The thyrotropin-releasing hormone (TRH) receptor was expressed in embryonic fibroblasts from mice lacking the α subunit of Gq and G11 (Fq/11 cells) to determine whether G protein coupling is necessary for agonist-dependent receptor internalization. Neither TRH nor agonists acting on endogenous receptors increased intracellular calcium unless the cells were co-transfected with the α subunit of Gq. In contrast, temperature-dependent internalization of [3H]MeTRH in Fq/11 cells was the same whether Gq,α was expressed or not. A rhodamine-labeled TRH analog and fluorescein-labeled transferrin co-localized in endocytic vesicles in Fq/11 cells, indicating that endocytosis took place via the normal clathrin pathway. Cotransfection with β-arrestin or V53D β-arrestin increased TRH-dependent receptor sequestration. Fq/11 cells were co-transfected with the TRH receptor and a green fluorescent protein (GFP)-β-arrestin conjugate. GFP-β-arrestin was uniformly distributed in the cytoplasm of untreated cells and quickly translocated to the periphery of the cells when TRH was added. A truncated TRH receptor that lacks potential phosphorylation sites in the cytoplasmic carboxyl terminus signaled but did not internalize or cause membrane localization of GFP-β-arrestin. These results prove that calcium signaling by the TRH receptor requires coupling to a G protein in the Gq family, but TRH-dependent binding of β-arrestin and sequestration do not.

Many G protein-coupled receptors undergo ligand-driven internalization through the clathrin pathway. Internalization or sequestration appears to modulate various aspects of signal transduction such as desensitization, resensitization, and activation of mitogen-activated protein kinases (1–3). A fundamental question regarding internalization of G-protein-coupled receptors is how the activated receptor is targeted to clathrin-coated pits in response to agonist binding. Recently, this question has been answered with the discovery that β-arrestin acts as an adaptor (4, 5), binding both to receptors and to clathrin (6, 7). Because β-arrestin binds most strongly to activated, phosphorylated receptors (5), and receptor phosphorylation is promoted by agonist binding, β-arrestin can discriminate between inactive and active receptors and target agonist-occupied receptors to clathrin-coated pits and the endocytic pathway.

Most receptors coupled to G proteins in the Gq family are sequestered following the binding of an agonist but not an antagonist (8). There are exceptions to this generalization, however, because some Gq-coupled receptors do not internalize at all, others internalize only in some cell contexts (9), and a few internalize when occupied by an antagonist (10, 11). It is uncertain if coupling to Gq proteins is required for receptor phosphorylation, β-arrestin recognition of agonist-occupied receptor, or receptor internalization.

Internalization of the receptor for thyrotropin-releasing hormone (TRH), 1 which signals through Gq and G11 to activate phospholipase C, has been studied extensively (12–18). The TRH receptor internalizes rapidly (∼2.5 min) through the clathrin pathway upon agonist binding (13). When its cytoplasmic, carboxyl-terminal tail is deleted, the TRH receptor binds ligand with high affinity and stimulates phospholipase C but does not internalize, showing that coupling to Gq is not sufficient to cause internalization (15, 17). It is not clear whether coupling to Gq is necessary for receptor sequestration. Several investigators have approached this question by characterizing the internalization of mutant receptors using antibodies rather than hormones to promote internalization and blocking signal pathways pharmacologically (15, 17). They have reached contradictory conclusions about whether receptor-G protein coupling is necessary for TRH receptor internalization.

In this report, we have taken advantage of a fibroblast cell line that lacks Gq and G11 to examine directly the role of these G proteins in the internalization of TRH receptor (19, 20). The Fq/11 cells were derived from the embryos of mice in which the α subunits of both Gq and G11 had been knocked out by targeted gene disruption. Our results show that Gq or G11 is absolutely required for calcium signaling but not for coupling between the activated TRH receptor and β-arrestin or for receptor internalization.

**EXPERIMENTAL PROCEDURES**

**Materials**—LipofectAMINE PLUS was from Life Technologies, Inc. Plasmids encoding the mouse TRH receptor (pCDM8mTRHR) and a truncated internalization-defective TRH receptor (pAd/CMVTRHIC33STOP) were gifts from Dr. Marvin C. Gershengorn (Cornell University, New York, NY). A plasmid-encoding mouse Gqα subunit (pCMV-Gqα) was a gift from Dr. Dianqing Wu (University of Rochester, Rochester, NY). The plasmid pRSVCAT encoding chloramphenicol acetyltransferase was from the American Type Culture Collection (Manassas, VA). Plasmids encoding wild type rat β-arrestin (pCMVβarr1), β-arrestin V53D (pDNAβarr1V53D), and a

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* The abbreviations used are: TRH, thyrotropin-releasing hormone; Rhod-TRH, rhodamine TRH; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid.
concentration of ionomycin to release Ca \(^{2+}\) and were cultured and transfected similarly. Fibroblasts were a gift from Dr. Dianqing Wu (University of Rochester, Rochester, NY) and were cultured and transfected similarly.

