Agonist-induced Internalization of the G Protein G_{11\alpha} and Thyrotropin-releasing Hormone Receptors Proceed on Different Time Scales*  

(Received for publication, November 26, 1997, and in revised form, April 30, 1998)  

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Using a combination of confocal immunofluorescence microscopy and subcellular fractionation, we demonstrate for the first time active internalization, trafficking, and down-regulation of a G protein α subunit subsequent to agonist occupation of a receptor. This proceeds on a much slower time scale than internalization of the corresponding receptor. In intact E2M11 HEK293 cells that express high levels of murine G_{11\alpha} and the rat thyrotropin-releasing hormone (TRH) receptor, the immunofluorescence signal of G_{11\alpha} was restricted almost exclusively to the plasma membrane. Exposure to TRH (10 μM) resulted first in partial relocation of G_{11\alpha} to discrete, segregated patches within the plasma membrane (10–60 min). Further exposure to TRH caused internalization of G_{11\alpha} to discrete, punctate, intracellular bodies (2–4 h) and subsequently to a virtually complete loss of G_{11\alpha} from plasma membranes and the cells (8–16 h). Short-term treatment with TRH followed by wash-out of the ligand allowed G_{11\alpha} immunofluorescence to be restored to the plasma membrane within 12 h. In subcellular membrane fractions, G_{11\alpha} was centered on plasma membranes, and this was not altered by up to 1–2 h of incubation with TRH. Further exposure to TRH (2–4 h) resulted in transfer of a significant portion of G_{11\alpha} to light-vesicular and cytosol fractions. At longer time intervals (4–16 h), an overall decrease in G_{11\alpha} content was observed.  

The capacity of agonist ligands to cause internalization of G protein-coupled receptors (GPCRs) has been actively studied for a number of years. Studies using fluorescent ligands, anti-receptor antibodies, antibodies to epitope tags introduced into the cDNA sequences of GPCRs, and a GPCR-Green fluorescent protein fusion protein have demonstrated many of the mechanisms that contribute to such processes (1–14). Despite the central role of G proteins in transducing GPCR-mediated signals, potential agonist-induced subcellular redistribution of heterotrimeric G proteins has not been widely examined. This is despite a large range of studies indicating that sustained treatment of cells with agonists at GPCRs can cause a reduction in total cellular levels of the G protein(s) activated by the receptor (15–23).  

We have recently produced a clonal cell line derived from human embryonic kidney, HEK, 293 cells, in which both the long isoform of the rat thyrotropin-releasing hormone (TRH) receptor and murine G_{11\alpha} are expressed to high levels (24). The principal mechanism of action of TRH receptors is via activation of phosphoinositidase C in a pertussis toxin-insensitive manner, a process which proceeds via interaction of the receptor with G proteins of the G_{11} family (23–26). Using this cell line and fractionation of cell homogenate on sucrose density gradient, we were able to demonstrate that long-term agonist treatment results in subcellular redistribution and down-regulation of this G protein (24).  

Herein we demonstrate the active internalization and trafficking of a phosphoinositidase C-linked G protein in response to agonist activation and that this proceeds on a much slower time scale than internalization of the TRH receptor.  

**EXPERIMENTAL PROCEDURES**  
**Materials**  

[3H]TRH (74 Ci/nmol) was from NEN Life Science Products (NET-577). Goat anti-rabbit IgG FITC conjugate (T-6005), goat anti-mouse IgG TRITC conjugate (T-5393) and phenylarsine oxide were from Sigma, sucrose (Aristar grade) was obtained from BDH. HEK-293 cells were obtained from the American Tissue Type Collection.  

