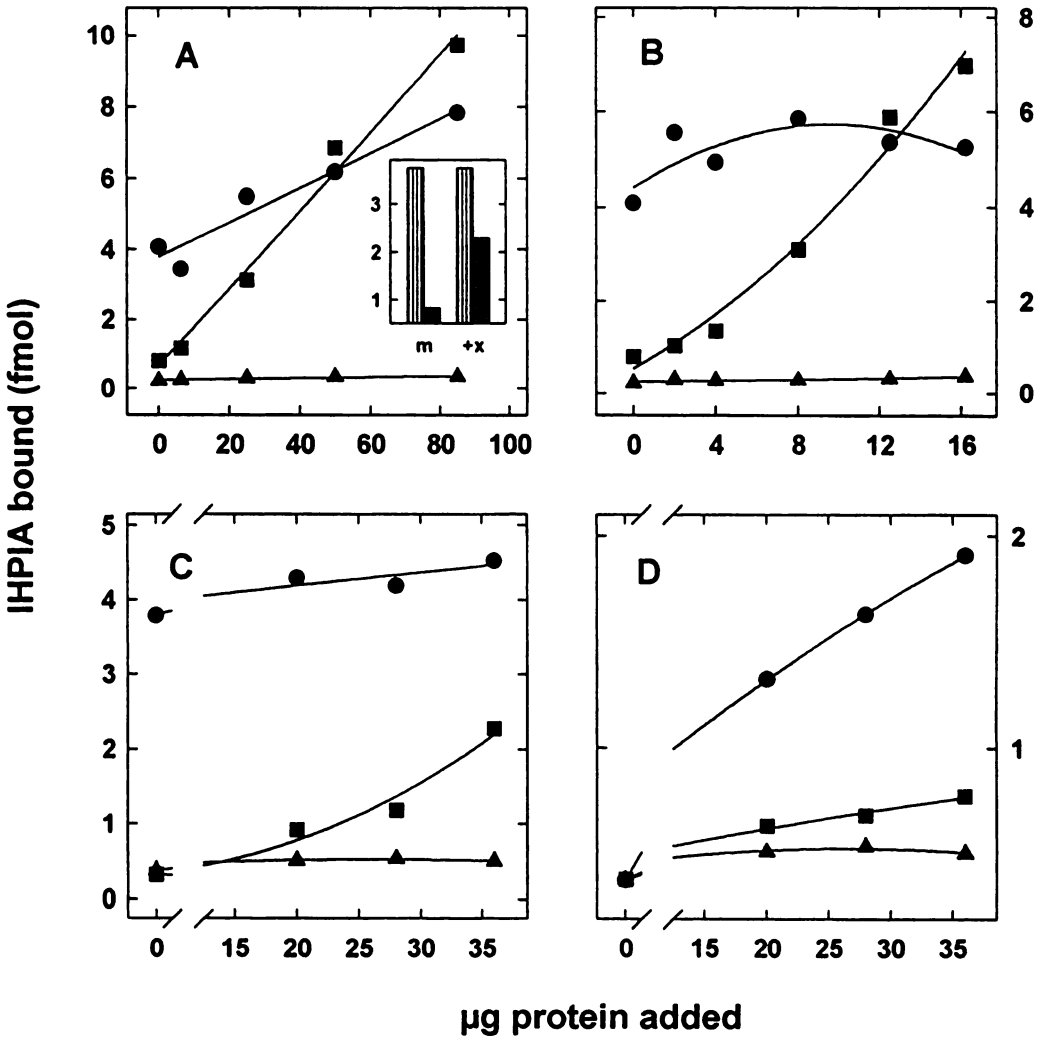

Fig. 6. Partial purification of the A_1 adenosine receptor coupling cofactor (resolution from soluble A_1) agonist high affinity binding and reconstitution of the coupling cofactor with human brain membranes. A solubilized extract from rat brain membranes was sequentially chromatographed over a DEAE and a Superose gel filtration column (see Experimental Procedures). The fractions containing peak activity of the coupling cofactor were pooled and concentrated and assayed, as outlined in the legend to Fig. 3. Aliquots were diluted with elution buffer to give the indicated amounts of protein in 5 µl (B) or 8 µl (C and D), respectively. Likewise, crude solubilized extract was diluted with solubilization buffer (A). To detergent-extracted rat brain membranes (~25 µg membrane protein), 5 µl of soluble protein was added, and [^{125}I]HPIA binding was assayed in a final volume of 50 µl (A and B). Human brain membranes (35 µg; C) and membranes that had been heat-inactivated for 3 hr at 60° (35 µg; D) were reconstituted with 8 µl of partially purified soluble protein, and binding assays were carried out in a volume of 80 µl. Equilibrium IHPIA binding was determined in the absence (●) or presence of either 3 µM GTPγS (■) or 1 µM CPA (▲). The reaction was carried out for 90 min at 25°. *Inset*, Human brain membranes were solubilized as described for rat brain membranes (see Experimental Procedures). Concentrated soluble extract (55 µg; +*x*) was combined with detergent-extracted rat brain membranes (*m*). IHPIA binding was determined in the absence (*striped bars*) or presence (*cross-hatched bars*) of 3 µM GTPγS.

tinct from receptor and G protein, its interaction with the receptor requires the presence of G proteins.