In colocalization experiments, cells were incubated in Hepes-buffered saline solution containing 170 mM Rhod-TRH and 5 \(\mu\)g/ml fluorescence-labeled transferrin (FITC-transferrin) at 37 °C for 1 h. Control experiments, in which cells were stained with Rhod-TRH or FITC-transferrin alone, showed that bleed-through was negligible.

To examine translocation of the \(\beta\)-arrestin-green fluorescent protein (GFP) conjugate (\(\beta\)arr2GFP), Fq/11 cells were transfected with 2 \(\mu\)g of pCDM8mTRHR or pAd/CMVTRHR335STOP and 0.4 \(\mu\)g of \(\beta\)arr2GFP/dish. About 24 h after transfection, cells were washed and incubated with Hepes-buffered saline solution at 37 °C. Cells were stimulated with 1 \(\mu\)M TRH for up to 20 min. Localization of \(\beta\)arr2GFP in living cells was followed microscopically with a 40× objective and a fluorescence filter. Microscopic results are typical of data obtained in 3 to 10 independent experiments.

RESULTS

We transfected Fq/11 cells with cDNA encoding the TRH receptor and a 2-fold higher concentration of either a control plasmid or plasmid encoding G\(\alpha\)\(q\) and then measured the ability of various agonists to increase the concentration of cytoplasmic free Ca\(^{2+}\), [Ca\(^{2+}\)], in individual cells loaded with the Ca\(^{2+}\) reporter Fura-2. Following transfection with the TRH receptor alone, Fq/11 cells failed to respond to TRH or to a mixture of agonists acting on endogenous receptors (thrombin, endothelin, bombesin, and bradykinin) (Figs. 1 and 2 and Table I). When transfected with the TRH receptor and the \(\alpha\) subunit of G\(q\), a significant number of cells responded to TRH (15%) and to the mixture (11%) (Figs. 1 and 2 and Table I). Subsequent experiments showed that bradykinin was responsible for most of the activity in the agonist mixture (Table I). TRH caused a consistently larger increase in [Ca\(^{2+}\)] than the mixture. Based on \([^{3}H]MeTRH binding, the number of TRH receptors expressed was within 50% of the same in cultures transfected with a control plasmid or plasmid encoding G\(\alpha\) (data not shown). To determine whether intracellular Ca\(^{2+}\) stores were adequate in the nonresponsive cells, we added the Ca\(^{2+}\) chelator BAPTA to reduce extracellular Ca\(^{2+}\) to ~4 nM and then added a low concentration of ionomycin to release Ca\(^{2+}\) from intracellular stores. All of the Fq/11 cells tested responded to ionomycin with a large increase in Ca\(^{2+}\) except those cells that had previously responded to TRH, which had predictably smaller responses because their Ca\(^{2+}\) stores had been partially depleted.

To determine whether internalization of the TRH receptor also requires G protein coupling, we measured sequestration of \([^{3}H]MeTRH\) to an acid/salt-resistant compartment in Fq/11 cells with and without transfection of G\(\alpha\)q. When Fq/11 cells were transfected with only the TRH receptor and then incubated with \([^{3}H]MeTRH\) at 37 °C, 32% of specifically bound hormone was acid/salt resistant, in comparison to only 12% when binding was performed at 0 °C (Fig. 3). When cells were transfected with the receptor and G\(\alpha\), the extent of internalization was the same (Fig. 3). Internalization of \([^{3}H]MeTRH\) was unaffected by treatment with pertussis toxin, indicating that G\(q\) and G\(i\) are not involved. We also transfected embryonic fibroblast cultures obtained from wild type mice of the same strain as the knockout mice (MEF cells) with the TRH receptor cDNA and followed \([^{3}H]MeTRH\) internalization. The wild type fibroblasts expressed TRH receptors at the same level as the Fq/11 cells, and they also internalized \([^{3}H]MeTRH\) by a pertussis toxin-insensitive pathway.