**Methods**  

**Generation and Isolation of Clone E2M11**  

Clone E2M11 of HEK 293 cells that stably express high levels of the rat TRH receptor and murine G_{11\alpha} was prepared as described before (24). Briefly, a full-length rat (long isoform) TRH receptor cDNA (2.2 kilobases) (27) was subcloned into the eukaryotic expression vector pcDNA1 (Invitrogen) which is driven by the cytomegalovirus (CMV) promoter. HEK-293 cells were cotransfected with linearized pcDNA1/TRH receptor and pSP neo (Invitrogen) using Lipofectin reagent (Life Technologies, Inc., Paisley, Strathclyde, UK). Resultant Geneticin-resistant clones were picked, and TRH receptor-containing clones were identified as those in which TRH produced a rise in total inositol phosphate production. Expression of the TRH receptor in membranes from these clones was assessed by the specific binding of [3H]TRH. Clone E2, which expresses some 14 pmol of the receptor/mg of membrane protein (28), was selected for further transfection with plasmid pCMV, into which a cDNA encoding murine G_{11\alpha} was inserted, and with the plasmid pBABE hygro, which allows expression of resistance to the antibiotic hygromycin B. Clones were selected on the basis of resistance to hygromycin B, and the continued expression of the TRH receptor and novel expression of murine G_{11\alpha} was examined (24).
Internalization of G_{11\alpha} by Long-term TRH Exposure

Cell Growth
Clone E2M11 cells were grown in tissue culture in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% (v/v) newborn calf serum and were maintained in the presence of Geneticin sulfate (800 μg/ml) and hygromycin B (200 μg/ml). Prior to confluency, they were split 1:5 into fresh tissue culture flasks or were harvested.

Production of Antisera
Antisera 452 and CQ are rabbit polyclonal antipeptide antisera that were raised in rabbits obtained from VELAZ, Prague (452) and New Zealand White rabbits (CQ). Rabbits were immunized with a synthetic peptide QNLKKEYNLV (C-terminal decapeptide conserved between G_\alpha and G_{11\alpha}) conjugated by the glutaraldehyde method to keyhole limpet hemocyanin (Calbiochem) as described before (29).

Immunofluorescence and Confocal Microscopy
E2M11 cells were grown on ethanol-sterilized glass coverslips. Before confluency, they were pre-treated in the absence or presence of 10 μM TRH for various times. The cells were washed three times with PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KHPO_{4}, 8.1 mM Na_{2}HPO_{4}, pH 7.2) (pH 7.5) at 37 °C and fixed with methanol for 5 min at 20 °C with acetone for 1 min at −20 °C. Cell membranes were permeabilized with 0.15% (w/v) Triton X-100 in PBS at room temperature (Rt) for three times with PBS at Rt for 5 min. G_{11\alpha} and G_\alpha are expressed to very similar levels by HEK 293 cells (23), and these are both activated by the TRH receptor in E2M11 cells (23, 24). The level of overexpression of G_{11\alpha} in E2M11 cells (24) means that although the antiserum used should identify G_{11\alpha} and G_\alpha equally, at least 90% of the immunological signal is derived from the stably introduced murine G_{11\alpha}. The G_{11\alpha} was labeled with antisera 452 (details above) for 1 h at 37 °C with a 1:500 dilution of the antisera in PBS with 0.2% (w/v) BSA. In the cases labeled with antiserum 452 (details above) for 1 h at 37 °C with a 1:500 dilution directed against these proteins with the anti-G_{11\alpha} and G_\alpha antisera. After primary labeling, coverslips were washed three times with PBS for 1 h at 37 °C. Finally, the coverslips were washed three times with PBS at Rt for 5 min, covered with 40% (v/v) glycerol in PBS, and laid on slides. Detection was performed using a Bio-Rad MRC 600 confocal laser scanning microscope. FITC (green color fluorescence) and TRITC (red color fluorescence) were exited at 488 and 567 nm, respectively.

