Inhibition of subunit dissociation and release of the stimulatory G-protein, G_s , by $\beta\gamma$ -subunits and somatostatin in S49 lymphoma cell membranes

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We examined the interaction between the stimulatory guanine-nucleotide-binding protein, G_s , and the inhibitory guanine-nucleotide-binding protein, G_i , in cell membranes of S49 lymphoma cells. In these cells, β -adrenergic receptors stimulate the activity of adenylate cyclase via G_s , whereas inhibition via somatostatin receptors is transduced by an inhibitory G-protein, G_i . Using an antibody that selectively recognizes α_s , the monomeric, but not the heterotrimeric, α -subunit of G_s , we quantified the extent of dissociation of G_s in a competitive e.l.i.s.a. Incubation of S49-cell plasma membranes with 0.1 μ M-isoprenaline, 100 μ M free Mg^{2+} and 100 μ M-GTP produced substantial subunit dissociation of G_s , which was reversible by addition of purified $\beta\gamma$ -subunit dimer or somatostatin. Somatostatin produced an immediate (without a lag) time- and concentration-dependent decrease in the concentration of dissociated G_s ($k_{inhib.}$ for somatostatin = 51 ± 12 nM) and in the activity of adenylate cyclase ($k_{inhib.}$ = 121 ± 20 nM). By contrast, after addition of a 10-fold molar excess of $\beta\gamma$ -dimer relative to α_s , there was a 2–3 min lag, after which the $\beta\gamma$ -dimer re-associated G_s . Isoprenaline-induced dissociation of G_s was accompanied by a release of α_s from the incubated membranes to a post-100 000 g supernatant, and somatostatin could reverse this release. Immunoblot analysis with both a C-terminal anti-peptide antibody and an antibody directed against a sequence near the N-terminal also showed release of α_s by the β -agonist and reversal by somatostatin. Membrane release of G_s by isoprenaline that could be blocked by somatostatin was also confirmed in reconstitution studies of supernatant fraction into cyc⁻ S49-cell membranes. We conclude that in native cell membranes somatostatin-induced activation of G_i dissociates G_i and interferes with the G_s activation cycle by providing $\beta\gamma$ -dimer, which acts to prevent or reverse formation of monomeric α_s . Because α_s can be released from the cell membrane, regulation of the local concentration of GTP-liganded dissociated α_s is likely to be an important factor in modulating the activity of adenylate cyclase.

INTRODUCTION

Hormone- and neurotransmitter-mediated signal transduction across the plasma membrane commonly involves the complex interaction of several discrete membrane proteins: receptors, guanine-nucleotide-binding regulatory (G-) proteins and effector systems, such as enzymes and ion channels (reviewed in [1–6]). For some effector systems, such as the enzyme adenylate cyclase, bidirectional control is achieved by classes of agonists that either stimulate or inhibit enzyme activity. However, the precise mechanism for such bidirectional control is incompletely understood.

Adenylate cyclase activity is regulated by stimulatory and inhibitory receptors through a pair of G-proteins, G_s and G_i respectively. The interaction between G_s and G_i is presumed to determine the extent of activation of the catalytic subunit of adenylate cyclase. G-proteins consist of an α -subunit, which confers the functional characteristics of each individual G-protein, and a hydrophobic $\beta\gamma$ -dimer that is identical or very similar in different G-proteins and which may anchor G-proteins to the plasma membranes [1–5]. The similarity in $\beta\gamma$ -subunits between different G-proteins, as well as data obtained in reconstituted phospholipid vesicles, have suggested that activation of G_i inhibits the stimulation of the catalytic subunit by α_s (α -subunit of G_s) by favouring the formation of an inactive heterotrimer $\alpha_s\beta\gamma$ [4]. However, other findings have suggested that $\beta\gamma$ or α_i (α -subunit of G_i) subunits may directly regulate the catalytic subunit [1,4–6].

