Immunoprecipitation of Adenylate Cyclase with an Antibody to a Carboxyl-Terminal Peptide from Gsα

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ABSTRACT: An antibody (RM) raised against the carboxyl-terminal decapeptide of the α subunit of the stimulatory guanine nucleotide regulatory protein (Gsα) has been used to study the interaction of Gsα with bovine brain adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1]. RM antibody immunoprecipitated about 60% of the solubilized adenylate cyclase preactivated with either GTP-γ-S or AlF_4^-. In contrast, RM antibody immunoprecipitated about 5% of the adenylate cyclase not preactivated (control) and 15% of the adenylate cyclase pretreated with forskolin. Adenylate cyclase solubilized from control membranes or GTP-γ-S preactivated membranes was partially purified by using forskolin–agarose affinity chromatography. The amount of Gsα protein in the partially purified preparations was determined by immunoblotting with RM antibody. There was 3-fold more Gsα detected in partially purified adenylate cyclase from preactivated membranes than in the partially purified adenylate cyclase from control membranes. Partially purified adenylate cyclase from preactivated membranes was immunoprecipitated with RM antibody and the amount of adenylate cyclase activity immunoprecipitated (65% of total) corresponded to the amount of Gsα protein immunoprecipitated. Only 15% of the partially purified adenylate cyclase from control membranes was immunoprecipitated. The presence of other G proteins in the partially purified preparations of adenylate cyclase was investigated by using specific antisera that detect Goα, Giα, and Gβ. The Gβ protein was the only subunit detected in the partially purified preparations of adenylate cyclase and the amount of Gβ was about the same in adenylate cyclase from preactivated membranes and from control membranes. Examination of the RM antibody immunoprecipitates from control and GTP-γ-S preactivated solubilized membranes failed to detect any of these G proteins. These results indicate that the complex between the adenylate cyclase catalytic subunit and the activated Gsα protein can be isolated by immunoprecipitation with an anti-Gsα antibody. There does not appear to be a specific association of Goα, Giα, or Gβ with the preactivated complex of the catalytic subunit and the activated Gsα subunit.

Hormone-sensitive adenylate cyclase is regulated by the interaction of hormone receptors with specific guanine nucleotide binding proteins (G proteins), which transduce the hormone signal to the adenylate cyclase catalytic subunit (Gilman, 1987; Spiegel, 1987). G proteins are heterotrimeric proteins consisting of α, β, and γ subunits. The α subunits bind guanine nucleotides and some of the α subunits are substrates for ADP ribosylation by bacterial toxins. The α subunits are believed to interact with effector enzymes or ion channels (Yatani et al., 1988). Different forms of the Gsα protein have been identified by cloning and at least two of these forms (45 and 52 kDa) can be identified in brain and other tissues (Gilman, 1987; Spiegel, 1987).

The complex of the catalytic subunit and the Gsα protein can be purified by chromatography on forskolin affinity columns using harsh washing conditions designed to decrease adsorption of nonspecific proteins to the matrix (Pfeuffer et al., 1985a,b; Pfeuffer & Metzger, 1982). These studies have demonstrated that there is an interaction between the Gsα protein and the catalytic subunit that is stable to membrane solubilization and affinity chromatography. Forskolin has also been shown to increase the interaction between the Gsα protein and adenylate cyclase even in the absence of activating ligands such as GTP-γ-S or AlF_4^- (Bouhelal et al., 1985). It has been suggested that forskolin and Gsα bind cooperatively to the adenylate cyclase catalytic subunit, although the nature of this interaction is still unknown (Nelson & Seamon, 1986; Seamon et al., 1985; Green & Clark, 1984). Other G proteins have not been shown to interact directly with the adenylate cyclase catalytic subunit. Hormonal inhibition of adenylate cyclase requires the mediation of a specific G protein, Gi (Katada et al., 1984). A mixture of Giα and Goα does not inhibit the purified adenylate cyclase catalytic subunit, while the addition of βγ will inhibit adenylate cyclase stimulated by Gsα (Smigel, 1986; Cerione et al., 1987). It has been suggested that hormonal inhibition of adenylate cyclase occurs primarily through the interaction of βγ with Gsα. However, a weak interaction of Gi with the catalytic subunit may occur under some conditions, since hormonal inhibition of adenylate cyclase is observed in cells that do not have Gsα (Hildebrandt & Birnbaumer, 1983).