Subcellular Fractionation on Sucrose Density Gradients
E2M11 HEK-293 cells (3 × 75 cm² flasks per sample) were harvested by low speed centrifugation, washed twice in 140 mM NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM MgCl_{2}, and 1 mM EDTA, and homogenized in 2.5 ml of 20 mM Tris-HCl (pH 7.4), 3 mM MgCl_{2}, and 1 mM EDTA using a Potter-Elvehjem (Teflon-glass homogenizer). Two ml of homogenate (after freezing at −80 °C for at least 1 h) was layered on the top of a discontinuous sucrose density gradient consisting of (from top to bottom) 19, 23, 27, 31, 35 (all 5 ml), and 43% (10 ml) (all w/v) sucrose, 20 mM Tris-HCl (pH 8.0), 3 mM MgCl_{2}, and 1 mM EDTA. The gradient was centrifuged for 30 min at 27,000 rpm in a Beckman SW 50 rotor and fractionated manually from the meniscus (fractions 1–7, 5 ml each). The first 5 ml (fraction 1) represented an interphase between the overlaid homogenate and 19% (w/v) sucrose. To separate the low density membranes (light-vesicles) from cytosol, fraction 1 was diluted 1:1 with redistilled water, centrifuged for 120 min at 50,000 rpm in Beckman Ti-50 rotor, and the resulting pellet (fraction IP) was suspended by rehomogenization in 0.3 ml of 20 mM Tris-HCl (pH 7.4), 3 mM MgCl_{2}, and 1 mM EDTA (TME buffer). The supernatant (fraction 1S) represented the cytosol fragment. The gradient fractions were frozen at −80 °C until use (see Ref. 24 for further details).

Immunoblotting of Sucrose Density Gradient Fractions
Sucrose density gradient fractions were precipitated with TCA (6% w/v, 1 h on ice), and the precipitates were solubilized in Laemmli buffer. Standard (10% w/v acrylamide, 0.28% w/v bisacrylamide) or urea (12.5% w/v acrylamide, 0.0625% w/v bisacrylamide containing 6 M urea) SDS-PAGE was carried out overnight at 60 V (standard gel) or 100 V (urea gel) as described before (24). Molecular mass determinations were based on prestained molecular mass markers (Sigma, SDS 7B).

RESULTS

Internalization of G_{11\alpha} after Long-term Agonist Treatment Detected by Confocal Fluorescence Microscopy—E2M11 cells grown on glass coverslips were prepared for immunofluorescence and confocal microscopy as described under “Experimental Procedures,” and G_{11\alpha} was detected using antisera 452 which identifies the extreme C-terminal decapeptide of this G protein. The immunofluorescence signal was essentially entirely restricted to the plasma membranes of E2M11 cells, forming a sharp homogeneous barrier (Fig. 1a). Dual labeling with an antibody directed against β-tubulin indicated this cytoskeletal protein to be distributed within the cytoplasm and the cells had large distinct nuclei (Fig. 1a). Addition of Long-term Agonist Treatment (10 μM, 16 h) prior to preparation of the cells for the immunofluorescence study resulted in a vastly different cellular distribution of the G protein (Fig. 1b). Very little of the G_{11\alpha} protein could be detected then at the plasma membrane, whereas the vast majority of the immunodetectable G_{11\alpha} had a punctate, intracellular location. By contrast, the cytoplasmic distribution pattern of β-tubulin was unchanged (Fig. 1b).
Time Course of Redistribution of \( \gamma_{11} \alpha \) Analyzed by Sucrose Density Gradient Centrifugation—E2M11 cells were untreated or treated with TRH (10 \( \mu \)M) for the same time intervals (10, 30, and 60 min and 2, 4, and 16 h) as described above for immunofluorescence analysis. The control and TRH-treated cells were collected by low speed centrifugation and then homogenized, and subcellular fractionation on sucrose density gradients was performed as described under “Experimental Procedures” and in Ref. 24. The cytosol (1S), light vesicular (1P and 2), plasma membrane (3 and 4), mitochondrial (5 and 6), and nuclear (8) fractions were precipitated by 6% TCA and resolved by 12.5% (w/v) acrylamide SDS-PAGE containing 6 M urea, and \( \gamma_{11} \alpha \) was detected by immunoblotting with 452 antiserum. The immunoblots were quantitated by densitometric scanning.

