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Somatostatin-Induced Stimulation of a High-Affinity GTPase in Membranes of S49 Lymphoma *cyc*$^-$ and H21a Variants

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SUMMARY

The influence of somatostatin was studied on GTPase activity in membranes of *cyc*$^-$ and H21a variants of S49 lymphoma cells, which are functionally defective in the guanine nucleotide site (N_s) mediating hormonal stimulation of the adenylate cyclase. Somatostatin, which inhibits adenylate cyclase in these membranes by a GTP-dependent process, caused a concomitant activation of a high-affinity GTPase (apparent $K_m \cong 0.2$ μM) by 40–50%. The hormone-stimulated GTPase also exhibited an apparent K_m value of about 0.2 μM. GTPase stimulation by somatostatin occurred without an apparent lag phase. There was a close correlation between adenylate cyclase inhibition and high-affinity GTPase stimulation induced by somatostatin. Various other peptide hormones studied and isoproterenol had no effect on GTP hydrolysis. Activation of the enzyme by somatostatin was reduced or abolished by pretreatment of the membranes with the SH reagent, *N*-ethylmaleimide. In membranes of wild-type S49 lymphoma cells, somatostatin caused an increase in GTPase activity similar to that in *cyc*$^-$ and H21a membranes. The data show that *cyc*$^-$ and H21a membranes, which are more or less defective in N_s, contain a hormone-sensitive, high-affinity GTPase and that the activation of this enzyme is closely related to adenylate cyclase inhibition by somatostatin. The data suggest that, similar to N_s, the activity state of the guanine nucleotide site (N_i), which apparently mediates somatostatin-induced inhibition of the adenylate cyclase, is controlled by a high-affinity GTPase.

INTRODUCTION

Stimulation of adenylate cyclase by hormones and neurotransmitters is apparently initiated by the replacement of GDP by GTP bound to the guanine nucleotide regulatory site, N_s, which subsequently causes activation of the catalytic moiety (1, 2). As shown for various cellular systems and hormonal factors, hormonal stimulation of adenylate cyclase is accompanied by an increase in GTP hydrolysis due to stimulation of a membrane-bound high-affinity GTPase (3–8). The hormone-stimulated GTP hydrolysis is largely reduced by stable GTP analogues (9) or cholera toxin (8, 10, 11), both of which can cause a persistent activation of the adenylate cyclase. On the basis of these findings, it has been suggested (10, 12) that the hydrolysis of N_s-bound GTP to GDP terminates hormonal stimulation of adenylate cyclase.

Similar to hormonal stimulation, inhibition of adenylate cyclase by hormones and neurotransmitters is a GTP-dependent process, and available evidence suggests that this inhibition is mediated by a distinct guanine nucleotide regulatory site, N_i (see refs. 13–16 for reviews). This evidence has been strongly corroborated by recent findings in *cyc*$^-$ variants of S49 lymphoma cells. The adenylate cyclase system of these variants is deficient in N_s (17), but the enzyme stimulated by forskolin or purified, preactivated N_s can be inhibited by stable GTP analogues (18, 19). Furthermore, the peptide hormone somatostatin decreases cyclic AMP levels and inhibits adenylate cyclase by a GTP-dependent process (20). Like hormonal stimulation, inhibition of adenylate cyclase by hormones is accompanied by an increase in GTP hydrolysis due to the stimulation of a membrane-bound high-affinity GTPase (21–25). In contrast to the N_s-associated GTPase stimulation, GTPase stimulation by hormones that inhibit adenylate cyclase is not affected by cholera toxin (26, 27), suggesting that two GTPases or two GTPase activation mechanisms are involved in hormonal regulation of adenylate cyclase activity. In order to prove this hypothesis, we attempted to determine whether somatostatin stimulates a GTPase in the N_s-deficient *cyc*$^-$ membranes. This question was additionally examined in membranes of H21a variants of S49 lymphoma cells, which are apparently only partially defective in N_s (28), and in membranes of wild-type cells. We report here that, similar to the situation in complete adenylate cyclase systems, inhibition of adenylate cyclase by a hor-

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mone in *cyc*⁻ and H21a membranes is accompanied by an increased GTP hydrolysis due to activation of a high-affinity GTPase.

