THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 31, Issue of August 2, pp. 28228–28237, 2002

Printed in U.S.A.

Dimerization and Phosphorylation of Thyrotropin-releasing Hormone Receptors Are Modulated by Agonist Stimulation*

Received for publication, April 30, 2002
Published, JBC Papers in Press, May 22, 2002, DOI 10.1074/jbc.M204221200

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Dimerization and phosphorylation of thyrotropin-releasing hormone (TRH) receptors was characterized using HEK293 and pituitary GHFT cells expressing epitope-tagged receptors. TRH receptors tagged with FLAG and hemagglutinin epitopes were co-precipitated only if they were co-expressed, and 10–30% of receptors were isolated as hemagglutinin/FLAG-receptor dimers under basal conditions. The abundance of receptor dimers was increased when cells had been stimulated by TRH, indicating that TRH either stabilizes pre-existing dimers or increases dimer formation. TRH increased receptor dimerization and phosphorylation within 1 min in a dose-dependent manner. TRH increased phosphorylation of both receptor monomers and dimers, documented by incorporation of ^{32}P and an upshift in receptor mobility reversed by phosphatase treatment. The ability of TRH to increase receptor phosphorylation and dimerization did not depend on signal transduction, because it was not inhibited by the phospholipase C inhibitor U73122. Receptor phosphorylation required an agonist but was not blocked by the casein kinase II inhibitor apigenin, the protein kinase C inhibitor GF109203X, or expression of a dominant negative form of G protein-coupled receptor kinase 2. TRH receptors lacking most of the cytoplasmic carboxyl terminus formed dimers constitutively but failed to undergo agonist-induced dimerization and phosphorylation. TRH also increased phosphorylation and dimerization of TRH receptors expressed in GHFT pre-lactotroph cells.

The TRH[1] receptor belongs to the superfamily of seven-transmembrane-helix G protein-coupled receptors (GPCRs) and plays a key role in maintaining proper function of the thyroid gland (1, 2). Two subtypes of TRH receptors, termed type 1 and 2, have been identified (3–5). Although both receptor types are detected in various tissues at different levels (6–8), the type 1 TRH receptor is primarily expressed in thyrotrophs and lactotrophs in the anterior pituitary gland, and its activation stimulates the secretion of TSH and prolactin, at least in part, by raising the intracellular calcium concentration (9, 10). The TRH receptor, once occupied by agonist, activates phospholipase C through $G_{q/11}$, leading to the formation of inositol 1,4,5-trisphosphate ($InsP_3$), which causes an elevation of intracellular calcium by mobilizing an $InsP_3$-sensitive Ca^{2+} store in the endoplasmic reticulum (10, 11).

In conventional models, GPCRs have been thought to function as monomers that bind one molecule of ligand and then activate one heterotrimeric G protein to turn on the cognate signaling pathway (12–16). However, evidence indicating that GPCRs can form homo- or heterodimers, pairing with the same receptor type, different subtypes within the same receptor family, or even distinct classes of receptors, has begun to emerge. Receptor dimerization has been documented for a wide variety of GPCRs including the β_2-adrenergic receptor (17, 18), Ca^{2+}-sensing receptor (19, 20), muscarinic m3 receptor (21), γ-aminobutyric acid $GABA_B$ receptor (22–25), opioid receptor (25–27), GnRH receptor (28, 29), and others (26, 30–36). The ligand binding and signaling properties of a number of GPCRs are modified as a result of receptor dimerization, suggesting functional relevance to this phenomenon (18, 22, 26, 31, 37–42). Thus, GPCR dimerization appears to underscore a novel mechanism of modulating GPCR-mediated signal transduction or mediating "cross-talk" between different receptor families.

Eidne and co-workers (43) recently concluded that the TRH receptor forms dimers. Using transiently transfected COS cells, they found that bioluminescence resonance energy transfer occurs between receptors labeled with luciferase and yellow fluorescent protein and that TRH increases energy transfer (43), implying that ligand binding either alters receptor conformation to bring the two reporter groups in closer proximity or promotes receptor dimerization. TRH receptors have also been reported to run at the molecular weight of dimers on SDS-PAGE (44). One caveat in the interpretation of these experiments is that the receptors were overexpressed, which would be expected to favor oligomerization.

In the present study, we characterize dimerization and phosphorylation of epitope-tagged TRH receptors biochemically using human embryonic kidney HEK293 cells and a pituitary cell model system, both expressing receptors at levels no higher than those typical of endogenous pituitary receptors. We show that TRH receptor monomers and dimers are isolated from non-stimulated cells and that TRH increases receptor phosphorylation and dimerization in a dose- and time-dependent manner independent of signaling.

EXPERIMENTAL PROCEDURES

Materials—HEK293 cells were obtained from the American Type Culture Collection. Sources of equipment and reagents were: pcDNA3 (Invitrogen), primers (synthesized by Genosys), DeepVent DNA polymerase (New England BioLabs), restriction enzymes and LipofectAMINE

* This work was supported by National Institutes of Health Grant DK19974, a Wilmot Cancer Research Fellowship (to C.-C. Z.), and a Pharmaceutical Manufacturers' Association Advanced Predoctoral Fellowship (to L. B. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TRH, thyrotropin-releasing hormone; TRHR, TRH receptor; GPCR, G protein-coupled receptor; GRK, GPCR kinase; PNGaseF, *N*-glycosidase F; HA, hemagglutinin; CKII, casein kinase II.

(Invitrogen), Geneticin, TRH, GF109203X (bisindolylmaleimide I), and protease inhibitor mixture (Calbiochem), alkaline phosphatase, M2 monoclonal anti-FLAG antibody, apigenin, and protein A-conjugated Sepharose 4B-CL beads (Sigma), peptide *N*-glycosidase F (PNGaseF) (Roche Molecular Biochemicals), U73122 (Biomol), chlordiazepoxide (ICN Pharmaceuticals, Inc.), HA11 monoclonal anti-HA antibody (Covance), horseradish peroxidase-conjugated anti-mouse IgG (Amersham Biosciences), wheat germ agglutinin (Vector Labs), Renaissance chemiluminescence reagent (PerkinElmer Life Sciences), [^{3}H]MeTRH, [^{32}P]orthophosphate, and [^{3}H]inositol (PerkinElmer Life Sciences), fura2/AM (Molecular Probes), and mini-gel electrophoresis system (Bio-Rad). GHFT cells (45) were provided by Dr. Richard N. Day (University of Virginia Medical School, Charlottesville, VA), plasmids encoding GRK2 and dominant negative GRK2-K220R were provided by Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA), and an HA-tagged β_2-adrenergic receptor was provided by Dr. Richard Clark (University of Texas Health Science Center, Houston, TX).

