0026-895X/93/040556-08$03.00/0

MOLECULAR PHARMACOLOGY, 43:556-563

Coupling of Prostaglandin E_1 Receptors to the Stimulatory GTP-Binding Protein G_s Is Enhanced in Neuroblastoma × Glioma (NG108–15) Hybrid Cells Chronically Exposed to an Opioid

H. AMMER and R. SCHULZ

Institute of Pharmacology, Toxicology, and Pharmacy, Universtity of Munich, D-8000 München 22, Germany

Received July 9, 1992; Accepted January 20, 1993

SUMMARY

This study investigates the functional state of the stimulatory GTP-binding protein G_s in neuroblastoma × glioma NG108–15 hybrid cells chronically exposed to an opioid. For this purpose, a novel *in situ* reconstitution protocol was established using membranes selectively depleted of G_s function by transient exposure to low pH and then reconstituted with purified exogenous stimulatory GTP-binding proteins. With prostaglandin E_1 (PGE_1) receptor-stimulated adenylate cyclase activity as an indicator, reconstituted membranes of cells previously rendered tolerant to the δ-opioid [D-Ala^2,D-Leu^5]enkephalin (DADLE) exhibited approximately 3-fold elevated cAMP generation upon stimulation with PGE_1, compared with nontolerant reconstituted cell membranes. This effect developed dose-dependently with respect to the opioid concentration used for pretreatment of the cells and was blocked by concomitant exposure to naloxone. In contrast, receptor-independent activation of G_s by the stable GTP analogue guanosine-5′-*O*-(3-thio)triphosphate did not reveal any difference in adenylate cyclase activity between reconstituted membranes of control and chronically DADLE-pretreated cells. Furthermore, the functional activity of endogenous G_s displayed no difference between control and DADLE-tolerant cells, as assessed in S49 *cyc*$^-$ reconstitution assays using sodium cholate extracts derived from NG108–15 membranes. The data presented suggest that the increase in PGE_1 receptor-mediated adenylate cyclase activity in opioid-tolerant/dependent NG108–15 hybrid cells most likely relates to enhanced coupling efficiency between the PGE_1 binding site (receptor) and G_s. Moreover, our results support the concept that supersensitivity to excitatory drugs reflects an adaptive mechanism of cells chronically exposed to an opioid.

Investigations into the molecular mechanisms underlying opioid tolerance and dependence have focused mainly on opioid receptors and their regulation of the cAMP-generating effector system (for review, see Refs. 1 and 2). A general finding of these investigations is that long term exposure to an opioid does affect both the opioid binding site (3, 4) and adenylate cyclase activity (5). Despite this information, the molecular basis of drug addiction is still poorly understood.

The discovery of G proteins (6) attracted attention in the opioid field, because opioid receptors control adenylate cyclase activity by pertussis toxin-sensitive G proteins (7). It was hypothized, therefore, that G proteins are likely to also adapt during chronic opioid actions and, thus, may contribute to the phenomena of tolerance and dependence (8). Our recent work supports this notion, because chronic opioid treatment of guinea pigs results in increased levels of certain inhibitory G proteins in the tolerant/dependent myenteric plexus (9, 10). Similar effects were observed for the human neuroblastoma SH-SY5Y cell clone (11) and for distinct regions of the rat brain (12, 13).

Hormone-sensitive adenylate cyclase is under the control of both inhibitory and stimulatory G proteins (G_i and G_s), suggesting a functional interdependence of these entities (6). In fact, prolonged activation of inhibitory A_1 adenosine receptors in cultured hamster smooth muscle cells enhances β_2-adrenoceptor-mediated activation of adenylate cyclase (14). It is thus speculated that the inhibitory opioids also may affect stimulatory control of adenylate cyclase in a similar manner. To test this hypothesis, the present experiments were conducted to determine the functional activity of G_s in the permanent neuroblastoma × glioma NG108–15 hybrid cell line (15) after chronic exposure to an opioid. These hybridomas carry opioid receptors of the δ type (16), and their prolonged activation has been

This work was supported by Deutsche Forschungsgemeinschaft (SFB 220) (R.S.).

ABBREVIATIONS: G protein, guanine nucleotide-binding regulatory protein; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DTT, dithiothreitol; Ro 20–1724, DL-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; PGE_1, prostaglandin E_1; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

demonstrated to cause desensitization of adenylate cyclase by multiple-step mechanisms (3, 16, 17). This adaptation, however, is not associated with alterations in the abundance of G proteins (18, 19). In addition, studies on PGE_1 receptors also did not reveal significant alterations of either the maximal number of binding sites or the ED_{50} of adenylate cyclase stimulation (3, 20).

One experimental approach to study G protein function *in situ* is based on the selective interaction of anti-G protein antibodies with carboxyl-terminal sequences of the G protein α subunit (21–24). However, in our initial experiments such antibodies, as well as Fab fragments thereof, failed to specifically interfere with signal transduction in NG108–15 cell membranes. Therefore, we established a novel reconstitution protocol to test the functional state of G_s *in situ*. This method is based on the selective inactivation of G_s function by short term exposure of cell membranes to pH 4.5 (25) and its subsequent reconstitution with purified exogenous G_s or $G_{s\alpha}$. Using this technique, we could demonstrate a considerable potentiation of PGE_1 receptor-mediated adenylate cyclase activity in cells chronically exposed to DADLE. This effect was attributed to enhanced coupling efficiency between PGE_1 receptors and their associated G proteins, because direct activation of G_s by GTPγS failed to reveal any difference in adenylate cyclase activity, compared with controls. The results obtained suggest that prolonged opioidergic inhibition of adenylate cyclase in NG108–15 cells causes functional adaptation in stimulatory control of adenylate cyclase activity, which might represent at least one biochemical mechanism underlying opioid tolerance/dependence.

Experimental Procedures

Cell culture and membrane preparation. Mouse neuroblastoma × glioma (NG108–15) hybrid cells were cultured in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum, 100 μM hypoxanthine, 1 μM aminopterin, and 17 μM thymidine, in a humified atmosphere of 5% CO_2 at 37°. Murine S49 *cyc*$^-$ lymphoma cells were grown in Dulbecco's modified Eagle medium containing 10% heat-inactivated horse serum. Cells were harvested and membranes were prepared as described (17). Membrane protein was adjusted to a concentration of 1 mg/ml in 5 mM Tris·HCl, pH 7.4, containing 1 mM EGTA and 1 mM DTT, and was stored in aliquots at −70°.