In the present studies we have used immunochemical and reconstitution assays in order to test whether somatostatin, an agonist active at an inhibitory receptor, alters the dissociation and release from cell membranes of G_s promoted by a stimulatory agonist.

EXPERIMENTAL

Antibody preparation and purification

Antibodies were generated in New Zealand White rabbits to a peptide conjugated to BSA, as described previously [7]. The amino acid sequence of the peptide corresponds to amino acids 28–42 in α_s . Rabbit sera were affinity-purified and characterized by immunoblots, as described [7]. Antibodies denoted GS-1 recognize α_s in both monomeric and heterotrimeric forms [7,8], whereas antibodies named GS-2 selectively detect monomeric α_s [8]; neither of the antibodies recognizes G_i , G_o , β -subunits or any other S49 lymphoma cell membrane proteins [7].

Two other antibodies recognizing α_s were utilized. Antibody 708R, to the C-terminal decapeptide of α_s [9], was kindly provided by Dr. Allen M. Spiegel (N.I.H., Bethesda, MD, U.S.A.), whereas LR4 was elicited against the same peptide as above but in an uncoupled state. In some experiments, LR4 was partially purified by overnight incubation at 4 °C with crude cyc⁻ membranes, and a post-100 000 g supernatant (1 h) was utilized as antiserum.

Abbreviations used: G-protein, guanine-nucleotide-binding regulatory protein; G_s , G-protein that stimulates adenylate cyclase; G_i , G-protein that inhibits adenylate cyclase.

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Membrane preparation and incubation

Wild-type S49 lymphoma cells were grown in suspension culture at $(1-2) \times 10^6$ cells/ml in Dulbecco's modified Eagle's medium and 10% (v/v) heat-inactivated horse serum. Plasma membranes were prepared and purified as described [10] by nitrogen cavitation and sucrose-gradient centrifugation. Membranes (15–50 μ g of protein/sample) were incubated at 37 °C with 100 nM (–)-isoprenaline and 100 μ M-GTP in 20 mM-Tris/HCl (pH 7.6)/1 mM-EDTA/25 mM-NaCl (TEN), and 1.09 mM-MgCl₂. The free Mg²⁺ concentration was calculated as 100 μ M [11]. At times indicated in the Figure legends, purified $\beta\gamma$ -subunits or somatostatin were added. Samples were withdrawn at the indicated times and snap-frozen in liquid N₂.

Assay of α_s

Samples were thawed and processed either by cholate extraction or by dilution and subsequent membrane separation by centrifugation [8]. In the former case, an equal volume of 2% (w/v) sodium cholate in TEN was added to the incubation mixture and was kept on ice for 1 h. The resulting extract was then analysed in a competitive e.l.i.s.a., which has been described and validated elsewhere [7,8,12]. In the latter case samples were diluted 3-fold, and centrifuged at 100 000 *g* at 4 °C for 1 h. The resulting membrane pellet and supernatant were analysed separately as previously described [8] in the competitive e.l.i.s.a.

Miscellaneous

Adenylate cyclase activity was determined from a modification of published procedures [10,13], and as detailed in the legend of Table 1. Protein concentration was determined as described [14]. $\beta\gamma$ -subunits were prepared as described [8,15]; as judged by SDS/PAGE and immunoblotting, the $\beta\gamma$ preparation did not contain α_s , α_o or α_i subunits (results not shown). Reconstitution of α_s into cyc[–] cell membranes was performed as described [16]. Non-linear-regression analysis was undertaken as described [17]. SDS/PAGE and immunoblotting were undertaken as previously described [7], except that 10%-polyacrylamide gels contained half of the usual concentration of bisacrylamide (final 0.13 g/100 ml rather than 0.27). This was found to give better resolution of α -subunits [18].