Epitope Tagging of the TRH Receptor—Type 1 rat TRH receptor was tagged at its amino terminus with either two repeats of hemagglutinin (HA) nonapeptide (YPYDVPDYA) separated by a Gly residue or with two repeats of a FLAG sequence (DYKDDDDK), also separated by a Gly residue, using polymerase chain reaction as described previously (46). The forward primer for HA tagging carried a *Bam*HI recognition site at its 5′ end followed by a Kozak sequence (47), HA-coding sequences, and a sequence derived from the first 18 nucleotides of the TRH receptor cDNA. The reverse primer was complimentary to the last 23 nucleotides (for making the full-length receptor) or to the region between nucleotides 978 and 1002, with the addition of a stop codon (for making a mutant receptor truncated after Leu-334) of the receptor cDNA with a flanking *Xba*I recognition site at its 5′ end. The fragments were amplified from a plasmid carrying type 1 rat TRH receptor cDNA (48), digested with *Bam*HI and *Xba*I, and subcloned into a mammalian expression vector, pcDNA3, yielding *p*2HA-TRHR and *p*2HA-CTTRHR, respectively, which encode full-length and carboxyl domain-truncated 2HA-tagged type 1 rat TRH receptors. Because the receptor containing two FLAG epitopes at the amino terminus did not localize to the cell membrane based on radioligand binding and immunolocalization, we introduced a prolactin signal peptide preceding the FLAG sequence to create *p*Prol2FLAG-TRHR. Immunocytochemical analysis showed that the receptor encoded by this construct was expressed primarily on the cell membrane. All sequences were confirmed by nucleotide sequencing.

Cell Cultures and Transfection—HEK293 cell monolayers were grown in 6-cm dishes containing Dulbecco's modified Eagle's medium (DMEM) with 7.5% fetal bovine serum as described previously (49). GHFT cells were grown in DMEM/F-12 medium supplemented with 10% fetal bovine serum. Medium was changed every 2–5 days and replaced with serum-free medium 4–12 h before the cells were challenged with stimuli. Results were not different when serum starvation was omitted. For transient transfection, cell monolayers at ~80% confluence were washed twice with serum-free medium and then overlaid with 1.6 ml/dish of transfecting complex prepared from 2 μg of plasmid DNA and 16 μl of LipofectAMINE in serum-free medium. After a 5-h incubation, cells were washed once with serum-containing medium, and culture was subsequently resumed in 2.5 ml of the same medium. Experiments were conducted 2 or 5 days after transfection. To create cell lines stably expressing TRH receptors, cells were transfected as described above and either 400 (GHFT cells) or 500 (HEK293 cells) μg/ml Geneticin was added to the culture medium to start selection 24 h after transfection (50). Geneticin-resistant colonies were amplified and then screened for expression of TRH receptors; pools were then cloned. The established cell lines were maintained in the same media as the parental lines.

Preparation of Cell Membranes—Cells in 6-cm dishes were detached and harvested in 1 ml/dish of buffer containing 155 mM NaCl, 10 mM HEPES, and 1 mM EDTA, pH 7.4. After centrifugation at 500 × *g* for 1 min, cell pellets were resuspended in 300 μl of homogenization buffer containing 5 mM Tris-HCl, 2 mM EDTA, pH 7.4, plus 1:200 protease inhibitor mixture and then disrupted by 30 passages through 25-gauge needles. The unbroken cells were removed after centrifugation at 500 × *g* for 2 min, and the resulting supernatant was centrifuged at 14,000 × *g* for 10 min. The pellets were resuspended in 50 μl of homogenization buffer, and protein concentrations were quantified. Finally, the sample was mixed with an equal volume of sample buffer containing 100 mM Tris-HCl, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, 20% glycerol, pH 6.8, for further analysis.

Immunopurification of TRH Receptors—Cells in 6-cm dishes were solubilized by incubation for 30 min in 1 ml/dish of ice-cold lysis buffer containing 150 mM NaCl, 50 mM Tris base, 1 mM EDTA, 1% Triton X-100, pH 8.0, plus 1:200 protease inhibitor mixture. In the experiments shown in Figs. 4–8, phosphatase inhibitors were also included (10 mM sodium fluoride, 10 mM sodium pyrophosphate, 100 nM sodium orthovanadate, and 100 nM okadaic acid). The cell lysates were centrifuged at 14,000 × *g*, 4 °C, for 10 min. The supernatant fractions were collected and then incubated for 2–18 h at 4 °C with HA11 (1:5,000), a monoclonal antibody against HA, or M2 (1:5000), a monoclonal antibody against FLAG. The incubation was continued for another 2 h in the presence of protein A-conjugated Sepharose CL-4B (protein A beads) (5 mg/sample). The beads were washed 3 times with 1 ml of lysis buffer and, finally, resuspended in sample buffer, usually 75 μl.

Receptor Deglycosylation and Dephosphorylation—For deglycosylation (51), TRH receptors were immunopurified from a 6-cm dish of cells, and then the protein A beads were boiled for 2 min in 10 μl of 1% SDS. After dilution with 90 μl of 20 mM phosphate-buffered saline (pH 7.2), 50 mM EDTA, 0.5% Nonidet P-40, and 10 mM NaN_3, the receptors were incubated at 37 °C for 10 h in the presence of 0.5 units of peptide PNGaseF. Alternatively, immunoprecipitates were incubated for 2 h at 37 °C in 50 μl of lysis buffer containing 0.14 M β-mercaptoethanol, 10 mM EDTA, 10 mM sodium azide, 5 mM EDTA, and 500 units of PNGaseF. For dephosphorylation (52), immunopurified receptors from a 6-cm dish of cells were resuspended in 50 μl of 10 mM Tris-HCl, pH 8.0, and then incubated with up to 100 units/ml alkaline phosphatase at 37 °C for 30 min. Where noted, receptors were absorbed for 2–18 h at 4 °C on wheat germ agglutinin (20-μl suspension/1 ml of cell lysate) and then deglycosylated. In all enzymatic steps, control lanes show preparations that were incubated identically but without enzyme.