The distribution of \( \gamma_{11} \alpha \) along the gradient fractions was not significantly altered by up to 1 h of incubation with TRH (Fig. 4). At longer time intervals (2–4 h), a significant portion of the plasma membrane-localized \( \gamma_{11} \alpha \) (fractions 3 and 4) was transferred to the low density end of the gradient, i.e. to the light vesicular fractions 1P and 2. The amount of \( \gamma_{11} \alpha \) proteins recovered in cytosol (fraction 1S) was increased in parallel. The decrease of the total immunoblot signal of \( \gamma_{11} \alpha \) in E2M11 cells (down-regulation), which was calculated as a sum of the \( \gamma_{11} \alpha \) content in all gradient fractions, after 16 h of incubation with TRH was some 50% as compared with controls (data not shown, but see Ref. 24). Thus, the results obtained by fractionation of E2M11 cell homogenate on sucrose density gradients supported strongly the data obtained by studies of intact cells by confocal fluorescence microscopy, i.e. the TRH-induced subcellular redistribution of \( \gamma_{11} \alpha \) proceeded as a long-term process that was not detectable earlier than 1 h after agonist exposure.

Time Course of TRH Receptor Internalization Measured by Radioligand Binding—To compare the time course of \( \gamma_{11} \alpha \) and TRH receptor internalization, the kinetics of \(^{3}H\)TRH binding were measured in acid-washed E2M11 cells according to both Hinkle and Kinsella (30) and Petrou et al. (31). According to protocol I, \(^{3}H\)TRH binding was measured by direct radioligand binding assays performed with untreated E2M11 cells (Fig. 5a); in protocol II, E2M11 cells were preincubated with unlabeled TRH to saturate the internal pool of TRH receptors before initiation of the internalization process. The remaining surface (membrane)-associated receptors were then determined by the \(^{3}H\)TRH binding reaction (Fig. 5b). Both approaches produced virtually the same results. In untreated cells (protocol I), about 73% of receptors were transferred to the acid-resistant (internalized) pool within 60 min of agonist exposure (half-time \( \sim 29 \) min). The cells pre-treated with unlabeled TRH exhibited very similar results—after 1 h of TRH exposure, about 70% of binding sites were lost from the surface (half-time \( \sim 25 \) min). After prolonged exposure (up to 16 h) of E2M11 cells to the hormone, surface membrane-associated receptors were almost completely depleted. Incubation of E2M11 cells with 10 \( \mu \)M TRH for 0.5 h followed by wash-out of TRH and

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**Fig. 1. Internalization of \( \gamma_{11} \alpha \) by long-term TRH treatment.** E2M11 cells grown on coverslips as described under “Experimental Procedures” were either untreated (a) or exposed to TRH (10 \( \mu \)M, 16 h) (b) and then prepared for immunofluorescence confocal microscopy to detect \( \gamma_{11} \alpha \) (green) and \( \beta \)-tubulin (brown).

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related fluorescence of \( \gamma_{11} \alpha \) was excluded and TRITC-labeled vimentin could be therefore analyzed with much higher resolution than on colored pictures. The native vimentin structure was changed from an extended fibrillar organization (Fig. 3a) into bundle-like coils which were closely allied to the nucleus (Fig. 3, b and c). In cells that had been treated for extended periods (4 h) with TRH, this process was partially reversed (Fig. 3d), and following 16 h of exposure to TRH, the pattern of vimentin immunostaining had returned to the initial state (Fig. 3e). This cytoskeletal reorganization was associated with a change of overall cell shape. The relaxed state with extended processes observed in control cells was changed to a rounded state that appeared shortly (0.5–1 h) after agonist addition.

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**FIG. 1.** Internalization of \( \gamma_{11} \alpha \) by long-term TRH treatment. E2M11 cells grown on coverslips as described under “Experimental Procedures” were either untreated (a) or exposed to TRH (10 \( \mu \)M, 16 h) (b) and then prepared for immunofluorescence confocal microscopy to detect \( \gamma_{11} \alpha \) (green) and \( \beta \)-tubulin (brown).
Internalization of $G_{11\alpha}$ by Long-term TRH Exposure
further incubation for 3 h without TRH resulted in recovery of 84% of receptors back to the surface (data not shown).