Chronic treatment of NG108–15 cells. In a number of cases, subconfluent monolayers from an individual passage were exposed to different concentrations of the δ-opioid receptor agonist DADLE for 3 days, with daily medium changes. Cells were washed three times with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline before membranes were prepared.

Antibody preparation. A peptide-specific antibody (S1) was raised against the extreme carboxyl-terminal decapeptide of $G_{s\alpha}$ (RMHLRQYELL; amino acids 385–394), according to the method of Simonds *et al.* (24). Selectivity of this antiserum was tested by Western blot analysis of recombinant G protein subunits expressed in *Escherichia coli*. In these experiments, antiserum S1 selectively binds to $G_{s\alpha}$, lacking cross-reactivity with the following G protein subunits: α_{i1-3}, α_{o1}, and α_{o2}. Antibody specificity was tested by blocking antibody binding with the peptide used for immunization. In NG108–15 membranes, antiserum S1 identifies a 45-kDa as well as a 42-kDa form of $G_{s\alpha}$, with the larger form being prominent. Affinity-purified antibodies were prepared as described (26).

Preparation of $G_{s\alpha}$. G_s proteins were isolated from cholate extracts of rabbit liver membranes according to the procedure of Sternweis *et al.* (27). The resulting preparations, however, were not suitable for application in reconstitution experiments in which receptor-mediated stimulation of adenylate cyclase *in situ* was examined. This is most likely due to their substantial contamination with other G proteins (G_i and G_o) and their excess of $\beta\gamma$ subunits. Hence, $G_{s\alpha}$ was isolated from this crude preparation by means of immunoaffinity chromatography. The procedure used essentially followed the method of Asano *et al.* (28), with the exception that affinity-purified S1 antibodies (3 mg/mg of CNBr-activated Sepharose 4B) were used to prepare the affinity gel. The fractions eluted were tested for their stimulatory activity in reconstitution experiments with G_s-depleted membranes. Fractions containing $G_{s\alpha}$ activity were combined and the buffer was changed to 20 mM Tris·HCl, pH 7.4, containing 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.1% cholate. The preparation was concentrated by ultrafiltration, and $G_{s\alpha}$ content was estimated by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gels, using bovine serum albumin as a standard (29).

Inactivation of G_s by low pH treatment. Exposure of membranes to low pH selectively eliminates the function of G_s (25). Membranes of NG108–15 cells (100 μg of protein) were pelleted (12,000 × *g*; 15 min), resuspended in 200 μl of ice-cold 50 mM sodium acetate buffer, pH 4.5, containing 5 mM $MgCl_2$ and 1 mM DTT, and kept on ice for 30 min. Thereafter, 800 μl of 50 mM Tris·HCl buffer, pH 7.4, containing 5 mM $MgCl_2$ and 1 mM DTT, were added to stop the reaction. Membranes ("low pH membranes") were collected as described above, and the pellet was resuspended in Tris buffer and immediately used for experiments.

Reconstitution of low pH membranes. G_s-depleted membranes (10 μg of protein/assay) were reconstituted on ice for 15 min with G_s or affinity-purified $G_{s\alpha}$ (100 fmol/assay, unless otherwise stated) before determination of adenylate cyclase activity. Heat-inactivated (90°; 10 min) G protein preparations or $G_{s\alpha}$ that had been prereacted with affinity-purified S1 antibodies on ice for 2 hr served as controls.

Preparation of detergent extracts and S49 cyc$^-$ reconstitution assay. Membranes of NG108–15 cells were extracted with 1% (w/v) sodium cholate for 1 hr at 4° essentially as described (30). Solubilized proteins were adjusted to a concentration of 1 mg/ml in 50 mM Tris·HCl, pH 8.0, containing 1 mM EDTA, 1 mM DTT, and 1% sodium cholate. Five-milliliter aliquots were used to reconstitute adenylate cyclase in S49 *cyc*$^-$ membranes (10 μg/assay). Activation of $G_{s\alpha}$ was accomplished by the inclusion of GTPγS (100 μM) in the reaction mixtures and enzyme activity was determined as described below.

Adenylate cyclase assay. Adenylate cyclase activity in NG108–15 membranes (10 μg/assay) was determined at 32° for 10 min (unless otherwise stated), as described by Vachon *et al.* (17). The assay system contained 40 mM Tris·HCl, pH 7.4, 0.2 mM EGTA, 0.2 mM DTT, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM ATP, 5 μM phosphocreatine, 5 units of creatine phosphokinase, 10 μM GTP, and a 30 μM concentration of the phosphodiesterase inhibitor Ro 20–1724, in a final volume of 100 μl. Stimulation of enzyme activity was assessed by the addition of either PGE_1 (1 μM) or GTPγS (100 μM). The amount of cAMP generated was measured by radioimmunoassay (31).

Miscellaneous methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were carried out as described by Lang and Schulz (9). Membrane protein was determined according to the method of Lowry *et al.* (32), using bovine serum albumin as a standard. Isolated IgG was photometrically quantified using the relation $A^{1\%}_{1cm,280} = 3.5$ (33).

Materials. cAMP antiserum was obtained from Bio-Yeda (Rehovot, Israel). The peptide used for immunization was purchased from American Peptide Co. (Santa Clara, CA). Cell culture reagents were from GIBCO BRL (Karlsruhe, Germany) or from PAN Systems (Aidenbach, Germany). The phosphodiesterase inhibitor Ro 20–1724 and keyhole limpet hemocyanin were from Calbiochem Corp. (San Diego, CA). All chromatography reagents were from Pharmazia-LKB (Freiburg, Germany). ATP, cAMP, GTP, creatine phosphokinase, and phosphocreatine were purchased from Boehringer (Mannheim, Germany). ^{125}I-cAMP tracer and naloxone were from DuPont-New England Nuclear

(Dreieich, Germany). DADLE was from Bachem Biochemica (Heidelberg, Germany). All other reagents were from Sigma (München, Germany) and of the highest quality available.

Results

To investigate the function of the stimulatory G protein in opioid-tolerant/dependent NG108–15 hybridomas, the cells were chronically exposed to DADLE and *in situ* reconstitution experiments were performed by using G_s-inactivated membranes and purified exogenous $G_{s\alpha}$.

Inactivation of $G_{s\alpha}$ in NG108–15 cell membranes. Exposure of striatal membranes to low pH has been reported to eliminate G_s function (25), whereas intrinsic adenylate cyclase activity remains unaffected (34). Fig. 1A demonstrates the time-dependent loss of cAMP production in NG108–15 membranes transiently exposed to pH 4.5. Maximal loss of both basal and PGE_1-stimulated adenylate cyclase activity was observed after 30 min. In contrast, δ-opioid receptor-mediated inhibition of adenylate cyclase was retained in low pH-treated membranes (Fig. 1B). The experiments demonstrate the selectivity of the method in inactivating G_s function without affecting G_{i2}, the G protein species mediating opioid inhibition of adenylate cyclase in these cells (22).

