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Inhibitory GTP-Binding Regulatory Protein G_{i3} Can Couple Angiotensin II Receptors to Inhibition of Adenylyl Cyclase in Hepatocytes

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SUMMARY

Angiotensin II can inhibit hormone-stimulated adenylyl cyclase in intact hepatocytes or in hepatic membrane preparations. Because the response can be blocked by pertussis toxin, the object of the present study was to determine which of the known variants of G_i can couple angiotensin II receptors to inhibition of adenylyl cyclase. The potential candidates were identified by probing RNA isolated from rat hepatocytes with cDNAs specific for the α subunits of known toxin-sensitive guanine nucleotide-binding regulatory proteins (G proteins). Hepatocytes contained no detectable RNA for the G_o or G_{i1} α subunits and similar levels of RNA coding for the G_{i2} and G_{i3} α subunits. To determine whether G_{i3} could couple angiotensin receptors to inhibition of cyclase, membranes were prepared from hepatocytes whose G proteins were fully ADP-ribosylated with pertussis toxin, and the G_{i3} holoprotein purified from rabbit liver was reconstituted into the membranes. The nature of the G_{i3} reconstituted into the membrane was assessed by immunoblotting with antibodies specific for the G_i α subunits. Reconstitution of 6–10 pmol of G_{i3}/mg of membrane protein into the toxin-treated membranes restored the ability of 10 nM angiotensin II to inhibit adenylyl cyclase. Because pertussis toxin has nonspecific effects, an assay was developed to measure the interaction of the angiotensin receptor with reconstituted G proteins in normal membranes. In the presence of Mg^{2+}, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) caused a reduction of the affinity of the angiotensin II receptor for ^{125}I-angiotensin II that was stable to washing and the detergents used to reconstitute G proteins into the membranes. Using this protocol to activate G proteins and "uncouple" receptors, the ability of the GDP-liganded form of G_i to restore high affinity binding was examined. Reconstitution of about 10–15 pmol of oligomeric G_{i3}/mg of membrane protein restored both the high affinity state of the angiotensin II receptor and the ability of GTPγS to shift the affinity to a lower state. The same shift in receptor affinity could be accomplished by reconstituting the G_{i3} α subunit, resolved free of $\beta\gamma$ subunits, into the membranes. Reconstitution of up to 50 pmol of G_s/mg of membrane protein had no effect on angiotensin II receptor affinity. The results suggest that a major form of G_i in hepatocytes is G_{i3} and that it can couple angiotensin receptors to inhibition of adenylyl cyclase.

Appreciation of the role of G proteins in transmembrane signaling is expanding rapidly (1–4). Elucidation of the actions of G_t in the transduction of light in the rod outer segment (5) and of G_s and G_i in mediating the effects of hormones on the adenylyl cyclase complex (6, 7) has provided important examples of how these proteins stimulate effector enzymes to generate second messengers within cells. The mechanism by which hormones regulate adenylyl cyclase uses two similar G proteins, G_s and G_i, to couple a variety of stimulatory or inhibitory receptors, respectively, to the catalytic unit that converts ATP to cyclic AMP (8). Although the role of G_s in the stimulation of adenylyl cyclase is well characterized (1, 6, 8), the exact role of G_i in mediating inhibition of adenylyl cyclase still needs clarification (4, 9–11).

Purification of different G proteins has demonstrated that they are heterotrimers consisting of α, β, and γ subunits (1–5). Molecular cloning has elucidated the primary structure of the α, β, and γ subunits of these proteins and demonstrated that G_s and G_i belong to a family of G proteins currently numbering more than 15 members, based on differences in their α subunits (4, 12). In addition, there is clear evidence for heterogeneity of the β and γ subunits (13–18). The reasons for the diversity of the various polypeptides comprising the G protein family are

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ABBREVIATIONS: G protein, guanine nucleotide-binding regulatory protein; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); BSA, bovine serum albumin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; angiotensin II, (Asp[1]-Ile[5])-angiotensin II; GABA, γ-aminobutyric acid; SDS, sodium dodecyl sulfate.

not completely understood. However, certain G proteins have been demonstrated to activate multiple effector enzymes (1, 3, 4), and some members of the family are differentially expressed in certain tissues (19). Evidence also exists to suggest that different isozymes of closely related members of the family may couple to distinct receptors (20, 21). The α subunits of the G proteins are thought to be responsible for the stimulation of effector enzymes, a point verified using purified or recombinant G_s or G_i α subunits to stimulate adenylyl cyclase (6, 22, 23) or muscarinic K^+ channels (4, 24, 25), respectively.

G_i is a member of the family that is thought to mediate inhibition of adenylyl cyclase (1, 7, 8), stimulation of phosphatidylinositol metabolism (26–28), activation of muscarinic K^+ channels (4, 24, 25), and inhibition of ligand-gated Ca^{2+} channels (21). Molecular cloning and direct purification have demonstrated that there are at least three G_i-like proteins with distinct α subunits, termed G_{i1}, G_{i2}, and G_{i3} (1, 4, 29). Previous work has shown that the α subunits of all three of these proteins appear to be able to couple to the muscarinic K^+ channel (24, 25, 30). Although the three forms of G_i are able to inhibit GTPγS-stimulated adenylyl cyclase in $S49^{cyc-}$ cell membranes reconstituted with G_s (30), the forms of G_i that couple hormone receptors to inhibition of adenylyl cyclase have not been clarified. Hepatic membranes contain glucagon receptors that are linked to adenylyl cyclase via G_s (1) and an abundance of angiotensin II receptors that couple to inhibition of adenylyl cyclase via one of the G_i proteins (31, 32). Thus, the hepatic membrane provides a useful experimental system in which to study the interaction of receptors and G_i with the cyclase moiety.

This work was undertaken to explore the role of one variant of G_i, G_{i3}, in coupling the angiotensin II receptor to inhibition of adenylyl cyclase. The basic experimental approach was to prepare plasma membranes from hepatocytes isolated from normal rats or from rats that had been pretreated with enough pertussis toxin to completely ADP-ribosylate the G_i in the membranes of the cells. These membranes were used in two assay systems designed to measure the ability of purified G_{i3} either to recouple the interaction of the angiotensin II receptor with the adenylyl cyclase complex in toxin-treated membranes or to regulate the affinity of the angiotensin II receptor in normal membranes. The results demonstrate that G_{i3} can couple the angiotensin II receptor to adenylyl cyclase.

Materials and Methods

Treatment of rats with pertussis toxin and isolation of hepatocytes. Hepatocytes were isolated from fasted, 150–200-g, male Wistar rats by perfusion of the liver with collagenase. The hepatocytes were collected, washed three times, diluted to a concentration of ~2 × 10^7 cells/ml in Krebs-Ringer bicarbonate buffer supplemented with 16 mM L-lactate and 4 mM pyruvate, and gassed with 95% O_2/5% CO_2 (33). To obtain hepatocytes whose G_i was completely ADP-ribosylated by pertussis toxin, intact rats were injected with 50 μg of toxin/100 g of body weight, in the peritoneal cavity, 72 hr before isolation of hepatocytes (32).

Hepatocyte membrane preparation. Membranes were prepared from freshly isolated hepatocytes obtained from normal or pertussis toxin-treated rats as described (32). Cells were pelleted at 50 × *g* for 3 min, and the pellet was homogenized in 5 mM EDTA, 10 mM HEPES, pH 7.4, with a Dounce homogenizer. Homogenates were centrifuged at 150 × *g* for 5 min to remove unbroken cells. The supernatant was centrifuged at 1500 × *g* for 15 min, resuspended in the initial volume of homogenization buffer, and centrifuged again at 3000 × *g* for 20 min. The pellets were resuspended in a minimal volume of buffer, adjusted to 42% (w/v) sucrose, and centrifuged on a sucrose gradient, in an SW50.1 rotor, at 82,000 × *g* for 2 hr. Plasma membrane fractions were collected from the top of the gradient, washed twice by resuspension and centrifugation at 15,000 × *g* for 15 min in 20 mM Tris, pH 7.5, and stored in liquid N_2. The extent of the ADP-ribosylation of the G_i in the membranes prepared from cells isolated from toxin-treated rats was determined as described (32). Only membranes that had >99% of the G_i modified by the toxin pretreatment were used in the reconstitution experiments described in Results. In pilot experiments testing the feasibility of uncoupling angiotensin II receptors from G proteins by treatment of membranes with GTPγS, a membrane preparation from frozen rat livers was employed. Fifty grams of rat liver were frozen in liquid N_2, coarsely pulverized, thawed in 250 ml of 20 mM Tris·HCl, pH 7.5, 1 mM EDTA, and homogenized in a blender. A plasma membrane fraction was purified on a sucrose gradient, as described above for membranes from rat hepatocytes (32).