RESULTS AND DISCUSSION

We have developed and purified rabbit anti-peptide antibodies directed against amino acids 28–42 in α_s . The antibodies have been employed in a competitive e.l.i.s.a. for quantification of α_s . One of these antibodies, termed GS-2 [8], can recognize monomeric dissociated α_s , but not α_s in its heterotrimeric form, G_s, whereas another antibody, GS-1, detects α_s in both states [7]. Using GS-2, we have demonstrated that plasma membranes prepared from wild-type S49 lymphoma cells contain monomeric α_s and that binding of a β -adrenergic agonist, isoprenaline, to β_2 -adrenergic receptors dissociates G_s with an absolute requirement for both guanine nucleotide [8,14] and Mg²⁺ ions (L. A. Ransnäs, J. R. Jasper, D. Leiber & P. A. Insel, unpublished work). We reasoned that these α_s antibodies might prove useful for defining the mechanism of action of G_i.

The molecular mechanisms of inhibition of adenylate cyclase activity by the inhibitory G-protein, G_i, have been a subject of controversy (see ref. [5] for recent review). Although $\beta\gamma$ -subunits arising from G_i activation have been proposed to interfere with G_s dissociation, and thereby indirectly inhibit the activity of adenylate cyclase, this hypothesis has not been fully accepted.

Direct effects of $\beta\gamma$ -subunits on the catalytic subunit of adenylate cyclase have also been proposed [1,3–6]. In plasma membranes from the cyc[–] mutant S49 lymphoma cell line, which lacks α_s , the α -subunit of G_i, α_i , has been found to exert a modest inhibitory influence on the activity of adenylate cyclase by a mechanism that appeared to be competitive with exogenously added α_s , although the latter studies utilized forskolin and high concentrations of Mg²⁺ in order to assess activity of adenylate cyclase [1]. This has raised questions about the physiological importance of this response to α_i in cells containing α_s .

In initial experiments, we investigated the effect of exogenously added $\beta\gamma$ -subunits on G_s dissociation. Plasma membranes from S49 lymphoma cells were incubated at 37 °C with 100 nM (–)-isoprenaline and 100 μ M-GTP at a physiological free Mg²⁺ concentration, 100 μ M, for 7 min, and then a 10-fold molar excess of purified $\beta\gamma$ -subunits was added. This concentration was chosen on the basis of previous estimates of relative stoichiometry of α_s and $\beta\gamma$ [7,19]. At 37 °C, maximally dissociated levels of α_s were obtained within 5 min incubation with isoprenaline, Mg²⁺ and GTP (Fig. 1) at an apparent rate of 1.50 ± 0.27 min^{–1}. The addition of exogenous $\beta\gamma$ -dimers reversed the dissociation of G_s. After an initial 2–3 min lag phase, a rapid decrease in the level of monomeric α_s occurred, and this decrease could be described by a single-component exponential decay. After the initial lag phase, the apparent rate of disappearance of monomeric α_s was 0.26 ± 0.06 min^{–1} (Fig. 1). These observations provide direct evidence that dissociation induced by a β -adrenergic agonist is a reversible event when assessed with 100 μ M-Mg²⁺ and 100 μ M-GTP, and that exogenously added $\beta\gamma$ -subunits can interfere with this dissociation in native cell membranes.

In order to investigate whether not only exogenously added $\beta\gamma$ -dimers, but also $\beta\gamma$ -dimers made available by receptor-induced activation (and dissociation [5,20]) of G_i, would inactivate G_s, we incubated plasma membranes treated as described above with the peptide hormone somatostatin (Fig. 2). In S49 lymphoma cells, somatostatin receptors mediate inhibition of the activity of adenylate cyclase through G_i [21]. Similar to the

Fig. 1. Reassociation of G_s in plasma membranes by addition of $\beta\gamma$ -dimers

