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VIP Induces the Translocation and Degradation of the α Subunit of G_s Protein in Rat Pituitary GH_4C_1 Cells

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It has been shown that G proteins are potential regulatory molecules in the transmembrane signaling cascade. The aim of this study was to examine the possibility of equivalent G-protein redistribution and/or down-regulation in a target cell upon agonist stimulation. Short-term (0-80 min) incubation of rat pituitary GH_4C_1 cells with vasoactive intestinal peptide (VIP, 0.1 μM) induced a decrease in the levels of $G_s\alpha$ in the membrane fraction, whereas immunoblot analysis and reconstitution assay of adenylyl cyclase clearly showed an increase in the amount of $G_s\alpha$ in the supernatant (cytosolic) fraction. The VIP-induced release of G proteins α subunits from membranes was specific for $G_s\alpha$. The VIP-dependent release of $G_s\alpha$ from membranes was blocked by a VIP-receptor antagonist, (N-Ac-Tyr, D-Phe)-GRF(1-29)-NH_2 (10 μM). Pituitary adenylate cyclase-activating polypeptide (PACAP) also stimulated the release of $G_s\alpha$ from membranes of GH_4C_1 cells. Furthermore, prolonged exposure of cells to VIP (0.1 μM) for 2-24 h caused a 21-40% decrease in $G_s\alpha$ from membranes and a 6% increase in total $G_s\alpha$ in the cytosolic fraction. The effect of VIP was dose-dependent with ED_{50} values of 81.6±20.0 nM for down-regulation and 2.5±0.3 nM for translocation of $G_s\alpha$. Concurrent treatment of GH_4C_1 cells with VIP and cycloheximide indicated that suppression of protein synthesis *de novo* did not mimic the effect of VIP. Moreover, the chase experiment of ^{35}S-labeled $G_s\alpha$ clearly demonstrated a more rapid rate of decay in the cells maintained in the presence of the agonist. These data indicate that VIP-receptor activates $G_s\alpha$ protein and induces the release of $G_s\alpha$ from membranes along with its down-regulation in cellular levels.

Key words: α subunit, down-regulation, stimulatory G protein (G_s), translocation, VIP.

The heterotrimeric ($\alpha\beta\gamma$) G proteins are involved in transmembrane signaling and mediation between the activated transmembranous receptors and submembranous effectors that generate subsequent intracellular signals (*1*). Of the three G protein subunits, β and γ remain tightly associated as a complex under non-denaturing conditions, while the α subunit dissociates from the $\beta\gamma$ complex following stimulation of the receptor, which induces the replacement of bound GDP by GTP on the α subunit and the subsequent activation of the effector system. G proteins locate on the cytoplasmic face of plasma membranes and their subunits behave essentially as integral membrane proteins. However, numerous studies suggest important functions for G proteins at other cellular locations in addition to the plasma membrane. Certain G proteins have been detached from intracellular membranes, such as the Golgi complex, and have been implicated in intracellular vesicle trafficking (*2*), whereas others associate with cytoskeletal structures, such as microtubules (*3*) and actin (*4*).

There are several reports, including ours, that the activation of G protein-linked receptors by agonists promotes the translocation of α subunits from plasma membranes to the cytosol where they regulate effectors (*5-8*). On the other hand, it has been reported that chronic exposure of cells to agonists that act on G protein-linked receptors can lead to alterations in cellular levels of G proteins as well as to down-regulation of the receptors (*9-11*). Therefore, we hypothesize that agonist activation of G protein-linked receptors results in the simultaneous translocation and down regulation of α subunits. However, evidence for both the translocation and down-regulation of the α subunit upon hormonal stimulation in intact cells is scarce. In the present experiment, we study the effect of sustained exposure of rat pituitary GH_4C_1 cells to vasoactive intestinal polypeptide (VIP) on cellular distribution and levels of G-proteins. Here we show that the translocation of the α subunit of the stimulatory G protein ($G_s\alpha$) from the membrane to the cytosolic fraction occurs upon

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Abbreviations: G proteins, guanine nucleotide-binding regulatory proteins; G_s and G_i, the G proteins that stimulate and inhibit adenylyl cyclase, respectively; G_o, the predominant brain G protein; $G_s\alpha$, $G_i\alpha$, and $G_o\alpha$, the α subunits of G_s, G_i, and G_o, respectively; $\alpha_{s\text{-}s}$, the short form of $G_s\alpha$; $\alpha_{s\text{-}l}$, the long form of $G_s\alpha$; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating peptide; GTPγS, guanine 5′-*O*-(γ-thio)triphosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

stimulation by VIP and PACAP, whose receptors are both coupled to G proteins (G_s), to transduce the signals for adenylyl cyclase and stimulate the secretion of growth hormone and prolactin in GH cell lines (*12, 13*). In addition, we have found that long-term treatment (24 h) of GH_4C_1 cells with VIP results in an apparent loss of $G_s\alpha$, suggesting its down-regulation.