After associating with the A_1 adenosine receptor/G protein complex, the coupling cofactor operates as a brake on signal amplification. All observations are consistent with the interpretation that the coupling cofactor traps the ternary complex of agonist/receptor/G protein (HRG) in the high affinity state. (i) In the presence of coupling cofactor, high affinity binding of the agonist radioligand is resistant to guanine nucleotides. (ii) Thermodynamic considerations imply that the affinity of the agonist-liganded receptor for the G protein increases progressively during the individual transition steps (HRG_{GDP}, HRG_{empty}, HRG_{GTP}), with the highest affinity being reached in the complex HRG_{GTP} (23, 24). In the presence of coupling cofactor, GTPγS increased high affinity agonist binding, suggesting that the signaling process has been arrested in the conformation HRG_{GTP} and that coupling cofactor prevented the subsequent destabilization of the receptor/G protein complex induced by subunit dissociation of the G protein. (iii) A block imposed at this transitional stage also accounts for the ability of coupling cofactor to inhibit the catalytic efficiency of the receptor. An alternative interpretation, which considers the coupling cofactor merely as a modulator of guanine nucleotide affinity, does not adequately explain the paradoxical increase in agonist affinity induced by GTPγS in the presence of coupling cofactor. (iv) GTPγS was 100-fold more potent in enhancing antagonist binding than in inhibiting agonist binding in native membranes. Removal of coupling cofactor by detergent extraction eliminated this difference in potency. Evidently, the coupling co-