EXPERIMENTAL PROCEDURES

Materials. ATP, AMP-P(NH)P,[1] and GTP were obtained from Boehringer Mannheim. Bacitracin, cholera toxin, isoproterenol, vasoactive intestinal peptide, angiotensin II, arginine vasopressin, and somatostatin were purchased from Sigma Chemical Company. Somatostatin was dissolved in 10 mM acetic acid containing bovine serum albumin (1 mg/ml), which solvent was also added to control tubes. Met-enkephalin was donated by Dr. B. Hamprecht, Würzburg, and forskolin by Dr. H. Metzger, Frankfurt. [α-^{32}P]ATP and [γ-^{32}P]GTP were prepared according to the method of Walseth and Johnson (29). All other materials were from previously described sources (23, 27).

Preparation of S49 lymphoma cell membranes. S49 Lymphoma wild-type cells and their *cyc*⁻ and H21a variants were grown in Dulbecco's modified Eagle's medium with 10% (v/v) horse serum. Cells were disintegrated by nitrogen cavitation in a medium containing 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4). The broken cells were supplemented with 3 mM EDTA and 15 mM β-mercaptoethanol and were centrifuged for 10 min at 200 × *g*. The supernatant fraction was recentrifuged for 10 min at 30,000 × *g*. The subsequent pellet fraction was resuspended in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) containing 0.1 mM EDTA and 5 mM β-mercaptoethanol, and was stored in small aliquots at −80°. After thawing, the membranes were pelleted again by centrifugation for 10 min at 30,000 × *g* and resuspended in the appropriate volumes of 10 mM triethanolamine HCl (pH 7.4) for GTPase and adenylate cyclase assays.

GTPase assay. GTPase activity was determined essentially as previously described (3). The reaction mixture contained, if not otherwise stated, [γ-^{32}P]GTP (about 0.1 μCi/tube) at the indicated concentrations, 3 mM $MgCl_2$, 0.1 mM ATP, 1 mM AMP-P(NH)P, 5 mM creatine phosphate as its tris(hydroxymethyl)aminomethane salt, creatine kinase (0.4 mg/ml), 1 mM 3-isobutyl-1-methylxanthine, 1 mM EDTA, bacitracin (1 mg/ml), bovine serum albumin (2 mg/ml) and 50 mM triethanolamine HCl (pH 7.4) in a final volume of 100 μl. Reactions were started by the addition of membrane preparations (5–10 μg of protein per tube) to the prewarmed reaction mixture and were conducted for 10 min at 25°. Reactions were terminated by the addition of 800 μl of ice-cold sodium phosphate buffer (20 mM, pH 2.0) containing 5% (w/v) activated charcoal. After centrifugation for 20 min at 10,000 × *g*, the radioactivity was determined in 500 μl of the supernatant fraction by measuring Cerenkov radiation. Release of $^{32}P_i$ in the absence of membranes was about 0.5% of added [γ-^{32}P]GTP.

Adenylate cyclase assay. Adenylate cyclase activity was determined in *cyc*⁻ and H21a membranes (10–50 μg of protein per tube) with essentially the same reaction mixture as used for the GTPase assay, with the exceptions that AMP-P(NH)P was omitted and that, instead of labeled GTP, [α-^{32}P]ATP (about 0.4 μCi/tube) was present. When adenylate cyclase and GTPase activities were studied in parallel experiments, 0.1 mM cyclic AMP was additionally present in either reaction mixture. The incubations were carried out for 10 min at 25°. Termination of reactions and isolation of cyclic AMP formed were carried out as described (30).

Protein was determined according to the method of Lowry *et al.* (31), with human serum albumin as standard. The experiments were performed in triplicate, with an intra-assay variation of less than 5% of the means; they were repeated at least twice with similar results as shown herein.

