The G protein-coupled thyrotropin-releasing hormone (TRH) receptor is phosphorylated and binds to β-arrestin after agonist exposure. To define the importance of receptor phosphorylation and β-arrestin binding in desensitization, and to determine whether β-arrestin binding and receptor endocytosis are required for receptor dephosphorylation, we expressed TRH receptors in fibroblasts from mice lacking β-arrestin-1 and/or β-arrestin-2. Apparent affinity for [3H]MeTRH was increased 8-fold in cells expressing β-arrestins, including a β-arrestin mutant that did not permit receptor internalization. TRH caused extensive receptor endocytosis in the presence of β-arrestins, but receptors remained primarily on the plasma membrane without β-arrestin. β-Arrestins strongly inhibited inositol 1,4,5-trisphosphate production within 10 s. At 30 min, endogenous β-arrestins reduced TRH-stimulated inositol phosphate production by 48% (β-arrestin-1), 71% (β-arrestin-2), and 84% (β-arrestins-1 and -2). In contrast, receptor phosphorylation, detected by the mobility shift of deglycosylated receptor, was unaffected by β-arrestins. Receptors were fully phosphorylated within 15 s of TRH addition. Receptor dephosphorylation was identical with or without β-arrestins and almost complete 20 min after TRH withdrawal. Blocking endocytosis with hypertonic sucrose did not alter the rate of receptor phosphorylation or dephosphorylation. Expressing receptors in cells lacking Goα and Go11, or inhibiting protein kinase C pharmacologically did not prevent receptor phosphorylation or dephosphorylation. Overexpression of dominant negative G protein-coupled receptor kinase-2 (GRK2), however, retarded receptor phosphorylation. Receptor activation caused translocation of endogenous GRK2 to the plasma membrane. The results show conclusively that receptor dephosphorylation can take place on the plasma membrane and that β-arrestin binding is critical for desensitization and internalization.

The type 1 thyrotropin-releasing hormone (TRH) receptor is a seven-transmembrane helix protein that regulates thyroid stimulating hormone and prolactin release from the anterior pituitary. Upon exposure to agonist, the TRH receptor stimulates phospholipase Cβ through coupling to the GTP-binding proteins Goα or Go11, leading to the formation of inositol 1,4,5-trisphosphate (IP3) and the subsequent release of Ca2+ from the endoplasmic reticulum. This signaling pathway ultimately leads to the release of thyroid stimulating hormone and is essential for proper thyroid function and targeted deletion of the type 1 TRH receptor results in hypothyroidism (1).

When activated, most G protein-coupled receptors (GPCRs) undergo phosphorylation, which is followed by receptor interaction with β-arrestins and desensitization and endocytosis (2). Phosphorylation is carried out by second messenger-activated kinases, such as protein kinase C, or by G protein-coupled receptor kinases (GRKs). Less is known about dephosphorylation, but it is believed to be a crucial step in the desensitization of GPCRs. Dephosphorylation of the well studied β2-adrenergic receptor is reported to take place in acidified endosomes (3).

As with most GPCRs, the activity of the TRH receptor is modulated by β-arrestins, which influence desensitization (4) and receptor trafficking (5–11). Following agonist binding, the TRH receptor becomes phosphorylated and rapidly recruits β-arrestin to the plasma membrane. The receptor–β-arrestin complex moves to pre-existing clathrin-coated regions (12) and undergoes rapid and extensive internalization (7, 13–16). Following endocytosis, TRH receptors and β-arrestin are colocalized in cytoplasmic vesicles. The two non-visual arrestins, β-arrestin 1 and β-arrestin 2, are ubiquitously expressed (17, 18) and capable of interacting with the TRH receptor (10, 19, 20). In general, GPCRs that internalize with β-arrestin are extensively degraded rather than recycled. The TRH receptor is an exception to this rule, because it recycles extensively following hormone withdrawal. Internalization of the TRH receptor is inhibited by dominant negative forms of β-arrestin and dynamin, as well as by truncation of the cytoplasmic tail of the receptor, which contains multiple phosphorylation sites. Mutant forms of β-arrestin that bind in a phosphorylation-independent manner promote agonist-independent internalization of TRH receptors lacking several potential phosphorylation sites in the carboxyl tail (14).