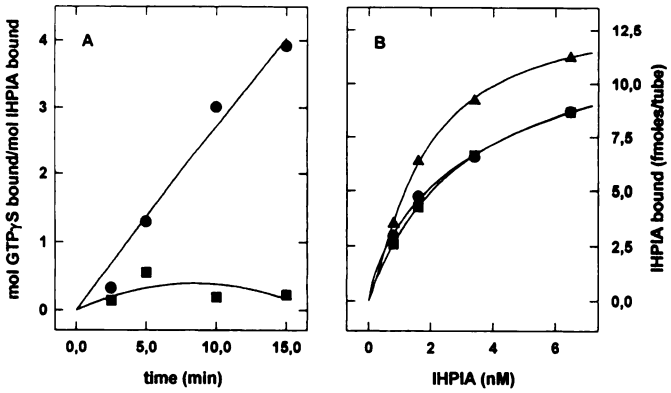

Fig. 7. Effect of partially purified coupling cofactor on the agonist-stimulated rate of [^{35}S]GTPγS binding (A) and on the saturation isotherm for [^{125}I]HPIA binding (B) to extracted rat brain membranes. A, Partially purified coupling cofactor (~15 μg protein) was added to detergent-extracted rat brain membranes (~20 μg) as outlined in the legend to Fig 3. [^{35}S]GTPγS binding was determined in 50 μl containing 20 mM HEPES·NaOH, pH 7.5, 0.5 mM EDTA, 0.1 mM GDP, 1.5 mM $MgCl_2$, and 150 mM NaCl in the presence of either 10 μM CPA or 1 μM DPCPX. The assay was initiated by the addition of 10 nM [^{35}S]GTPγS (specific activity, 50 nCi/fmol) and was carried out for the indicated periods. Shown is the A_1 agonist-induced increment in GTPγS binding (see Fig. 2A) in extracted membranes without (●) or with (■) the addition of partially purified coupling cofactor. These numbers were normalized for the number of A_1 adenosine receptors determined by [^{125}I]HPIA saturation binding in extracted and reconstituted membranes. The results are representative of two experiments. B, Saturation binding was carried out at the indicated concentrations of [^{125}I]HPIA in a volume of 40 μl containing 20 mM HEPES·NaOH, pH 7.5, 0.5 mM EDTA, 5 mM $MgCl_2$, and detergent-extracted membranes (~20 μg) combined with 4 μl of soluble protein from fractions 17–20 (10 μg; compare with Fig. 7, ■) or with 4 μl ultrafiltrate (●). The soluble fraction was supplemented with purified holo-G_{i1} as described in legend to Fig. 5 (final concentration = 0.1 μM). [^{125}I]HPIA binding to reconstituted membranes was performed in the absence (■) or presence (▲) of 3 μM GTPγS. The incubation was for 90 min at 25°. Nonspecific binding was assessed in the presence of 1 μM XAC.

factor interacts preferentially with the agonist-bound receptor. It is therefore intriguing to speculate that the agonist-induced conformational change in the receptor/G protein complex increases the affinity for both GTP (via GDP release) and the coupling cofactor.

An additional surprising finding was that the human brain A_1 adenosine receptor does not interact with the coupling cofactor in the membranes. The presence of coupling cofactor was clearly demonstrated on reconstitution of human brain extracts with rat acceptor membranes. In addition, rat brain coupling cofactor can interact with the human A_1 adenosine receptor, but apparently the concentrations required are higher than those present in human brain membranes. We therefore conclude that in human brain membranes the high guanine nucleotide sensitivity is due to the low affinity of the A_1 adenosine receptor for the coupling cofactor and not to a lack of the component itself.