Electrophoresis and Immunoblots—Membrane preparations or immunopurified receptors were boiled for 2 min and resolved along with prestained molecular mass markers on 10% SDS-PAGE as described previously (36). When the boiling step was omitted and samples were run without heating or after heating to 37 °C, the ratio of monomers to dimers was not altered, but much of the receptor did not enter the gel. Proteins were transferred onto a nitrocellulose membrane, which was then subjected to two sequential 1-h incubations with primary (1:10,000 HA11, or M2) and secondary (1:2,000 horseradish peroxidase-conjugated anti-mouse IgG) antibodies, respectively, and immunoreactivity was detected by chemiluminescence. All blots are representative of experiments repeated 2–6 times. In some experiments, the apparent intensity of TRH receptor bands increased after TRH treatment. Control experiments confirmed that equal amounts of protein had been loaded per lane and that all immunoreactive receptors had been solubilized by lysis buffer and entered the gel, suggesting that the immunoreactivity of receptor may be increased after TRH treatment.

^{32}P Labeling—Cells on 6-cm dishes were washed twice and incubated for 1–2 h in phosphate-free Dulbecco's modified Eagle's medium. Cells were then incubated for 3–4 h in the same buffer containing 0.2–0.5 mCi/ml [^{32}P]orthophosphate, washed 3 times, lysed, and treated as described above in buffer with protease and phosphatase inhibitors. Immunoprecipitates from each dish were suspended in 50–65 μl of sample buffer, in some cases after deglycosylation with PNGaseF or dephosphorylation with potato acid phosphatase; 5–10 μl were subsequently used for Western blots, and 10–30 μl were used for phosphorimaging. Lanes contained equal amounts of trichloroacetic acid-precipitable ^{32}P. Samples were transferred to nitrocellulose paper and either immunoblotted or analyzed for ^{32}P-containing bands on a Molecular Dynamics PhosphorImager.

Other Methods—To identify TRH receptors by immunocytochemistry, cells were grown on glass coverslips, fixed with paraformaldehyde, and stained with HA11 (1:1000) followed by rhodamine-labeled anti-mouse IgG (1:200) as previously described (49). To measure calcium responses, cells were grown on glass coverslips and loaded with fura2/AM in buffered Hanks' balanced salt solution at room temperature. The cells were washed and incubated in the same buffer at 37 °C. Cells were alternately excited with 340- and 380-nm light, and fluorescence emission was measured at 490 nm; 340/380 fluorescence ratios were determined every 1200 ms (53). Proteins were determined by the Lowry or Bradford methods using bovine serum albumin as standard. Specific binding of 10 nM [^{3}H]MeTRH was measured by incubating cells with radioligand with or without a 1000-fold molar excess of unlabeled TRH for 1 h at 37 °C, washing dishes 4 times, lysing cells, and counting. To measure TRH-stimulated inositol phosphate formation, cells were metabolically labeled for 48 h with 2 μCi/ml [^{3}H]inositol and then incubated for 30 min in medium containing 10 mM LiCl with or without 1 μM TRH. Cells were washed, and lipids and inositol sugars were extracted and separated on anion exchange columns (54).

TABLE I
Expression of TRH receptors

Specific binding of [^{3}H]MeTRH to various cell lines stably expressing rat TRH receptors tagged with double HA or double FLAG epitopes at the N termini was measured by incubating dishes for 1 h at 37 °C with 10 nM [^{3}H]MeTRH with or without 1 μM unlabeled TRH. Values are the averages of measurements made over the course of the studies described here.

Cell line	Receptor	TRH receptors
		pmol/mg protein
HEK293	HA-TRHR	0.65
HEK293	FLAG-TRHR	0.65
HEK293	HA-TRHR-Δ335–412	0.10
GHFT	HA-TRHR	0.03

RESULTS

Expression of TRH Receptors in HEK293 Cells—We initially developed stable cell lines of HEK293 cells expressing epitope-tagged TRH receptors because this cell type has high transfection efficiency and wide application in studies involving GPCRs. The stable lines expressing HA- or FLAG-tagged TRH receptors bound between 0.5 and 0.8 pmol of [^{3}H]MeTRH/mg of protein at 10 nM radioligand over the course of these studies (Table I). These expression levels are comparable with those of pituitary GH3 cells, which express endogenous receptors and bind 0.5–1 pmol [^{3}H]MeTRH/mg.

Transfection of HEK293 cells with a plasmid encoding a TRH receptor with two amino-terminal HA epitopes, p2HA-TRHR, resulted in the appearance of three bands on immunoblot that were immunoreactive to HA11, a mouse monoclonal anti-HA antibody (Fig. 1*A*, *lane 2*). These bands were not present in control cells (Fig. 1*A*, *lane 1*), indicating that they represent the TRH receptor protein. Based on densitometry, the relative abundance of these bands in different experiments averaged 1:0.6:0.3 (*bottom/middle/top*) under the standard reducing and denaturing conditions used for SDS-PAGE. The bottom band, which is the predominant one, displayed an apparent molecular mass of ~ 65 kDa (Fig. 1*A*, *lane 2*). The type 1 TRH receptor contains two conserved *N*-linked glycosylation sites, and the receptor is absorbed to wheat germ agglutinin (55). In fact, pretreatment of the samples with PNGaseF, a glycosidase, to remove all *N*-linked polysaccharide groups increased its mobility to ~ 47 kDa (Fig. 1*B*, *lane 2*), which is very close to the calculated molecular mass of epitope-tagged type 1 TRH receptors, 49 kDa. This suggests that the bottom band represents glycosylated receptor monomers. PNGaseF treatment increased the gel mobility of the middle band from ~157 to ~ 100 kDa (Fig. 1*B*, *lane 2*), close to the predicted size of receptor dimers. The receptor never ran as a tight band regardless of the amount of PNGaseF used, possibly due to basal phosphorylation, palmitoylation, or other post-translational modification. The proportion of the various receptor bands was not altered by increasing the dithiothreitol concentration to 400 mM or by reduction and carboxymethylation with or without deglycosylation (data not shown). Under reducing conditions, the relative abundance of apparent receptor monomers, dimers, and higher oligomers was not affected by as much as 4% SDS with or without 8 M urea. When either SDS or dithiothreitol was omitted from the electrophoresis buffer, TRH receptors formed high molecular weight aggregates retained on the top of the gels.