A number of critical questions remain unanswered for the TRH receptor and many other GPCRs. One is whether the receptor becomes uncoupled from G proteins as a consequence of phosphorylation, β-arrestin association, or both. Another is whether phosphorylation and reactivation of the receptor require endocytosis, which would be predicted if the relevant phosphatase acts only on receptors in acidified endosomes, or whether dephosphorylation can take place at the plasma membrane. An additional uncertainty is whether the association of receptor with β-arrestin prolongs desensitization by blocking access of phosphatases to phosphorylated residues on the receptor. Here, we take advantage of fibroblasts from mice lacking β-arrestin 1 (βArr1KO), β-arrestin 2 (βArr2KO), or β-arrestins 1 and 2 (βArr1/2KO) and from wild-type (wt) littermates (21) to address these questions and analyze...
the roles of β-arrestins in desensitization, internalization, and dephosphorylation of the TRH receptor.

EXPERIMENTAL PROCEDURES

Cell Cultures and Transfection—Mouse embryo fibroblasts (MEFs) from mice lacking β-arrestins were provided by Dr. Robert Lefkowitz (Duke University, Durham, NC). Goα11 KO MEFs derived from embryos lacking the α subunits of Gαq/11 were provided by Dr. Melvin Simon (California Institute of Technology, Pasadena, CA). MEFs, COS, and CHO cells were maintained in Dulbecco’s modified Eagle’s medium/F-12 with 5–10% fetal bovine serum and grown as monolayers in humidified 95% air and 5% CO2 at 37 °C.

We transiently transfected cells using Lipofectamine (Invitrogen) or FuGENE (Roche Diagnostics) following manufacturers’ instructions. Cells were transfected with plasmids encoding TRH receptors tagged at the amino terminus with either hemagglutinin (HA) or FLAG epitopes (13), β-arrestin 2 (provided by Dr. Marc Caron, Duke University, Durham, NC), β-arrestin 1 (provided by Dr. Vsevolod Gurevich, Vanderbilt University, Nashville, TN), β-arrestin 1 ΔLIEFD/E391A and wild type and K220R GRK2 (provided by Dr. Jeffrey Benovic, Thomas Jefferson University, Philadelphia, PA), green fluorescent protein (GFP) and/or HcRed (Clontech, Palo Alto, CA). When cells were transfected with cDNA encoding arrestins, a plasmid encoding GFP, a soluble protein of similar size, was used as a control. Cells were maintained in serum-containing media until assayed 24–48 h after transfection. Transfection efficiencies, which were estimated by transfecting with cDNA encoding GFP and staining nuclei with cell-permeable Hoechst 33342 (Molecular Probes, Eugene, OR), varied from 11 to 19% in β-arrestin MEFs, 8–11% in Goα11 KO MEFs, and 45–55% in COS cells at different times. We found no consistent differences in transfection efficiencies between βArr1KO, βArr2KO, βArr1/2KO, and wt MEFs. βArr1/2KO cells stably expressing GFP-tagged TRH receptor were generated by co-transfecting with pTK-Hyg plasmid (Clontech) conferring resistance to hygromycin B, then adding 250 μg/ml hygromycin B (Invitrogen) 48 h after transfection and selecting stable clones.

Radioligand Binding—To measure specific TRH binding, cells were incubated in serum-free Dulbecco’s modified Eagle’s medium/F-12 or Hanks’ balanced salt solution containing [3H]MeTRH (72 Ci/mmol, PerkinElmer Life Sciences) for 30–90 min at 37 °C. Cells were then washed on ice three times with ice-cold saline and collected in 0.1% SDS, and radioactivity was measured by liquid scintillation counting. Protein concentrations were determined by using the Lowry method with bovine serum albumin as a standard. For Scatchard analysis, cells were incubated for 60 min with 0.67–20 nM [3H]MeTRH. Nonspecific binding, which was <5% of total binding, was measured in mock transfected dishes in all experiments.

Inositol Phosphate Accumulation—To measure total inositol phosphate accumulation, we labeled transfected cells either immediately or 24 h after transfection with 2–5 μCi/ml myo-[3H]inositol overnight in F-10 media with 5% fetal bovine serum. Cells were treated with 10 mM LiCl with or without TRH at 37 °C. Dishes were washed on ice, washed three times with ice-cold saline, and then incubated for 90 min in 50 mM formic acid at 4 °C. [3H]inositol phosphates were subsequently isolated by ion exchange chromatography (22).

Radioassay of IP3—IP3 mass was measured by treating 35-mm dishes with 1 μM TRH in Dulbecco’s phosphate-buffered saline solution for various times at 37 °C. Dishes were then placed on ice, and IP3 was extracted by addition of 3% trichloroacetic acid. Samples were extracted with 3.1 trifluorotrichloroethane/pcmethylamine, and IP3 was quantified using the Biotrak Assay System from Amersham Biosciences (Buckinghamshire, UK) according to the manufacturer’s instructions.