RESULTS

Similar to findings in various other membrane preparations (3–6, 21–23), GTP hydrolysis in *cyc*⁻ membranes were catalyzed by at least two GTPases, characterized by high and low affinities for the substrate, GTP (Fig. 1). The low-affinity GTPase exhibited an apparent K_m value of more than 50 μM GTP, and its activity was not affected by somatostatin (data not shown). The activity of the high-affinity GTPase was estimated as described by Cassel and Selinger (3) by subtracting the amount of [γ-^{32}P] GTP hydrolysis measured in the presence of 30 μM unlabeled GTP from the [γ-^{32}P]GTP hydrolysis measured at low GTP concentrations. The apparent K_m value of the high-affinity GTPase was approximately 0.2 μM (Fig. 2). Somatostatin (1 μM) increased the activity of the high-affinity GTPase by 40–50%. The hormone-stimulated GTPase also exhibited an apparent K_m value of about 0.2 μM. As shown in Fig. 3, GTP hydrolysis catalyzed by the high-affinity GTPase in *cyc*⁻ membranes was linear as a function of incubation time for up to 15 min at 25°. Furthermore, the somatostatin-induced stimulation of GTP hydrolysis occurred without an apparent lag phase.

In the presence of 50 μM forskolin and 0.3 μM GTP, somatostatin (1 μM) decreased *cyc*⁻ adenylate cyclase activity by about 20% (Fig. 4), as observed before (20). Under almost identical conditions, with the exception that AMP-P(NH)P (1 mM) was additionally present, the influence of somatostatin was studied on GTPase activity. Somatostatin caused a concentration-dependent increase in the high-affinity GTPase activity. Half-maximal and maximal activations were observed at about 10 and 100 nM somatostatin, respectively.

H21a Variants of S49 lymphoma cells and H21a membranes, like those of *cyc*⁻ are unresponsive to stimulation of cyclic AMP synthesis by hormones, stable GTP analogues, and cholera toxin (28). In contrast to *cyc*⁻ but like wild-type membranes, GTP modulates agonist binding to beta-adrenoceptors, and cholera toxin specifically catalyzes ADP ribosylation of H21a membrane proteins (32); these findings suggest that H21a membranes contain a partially functional N_s. GTP hydrolysis in H21a membranes was also catalyzed by high- and low-affinity GTPases. As observed in *cyc*⁻ membranes, somatostatin

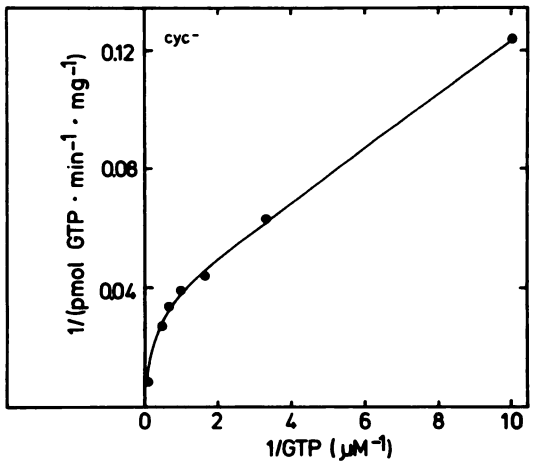

FIG. 1. *Lineweaver-Burk plot of GTP hydrolysis in cyc⁻ membranes*

GTP hydrolysis was studied in *cyc*⁻ membranes at various concentrations of GTP as described under Experimental Procedures. The reciprocal values of total GTPase activity are given.

[1] The abbreviation used is: AMP-P(NH)P, adenyl 5′-yl-imidodiphosphate.

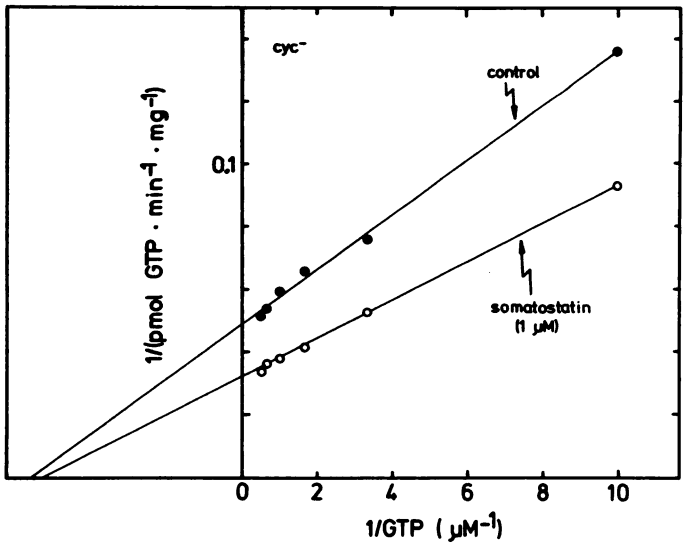

FIG. 2. *Lineweaver-Burk plot of control and somatostatin-stimulated high-affinity GTPase in cyc⁻ membranes*

GTP hydrolysis was determined in *cyc*⁻ membranes at various concentrations of GTP without and with 1 μM somatostatin. High-affinity GTPase activity was estimated as described (3) by subtracting the amount of GTP hydrolyzed in the presence of 30 μM GTP from the total amount of [γ-^{32}P]GTP hydrolyzed at the indicated concentrations of GTP.