Reconstitution of low pH-pretreated NG108–15 membranes with stimulatory G proteins. The functional reconstitution of PGE_1 receptor-stimulated adenylate cyclase activity in G_s-depleted membranes required highly purified and biologically active G_s or $G_{s\alpha}$ preparations. Substitution of low pH-pretreated membranes with stimulatory G proteins resulted in a concentration-dependent recovery of cAMP generation (Fig. 2) after exposure to either PGE_1 (1 μM) or GTPγS (100 μM). Under basal conditions, addition of $G_{s\alpha}$ had no effect or only negligible effect on adenylate cyclase activity (data not shown). The amount of $G_{s\alpha}$ required to bring about maximal adenylate cyclase stimulation was about 200 fmol/10 μg of membrane protein. Under these conditions, PGE_1 (1 μM) and GTPγS (100 μM) stimulated adenylate cyclase activity to similar extents. In contrast, when nonsaturating amounts of $G_{s\alpha}$ were used GTPγS (100 μM) proved to be more potent in elevating cAMP levels, compared with receptor-mediated activation with PGE_1 (1 μM). To detect discrete changes in receptor-mediated activation of reconstituted adenylate cyclase, the experiments were conducted using 100 fmol of the stimulatory G protein, a concentration that evokes approximately half-maximal stimulation of adenylate cyclase in this system (Fig. 2). Under these experimental conditions, replicates of experiments produced qualitatively similar results, although the maximal extent of cAMP production varied within individual experiments. This variability was due to differences in the biologic activity of $G_{s\alpha}$ preparations used, most probably resulting from denaturation of $G_{s\alpha}$ during purification and storage of the protein. The functional reconstitution of receptor-mediated stimulation of adenylate cyclase activity was completed within <15 min (Fig. 3). Therefore, all subsequent reconstitution experiments designed to determine receptor-mediated stimulation of adenylate cyclase activity were conducted 15 min after $G_{s\alpha}$ was added to the membranes. In contrast, receptor-independent activation of $G_{s\alpha}$ required no reconstitution period and resulted in maximal cAMP levels almost immediately after the addition of GTPγS (Fig. 3).

Specificity of adenylate cyclase reconstitution for $G_{s\alpha}$ was investigated in control experiments by the use of S1 antibodies. Preincubation of $G_{s\alpha}$ (100 fmol) with increasing amounts of S1 antibodies (1–100 ng) before reconstitution into low pH-exposed membranes dose-dependently abolished PGE_1 receptor-mediated reconstitution of adenylate cyclase activity (Fig. 4). Furthermore, saturation of the antibody with its cognate peptide blocked antibody binding to $G_{s\alpha}$ and, thus, reconstitution

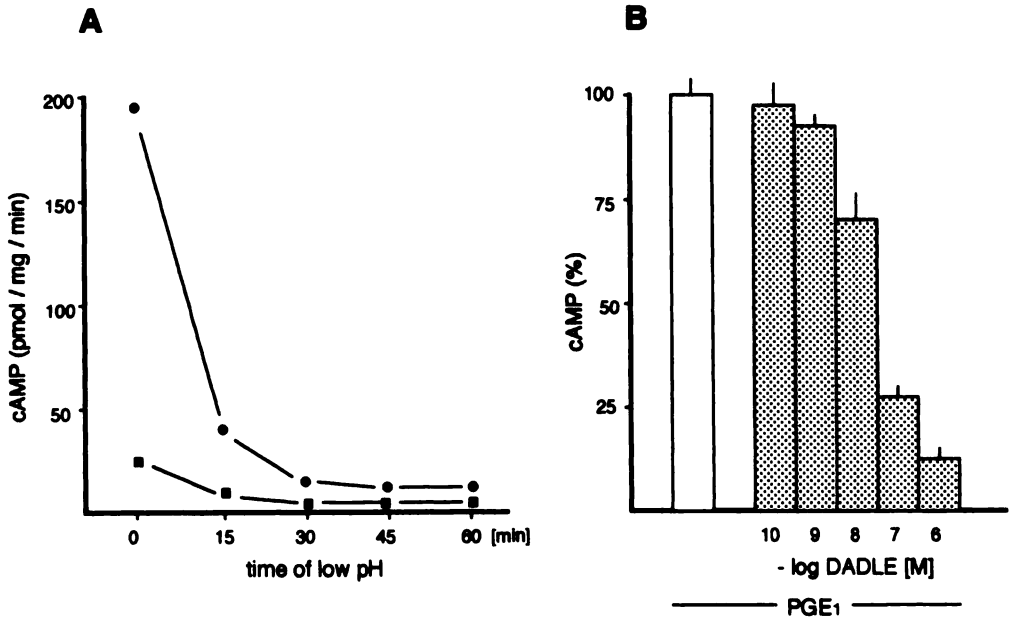

Fig. 1. Selective inactivation of G_s activity in NG108–15 cell membranes by low pH exposure. A, NG108–15 membranes were prepared and transiently exposed to pH 4.5 for the times indicated, as described in Materials and Methods. Basal (■) or PGE_1 (1 μM)-stimulated (●) adenylate cyclase activity was measured for 10 min at 32°. Adenylate cyclase activity is expressed as pmol of cAMP formed/min/mg of membrane protein. Each data point represents the mean value of duplicate determinations. B, Ability of DADLE to inhibit adenylate cyclase activity in low pH membranes. Aliquots of G_s-depleted NG108–15 membranes (10 μg) were assayed in the presence of PGE_1 (1 μM) and various concentrations of DADLE (▣). Inhibition of adenylate cyclase is expressed as percentage of cAMP formed, compared with controls (□) measured in the absence of DADLE. Values represent means ± standard errors of triplicate determinations.