Immunoprecipitation and Deglycosylation—CHO cells expressing HA-tagged TRH receptor in 35-mm dishes or MEFs transfected in 60-mm dishes were treated as detailed. Immunoprecipitation of receptors was carried out as described (13). Briefly, cells were lysed on ice in 1 ml of lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% Triton X-100, pH 8.0) containing 1:1000 protease inhibitor mixture set III (Calbiochem) and phoshpatase inhibitors (10 mM sodium fluoride, 10 mM sodium pyrophosphate, and 100 mM sodium orthovanadate). After centrifugation, supernatants were incubated 12–18 h with 1:5000 monoclonal HA11 antibody (Covance, Berkeley, CA). After 1-h incubation with protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA), samples were washed four times with 1 ml of lysis buffer. Deglycosylation was performed using peptide N-glycosidase F (New England Biolabs, Beverly, MA) exactly as instructed by the manufacturer, and the reaction was terminated by addition of 5× sample buffer (250 mM Tris-HCl, 500 mM dithiothreitol, 10% SDS, 0.5% bромphenol blue, 50% glycerol, pH 6.8).

Electrophoresis and Immunoblotting—Immunoprecipitated and deglycosylated proteins were separated on a 10% polyacrylamide gel by SDS-PAGE as described (23), except that SDS-PAGE was performed at 85–110 V, and in one experiment samples were loaded onto 10–20% PAGEr Gold Tris-Glycine gels (Cambrex, Baltimore, MD) and run at 125 V.

Phosphorylation/Dephosphorylation—To measure TRH receptor phosphorylation, we treated transfected cells with 10 nM TRH for either 5 or 45 min, washed them three times with saline to remove excess agonist, then allowed the cells to recover for different times before they were harvested. We immunoprecipitated HA-tagged TRH receptors, deglycosylated, ran SDS-PAGE, and observed the mobility shift due to TRH receptor phosphorylation. To quantify and compare dephosphorylation rates in different cell types, the relative mobility of TRH receptor bands was measured using NIH Image version 1.63 or LabWorks Analysis Software (UVp, Upland, CA). Densitometry profiles graphing distance traveled versus density were generated for each lane. The mobility of the peak density was determined, and the relative mobility of TRH receptors in each lane was defined by assigning a value of 0 to the distance traveled by untreated TRH receptors and a value of 1 to the distance traveled by TRH receptors treated for 5 min with TRH.

Alkaline Phosphatase Treatment—To confirm that TRH receptor up-shift is due to phosphorylation, we immunoprecipitated HA-tagged TRH receptors from stably transfected CHO cells and incubated them with 0, 4, 20, or 100 units/ml calf intestine alkaline phosphatase (Calbiochem) for 1 h at 37 °C. Samples were then deglycosylated and separated by SDS-PAGE as described above.

Internalization—To follow internalization of the TRH receptor, we measured the acid resistance of specifically bound [3H]MeTRH. Cells were incubated in 5 nM [3H]MeTRH then washed on ice with ice-cold saline. Surface ligand was extracted with ice-cold acid/salt buffer (0.2 M acetic acid, 0.5 M NaCl, pH 2.5) and internalized ligand was extracted by solubilizing the cells in 0.1% SDS. Internalization was also measured by an enzyme-linked immunosorbent assay using minor modifications of the procedure described by Song and Hinkle (24).

We also imaged receptors in βArr1/2KO MEFs stably expressing a GFP-tagged TRH receptor and transiently transfected with β-arrestin 2 and HcRed. Cells were plated on ECL (Upstate, Chicago, IL)-coated coverslips, transfected, stimulated with 1 μM TRH, and imaged on a fluorescence microscope as described previously (9).

