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Stimulation of β-adrenergic receptors of S49 lymphoma cells redistributes the α subunit of the stimulatory G protein between cytosol and membranes

(competitive enzyme-linked immunosorbent assay/adenylyl cyclase/desensitization)

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ABSTRACT **The stimulatory guanine nucleotide-binding protein (G_s), which links cell-surface receptors to second-messenger effector systems, is assumed to be confined to plasma membranes. In the current studies we tested whether G_s redistributes within cells by treating S49 lymphoma cells with the β-adrenergic agonist isoproterenol, then separating cytosol and crude membrane fractions (defined as pellet and supernatant, respectively, after centrifugation for 1 hr at 150,000 × *g*), and assaying fractions for the α subunit of G_s (α_s) using a competitive ELISA and reconstitution techniques. Under basal conditions, a small (10%) pool of α_s was identified in supernatant fractions of S49 cells. The size of this pool decreased in the first 15 min after agonist treatment of cells. This decrease was blocked by a β-adrenergic receptor antagonist and did not occur in an S49 variant, UNC, which lacks functional interaction between receptors and G_s. The size of the α_s pool in supernatant fractions increased to almost 50% of total cellular α_s during a 1-hr incubation of cells with isoproterenol. Before isoproterenol treatment only the competitive ELISA was sensitive enough to detect cytosolic α_s, whereas at later time points (≥30 min) the presence of α_s in the cytosol was confirmed by both immunoblotting and by reconstitution of adenylyl cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] in G_s-deficient membranes derived from cyc⁻S49 cells. In contrast to membrane α_s, cytosolic α_s did not require activation (e.g., by AlF_4^-) in the reconstitution assay to stimulate adenylyl cyclase. Use of an antibody that selectively recognizes monomeric dissociated α_s, but not heterotrimeric α_s, indicated that cytosolic α_s is monomeric. These data indicate that α_s is not exclusively localized to the plasma membrane and that agonist treatment redistributes this protein within target cells.**

Guanine nucleotide-binding regulatory proteins, G proteins, constitute a family of homologous proteins coupling cell-surface membrane receptors to various second-messenger systems (1–4). Adenylyl cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] resides in the plasma membrane and is activated by the stimulatory G protein (G_s). Each of the G proteins consists of three subunits designated α, β, and γ. The α subunit (α_s) contains the GTP-binding site, possesses a GTPase activity, and is ADP-ribosylated by bacterial toxins. Hormone-liganded receptors are proposed to activate G_s by exchanging G_s-bound GDP for GTP, leading to dissociation of G_s, and the GTP-liganded α_s of G_s, α_s–GTP, then activates adenylyl cyclase until bound GTP is hydrolyzed to GDP. The βγ subunits of G_s are believed to constitute the membrane anchorage for α_s, whereas N-myristoylation seems to play a role for other G proteins (5). Although divergent results have been published (6), G_s is generally assumed to be confined to plasma membranes and perhaps to other membrane fractions (2, 7, 8). Recent data obtained from ADP-ribosylation studies using cholera toxin-treated enterocytes indicated a differential localization of adenylyl cyclase and G_s and suggested that α_s must traverse the cytosol of enterocytes to produce its pathogenetic effects in cholera (9). Cholera toxin-induced ADP-ribosylation of G_s in rat liver membranes has been reported to release α_s from plasma membranes (10). However, physiological stimuli have not been demonstrated to alter the intracellular distribution of G_s.

In the present report we show that the cytosol of S49 lymphoma cells, a widely used model system for the study of the β-adrenergic receptor–G_s adenylyl cyclase pathway (2), contains considerable amounts of α_s and that the cytosolic content of α_s is modulated by β-adrenergic receptor stimulation. Incubation of wild-type S49 cells with the β-adrenergic receptor agonist isoproterenol resulted in the redistribution of about half of the cellular content of α_s to the cytosol from membrane fractions. These results imply that α_s is not exclusively localized in the plasma membrane and that hormones and neurotransmitters can regulate cellular distribution of this protein.

MATERIALS AND METHODS

Materials. All chemicals used were of the highest analytical reagent grade commercially available. Sodium cholate (Sigma) was purified as described elsewhere (11).