The carboxyl-terminal region of G proteins has been postulated to be important for the interaction of G proteins with receptors (Masters et al., 1986). Antibodies raised against a carboxyl-terminal decapeptide from Gi have been produced and inhibit the receptor-dependent inhibition of adenylate cyclase in human platelet membranes (Simonds et al., 1989a). An antibody raised against a decapeptide derived from the carboxyl terminus of Gsα has been produced (RM antibody) and inhibits the isoproterenol activation of adenylate cyclase in turkey erythrocytes (Simonds et al., 1989b). Preliminary

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data also indicated that this antibody could immunoprecipitate activated complexes of adenylate cyclase and Gsα (Simonds et al., 1989b). We have used this antibody to further characterize the interaction of G proteins with adenylate cyclase. The ability of RM antibody to immunoprecipitate adenylate cyclase under different preactivation conditions has been assessed and shown to require ligands that activate Gsα directly. The presence of other G protein subunits in RM antibody immunoprecipitates of preactivated adenylate cyclase was examined by using subunit specific antibodies. There does not appear to be any stable association of Gi, Go, or Gβ with adenylate cyclase that has been partially purified by forskolin affinity chromatography.

Experimental Procedures

Materials. Affi-Gel 10 was from Bio-Rad; Lubrol-PX was from Pierce Chemical Co.; guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S), pepstatin A, and leupeptin were from Boehringer Mannheim; benzamidine, Tween 60, and phenylmethanesulfonyl fluoride (PMSF) were from Sigma Chemical Co.; crystallized bovine serum albumin (BSA) was from Miles Diagnostics; protein A–agarose was from Calbiochem; nonimmune rabbit IgG (heavy and light chain) was from Cappell; [α-^{32}P]ATP, [^{3}H]cAMP, and [^{125}I]protein A were from New England Nuclear; and ^{14}C molecular weight standards were from Bethesda Research Laboratories.

Membrane Preactivation and Detergent Solubilization of Proteins. Crude bovine brain membranes were prepared by modifications of the method of Sternweiss and Robishaw (1984). Bovine brains were immersed in ice-cold homogenization buffer [10 mM Tris-HCl, pH 7.4, 0.32 M sucrose, 10 mM EDTA, 1 mM DTT, 1 mM benzamidine, 10^{-6} M pepstatin A, 10^{-6} M leupeptin, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF)] as soon as possible after sacrifice. All subsequent manipulations were carried out at 0–4 °C. The meninges and as much white matter as possible were removed. Pieces from four brains were initially disrupted in 2 L of homogenization buffer with two 15-s pulses in a Waring blendor. The resultant material was passed through a Yamato LH-41 homogenizer equipped with a loose pestle. Homogenization was carried out at 1800 rpm. Immediately beforehand, the mixture was brought to 0.5 mM PMSF with a 200 mM stock dissolved in 1-propanol. The homogenate was filtered through cheese cloth and centrifuged at 20000*g* for 30 min at 4 °C. The supernatants were aspirated and the pellets suspended to original volume in homogenization buffer. This mixture was passed through the homogenizer and centrifuged as above but for 40 min. Resuspension, homogenization, and centrifugation was repeated once. The material was then suspended and homogenized before being aliquoted, frozen immediately in liquid nitrogen, and stored at −80 °C. Thawed membranes were washed and resuspended in NMT buffer (150 mM NaCl, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5) as described (Simonds et al., 1989b). The membranes were incubated for 30 min at 30 °C with buffer alone (control) or with 100 μM forskolin, 100 μM GTP-γ-S, or 10 μM $AlCl_3^-$ and 10 mM NaF (AlF_4^-). The membranes were washed and resuspended in NMT buffer and then solubilized by stirring with an equal volume of 2% Lubrol-PX in NMT buffer for 40–60 min at 4 °C. The solubilized membranes were then centrifuged in a Ti 70 rotor at 35000 rpm for 90 min at 4 °C. Solubilized proteins were recovered in the supernatants, aliquoted, and frozen at −80 °C.

Production of Antibodies. Antibodies were produced in rabbits against the RM peptide (the carboxyl-terminal decapeptide) of the Gsα protein (Simonds et al., 1989b). The antibodies were affinity purified from crude antiserum on a RM peptide–agarose column (Simonds et al., 1989b). Anti-Gβ antibodies were produced in rabbits against an amino-terminal decapeptide of the Gβ protein subunit (Goldsmith et al., 1988a). Anti-Go antibodies were produced in rabbits against the carboxy-terminal decapeptide of the Go protein subunit (Goldsmith et al., 1988b). Anti-Gi antibodies, AS_7, were produced in rabbits against the carboxyl-terminal decapeptide of the Gi protein subunit (Goldsmith et al., 1987).

Immunoprecipitation. Detergent extracts of bovine brain membranes were incubated with RM antibody (25 μg/mL) or nonimmune rabbit IgG (25 μg/mL) for 4 h at 4 °C as described (Simonds et al., 1989b). Protein A–agarose was suspended with an equal volume of PBS containing 0.02% sodium azide. Prior to use, the protein A–agarose was equilibrated with 1% Lubrol-PX in NMT buffer to the same concentration. The suspension was added to the antibody mixture and incubated for 30 min on a rotator at 4 °C. The mixture was microfuged at full speed for 30 s at 4 °C. The supernatants were removed and the pellets were washed once with 1.0 mL of 1% Lubrol-PX in NMT buffer. The pellets were resuspended in buffer containing 5 mg/mL BSA for adenylate cyclase assays or in electrophoresis sample buffer for Western blotting and detection by RM antibody as described below.