(1 μM) increased the activity of the high-affinity GTPase by 40%–50% (Fig. 5). Half-maximal activities of the basal and hormone-stimulated GTPase were observed at about 0.3 μM GTP. Somatostatin-stimulated GTP hydrolysis in H21a membranes occurred without an apparent lag phase and was linear with time for up to 15 min of incubation (see Fig. 3).

In the presence of forskolin (50 μM) and GTP (0.6 μM), somatostatin caused a concentration-dependent inhibition of H21a adenylate cyclase by maximally 25% (Fig. 6). Stimulation of the high-affinity GTPase was observed at very similar concentrations of the hormone. Half-maximal effects on both enzymes were observed at about 10 nM somatostatin. In contrast to the effect of somatostatin on the high-affinity GTPase activity, other peptide hormones such as angiotensin II, arginine vasopressin, ad-

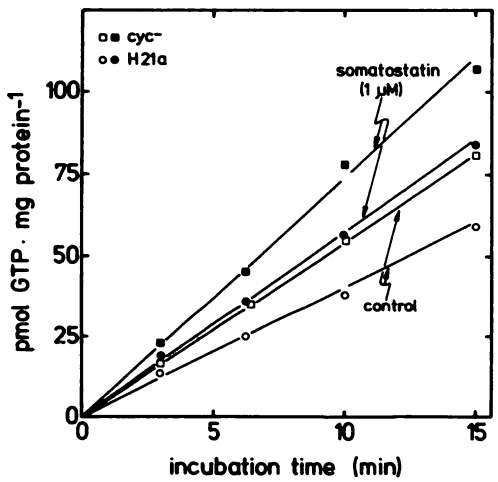

FIG. 3. *Time course of control and somatostatin-stimulated GTP hydrolysis in cyc⁻ and H21a membranes*

GTP hydrolysis was studied in *cyc*⁻ and H21a membranes in the absence and presence of 1 μM somatostatin as indicated. Given is the GTP hydrolysis by the high-affinity GTPase measured at 0.3 μM GTP.

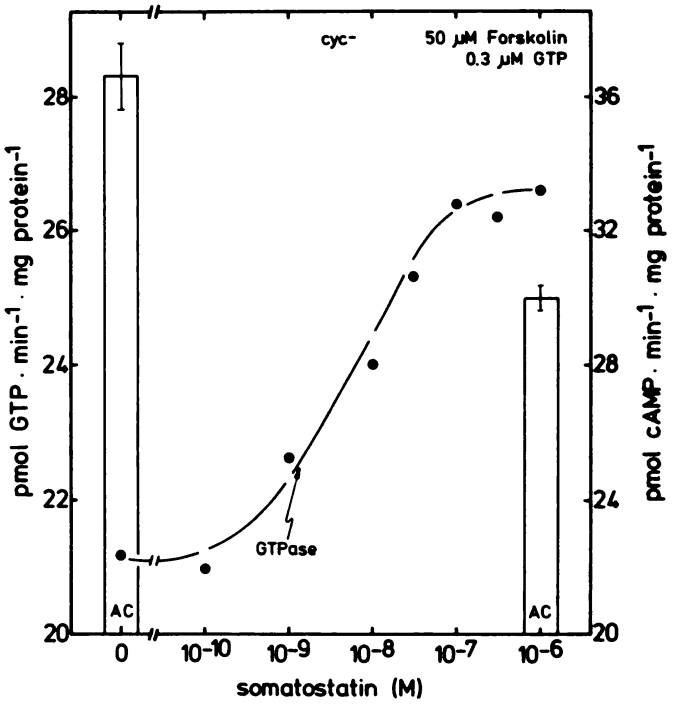

FIG. 4. *Influence of somatostatin on high-affinity GTPase and adenylate cyclase in cyc⁻ membranes*

High-affinity GTPase activity, indicated on the left ordinate, was determined at increasing concentrations of somatostatin. Adenylate cyclase (*AC*) activity, indicated on the right ordinate, was determined in the absence and presence of 1 μM somatostatin and is given in the form of *bars* as means ± standard error of the mean ($n = 3$). Incubation was carried out for 15 min with 0.3 μM GTP and 50 μM forskolin.