Oligomerization of TRH Receptors—To clarify whether the higher molecular weight bands were derived from receptor oligomerization or association with other unknown proteins, we conducted differential immunoprecipitation in which two different epitope-tagged receptors were co-expressed and subjected to immunoprecipitation with the antibody against one

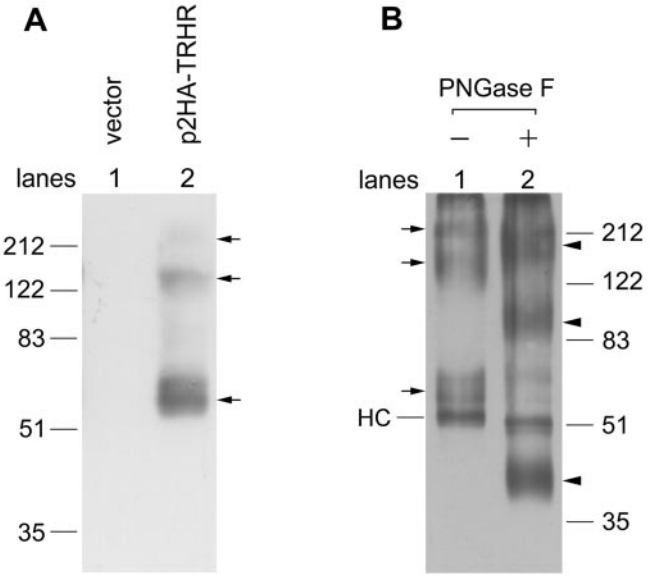

FIG. 1. **Expression of TRH receptors.** *A*, membrane preparations (5 μg/lane) from control HEK293 cells (*lane 1*) and cells stably transfected with *p*2HA-TRHR (*lane 2*) were resolved by 10% SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with anti-HA antibody, HA11. *B*, HA11-derived immunoprecipitates from HEK293 cells stably transfected with *p*2HA-TRHR were treated without (−) or with (+) 5 units/ml PNGaseF for 10 h at 37 °C and then analyzed by immunoblot with HA11. The migration positions of the HA-immunoreactive proteins before and after PNGaseF treatment are denoted by *arrows* and *arrowheads*, respectively. Molecular mass markers (in kDa) and immunoglobulin heavy chain (*HC*) are also indicated.

tag and then resolved on SDS-PAGE and immunoblotted with antibody against the other. As shown in Fig. 2*A*, when FLAG- and HA-tagged TRH receptors were transiently co-expressed in HEK293 cells, FLAG-tagged TRH receptors were clearly present in HA antibody-derived immunoprecipitates and vice versa. Based on densitometric analysis, we estimated that as much as 30% of total HA-tagged receptors were co-immunoprecipitated by anti-FLAG antibody, and approximately 10% of FLAG-tagged receptors were co-immunoprecipitated by anti-HA antibody. This disparity is presumably the result of differences in the expression levels of the two receptors or the affinities of immunoprecipitating antibodies. The co-immunoprecipitated TRH receptors ran as a group of bands in the immunoblots, with the higher molecular mass bands predominant (Fig. 2*A*, *lane 3*). Only receptors in a multi-receptor complex would be detected in this protocol, and the higher molecular weight bands could represent receptor trimers or tetramers or receptors tightly associated with other proteins. The TRH receptor oligomers were quite stable, since less than half of the receptor complexes dissociated into monomers under the reducing and denaturing conditions used for SDS-PAGE. When cells were separately transfected with FLAG- and HA-tagged receptors and then mixed before lysis and immunoprecipitation, no FLAG-tagged receptor was immunoprecipitated with antibody to HA and vice versa (Fig. 2*A*, *lane 4*), showing that the interaction between receptors takes place in the cell and not during sample preparation.

In the transient expression system used in the experiment shown in Fig. 2*A*, receptors ran very close to the predicted molecular mass of unmodified TRH receptor monomers and dimers (monomer ahead of the immunoglobulin heavy chain), and enzymatic deglycosylation did not increase their gel mobility measurably (data not shown), indicating that receptors were not heavily glycosylated and that they can dimerize without extensive carbohydrate addition. To determine whether

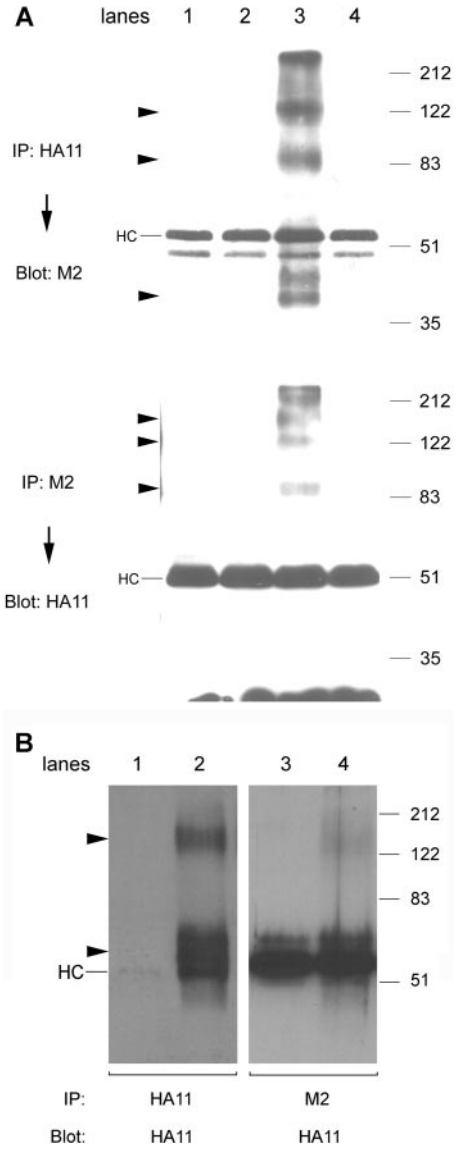

FIG. 2. **Co-immunoprecipitation of HA-tagged TRH receptors with FLAG-tagged TRH receptors.** *A*, TRH receptors were immunoprecipitated (*IP*) with anti-HA antibody, HA11 (*top panel*), or anti-FLAG antibody, M2 (*bottom panel*), from HEK293 cells transiently transfected with *p*Prol2FLAG-TRHR (*lane 1*), *p*2HA-TRHR (*lane 2*), or both (*lane 3*). Samples in *lane 4* were prepared by mixing and then solubilizing, before immunoprecipitation, two identical aliquots of cell suspensions that were derived from and equal to half the amount of samples analyzed in *lanes 1* and *2*, respectively. Proteins were resolved on SDS-PAGE and probed with M2 and HA11, respectively, in immunoblot analyses. *B*, HEK293 cells stably expressing FLAG-tagged TRH receptors were transiently transfected with *p*cDNA3 (*lanes 1* and *3*) or *p*2HA-TRHR (*lanes 2* and *4*). Five days after transfection, TRH receptors were immunoprecipitated with anti-HA antibody, HA11 (*lanes 1* and *2*), or anti-FLAG antibody, M2 (*lanes 3* and *4*) and then probed in immunoblot with anti-HA antibody, HA11. *Arrowheads* denote TRH receptors.