Isoproterenol Treatment of S49 Lymphoma Cells. S49 lymphoma cells were grown in suspension in Dulbecco's modified Eagle's medium containing 10% heat-inactivated horse serum and were used in experiments when at logarithmic phase (≈10^6 cells per ml). Cells were incubated at 37°C with (−)-isoproterenol in cell culture medium to which was added superoxide dismutase (EC 1.15.1.1) at 10 μg/ml and catalase (EC 1.11.1.6) at 10 μg/ml, which block degradation of isoproterenol and maintain cell viability (12). Incubation was terminated by adding cold (4°C) cell culture medium and immediately centrifuging (900 × *g*) cells for 10 min. In some experiments 10 μM (−)-propranolol, a β-adrenergic receptor antagonist, was also included to terminate β-adrenergic receptor stimulation. The pelleted cells (2×10^8) were dissolved in 50 ml of 137 mM NaCl/20 mM *N*-2-hydroxyethylpiperazine-N^1-2-ethanesulfonate (Hepes, pH = 7.4)/2 mM $MgCl_2$/1 mM EDTA and centrifuged at 600 × *g* for 10 min at 4°C. This last step was repeated once before the washed cells were brought up in 50 ml of the buffer and subjected to nitrogen cavitation at 400 psi (1 psi = 6.9 kPa) for 20 min at 4°C. The homogenate was spun at 900 × *g* for 5 min at 4°C to remove unbroken cells and nuclei. The supernatant was centrifuged for 1 hr at 150,000 × *g* at 4°C or in some experiments 2 hr at 250,000 × *g*. The pellet was suspended in

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Abbreviations: G_s, stimulatory guanine nucleotide-binding protein; α_s, the α subunit of G_s.

20 mM Tris·HCl, pH = 8.0/1 mM EDTA/25 mM NaCl (TEN buffer) and was denoted crude membranes. The supernatant was considered to be a cytosolic fraction. Protein concentration was analyzed according to Lowry *et al.* (13).

Quantitation of G_s by Immunoassay. A competitive enzyme-linked immunosorbent assay (ELISA) based on antipeptide antibodies against amino acids 28–42 in α_s was used for quantitative purposes as described (14). Briefly, membrane or cytosol samples were extracted with 1% sodium cholate in TEN buffer for 1 hr on ice and centrifuged at 15,000 × *g* at room temperature for 5 min. The supernatant was diluted 5-fold with 1.25% Tween-20 and incubated with affinity-purified antibody GS-1 (diluted 1:600) or GS-2 (diluted 1:2000) at room temperature for 1 hr. Unbound and α_s-bound antibodies were then separated by an ELISA by using microtiter plates coated with the peptide used for immunization of rabbits. Microtiter-bound primary antibody (GS-1 or GS-2) was quantitated by a peroxidase-coupled goat anti-rabbit IgG antibody. α_s purified from rabbit liver (15) was used as standards to quantitate α_s in the S49 cell fractions. For immunoblot assays, SDS/PAGE was performed on 11% resolving gels, and electrophoretic transfer and immunoblot assays were conducted as described (14), except that serum containing antibody was used.

Reconstitution Assay of G_s. Samples were activated by 20 μM $AlCl_3$/10 mM $MgCl_2$/10 mM NaF, reconstituted (16) with G_s-deficient membranes prepared (17) from the cyc$^-$ S49 lymphoma cells, and adenylyl cyclase activity was determined as described (18).