SDS–PAGE and Western Blots. Protein samples to be analyzed were precipitated by adding 1/10 vol of 0.15% deoxycholate and incubating for 10 min at room temperature, followed by adding 1/10 vol of 72% trichloroacetic acid (Peterson, 1983). The samples were microfuged at full speed for 15 min and the supernatants were removed. Ice-cold acetone was added to each sample and the samples were kept on ice for 10 min. The samples were microfuged for 5 min and the supernatants were removed. Electrophoresis sample buffer (Laemmli, 1970) containing 1% SDS was added to each sample and the samples were heated for 10 min at 55 °C. The proteins were then separated on 10% SDS–polyacrylamide gels (Laemmli, 1970). The gels were run at a constant 200 V by using the Bio-Rad Mini-Protean II apparatus. The proteins were electrophoretically transferred onto Immobilon at 50 V for 1 h by using an LKB Midget MultiBlot apparatus with 25 mM Tris and 10 mM glycine buffer in 10% methanol. Membranes were blocked with 3% gelatin in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) for 1 h, incubated overnight with 5 μg/mL RM antibody or 1:200 dilutions of crude antisera in TBS with 1.0% gelatin for the antibodies against the Go, Gi, and Gβ proteins, washed with TBS containing 0.05% Tween 20 for 10 min, washed with TBS for 10 min, and incubated with approximately 0.2 μCi/mL recombinant [^{125}I]protein A in TBS with 1.0% gelatin for 1 h. The blots were washed with TBS containing 0.05% Tween 20 for 10 min and with TBS for 10 min, dried, and exposed to X-ray film.

Adenylate Cyclase Assay. Incubations were in a total volume of 250 μL containing 50 mM Tris-HCl buffer, pH 7.5, 1.0 mM 3-isobutyl-1-methylxanthine, 5 mM $MgCl_2$, and 0.1 mM ATP. Each assay mixture contained 1 μCi of [α-^{32}P]ATP and an ATP-regenerating system of 5 units of creatine kinase and 2 mM creatine phosphate. Assays were carried out at 30 °C for 10 min and terminated by the addition of 0.5 mL of 10% trichloroacetic acid. Carrier cyclic AMP solution (final concentration 0.5 mM) containing about 20000 cpm of cyclic [^{3}H]AMP was added, and cyclic AMP was isolated and analyzed as described by Salomon et al. (1974). Assays were carried out in triplicate.

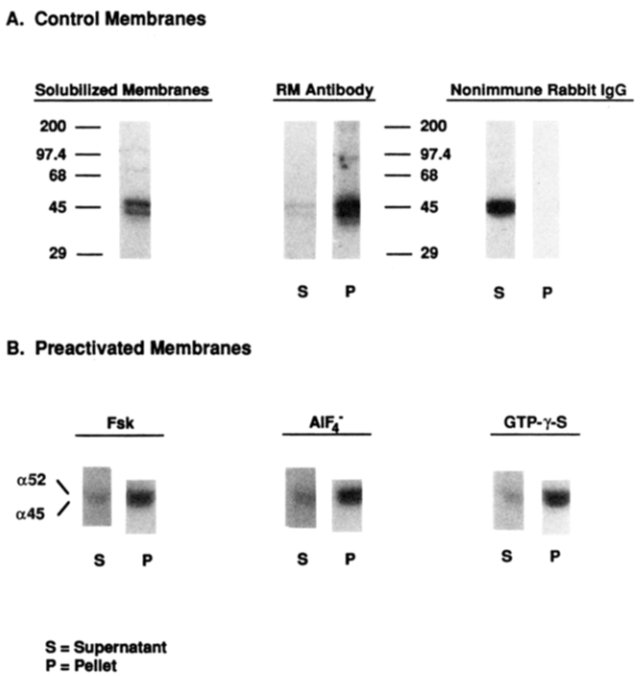

FIGURE 1: Detection of Gsα after immunoprecipitation of solubilized proteins from control and preactivated membranes. (A) Control membranes were solubilized as described under Experimental Procedures, and aliquots (250 μL containing 400 μg of protein) were incubated with 25 μL of NMT buffer containing RM antibody or nonimmune IgG antibody so that the final concentration of antibody was 25 μg/mL. After overnight incubation, 200 μL of the protein A–agarose suspension was added and allowed to incubate for 30 min, and then the solution was centrifuged and the pellet (P) and supernatant (S) were run on 10% SDS–polyacrylamide gels and transferred to Immobilon. Gsα was detected with RM antibody as described under Experimental Procedures. (B) Membranes were pretreated with 100 μM forskolin, 10 μM AlF_4^-, or 100 μM GTP-γ-S, solubilized, immunoprecipitated with RM antibody, and analyzed for Gsα as in (A). The ^{14}C molecular weight standards were myosin (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