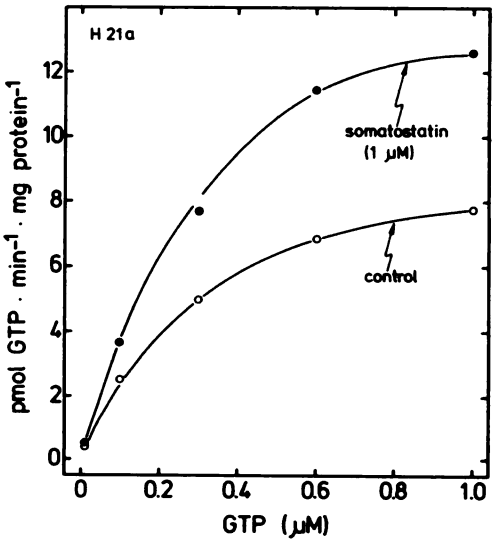

FIG. 5. *Influence of somatostatin on high-affinity GTPase in H21a membranes*

High-affinity GTPase activity was determined in H21a membranes at the indicated concentrations of GTP in the absence and presence of 1 μM somatostatin. The activity of the high-affinity GTPase was estimated as described in the legend to Fig. 1. The concentration of AMP-P(NH)P was 3 mM.

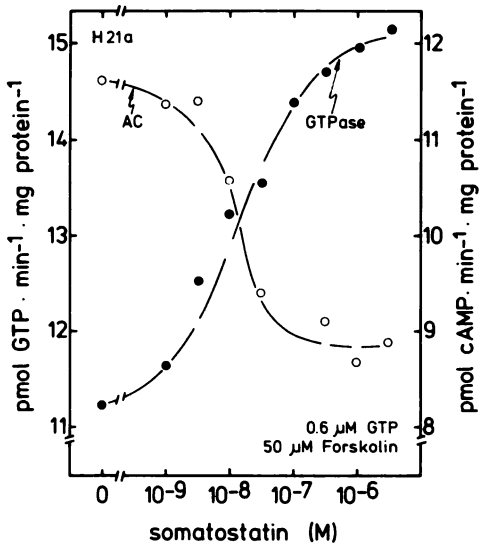

FIG. 6. *Influence of somatostatin on high-affinity GTPase and adenylate cyclase in H21a membranes*

High-affinity GTPase activity, indicated on the left ordinate, and adenylate cyclase (*AC*) activity, indicated on the right ordinate, were determined in H21a membranes at increasing concentrations of somatostatin. Incubation was carried out for 15 min with 0.6 μM GTP, 50 μM forskolin, and 3 mM AMP-P(NH)P.

renocorticotropic hormone, vasoactive intestinal peptide, and met-enkephalin (each 1 μM) had no significant effect on the enzyme activity (Fig. 7). Furthermore, under the assay conditions used, isoproterenol (100 μM), which stimulates adenylate cyclase in S49 lymphoma wild-type membranes but not in H21a membranes (28), was also without effect on the H21a GTPase activity.

When H21a membranes were pretreated with cholera toxin (100 μg/ml, in the presence of 2 mM NAD) for 10 min at 30°, there was no significant reduction of the somatostatin-induced GTPase stimulation (data not shown). However, pretreatment of the membranes for 10 min at 30° with the SH reagent, *N*-ethylmaleimide, impaired the GTPase stimulation induced by somatostatin (1 μM) in a concentration-dependent manner (Fig. 8). After treatment with 300 μM *N*-ethylmaleimide, the stimulatory effect of somatostatin was completely abolished and control GTPase activity was slightly reduced.