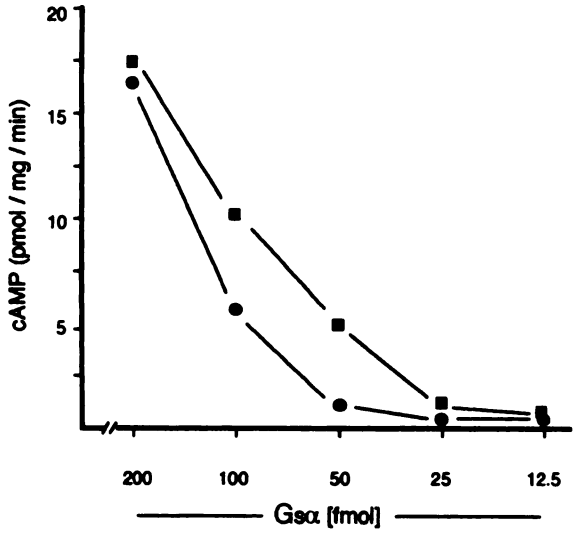

Fig. 2. Reconstitution of low pH membranes with exogenous $G_{s\alpha}$. G_s-depleted membranes (10 μg of protein) were mixed with various amounts of affinity-purified $G_{s\alpha}$ (12.5–200 fmol) for 15 min on ice, in a total volume of 30 μl. Controls (no G protein added) were handled in a similar manner. Adenylate cyclase activity was measured in the presence of either 1 μM PGE_1 (●) or 100 μM GTPγS (■) for 20 min at 32°. Enzymatic activity is expressed as pmol of cAMP formed/min/mg of membrane protein. The data shown are mean values derived from a typical experiment done in triplicate, with a variability of <5%.

in situ was facilitated, as indicated by the production of cAMP. These experiments clearly demonstrate that the recovery of cAMP production in membranes exposed to low pH is indeed due to the incorporation of exogenous $G_{s\alpha}$.

Effect of $G_{s\alpha}$ in low pH membranes derived from NG108–15 cells chronically exposed to DADLE. NG108–15 cells were chronically exposed to DADLE (100 nM) for 3 days. This treatment has been demonstrated to produce a high degree of tolerance in these cells (18). Membranes were prepared and depleted of G_s function by exposure to low pH. Membrane aliquots (10 μg of protein) were reconstituted with 100 fmol of affinity-purified $G_{s\alpha}$ and adenylate cyclase was stimulated by the addition of PGE_1. Fig. 5 demonstrates that control cells (no drug treatment) displayed a moderate increase in cAMP generation when stimulated via PGE_1 receptors. In contrast, reconstituted membranes of cells rendered tolerant to the opioid exhibited a potentiation of adenylate cyclase stimulation upon challenge with PGE_1. Attempts to block this effect by incubating the cells simultaneously with naloxone (1 μM; 3 days) proved only partially successful, because higher concentrations of naloxone (10–100 μM) by itself affected adenylate cyclase activity. Cells pretreated with naloxone alone (1 μM; 3 days) responded to PGE_1 as did control membranes. In contrast to the studies with PGE_1, receptor-independent activation of $G_{s\alpha}$ by GTPγS (100 μM) resulted in similar cAMP levels in membranes of both control cells and cells chronically exposed to DADLE (Fig. 5).

In an extension of these experiments, reconstitution experiments were conducted with NG108–15 membranes chronically exposed to different concentrations of DADLE (1–100 nM; 3 days) and reconstituted with various amounts of $G_{s\alpha}$. The data in Fig. 6 clearly demonstrate that the elevated PGE_1 response depends both on the opioid concentration used for pretreatment and on the amount of $G_{s\alpha}$ used for reconstitution.

Determination of endogenous $G_{s\alpha}$ activity. The functional activity of endogenous $G_{s\alpha}$ was assessed by the ability of sodium cholate extracts to reconstitute adenylate cyclase activity in membranes of G_s-deficient S49 *cyc*$^-$ cells. Under the assay conditions used, adenylate cyclase activity proved to be linear within a range of 0.5–10 μg of protein extract (data not shown). Generation of cAMP was observed only in the presence, and not in the absence, of GTPγS (100 μM) in the assay. As shown in Table 1, detergent extracts of control and DADLE-tolerant NG108–15 hybrid cells did not differ in their efficiency in activating adenylate cyclase in membranes of S49 *cyc*$^-$ cells.

Determination of intrinsic adenylate cyclase activity in control and DADLE-pretreated cells. To examine intrinsic adenylate cyclase activities in native and chronically DADLE-exposed NG108–15 hybrid cells, enzyme activity in particulate membrane preparations was assayed in the presence of 5.6 mM Mn^{2+} and 100 μM forskolin (35). As shown in Table 2, no difference between control and DADLE-pretreated cell membranes was evident at the effector level.

Discussion

The aim of the present study was to investigate possible adaptations in the stimulatory pathway of adenylate cyclase in

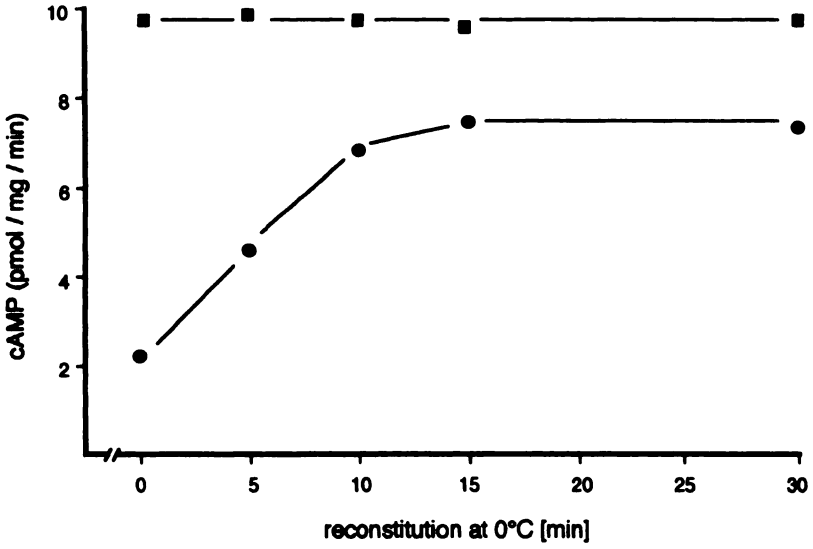

Fig. 3. Time course of reconstitution in low pH membranes. Aliquots of G_s-depleted NG108–15 membranes (10 μg) were reconstituted with 100 fmol of affinity-purified $G_{s\alpha}$ on ice for the times indicated before incubation for assay of adenylate cyclase activity (20 min, 32°). Receptor-mediated stimulation of adenylate cyclase was with 1 μM PGE_1 (●), whereas receptor-independent activation of $G_{s\alpha}$ was accomplished in the presence of 100 μM GTPγS (■). Adenylate cyclase activity is expressed as pmol of cAMP formed/min/mg of membrane protein. Each data point represents the mean value of a single experiment, with triplicate determinations.