RESULTS

Occupancy of β-adrenergic receptors on wild-type S49 lymphoma cells by isoproterenol, epinephrine, and other agonists causes rapid stimulation followed by subsequent decrease in cAMP accumulation and adenylyl cyclase activity (17, 19, 20). In the current studies we quantitated levels of G_s by competitive ELISA, based on antipeptide antibodies specific for α_s (14), after S49 cells were treated with 1 μM (−)-isoproterenol, a maximally effective concentration in stimulating adenylyl cyclase in these cells (17, 19). Under "basal" conditions—i.e., in the absence of agonist treatment (other than what might occur from hormones or factors present in cell culture medium and 10% heat-inactivated horse serum), we detected 40.6 ± 14.4 pmol of α_s per 2 × 10^8 cells in wild-type S49 cell membranes and 5.0 ± 2.1 pmol of α_s per 2 × 10^8 cells in the 150,000 × *g* supernatant (cytosolic fraction). When S49 cells were treated with isoproterenol, levels of α_s in crude membranes increased slightly during the first 15 min to 52.2 ± 13.6 pmol per 2 × 10^8 cells, whereas more prolonged treatment with isoproterenol decreased α_s levels in the membranes (23.2 ± 6.1 pmol of α_s per 2 × 10^8 cells at 50 min) (Fig. 1). These results suggested that isoproterenol promoted a redistribution of G_s within the cells. Indeed, assay of the 150,000 × *g* supernatant (cytosolic fraction) demonstrated opposite changes in the level of α_s to those found in crude membranes in response to the isoproterenol stimulation of the cells (Fig. 1 *Right*). Thus, the cytosolic fractions were largely depleted of α_s at 15 min but showed 4-fold increases over basal levels after 50-min treatment with isoproterenol. The β-adrenergic antagonist propranolol blocked these responses (Fig. 1 *Insets*). Isolation of the cytosolic fraction at even higher *g* force (250,000 × *g* for 2 hr) yielded similar findings. As a percentage of total detectable cellular α_s, the cytosolic α_s constituted 11.2 ± 4.5% before addition of 1 μM isoproterenol, fell to 2.8 ± 2.5% within 15 min after isoproterenol treatment, and then increased to 46.7 ± 11.7% after 50 min of receptor stimulation. Termination of the treatment of cells with addition of propranolol instead of cooling and centrifugation did not influence the results (data not shown). Fig. 2 shows that the decrease in cytosolic α_s

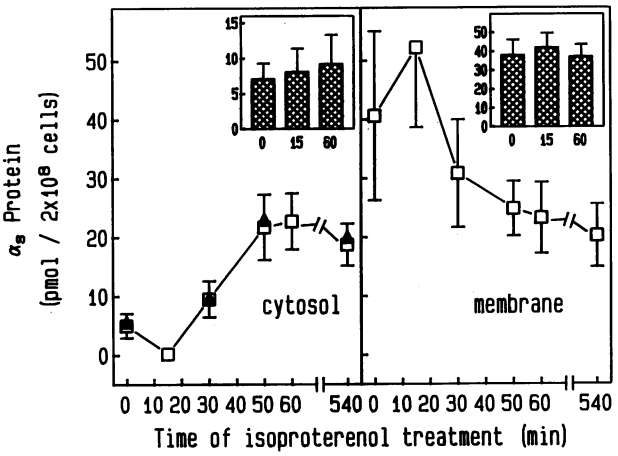

FIG. 1. Time-dependent changes in α_s levels in S49 lymphoma cell membranes (*Right*) and cytosol (*Left*) prepared after treatment with 1 μM (−)-isoproterenol. Membranes and cytosol were assayed for total α_s (□) using antibody GS-1 and samples prepared from ≈2 × 10^8 S49 lymphoma cells at each time point. Results are the average ± 1 SD from four independent experiments run in quadruplicate and are expressed as pmol of α_s per 2 × 10^8 cells. Assay of cytosol by antibody GS-2, which recognizes dissociated (monomeric) α_s (21), is shown at *Left* as closed triangles. (*Insets*) Measurement of total α_s after treatment of cells with isoproterenol in the presence of 1 μM (−)-propranolol is shown for both cytosol (*Left*) and membranes (*Right*).

during the first 15 min after isoproterenol treatment occurred in a concentration-dependent manner and did not occur in an S49 variant, UNC, which has an altered α_s protein that prevents functional interaction with β-adrenergic receptors (22).