Purification of G proteins. Rabbit liver G_{i3} was purified by a modification of the method described by Bokoch *et al.* (34). Because the normal procedures do not resolve the three different G_i forms (30, 35), G_{i3} was purified as follows. The G_i in four preparations was pooled after purification through the DEAE and ACA-34 columns. The position of G_i on the columns was monitored by GTPγS binding and pertussis toxin labeling. The peak fractions from the ACA-34 column were pooled and chromatographed over another ACA-34 column and a heptylamine-Sepharose column. The peak fractions from the heptylamine column, containing >50% of the total pertussis toxin labeling and GTPγS-binding activity, were applied to a hydroxyapatite column and eluted as described (34). The peak fraction from the hydroxyapatite column was used in these experiments. Selection of fractions by this procedure removed G_{i2} from G_{i3}, and the preparation contained no detectable G_{i1} or G_{i2} by Western blotting (see Fig. 2). The α subunit of G_{i3} was resolved from its $\beta\gamma$ subunits as described (22). The G_{i3} was stored in 20 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Lubrol. This material was diluted about 25-fold into each of the reconstitution assays described below.

Human placental G_i was purified by following the procedure of Evans *et al.* (36); platelet G_i was isolated from membranes prepared from recently outdated human platelets. The membranes were prepared as described by Katada *et al.* (7). The G_i fractions that were labeled with pertussis toxin and [^{32}P]NAD^+ were isolated from the platelet membranes exactly as described for human placental G_i by Evans *et al.* (36). Both placental and platelet G_i were further purified with a NaCl elution from a DEAE column after the hydroxyapatite step, to resolve G_{i2} from G_{i3}. Bovine brain G_o/G_i was prepared by a modification of the procedure used by Sternweis and Robishaw (37). The purification was monitored via GTPγS binding, pertussis toxin labeling, and Western blotting with β subunit antibodies. However, rather than pooling the $G_o\alpha$ subunit resolved from the $\beta\gamma$ subunits on the heptylamine-Sepharose column, the oligomeric protein ($\alpha\beta\gamma$) was pooled.

Western blotting. Western blots using antibodies specific for certain G protein α subunits were performed as follows. About 400 ng of the G protein of interest were loaded on a 12%, one-dimensional, SDS gel and run for 30–40 min past the time when the dye front had run off the bottom of the gel. The gels were cut into six identical sections; one section was stained with silver (38) and the other five were transferred to nitrocellulose at 40 V overnight in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3 (39). The nitrocellulose strips were blocked for 1 hr with 50 mM Tris, pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 5% (w/v) nonfat dry milk, 0.2% (v/v) NP-40, before probing for 1 hr with antisera specific for the different G protein α subunits, at dilutions of 1/500 to 1/2000 (21, 35, 40). The blots were washed three times in the aforementioned blocking solution, with SDS added to 0.2% (w/v) and NP-40 raised to 2.0% (v/v). The bound primary antibodies were identified with goat anti-rabbit ^{125}I-labeled IgG Fab fragments in the high detergent solution, washed, and dried (41). Autoradiographs

were made from the dried blots by exposing them to Kodak XAR-5 or XK-1 film for 1–2 days.

Isolation of RNA and Northern blotting. Nucleic acid was extracted from fresh rat hepatocytes or rat brains via homogenization with 4 M guanidine isothiocyanate, followed by separation of RNA from DNA on a cesium chloride gradient (42). The RNA was separated by electrophoresis on a denaturing 1.2% agarose gel and blotted to a Zetabind (Cuno) nylon membrane (43). The membranes were hybridized at 65° overnight with Church hybridization buffer (44) containing ^{32}P-labeled probe. The full-length cDNA probes for the α subunits of G_{i1}, G_{i2}, and G_{i3} were labeled with [α-^{32}P]ATP using the random-primer method (PrimeTime, IBI). The 5′ end of the G_o clone was removed before labeling, to reduce hybridization that was not specific for the α subunit of G_o. Unincorporated nucleotides were removed from the DNA by chromatography on a DuPont NENSORB column. The percentage of incorporation and specific activity of the cDNA probes were determined as follows. The PrimeTime reaction mix was diluted 1/100, and 1 μl of the mixture was added to 125 μl of 200 mM EDTA, 20 μl of 10 mg/ml sonicated herring sperm DNA, and 105 μl of water. Twenty five microliters of the solution were absorbed onto a glass filter to measure total cpm added. One milliliter of 1 M HCl/0.1% sodium pyrophosphate was added to the remaining solution, and the solution was placed on ice for 10 min. The precipitated DNA was filtered onto a glass filter and rinsed with HCl/sodium pyrophosphate, followed by cold 95% ethanol. The filters were counted in a liquid scintillation counter to measure the radioactivity incorporated into the cDNA. The probes were diluted with the appropriate cDNA to equal specific activities, based on the amount of DNA added to the original reaction and the counts recovered on the filters. The blots were washed at 65° with two solutions of increasing stringency, dried, and subjected to autoradiography at −70° with Kodak XAR-5 film and DuPont Cronex intensifying screens.

Preparation of monoiodinated ^{125}I-Sar^1-Ile^8-angiotensin II. Approximately 40 nmol of Sar^1-Ile^8-angiotensin II were incubated with 2 mCi of carrier-free ^{125}I, in a total reaction volume of 150 μl containing 66 mM $NaPO_4$, pH 7.2, 0.16% β-D-glucose, and 25 μl Enzymobeads (Bio Rad), at 21° for 25 min. The reaction was terminated by immersion of the tube in ice and centrifugation of the mixture at 13,000 × g for 2 min to remove the beads. The beads were washed with 150 μl of 0.2 M $NaPO_4$, pH 7.2, and centrifuged again to elute more iodinated peptide from the beads; the supernatants were combined and injected onto a Waters reverse phase C-18 column. The unreacted peptides and mono- and diiodinated peptides were resolved with a linear gradient of 0–42% acetonitrile containing 0.1% trifluoroacetic acid. About 70–80% of the iodine incorporated was recovered as the monoiodinated peptide (45). The specific activity of the monoiodinated peptide was calculated from its mass (based on its absorbance at 209 nm, as read on the flow detector), assuming the theoretical maximal incorporation of ^{125}I (2200 Ci/mmol).

Ligand binding assays. The binding of ^{125}I-angiotensin II or ^{125}I-Sar^1-Ile^8-angiotensin II to its receptor was performed in a buffer consisting of 20 mM Tris, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, and 0.2% crystalline BSA, as described (46). Binding isotherms were performed using ^{125}I-angiotensin II at concentrations ranging from 0.12 to 1.2 nM. When a ligand concentration greater than 1.2 nM was required, unlabeled angiotensin II was added to achieve the concentrations indicated in the figures. Reactions were initiated by the addition of 10–20 μg of membrane protein to the binding buffer, followed by incubation at 22° for 60 min. When desired, a final concentration of 10^{-4} M GTPγS was added to the incubations. Bound ligand was separated from free ligand by centrifugation at 13,000 × g for 1 min in an Eppendorf centrifuge, and the supernatant containing free ligand was removed by vacuum aspiration. The membrane pellet was washed once with 350 μl of ice-cold 20 mM Tris, pH 7.5, containing 0.2% fraction V BSA, and was counted at an efficiency of 75% in a Beckman Gamma 4000 counter (46).

Modification of angiotensin receptor affinity with GTPγS and reconstitution of G_{i3}. To pretreat the membranes with GTPγS and cause a stable shift of the high affinity angiotensin II binding sites to low affinity sites, hepatocyte membranes were incubated at a concentration of approximately 2.5 mg/ml in binding buffer (without crystalline BSA), with or without 0.1–1 mM GTPγS, for 30–45 min at 30°. Excess guanine nucleotide was removed by dilution of the membranes 20-fold in ice-cold 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, followed by centrifugation at 12,000 × g in a Beckman JA-21 rotor for 20 min at 4°. The wash was repeated once, and the pellet was resuspended in binding buffer at a concentration of 0.7–1.0 mg of membrane protein/ml. At this point, the membranes could be used in the normal binding assay or included in the reconstitution protocols described below. To determine whether the wash protocol removed bound GTPγS, the magnitude of the affinity shift before and after the procedure was compared. Usually, >95% of the shift was retained. After GTPγS treatment, purified G_{i3} was diluted 26-fold into a suspension containing hepatocyte membranes (0.5–0.8 mg of protein/ml), in a buffer containing 20 mM Tris, pH 7.5, 60 mM NaCl, 12 mM $MgCl_2$, and 0.04% cholate. The final concentration of Lubrol transferred with the G_{i3} was 0.004%. This mixture was incubated 10 min at 0° before the start of the binding assay described above.