Partial Purification of Adenylate Cyclase. Forskolin–agarose columns were prepared as described by Pfeuffer et al. (1985b). Forskolin–agarose columns were equilibrated with buffer containing 10 mM MOPS, pH 7.5, 1.0 mM EDTA, 1.0 mM $MgCl_2$, 1 mM Tween 60 detergent, and 0.1 M NaCl. Lubrol-PX extracts of control membrane or membranes preactivated with 100 μM GTP-γ-S were individually applied to the columns overnight. The columns were washed with 10 column volumes of equilibration buffer containing 0.5 M NaCl. Adenylate cyclase was specifically eluted with 100 μM forskolin in buffer containing 0.5 M NaCl. Fractions were assayed for adenylate cyclase in the presence of 100 μM forskolin and 5 mM $MnCl_2$. Forskolin-eluted fractions containing adenylate cyclase activity were pooled, concentrated 5–8-fold on an Amicon YM 30 membrane, aliquoted, and frozen at −80 °C. This procedure yielded final specific activities of 5.34×10^4 (pmol/min)/mg and 8.14×10^4 (pmol/min)/mg for adenylate cyclase from control membranes and GTP-γ-S preactivated membranes, respectively.

RESULTS

Immunodetection of Gsα Protein by RM Antibody. Experiments were carried out to determine the optimal concentrations of RM antibody and protein A–agarose for complete immunoprecipitation of Gsα (data not shown). A concentration of 25 μg/mL RM antibody produced the most efficient immunoprecipitation of Gsα. Higher concentrations of RM antibody (up to 100 μg/mL) actually resulted in less Gsα being immunoprecipitated. The amount of protein A–agarose that produced the best immunoprecipitation was 200 μL per 500 μL total incubation volume (i.e., that volume containing solubilized proteins and RM antibody).

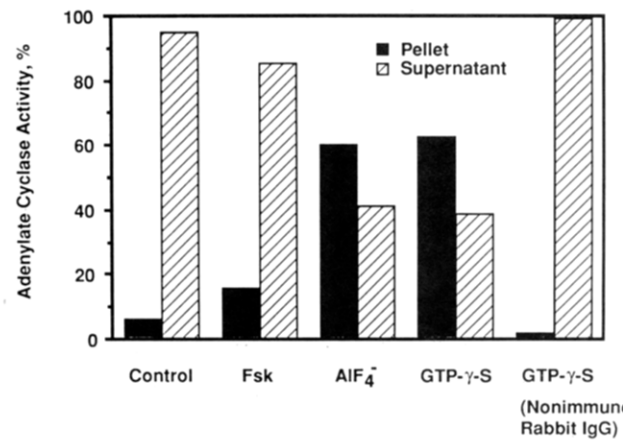

FIGURE 2: Measurement of adenylate cyclase activity after immunoprecipitation of control and preactivated solubilized membranes. Control and preactivated solubilized membranes were immunoprecipitated with RM antibody exactly as described in the legend to Figure 1. Membranes preactivated with 100 μM GTP-γ-S were also immunoprecipitated with nonimmune IgG. The adenylate cyclase activity was measured in triplicate, as described under Experimental Procedures, in the supernatants and pellets after immunoprecipitation.

Lubrol-PX-solubilized proteins from control membranes were immunoprecipitated with RM antibody or nonimmune rabbit IgG. The amount of Gsα in the pellets and supernatants was determined by Western blotting and detection with RM antibody. Both the 52-kDa and 45-kDa forms of the Gsα protein were detected by the RM antibody in the solubilized proteins from control membranes (Figure 1A). Virtually all of the Gsα was immunoprecipitated from solubilized control membranes with RM antibody and very little Gsα remained in the supernatant. In contrast, virtually all of the Gsα protein remained in the supernatant of the nonimmune rabbit IgG immunoprecipitate (Figure 1A).

The effects of various activators of adenylate cyclase on the ability of RM antibody to immunoprecipitate Gsα were determined. Membranes were preactivated with 10 μM AlF_4^-, 100 μM forskolin, or 100 μM GTP-γ-S prior to Lubrol-PX solubilization. Immunoprecipitation of these preactivated preparations with RM antibody showed results similar to immunoprecipitation of the control membranes. Gsα protein was detected predominantly in the pellets of all the immunoprecipitates, regardless of the agent used for preactivation (Figure 1B). Nonimmune rabbit IgG failed to immunoprecipitate Gsα protein from GTP-γ-S preactivated solubilized membranes (data not shown).