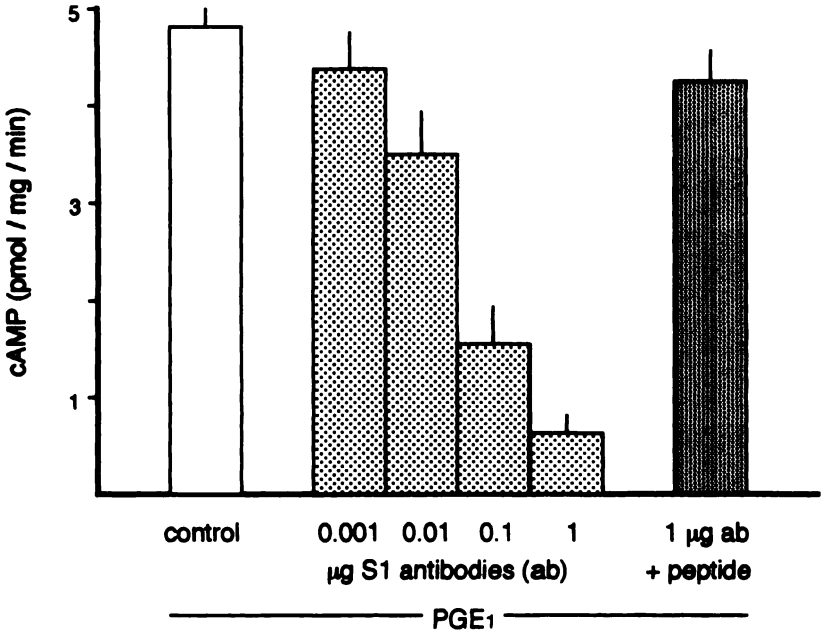

Fig. 4. Precipitation of the stimulatory effect of $G_{s\alpha}$ in low pH membranes by anti-$G_{s\alpha}$ antibodies. Aliquots of low pH-treated membranes (10 µg of protein) were reconstituted for 15 min on ice with either 100 fmol of affinity-purified $G_{s\alpha}$ (□) or similar amounts of $G_{s\alpha}$ that had been prereacted for 2 hr on ice with various amounts of affinity-purified S1 antibodies (▨) (0.001–1 µg/100 fmol of $G_{s\alpha}$). After reconstitution, adenylate cyclase activity was determined in the presence of 1 µM PGE_1 for 20 min at 32°. Precipitation of $G_{s\alpha}$ by S1 antibodies was prevented by coincubation of $G_{s\alpha}$ with a 10-fold molar excess of the peptide used for immunization (▩). Adenylate cyclase activity is expressed as pmol of cAMP formed/min/mg of membrane protein. The data shown are mean values ± standard errors of triplicate determinations from one representative experiment.

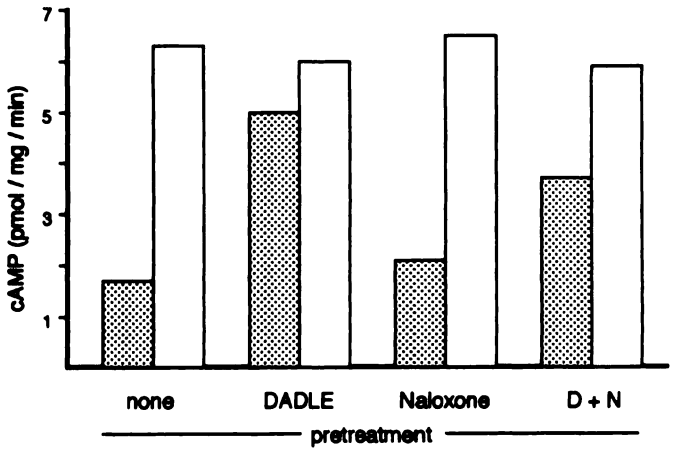

Fig. 5. Effect of $G_{s\alpha}$ in low pH membranes derived from NG108–15 cells chronically exposed to opioids. NG108–15 cells were treated with either 100 nM DADLE, 1 µM naloxone, or both drugs (*D+N*) *in vivo* for 3 days before membranes were prepared and depleted of G_s activity. Controls (*none*) received no drug treatment. Adenylate cyclase was reconstituted with 100 fmol of affinity-purified $G_{s\alpha}$ for 15 min on ice before PGE_1 (1 µM)-mediated (□) or GTPγS (100 µM)-mediated (□) stimulation of adenylate cyclase activity was determined. Adenylate cyclase activity is expressed as pmol of cAMP formed/min/mg of membrane protein. The data shown represent mean values of one typical experiment carried out in triplicate, with a variability of <5%. Three additional experiments produced qualitatively similar results.

a neuronal hybrid cell line (NG108–15) chronically exposed to an opioid. For this purpose, a novel experimental design was developed to investigate the functional state of $G_{s\alpha}$ *in situ*. This method is based on the selective inactivation of G_s function by transient exposure of the membranes to low pH, as originally described for rat striatal membranes (25). To study mechanisms of transmembrane signaling from excitatory receptors to adenylate cyclase, the inactivated system was reconstituted with affinity-purified $G_{s\alpha}$ from rabbit liver. Therefore, the extent of cAMP recovery upon receptor activation may be considered a measure of receptor/G protein coupling. Using this approach, it is now possible to overcome the limitations in the analysis of functional interactions between excitatory receptors and G_s *in situ*, as found in the determination of low-K_m GTPase in most cellular systems (36). Analogous reconstitution techniques using pertussis toxin (37) or *N*-ethylmaleimide-inactivated membranes (38) have been described previously for the functional analysis of inhibitory receptors and their coupling to various G proteins.

The functional reconstitution of adenylate cyclase in low pH-inactivated membranes, as conducted in this study, proved to be time- and dose-dependent as well as specific for $G_{s\alpha}$. However, when receptor-mediated stimulation of adenylate cyclase activity was investigated, this technique required highly purified and biologically active $G_{s\alpha}$, because contamination of the preparations with inhibitory (or as yet unidentified) G proteins or the presence of an excess of βγ subunits unpredictably influenced adenylate cyclase activity. We, therefore, isolated $G_{s\alpha}$ from crude G_s preparations by means of affinity purification. This procedure yielded highly purified $G_{s\alpha}$, but the amounts recovered were very small. Although all purification steps were carried out under nondissociating conditions, the material eluted contained only $G_{s\alpha}$ and not βγ subunits. This observation was somewhat surprising, suggesting conformational changes of solubilized G_s upon binding to the carboxyl-terminal S1 antibody, which in turn may promote dissociation from βγ subunits. Moreover, stability of these $G_{s\alpha}$ preparations was low, even at −70°, because we had to eliminate from the buffers AlF_4^-, which by itself activates $G_{s\alpha}$ under our experimental conditions. Because of these problems, several different batches of $G_{s\alpha}$ were required to perform the experiments described in this study. Although replicates of individual experiments produced qualitative similar data, the factors described caused variability in the maximal extent of cAMP generation.