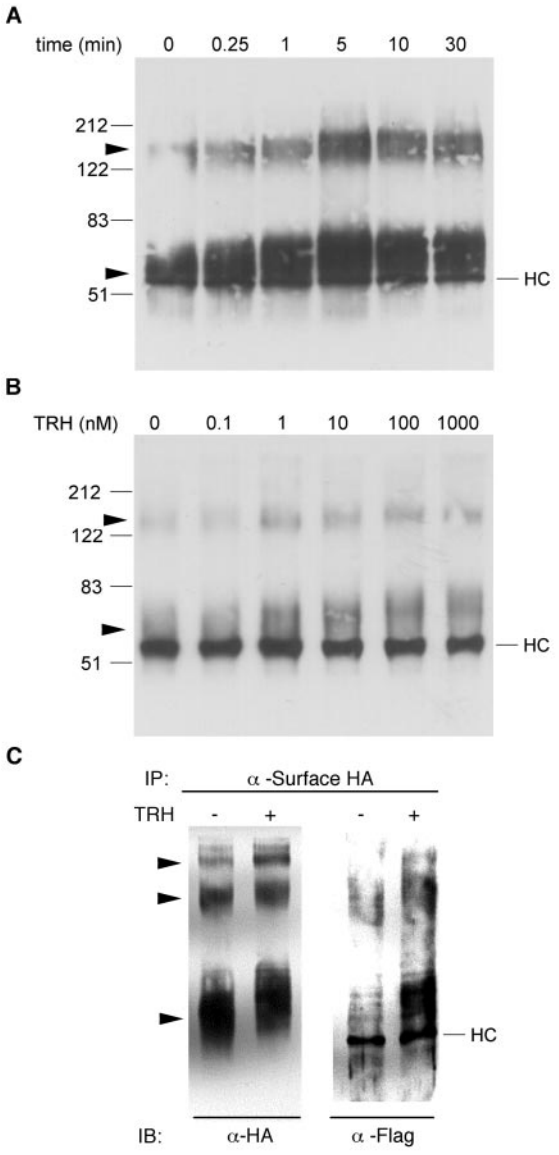

FIG. 3. **Time course and dose dependence of ligand-modulated TRH receptor dimerization.** Monolayers of HEK293 cells stably expressing HA-tagged TRH receptors were stimulated with 1 μM TRH for 0, 0.25, 1, 5, 10, or 30 min (*A*) or 0, 0.1, 1, 10, 100, or 1000 nM TRH for 5 min (*B*) and then subjected to immunoprecipitation and immunoblotting with anti-HA antibody, HA11. *C*, HEK293 cells were co-transfected with HA- and FLAG-tagged TRH receptors, as described in the legend to Fig. 2, and stimulated with or without TRH for 5 min. Dishes were then incubated on ice with HA11 anti-HA antibody (1:1000 in Hanks' balanced salt solution) for 1 h to label surface receptors. Cells were lysed and washed extensively, and antibody-receptor complexes were pelleted with protein A/G beads and then immunoblotted (*IB*) with HA11 anti-HA (*left*) or M2 anti-FLAG (*right*) antibody. *HC* denotes immunoglobulin heavy chain, and *arrowheads* denote TRH receptors. *IP*, immunoprecipitation.

mature, glycosylated receptor also dimerizes, we took advantage of our observation that by 5 days after transient transfection, the density of HA-tagged TRH receptors was low, but the receptors were fully glycosylated and localized on the plasma membrane based on mobility during SDS-PAGE and immunocytochemistry. FLAG-tagged receptors were heavily glycosylated only when expressed in stable cell lines. We therefore used stable cell lines expressing FLAG-tagged TRH receptors, transfected them with plasmid encoding HA-tagged receptors, and waited for 5 days so that both receptors were glycosylated. As shown in *lane 4* of Fig. 2*B*, glycosylated HA-tagged TRH receptor monomers and dimers (monomer above the immunoglobulin heavy chain) were present in FLAG immunoprecipitates. Again, the HA-tagged TRH receptors were only seen in the co-transfected cells (Fig. 2*B*, *lanes 2* and *4* but not *lanes 1* and *3*).

Modulation of TRH Receptor Dimers by Agonist—To address the physiological relevance of TRH receptor dimerization, we asked whether this process is regulated by receptor activation. As shown in Fig. 3*A*, stimulation of cells stably expressing HA-tagged TRH receptors with 1 μM TRH increased the pro-

portion of receptors running at the molecular weight of dimers. This effect became apparent as early as 1 min and reached a maximum at 5 min. The half-time ($t_{1/2}$) for the maximal enhancement of receptor dimerization was less than 1 min based on multiple experiments in which dimerization was measured at times from 15 s to 30 min after TRH addition. The dose dependence of TRH-induced receptor dimerization is shown in Fig. 3*B* and is consistent with the K_d for TRH, 10 nM. Based on densitometry, TRH increased the proportion of receptor running as apparent dimer by 50–100% in different experiments.

The ability of TRH to stimulate dimer formation was also tested by co-immunoprecipitation. Cells were transiently transfected with HA- and FLAG-tagged TRH receptors, exposed to either TRH or vehicle for 5 min, and then incubated with anti-HA antibody at 0 °C to label plasma membrane receptors selectively. TRH treatment did not change the total amount of HA-tagged receptor on the cell surface, although it caused a clear upshift in both the apparent monomer and dimer bands, suggestive of phosphorylation (*left lanes* in Fig. 3*C*). However, TRH treatment did increase the amount of FLAG-tagged receptor associated with surface HA receptor by 55 and 100% in different experiments, confirming that the proportion of dimer was increased by hormone binding (*right lanes* in Fig. 3*C*).