MATERIALS AND METHODS

Cell Culture and Hormonal Treatment—GH_4C_1 cells were grown in monolayer culture in Ham's F-10 medium supplemented with heat-inactivated 15% horse serum and 2.5% fetal calf serum. Incubations of GH_4C_1 cells with VIP were carried out in serum-free Ham's F-10 medium supplemented with 5% lactalbumin hydrolysate (F-10-lh) to prevent degradation of the peptide by serum proteases, and the reactions were terminated by aspirating the medium. The cells were then washed twice with phosphate-buffered saline (PBS), harvested by centrifugation, suspended in 2 volumes of ice-cold 20 mM Tris-HCl, pH 7.5, containing 1 mM $MgCl_2$, 2 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, and 0.1 mM benzamidine, and homogenized in a Dounce glass homogenizer with 15 strokes of pestle B. After removal of the undisrupted cells and nuclei by 5-min centrifugation at $100\times g$ at 4°C, the supernatant was further centrifuged at $274,000\times g$ for 30 min at 4°C to obtain the membrane-rich fraction. The pellet (particulate fraction) was resuspended in homogenization buffer, and the suspended particulate and supernatant (cytosolic fraction) were each centrifuged again at $274,000\times g$ for 30 min to avoid cross-contamination. The cytosolic and particulate fractions were used for the assay of G_s activity. Proteins in the cytosolic fraction were precipitated by the addition of deoxycholate/TCA (0.0125% deoxycholate/6% trichloroacetic acid, w/v) for immunoblot experiments. S49 cyc^- lymphoma cells were kindly supplied by Dr. S. Nagata (Osaka Bioscience Institute, Osaka). The cells were grown in RPM1640 medium containing 10% fetal calf serum and maintained at densities between 2×10^5 and 1×10^6 cells/ml. Membranes were prepared as described (*6*).

Immunoblot Analysis—Membrane fractions and the cytosolic fractions were subjected to SDS-polyacrylamide (10%) gel electrophoresis, then electrophoretically transferred to nitrocellulose membranes. The membranes were blocked for 1 h with 5% dried skim-milk in Tris-buffered saline (TBS consisting of 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20), then washed 4 times in TBS. After washing, the nitrocellulose membranes were incubated overnight with the primary antiserum (RM/1 diluted 1:200 in TBS), which recognizes two distinct forms of $G_s\alpha$, a long form ($\alpha_{s\text{-}l}$) and a short form ($\alpha_{s\text{-}s}$). In GH_4C_1 cells, $\alpha_{s\text{-}l}$ (52 kDa) is the major form of the $G_s\alpha$ protein seen on Western blots. The membranes were washed again as described above, incubated with the second antibody (goat anti-rabbit IgG coupled to alkaline phosphatase, diluted 1:2,000 in TBS) for 1 h, subjected to the same series of washes, and finally developed with a Vectastain Kit II (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's instructions. The blots were analyzed using an ACI Imagepro computer-assisted video image analysis system (ACI Japan, Kanagawa). It was confirmed that the labeling intensity of each band was directly proportional to the amount of membrane proteins (from 0.5 to 3.0 μg) or cytosolic protein (from 1 to 10 μg) for RM/1 antiserum in the assay (data not shown).

Assay for G_s Activity—G_s activities in the membrane and cytosolic fractions from VIP-treated GH_4C_1 cells were assessed for their ability to complement the $G_s\alpha$-deficiency of S49 mouse lymphoma cyc^- membranes as described (*6*). The membranes derived from VIP-treated cells were extracted with sodium cholate (1%, w/v) for 1 h on ice, centrifuged at $274,000\times g$ for 30 min, and the supernatant was taken as the detergent extract. The supernatant fraction (30 μl) or detergent extract (20 μl, 2 μg) was added to cyc^- membranes (60 μg) and incubated for 20 min at 30°C in a total volume of 100 μl of 50 mM HEPES-NaOH, pH 8.0, containing 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM 3-isobutyl-1-methylxanthine, 1 mM ATP, and 1 μM GTPγS; GTPγS was added to obtain full adenylyl cyclase activity in the reconstitution assay. The reaction was terminated by the addition of 49 μl of 1 N HCl. cAMP formation was measured by radioimmunoassay in the Yamasa cAMP [^{125}I] assay system.

Turnover Studies—For labeling of cellular proteins with [^{35}S], cells were trypsinized from the flasks and seeded in 60-mm-diam culture plates following the methods of Mitchell *et al.* (*10*). At about 60% confluency, the culture medium was then replaced with Ham's F-10 lacking methionine and cysteine, and cells were incubated in this medium supplemented with 5% dialyzed fetal calf serum for 2 h. This medium was then replaced with 5 ml of Ham's F10 supplemented with 5% dialyzed fetal calf serum and containing 5% of the normal methionine and cysteine concentration plus 30-50 μCi/ml of [^{35}S]methionine/[^{35}S]cysteine (EXPRESS™, NEN, 1,175 Ci/mmol). After the labeling period (between 20 and 40 h), the labeling medium was removed and cells were subsequently maintained in the standard F-10-lh medium in the presence or absence of VIP. At appropriate times, cells were harvested and a homogenized in 10 mM Tris/HCl/0.1 mM EDTA/1 mM $MgCl_2$, pH 7.5, using a hand Teflon-on-glass homogenizer. The crude membrane fractions thus prepared were resuspended in 1% SDS, heated to 100°C for 5 min to denature proteins, then diluted to 0.1% SDS with RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 8 μg/ml PMSF, 2 μg/ml each of leupeptin, and aporotinin). The mixture was centrifuged at 15,000 rpm in an Eppendorf centrifuge (Beckman Instruments, Westbury, NY) for 10 min, and the clarified supernatant was used as membrane lysate. In some experiments, membrane lysate was precleared by incubation with Pansorbin at 4°C for 1 h, and the supernatant (10-40 μg of protein, 100 μl) was subjected to immunoprecipitation by addition of the specific G-protein antiserum, RM/1 (15 μl at 1:30 dilution) and incubated at 4°C overnight on a rotator. Forty microliters of 25% (v/v) Protein A-Sepharose suspension in 0.9% NaCl was added, and the mixture was incubated for 2 h at 4°C. Immune complexes were then recovered by centrifugation (7,000 rpm, 2 min at 4°C) and washed by resuspension-centrifugation three times each with RIPA buffer and once with 0.1% NP-40, 10 mM Tris-HCl, pH 7.4. The antigen and antibody were eluted with the SDS/PAGE sample buffer and analyzed by 10% SDS/PAGE. Following electrophoresis, gels were stained with