Further immunological studies were undertaken to confirm that isoproterenol treatment had translocated α_s between membrane and cytosol. Immunoblots of cytosol taken after 30-min stimulation with 1 μM (−)-isoproterenol demonstrated the appearance of a protein band with an apparent molecular mass of 45 kDa, a size identical with that seen for purified α_s and for membrane α_s in S49 cells (14); this band was not detectable at earlier time points (Fig. 3). The affinity-purified antibody used in the competitive ELISA for α_s, denoted GS-1, has previously been shown to detect only α_s and not α_i, α_o, or other proteins in membranes derived from S49 lymphoma cells (14). The finding of the new protein band at 45 kDa in the immunoblot of cytosol provides strong

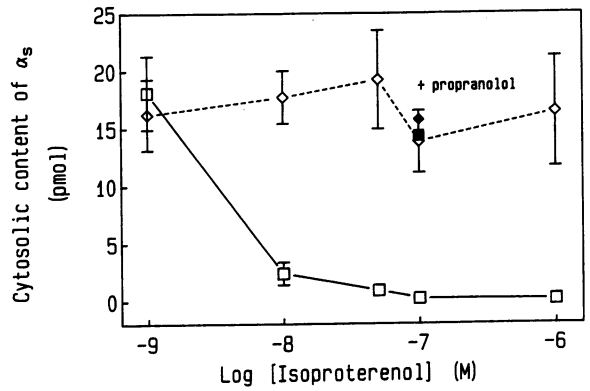

FIG. 2. Concentration–response relationship for isoproterenol-promoted depletion of cytosolic α_s from S49 cells. Cells (8 × 10^8) were incubated with various concentrations of (−)-isoproterenol for 10 min in the absence (open symbols) or presence (closed symbols) of 10 μM (−)-propranolol, and cytosol fractions were prepared. Total α_s was determined by competitive ELISA with antibody GS-1 of cytosol prepared from wild-type (□) and the UNC (◇) S49 cells.

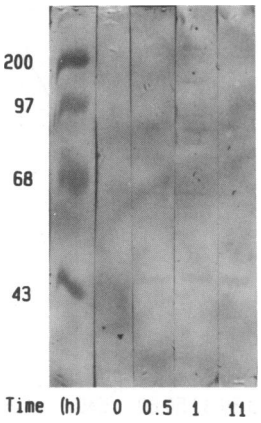

FIG. 3. Immunoblot of cytosolic fractions prepared from S49 cells treated with isoproterenol for various time periods. Cytosol prepared from S49 lymphoma cells treated with isoproterenol for 0 (lane 2), 0.5 (lane 3), 1 (lane 4), and 11 hr (lane 5) as well as prestained molecular mass markers (lane 1; myosin, phosphorylase b, bovine serum albumin, ovalbumin) were subjected to SDS/PAGE on 11% resolving gel and electrotransferred to nitrocellulose filter. Immunoblot assays with rabbit serum containing antipeptide α_s antibodies were undertaken as described.

evidence that α_s levels were increased in cytosol by isoproterenol treatment. However, the band seen was too faint to allow precise quantitation. The 10-fold larger volume of cytosol assayed compared with corresponding membranes probably explains why even though the cytosolic α_s content (as detected by the ELISA) constituted ≈40–50% of total cellular α_s, it was just barely detectable by immunoblot analysis; this amount corresponds per volume to 4–5% of the membrane concentration of α_s. Both cytosol and membranes derived from the G_s-deficient cyc$^-$ mutant S49 lymphoma cell line, lacking mRNA for α_s (23), neither competed in the ELISA nor contained any protein band by immunoblotting (ref. 14 and data not shown).

As a further means to show redistribution of α_s independent of antibodies, we assessed adenylyl cyclase activation in α_s-deficient membranes derived from cyc$^-$ S49 cells after reconstitution with membranes or cytosol obtained from wild-type S49 cells treated with isoproterenol (Fig. 4). Although reconstitutive activity was barely detectable in cytosol prepared from cells treated with isoproterenol for <30 min, after longer stimulation with 1 μM isoproterenol the cytosol was able to reconstitute adenylyl cyclase activity in cyc$^-$ membranes. Reconstitution of cyc$^-$ with wild-type S49 membrane extracts was less effective when the membrane extracts were derived from cells treated with isoproterenol >30 min. Moreover, although reconstitution of adenylyl cyclase activity in cyc$^-$ membranes by wild-type membrane extracts was completely dependent on activation by F^-, the cytosolic fractions did not require activation by F^-, and stimulation of adenylyl cyclase was even more prominent in the absence of activation of cytosol by F^-, probably because of the activation of the G_i protein in cyc$^-$ membranes by F^- (2). The cytosol itself did not contain any adenylyl cyclase activity (data not shown).