Effect of Preactivation on Immunoprecipitation of Adenylate Cyclase by RM Antibody. Adenylate cyclase activity was measured in the supernatants and the pellets after immunoprecipitation of control and preactivated solubilized membranes with RM antibody. The activity was assayed in the presence of 100 μM forskolin and is represented as a percentage of the total activity recovered (Figure 2). Most of the adenylate cyclase activity was in the supernatants of the RM antibody immunoprecipitates from control membranes or membranes preactivated with 100 μM forskolin, with only 5% and 15% of the total adenylate cyclase activity measured in the immunoprecipitated pellets, respectively. In contrast, immunoprecipitation of membranes preactivated with 10 μM AlF_4^- or 100 μM GTP-γ-S resulted in a greater proportion of total activity being measured in the immunoprecipitated

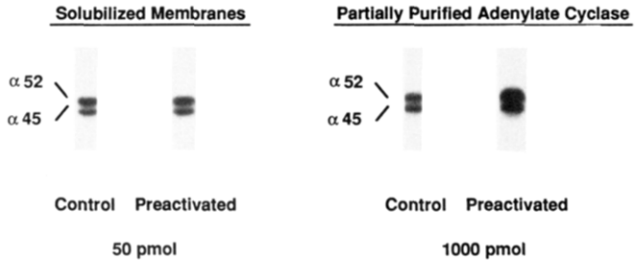

FIGURE 3: Detection of Gsα protein in solubilized membranes and partially purified adenylate cyclase. Adenylate cyclase activity, 50 pmol/min, from control and 100 μM GTP-γ-S preactivated solubilized membranes and 1000 pmol/min of adenylate cyclase activity from partially purified preparations of adenylate cyclase from control and 100 μM GTP-γ-S preactivated solubilized membranes were TCA/DOC precipitated for gels as described under Experimental Procedures. The samples were run on 10% SDS–polyacrylamide gels and Gsα was detected with RM antibody.

pellets, 60% and 65%, respectively. Nearly all of the adenylate cyclase activity from GTP-γ-S preactivated membranes and control membranes was detected in the supernatants after immunoprecipitation with nonimmune rabbit IgG (Figure 2 and data not shown for control membranes).

Detection of Gsα Protein in Partially Purified Preparations of Adenylate Cyclase. Adenylate cyclase from control Lubrol-PX-solubilized membranes and membranes solubilized after preactivation with 100 μM GTP-γ-S was partially purified by using a forskolin–agarose column. The amount of Gsα protein in crude solubilized membranes and partially purified preparations was estimated by immunodetection with RM antibody on Western blots (Figure 3). The large amount of total protein in the crude solubilized preparations limited the amount of adenylate cyclase activity that could be applied to the gels for Gsα determination. Thus, 50 pmol/min of enzyme activity from crude solubilized membranes was used for the Gsα determination while 1000 pmol/min of enzyme activity was used for the partially purified preparations. Densitometric scanning of the exposed X-ray film from the Western blots indicated that there were approximately equal amounts of Gsα protein in both control and 100 μM GTP-γ-S preactivated preparations of crude solubilized membranes (Figure 3). Therefore, preactivation of membranes had no effect on the amount of Gsα solubilized from crude membranes. In contrast, there was roughly three times more Gsα protein in the partially purified preparation from GTP-γ-S preactivated solubilized membranes than in the partially purified preparation from control solubilized membranes (Figure 3).

Detection of Gsα Protein and Measurement of Adenylate Cyclase Activity after Immunoprecipitation of Partially Purified Preparations of Adenylate Cyclase. Partially purified preparations of adenylate cyclase from control membranes and membranes preactivated with GTP-γ-S were immunoprecipitated with RM antibody or nonimmune rabbit IgG. Adenylate cyclase activity and Gsα protein were measured in the supernatants and pellets after immunoprecipitation. Adenylate cyclase activity was assayed in the presence of 100 μM forskolin and is represented as a percentage of the total activity recovered. The Gsα protein was analyzed by Western blot with RM antibody.

As noted above, less Gsα protein was detected in partially purified adenylate cyclase from control membranes than in that from preactivated membranes (Figure 4A). The pellet from the RM antibody immunoprecipitation of the partially purified adenylate cyclase from control membranes contained 50% of the Gsα protein and only 15% of adenylate cyclase