Because the TRH receptor normally internalizes in a clathrin-dependent manner, we compared internalization of FITC-transferrin, a classical marker for the coated pit pathway, and a bioactive, rhodamine-labeled TRH analog, Rhod-TRH, in Fq/11 cells. Rhod-TRH fluorescence in Fq/11 cells was dim, consistent with the low level of \([^{3}H]MeTRH\) binding. When Fq/11 cells were transfected with only the TRH receptor and incubated with Rhod-TRH at 0 °C, fluorescence was on the cell surface (Fig. 4). Incubation with excess TRH blocked the membrane fluorescence, indicating that it was because of receptor-bound Rhod-TRH. When these cells were incubated with Rhod-TRH at 37 °C, Rhod-TRH was concentrated inside the cell, indicating that internalization of the liganded receptor had taken place. The bright specks that can be seen in all panels were associated with transfection reagents and were also seen in mock-transfected cells (data not shown). Internalization of FITC-transferrin was rapid and extensive, with nearly all the label in an endocytic compartment by 5 min (data not shown). Cells were also incubated simultaneously with Rhod-TRH (red) and FITC-transferrin (green) at 37 °C. There was extensive colocalization of the two labels, shown in yellow and orange (Fig. 5). These findings all indicate that TRH drives internalization of its receptor via the normal coated pit pathway without G protein interaction.

To establish whether overexpression of \(\beta\)-arrestin would increase sequestration, we transfected Fq/11 cells with the TRH receptor with or without \(\beta\)-arrestin. Cells transfected with the receptor and \(\beta\)-arrestin internalized more \([^{3}H]MeTRH\) (56%)...
than cells transfected with receptor alone (32%) (Fig. 6), suggesting that β-arrestin was functioning as an adaptor in the absence of G protein coupling. A mutant β-arrestin, V53D, has been shown to act in a dominant negative manner in some settings but not in others. V53D β-arrestin increased internalization of [3H]MeTRH as effectively as wild type arrestin (Fig. 6).

To determine whether G protein coupling is needed for β-arrestin binding, we transfected Fq/11 cells with the TRH receptor and a GFP-β-arrestin conjugate and followed the localization of GFP-β-arrestin before and after activating the receptor. GFP-β-arrestin was uniformly distributed in the cytoplasm of untreated cells (Fig. 7). Following the addition of TRH, GFP-β-arrestin quickly translocated to the periphery of the cells, suggesting that it bound to the agonist-occupied receptor in the absence of G protein interaction. Once translocated to the membrane, much of the GFP-β-arrestin remained there for up to 1 h, indicating that it was not able to move again (Fig. 8).

We have found that agonist mixtures activating endogenous receptors in transfected 293 cells or GH3 cells do not cause translocation of GFP-β-arrestin, although they cause
responses comparable with those evoked by TRH. These results suggest that the TRH receptor is more effective than most in translocating GFP-β-arrestin. We also evaluated the effect of a TRH antagonist, chlordiazepoxide, on GFP-β-arrestin localization. Chlordiazepoxide (10 μM) did not cause translocation of GFP-β-arrestin by itself but blocked GFP-β-arrestin translocation initiated by a low dose of TRH, 10 nM (Fig. 9). When added at 10 μM, TRH overcame the effect of the antagonist and caused GFP-β-arrestin to move to the membrane. The K_d values of TRH and chlordiazepoxide for the TRH receptor are ~10 nM and 3 μM, respectively. β-Arrestin is believed to bind more tightly to phosphorylated receptors (6, 7), and although the phosphorylation sites on the TRH receptor have not been mapped, most potential phosphorylation sites are found in the intracellular carboxyl-terminal tail after residue 341. We therefore transfected cells with GFP-β-arrestin and a truncated form of the TRH receptor (C335STOP) that lacks these potential phosphorylation sites. The truncated receptor was expressed and capable of transducing a signal, because TRH increased [Ca^{2+}]_i if G_qα was cotransfected with the C335STOP receptor (Table I). Activation of the truncated TRH receptor with TRH did not cause GFP-β-arrestin to move to the membrane, however (Fig. 7).

Because it has been suggested that a receptor-β-arrestin complex can signal directly to the mitogen-activated protein kinase pathway (3), we asked whether TRH can activate mitogen-activated protein kinase in Fq/11 cells expressing the TRH receptor with and without G_qα. We did not detect significant

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2 R. Yu and P. M. Hinkle, unpublished observations.
TRH effects on the abundance of phosphorylated mitogen-activated protein kinases on Western blots using cells that had been serum-starved for 24 h and treated with 1 μM TRH for up to 20 min, regardless of expression of Gqα. Because background mitogen-activated protein kinase activity was high and TRH receptor levels were low, these results do not rule out the possibility that TRH activates the mitogen-activated protein kinase pathway weakly in transfected Fq/11 cells, but any response must be very small.