G_s is a hydrophobic protein not soluble in the absence of detergents, although monomeric α_s is soluble in the absence of $\beta\gamma$ subunits (1, 3, 21). These considerations suggested that cytosolic α_s might be monomeric and not heterotrimeric. This possibility was also suggested by the requirement of membranes but not cytosol for F^- [which dissociates the $\alpha\beta\gamma$ heterotrimer (1–3)] for reconstitution of adenylyl cyclase. We tested this idea by use of the competitive ELISA and using a second antibody, GS-2, which selectively recognizes dissociated α_s (24). The results confirmed that all detected α_s in the cytosol was monomeric (Fig. 1).

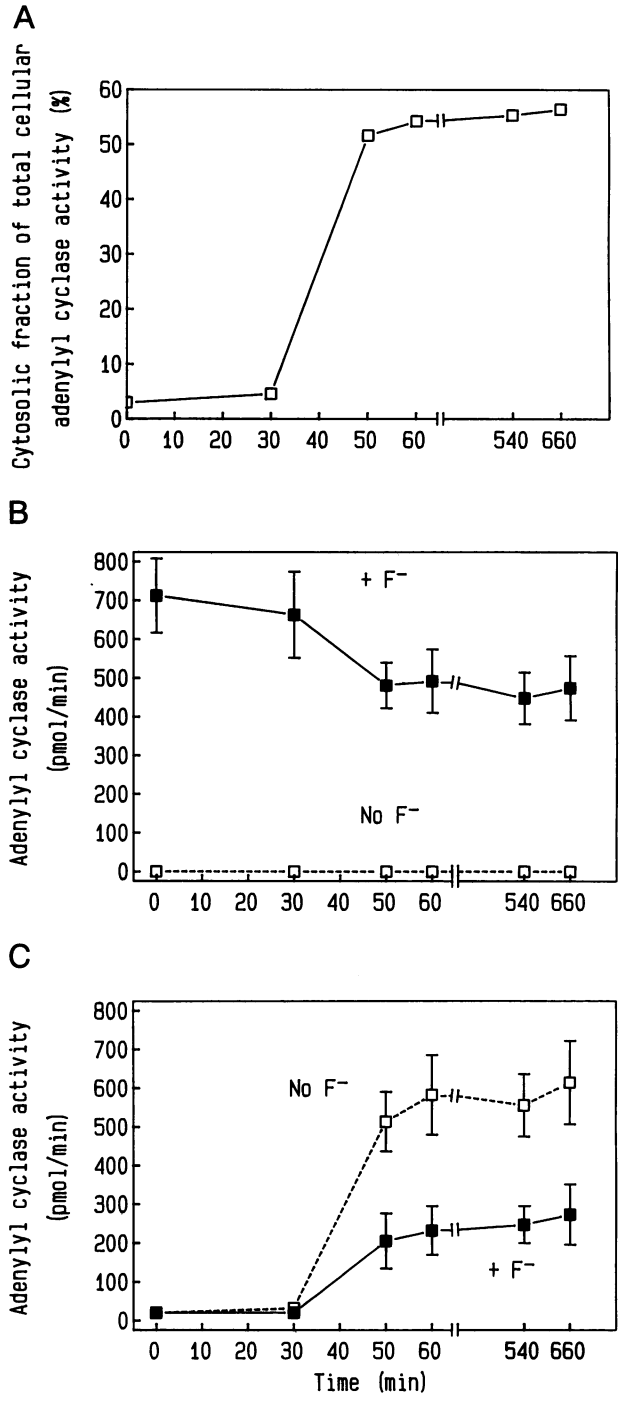

FIG. 4. Reconstitution of adenylyl cyclase activity by using cytosol and membrane fractions prepared from S49 cells incubated with isoproterenol. Adenylyl cyclase activity was determined in cyc$^-$S49 cell membranes after reconstitution with membranes or cytosol prepared from S49 lymphoma cells treated with 1 μM (−)-isoproterenol for various time periods. (*A*) Cytosolic reconstitution ability is shown as percent of total reconstitution capacity. (*B* and *C*) Total adenylyl cyclase activity is shown for membrane (*B*) and cytosolic (*C*) fractions prepared from 2×10^8 cells. Samples were activated with or without 20 μM $AlCl_3$/10 mM $MgCl_2$/10 mM NaF before reconstitution with cyc$^-$ membranes. Results are shown as the average ± 1 SD for three independent experiments run in duplicate.

DISCUSSION

Several years ago preliminary evidence was presented (25, 26) suggesting that activation of G proteins might cause the α subunits to be released from plasma membrane. In contrast

to other G proteins (5), the mechanism proposed for membrane anchorage of α_s is the $\beta\gamma$ subunit (2, 21). The $\beta\gamma$ subunits are hydrophobic and have been shown to associate with phospholipid vesicles (21) and to induce stoichiometric binding of the α subunit of G_o to vesicles containing $\beta\gamma$ subunits. Although the same experiment has not been reported for α_s, other evidence shows that monomeric α_s is a less hydrophobic protein than G_s and is soluble in the absence of detergent (1, 3, 21). The current results appear to confirm the previously proposed hypothesis (25, 26), as well as the recent suggestion that G_s moves into aqueous compartments when β-adrenergic receptors activate adenylyl cyclase (27). The results are also consistent with findings observed for the activation of another G protein, retinal transducin (1), and findings observed for release of α_s in S49 membrane preparations (24) and of α_o in NG108-15 cell membranes (28).