Several proteins are known to interfere with receptor/G protein coupling; these are candidates for mediating the effects of the coupling cofactor. Phosducin, arrestin, and their related homologues as well as the microfilament protein tubulin are among the best-characterized proteins that modulate the interaction between receptors and G proteins. Phosducin and arrestin are readily distinguished from coupling cofactor. Phosducin (and phosducin-like proteins) has been identified in the cytosol of cerebral cortex; G protein βγ subunits bind to phosducin and are supposed to target phosducin to the cell membrane (25–27). The association of phosducin with G proteins inhibits the receptor-dependent activation of effectors. The fact that active phosducin was detected in and purified from cytosolic fractions (28) rules out that phosducin and coupling cofactor are the same protein; concentrated preparations from rat brain cytosol did not contain any coupling cofactor activity (not shown). β-Arrestin and the retinal counterpart arrestin are cytosolic cofactors that mediate receptor desensitization. These proteins bind with high affinity to phosphorylated receptors and compete directly with G proteins (see Ref. 29 for a review). This is incompatible with coupling cofactor activity, which is enhanced by G proteins. Although agonist-stimulated receptor phosphorylation was demonstrated to occur, the A_1 adenosine receptor appears to be a poor target for protein kinases with very few phosphorylation consensus sites (3, 30). Tubulin was demonstrated to bind to G proteins and augment effector stimulation, indicating that it may even enhance subunit dissociation (31, 32). Likewise, reports on stimulation of leukocytes indicate that G proteins adhere to the actin filament network and are released on fMLP receptor or direct guanine nucleotide-induced activation (33). The effects of these microfilament proteins are the opposite of coupling cofactor activity. Moreover, tubulin-dependent alterations in G protein-mediated signal transduction are sensitive to microtubule dissolving agents (34). The addition of colchicin and vinblastine to native rat brain membranes, however, did not alter the GTPγS-refractoriness of [^{125}I]HPIA binding (not shown). Other structural proteins like spectrin, dynamin, and caveolin are believed to participate in the organization of receptor/G protein coupling; speculations have focused on the possibility that such a higher level of organization contributes to the specificity of receptor/G protein interaction (see Ref. 35 for a review). The possibility exists that coupling cofactor activity is exerted by one of these protein components of the cytoskeleton. Obviously, unequivocal assignment of coupling cofactor activity to a protein requires its purification to homogeneity; this is being attempted.

Further instances for restrained signaling efficiency include the mammalian A_2 adenosine receptor and the β-adrenoceptor in avian erythrocytes. In both receptors, structural domains of the receptor itself are responsible for the resistance of the ternary complex to guanine nucleotide modulation (14, 36). It is not known whether these domains (e.g., the extended carboxyl-terminal domain in the avian β-adrenoceptor) make contact with the G protein or with an additional component, such as the coupling cofactor. The GTP sensitivity of agonist binding to the 5-hydroxytryptamine$_2$ receptor depends on the cell line used to express the cloned receptor; in a human kidney cell line (293), the entire receptor population was resistant to GTP analogues, whereas in a mouse cell line (NIH 3T3 fibroblasts), a 70% fraction and a 30% fraction of the receptor population in rat brain membranes did not respond (37). In these experiments, guanine nucleotide refractoriness cannot be solely specified by the receptor/G protein interaction but suggests that differences are in the expression levels or the affinity of an additional component for the receptor. The analogy to our observations, in particular with the human brain A_1 adenosine receptor, are obvious. In addition, we have observed that the the guanine nucleotide sensitivity of the striatal D_2 dopamine receptor is

also enhanced by removal of an inhibitory constraint in a manner similar to the A_1 adenosine receptor.[1] Therefore, the coupling cofactor appears to associate with several receptors. The inhibition imposed by coupling cofactor may be relieved or enhanced by a concomitant signal activated by a second receptor and thus may be involved in cross-talk between receptors. A membrane-delineated, reciprocal inhibition of receptor/G protein coupling that specifically modulates the GTP sensitivity of agonist binding has been noted between A_{2a} adenosine receptors and D_2 dopamine receptors in striatal membranes (38).