0.1% Coomassie Blue, dried, and exposed to phosphor storage-screen autoradiography according to the manufacturer's instructions using a Fujix Bio-imaging Analyzer.

Additional Procedures—The protein contents of the membrane and cytosolic fractions were determined by use of a protein assay kit (Bio-Rad Laboratory) with a bovine serum albumin (BSA) as the standard. RM/1, AS/7, and QL antisera for $G_s\alpha$, $G_{i2}\alpha$ and $G_{q/11}\alpha$, respectively, were obtained from New England Nuclear (Boston, MA, USA), and antiserum for $G_o\alpha$ was kindly gifted by Dr. T. Asano (Aichi Human Service Center, Aichi). VIP, a VIP antagonist (N-Ac-Tyr,D-Phe)-GRF(1-29)-NH_2, and PACAP were from Peptide Institute (Osaka). The standard of $G_s\alpha$ was purchased from Calbiochem. (California, USA). The results presented represent three or more experiments performed under identical conditions. Statistical comparisons were performed by Student's *t*-test.

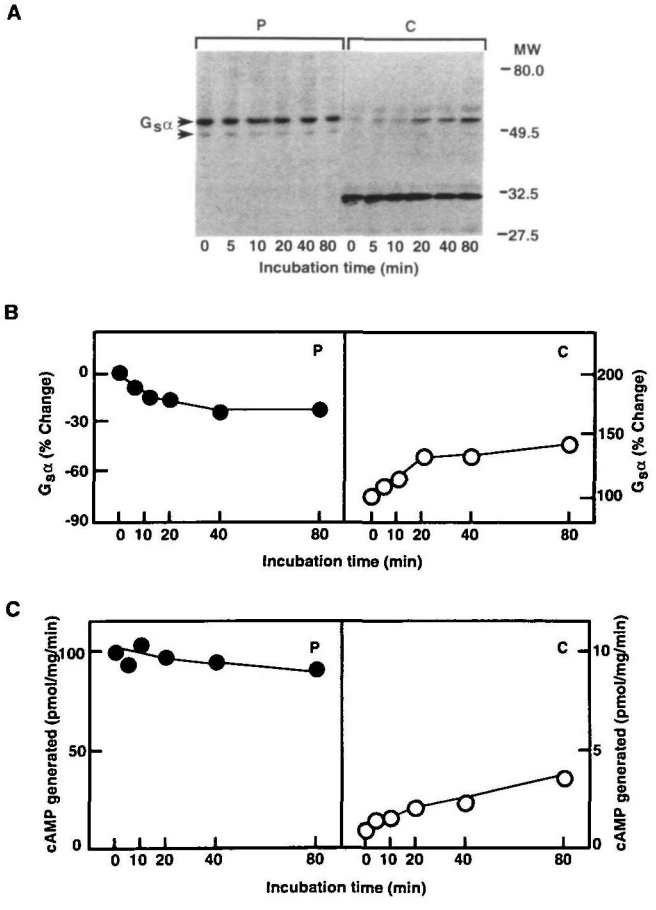

Fig. 1. **Effects of VIP on the translocation of $G_s\alpha$ from the membrane to the cytosol in GH_4C_1 cells.** Panel A: Cells were incubated at 37°C in the presence of 0.1 μM VIP for the times indicated and separated into membrane (particulate, P) and cytosolic (C) fractions by centrifugation as described in "MATERIALS AND METHODS." The particulate fraction (2.0 μg proteins from 1×10^5 cells) and cytosolic fraction (10 μg proteins from 7.1×10^5 cells) were subjected to SDS-PAGE and immunoblotted with RM/1 as the first antibody. Panel B: Densitometric tracings of the results in Panel A. Panel C: The supernatant and membrane fractions were treated with 1% sodium cholate in a final volume of 130 μl for 1 h on ice. Detergent extracts (20 μl) were mixed with S49 cyc$^-$ membranes (60 μg proteins) and adenylyl cyclase activity was assayed in a total volume of 100 μl in the presence of 1 μM GTPγS for 30 min at 37°C. The graphs are representative of three experiments with comparable results. Each point is the average of duplicate determinations.