After the reconstitution technique was established, excitatory signal transduction mechanisms were examined in opioid-tolerant/dependent NG108–15 cells. These cells do not display quantitative changes in the abundance of $G_{s\alpha}$ (19) or in the intrinsic activity of the catalytic moiety of adenylate cyclase (Table 2). However, our data revealed a supersensitive respon-

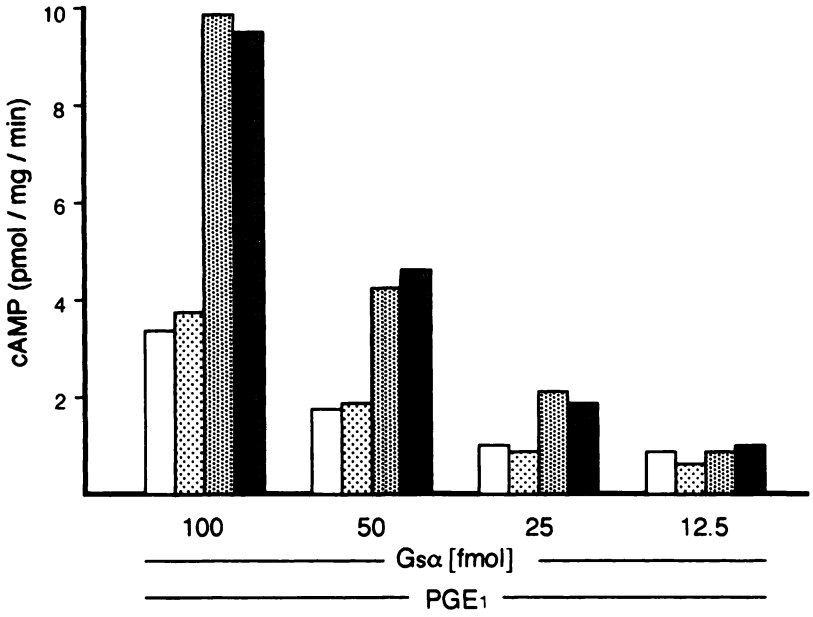

Fig. 6. Effect of various concentrations of DADLE used for pretreatment of NG108–15 cells on $G_{s\alpha}$-reconstituted adenylate cyclase. NG108–15 cells were either untreated (□) or chronically exposed to various concentrations of DADLE (▨, 1 nM; □, 10 nM; ■, 100 nM) for 3 days before low pH membranes were prepared. Aliquots (10 μg of protein) were reconstituted with increasing amounts of affinity-purified $G_{s\alpha}$ (12.5–100 fmol) for 15 min on ice before adenylate cyclase was measured in the presence of 1 μM PGE_1 for 20 min at 32°. Stimulation of adenylate cyclase activity is expressed as pmol of cAMP formed/min/mg of membrane protein. The data shown represent mean values of one typical experiment carried out in triplicate, with a variability of <5%. Three additional experiments produced qualitatively similar results.

TABLE 1

Determination of functional activity of endogenous $G_{s\alpha}$

Membranes of control and DADLE (100 nM; 3 days)-pretreated NG108-15 cells were extracted with 1% (w/v) sodium cholate for 1 hr at 4°. Reconstitution of GTPγS (100 μM)-stimulated adenylate cyclase activity with 10 μg of S49 *cyc*⁻ membranes was performed with 5 μg of detergent extract, as described in Experimental Procedures. Enzyme activity was determined at 32° for 15 min and is expressed as the pmol of cAMP formed/min/mg of membrane protein. Results are presented as means ± standard errors from triplicate determinations.

Sodium cholate extract	Adenylate cyclase activity[a]
	pmol of cAMP/min/mg of cyc⁻ membranes
Control cells	28.44 ± 1.2
DADLE-pretreated cells (100 nM; 3 days)	28.23 ± 0.4

[a] Determined in the presence of 100 μM GTPγS.

TABLE 2

Intrinsic adenylate cyclase activity in NG108-15 cells chronically exposed to DADLE *in vivo*

NG108-15 cells were treated for 3 days with DADLE at the concentrations indicated before membranes were prepared as described in the text. Intrinsic activity of adenylate cyclase was determined in the presence of 5.6 mM Mn^{2+} and 100 μM forskolin for 10 min at 32°. Enzymatic activity is expressed as the pmol of cAMP formed/min/mg of membrane protein. The data shown are mean values ± standard errors of a representative experiment carried out in triplicate.

Pretreatment (3 days)	cAMP
	pmol/mg/min
None	203.2 ± 6
DADLE, 1 nM	199.4 ± 4
DADLE, 10 nM	204.8 ± 3
DADLE, 100 nM	206.5 ± 4

siveness of G_s-reconstituted low pH membranes derived from DADLE-tolerant cells upon challenge with PGE_1. The notion that this adaptation is normally masked in intact membranes of tolerant cells suggests that distinct experimental conditions are required to uncover this effect. This hypothesis is strengthened by a recent report on the stoichiometry of receptor/G_s/adenylate cyclase interactions in murine S49 lymphoma cells (39), which proposes that G_s is in large excess over both stimulatory receptors and adenylate cyclase. This is in line with our observation that the addition of exogenous G_s to intact cell membranes does not further elevate adenylate cyclase activity. According to this hypothesis, it could be speculated that subtle alterations in the stimulatory pathway of adenylate cyclase may become evident only when the amount of G_s becomes limited within the flow cascade between receptor and effector. Indeed, the differences in receptor-mediated adenylate cyclase activities between control and chronically DADLE-pretreated cells were only observed when nonsaturating amounts of G_s were used in reconstitution assays. Therefore, the failure of Boyd *et al.* (19) to observe an altered G_s activity in tolerant NG108–15 cells may be due to their specific experimental conditions.