Translocation of GRK2—CHO cells stably transfected with TRH receptor in 6-cm dishes were incubated for 1 h in Hanks’ balanced salt...
TRH Receptor Phosphorylation and Dephosphorylation

FIGURE 1. TRH binding and responses in MEFs. A and B, wt, βArr1KO, βArr2KO, or βArr1/2KO MEFs were transfected with TRH receptor. A, TRH receptor concentrations were compared by measuring specific binding of 5 nM [3H]MeTRH. B, cells from the same experiment as in A were metabolically labeled with [3H]inositol overnight and then incubated for 30 min with 10 mM LiCl with or without 1 μM TRH and total [3H]inositol phosphates were determined. [3H]inositol phosphates in unstimulated wt, βArr1KO, βArr2KO, or βArr1/2KO MEFs were 800, 505, 417, and 800 cpm, respectively. C, wt MEFs were transfected with TRH receptor, and βArr1/2KO MEFs were co-transfected with TRH receptor and either β-arrestin 2 or GFP (as control). TRH-stimulated [3H]inositol phosphate production was measured. [3H]inositol phosphates were 793, 1182, and 1058 cpm in unstimulated wt MEFs or βArr1/2KO MEFs without or with β-arrestin 2, respectively. D and E, βArr1/2KO MEFs were co-transfected with TRH receptor and either control plasmid (open circles) or β-arrestin 2 (filled diamonds). D, TRH-stimulated [3H]inositol phosphates were measured. [3H]inositol phosphate production in unstimulated cells was 2343 (βArr2) and 2119 (control) cpm. E, cells were stimulated by addition of 1 μM TRH for 0–60 s, and IP3 mass was measured.

solution then stimulated with 1 μM TRH. Cells were placed on ice, and 3 ml of ice-cold TM buffer (20 mM Tris-HCl, 2 mM MgCl2, pH 7.6) was added, dishes were scraped, and cells were allowed to swell for 20 min before being briefly vortexed and then centrifuged at 3000 × g for 10 min at 4 °C to separate cytosolic (supernatant) and membrane (pellet) fractions. Pellets were resuspended by vortexing vigorously in 1 ml of TM buffer. GRK2 was immunoblotted with 1:500 polyclonal anti-GRK2 (C-15) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-rabbit horseradish peroxidase-linked secondary.

Other—All experiments were repeated a minimum of three times except those shown in Figs. 1E and 8A, which were performed twice. All points represent the mean and standard error of at least triplicate determinations, except in Fig. 1A and the βArr1/2KO points in Fig. 6C, which show the mean and range of duplicate determinations. Where error bars are not visible, they were within symbol size.

RESULTS

Desensitization of the TRH Receptor by β-Arrestins—To assess the role of β-arrestins in the desensitization of TRH receptor signaling through phospholipase Cβ, we expressed the TRH receptor in wt, βArr1KO, βArr2KO, or βArr1/2KO MEFs. When cells were incubated with 5 nM [3H]MeTRH, specific binding per milligram of protein was consistently higher in cells expressing at least one β-arrestin subtype (Fig. 1A). We metabolically labeled cells from the same experiment with [3H]inositol and measured TRH-stimulated production of [3H]inositol phosphates over 30 min. Expression of either β-arrestin reduced TRH-stimulated production of [3H]inositol phosphates (Fig. 1B). In comparison to the response in the absence of either β-arrestin, the TRH response was inhibited by 84% in wt cells expressing both β-arrestins, 48% in cells expressing only β-arrestin 1 and by 71% in cells expressing only β-arrestin 2. This result agrees with the report that β-arrestin 2 interacts with the TRH receptor more effectively than β-arrestin 1 based on the ability to promote endocytosis (20). β-Arrestins 1 and 2 are present at approximately equal concentrations in the cell lines used (21).

To eliminate the chance that differences in inositol phosphate production could be due to differences in the cell types rather than the activity of the TRH receptor, we also co-transfected βArr1/2KO MEFs with TRH receptor and β-arrestin 2. β-Arrestin 2 reduced the TRH response to that seen in wt cells in the same experiment (Fig. 1C). The magnitude of the TRH response varied among experiments, presumably due to variation in transfection efficiency, but in every instance expression of β-arrestin greatly reduced the TRH-stimulated increase in [3H]inositol phosphates. In numerous experiments using Western blots and enzyme-linked immunosorbent assays with and without permeabilization to quantify total and surface receptors we found that the concentration of TRH receptors was either not changed or slightly increased by co-expression of β-arrestin.

The effect of TRH concentration on total inositol phosphate production in βArr1/2KO co-transfected with TRH receptor and either β-arrestin 2 or control plasmid is shown in Fig. 1D. The dose-response curve for MEFs with β-arrestin 2 was shifted to the left, but the maximal response was lower.