RESULTS

Effect of VIP on the Translocation of $G_s\alpha$ in GH_4C_1 Cells—GH_4C_1 cells were incubated with VIP for various periods, after which the reactions were terminated by aspirating the medium. The levels of $G_s\alpha$ in the crude membranes decreased during the first 10 min of incubation with VIP (0.1 μM) and continued to decrease gradually to $75.9\pm7.3\%$ ($n=4$, $p<0.05$) of control values at 80 min (Fig. 1, A and B). In contrast, the increase in α subunits in the cytosolic fraction began after 5 min of incubation and continued through 80 min (Fig. 1A). Although the 30 kDa protein detected in the cytosolic fraction by the anti-$G_s\alpha$ antibody RM/1 decreased in a time-dependent manner, the relationship between hormonal treatment and the appearance of the 30 kDa protein is not clear. In parallel with the release of α subunits into the cytosolic fraction, cytosol gained the ability to reconstitute adenylyl cyclase activity in the membranes derived from $G_s\alpha$-deficient S49 cyc$^-$ cells (Fig. 1C). However, no significant appearance of $G_{i2}\alpha$, $G_o\alpha$, and $G_{q/11}\alpha$ subunits was revealed by immunoblotting of the cytosolic protein sample of VIP-treated GH_4C_1 cells compared to that of the untreated cells (Fig. 2). In a typical example, as displayed in Fig. 3, sustained challenge of the GH_4C_1 cells with VIP (0.1 μM, 80 min) resulted in the appearance of cytosolic immunodetectable $G_s\alpha$ at a levels of to 4.8 μg/mg cytosolic proteins, as estimated by comparison with known levels of $G_s\alpha$ (Calbiochem) following immunoblotting with antiserum RM/1 (Fig. 3). On the other hand, membrane-associated $G_s\alpha$ decreased from 26.6 to 18.0 μg/mg membrane proteins. Another receptor agonist, PACAP, a member of the secretin-VIP family, which elevates the cAMP content of GH_4C_1 cells through type III-PACAP/VIP receptor (*14*), was tested for the release of $G_s\alpha$ from membranes of GH_4C_1 cells. PACAP (0.1 μM) also induced the release of $G_s\alpha$ from GH_4C_1 cell

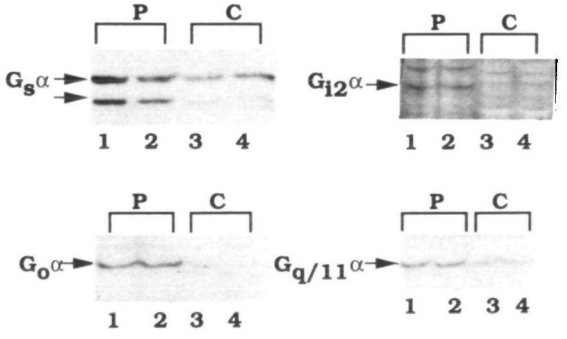

Fig. 2. **Specificity of the effect of VIP for $G_s\alpha$ translocation in GH_4C_1 cells.** GH_4C_1 cells were lysed by suspending in hypotonic buffer after incubation in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 0.1 μM VIP for 80 min. In all cases, cellular proteins were separated into particulate (P) and cytosolic (C) fractions as described under "MATERIALS AND METHODS." Equal volumes of membrane and cytosolic fractions (3.5×10^5 cells for $G_s\alpha$, 10×10^5 cells for $G_{i2}\alpha$, 15×10^5 cells for $G_o\alpha$, and 7.5×10^5 cells for $G_{q/11}\alpha$) were resolved by SDS-PAGE (10%), and representative immunoblots with anti-$G_s\alpha$, anti-$G_{i2}\alpha$, anti-$G_o\alpha$, and anti-$G_{q/11}\alpha$ antibodies were shown.

membranes to the cytosolic fraction, where it was able to stimulate adenylyl cyclase activity in cyc$^-$ membranes (Fig. 4). The VIP-induced increases in $G_s\alpha$ in the cytosol were blocked by the prior addition of a VIP-antagonist (N-Ac-Tyr,D-Phe)-GRF(1-29)-NH_2 (10 μM) (Fig. 4) (*15*).

Effect of VIP on the Down-Regulation of $G_s\alpha$ in GH_4C_1 Cells—Immunoblotting assay showed that prolonged exposure of GH_4C_1 cells to VIP caused a marked 40.0±3.4% ($n=4$, $p<0.01$) decrease in the detectable levels of $G_s\alpha$ in membranes with a continuous increase in the level of cytosolic $G_s\alpha$ (Fig. 5). Analysis of the time course of 0.1 μM VIP-mediated changes in $G_s\alpha$ demonstrated a maximal reduction in membrane levels of $G_s\alpha$ after 8 h (Fig. 5). Densitometric determination showed that the cytosol fraction of unstimulated cells contained 2.7% of the total cellular $G_s\alpha$, while 6.7% was found in the cytosol fraction of VIP-stimulated cells, a 2.5-fold increase. These results indicate that VIP induced the translocation of 4.0% of total cellular $G_s\alpha$ from the membrane to the cytosol. However, since 40% of membrane-bound $G_s\alpha$ (39% of total cellular $G_s\alpha$) was lost from membranes as described above, the remaining 35% of the total $G_s\alpha$ disappeared, namely, was degraded. The amount of $G_s\alpha$ down-regulated is slightly less than the 50% reduction caused by exposure of NG108-