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References

1. Mahan L. C., L. D. McVittie, E. M. Smyk-Randall, H. Nakata, F. J. Monsma, C. R. Gerfen, and D. R. Sibley. Cloning and expression of an A_1 adenosine receptor from rat brain. *Mol. Pharmacol.* **40**:1–7 (1991).
2. Reppert, S. M., D. R. Weaver, J. H. Stehle, and S. A. Rivkees. Molecular cloning and characterization of a rat A_1 adenosine receptor that is widely expressed in brain and spinal cord. *Mol. Endocrinol.* **5**:1037–1048 (1991).
3. Olah, M., H. Ren, J. Ostrowski, K. A. Jacobson, and G. L. Stiles. Cloning, expression and characterization of the unique bovine A_1 adenosine receptor. *J. Biol. Chem.* **267**:10764–10770 (1992).
4. Libert, F., S. N. Schiffmann, A. Lefort, M. Parmentier, C. Gerard, J. E. Dumont, J.-J. Van der Haegen, G. Vassart. The orphan receptor cDNA RDC7 encodes an A_1 adenosine receptor. *EMBO J.* **10**:1677–1682 (1991).
5. Townsend-Nicholson, A., and J. Shine. Molecular cloning and characterisation of a human brain A_1 adenosine receptor cDNA. *Mol. Brain Res.* **16**:365–370 (1992).
6. Olah, M. E., K. A. Jacobson, and G. L. Stiles. Identification of an adenosine receptor domain specifically involved in binding of 5′-substituted adenosine agonists. *J. Biol. Chem.* **269**:18016–18020 (1994).
7. Freissmuth, M., E. Selzer, and W. Schütz. Interactions of purified bovine brain A_1 adenosine receptors with G-proteins. *Biochem. J.* **275**:651–656 (1991).
8. Freissmuth, M., W. Schütz, and M. E. Linder. Interactions of the bovine brain A_1 adenosine receptor with recombinant G protein α subunits. *J. Biol. Chem.* **266**:17778–17783 (1991).
9. Jockers R., M. E. Linder, M. Hohenegger, C. Nanoff, B. Bertin, A. D. Strosberg, S. Marullo, and Freissmuth, M. Species difference in the G protein selectivity of the human and the bovine A_1 adenosine receptor. *J. Biol. Chem.* **269**:32077–32084 (1994).
10. Nakata, H. Biochemical and immunological characterization of A_1 adenosine receptors purified from human brain membranes. *Eur. J. Biochem.* **206**:171–177 (1992).
11. Stiles G. L. The A_1 adenosine receptor. solubilization and characterization of a guanine nucleotide-sensitive form of the receptor. *J. Biol. Chem.* **260**:6728–6732 (1985).
12. 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).
13. Ströher M., C. Nanoff, and W. Schütz. Differences in the GTP-regulation of membrane-bound and solubilized A_1 adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **340**:87–92 (1989).
14. Nanoff, C., K. A. Jacobson, and G. L. Stiles. The A_2 adenosine receptor: guanine nucleotide modulation of agonist binding is enhanced by proteolysis. *Mol. Pharmacol.* **39**:130–135 (1991).
15. Nanoff, C., and G. L. Stiles. Solubilization and characterization of the A_2 adenosine receptor. *J. Recept. Res.* **13**:961–973 (1993).
16. Florio, V. A., and P. C. Sternweis. Reconstitution of resolved muscarinic cholinergic receptors with purified GTP-binding proteins. *J. Biol. Chem.* **260**:3477–3483 (1985).
17. Linden, J. Purification and characterization of (-)[^{125}I]hydroxyphenylisopropyladenosine, an adenosine R-site agonist radioligand and theoretical analysis of mixed stereoisomer radioligand binding. *Mol. Pharmacol.* **26**: 414–423 (1984)
18. Casey, P. J., M. P. Graziano, and A. G. Gilman. G protein *beta gamma* subunits from bovine brain and retina: equivalent catalytic support of ADP-ribosylation of *alpha* subunits by pertussis toxin but differential interactions with G_s *alpha*. *Biochemistry* **28**:611–616 (1989).
19. Mumby, S. M., and M. E. Linder. Myristoylation of G protein α subunits. *Meth. Enzymol.* **237**:254–267 (1994).