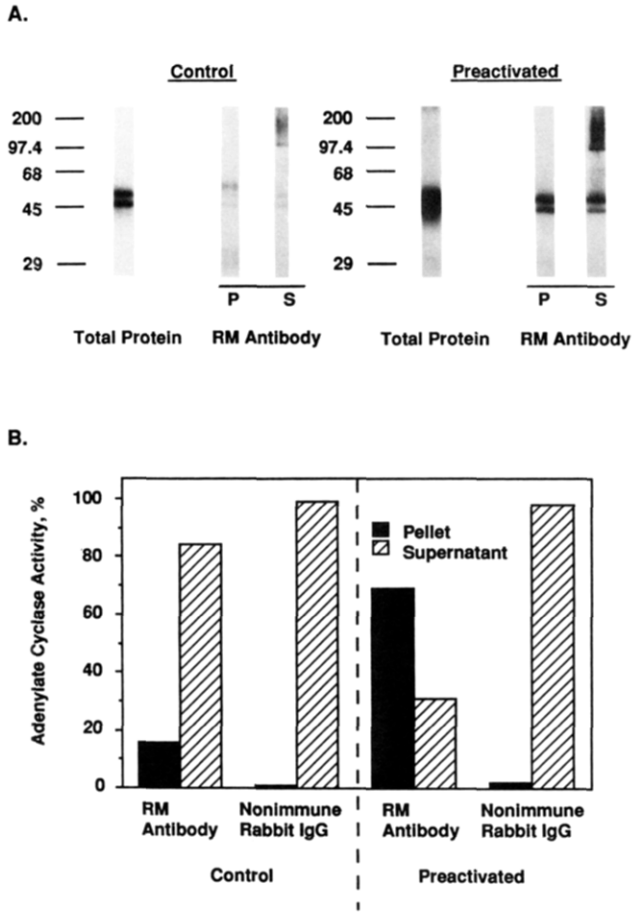

FIGURE 4: Detection of Gsα protein and measurement of adenylate cyclase activity after immunoprecipitation of partially purified preparations of adenylate cyclase. Control and preactivated (100 μM GTP-γ-S) adenylate cyclase was partially purified on a forskolin–agarose column. The partially purified preparations (240 μL containing 8 μg of protein) were immunoprecipitated by adding 10 μL of RM antibody solution (0.6 mg/mL) to achieve a final concentration of 25 μg/mL. After overnight incubation, 100 μL of protein A–agarose suspension was added and incubated for 30 min. The suspensions were centrifuged and the pellets and supernatants treated as described under Experimental Procedures. (A) The total protein in the two partially purified preparations before immunoprecipitation and the remaining protein in the supernatants (S) and pellets (P) after the immunoprecipitation with RM antibody were precipitated with TCA/DOC, run on 10% SDS–polyacrylamide gels, and Gsα protein was detected with RM antibody. (B) Adenylate cyclase activity was measured in triplicate in the supernatants (S) and pellets (P) after immunoprecipation with both RM antibody and nonimmune IgG.

activity (Figure 4). Thus, there was no relation between the amount of Gsα protein or adenylate cyclase activity immunoprecipitated. The pellet from the RM antibody immunoprecipitation of the partially purified adenylate cyclase from preactivated membranes contained about 50% of the Gsα protein and 65% of the total adenylate cyclase (Figure 4). Therefore, in this preparation there was a closer relation between the amount of Gsα protein and the adenylate cyclase activity immunoprecipitated. Less than 2% of the total adenylate cyclase was measured in the pellets after immunoprecipitation of control or partially purified adenylate cyclase with nonimmune rabbit IgG (Figure 4B).

Immunodetection of Giα, Goα, and Gβ Proteins in Solubilized and Partially Purified Membrane Preparations. Antisera against the Goα, Giα, and Gβ proteins were used to characterize crude solubilized membranes and partially purified preparations of adenylate cyclase (Figure 5). Western blots were performed with each of the different antibodies on gels loaded with 50 pmol of adenylate cyclase from crude