To determine how rapidly β-arrestins desensitize TRH signaling, we measured IP3 mass by radioreceptor assay 10, 30, or 60 s after addition of TRH or vehicle. It was not feasible to measure the TRH-induced increase in total inositol phosphates at early time points because the signal was too small. At 10 s, TRH caused a 7.6-fold increase in IP3 in βArr1/2KO cells compared with a 3.4-fold increase in the same cells co-transfected with β-arrestin 2 (Fig. 1E). The increase in IP3 in wt MEFs was similar to that seen in βArr1/2KO cells overexpressing β-ar-
restin 2 (data not shown). These results show that β-arrestin powerfully inhibits TRH signaling within 10 s.

Expression of TRH Receptors in MEFs—To study the role of β-arrestins in the regulation of the TRH receptor, we transiently expressed the receptor in βArr1KO, βArr2KO, βArr1/2KO, and wt MEFs. Because β-arrestins increased [3H]MeTRH binding (Fig. 1A) and have been reported to increase the binding affinity of receptors for their agonists (25, 26), we carried out equilibrium binding studies in live TRH receptor and either β-arrestin 1, βArr1ΔLIEFD/F391A, or control plasmid. B, cells were treated with or without 100 nm TRH for 30 min, then internalization of TRH receptors was measured by enzyme-linked immunosorbent assay. C, TRH-stimulated [3H]inositol phosphates were determined as described for Fig. 1. [3H]inositol phosphates in unstimulated MEFs transfected with control plasmid, β-arrestin 1, or βArr1ΔLIEFD/F391A were 363, 478, and 452 cpm, respectively. D, specific binding of [3H]MeTRH was measured.

TRH Receptor Phosphorylation and Dephosphorylation

Impaired Internalization in Cells Lacking β-Arrestins—To visualize TRH receptor internalization after agonist treatment, a line of βArr1/2KO MEFs stably expressing a GFP-tagged TRH receptor was prepared. These cells were co-transfected with β-arrestin 2 and HcRed, allowing easy identification of successfully transfected (red) cells (Fig. 3A). In cells expressing β-arrestin 2, TRH caused distinct receptor internalization by 10 min and extensive endocytosis after 30 min, as indicated by the punctate appearance of GFP-TRH receptor in endosomes. In contrast, TRH caused minimal changes in receptor localization in cells lacking any β-arrestin.

We also measured internalization in wt and βArr1/2KO MEFs transiently transfected with TRH receptor with or without β-arrestin 2 by measuring the percent of specifically bound [3H]MeTRH in an acid-resistant (internalized) form at intervals. Internalization was slower and less extensive in the absence of β-arrestins, reaching a maximum of only 25% in 15 min in βArr1/2KO cells compared with 45% in wt MEFs and 65% in βArr1/2KO MEFs overexpressing β-arrestin 2 (Fig. 3B).

Effect of β-Arrestin Concentration—To determine the dependence of desensitization and internalization on β-arrestin concentration, βArr1/2KO MEFs were transiently co-transfected with TRH receptors and increasing amounts of β-arrestin 2. Internalization after 30 min was measured as a percentage of specifically bound [3H]MeTRH resistant to a low pH, high salt wash (Fig. 4A). Internalization was slower and less extensive in the absence of β-arrestins, reaching a maximum of only 25% in 15 min in βArr1/2KO cells compared with 45% in wt MEFs and 65% in βArr1/2KO MEFs overexpressing β-arrestin 2 (Fig. 3B).

Phosphorylation and Dephosphorylation of TRH Receptor—There are 38 serine and threonine residues on the cytoplasmic surface of the rat TRH receptor that are potential phosphorylation sites, using the membrane boundaries suggested by Gershengorn and Osman (28). Although the carboxy-terminal tail is required for phosphorylation (13, 14), the
sites of agonist-induced phosphorylation have not been definitively identified. To measure receptor phosphorylation, we took advantage of the finding that phosphorylated TRH receptor migrates more slowly than non-phosphorylated TRH receptor on SDS-PAGE, as shown for CHO cells by 32P labeling (13).

We incubated CHO cells stably expressing HA-tagged TRH receptors (8) for 5 min with different concentrations of TRH, then immunoprecipitated receptors, deglycosylated, and resolved on SDS-PAGE. When immunoprecipitated receptor was incubated with alkaline phosphatase prior to deglycosylation, the mobility of the receptor reverted to that seen in naïve cells, confirming that the up-shift results from phosphorylation (Fig. 5A). Importantly, the TRH-dependent change in mobility of the homodimer and monomer bands of the TRH receptor was parallel in this and all other experiments. Because the overlapping mobility of the homodimer and monomer bands of the TRH receptor was also toxic. As shown in Fig. 5B, the fraction of specifically bound hormone internalized was measured using resistance to an acid/salt wash.