Adenylyl cyclase assay and reconstitution of G_{i3}. Conversion of ATP to cAMP was measured as described (31). The assay was performed in a solution consisting of 0.5 mM [α-^{32}P]ATP (1×10^6 cpm/tube), 50 mM Tris, pH 8, 0.4 mM 3-isobutyl-1-methylxanthine, 1 mM EDTA, 2–10 mM $MgCl_2$, 10 units/ml creatine phosphokinase, 10 mM phosphocreatine, 0.1 mg/ml crystalline BSA, 0.1 mM GTP, and 250 mM LiCl. GTP and lithium were used to provide a slight stimulation of basal cyclase activity, so that the inhibitory effects of angiotensin could be observed (31). Agonists were added at the beginning of each incubation, and the reaction was continued for 10–12 min at 30°. Each reaction was stopped by the addition of a solution containing 0.25% SDS, 5 mM ATP, and 17.5 μM cAMP. The [^{32}P]cAMP formed during the assay was separated from [α-^{32}P]ATP by chromatography on columns of Dowex AG50-WX4 and alumina. Calculations of the amount of [^{32}P]cAMP formed were determined based on the overall column recovery, as measured with a [^{3}H]cAMP standard, and the specific activity of the [α-^{32}P]ATP (31). To reconstitute the ability of angiotensin to inhibit adenylyl cyclase in toxin-pretreated membranes, purified rabbit liver G_{i3} was diluted 24-fold into a suspension of hepatocyte membranes, at a concentration of 0.9 mg/ml, in a buffer of 20 mM Tris, pH 7.5, 60 mM NaCl, and 12 mM $MgCl_2$. The final concentration of Lubrol was 0.004%. G_{i3} was allowed to insert into the membranes during a 20-min incubation at 30°. Reconstituted membranes were then held on ice for a time ranging from 2 to 15 min, until they were added to the adenylyl cyclase assay described above. Control experiments demonstrated that the reconstitution protocols did insert G_{i3} into the membranes. A 41-kDa band was not visible in membranes from pertussis toxin-treated rats incubated *in vitro* with toxin and [^{32}P]NAD$^+$ (32). However, after reconstitution of native G_{i3} into the toxin-treated membrane, followed by washing to remove excess G_{i3}, a 41-kDa band was labeled with toxin and [^{32}P]NAD$^+$. Quantitation of the amount of G_{i3} inserted into the membranes estimated that >80% of the added G protein was recovered in the membrane.

Materials. The peptide antibodies to the α subunits of G_{i1}, G_{i2}, G_{i3}, and G_o and the G_{com} sequence (21, 35, 40) were generously provided by Dr. Suzanne Mumby of the University of Texas Health Science Center at Dallas. The cDNA clones for the α subunits of G_{i1}, G_{i2}, G_{i3}, and G_o were kindly provided by Dr. Randall Reed of Johns Hopkins University (29). The [α-^{32}P]ATP for adenylyl cyclase assays was prepared by the University of Virginia Diabetes Center, according to the method of Johnson and Walseth (47). Pertussis toxin was generously provided by Dr. Erik L. Hewlett of the University of Virginia. Other reagents were obtained from the following suppliers: Enzymobeads (lactoperoxidase coupled to beads), Bio-Rad; ^{125}I, [^{32}P]NAD$^+$, ^{125}I-angiotensin II, and NENSORB, New England Nuclear; cholic acid, L-dimyristoyl phosphatidylcholine, creatine kinase (type I, lyophilized from rabbit mus-

cle), 3-isobutyl-1-methylxanthine, creatine phosphate (di-Tris salt), dithiothreitol, ATP (disodium salt), GTP, Tris, EDTA, fraction V BSA, LiCl, NAD^+ (sodium salt, grade VII), and ovalbumin, Sigma; angiotensin II and Sar^1-Ile^8-angiotensin II, Peninsula Laboratories; thymidine, Aldrich; crystalline BSA, Miles Laboratories; sucrose (density gradient-grade), Schwarz/Mann; HEPES, Calbiochem-Behring; and GTPγS (tetralithium salt), Boehringer Mannheim.

Calculations and expression of results. The number of angiotensin II binding sites and the K_d values for the binding isotherms were determined by computer fitting of the data to a one- or two-site model, using a nonlinear least-squares curve-fitting program, as described (46). Averaged data are presented as the mean ± standard error in Figs. 3 and 4. Data presented as representative (Figs. 1, 5, 6, and 7) were repeated three or more times.

Results

Identity of the G_i proteins present in hepatocytes. Studies using antibodies specific for the various G_i α subunits have suggested that G_i preparations from rabbit liver contain both G_{i2} and G_{i3} (35), and analysis of liver mRNA using oligonucleotide probes specific for the various G protein α subunits has suggested that liver contains high levels of G_{i3} (48). One problem common to these studies is that the starting material for the experiments was whole liver, an organ containing multiple cell types, and thus the complement of G_i α subunits in hepatocytes themselves was not determined. It was important to examine the endogenous G proteins present in isolated hepatocytes before testing the nature of the G_i able to reconstitute the ability of angiotensin II to inhibit adenylyl cyclase in hepatocyte membranes. Therefore, RNA prepared from isolated hepatocytes was probed with ^{32}P-labeled cDNAs corresponding to the α subunits of the four known pertussis toxin-sensitive G proteins, G_o, G_{i1}, G_{i2}, and G_{i3}. The levels of the RNA for these proteins in hepatocytes was compared with those in rat brain, a tissue known to have an abundance of G proteins (37). An autoradiograph of a Northern blot from brain and hepatocyte RNA probed with full-length cDNAs for all four G protein α subunits, which were labeled to equal specific activity (see Materials and Methods), is presented in Fig. 1. The figure demonstrates that whole rat brain contains RNA for the α subunits of all four G proteins, whereas hepatocytes contain RNA for only the G_{i2} and G_{i3} α subunits. Because the probes were labeled to equal specific activity, the figure provides a rough comparison of the levels of the mRNA for the four α subunits in each tissue. Clearly, the mRNAs for G_{i2} and G_{i3} exist in liver in roughly equal amounts, and whole brain contains higher levels of mRNA for G_o, G_{i1}, and G_{i2} than does liver.

Based on the results presented in Fig. 1, both G_{i2} and G_{i3} are candidates for the G protein that links angiotensin II receptors to inhibition of adenylyl cyclase in hepatocytes. Initial studies to verify this hypothesis were undertaken with G_{i3}, because only G_{i3} of hepatic origin was available in the quantities required to complete the experiments (see Materials and Methods). Fig. 2 presents evidence that the purified G protein used in the reconstitution experiments shown in Figs. 3–7 is indeed G_{i3} and is free of G_{i2}. Purified G proteins from four different tissues were resolved on six identical sections of a 12% polyacrylamide gel. One section of the gel was stained with silver and the other five were transferred to nitrocellulose before blotting with G protein antibodies. Fig. 2, *upper left*, shows the purity of the preparations, as judged by staining with silver, and the slight differences in the migration of the α and β subunits of G_o and the G_is from brain, placenta, platelet, and liver, respectively. The brain, placenta, and platelet G protein preparations were used as controls. Note that the α subunit of platelet G_i migrates slightly faster (at 40 kDa) than those from placenta or liver, which migrate at 41 kDa. The other four panels in Fig. 2 show the nature of these proteins, as determined by Western blotting with peptide antisera that recognize specific epitopes of the known G proteins (21, 35, 40). Note that the platelet protein only reacts with the G_{com} and G_{i2} antisera, indicating that this protein is G_{i2}. The purified liver G_i only reacts with the G_{com} and G_{i3} antisera, verifying that the purified G_i preparation used in the experiments presented in Figs. 3–7 is G_{i3}. An antisera specific for G_{i1} (I-355) did not react with the either the platelet or the liver protein (data not shown, but see details in legend to Fig. 2). These data indicate that the G_i purified from rabbit liver and used in the experiments presented below is G_{i3}.

Restoration of angiotensin inhibition of cyclase in intoxicated membranes by reconstitution of G_{i3}. To determine whether G_{i3} could couple the angiotensin II receptor to inhibition of adenylyl cyclase, membranes were prepared from hepatocytes in which >99% of their G_i was modified by ADP-ribosylation (see Materials and Methods for details). The effect of complete modification of G_i on angiotensin II-mediated inhibition of adenylyl cyclase activity is presented in Fig. 3A. Note that concentrations of angiotensin II between 10^{-10} and 10^{-6} M do not inhibit adenylyl cyclase in membranes prepared from intoxicated hepatocytes (Fig. 3A), whereas the peptide produces a 30–35% inhibition, with an EC_{50} of ~10^{-8} M, in control membranes (Fig. 3B). Reconstitution of about 7 pmol of G_{i3}/mg of membrane protein into the toxin-modified membranes restores the ability of angiotensin II to inhibit adenylyl cyclase (Fig. 3A). Note that the magnitude of the inhibition (about 30–35%) and the EC_{50} (10^{-8} M) observed in the intoxicated membranes reconstituted with G_{i3} are similar to those observed in control membranes.