The demonstrated adenylate cyclase supersensitivity in $G_{s\alpha}$-reconstituted membranes derived from opioid-tolerant cells raises the question of whether altered receptor/G protein or G protein/effector coupling is responsible for the enhanced cAMP production. This issue was investigated by comparing receptor-mediated and receptor-independent activation of adenylate cyclase in reconstituted low pH membranes. Our data demonstrate that only PGE_1, and not the stable GTP analogue GTPγS, results in the potentiation of enzyme activity in opioid-tolerant cells (Fig. 5). This finding suggests more efficient coupling between PGE_1 receptors and G_s than between activated $G_{s\alpha}$ and the effector molecule. Such a mechanism is likely in view of previous reports that revealed no alterations in the level of PGE_1 receptors in opioid-tolerant cells. In particular, neither the maximal number of PGE_1 binding sites, the ED_{50} of PGE_1-stimulated adenylate cyclase, nor the maximal degree of adenylate cyclase stimulation is significantly changed (3, 20). Because both the level (18, 19) and the functional activity of endogenous $G_{s\alpha}$ (Table 2) remain unaffected in DADLE-tolerant NG108–15 cells, we suggest that enhanced coupling efficiency between PGE_1 receptors and G_s most likely accounts for the increase in adenylate cyclase activity found in reconstituted membranes derived from DADLE-tolerant cells.

Alterations in excitatory signal transduction pathways have been proposed to represent at least one possible adaptive mechanism by which cells respond to persistent activation of inhibitory receptors. For example, prolonged exposure of cultured DDT_1-MF-2 hamster smooth muscle cells to an A_1 adenosine

receptor agonist enhances responsiveness of adenylate cyclase to β_2-adrenergic receptors (14). In addition to several alterations of components involved in the inhibitory control of adenylate cyclase (i.e., receptor and G proteins), an increase in the level of β_2 receptor mRNA is observed in this cell line. The stimulatory branch of the adenylate cyclase pathway is also a target for alterations in rat cerebral cortex chronically exposed to antidepressants. In this tissue, treatment with tricyclic antidepressants results in down-regulation of β_2 receptors, which is functionally compensated for by an increased coupling of $G_{s\alpha}$ to adenylate cyclase (40). In an extension of these possibilities, our finding provides an additional example of cross-regulation of the stimulatory pathway of adenylate cyclase. However, the underlying biochemical mechanisms seem to be different, because adenylate cyclase supersensitivity is not accompanied by alterations in either the absolute amount or the intrinsic activity of the components involved.

Our finding also supports the concept of increased excitatory activity in opioid-tolerant/dependent cells (41), which is manifested as elevated cAMP production upon withdrawal of the drug (42). As a consequence, this hypothesis would imply that chronic opioid exposure leads to the establishment of a new equilibrium within the complex arrangement of intracellular signaling mechanisms. Such a newly established equilibrium would easily explain why DADLE-tolerant NG108–15 hybrid cells do not exhibit significant changes in the regulation of adenylate cyclase activity (3, 20), although adaptive processes have taken place within intracellular signaling mechanisms. Under certain conditions the hidden adaptations may become evident, for instance in the case where one of the signal transduction pathways involved becomes limited. Therefore, our experimental approach is suitable for uncovering such discrete changes in the fine tuning of the stimulatory signal transduction pathways, which are normally masked under "physiologic conditions." In this view, the suggestion of enhanced coupling efficiency between excitatory PGE_1 receptors and their associated stimulatory G proteins represents one possible adaptation by which cells may respond to prolonged exposure to opioids and could contribute to the phenomenon of dependence. Nevertheless, the biochemical mechanisms underlying opioid tolerance/dependence seem to be more complex, because additional adaptations have also been observed for inhibitory G proteins.

Acknowledgments

The authors would like to thank Dr. J. M. Helmreich (Würzburg, Germany) for providing G_s preparations used in preliminary experiments, Dr. F. J. Klinz (Berlin, Germany) for donation of *E. coli* lysates containing recombinant G proteins, and Dr. W. Rosenthal (Berlin) for the gift of cAMP antiserum used in initial experiments. We would also like to thank Dr. V. Breuninger (this institute) for raising S1 antiserum.