The Concentration Dependence of TRH-induced Redistribution of G\(_{11\alpha}\) — The agonist concentrations used in these (Figs. 1–4) as well as previous experiments to demonstrate down-regulation and/or redistribution of G proteins (for review, see Ref. 32), undoubtedly represents a supramaximal dose when compared with physiological hormone concentrations. Therefore, E2M11 cells were treated with increasing concentrations of TRH (0.1 nM–10 \(\mu\)M, 16 h), and the membrane-cytosol balance of G\(_{11\alpha}\) was measured after centrifugation of the cell homogenate for 1 h at 250,000 \(\times\) g (Fig. 6). The first decrease in membrane-bound G\(_{11\alpha}\) was observed with 1 nM TRH, with half-maximal response at 5 and 3 nM TRH for membrane (decrease in content) and cytosolic (increase in content) G\(_{11\alpha}\), respectively. Distribution of G\(_{11\alpha}\) was not substantially altered by further increase of TRH concentration from 0.1 to 10 \(\mu\)M (Fig. 6).

Recovery of Plasma Membrane-associated G\(_{11\alpha}\) following Removal of TRH and Effect of Cytoskeletal Inhibitors — In preliminary experiments to determine the minimum time of TRH exposure sufficient to induce marked internalization of G\(_{11\alpha}\), E2M11 cells were treated with TRH (10 \(\mu\)M) for 5, 10, 20, and 30 min, washed three times with DMEM to remove the agonist and further incubated for 3 h without TRH. The combination of 0.5 h TRH(+) and 3 h TRH(−) incubation periods was found to be sufficient to induce marked internalization of G\(_{11\alpha}\) (Fig. 7b).

**Fig. 2.** Time course of TRH-induced internalization and redistribution of G\(_{11\alpha}\) studied by confocal fluorescence microscopy of intact cells. E2M11 cells grown on coverslips as described under “Experimental Procedures” were either untreated (a) or exposed to TRH (10 \(\mu\)M) for 10 min (b), 30 min (c), 60 min (d), 2 h (e), 4 h (f), or 16 h (g). The cells were then immunostained to detect G\(_{11\alpha}\) (green) or vimentin (red).

**Fig. 3.** Alteration of vimentin structure during TRH exposure. E2M11 cells were prepared as described in the legend to Fig. 2. The FITC-related fluorescence of G\(_{11\alpha}\) was excluded by a cut-off filter, and the TRITC signal of vimentin was detected on black and white film. Control cells (a), and cells treated with 10 \(\mu\)M TRH for 30 min (b), 1 h (c), 4 h (d), and 16 h (e).
when compared with control signal (Fig. 7a). Prolongation of the incubation period without TRH (after constant 0.5 h TRH exposure) to 5 h (Fig. 7c), 8 h (Fig. 7d), and 12 h (Fig. 7e) was associated with a step-wise increase of $G_{11 \alpha}$ immunofluorescence such that after 12 h, the plasma membrane was again richly endowed with a uniform population of $G_{11 \alpha}$. Further prolongation of recovery period up to 16 h did not further increase the plasma membrane-associated fluorescence signal of $G_{11 \alpha}$ (Fig. 7f).

Recovery of $G_{11 \alpha}$ immunofluorescence signal obtained after 0.5 h TRH (+) and 8 h TRH(−) incubation periods was significantly blocked by 100 $\mu$M cycloheximide (Fig. 7g). Therefore, recovery of plasma membrane $G_{11 \alpha}$ seems to require de novo protein synthesis. The cytoskeletal inhibitors nocodazole (microtubules), cytochalasin B (microfilaments), and colcemide (microtubules and intermediate filaments) exhibited clear inhibitory effects on internalization of $G_{11 \alpha}$ in response to TRH (data not shown).

Inositol Phosphate Generation—E2M11 cells prelabeled with $[^3]$Hinositol (1 $\mu$Ci/ml) were incubated with TRH (10 $\mu$M) for 0, 0.5, 1, 2, 8, and 16 h, and production of $[^3]$Hinositol phosphates was subsequently measured over a 10-min period following wash-out of TRH and reexposure to TRH (10 $\mu$M). In the absence of preexposure to the ligand, addition of TRH resulted in a 3.9 ± 0.1 (n = 2) fold stimulation of $[^3]$HIP generation (Table I).