We tested the requirement for signal transduction by pretreating cells with the phospholipase C inhibitor U73122. In this experiment, receptors were enriched on wheat germ agglutinin and then deglycosylated before SDS-PAGE and immunoblotting, avoiding an immunoprecipitation step. Blocking the TRH signal pathway did not alter the agonist-induced mobility shifts seen in response to either intermediate (10 nM) or maximally effective (1 μM) concentrations of TRH (Fig. 4*A*). To confirm the effectiveness of U73122, we evaluated TRH responses in individual cells loaded with the calcium indicator fura2 (Fig. 4*B*). Calcium responses to TRH were inhibited by U73122 as was TRH-stimulated inositol phosphate production (Fig. 4*C*). U73122 did not affect ligand-induced internalization of the receptor (data not shown).

Dimerization and Phosphorylation of an Internalization-deficient Mutant TRH Receptor—A carboxyl-terminally truncated mutant TRH receptor, which lacks residues 335–412, binds TRH (Table I) and generates a calcium signal but is not able to recruit β-arrestin or undergo agonist-induced internalization (56, 57). As shown in Fig. 5, truncated receptors ran as apparent monomers and oligomers when isolated from stably transfected HEK293 cells. In contrast to results with the full-length receptor, TRH stimulation did not modulate dimerization of the truncated receptors or cause a shift in gel mobility in any experiment. These data suggest that the carboxyl domain of the TRH receptor, although not required for formation of constitutive receptor oligomers, is necessary for agonist-induced receptor dimerization and phosphorylation. When full-length receptors were expressed together with truncated receptors, dimers of intermediate size were seen (data not shown).

Phosphorylation of TRH Receptors—Alkaline phosphatase collapsed the receptor bands from TRH-treated cells to the mobility of receptors from naïve cells, indicating that the upshift resulted from agonist-induced phosphorylation (Fig. 6*A*). The inverse agonist chlordiazepoxide did not affect receptor mobility by itself but did block the TRH-induced upshift, providing further evidence that an activated receptor is required (Fig. 6*B*).

The ability of several kinase inhibitors to prevent TRH-stimulated receptor phosphorylation is shown in Fig. 6*C*. Receptors were deglycosylated before electrophoresis to exaggerate the mobility shift, and no immunoprecipitation step was included. TRH treatment led to the expected upshift in monomer and dimer bands. Two monomer bands could sometimes be resolved in TRH-treated lanes and may represent different phosphorylation states. Because TRH activates protein kinase C and there are potential protein kinase C phosphorylation sites in the cytoplasmic region of the receptor, we tested the effect of the protein kinase C inhibitor GF109203X, which did not prevent the TRH-dependent mobility shift. Because Hanyaloglu *et al.* (58) report that phosphorylation of TRH receptors

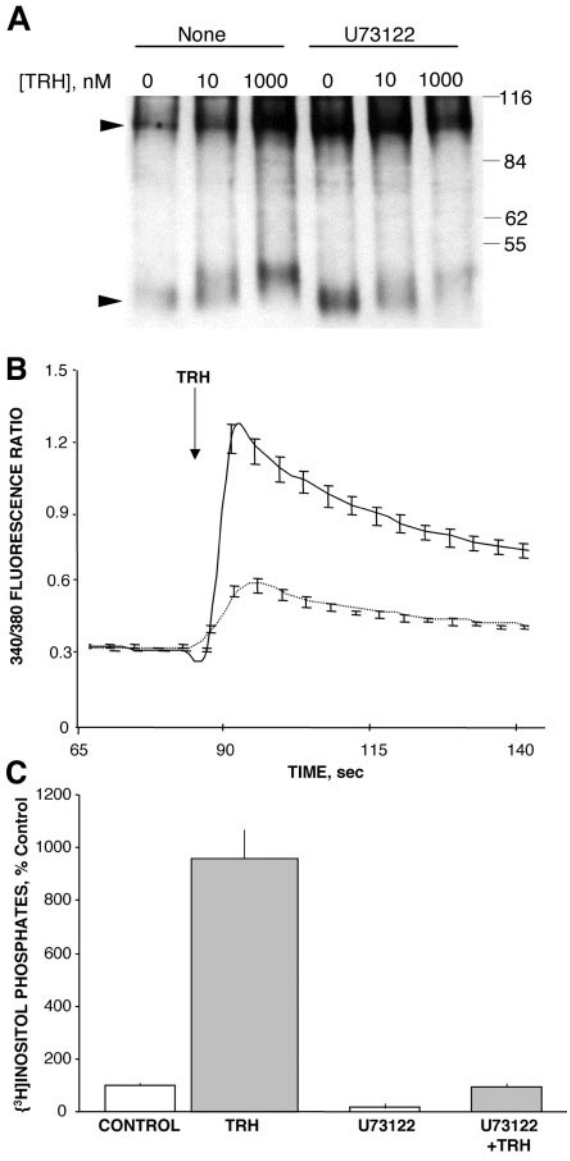

FIG. 4. **Effects of U73122 on TRH receptor dimerization and calcium signaling.** *A*, monolayers of HEK293 cells stably expressing HA-tagged TRH receptors were pretreated for 1 h with vehicle or 10 μM U73122 and then stimulated with 0, 10 nM, or 1 μM TRH for 5 min in the continued presence of drugs. The cells were solubilized, and receptors were absorbed on wheat germ agglutinin and deglycosylated before electrophoresis and immunoblotting with HA11 anti-HA antibody without immunoprecipitation. *B*, cells were treated with vehicle or 10 μM U73122 during loading with fura2 and calcium imaging. Calcium responses of individual cells were followed by measuring the 340/380 fluorescence ratio. At the time noted by the *arrows*, 1 μM TRH was added. Curves shown represent the mean ± S.E. of responses from 30–40 cells. *Solid line* shows control, and *broken line* U73122-treated cells. *C*, cells were metabolically labeled with [^{3}H]inositol and incubated with either vehicle or 10 μM U73122 for 2 h when 10 mM LiCl and either vehicle or TRH was added for 30 min. Shown are the mean and S.E. of triplicate determinations of total ^{3}H-labeled inositol phosphates, normalized to ^{3}H-labeled lipids. *Arrowheads* denote TRH receptors.