References

1. Johnson, S. M., and W. W. Fleming. Mechanisms of cellular adaptive sensitivity changes: applications to opioid tolerance and dependence. *Pharmacol. Rev.* **41**:435–488 (1989).
2. Childers, S. R. Opioid receptor-coupled second messenger systems. *Life Sci.* **48**:1991–2003 (1991).
3. Law, P. Y., D. S. Hom, and H. H. Loh. Opiate receptor down-regulation and desensitization in neuroblastoma × glioma NG 108–15 hybrid cells are two separate cellular adaptation processes. *Mol. Pharmacol.* **24**:413–424 (1983).
4. Rothman, R. B., J. B. Long, V. Bykov, H. Xu, A. E. Jacobsen, K. C. Rice, and J. W. Holaday. Upregulation of opioid receptor complex by the chronic administration of morphine: a biochemical marker related to the development of tolerance and dependence. *Peptides* **12**:151–160 (1991).
5. Musacchio, J. M., and D. L. Greenspan. The adenylate cyclase rebound response to naloxone in the NG108–15 cells: effects of etorphine and other opiates. *Neuropharmacology* **25**:833–837 (1986).
6. Rodbell, M. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature (Lond.)* **284**:17–22 (1980).
7. Kurose, H., T. Katada, T. Amana, and M. Ui. Specific uncoupling by islet-activating protein, pertussis toxin, of negative signal transduction via *alpha*-adrenergic, cholinergic, and opiate receptors in neuroblastoma × glioma hybrid cells. *J. Biol. Chem.* **258**:4870–4875 (1983).
8. Collier, H. O. J. Cellular aspects of opioid tolerance and dependence, in *Opioids: Past, Present and Future* (J. Hughes, H. O. J. Collier, M. J. Rance, and M. B. Tyres, eds.). Taylor & Francis Ltd., London, 109–125 (1984).
9. Lang, J., and R. Schulz. Chronic opiate receptor activation *in vivo* alters the level of G-protein subunits in the guinea-pig myenteric plexus. *Neuroscience* **32**:503–510 (1989).
10. Ammer, H., L. Nice, J. Lang, and R. Schulz. Regulation of G proteins by chronic opiate and clonidine treatment in the guinea pig myenteric plexus. *J. Pharmacol. Exp. Ther.* **258**:790–796 (1991).
11. Ammer, H., and R. Schulz. G protein levels in neuronal cell lines chronically exposed to opioids, in *Abstracts of the International Narcotics Research Conference, Keystone, Colorado* (H. Akil, ed.). International Narcotics Research Conference, Ann Arbor, 124 (1992).
12. Nestler, E. J., J. J. Erdos, R. Terwilliger, R. S. Duman, and J. F. Tallman. Regulation of G proteins by chronic morphine in the rat locus coeruleus. *Brain Res.* **476**:230–239 (1989).
13. Terwilliger, R. Z., D. Beitner-Johnson, K. A. Sevarino, S. M. Crain, and E. J. Nestler. A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. *Brain Res.* **478**:100–110 (1991).
14. Hadcock, J. R., J. D. Port, and C. C. Malbon. Cross-regulation between G-protein-mediated pathways: activation of the inhibitory pathway of adenylate cyclase increases the expression of β_2-adrenergic receptors. *J. Biol. Chem.* **266**:11915–11922 (1991).
15. Klee, W. A., and M. Nirenberg. A neuroblastoma × glioma hybrid cell line with morphine receptors. *Proc. Natl. Acad. Sci. USA* **71**:3474–3477 (1974).
16. Law, P. Y., D. S. Hom, and H. H. Loh. Loss of opiate receptor activity in neuroblastoma × glioma NG108–15 hybrid cells after chronic opiate treatment. *Mol. Pharmacol.* **22**:1–4 (1982).
17. Vachon, L., T. Costa, and A. Herz. GTPase and adenylate cyclase desensitize at different rates in NG108–15 cells. *Mol. Pharmacol.* **31**:159–168 (1987).
18. Lang, J., and T. Costa. Chronic exposure of NG108–15 cells to opiate agonists does not alter the amount of the guanine nucleotide-binding proteins G_i and G_o. *J. Neurochem.* **53**:1500–1506 (1989).
19. Boyd, R. S., L. E. Donnelly, and J. MacDermot. Opiate-dependent changes of adenylate cyclase to stimulatory agonists and 5′-guanylylimidodiphosphate are independent of G protein abundance and eukaryotic ADP-ribosyltransferase activity in NG108–15 cells. *J. Neurochem.* **58**:688–693 (1992).
20. Traber, J., R. Gullis, and B. Hamprecht. Cyclic nucleotides in cell cultures of the nervous system. *Fed. Proc.* **41**:165–177 (1975).
21. Cerione, R. A., S. Kroll, R. Rajaram, C. Unson, P. Goldsmith, and A. M. Spiegel. An antibody directed against the carboxyl-terminal decapeptide of the α subunit of the retinal GTP-binding protein, transducin. *J. Biol. Chem.* **263**:9345–9352 (1988).
22. McKenzie, F. R., and G. Milligan. δ-Opioid receptor mediated inhibition of adenylate cyclase is transduced specifically by the guanine nucleotide-binding protein G_{i2}. *Biochem. J.* **267**:391–398 (1990).
23. Simonds, W. F., P. K. Goldsmith, J. Codina, C. G. Unson, and A. M. Spiegel. G_{i2} mediates α_2-adrenergic inhibition of adenylyl cyclase in platelet membranes: *in situ* identification with G_s C-terminal antibodies. *Proc. Natl. Acad. Sci. USA* **86**:7809–7813 (1989).
24. Simonds, W. F., P. K. Goldsmith, C. J. Woodard, C. G. Unson, and A. M. Spiegel. Receptor and effector interactions of G_s: functional studies with antibodies to the α_s carboxyl-terminal decapeptide. *FEBS Lett.* **249**:189–194 (1989).
25. Childers, S. R., and G. LaRiviere. Modification of guanine nucleotide-regulatory components in brain membranes. *J. Neurosci.* **4**:2764–2771 (1984).
26. Mumby, S., I.-H. Pang, A. G. Gilman, and P. C. Sternweis. Chromatographic resolution and immunologic identification of the α40 and α41 subunits of guanine nucleotide-binding regulatory proteins from bovine brain. *J. Biol. Chem.* **263**:2020–2026 (1988).
27. Sternweis, P. C., J. K. Northup, M. D. Smigel, and A. G. Gilman. The regulatory component of adenylate cyclase. *J. Biol. Chem.* **256**:11517–11526 (1981).
28. Asano, T., R. Morishita, and K. Kato. Immuno-affinity purification and characterization of the α subunits of G_o type G proteins from various bovine tissues. *J. Biochem. (Tokyo)* **110**:571–574 (1991).
29. Codina, J., D. J. Carty, L. Birnbaumer, and R. Iyengar. Purification of G proteins. *Methods Enzymol.* **195**:177–188 (1991).
30. McKenzie, F. R., and G. Milligan. Prostaglandin E_1-mediated, cyclic AMP-independent, down-regulation of $G_{s\alpha}$ in neuroblastoma × glioma hybrid cells. *J. Biol. Chem.* **265**:17084–17093 (1990).
31. Frandsen, E. K., and G. Krishna. A simple ultrasensitive method for the assay of cyclic AMP and cyclic GMP in tissues. *Life Sci.* **18**:529–542 (1977).
32. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
33. Peterson, G. L. Determination of total protein. *Methods Enzymol.* **91**:95–119 (1983).
34. Childers, S. R. Opiate-inhibited adenylate cyclase in rat brain membranes

depleted of G_s-stimulated adenylate cyclase. *J. Neurochem.* **50**:543–553 (1988).
35. May, D. C., E. M. Ross, A. G. Gilman, and M. D. Smigel. Reconstitution of catecholamine-stimulated adenylate cyclase activity using three purified proteins. *J. Biol. Chem.* **260**:15829–15833 (1985).
36. Milligan, G. Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide-binding proteins. *Biochem. J.* **255**:1–13 (1988).
37. Katada, T., G. M. Bokoch, J. K. Nothup, M. Ui, and A. G. Gilman. The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. *J. Biol. Chem.* **259**:3568–3577 (1984).
38. Shinoda, M., T. Katada, and M. Ui. Selective coupling of purified α-subunits of pertussis toxin-substrate GTP-binding proteins to endogenous receptors in rat brain membranes treated with *N*-ethylmaleimide. *Cell. Signalling* **2**:403–414 (1990).
39. Alousi, A. A., J. R. Jasper, P. A. Insel, and H. J. Motulsky. Stoichiometry of receptor-G_s-adenylate cyclase interactions. *FASEB J.* **5**:2300–2303 (1991).
40. Ozawa, H., and M. M. Rasenick. Coupling of the stimulatory GTP-binding protein G_s to rat synaptic membrane adenylate cyclase is enhanced subsequently to chronic antidepressant treatment. *Mol. Pharmacol.* **36**:803–808 (1989).
41. Collier, H. O. J. A general theory of the genesis of drug dependence by induction of receptors. *Nature (Lond.)* **205**:181–182 (1965).
42. Sharma, S. K., W. A. Klee, and M. Nirenberg. Morphine receptors as regulators of adenylate cyclase activity. *Proc. Natl. Acad. Sci. USA* **72**:590–594 (1975).

Send reprint requests to: H. Ammer, Institute of Pharmacology, Toxicology and Pharmacy, University of Munich, Königinstrasse 16, W-8000 München 22, Germany.