To determine the amount of G_{i3} needed to recouple angiotensin II-mediated inhibition of adenylyl cyclase, 3 to 50 pmol of G_{i3} were reconstituted/mg of toxin-modified membranes, and the cyclase activity was assayed in the presence and absence of a maximal concentration of angiotensin II, 10^{-6} M. The data in Fig. 4 show that recovery of hormonal inhibition is observed when ~3 pmol of G_i/mg of membrane protein are reconstituted. Fig. 4, *inset*, shows that the degree of inhibition reaches a maximum when about 6–10 pmol of G_{i3} are reconstituted/mg of membrane protein. The maximum inhibition observed in these experiments is about 25 ± 6% over the range of 10–50 pmol of G_{i3} added/mg of membrane protein (Fig. 4, *inset*). Note that, as higher amounts of G_i are inserted into the membranes, the GTP/LiCl-stimulated levels of adenylyl cyclase gradually decrease (Fig. 4). Similar behavior was observed in experiments using G_i reconstituted into forskolin-stimulated platelet membranes (7). One possible explanation for this result may be that excess βγ subunit is added to the membranes with the G_{i3}, causing an inhibition of adenylyl cyclase activity (7). Overall, the results presented in Figs. 3 and 4 argue that exogenously added G_{i3} repairs the defect produced by pertussis toxin in hepatic membranes and recouples the angiotensin II receptor to inhibition of adenylyl cyclase. More importantly, the amount of G_i that needs to be reconstituted into the intoxicated membranes to restore maximal hormonal inhibition, 6–10 pmol/mg

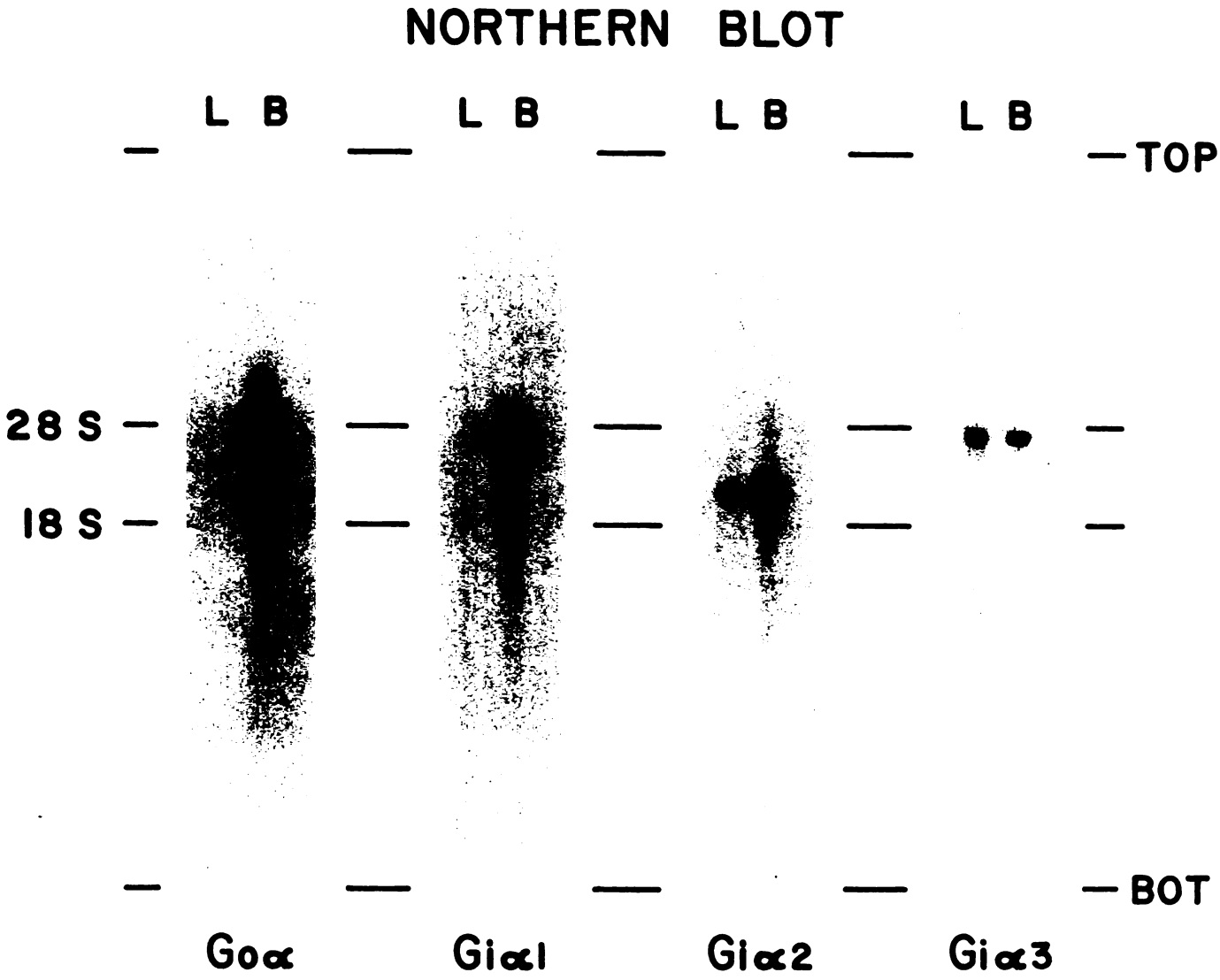

Fig. 1. Northern blot of RNA isolated from rat hepatocytes and rat brain, probed with cDNAs corresponding to the α subunits of G_o, G_{i1}, G_{i2}, and G_{i3}. Seven micrograms of total RNA from rat hepatocytes (*L*) or brain (*B*) were separated by agarose gel electrophoresis and blotted onto a nylon membrane (Zetabind). The blots were probed with cDNAs coding for the α subunits of G_o, G_{i1}, G_{i2}, and G_{i3} labeled with [α-^{32}P]ATP and adjusted to approximately equal specific activity (see Materials and Methods). The probe used is indicated below each panel. *TOP* and *BOT*, beginning and end of electrophoresis, respectively. The positions of the 18 S and 28 S ribosomal RNA bands are indicated by the *horizontal lines* beside each pair of lanes. Autoradiographs were made from the blots with Kodak XAR-5 film.

of membrane protein, is close to the amount of endogenous G_i in the membrane (about 4 pmol/mg of membrane protein), as determined by ADP-ribosylation *in vitro* (32).

Stable GTPγS shift of receptor affinity. Although the effect of pertussis toxin on the α subunits of G_i proteins is highly specific, nonspecific effects of the toxin have been identified (49). Therefore, to assess the direct interaction of angiotensin II receptors with G_{i3} in normal membranes, a more general technique was developed to modify the coupling between receptors and G proteins in purified plasma membranes. This technique takes advantage of two properties of the G protein/receptor system. First, in membranes treated with GTPγS, the endogenous G protein α subunits dissociate from their $\beta\gamma$ subunits and receptors coupled to the G protein display a substantially decreased affinity for agonists (1, 4). Presumably this occurs when the receptors become uncoupled from the G protein α subunit, which, in its GDP-liganded form, induces the high affinity state of the receptor (1, 4). Second, GTPγS binds to G proteins with very high affinity and is not easily removed even by repeated washes if 10–20 mM Mg^{2+} is included in the wash buffer (34). These observations suggest that it may be possible to produce a stable dissociation of angiotensin II receptors from their relevant G proteins by treatment of control membranes with GTPγS and Mg^{2+}. The stability and extent of the response could be monitored by measuring the affinity of the angiotensin II receptor for ^{125}I-angiotensin. In theory, if the receptor in a GTPγS-"uncoupled" membrane is presented with the correct, exogenously added, G protein in the GDP-liganded state, it will revert to its higher affinity state and the binding isotherm will be shifted to the left. This response would indicate functional receptor-G protein coupling.