For comparison, the effect of somatostatin was studied on GTP hydrolysis in membranes of wild-type S49 lymphoma cells. As shown in Table 1, somatostatin (1 μM) increased total GTPase activity measured at 0.5 μM GTP by 40–50%. In the presence of 30 μM unlabeled GTP, somatostatin had no effect on GTP hydrolysis, as was also observed in *cyc*⁻ and H21a membranes.

DISCUSSION

The data presented show that the hydrolysis of GTP in membranes of *cyc*⁻ and H21a variants of S49 lymphoma cells was due to at least two GTPase activities: a low-affinity GTPase (apparent $K_m \geqq 50$ μM) and a GTPase with high affinity for GTP (apparent $K_m \cong 0.2$ μM). As described for various other membrane systems (3–6, 21–23), only the high-affinity GTPase was hormone-sensitive. Somatostatin, which has been shown to inhibit the *cyc*⁻ adenylate cyclase by a GTP-dependent process (20), increased the activity of the high-affinity GTPase in *cyc*⁻ and H21a membranes by maximally 40–50%. The hormone-stimulated GTPase exhibited an affinity for

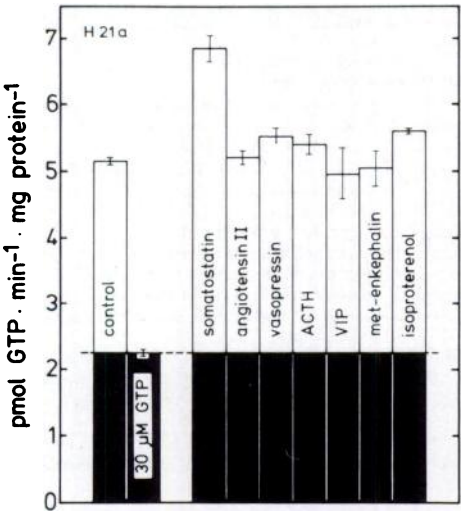

FIG. 7. *Influence of various hormonal factors on GTP hydrolysis in H21a membranes*

GTP hydrolysis was determined in H21a membranes with 0.3 μM [γ-^{32}P]GTP in the absence (control) and presence of somatostatin, angiotensin II, arginine vasopressin, adrenocorticotropic hormone (*ACTH*), vasoactive intestinal peptide (*VIP*), met-enkephalin (1 μM each), or isoproterenol (100 μM) as indicated. Black portions of the bars indicate GTP hydrolysis due to low-affinity GTPase as measured in the presence of 30 μM GTP. The low-affinity GTPase was not affected by any of the factors studied. Values are given as means ± standard error of the mean ($n = 3$).

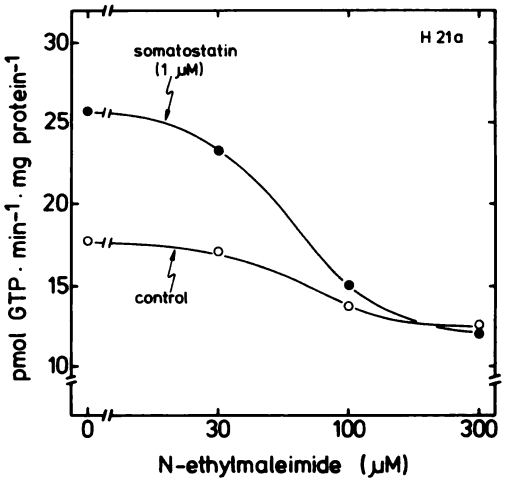

FIG. 8. *Influence of N-ethylmaleimide on stimulation of GTP hydrolysis by somatostatin in H21a membranes*

H21a membranes (1 mg/ml) were pretreated without and with *N*-ethylmaleimide at the indicated concentrations for 10 min at 30° in a reaction mixture containing 50 μM ATP, 3 μM GTP, 2 mM $MgCl_2$, 1 mM EDTA, 5 mM creatine phosphate, creatine kinase (0.4 mg/ml), bovine serum albumin (2 mg/ml), and 50 mM triethanolamine HCl (pH 7.4) in a final volume of 1 ml. The reaction was stopped by the addition of 100 μl of 30 mM dithiothreitol. The membranes were then pelleted by centrifugation for 5 min at 10,000 × *g*, resuspended in 10 mM triethanolamine HCl (pH 7.4), and immediately used for GTPase assay. Total GTPase activity was determined for 10 min at 30° with 0.5 μM GTP, 50 μM forskolin, and 1 mM cyclic AMP in the absence and presence of 1 μM somatostatin.