We asked whether β-arrestins affect the phosphorylation state of the TRH receptor by expressing HA-tagged TRH receptors in wt and βArr1/2KO MEFs. Cells were exposed to TRH for either 5 or 45 min and then washed to remove excess agonist and allowed to recover for different times before cells were harvested. In both cell types, TRH treatment caused a marked up-shift due to phosphorylation within 15 s and TRH receptor remained phosphorylated for at least an hour as long as TRH was present (Fig. 6A and data not shown). Between 5 and 10 min after TRH was removed, the TRH receptor band was halfway between fully phosphorylated (up-shifted) and non-activated (non-shifted) forms (compare lanes 5 and 6 with lanes 2–4, Fig. 6A). Following TRH withdrawal, nearly complete dephosphorylation was seen by 20 min in both wild-type and βArr1/2KO cells. Data from three independent experiments were averaged by normalizing mobility of the bands and showed a consistent time course and pattern of phosphorylation (Fig. 6B).

It is reported that dephosphorylation of the β2-adrenergic receptor depends on the movement of receptor to an acidified endosome (3). We asked whether internalization is required for the dephosphorylation of TRH receptor by blocking endocytosis with 0.44 mM sucrose, which prevents formation of clathrin-coated vesicles (29), in wt and βArr1/2KO MEFs. Dishes were again incubated with TRH, washed, and incubated without peptide for various times. Blocking internalization with hypertonic sucrose did not alter the extent of phosphorylation or the rate of dephosphorylation (Fig. 6C) but effectively blocked the internalization of [3H]MeTRH in wt MEFs, as shown in TABLE ONE.

At least five of the Ser/Thr residues in the cytoplasmic surface are potential substrates for protein kinase C, which becomes active after the TRH receptor activates phospholipase Cβ through Gαq11 signaling. To test whether phosphorylation of the TRH receptor depends on signal transduction and subsequent activation of protein kinase C or other downstream kinases, we expressed the receptor in MEFs from mice lacking both Gαq and Gα11. The TRH receptor does not generate a calcium signal in these cells but does undergo hormone-dependent endocytosis (16). When the TRH receptor was expressed in these
GRK2 on TRH-dependent incorporation of 32P into receptors, mobility shift, or receptor internalization (13). Dominant negative GRK2 probably had no effect in that study because the cells were exposed to 1 μM TRH for 5 min, conditions where the inhibitory effect would have been overcome based on data shown in Fig. 8A. In wt and Arr1/2KO cells, and filled squares show wild-type cells.

FIGURE 5. Effects of alkaline phosphatase, phosphatase inhibitor, and TRH on TRH receptor mobility. A–C, CHO cells stably expressing HA-tagged TRH receptor were used. A, cells were treated for 5 min with or without 1 μM TRH, and then receptors were immunoprecipitated and incubated with calf intestine alkaline phosphatase (CIAP) for 1 h at 37 °C as shown and deglycosylated and resolved on SDS-PAGE. B, cells were incubated with 10 nM calyculin A or vehicle (0.2% Me2SO) for 5 min, then 100 nM TRH was added or not for 5 min. C, cells were treated for 10 to 5 min with the concentrations of TRH shown. In B and C, receptors were immunoprecipitated and deglycosylated.

Many GPCRs are phosphorylated by GRKs, a family of protein kinases that recognize activated receptors (30). Of the seven known GRKs, four are ubiquitously expressed: GRK2, -3, -5, and -6. GRK2 and -3 are found in the cytosol in unstimulated cells and are recruited to the plasma membrane by activated G protein βγ subunits. When wild-type or dominant negative K220R (kinase-dead) GRK2 and TRH receptor were co-expressed in βArr1/2KO MEFs, dominant negative GRK2 inhibited phosphorylation of the TRH receptor caused by 100 nM TRH over short time periods, 20–60 s, evidenced by the lack of an up-shift in lanes 4 and 6 of Fig. 8A. Note that at 3 and 9 min, TRH receptors had become phosphorylated (lanes 8 and 10). In a previous study using HEK293 cells expressing TRH receptors, we saw no effect of K220R GRK2 on TRH-dependent incorporation of 32P into receptors, mobility shift, or receptor internalization (13). Dominant negative GRK2 probably had no effect in that study because the cells were exposed to 1 μM TRH for 5 min, conditions where the inhibitory effect would have been overcome based on data shown in Fig. 8A.