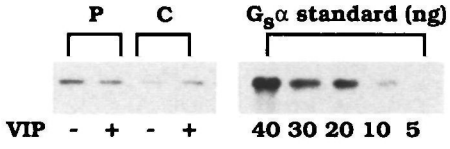

Fig. 3. **Quantitation of $G_s\alpha$ levels in VIP-treated GH_4C_1 cells.** Known quantities of commercially available $G_s\alpha$ (Calbiochem.) were resolved by SDS-PAGE together with membranes (P, 0.5 μg) and cytosolic fractions (C, 2 μg) of untreated or treated with VIP (0.1 μM VIP for 80 min) as in Fig. 2.

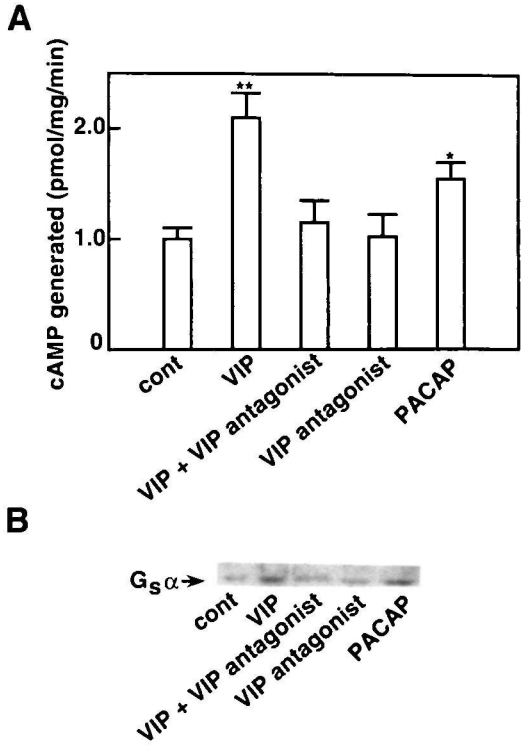

Fig. 4. **Release of $G_s\alpha$ from membranes to the cytosolic fraction upon activation of G_s.** GH_4C_1 cells were incubated at 37°C for 60 min in the presence of 0.1 μM VIP or 0.1 μM PACAP. The effect of antagonist was assessed by pre-incubating the cells for 30 min with 0.1 μM VIP antagonist, (N-Ac-Tyr,D-Phe)-GRF(1-29)-NH_2. The cytosolic fractions were prepared as described in the legend to Fig. 1. Panel A: Adenylyl cyclase activity was determined in S49 cyc$^-$ cell membranes after reconstitution with the cytosolic fractions. Panel B: Western blot for $G_s\alpha$ in the cytosolic fractions. Values are means±SE of three experiments performed in duplicate. $^*p<0.05$ and $^{**}p<0.01$ *versus* control levels.

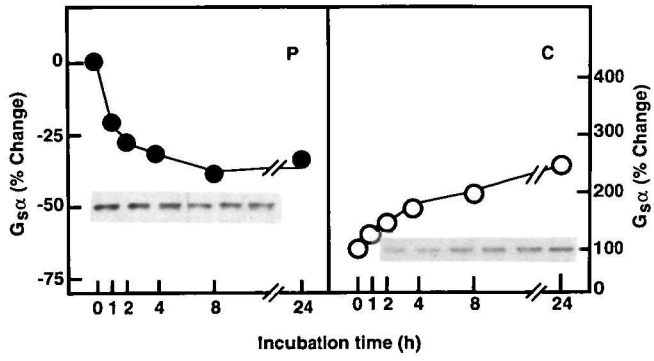

Fig. 5. **Translocation and down-regulation of $G_s\alpha$ upon prolonged exposure of GH_4C_1 cells to VIP.** GH_4C_1 cells were treated with 0.1 μM VIP for up to 24 h. $G_s\alpha$ levels in the membranes (P, 0.8 μg proteins from 0.4×10^5 cells) and cytosol (C, 5 μg proteins from 3.5×10^5 cells) were assessed by immunoblotting with RM/1 as the first antibody. Representative immunoblot examples are shown. Three similar experiments gave similar results.