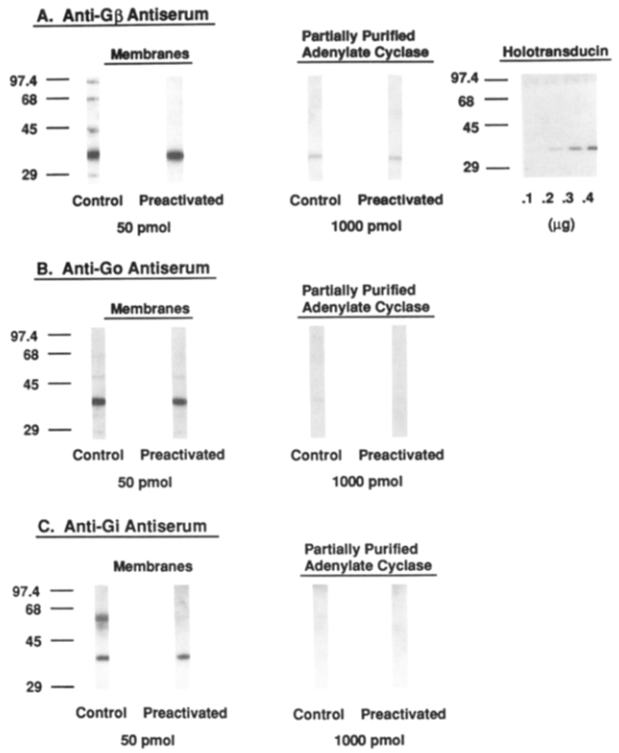

FIGURE 5: Detection of Gβ, Go, and Gi proteins in detergent-solubilized membrane proteins and in partially purified adenylate cyclase. Adenylate cyclase activity, 50 pmol/min, solubilized from control and 100 μM GTP-γ-S preactivated membranes and partially purified adenylate cyclase, 1000 pmol/min, from control and 100 μM GTP-γ-S preactivated membranes were precipitated with TCA/DOC and run in triplicate on separate 10% SDS–polyacrylamide gels as described under Experimental Procedures. Western blots of the gels were detected with anti-Gβ (A), anti-Go (B), or anti-Gi (C) antisera. (A) Solution of holotransducin, 0.9 mg/mL, was diluted with PBS. Four different concentrations of the dilute holotransducin were precipitated with TCA/DOC and run on the gel, which was then detected by using the anti-Gβ antiserum. ^{14}C molecular weight standards were run on the same lane as the control membranes and the molecular weights of the standards are indicated adjacent to the autoradiogram. (B) Some bands are observed in the control and preactivated membranes that are very light compared to the intense staining of the Go protein. These are not observed in the blots of the partially purified adenylate cyclase. (C) A band is observed at about 68 kDa in the control membrane lane. This band is not consistently observed in Western blots with this antibody. In some experiments a very light band was observed at 68 kDa in both control and preactivated membranes.

solubilized membranes and 1000 pmol of adenylate cyclase from partially purified preparations (assayed with 100 μM forskolin and 5 mM $MnCl_2$). There was no difference in the amount of Goα, Giα, or Gβ protein in control or GTP-γ-S preactivated solubilized membrane. Preactivation therefore did not affect the amount of these proteins that was solubilized from the membranes.

As previously shown, about 3-fold more Gsα was detected in partially purified adenylate cyclase from preactivated membranes than in partially purified nonpreactivated adenylate cyclase from control membranes (Figures 3 and 4). A trace amount of Goα protein was detected in the partially purified preparation from control membranes (Figure 5B). No Giα protein was detected in either partially purified preparation of adenylate cyclase (Figure 5C). In contrast, roughly equal amounts of Gβ protein were detected in both partially purified preparations of adenylate cyclase (Figure 5A). On the basis of densitometric scanning of a known range of concentrations of holotransducin, the concentration of Gβ protein could be estimated in the two partially purified preparations. There was approximately 130 ng of Gβ protein per 1000 pmol/min of adenylate cyclase. The antibodies were also used to check for the presence of Goα, Giα, and Gβ proteins in pellets after immunoprecipitation by RM antibody from control and GTP-γ-S preactivated solubilized membranes. Western blot analysis with the different antibodies failed to detect any of these proteins in the immunoprecipitated pellets (data not shown). Analysis of the pellets immunoprecipitated by RM antibody from the partially purified preparations with anti-Gβ antibody also failed to detect the presence of Gβ protein (data not shown).

Discussion

The RM antibody has been used to examine the interactions of the Gsα protein with adenylate cyclase. The antibody, which was raised against a decapeptide from the carboxyl-terminal portion of the Gsα protein, immunoprecipitates Gsα and interferes with β-receptor activation of adenylate cyclase in turkey erythrocyte membranes (Simonds et al., 1989b). However, the antibody did not block the binding of Gsα to the catalytic subunit of adenylate cyclase and can therefore be used to immunoprecipitate and study activated complexes of Gsα and the catalytic subunit of adenylate cyclase.

The state of activation of Gs did not appear to affect the ability of the RM antibody to immunoprecipate either the 45-kDa or the 52-kDa form of Gsα. Preactivation of G proteins in membranes with GTP-γ-S or AlF_4^- would be expected to activate G proteins and promote the dissociation of Gsα and Gβγ (Gilman, 1987; Spiegel, 1987). However, the amount of Gsα protein immunoprecipitated from solubilized control or preactivated membranes was the same. Therefore, ligand activation of Gs did not affect the ability of the RM antibody to recognize the C-terminal epitope of Gsα. Thus this epitope was accessible to the RM antibody under GDP liganded conditions as well as when the site was occupied with GTP-γ-S or the activating form of AlF_4^-.