In the present report we examined levels of α_s using both ELISA and reconstitution in crude membranes and cytosol (defined as the supernatant after 1-hr centrifugation at 150,000 × *g*). Our ELISA, based on antipeptide antibodies specific for α_s, detected considerable amounts of dissociated α_s in the cytosol, about 11% of the total cellular content before hormonal treatment. The concentration of α_s in the cytosol was, however, below the limit that was readily detectable by either immunoblot or reconstitution assays. Isoproterenol stimulation of S49 lymphoma cells for <15 min induced a redistribution of α_s from the cytosol to the membrane fraction. This redistribution requires interaction of β-adrenergic receptors and a functional G_s (Fig. 2). Defining the precise molecular mechanisms responsible for this redistribution will require further study. It is intriguing that this agonist-promoted redistribution is similar to that reported for a β-adrenergic receptor kinase that phosphorylates β-adrenergic receptors in a cyclic AMP-independent manner (29).

Isoproterenol stimulation of cells that lasted >30 min produced a substantial release of α_s from membranes to the cytosol, as detected by ELISA, immunoblots, and reconstitution of adenylyl cyclase in G_s-deficient membranes. Thus, all the approaches used here confirm that this longer stimulation of S49 cells redistributed α_s from membrane to cytosol, perhaps as a consequence of activation-induced changes in the protein. The finding that α_s of the cytosol from cells incubated with isoproterenol >30 min can reconstitute adenylyl cyclase in the absence of activation by F^- suggests that α_s in cytosolic fraction may be activated compared with α_s in the membrane fraction.

There are several implications of the findings that considerable levels of α_s are found in the cytosol. These include the possibility that α_s may interact with other organelles and components of the cell in addition to plasma membrane proteins. Such interaction may relate to previous observations of the isolation of α_s in membrane fractions other than the plasma membrane (7, 8). The substantial depletion of G_s levels from membranes after isoproterenol stimulation also provides another mechanism to account for heterologous desensitization in which cells treated with a particular agonist lose response to multiple classes of agonists (20, 30, 31). In addition, agonist-induced changes in the stoichiometry between membrane levels of α_s and $\beta\gamma$ may conceivably alter the susceptibility of adenylyl cyclase to inhibition by other classes of agonists. At a minimum, the results imply that previous notions that have considered G_s to exist exclusively as a membrane protein must be reevaluated.

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