Table 1 presents data from a representative experiment examining the conditions needed to produce a stable shift in the affinity of the angiotensin II receptor after treatment with GTPγS and Mg^{2+}. The experiments were performed with 0.25 nM angiotensin II, a concentration near the K_d for high affinity

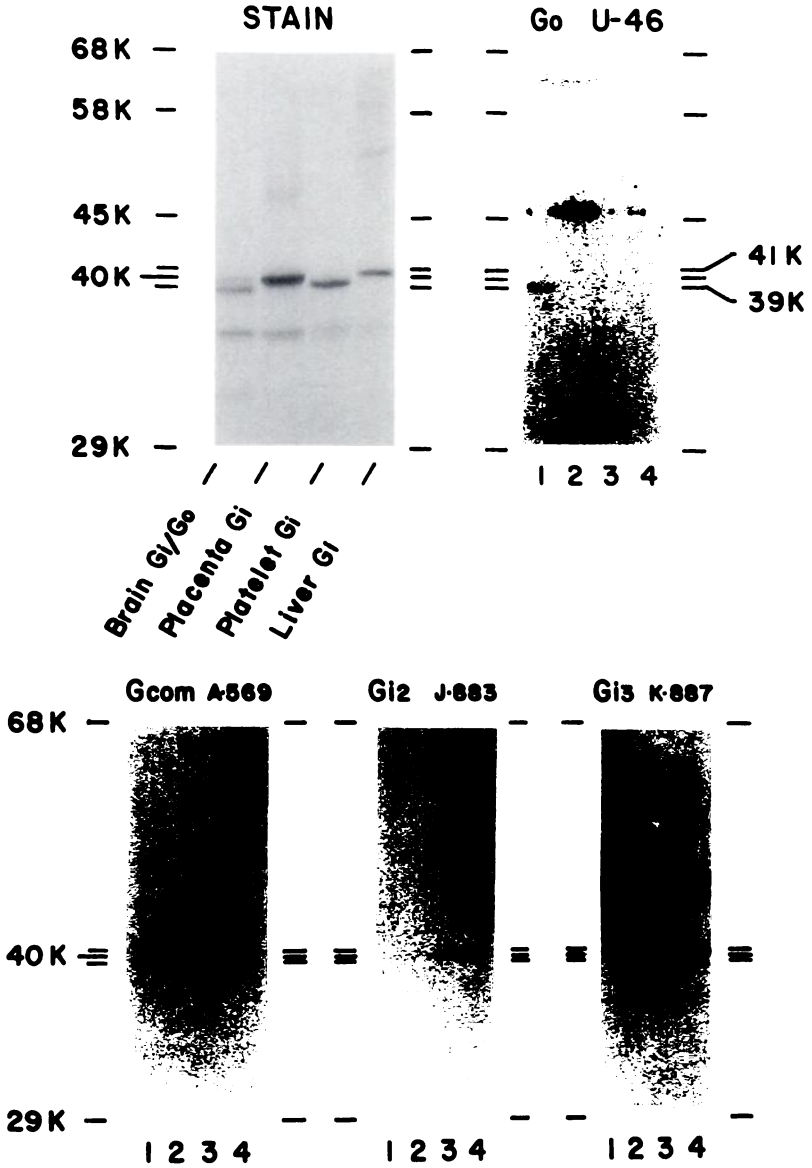

Fig. 2. The purified G_i used in these experiments is G_{i3}, as determined by Western blotting. Four preparations of G proteins from brain (G_o/G_i), placenta, platelet, and liver were subjected to electrophoresis on a 12% polyacrylamide gel. One panel of the gel was stained with silver, and five others were transferred to nitrocellulose before Western blotting with $G_{i\alpha}$-specific antibodies. *Upper left* (*STAIN*), the stained gel shows the slightly different migrations of the α subunits of G_o, G_{i2}, and G_{i3}. *Upper right* (*Go U-46*), one panel of the nitrocellulose was blotted with the U-46 antibody specific for the α subunit of G_o. *Lower left* (*Gcom A-569*), another panel of the nitrocellulose was blotted with the A-569 antibody, which recognizes an epitope common to α subunits of the pertussis toxin-sensitive G proteins. *Lower center* (*Gi2 J-883*), a third panel of the nitrocellulose was blotted with the J-883 antibody specific for the α subunit of G_{i2}. Note that only the platelet G_i is recognized. *Lower right* (*Gi3 K-887*), the final panel of the nitrocellulose was blotted with the K-887 antibody, which recognizes the α subunit of G_{i3}. Note that the liver preparation is recognized by only the G_{com} and G_{i3} antibodies. A similar blot using an antibody specific for the α subunit of G_{i1} (I-355) was negative for the liver preparation but did react with the placenta preparation (not shown).

angiotensin binding to hepatic membranes (46). In these experiments, the membranes were preincubated with buffer alone or with GTPγS in the presence of Mg^{2+}, for 30 min at 30°, and were washed twice before the incubation with iodinated ligand to measure receptor number. Note that, in membranes not pretreated with the nucleotide, inclusion of GTPγS in the binding reaction produced an ~66% decrease in the amount of angiotensin II bound to its receptor (from 61 to 21 fmol of angiotensin/mg of protein). This is indicative of a shift in the binding isotherm to the right (see Fig. 5). However, in membranes pretreated with GTPγS, angiotensin II binding is low (29 fmol of angiotensin/mg of protein) and is not greatly reduced by inclusion of the nucleotide in the binding assay (25 fmol of angiotensin/mg of protein), indicating that GTPγS remained bound to most of the G proteins in the membranes during the washes before the binding reaction was initiated.

Sar^1-Ile^8-Angiotensin II is an antagonist at the angiotensin II receptor site in the liver, and its binding is not affected by GTP analogues (31). Using 0.25 nM ^{125}I-Sar^1-Ile^8-angiotensin II as a ligand in the binding reaction, both control and GTPγS-pretreated membranes appear to retain a full complement of angiotensin II binding sites, ~60 fmol/mg of protein. Inclusion of GTPγS during the preincubation or the assay itself produces only a small decrement in the apparent number of receptors. However, the sites labeled by ^{125}I-Sar^1-Ile^8-angiotensin II were indeed angiotensin receptors, in that binding could be reduced to nonspecific levels with excess unlabeled angiotensin II (see Table 1 legend). Other control experiments demonstrated that there was no detectable effect of GTPγS if the incubation was carried out at 0° and that the effect of GTPγS was essentially complete in 30 min at 30°, as judged by the observation that addition of agonist at this time to increase the rate of guanine nucleotide exchange did not increase the effect of GTPγS on angiotensin II receptor affinity (data not shown). The same affinity shift could be achieved with a 5-min incubation if angiotensin, GTPγS, and Mg^{2+} were included in the assay. However, in all reconstitution experiments described below, a 30-min incubation period and only GTPγS and Mg^{2+} were used, to exclude the possibility that angiotensin II added during the shift protocol remained bound to the receptor.

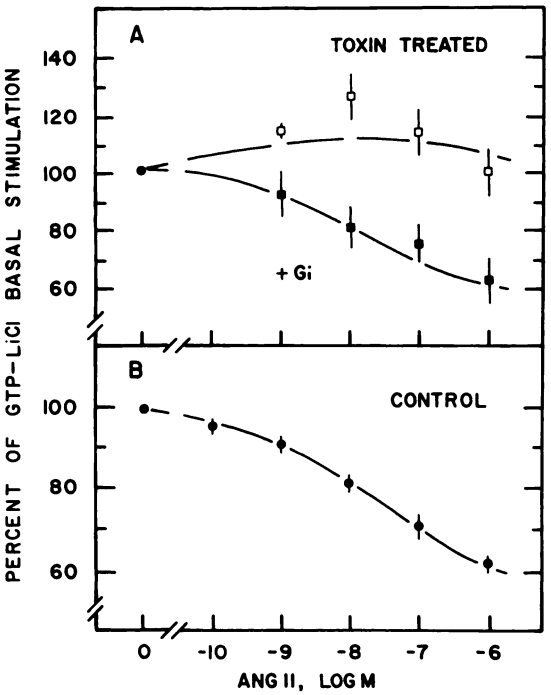

Fig. 3. Angiotensin II (*ANG II*) inhibition of adenylyl cyclase is restored by reconstitution of G_{i3} into hepatocyte membranes. A, Effects of angiotensin on adenylyl cyclase activity in membranes prepared from hepatocytes isolated from pertussis toxin-treated rats. Reconstitution of either 7 pmol of G_{i3}/mg of membrane protein (■) or 0.004% Lubrol alone (□) to membranes from pertussis toxin-treated rats was performed as described in Materials and Methods. GTP/LiCl was added to provide stimulation of adenylyl cyclase activity that was subsequently inhibited by the indicated concentrations of angiotensin II. The basal level of adenylyl cyclase was 8.7 ± 1.0 pmol/min/mg. GTP/LiCl. Increased the activity 3.9-fold, to 33.7 ± 3.5 pmol/min/mg of protein. This level of activity was about 12% of the maximum stimulation generated by 1 μM glucagon (data not shown). Results are expressed as the percentage of the activity measured in the absence of angiotensin II from three completely ADP-ribosylated hepatocyte membrane preparations. Restoration of the ability of angiotensin II to inhibit adenylyl cyclase after G_{i3} addition was statistically significant at 10^{-8} M and 10^{-6} M angiotensin II ($p < 0.05$ by paired t test). B, Effect of angiotensin II on adenylyl cyclase in membranes from normal rats. Data are from three normal rat hepatocyte membrane preparations. All points are averages ± standard errors.