TABLE 1

Influence of somatostatin on GTP hydrolysis in membranes of wild-type S49 lymphoma cells

GTP hydrolysis was studied in wild-type membranes with either 0.5 or 30.5 μM [γ-^{32}P]GTP in the absence and presence of 1 μM somatostatin. Forskolin (20 μM) was present under each condition. Values are means ± standard error of the mean of two separate experiments performed in triplicate.

	0.5 μM GTP	30.5 μM GTP
	pmoles GTP hydrolyzed·min^{-1}*·mg protein*$^{-1}$	
Control	20.2 ± 0.3	695 ± 6
Somatostatin	29.0 ± 0.2	695 ± 12

GTP similar to that of the basal unstimulated enzyme. This finding suggests that the basal and the hormone-stimulated high-affinity GTPase represent a single catalytic activity, although it cannot be excluded that the activity stimulated by somatostatin is only a part of the total "basal" GTPase activity. The GTPase stimulation was specific for somatostatin, since various other peptide hormones had no effect. Furthermore, isoproterenol, which stimulates adenylate cyclase in S49 lymphoma wild-type but not in *cyc*⁻ and H21a membranes (17, 28), did not cause an increase in GTP hydrolysis. There was a close correlation between the somatostatin-induced activation of the high-affinity GTPase and the adenylate cyclase inhibition induced by this hormone, both with regard to the concentration-response curves of somatostatin and the concentrations of GTP required for half-maximal GTPase activity (0.2 μM) and half-maximal somatostatin-induced adenylate cyclase inhibition (0.1 μM).

Recent studies in adipocyte and platelet membranes (21, 23, 26, 27, 33) suggest that two distinct GTPases or two distinct activation mechanisms of one GTPase are involved in the stimulation of GTP hydrolysis by adenylate cyclase stimulatory and inhibitory hormones. This assumption is based on the following findings. First, maximal stimulations of GTP hydrolysis by hormonal factors affecting adenylate cyclase activity in an opposite manner were additive, whereas the combination of different adenylate cyclase-inhibiting hormones was not additive with regard to GTPase activity. Second, treatment of intact cells or membranes with the alkylating agent *N*-ethylmaleimide reduced or abolished GTPase stimulation by adenylate cyclase-inhibiting hormones, whereas the N_s-dependent GTPase stimulation was largely insensitive to this treatment (3, 8, 26). Finally, cholera toxin treatment inhibited GTPase stimulation by hormones that stimulate adenylate cyclase (8, 10, 11), whereas the toxin had no effect on the high-affinity GTPase activation by hormones that inhibit adenylate cyclase. The data presented herein that somatostatin stimulates a high-affinity GTPase in S49 lymphoma *cyc*⁻ and H21a variant membranes, which are more or less defective in N_s, corroborate the hypothesis of two GTPases or of two activation mechanisms of one GTPase. In agreement with the above-mentioned findings, the somatostatin-induced GTPase activation was not affected by cholera toxin. Furthermore, after pretreatment with *N*-ethylmaleimide, GTPase stimulation by somatostatin was reduced or abolished, although it cannot be excluded that the SH reagent may have inactivated the somatostatin receptor. That the somatostatin-induced GTPase stimulation in *cyc*⁻ and H21a membranes was not due to an "abnormal" N_s in these cells is indicated by the finding that somatostatin also increased GTP hydrolysis in membranes of wild-type S49 lymphoma cells.

The data reported herein show that in the N_s-defective *cyc*⁻ and H21a membranes a hormone-sensitive GTPase is present and that the activity of this enzyme is closely related to the adenylate cyclase inhibition induced by the hormone. In analogy to the GTPase cycle postulated by Cassel *et al.* (12) for adenylate cyclase stimulation via N_s, it is thus feasible that the activity state of N_i, the guanine nucleotide site apparently mediating adenylate cyclase inhibition (13–16), is also controlled by a high-affinity GTPase. It is presently not clear whether there are two GTPases, one N_s-associated and one N_i-associated, or whether there is only one GTPase, activated by different mechanisms and controlling the activity states of both N_s and N_i.

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