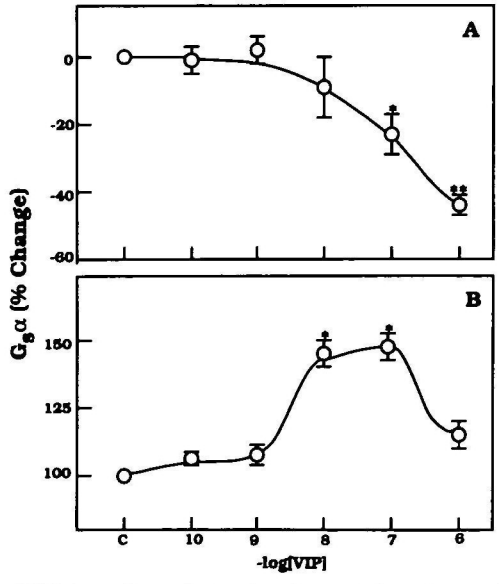

Fig. 6. **VIP dose-dependency for the translocation and down-regulation of immunoreactive $G_s\alpha$.** GH_4C_1 cells were incubated with various concentrations of VIP for 24 h to analyze the down-regulation of $G_s\alpha$ in the membrane fraction (A) and for 20 min to analyze its translocation into the cytosolic fraction (B). Aliquots of membrane and cytosolic fractions as described in Fig. 5, were subjected to SDS-PAGE and immunoblotted with RM/1 as the first antibody. The graph shows data representative of three experiments with comparable results. $^*p<0.05$ and $^{**}p<0.01$ *versus* control levels.

TABLE I. **Effect of cycloheximide on VIP-induced loss of $G_s\alpha$ from membranes of GH_4C_1 cells.** GH_4C_1 cells were treated with cycloheximide (100 μg) or VIP (0.1 μM), or a combination of these agents. In the combination experiments, cycloheximide was added 30 min before VIP. Membranes (2 μg) prepared from the cells were immunoblotted for either $G_s\alpha$ or $G_{i2}\alpha$ as described under "MATERIALS AND METHODS," and the developed immunoblots were quantified by laser densitometry. Results are presented from a single experiment. A further experiment reproduced similar data.

Treatment	G-proteins	
	$G_s\alpha$ (% of untreated)	$G_{i2}\alpha$ (% of untreated)
VIP (8 h, 0.1 μM)	61.8±4.3	94.9±3.2
Cycloheximide (8.5 h, 100 μg/ml)	86.9±4.2	87.7±6.9
VIP (8 h, 0.1 μM) +cycloheximide (8.5 h, 100 μg/ml)	44.9±12.7	86.0±10.9

15 cells to prostaglandin E_1 (*9*). However, in our experiments using cholera toxin as the stimulant, the percentage loss of $G_s\alpha$ was 75-85% after 5 h of incubation, and half-maximal loss of $G_s\alpha$ was occurred after 1.8±0.5 h ($n=3$), which is similar to the result in a closely related cell line, GH_3 (*16*). We also found that cholera toxin increased soluble $G_s\alpha$ in the cytosolic fraction by 187±18% ($n=3$) of control values after 1 h of incubation.

Treatment of GH_4C_1 cells with VIP (0.1 μM, 24 h) produced a substantial down-regulation of levels of membrane-bound $G_s\alpha$ (Fig. 5). In contrast, VIP rapidly induced a shift of $G_s\alpha$ from the membrane fraction to the cytosolic fraction (Fig. 1). Therefore, we examined the dose-dependency on VIP of the down-regulation of $G_s\alpha$ by treatment of GH_4C_1 cells for 24 h, and that of translocation by treatment for 20 min. As shown in Fig. 6A, the effect of VIP on the down-regulation of $G_s\alpha$ was dose-dependent, with half-maximal reduction in membrane-bound $G_s\alpha$ at 81.6±20.0 nM ($n=3$) ligand. On the other hand, VIP showed dose-dependent and bell-shaped activity on the translocation of $G_s\alpha$ (Fig. 6B), with an ED_{50} value of 2.5±0.3 nM ($n=3$), which is similar to that of 3 nM for the stimulation of cAMP accumulation by VIP in GH_4C_1 cells (*12*). This concentration of 81.6 nM ligand required for half-maximal reduction in membrane-bound $G_s\alpha$ is significantly less than that required for the VIP-stimulated appearance of cytosolic $G_s\alpha$ ($p<0.01$).

We assessed whether blockade of $G_s\alpha$ synthesis *de novo* would mimic the VIP-induced down-regulation of the G-protein. Incubation of GH_4C_1 cells for 8 h with 100 μg/ml cycloheximide, which is sufficient to abolish *de novo* protein synthesis (*17*), reduced membrane-associated levels of $G_s\alpha$ by 13.1% (Table I). This effect was considerably smaller than the decrease produced by VIP. In the same experiment, treatment of the cells with VIP (0.1 μM, 8 h) reduced membrane levels of $G_s\alpha$ by 38.2% in the absence of cycloheximide and by 55.1% in the presence of cycloheximide, suggesting that the individual effects of the two agents were simply additive. These results indicated that complete suppression of *de novo* protein synthesis could not mimic the effects of VIP, and hence even complete inhibition of transcription of the $G_s\alpha$ gene and/or translation of pre-existing mRNA could not account for the agonist-induced effects. Even if transcription was completely blocked and translation of pre-existing mRNA was abolished, the levels of $G_s\alpha$ would be expected to be decreased by only