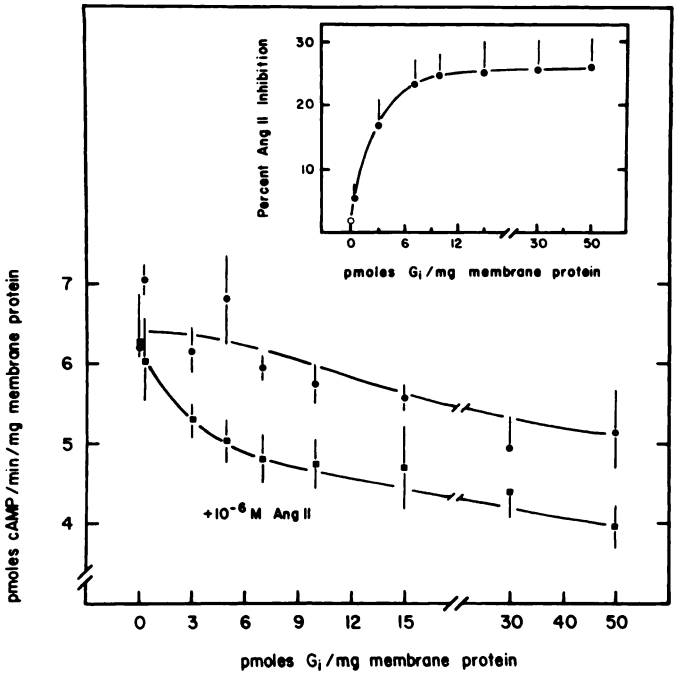

Fig. 4. Concentration of G_{i3} needed to restore the angiotensin II inhibition of adenylyl cyclase activity. Concentrations of G_{i3} from 0 to 50 pmol/mg of membrane protein were reconstituted into membranes prepared from hepatocytes isolated from pertussis toxin-treated rats. The detergent concentration, 0.004% Lubrol, was kept constant with the different amounts of added G_{i3}. Membranes were then diluted 2-fold into the adenylyl cyclase assay, which was performed in the presence of 250 mM LiCl, 0.1 mM GTP, and 5 mM Mg^{2+}, with (■) and without (●) 10^{-6} M angiotensin II, for 12 min at 30°. The points represent the mean ± standard error of triplicate determinations. *Inset*, the data represented by the *lines* in the main panel were replotted to show the percentage of inhibition of cyclase activity caused by 10^{-6} M angiotensin II as a function of the concentration of G_{i3} reconstituted into the membrane. The magnitude of the inhibition at each G_{i3} concentration was determined by measuring the difference between the lines drawn through the data in the main panel. The error bars at each point were calculated (as standard error in percentage) from the errors displayed for the data in the main panel.

Fig. 5, *top*, presents the effect of GTPγS on the angiotensin II binding isotherm in membranes prepared from normal hepatocytes. Note that GTPγS shifts the ^{125}I-angiotensin II binding curve dramatically to the right. In keeping with previous results (46), computer analysis of the curves to obtain the K_d and B_{max} values gave a best fit to a two-site model, with parameters as follows (control): n_1 = 102 fmol/mg of protein, K_{d_1} = 0.19 nM, n_2 = 230 fmol/mg of protein, and K_{d_2} = 0.9 nM. In the presence of GTPγS the parameters were n_1 = 0, K_{d_1} = 0, n_2 = 150 fmol/mg of protein, and K_{d_2} = 1.0 nM. Based on the computer analysis, the functional effect of GTPγS is to eliminate binding to the high affinity sites. The GTPγS-altered curve shown in Fig. 5, *top*, was obtained by incubation of the membranes with GTPγS for the full incubation time, separation of the bound ligand from the free, and counting. However, at the end of the incubation, the membranes can be diluted 30-fold and washed in a buffer containing 10 mM Mg^{2+} and the binding assayed again with no change in the position or shape of the GTPγS curve. Moreover, addition of the final concentration of detergent present in the reconstitution experiments,

TABLE 1

Demonstration of a stable modification of angiotensin II receptor affinity in hepatic membranes

Rat hepatic membranes were incubated at 5 mg of protein/ml at 30° for 30 min with the indicated radioactive ligand and no GTPγS (membranes plus buffer) or with 100 μM GTPγS and 20 mM Mg^{2+}. After 30 min of incubation, the membranes were centrifuged at 13,000 × *g*, washed, and resuspended to about 2 mg of protein/ml. The number of angiotensin receptors was then determined with the indicated concentration of radioactive ligand, as described in Materials and Methods. Each binding assay was performed in the presence or absence of 10 μM GTPγS. Values are the means of duplicate determinations, which differed by <10%. Regardless of the identity of the radioactive ligand used, nonspecific binding was measured in the presence of 100 nM angiotensin II and was about 7 fmol/mg of membrane protein under all experimental conditions. The results shown are corrected for nonspecific binding and are representative of four similar experiments.

^{125}I-Ligand	Bound ligand	
	Membranes + buffer (decrease)	Membranes + 10^{-4} M GTPγS (decrease)
	fmol/mg of membrane protein	
0.25 nM Angiotensin II	61.4	29.3
+ 10^{-5} M GTPγS	21.4 (66%)	25.2 (13%)
0.25 nM Sar^1-Ile^8-angiotensin II	54.9	57.7
+ 10^{-5} M GTPγS	47.3 (13%)	61.3 (0%)

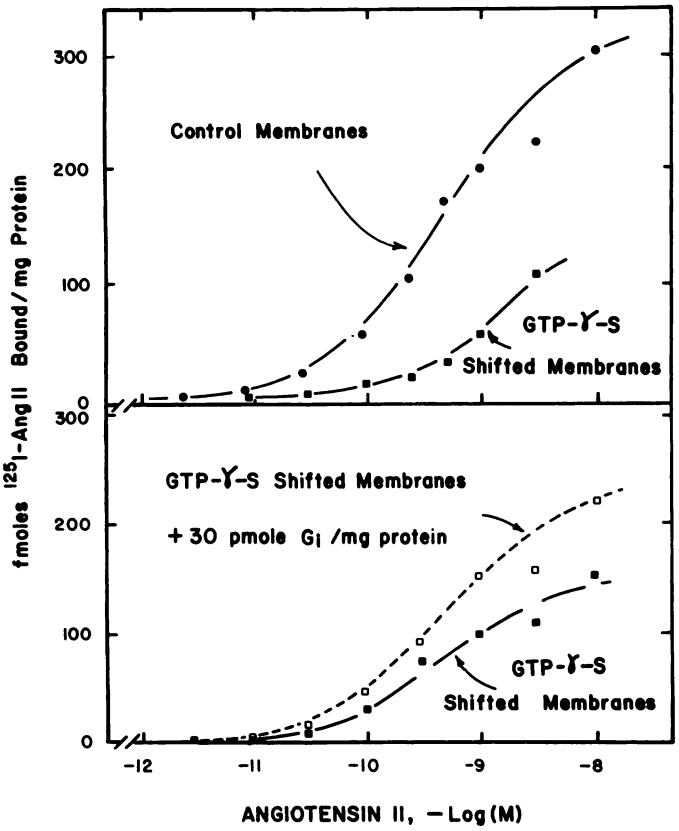

Fig. 5. Effect of GTPγS and reconstituted G_{i3} on the affinity of the angiotensin II receptor. *Top*, ^{125}I-angiotensin II binding isotherms were performed at 22° for 60 min in the presence (■) and absence (●) of 100 μM GTPγS without detergent present. *Bottom*, membranes were treated with 1 mM GTPγS for 30 min in the presence of 10 mM Mg^{2+} and washed, and then 30 pmol of G_{i3}/mg of membrane protein were reconstituted into the membrane. The concentration of detergent present in the reconstitution procedure (0.004% Lubrol/0.004% cholate) was diluted 8-fold when membranes were added to the binding assays. Points represent the average of duplicate determinations corrected for nonsaturatable binding. The figure is representative of three such experiments.

0.004% cholate, caused only a small dimunition in B_{max} values. The GTPγS curve presented in Fig. 5, *bottom*, is representative of the stability of the ^{125}I-angiotensin II binding isotherm in membranes that were washed and treated with detergent in this manner. The computer-generated binding parameters for this GTPγS-shifted curve are not statistically different from those obtained without washing and detergent treatment.