To rule out the possibility that the involvement of GRK2 in TRH receptor phosphorylation was the result of overexpression, we asked whether endogenous GRK2 translocated from the cytosol to the plasma membrane in response to TRH. CHO cells stably expressing TRH receptors were incubated with TRH for various times before cells were placed on ice, homogenized, and membrane and cytoplasmic fractions separated and analyzed for GRK2 by immunoblotting. GRK2 moved transiently from the cytosol to the plasma membrane, with peak membrane levels found 3–10 s after TRH addition in different experiments (Fig. 8, B and C).

FIGURE 6. Effects of arrestins on TRH receptor phosphorylation and dephosphorylation. A, wt or βArr1/2KO MEFs transfected with HA-tagged TRH receptor were treated with 100 nM TRH for 0–45 min. After 5 min, some dishes were washed to remove TRH and then allowed to recover in the absence of hormone for 0–45 min. TRH receptors were immunoprecipitated, deglycosylated, and resolved on SDS-PAGE. C, cells were incubated as in A, except that 0.44 M sucrose was added 20 min before and during the experiment. B and C, densitometric analysis of gels was performed as described under “Materials and Methods.” Open circles show βArr1/2KO cells, and filled squares show wild-type cells.

DISCUSSION

In this report, we have demonstrated the essential role of β-arrestins in both TRH signaling and TRH receptor trafficking by expressing the receptor in embryonic fibroblasts from normal animals or from mice lacking β-arrestins 1 and 2. The concentration of TRH receptor protein was similar in knock-out and wild-type fibroblasts, but the apparent affinity of receptors for TRH was 8-fold lower in β-arrestin knock-out cells. The increased affinity in intact cells is not due to internalization, because a mutant β-arrestin that does not support internalization still decreased the apparent Kd of the TRH receptor. Accordingly, the rate of dissociation of [3H]MeTRH from βArr1/2KO MEFs is much faster than from wt MEFs (data not shown). Our results in experiments using intact cells agree with reports for four other GPCRs that the β-arrestin-phosphoreceptor complex has higher affinity for agonist than the receptor alone (25, 26).

The diminished TRH signal strength with β-arrestin expression in the present studies implies that there is strong desensitization of TRH
TRH receptor phosphorylation and dephosphorylation

![Image](https://example.com/figure7.png)

**TABLE ONE**

| Effect of sucrose on internalization of [3H]MeTRH in MEFs |
|-----------------|-----------------|
| MEFs transfected with HA-tagged TRH receptor were incubated in media with or without 0.44 s sucrose for 20 to 30 min when media with 5 nM [3H]MeTRH was added for 30 min. Internalization was measured by acid/salt resistance. |
| Cell type | Sucrose | Internalization % |
|----------|---------|--------------------|
| wt       | No      | 44.6 ± 0.5         |
| wt       | Yes     | 14.0 ± 1.1         |
| βAr1/2KO | No      | 20.8 ± 1.1         |
| βAr1/2KO | Yes     | 11.4 ± 0.6         |

FIGURE 7. Phosphorylation and dephosphorylation of the TRH receptor in the absence of signaling. A, Gsα11KO MEFs were transfected with HA-tagged TRH receptors and treated with or without 100 nM TRH for 5 min. One dish was washed to remove TRH and allowed to recover for 20 min. B, CHO cells stably expressing HA-tagged TRH receptor were incubated for 1 h with or without 1 μM GF109203 or 30 min with or without 1 μM phorbol 12-myristate 13-acetate, and then 100 nM TRH was added or not for 5 min before lysing the cells. Receptors in A and B were immunoprecipitated, deglycosylated, and resolved on SDS-PAGE.

The inositol phosphate response to TRH was decreased by 84% in cells expressing endogenous levels of arrestins compared with the response in cells expressing no arrestin, when equivalent numbers of receptors were activated. Because transfection efficiencies were under 20% and non-transfected cells contributed to background inositol phosphate levels, it is likely that the TRH response and its desensitization are significantly underestimated.

There have been considerable discrepancies among reports of TRH receptor desensitization. When desensitization has been measured by comparing the accumulation of [3H]-labeled inositol phosphates over time in cells that have or have not been previously exposed to TRH, the extent of desensitization has varied from 0 to 50% in pituitary cells (31, 32) and 0 to 15% in COS, HeLa, KB, and HEK293 cells (32). When the peak increases in IP$_3$ mass (4) and calcium (33) have been measured, more profound TRH receptor desensitization has been detected. Our current results, obtained with the entirely different approach of comparing responses with and without β-arrestin, strengthen the conclusion that the TRH signaling pathway is subject to powerful desensitization.