Sucrose-gradient-purified S49 lymphoma cell membranes were incubated at 37 °C with 100 nM (–)-isoprenaline and 100 μ M-GTP in 20 mM-Tris/HCl (pH 7.6)/1 mM-EDTA/25 mM-NaCl (TEN buffer) and 1.09 mM-MgCl₂. The free Mg²⁺ concentration was calculated as 100 μ M. Samples were withdrawn at the indicated times and snap-frozen in liquid N₂. A 10-fold molar excess of purified $\beta\gamma$ -dimers (200 pmol/mg) over α_s was added after 7 min in some experiments (■); in others only buffer was added (□). Frozen samples were processed as described in the Experimental section, and extracts were analysed for monomeric α_s as described in a competitive e.l.i.s.a. utilizing antibody GS-2. Three independent experiments were run in quadruplicate, and results are expressed as means $\pm$ 1 s.d.

Fig. 2. Reassociation of G_i in plasma membranes by stimulation of somatostatin receptors

Membranes were activated as in Fig. 1 by 100 nM-isoprenaline and 100 μ M-GTP, and after 7 min 1 μ M-somatostatin was added ($\bullet$). In some experiments only buffer ($\square$) was added. Samples were taken at indicated times and analysed for monomeric α_s as described. Three independent experiments were analysed in quadruplicate, and results are expressed as means $\pm$ 1 S.D.

observations with exogenously added $\beta\gamma$, addition of somatostatin reversed the dissociation of G_s . Somatostatin-induced reassociation occurred without a lag phase and followed a monoexponential time course, with an apparent rate of $0.17 \pm 0.02 \text{ min}^{-1}$. Maximum responses to stimulation of somatostatin receptors and addition of exogenous $\beta\gamma$ -subunits were very similar and non-additive (results not shown). In view of that, and since the kinetics of reassociation were similar for somatostatin stimulation and that produced by exogenous $\beta\gamma$ -dimers (after the initial lag phase), the results suggest that dissociation of G_i into its α_i and $\beta\gamma$ subunits may be rate-limiting for reassociation of G_s . The lag phase observed with exogenous addition of $\beta\gamma$ subunits probably results from the time required for entry and orientation of these proteins into the appropriate membrane environment where α_s is located. Somatostatin presumably liberates endogenous $\beta\gamma$ and therefore does not require the transit of exogenous $\beta\gamma$ and α_s subunits within the membrane.

A concentration-response relationship for stimulation by somatostatin and the levels of monomeric α_s was established by first incubating membranes for 7 min with 100 nM-isoprenaline and 100 μ M-GTP and then adding various concentrations of somatostatin for another 15 min incubation period (Fig. 3). Two different kinds of quantification were performed: (1) direct determination of monomeric α_s by employing antibody GS-2 (Fig. 3a) and (2) separation by centrifugation of membranes and incubation buffer, followed by quantification of total α_s , i.e. monomeric and heterotrimeric in both the membrane and buffer phase (Fig. 3b) by dissociating all G_s present by treatment with 20 μ M- Al^{3+} , 10 mM- Mg^{2+} and 10 mM- F^- . The latter approach utilizes differences in hydrophobicity between $\beta\gamma$ and α_s rather than selective antibody recognition and provides a means of excluding the possibility that antibody affinity for G_s changes in our experiments owing to some factor unrelated to G_s dissociation. Somatostatin promoted reassociation of G_s with a K_D of $51 \pm 12 \text{ nM}$. The buffer phase, which, on the basis of solubility constraints [14,22], contains only monomeric α_s , displayed a decrease in α_s (Fig. 3b) virtually identical with the decreasing levels of monomeric α_s demonstrated in Fig. 3(a). The membrane phase showed the complementary response, thus confirming that the decrease in α_s in the buffer phase was

Fig. 3. Concentration-response relationship for somatostatin-induced reassociation of G_i