When about 30 pmol of G_{i3}/mg of membrane protein are reconstituted into the membranes, the ^{125}I-angiotensin II binding isotherm is shifted back to the left (Fig. 5). Computer analysis of this curve gives parameters of n_1 = 70 fmol/mg of protein, K_{d_1} = 0.16 nM, n_2 = 170 fmol/mg of protein, and K_{d_2} = 0.7 nM. The curve is not shifted completely back to the control position, but the result demonstrates that G_{i3} added to the membranes is able to restore high affinity binding, presumably by recoupling to some angiotensin II receptors in the membrane. Indeed, the computer analysis shows that it is exclusively the high affinity sites that are restored by reconstitution of G_{i3} into the membrane. The fact that the binding isotherm is not completely restored to the control position may be due to a number of reasons. Because angiotensin II receptors in the hepatic membrane are able to couple to G proteins other than G_{i3} (50), additional proteins such as G_{i2} or G_q (12, 51) may be required for a complete restoration. Alternatively, the reconstitution protocol may adversely affect the interaction of the receptor and G proteins.

In order to determine the amount of G_{i3} needed to restore high affinity binding, experiments similar to those in Fig. 5 were performed with one concentration of ^{125}I-angiotensin II, 0.3 nM. This concentration was chosen because it labels a large percentage of the high affinity binding sites. These experiments are presented in Figure 6. In membranes treated with detergent but not GTPγS, there were approximately 86 fmol of ^{125}I-angiotensin II bound/mg of membrane protein (black square, Fig. 6). When the membranes were pretreated with GTPγS as described above, binding was lowered to 36 fmol/mg of membrane protein. This point is shown in Fig. 6 as the level of binding obtained in the absence of any added G_{i3}. As increasing concentrations of G_{i3} are reconstituted into the membranes, binding increases until it reaches ~75 fmol/mg of protein (about 90% of the original value) when about 30 pmol of G_i/mg of membrane protein are reconstituted (Fig. 6). Because the recovery of high affinity binding sites could be due to the exogenously added G protein removing GTPγS from the endogenous G proteins, controls were performed by reconstituting similar concentrations of G_s into the membranes. Given that G_s and G_i have a similar affinity for GTP (21, 52, 53), it could also remove endogenous GTPγS from membrane G proteins; however, there is no biochemical evidence that G_s can couple to angiotensin II receptors. Clearly, G_s did not alter binding of ^{125}I-angiotensin II (Fig. 6). As might be expected, the ability of guanine nucleotides to lower angiotensin binding was agonist specific. Binding of the radiolabeled antagonist ^{125}I-Sar^1-Ile^8-angiotensin II was not altered by the GTPγS treatment or the reconstitution of G_i into GTPγS-treated membranes (data not shown).

Effect of the resolved α subunit of G_{i3} on binding. To determine whether the α or the βγ subunits of G_{i3} were respon-

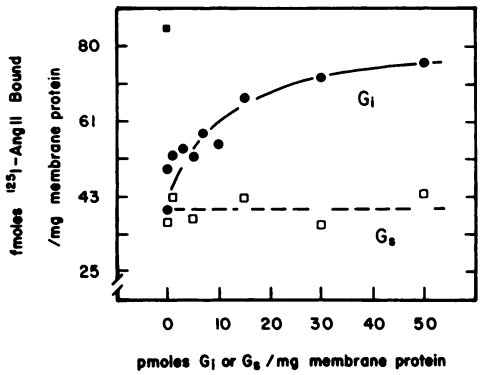

Fig. 6. Concentration of G_{i3} needed to restore high affinity ^{125}I-angiotensin II binding to GTPγS-treated membranes. The affinity of the angiotensin II receptor was decreased by the addition of GTPγS to membranes treated with detergent alone. GTPγS caused a decrease in the amount of ^{125}I-angiotensin II bound from 86 fmol/mg of membrane protein (■) to 35 fmol/mg of protein, as indicated on the figure by the points at 0 pmol of G_{i3} or G_s reconstituted/mg of membrane protein. After the GTPγS treatment, the indicated amount of G_{i3} or G_s was reconstituted into the membranes. Controls received detergent alone. Binding assays, containing 0.3 nM ^{125}I-angiotensin II, were initiated by the dilution of membranes approximately 8-fold into the binding assay. Results presented are typical of four such experiments in which G_{i3} was reconstituted into the membrane or two control experiments using G_s. Each point was corrected for nonsaturable binding and represents the average of duplicate determinations.

sible for the ability of G_{i3} to restore high affinity ^{125}I-angiotensin II binding, experiments were performed with the resolved 41-kDa G_{i3} α subunit. Fig. 7 demonstrates that the G_{i3} α subunit also restores high affinity binding in GTPγS-treated membranes incubated with 0.3 nM ^{125}I-angiotensin II. Note that, in the control membranes, GTPγS decreases binding about 63%, from 200 fmol bound/mg of protein to about 75 fmol/mg of protein. In membranes treated with GTPγS, washed, and then incubated with ^{125}I-angiotensin II, the level of binding is similar, ~60 fmol/mg of protein. Addition of GTPγS to the incubation does not decrease binding further (Fig. 7). Reconstitution of about 40 pmol of G_{i3} α subunit/mg of membrane protein increases binding from 58 to 156 fmol of ^{125}I-angiotensin II bound/mg of membrane protein (Fig. 7). This high affinity binding is now guanine nucleotide sensitive, decreasing to 39 fmol of ^{125}I-angiotensin II bound/mg of membrane protein in the presence of newly added 100 μM GTPγS. This implies that a functionally coupled angiotensin II receptor-G protein system was achieved by addition of the α subunit of G_{i3} alone.

Discussion

Analysis of cDNA clones corresponding to the family of G proteins indicates that the family contains groups of very similar proteins, based on the structure of their α subunits. Thus, there are at least four types of G_s, three nearly identical G_i forms that are substrates for pertussis toxin, four variants of G_o, and two retinal-specific G_t forms (1, 3, 4). Based on the existence of cDNA clones, the family also contains α subunits for proteins such as G_z, G_{11}, and G_q (12, 54, 55). Purification of G proteins with careful attention to the existence of these multiple forms has yielded purified proteins with α subunits that appear to correspond to the cDNAs (4, 30, 51, 56, 57). These results, combined with the data demonstrating that the α subunits of the G proteins are most likely involved in their signaling role (4), have lead to the suggestion that the multiple α subunits may be involved in regulating different signaling pathways in the cell. This view is reinforced by a large body of data showing that pertussis toxin can block a number of different signaling pathways, including inhibition of adenylyl cyclase

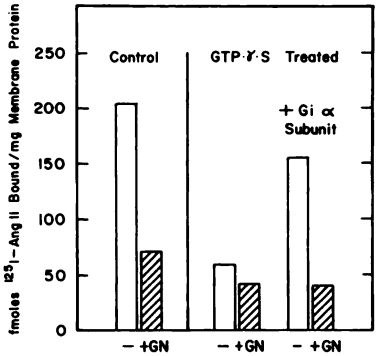

Fig. 7. Reconstitution of the resolved α subunit of G_{i3} into GTPγS-treated membranes. Membranes were incubated with (*GTPγS Treated*) or without (*Control*) 100 μM GTPγS for 30 min at 30°. This was followed by washing and incubation of the membranes for 10 min at 30° with 0.04% cholate or with approximately 45 pmol of the α subunit of G_{i3}/mg of membrane protein. Membranes were then diluted 8-fold into the binding assay containing 0.3 nM ^{125}I-angiotensin II. Assays were done in the presence (+*GN*) or absence (−) of 100 μM GTPγS regardless of the initial presence of GTPγS. The data represent the mean of duplicate determinations, and the figure is representative of three such experiments.

(1, 2), activation of K^+ channels (4), inhibition of Ca^{2+} channels (21), and stimulation of phosphatidylinositol metabolism (28). Thus, major questions revolve around the roles of the multiple variants of the G proteins. A great deal of attention has been focused on the G_i forms and G_o, because pertussis toxin has been observed to block many different signaling pathways.

Whereas G_i was originally thought to be involved only in inhibition of adenylyl cyclase (7, 9), it is becoming clear that G_i can mediate at least three other membrane signaling functions, i.e., inhibition of Ca^{2+} channels, activation of K^+ channels, and stimulation of phospholipase C. For example, convincing evidence demonstrates that all three variants of G_i can reconstitute muscarinic stimulation of K^+ channels. Using patch-clamp techniques, both tissue-derived and recombinant G_{i1}, G_{i2}, and G_{i3} have been shown to reconstitute muscarinic activation of K^+ channels in membranes from chick atrial cells that were pretreated with pertussis toxin to inactivate the endogenous G_i forms and G_o (4, 24, 30). Although there has been controversy regarding the role of the α versus the $\beta\gamma$ subunits in this system (4, 58), the data clearly document a place for G_i in this signaling event. However, the observation that all variants of G_i are able to reconstitute activation of the K^+ channel (24) or inhibition of the Ca^{2+} channel (21) raises questions about the assumption that different α subunits may mediate distinct signaling events. Recent experiments showing that recombinant 52-kDa and 45-kDa forms of G_s α subunits can both reconstitute β-adrenergic stimulation of adenylyl cyclase in S49^{cyc-} cells and stimulate Ca^{2+} channel activity further illustrate the problem (59). Some form of G_i also appears to be involved in coupling certain receptors to phospholipase C in neutrophils and brain (60, 61). For example, in HL-60 cells, treatment with pertussis toxin inhibits formyl-Met-Leu-Phe-stimulated inositol phosphate release from membrane preparations, and reconstitution of a brain G_i preparation reverses the inhibition (60). However, the isoforms of G_i able to couple receptors to phospholipase C have not been rigorously investigated (60, 61).