The state of activation of Gs was an important variable for the coprecipitation of adenylate cyclase with the Gsα protein by the RM antibody. A small percentage (about 15%) of adenylate cyclase from nonpreactivated control membranes was reproducibly coprecipitated with RM antibody. This suggests there was a small amount of Gsα that was tightly associated with adenylate cyclase even under conditions where Gs was not activated by the addition of GTP-γ-S or AlF_4^-. These observations were further substantiated by studying partially purified preparations of adenylate cyclase. Adenylate cyclase from nonpreactivated membranes was partially purified on a forskolin affinity column and contained a detectable amount of Gsα. Approximately 15% of the partially purified adenylate cyclase from the nonpreactivated membranes coprecipitated with RM antibody. Other data suggested there was a population of adenylate cyclase that was tightly associated with the Gs protein even in the absence of activating ligands. It was shown that preactivation of the Gsα protein is associated with an increase in the number of high-affinity forskolin binding sites and it was proposed that the high-affinity forskolin binding sites were representative of a complex between Gsα and the catalytic subunit of adenylate cyclase (Nelson & Seamon, 1986; Seamon et al., 1985). However, high-affinity binding sites for forskolin could be observed in rat brain membranes and human platelets even in the absence of preactivation (Nelson & Seamon, 1986; Seamon et al., 1985), suggesting that there might be a basal level of tight association between Gsα and the catalytic subunit that occurred in the absence of preactivation.

The amount of adenylate cyclase that coprecipitated with RM antibody was greater in the partially purified preparation

of adenylate cyclase from GTP-γ-S preactivated membranes than from control membranes. This was consistent with the greater amount of Gsα protein in the partially purified preparations from preactivated membranes than in preparations from control membranes. The complex of Gsα and the catalytic subunit formed upon preactivation was not disrupted by purification since the proportion of adenylate cyclase immunoprecipitated from crude solubilized preactivated membranes was the same as the proportion immunoprecipitated from partially purified preactivated adenylate cyclase, about 65% of the total adenylate cyclase. These results supported the speculation that adenylate cyclase exists in an equilibrium between the uncomplexed catalytic subunit and the complex between the catalytic subunit and Gsα (Mollner & Pfeuffer, 1988). Activators of Gα, e.g., GTP-γ-S, shifted the equilibrium toward the complex of Gsα with the catalytic subunit and this complex was tight enough to allow the copurification and coprecipitation of the two proteins with RM antibody.

The catalytic subunit can be purified to homogeneity without coelution with Gsα by using protocols employing harsh washing conditions (Pfeuffer et al., 1985b; Smigel, 1986). The purification protocol used in this paper involved relatively mild washing with only 0.5 M NaCl in order to maintain the interaction between the catalytic subunit and G proteins even in the absence of preactivation. Although forskolin has been reported to enhance the interaction of Gsα with the catalytic subunit in the absence of activating ligands (Bouhelal et al., 1985), forskolin did not promote a tight enough association between Gsα and adenylate cyclase to allow coprecipitation by RM antibody.

Antisera against Goα, Giα, and Gβ were used to determine if these G protein subunits copurified with the adenylate cyclase complex. Goα, Giα, and Gβ were all present in solubilized membranes and the amount of each protein detected by Western blotting with the specific antiserum was the same regardless of whether or not the membranes were preactivated. Even though Go is the predominant G protein present in the brain, there was only a minimal amount of Goα detected in the partially purified preparation of adenylate cyclase from control membranes. There was no Giα protein detected in either purified preparation. However, Gβ was detected in the partially purified preparations of adenylate cyclase from both control and preactivated membranes. Since there were approximately equal amounts of the Gβ protein in both partially purified preparations, in contrast to the different amounts of Gsα protein, it was difficult to determine if the presence of the protein was a result of complex formation to Gsα or the adenylate cyclase catalytic subunit or simply a result of the protein binding to the forskolin–agarose column. We have previously observed that a number of different cytoskeletal proteins bind to forskolin affinity columns and appear to elute as a large macromolecular complex, possibly as a result of aggregation (Moos and Seamon, unpublished results). This might account for the coelution of Gβ with adenylate cyclase from forskolin–agarose columns. Western blot analysis of RM antibody immunoprecipitates from both control and preactivated solubilized membranes with the different antibodies also failed to detect the presence of Goα, Giα, or Gβ protein (data not shown). Therefore, these proteins did not appear to be associated with the Gsα adenylate cyclase complex formed under basal or preactivated conditions.

RM antibody has been shown to be a powerful reagent for studying the effector coupling mechanism of adenylate cyclase (Simonds et al., 1989b). RM antibody interfered with the receptor-dependent activation of adenylate cyclase. Furthermore, the complex between adenylate cyclase and Gsα that was present under basal conditions or formed as a result of GTP-γ-S preactivation did not appear to be associated with two other G proteins, namely, Go and Gi. Although Gβ did coelute with adenylate cyclase from forskolin–agarose columns, Gβ did not coimmunoprecipitate with the adenylate cyclase Gsα complex. RM antibody will prove to be of great utility in isolating preactivated complexes of adenylate cyclase for further structural studies.

Registry No. Adenylate cyclase, 9012-42-4.

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