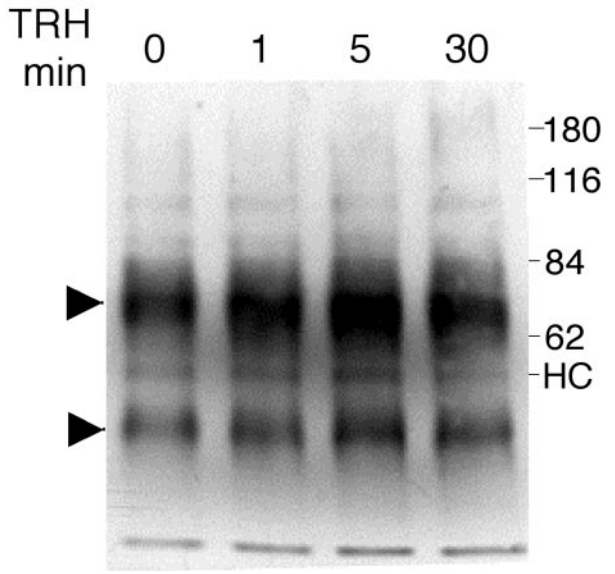

FIG. 5. **Dimerization and phosphorylation of the carboxyl-terminally truncated mutant TRH receptor.** Monolayers of HEK293 cells stably expressing HA-tagged CT-TRH receptors were stimulated with 1 μM TRH for 0, 1, 5, or 30 min. Cells were then lysed, immunoprecipitated and immunoblotted with HA11 anti-HA antibody. *Arrowheads* denote TRH receptors. *HC*, heavy chain.

by CKII is important in arrestin recruitment, we also tested apigenin, a CKII inhibitor. Apigenin did not prevent the TRH-induced shift in receptor mobility on SDS-PAGE at 100 μM or at doses up to 400 μM, when toxicity became apparent. Again, the phospholipase C inhibitor U73122 did not prevent receptor phosphorylation.

These findings suggest that receptor phosphorylation is carried out by a kinase that recognizes the agonist-receptor complex such as a G protein-coupled receptor kinase (GRK). The effects of overexpressing wild type and dominant negative forms of GRK2 are shown in Fig. 7*A*. Neither the wild type GRK2 nor the K220R mutant GRK2 affected receptor mobility shifts caused by TRH. To confirm that the GRK proteins were effective, we co-transfected them into HEK293 cells with epitope-tagged TRH or β_2-adrenergic receptors and incubated cells for 30 min with agonists before fixing and immunolocalizing receptors and scoring. TRH and β_2-adrenergic receptors were localized to the plasma membrane of about 90% of untreated cells. In cells exposed to 1 μM TRH for 30 min, receptors were localized in vesicles in approximately the same fraction of cells regardless of the expression of GRKs (Fig. 7*C*). The effects of the GRKs were pronounced in cells expressing the β_2-adrenergic receptor, however. Treatment with 100 μM isoproterenol caused redistribution of receptors to endocytic vesicles in 96% of cells expressing wild type GRK, 45% of cells expressing dominant negative GRK, and 81% of mock-transfected cells (Fig. 7*B*). The results indicate that the GRK proteins were expressed adequately and suggest that the activated TRH receptor is effectively phosphorylated by endogenous GRKs under all conditions.

To document phosphorylation directly, we labeled cells expressing HA-tagged TRH receptors with [^{32}P]orthophosphate and incubated with or without TRH before immunopurifying receptors. TRH stimulated the incorporation of ^{32}P into both receptor monomers and dimers (Fig. 8*A*). The ^{32}P-labeled receptor ran more slowly than receptor from unstimulated cells, confirming that the TRH-induced upshift is due to phosphorylation. After deglycosylation with PNGaseF, receptor migrated at molecular weights close to those predicted for unmodified monomers and dimers, and the mobility shift caused by TRH

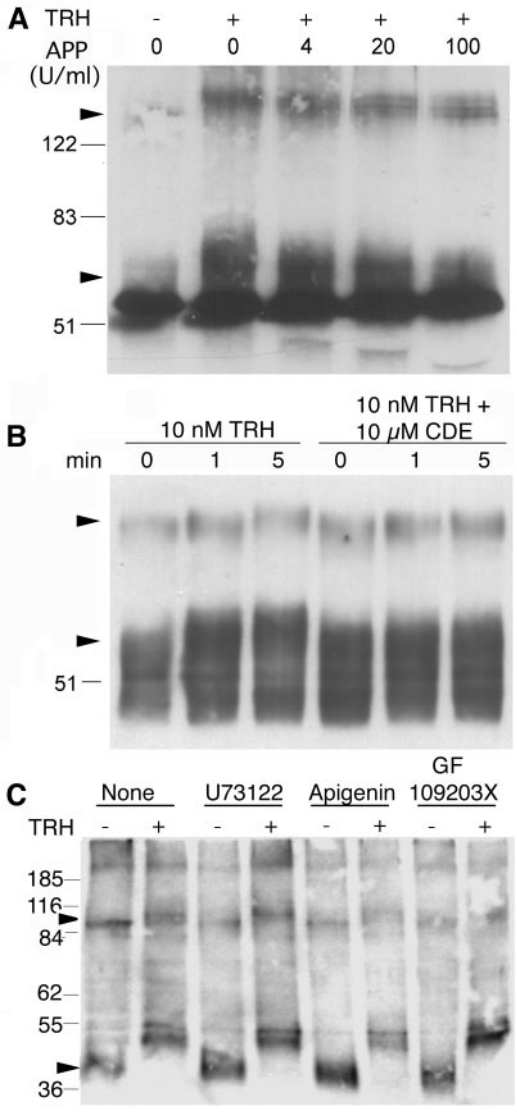

FIG. 6. **TRH receptor phosphorylation.** *A*, monolayers of HEK293 cells stably expressing HA-tagged TRH receptors were untreated (*left lane*) or stimulated with 1 μM TRH for 5 min and then subjected to immunoprecipitation with anti-HA antibody, HA11. The immunopurified receptors were incubated with 0, 4, 20, or 100 units (*U*)/ml alkaline phosphatase (*APP*) for 1 h at 37 °C and were then analyzed in immunoblots with HA11. *B*, cells were preincubated for 2 h with vehicle or 10 μM chlordiazepoxide (*CDE*) and then incubated with 10 nM TRH for 0, 1, or 5 min. Samples were analyzed as in *A*. *C*, cells were preincubated for 1 h with no inhibitor, 10 μM U73122, 100 μM apigenin, or 10 μM GF109203X and then treated with or without 1 μM TRH for 5 min in the continued presence of drug. Receptors were absorbed to wheat germ agglutinin, deglycosylated, and immunoblotted with anti-HA antibody without immunoprecipitation. *Arrowheads* denote TRH receptors.

was more evident. Phosphatase treatment removed ^{32}P from receptor bands (data not shown). Bands remained broad even after treatment with PNGaseF, phosphatase, and 1 M hydroxylamine, which is expected to remove palmitoyl esters (hydroxylamine data not shown), suggesting that there may be additional post-translational modifications. As predicted by results shown above, the phospholipase C inhibitor U73122 did not prevent the TRH-stimulated incorporation of ^{32}P into receptors (Fig. 8*B*). Likewise, overexpression of wild type or dominant negative GRK2 caused no change in the incorporation of ^{32}P into TRH receptors (Fig. 8*C*).