**Fig. 4.** Time course of redistribution of $G_{11 \alpha}$ studied by density gradient centrifugation of cell homogenate. The same amounts of E2M11 cells (3 × 75 cm² flasks) were untreated (0 time) or treated with TRH (10 $\mu$M) for 10 and 30 min and 1, 2, 4, and 16 h. The cells were subsequently homogenized, and subcellular fractions were prepared by sucrose density centrifugation as described under "Experimental Procedures." The fractions were concentrated by TCA precipitation (6%, 1 h on ice), resolved by urea-containing SDS-PAGE, and immunoblotted with antiserum 452. The immunoblot signal of $G_{11 \alpha}$ was quantified by densitometric scanning and expressed in arbitrary units. a, the amount of $G_{11 \alpha}$ in fractions 1S (○), 1P (●), 3 (●), 4 (●), and 5 (●). b, the relative amount of $G_{11 \alpha}$ in fractions 1S, 1P, 3, 4, and 5 expressed as percentage of the control values (in corresponding fractions prepared from TRH-ununtreated cells). The data represent the average (± S.E.) of densitometric scanning of gradient fractions obtained from typical fractionation procedures.

**Fig. 5.** Time course of TRH receptor internalization. a, the $[^3]$HTRH binding assay was performed according to protocol I described under "Experimental Procedures." ●, Total binding; ■, binding after acid wash (internalized pool); ○, radioactivity solubilized by acid/salt wash. b, E2M11 cells were preincubated for 0, 2, 5, 10, 15, 20, 60, and 120 min with unlabeled TRH, washed once with acid/salt solution, and further incubated with 10 nM $[^3]$HTRH for 90 min as described under "Experimental Procedures" (protocol II). The data represent the average (± S.E.) of three independent experiments carried out in duplicate.

**Fig. 6.** Dose-response curve of TRH-induced redistribution of $G_{11 \alpha}$. Homogenate prepared from the same amount (1 × 75 cm² flask) of control and hormone-exposed E2M11 cells (incubation for 16 h with 0.1 nM-10 $\mu$M TRH) was centrifuged for 1 h at 250,000 × g, and the membrane-cytosol balance (sediment versus supernatant) of the $G_{11 \alpha}$ protein was assessed by SDS-PAGE and immunoblotting as described under "Experimental Procedures" and the legend to Fig. 3. The data represent the average (± S.E.) of three experiments performed in duplicate.

E2M11 cells preincubated with 10 $\mu$M TRH for 0.5 h, which were subsequently allowed to recover for 3 h in medium without the hormone (Table II).
FIG. 7. Recovery of the internalization of $G_{11\alpha}$ and its blockade by cycloheximide. E2M11 cells grown on coverslips were untreated (a) or exposed to TRH (10 $\mu$M, 30 min) (b–f). TRH was then removed for 3 (b), 5 (c), 8 (d), 12 (e), and 16 (f) h prior to preparation of the cells for $G_{11\alpha}$ immunofluorescence analysis. In Fig. 7g, cycloheximide (100 $\mu$M) was included in the course of an 8-h recovery period after 30 min of TRH exposure.
**TABLE I**

| First incubation with TRH, time length (h) | Subsequent TRH addition, fold stimulation over basal level |
|------------------------------------------|----------------------------------------------------------|
| 0                                       | 3.9                                                      |
| 0.5                                     | 1.0                                                      |
| 1                                       | 1.0                                                      |
| 2                                       | 1.0                                                      |
| 8                                       | 1.1                                                      |
| 16                                      | 1.0                                                      |

**TABLE II**

| First incubation with TRH, time length (h) | Subsequent TRH addition, fold stimulation over basal level |
|------------------------------------------|----------------------------------------------------------|
| 3.5, TRH(+)                              | 1.0                                                      |
| 0.5, TRH(+) and 3, TRH(−)                | 1.2                                                      |

**DISCUSSION**

Continuous exposure of cells to agonists often causes a rapid waning of the stimulated response, an effect which is termed desensitization, refractoriness, tolerance, or tachyphylaxis. Agonist-promoted desensitization appears to be a general homeostatic mechanism by which target cells modulate responsiveness to agents acting on cell surface receptors, and molecular mechanism(s) of this process may be located at any step of the signal transmission pathway which is initiated by a receptor and proceeds into the cells. Among these, receptor-based regulations of desensitization have been studied most intensively (see Refs. 33–35 for reviews).