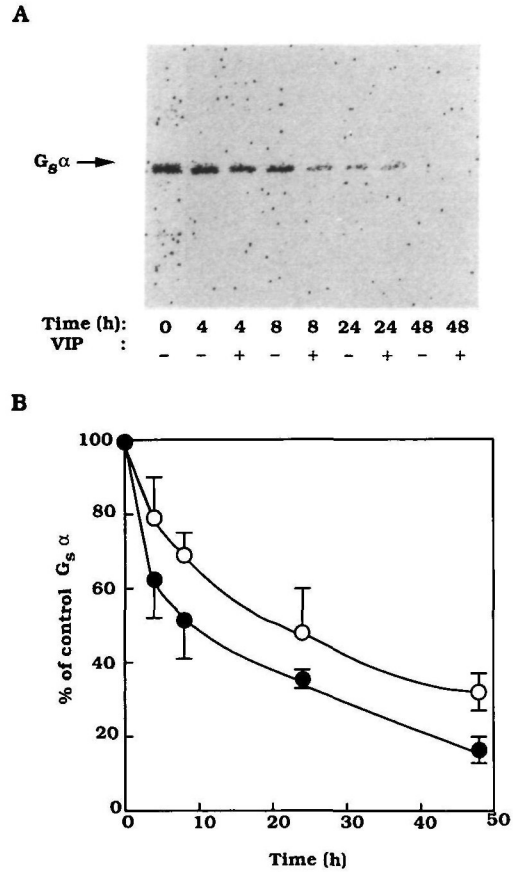

Fig. 7. **The turnover of $G_s\alpha$ in control and VIP-treated GH_4C_1 cells.** GH_4C_1 cells were labeled with [^{35}S]methionine/cysteine, then the turnover rate of $G_s\alpha$ was measured by immunoprecipitation with antiserum as RM/1 as described in "MATERIALS AND METHODS" after incubation of cells for various periods in the presence (+) or absence (−) of VIP (0.1 μM). (A) A fluorogram from a typical experiment is displayed. (B) Quantitative analysis of the effect of VIP on the turnover of $G_s\alpha$. Data of three individual experiments were quantitated and are displayed as means±SEM of three individual experiments. ○, control; ●, VIP-treated.

13% over this time period. As a control for this experiment, the effects of cycloheximide treatment on membrane-associated levels of $G_{i2}\alpha$ were measured in parallel. Cycloheximide treatment for 8 h caused a loss of 12.3% of immunodetectable $G_{i2}\alpha$ (Table I). As expected, the co-addition of VIP did not modify the effects of cycloheximide on cellular levels of $G_{i2}\alpha$.

We next examined the relative rate of $G_s\alpha$ degradation in GH_4C_1 cells in the presence and absence of VIP. We used immunoprecipitates from metabolically labeled cells to compare the turnover rates of $G_s\alpha$ from untreated and agonist-treated cells. Immunoprecipitation of [^{35}S]methionine/cysteine-labeled proteins in the membrane fraction of GH_4C_1 cells with RM/1, a specific antibody for $G_s\alpha$, showed a clear protein band of $G_s\alpha$ at 52 kDa (Fig. 7A). The indicated ^{35}S-labeled band was not present in immunoprecipitates with preimmune serum (data not shown). Figure 7A shows representative autoradiographs for agonist-treated and untreated cells, and Fig. 7B depicts the time-courses of $G_s\alpha$ degradation, as quantitated by analysis of the corresponding bands. In untreated cells, the

measured disappearance of ^{35}S-labeled $G_s\alpha$ was adequately described by a monoexponential curve with a half-time ($t_{0.5}$) of 8.8 ± 3.0 h ($n=3$), which is similar to that of 13 h in a closely related cell line, GH_3 (*18*). When the cells were treated with VIP a much faster pattern of $G_s\alpha$ degradation was observed. Upon addition of the agonist, the rate of degradation initially increased markedly ($t_{0.5} = 5.4 \pm 0.2$ h, $n=3$). However, in the continued presence of the agonist, the enhanced rate of degradation was not maintained: beyond 8 h of treatment with VIP, degradation of $G_s\alpha$ returned to a rate close to that observed in untreated cells.

DISCUSSION

The hormonally controlled regulation of cellular levels of α subunit of G_s has been noted in several situations. Stimulation of isopreterenol receptors (*5, 7*) or prostacyclin receptors (*6*) induces the translocation of $G_s\alpha$ subunits from membranes to cytosol, and stimulation of prostaglandin receptors also induces the down-regulation of $G_s\alpha$ in membranes (*9, 19, 20*). The relationship between the translocation and down-regulation of $G_s\alpha$ in these cases is, however, poorly understood. Levis and Bourne (*7*) showed that isopreterenol causes a rapid shift of 20% of epitope-tagged wild-type $G_s\alpha$ from the membrane-bound to the soluble compartment, but does not accelerate the turnover of epitope-tagged $G_s\alpha$ when expressed in S49 cyc$^-$ cells. They also showed that a $G_s\alpha$ subunit containing a mutation which keeps it permanently activated is degraded faster than wild-type $G_s\alpha$ (*7*). In contrast, agonist activation of an IP prostanoid receptor results in the down-regulation of a similarly epitope-tagged $G_s\alpha$ variant when expressed in neuroblastoma NG108-15 cells (*20*). Further, McKenzie and Milligan (*9*) reported that the prostaglandin E_1-induced reduction in membrane-associated $G_s\alpha$ is not accompanied by a compensating increase in immunoreactive $G_s\alpha$ in the cytosolic fraction of NG108-15. Conversely, the intracellular levels of G-proteins may depend on the type, strength and frequency of external signals.