The present experiments were undertaken to examine whether G_{i3} could couple to the angiotensin II receptor in membranes prepared from isolated hepatocytes. To date, the signaling systems known to couple to angiotensin II receptors in this membrane include inhibition of adenylyl cyclase (31) and stimulation of phosphatidylinositol breakdown (50). Only inhibition of adenylyl cyclase is blocked by pertussis toxin in these cells (32, 50, 62), suggesting that membranes purified from toxin-treated hepatocytes are a rational assay system for examining the role of G_i in liver. Two stages of the work are presented here, first, an evaluation of the content of the known pertussis toxin-sensitive G proteins, G_{i1}, G_{i2}, G_{i3}, and G_o, in hepatocytes, as judged by examination of mRNA using full-length cDNA clones labeled to equal specific activity, and, second, an evaluation of the ability of G_{i3} to recouple to angiotensin II receptors in hepatocyte membranes using two protocols, i.e., reconstitution of the inhibition of adenylyl cyclase in pertussis toxin-modified membranes and restoration of high affinity angiotensin II receptor binding after the shift in receptor affinity induced by GTPγS.

There was not a detectable amount of mRNA for G_o in the hepatocytes, suggesting that there is little if any G_o in the hepatocyte membranes. Although the level of mRNA for a specific protein is not an entirely accurate reflection of the

amount of the protein expressed in the cell, this result is consistent with previous work showing that there is no G_o in liver, as judged by ADP-ribosylation (32) or purification (37). Hepatocytes have no mRNA for G_{i1} and levels of mRNA for G_{i2} or G_{i3} that are comparable to those in brain, a tissue with high levels of G proteins. The role of G_{i3} in hepatic function was examined in the present experiments because of the availability of G_{i3} purified from rabbit liver.

The data in Figs. 3 and 4 demonstrate that a purified preparation of rabbit liver G_{i3} is able to reconstitute the angiotensin II inhibition of adenylyl cyclase in hepatocyte membranes whose endogenous G_i has been completely modified by pertussis toxin. The concentration dependence of angiotensin II-mediated inhibition of adenylyl cyclase was nearly identical in control and reconstituted membranes. Perhaps more notable is the result that reconstitution of the maximal inhibition requires addition of only 6–12 pmol of G_{i3}/mg of membrane protein. Although the exact amount of the G_i inserted into the membrane in the reconstitution protocol is not known, control experiments demonstrated that at least 80% of the added G_i is removed from solution in the protocol (see Materials and Methods). Accordingly, the amount of G_i needed to restore hormonal inhibition of adenylyl cyclase is very similar to the total G_i concentration in the membrane (4–6 pmol/mg of membrane protein), as measured by ADP-ribosylation in the presence of [^{32}P]NAD$^+$ (32). This result, combined with the data in Fig. 2 indicating that the purified G_{i3} used in these experiments is essentially free of G_{i2}, provides strong evidence that G_{i3} is a candidate for coupling angiotensin II receptors to inhibition of adenylyl cyclase in the hepatocyte.

It is important to emphasize that, although G_{i3} can couple to the angiotensin II receptor, there are a number of other G proteins in liver that should be able to couple to this receptor. For example, hepatocytes also contain G_{i2} (Fig. 1), and there is evidence that a pertussis toxin-insensitive G protein, now termed G_q, is most likely involved in coupling the angiotensin II receptor to stimulation of phospholipase C (50, 51, 57). The ability of the three different isoforms of G_i to couple to different effectors or receptors has been explored rigorously in four systems, i.e., the muscarinic K^+ channel (24, 30), the bradykinin-inhibited Ca^{2+} channel (21), the dopamine D_2 receptor (63), and GTPγS-stimulated cyclase (30). Although the D_2 receptor did show a preference for the G_{i2} subtype, in each of these systems all of the isoforms of G_i were functional. The ability of the isoforms of G_i to inhibit adenylyl cyclase has also been explored in platelet membranes, using an immunological approach. In platelets where G_{i2} appeared to be the most abundant G_i species, a peptide antiserum specific for G_{i2} blocked α_2-adrenergic inhibition of adenylyl cyclase, whereas an antiserum specific for G_{i3} was ineffective (64). This result suggests that G_{i2} can couple receptors to inhibition of adenylyl cyclase. Because there are approximately equal amounts of G_{i2} and G_{i3} in hepatocytes (see Fig. 1), it seems reasonable to assume that G_{i2} will also be able to couple to the angiotensin II receptor in the hepatic membrane. Future experiments will explore this possibility using recombinant G proteins.

Because the $\beta\gamma$ subunits of the G proteins can clearly mediate inhibition of adenylyl cyclase in the absence of α subunits (65), it was important to provide independent evidence that the α subunit of G_{i3} could couple to the angiotensin II receptor. In this study, the interaction of the receptor and the G protein was studied by examining the ability of the reconstituted protein to regulate receptor affinity in a GTP-dependent manner. This protocol has some advantages over those used in the experiments with pertussis toxin. For example, the entire procedure is carried out in a period of minutes, as opposed to treatment of rats with toxin for 3 days, and the controls are from the same membrane preparation. Treatment of the membranes with GTPγS is not selective for particular G proteins. However, because the ligands used to measure angiotensin II receptor affinity are selective, the protocol measures only the interaction of the angiotensin II receptor with G proteins. Figs. 5–7 provide data showing that purified G_{i3} can restore a high affinity state of the angiotensin II receptor that had been converted to a stable, low affinity state by pretreatment of the membranes with GTPγS. This effect is also produced with concentrations of G_{i3} (6–10 pmol/mg of membrane protein) near those in the normal membrane (4 pmol/mg of membrane protein). Importantly, reconstitution of up to 50 pmol/mg of membrane protein of G_s fails to restore the high affinity state of the angiotensin II receptor, and there is no biochemical evidence for interaction of the angiotensin II receptor with G_s. Finally, the α subunit of G_{i3} alone can reconstitute high affinity angiotensin II binding (Fig. 7). Although it is likely that the α subunit of G_{i3} recombines with free $\beta\gamma$ subunit in the membranes upon reconstitution and interacts with the receptor as the holoprotein, the observation that the free α subunit can restore the high affinity state of the receptor clearly indicates that it is not the $\beta\gamma$ subunit alone that is responsible for the effect observed.

Asano *et al.* (66) reported similar shifts in receptor affinity, using pertussis toxin-treated membranes from bovine cerebral cortex. A mixture of bovine brain G_i and G_o could restore high affinity GABA binding almost to the level seen in the control membranes. However, these experiments required very high concentrations of G protein (up to 100 pmol/mg of membranes protein) for a full restoration of high affinity GABA binding (66). Using either pertussis toxin or *N*-ethylmaleimide to disrupt the interaction between endogenous G proteins and receptors, G_o and/or G_i have been observed to restore functional coupling to μ-opiate receptors (67), κ-opiate receptors (68), kyotorphin receptors (61), and prostaglandin receptors (69). In theory, the ability of GTPγS to uncouple receptors from their G proteins can be used to examine the interaction of many different receptor types, by taking advantage of the specificity of different ligands for their receptors. A major advantage of this protocol is that it should be effective with receptors that are linked to pertussis toxin-insensitive G proteins and does not require potentially nonspecific inhibitors such as *N*-ethylmaleimide. Thus, the protocols described in Figs. 5–7 have general utility for examining the interaction of G proteins and receptors in native membrane preparations.

In summary, the present experiments have demonstrated functional reconstitution of angiotensin II receptors with rabbit liver G_{i3} in pertussis toxin-treated membranes and clearly indicate that G_{i3} and angiotensin II receptors interact when reconstituted into native membranes. In combination with the profile for G protein gene expression in rat hepatocytes, these results suggest that the endogenous G_{i3} in rat hepatic membranes can mediate angiotensin II inhibition of adenylyl cyclase. However, as the family of G proteins continues to expand

(12, 54, 55), other G proteins may be discovered that are able to function in the role examined in these experiments.

Acknowledgments

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