Agonist-induced phosphorylation of GPCRs and subsequent subcellular redistribution, sequestration, internalization, recycling, and finally down-regulation have frequently been found to be directly related to desensitization of hormone action. By contrast to GPCRs, relatively little attention has been devoted to the role of the subcellular redistribution of heterotrimeric G proteins in such phenomena. However, isoproterenol-stimulation of β2-adrenergic receptors in S49 lymphoma cells redistributed the Gαα subunits from membranes to cytosol (18). Such agonist-induced membrane-cytosol shifts of the α subunits of heterotrimeric G proteins have also been demonstrated for other systems including the prostatcyclic receptor and Gαα (36) and somatostatin receptor and Gαα (37). It has also been shown that agonist exposure can induce a transfer of Gαα subunits from plasma membranes to light vesicular membrane fractions which were recovered either by sucrose density gradient or differential centrifugation (see Ref. 24 for details). Moreover, Crouch (38) has indicated a capacity of mitogen-activated Gαα to be redistributed from the plasma membrane to the nucleus and for adrenaline to cause a redistribution of Gαα in platelets (39). Direct examination of the dynamics of internalization of heterotrimeric G protein induced by an agonist, however, has been missing.

Although not examined as widely as regulation of GPCR levels, agonist-mediated down-regulation of G protein α subunits has been observed in a number of systems (see Ref. 32 for review). In general, down-regulation of G proteins requires extended periods of cellular exposure to the agonist, is restricted to the G protein(s) expected to be activated by the agonist-GPCR complex, and requires the cell or tissue to express relatively high levels of the GPCR. This last point can be understood on the basis that the cellular levels of G proteins are usually far higher than the levels of any particular GPCR. Thus, even when considering the capacity of an agonist-occupied GPCR to catalytically activate the G protein population, the fraction of the cellular pool of a specific G protein which becomes activated by a GPCR is likely to be small unless GPCR levels are high. In the studies which have examined the mechanisms responsible for agonist-mediated reduction in cellular G protein levels, an enhanced rate of degradation of the cognate G protein, but not of other cellular G proteins which are not activated by that agonist, has routinely been observed (18–22). By contrast, relatively few studies have provided evidence for decreased rates of G protein synthesis or reduction in mRNA levels (see Ref. 32 for details).

Even in the case of widely studied GPCRs, it has not been clear whether sequestration is or is not a prerequisite for agonist-induced down-regulation. The process of sequestration, in which receptors remain detectable by lipophilic receptor ligands but become increasingly inaccessible to hydrophilic, membrane-impermeable ligands (40–41), was proposed to represent internalization of receptors into an intracellular compartment and to serve as a prerequisite for down-regulation. Direct demonstration of dynamic internalization and recycling pathways by confocal fluorescence microscopy was first achieved for β2-adrenergic receptors (1–2, 33) and has subsequently been replicated for a variety of other receptors (3–14). As such, in the current studies, we have utilized a combination of cell biology using confocal fluorescence microscopy and biochemical analyses of the subcellular localization of the G protein G11α following addition of TRH to cells expressing both the TRH receptor and G11α. Using these approaches, we have demonstrated sequestration, internalization, subcellular redistribution, and finally down-regulation of G11α protein as subsequent steps of TRH action and have observed good agreement of results between these two widely different methodological approaches. These observations provide new and original insights into the role of G protein regulation which are likely to contribute to long-term mechanisms of desensitization of hormone effect. Desensitization of the TRH receptor was clearly demonstrated in our studies by measurement of inositol phosphate generation in E2M11 cells that were pre-treated with TRH for various time intervals (0.5–16 h). 0.5 h was sufficient to induce complete desensitization of the inositol phosphate response to subsequent addition of TRH (Table I).