Membranes were activated by 100 nM-isoprenaline and 100 μ M-GTP as in Fig. 1, and after 7 min somatostatin was added to give the indicated concentrations. The incubation then proceeded for 15 min. In (a) monomeric α_s was determined as in Fig. 1, and in (b) the membrane pellet ($\square$) and supernatant ($\blacksquare$) were analysed separately either after a 30 min incubation with 20 μ M- AlCl_3 , 10 mM- MgCl_2 and 10 mM- NaF (AMF) with antibody GS-2 or directly using GS-1, respectively, in the competitive e.l.i.s.a., as described. Three independent experiments were analysed in quadruplicate, and results are shown as means $\pm$ 1 S.D. In (b) only the findings with GS-1 after AMF-treatment are shown; GS-2 gave similar results (results not shown).

matched by an increase in the membrane phase. These findings, taken together, are consistent with the conclusion that the responses observed reflect differences in G_s -subunit dissociation and exclude the possibility that somatostatin may change, directly or indirectly, the antibody affinity for monomeric α_s . In addition, these findings indicate that somatostatin not only induces reassociation of G_s but also reverses release of α_s subunits from the membrane.

In order to verify these observations in a manner independent of antibody GS-2, we utilized two other antisera directed against α_s , 708R, kindly provided by Dr. Allen M. Spiegel [9], and LR4, produced in our own laboratory. Membrane release of α_s was monitored by an immunoblot technique utilizing antisera 708R and LR4 and the 100 000 g supernatant of the incubation mixture. Stimulation of the membranes by isoprenaline and sequential addition of 10 μ M-somatostatin showed a pattern of hormone-induced membrane release of α_s , followed by a reversal (Fig. 4). This pattern was observed with both 708R and LR4 antisera, although the 708R antibody appears to be more effective at detecting the 52 kDa form of α_s in these cells. This agrees with our earlier observations regarding antibodies directed against amino acids 28-42 [7].

As a means to confirm the conclusions regarding membrane release of α_s independent of use of antibodies, we assessed the ability of supernatant fractions to reconstitute adenylate cyclase

Fig. 4. Redistribution of α_s between membranes and incubation buffer

Crude (not purified on a sucrose gradient) wild-type S49 cell membranes (120–140 μ g of protein) were incubated as in Fig. 1 in the absence (1) or the presence of 1 μ M-isoprenaline for 7 min (2) or 15 min (5) or with 5 μ M-somatostatin for 8 min (3). In some experiments, the membranes were incubated with isoprenaline (1 μ M) for 7 min before 5 μ M-somatostatin was added for 8 min (4). Incubation was terminated by a 2-fold dilution with cold TEN buffer. Samples were separated into membrane-associated (P) and supernatant (S) fractions. The different fractions were resolved by SDS/PAGE and immunoblot with 1/100 dilution of the partially purified antiserum LR4 (a) or antiserum 708R (b). The immunoblots for (a) and (b) are representative of results obtained in three separate experiments.

activity of membranes from *cyc*[−] S49 cells that lack α_s . As shown in Table 1, supernatant fractions prepared from membranes incubated with isoprenaline plus GTP had enhanced levels of α_s , and these levels were decreased in supernatant fractions prepared from membranes incubated with isoprenaline plus somatostatin. Thus functional α_s is released from wild-type S49 cells membranes by the β -agonist, and somatostatin can block this release.

The current findings obtained by using immunological and functional assessment of α_s thus provide evidence for G_s -subunit dissociation/reassociation and membrane release/association in target cells. This evidence, which is supported by other reports of release of α_s from the plasma membranes to an incubation mixture [8,23–25] or in intact cells to the cytoplasm [14], suggests that release of α_s from the membrane is a consequence of activation of G_s . Other studies have suggested that stimulation of adenylate cyclase is associated with membrane release of a small-molecular-mass protein recognized by anti- α_s antisera [26]. These results contrast with findings suggesting that ADP-ribosylation of α_s by cholera toxin fails to release α_s from the membrane [27].