In the present study, we observed that VIP (0.1 μM) induces the simultaneous translocation of $G_s\alpha$ from the membrane to cytosol and $G_s\alpha$ down-regulation in GH_4C_1 cells. The α subunits in the cytosolic fraction derived from VIP-treated cells are capable of activating adenylyl cyclase in $G_s\alpha$-deficient S49 cyc$^-$ cell membranes (Fig. 1). Our results show that the VIP-stimulated increase in dissociated $G_s\alpha$ is a receptor-dependent event, since the release is blocked by the prior addition of the VIP antagonist (N-Ac-Tyr,D-Phe)-GRF(1-29)-NH_2 (Fig. 4). The present data are consistent with results showing that β-adrenoreceptor agonist or prostacyclin receptor agonist induces the membrane-to-cytosol translocation of $G_s\alpha$ (*5-7*) and that the released $G_s\alpha$ reconstitutes the adenylyl cyclase activity in S49 cyc$^-$ cell membranes (*5, 6*). Furthermore, the ED_{50} value for VIP-mediated translocation of $G_s\alpha$ is close to that of stimulating cAMP accumulation (*12*), which suggests that a second effect of α_s activation, agonist-induced translocation from plasma membrane to cytosol, is necessary to activate the effector system, adenylyl cyclase (*21*). Although the precise molecular mechanism for the release of α subunits is still unclear, it has been reported that the binding of palmitate to Cys-3 of $G_s\alpha$ is required for both membrane association and the ability to mediate the hormonal stimulation of adenylyl cyclase (*22-24*), while nonpalmitoylated mutants of $G_s\alpha$ remain associated with the plasma membrane (*25, 26*). It was also reported that the carboxyl-terminal domain of $G_s\alpha$ is necessary for anchoring the activated form to the plasma membrane (*27, 28*), and that a soluble $G_s\alpha$ mutant has the ability to activate adenylyl cyclase in intact COS cells and after reconstitution with cyc$^-$ membranes (*29*). It is certain that palmitoylation is necessary, but not sufficient, for the membrane attachment and signaling functions of α subunits. Further studies are needed to identify the biochemical mechanisms involved in membrane localization and the activation of adenylyl cyclase.

On the other hand, chronic treatment of GH_4C_1 cells with VIP induced a 39% decrease in the total cellular levels of $G_s\alpha$ in the membrane and a 4% increase in the total cellular levels in the cytosol (Fig. 5). This means that 35% of cellular $G_s\alpha$ is degraded. Co-addition of the protein synthesis inhibitor cycloheximide with VIP provided an additional reduction in cellular $G_s\alpha$ levels (Table I). Measurement of the rate of degradation of $G_s\alpha$ in untreated cells and in cells maintained in the presence of VIP revealed that VIP treatment led to a rapid increase in the rate of degradation of $G_s\alpha$ (Fig. 7). It seemed most likely that enhanced protein degradation was responsible for the agonist-induced reduction in G protein. Also, we found that the amount of $G_s\alpha$ in the cytosolic fraction remained nearly constant during incubation of cells with VIP for 8-24 h (Fig. 5). This result is compatible with that obtained for isoproterenol stimulation, during which the abilities of the cytosolic fraction to reconstitute adenylyl cyclase activity remained constant up to 11 h of incubation (*5*). Wedegaertner *et al.* presented immunocytochemical evidence that replacing the agonist with an antagonist allows cytosolic $G_s\alpha$ to return to the plasma membrane (*30*). These findings together suggest that cytosolic $G_s\alpha$ is active and stable, and does not return to the membrane fraction unless the agonist is removed from the medium. Furthermore, our results suggest that the hormonally stimulated $G_s\alpha$ translocation and down-regulation occur independently, since we found a difference in the half-maximal doses of VIP for the translocation (2.5 nM) and down-regulation (81.6 nM) of $G_s\alpha$ in GH_4C_1 cells. Therefore, it cannot be inferred that the soluble pool of $G_s\alpha$ represents the route for the accelerated degradation of $G_s\alpha$. Levis and Bourne (*7*) showed that cholera toxin induced a 3.5-fold increase in the rate of degradation of mutant, HA-α_s-G226A, but did not cause it to move into the soluble fraction. This is compatible with our result that the loss of membrane attachment is not responsible for the accelerated degradation of α subunits in response to activation.

VIP, originally isolated and characterized from porcine intestine, localizes in neurons and endocrine cells of the central and peripheral nervous systems, where it serves a variety of functions including the regulation of exocrine secretion and cell growth (*31, 32*). VIP receptors are coupled *via* a G protein (G_s) to the activation of membrane-bound adenylyl cyclase and are readily desensitized by exposure to high concentrations of the homologous peptide, resulting in a virtual loss of cAMP production (*33*). The VIP-induced down-regulation of $G_s\alpha$ found in this study may be responsible for these processes. On the other hand, VIP shows trophic and mitogenic effects on embryonic

neural tissues (*31*) and non-small lung cancer cell lines (*32*). It is possible that the VIP-induced translocation of $G_s\alpha$ is relevant to the action of VIP, especially to its long-term effect on cell growth. This is the first report of the translocation and down-regulation of $G_s\alpha$ following VIP-treatment of rat pituitary cells.

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