In pituitary cells expressing TRH receptors, continuous exposure to TRH causes an increase in IP$_3$ concentration that peaks within 10 s, but then falls within 1 min to remain at approximately twice the basal level for at least 10 min (4). Desensitization of the TRH response is rapid and controlled upstream of phospholipase C activity (4, 34), most likely because the receptor is uncoupled from G protein as a consequence of phosphorylation and β-arrestin binding. In the present experiments, we anticipated that IP$_3$ production might begin at similar rates in cells with or without β-arrestin, and then slow down in cells expressing β-arrestin.

Instead, we found that the ability of TRH to increase IP$_3$ was much lower in cells expressing β-arrestins as early as 10 s, and the difference was maintained over at least 30 min. Thrombin-mediated increases in inositol phosphates are likewise inhibited by β-arrestin expression (35).

The TRH receptor was completely phosphorylated within 10 s in both wild-type and arrestin knock-out cells, so it is β-arrestin binding, not simply phosphorylation, that accounts for the lower signaling in cells expressing β-arrestins. TRH receptor activation, recruitment of receptor kinase, phosphorylation, and β-arrestin translocation all take place within seconds in cells expressing endogenous levels of β-arrestin. These results are consistent with a report by Groarke et al. (7), who found that GFP-tagged β-arrestin 1 translocated to the plasma membrane within 30 s upon addition of TRH. Similar concentrations of β-arrestin were required for desensitization and internalization of receptors in arrestin-null cells. Additionally, because TRH responses were inhibited by expression of either β-arrestin 1 or β-arrestin 2, we can conclude that either arrestin is capable of causing desensitization. Desensitization is reduced when the cytoplasmic tail of the receptor is removed or shortened, consistent with the importance of this region for β-arrestin binding (11, 36). These results provide conclusive evidence that β-arrestin is critical for the rapid desensitization of the TRH receptor seen within seconds and the prolonged desensitization seen over 30 min.

Internalization of the TRH receptor has been reported to require clathrin, dynamin, and β-arrestin (5–8, 10, 19), and we found that internalization was much slower in MEFs lacking β-arrestin than in wild type cells. There was, however, some internalization of the TRH receptor in...
cells devoid of β-arrestins as measured by resistance to low pH and microscopy. Hypertonic sucrose blocked the small amount of TRH-dependent receptor internalization in β-arrestin knock-out cells, implicating a clathrin- and dynamin-requiring pathway. Some GPCRs that require β-arrestin to desensitize can undergo β-arrestin-independent but clathrin/dynamin-dependent internalization (35, 37, 38), and it is possible that a similar pathway accounts for the low level of TRH receptor internalization in β-arrestin knock-out cells.

Treatment of phosphorylated TRH receptors with increasing concentrations of alkaline phosphatase exposed a form with mobility intermediate between the fully phosphorylated and non-activated forms. This mid-shifted band was also seen in cells allowed to recover after agonist treatment, so it appears to be a normal intermediate in TRH receptor processing. Our observation of three mobilities implies that the TRH receptor has at least two phosphorylation states, possibly following a pattern of hierarchical phosphorylation similar to that recognized in a growing number of GPCRs (39–43).

It has been shown that the β2-adrenergic receptor is dephosphorylated by a membrane-associated phosphatase that only dephosphorylates receptors on acidic endosomes where the low pH allows for an appropriate receptor conformation (3). Internalization, therefore, is necessary for β2-adrenergic receptor dephosphorylation. This does not appear to be the case for the TRH receptor, because internalization of receptors in β-arrestin knock-out cells was greatly impaired while dephosphorylation was unaffected in comparison to wild-type cells. Furthermore, inhibiting internalization with hypertonic sucrose did not retard dephosphorylation. Our results are similar to that found with the D1 dopamine receptor (44), which becomes dephosphorylated at the plasma membrane.

In summary, we have shown that essentially all TRH receptors become phosphorylated very rapidly in response to TRH but that it is the binding of β-arrestins that is critical for desensitization. Strong desensitization of TRH receptors occurs within seconds, and both β-arrestin 1 and β-arrestin 2 are effective. Although β-arrestins are required for extensive internalization of the TRH receptor, they do not affect the rate of receptor dephosphorylation, which can take place while the receptor is localized on the plasma membrane. Additional work is needed to identify the sites of phosphorylation that are required for β-arrestin binding as well as to explore possible involvement of additional kinase(s) and to identify the phosphatase(s) involved.

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TRH Receptor Phosphorylation and Dephosphorylation

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