Thus receptor-induced activation/deactivation of G_s involves a GDP/GTP exchange cycle, a subunit dissociation/reassociation cycle, and a membrane release/association mechanism. Observed levels of monomeric α_s following activation of G_s considerably exceed the expected concentrations of GTP-liganded α_s , based on rate constants obtained in experiments with purified G-proteins [11,28]. These previous studies indicated that only about 10–15% of α_s is liganded to GTP at steady state after receptor-promoted activation. Given this observation, our findings imply that monomeric α_s might exist in both a GDP- and a GTP-liganded state. The existence of active (i.e. GTP-liganded) and inactive (GDP-liganded) forms of monomeric α_s

Table 1. Membrane release of α_s as assessed by reconstitution into S49 *cyc*[−] cell membranes

Wild-type S49 cell membranes (43000 *g* fraction; 130 μ g of protein) were incubated for 15 min at 37 °C with 100 μ M-GTP with or without 1 μ M-isoprenaline in the absence or presence of 1 μ M-somatostatin. To stop the reaction, the samples were diluted with 90 μ l of ice-cold buffer and immediately spun at 150000 *g* at 2 °C for 1 h. The supernatant fractions and the pellets were then treated with 1% purified sodium cholate (final volume 130 μ l) for 1 h on ice. Samples (20 μ l) of supernatant and pellet fractions were mixed with 30 μ g of protein from S49 *cyc*[−] membrane (43000 *g*), and adenylate cyclase activity was assayed in a total volume of 100 μ l in the presence of 10 mM-NaF+20 μ M-AlCl₃ for 10 min at 37 °C. In ^a somatostatin (final concn. 66 nM) was added during the reconstitution in order to rule out a role of somatostatin carried over from the membrane incubation (at 66 nM final concn.) in the cyclase assay in producing the decrease in reconstitution of *cyc*[−] membranes. The data shown are means $\pm$ S.D. derived from three separate experiments. * $P \leq 0.05$ by paired *t* analysis for isoprenaline + GTP versus GTP, and for somatostatin + isoprenaline + GTP versus isoprenaline + GTP.

	Cyclic AMP (pmol/mg of <i>cyc</i> [−] protein)			
	Basal	Isoprenaline	Isoprenaline + somatostatin	Isoprenaline + somatostatin ^a
Supernatant	4 $\pm$ 0.3	25 $\pm$ 1.5*	6.5 $\pm$ 0.5*	24.1 $\pm$ 1.6*
Cholate extract	54 $\pm$ 6	36.5 $\pm$ 4*	52 $\pm$ 5.5*	37.5 $\pm$ 4.5*

argues that absolute total levels of dissociated α_s might not correlate precisely with the activity of adenylate cyclase. Even so, the concentration–response relationship for somatostatin-induced inhibition of G_s dissociation (51 $\pm$ 12 nM; Fig. 3a) is similar to that for the inhibition of isoprenaline-stimulated activity of adenylate cyclase (121 $\pm$ 20 nM; results not shown). Moreover, in other studies (results not shown), we have found that somatostatin treatment can block agonist-promoted release of α_s from membranes to cytosol of intact S49 cells.

In summary, we have shown that addition of $\beta\gamma$ subunits or the peptide hormone somatostatin interferes with β -adrenergic-agonist-stimulated G_s -subunit dissociation in S49 plasma membranes. In addition, somatostatin can block or reverse release of α_s from the membrane. We conclude that somatostatin, by providing $\beta\gamma$ subunits, inhibits dissociation and reverses membrane release of α_s , and this action of somatostatin (via G_i) is likely to contribute to inhibition of adenylate cyclase activity. On the basis of the similarity in kinetics for reassociation of G_s by exogenous $\beta\gamma$ and somatostatin, subunit dissociation would also appear to be an important facet of G_i activation as well. Our results do not rule out additional actions of dissociated α_i in G_i -mediated responses [1,4–6,29], but the findings strongly suggest that subunit dissociation and provision of $\beta\gamma$ subunits is likely to be more important than α_i for G_i action on adenylate cyclase activity.

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