20. Carty D. J. Pertussis toxin-catalyzed ADP-ribosylation of G proteins. *Methods Enzymol.* **237**:63–70 (1994).
21. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
22. Ramkumar, V., and Stiles, G. L. Reciprocal modulation of agonist and antagonist binding to A_1 adenosine receptors by guanine nucleotides is mediated via a pertussis toxin-sensitive G protein. *J. Pharmacol. Exp. Ther.* **246**:1194–1200 (1988).
23. Birnbaumer, L., J. Abramowitz, and A. M. Brown. Receptor-effector coupling by G proteins. *Biochim. Biophys. Acta* **1031**:163–224 (1990).
24. Schütz W., and M. Freissmuth. Reverse intrinsic activity of antagonists on G protein-coupled receptors. *Trends Pharmacol. Sci.* **13**:376–380 (1992).
25. Lee R. H., B. S. Lieberman, and R. N. Lolley. A novel complex from bovine visual cells of a 33000-Dalton phosphoprotein with β- and γ-transducin: Purification and subunit structure. *Biochemistry* **26**:3983–3990 (1987).
26. Hekman M., P. H. Bauer, P. Sohlemann, and M. J. Lohse. Phosducin inhibits receptor phosphorylation by the *beta*-adrenergic receptor kinase in a PKA-regulated manner. *FEBS Lett.* **343(2)**:120–124 (1994).
27. Miles, M. F., S. Barhite, M. Sganga, and M. Elliott. Phosducin-like protein: an ethanol-responsive potential modulator of guanine nucleotide-binding protein function. *Proc. Natl. Acad. Sci. USA* **90**:10831–10835 (1993).
28. Bauer P. H., S. Müller, M. Puzicha, S. Pippig, B. Obermaier, E. J. Helmreich, and Lohse, M. J. Phosducin is a protein kinase A-regulated G protein regulator. *Nature (Lond.)* **358**:73–76 (1992).
29. Hausdorff, W. P., M. G. Caron, and Lefkowitz, R. J. Turning off the signal: desensitization of β-adrenergic receptor function. *FASEB J.* **4**:2881–2889 (1991).
30. Ramkumar, V. M., E. Olah, K. A. Jacobson, and Stiles, G. L. Distinct pathways of desensitization of A_1- and A_2 adenosine receptors in DDT_1 MF-2 cells. *Mol. Pharmacol.* **40**:639–647 (1991).
31. Wang N., and Rasenick, M. M. Tubulin-G protein interactions involve microtubule polymerization domains. *Biochemistry* **30**:10957–10965 (1991).
32. Popova J. S., G. L. Johnson, and M. M. Rasenick. Chimeric $G_{\alpha s}/G_{\alpha i2}$ proteins define domains on $G_{\alpha s}$ that interact with tubulin for β-adrenergic activation of adenylyl cyclase. *J. Biol. Chem.* **269**:21748–21754 (1994).
33. Särndahl E., G. M. Bokoch, O. Stendahl, and Andersson, T. Stimulus-induced dissociation of α subunits of heterotrimeric GTP-binding proteins from the cytoskeleton of human neutrophils. *Proc. Natl. Acad. Sci. USA* **90**:6552–6556 (1993).
34. Leiber D., J. R. Jasper, A. A. Alousi, J. Martin, D. Berstein, and P. A. Insel. Alteration in G_s-mediated signal transduction in S49 lymphoma cells treated with inhibitors of microtubules. *J. Biol. Chem.* **268**:3833–3837 (1993).
35. Neubig R. R. Membrane organization in G-protein mechanisms. *FASEB J.* **8**:939–946 (1994).
36. Parker, E. M., and E. M. Ross. Truncation of the extended carboxyl-terminal domain increases the expression and regulatory activity of the avian β-adrenergic receptor. *J. Biol. Chem.* **266**:9987–9996 (1991).
37. Szele, F. G., and D. B. Pritchett. High affinity agonist binding to cloned 5-hydroxytryptamine$_2$ receptors is not sensitive to GTP-analogs. *Mol. Pharmacol* **43**:915–920 (1993).
38. Ferré, S., G. v. Euler, B. Johansson, B. B. Fredholm, and K. Fuxe. Stimulation of high affinity adenosine A_2 receptors decreases the affinity of dopamine D_2 receptors in rat striatal membranes. *Proc. Natl. Acad. Sci. USA* **88**:7238–7244 (1991).

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