The mechanisms of G protein internalization and the extent to which internalization of G11α proceeds via clathrin-dependent or other endocytic pathways remains to be examined in the future. Treatment with hypertonic sucrose that has been used to interfere with clathrin-mediated endocytosis and internalization of TRH receptors (31, 42) appeared to produce a nonspecific alteration in the pattern of G11α immunofluorescence (data not shown). Clearly, G11α internalization does not accompany the TRH receptor along its way into the cell interior as more than 70% of receptors were transferred into an acid wash-resistant, intracellular pool within the first 60 min of incubation with agonist (Fig. 5). At this time, no significant level of G11α was internalized in response to addition of TRH. These findings clearly indicate an uncoupling of G11α from the
TRH receptor in the course of the internalization process. The possibility that clathrin-dependent endocytosis is not involved in G11α internalization, therefore, has to be considered. Multiple G protein α subunits have been identified in caveoleae (43–45), and it has been shown that β-adrenergic receptors redistribute to caveolin-rich membrane domains in response to agonist stimulation (46–47). A similar scenario has been reported following addition of a muscarinic acetylcholine receptor agonist to cardiac myocytes, which resulted in movement of a proportion of the M2 muscarinic acetylcholine receptor population to a caveolar location and the subsequent interaction of the receptor with caveolin-3, a muscle-specific form of caveolin (48).

Although there has been an ongoing discussion as to whether β-adrenergic receptor is internalized via clathrin-coated pits or caveoleae (49–50), which serves as a good example of how difficult it has been to decide between these two alternative pathways of endocytosis, recent studies using GTP binding mutants of dynamin, a GTPase which plays a key role in pinching off of clathrin-coated endocytic vesicles from the plasma membrane, have shown this to interfere with agonist-induced sequestration of the β2-adrenergoreceptor when expressed in HEK 293 cells but not with internalization of the angiotensin II type 1A receptor in the same cells (5), arguing both for a specific role of clathrin-coated vesicles in the regulation of the β2-adrenoceptor and that this is not a pathway used universally by GPCRs. Analysis at the ultrastructural level using immunogold electron microscopy, such as that recently achieved for the cholecystokinin receptor (51), may, in time, provide an alternative approach to examine different membrane components in a single type of endocytic vesicle.

The results generated in this work display a host of other interesting features. There has been great interest in potential nonuniformity of plasma membrane G protein distribution and in the potential roles for cytoskeletal elements in regulating G protein distribution and function (52–56). However, in the absence of agonist, the cellular G11α in E2M11 cells was both highly concentrated at the plasma membrane and remarkably uniform in distribution around the plasma membrane (Fig. 1a). The double labeling procedures to detect β-tubulin and vimentin, were not able to detect any marked degree of association of G11α with elements of the cytoskeleton as has recently been reported in WRK cells (56). Indeed, the cellular distribution of β-tubulin was essentially unaffected during the process of TRH-induced G11α redistribution (Fig. 1) and although there were marked alterations in the pattern of vimentin immunostaining (Fig. 3), this seemed to be separate from that observed for G11α. On the other hand, the lack of structural evidence for G11α protein-cytoskeleton interaction does not mean that the two processes are functionally independent. Treatment of E2M11 cells with the cytoskeleton inhibitors nocodazole, cytochalasin B, and colcemide resulted in significant inhibition of G11α internalization.

In conclusion, these studies provide a dramatic new insight into the internalization, cellular distribution, and down-regulation of the phosphoinositidase C-linked G protein G11α in response to the presence of an agonist at a GPCR coupled to this G protein, and they demonstrate clearly that the series of recent studies on agonist regulation of GPCR distribution should be extended to analysis of their G protein partners.

Acknowledgments—We thank Dr. P. Draber, Institute of Molecular Genetics, AV CR for the anti-vimentin and anti-tubulin antibodies; Dr. L. Kujinova, Division of Biomathematics, Institute of Physiology, AV CR for excellent assistance with the confocal microscopy; and Dr. H. Kowarz, 1st Medical Faculty, Charles University, for preparation of 452 antiseraum.

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