**DISCUSSION**

Previous studies have led to contradictory conclusions concerning the requirement for G proteins in the sequestration of G protein-coupled receptors coupled to the Gq/G11 family (15, 17, 24, 25). By using cells lacking the α subunits of Gq and G11, we have obtained definitive evidence that the TRH receptor undergoes agonist-dependent sequestration in the absence of its normal signal transducers, Gq and G11. It is improbable that the receptor internalized as a result of coupling to some other G protein. Although the TRH receptor has been reported to exert effects on ion channels via Gs and Gq, pertussis toxin did not alter agonist-dependent internalization in Fq/11 cells. The TRH receptor has been reported to couple to phospholipase C via Gi in oocytes (29), but neither TRH nor bradykinin evoked any calcium response in Fq/11 cells unless the cells were transfected with Gqα. The TRH receptor was not overexpressed in mouse fibroblasts. Assuming that 15% of Fq/11 cells expressed receptors, because 15% of the cells exhibited a calcium response to TRH when Gqα was co-transfected, we calculate that there were no more than 30,000 TRH binding sites/cell. This is below the level in pituitary cells (at least 50,000) and well below the levels in other transfected cell models (HEK, COS, HeLa, Chinese hamster ovary, C6) (16, 30, 31).

Petrou et al. (15) suggested that TRH receptor internalization involves a Gq-independent pathway requiring the CNC sequence in the carboxyl-terminal tail and perhaps an additional, Gq-dependent pathway, based on the study of mutant receptors and the finding that antibody to an extracellular, amino-terminal epitope tag induced internalization but not signaling. They also suggested that Gqα and the TRH receptor internalize in the same vesicles. Milligan and co-workers (32) find that TRH down-regulates Gqα and causes an increased accumulation of Gqα in endocytic vesicles, but they do not think that the receptor and Gqα cointernalize because of large discrepancies in the half-times for receptor and Gqα internalization. Our results prove that Gqα is not needed for internalization but do not establish whether the receptor co-internalizes with Gqα when the appropriate G protein is available. Gershengorn and co-workers (18) suggest that G protein internalizes with its G protein partner (30) when the appropriate G protein is available. U73122 exerts multiple effects on calcium homeostasis and other processes (33–35) and may have impaired TRH-driven internalization by a nonspecific action. Uncertainty regarding the requirement for Gq coupling is not limited to the TRH receptor, because U73122 inhibits the internalization of muscarinic receptors (25), and sequestration of bombesin receptors has been reported to require G protein interaction (24).

It was not previously known whether receptors needed to couple to Gq either to promote an activated receptor conformation or to promote phosphorylation by receptor kinases to bind β-arrestin. Our results, which show that GFP-β-arrestin moves to the membrane in response to TRH in Fq/11 cells, confirm the role of β-arrestin in TRH receptor sequestration and prove that the process does not require G protein interaction. By demonstrating that GFP-β-arrestin does not bind to the carboxyl-terminal-truncated C335STOP receptor, we have established the molecular basis for its failure to internalize. These results suggest that phosphorylation sites between Thr-342 and Ser-391 are important for β-arrestin binding and receptor sequestration. Although β-arrestin appears to be involved in the internalization of most G protein-coupled receptors, there are exceptions, including the Gq-coupled M1 and M3 muscarinic receptors (36).

The maximal extent of TRH receptor sequestration was relatively low in the Fq/11 cells and in wild type mouse fibroblasts, below 40%, whereas the extent of internalization is above 80% when the receptor is expressed in HEK293, HeLa, or C6 cells (16, 18). Furthermore, TRH receptor sequestration in Fq/11 cells was increased by overexpression of β-arrestin. Because the extent of internalization correlates with the level of

**Fig. 9. Effect of the TRH antagonist chlordiazepoxide on GFP-β-arrestin localization.** Fq/11 cells were transfected with GFP-β-arrestin conjugate and TRH receptor. The following drugs were added in sequence to transfected cells: 10 μM chlordiazepoxide, 10 nM TRH, and 10 μM TRH at 37 °C. Shown are two cells before treatment (A), 20 min after the addition of 10 μM chlordiazepoxide (B), 1 min after 10 nM TRH (C), and 1 min after 10 μM TRH (D). Note that the two cells moved and changed shapes slightly during the 20-min chlordiazepoxide treatment, as do control cells, but the localization of GFP-β-arrestin did not change.

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expression of β-arrestin and receptor kinases in a number of cell lines (37), it seems likely that β-arrestin was expressed at low levels in the Fq/11 cells. The failure of the V53D mutant β-arrestin to act as a dominant negative is not completely surprising, because the dominant negative effect is only partial for the β2-adrenergic receptor (5, 7).

Previous work had shown that internalization of Gs- and Gq,G11-coupled receptors can occur without G protein involvement. The β2-adrenergic receptor can internalize in cells lacking Gαs (38, 39), and at least some Gq,G11-coupled receptors can internalize normally in pertussis toxin-treated cells (40). We have now shown that β-arrestin binding and internalization of Gq-coupled receptors can also occur in the absence of G protein coupling. β-Arrestin apparently discriminates between the agonist-activated and the inactive TRH receptor either by recognizing the active conformation of the receptor or by recognizing a protein that interacts with the activated receptor. Further work will be required to establish the molecular basis of this process.

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