Dimerization of TRH Receptors in Pituitary Cells—We were unable to study oligomerization of native receptors in pituitary cells using antibodies against receptor peptides that had

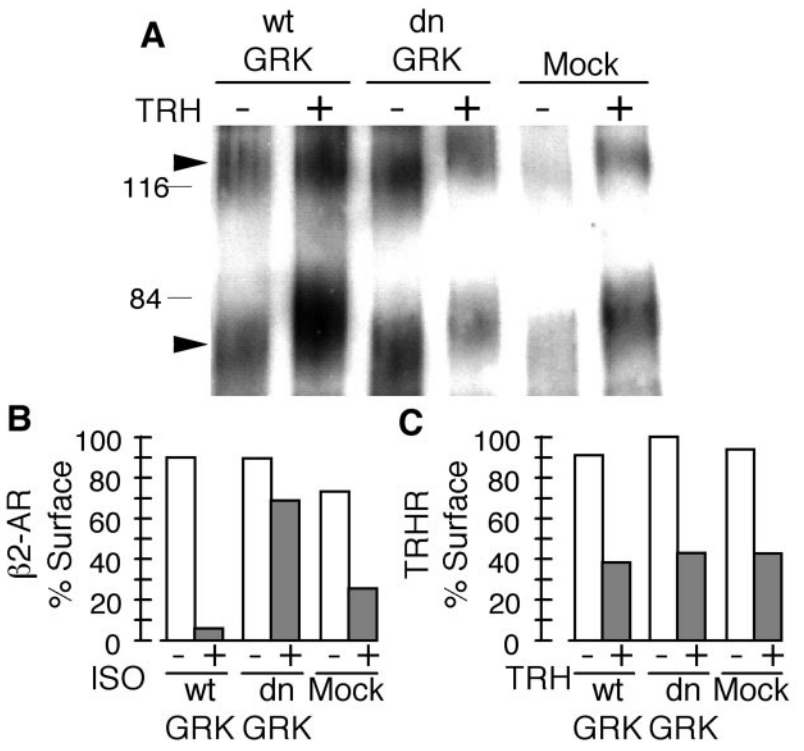

FIG. 7. **Effect of GRKs on TRH receptor phosphorylation.** *A*, monolayers of HEK293 cells were co-transfected with plasmids encoding HA-tagged TRH receptor and either wild type GRK2 (*wt GRK*), dominant negative GRK2-K220R (*dn GRK*), or empty vector (*Mock*). The cells were stimulated with or without 1 μM TRH for 5 min and lysed and immunoblotted with HA11 anti-HA antibody without immunoprecipitation. *Arrowheads* denote TRH receptors. *B* and *C*, HEK293 cells were co-transfected with either HA-tagged β_2-adrenergic receptor (*B*) or HA-tagged TRH receptor together (*C*) with wild type GRK2, dominant negative GRK2-K220R, or empty vector. *B*, cells expressing β_2-adrenergic receptor were incubated for 30 min with or without 100 μM isoproterenol before the cells were fixed and stained with monoclonal anti-HA antibody. *C*, cells expressing TRH receptors were incubated for 30 min with or without 1 μM TRH before immunostaining. Slides were scored for the fraction of cells with receptors predominantly on the plasma membrane or cytoplasmic vesicles by observers unaware of the treatment group; 22–84 cells were scored. In each case, the *open bars* show untreated cells, and the *dark bars* show cells after agonist stimulation.

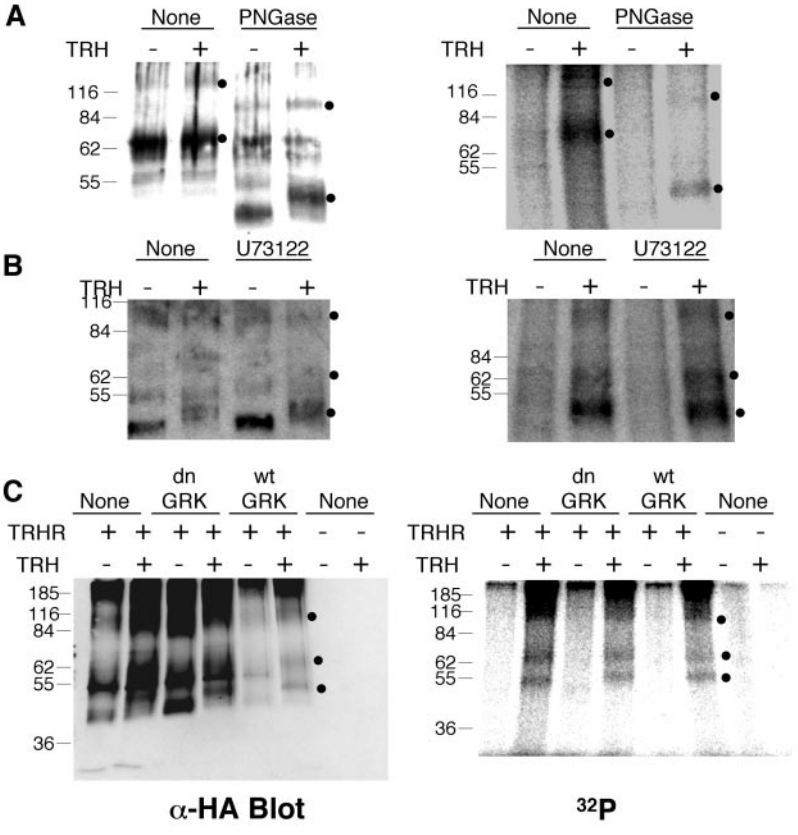

FIG. 8. **TRH stimulation of incorporation of ^{32}P into receptors.** *A*, HEK293 cells stably transfected with HA-tagged TRH receptors were labeled with [^{32}P]orthophosphate and then treated with or without 1 μM TRH for 5 min. Cells were lysed and immunoprecipitated with HA11 anti-HA antibody. Immunoprecipitates were incubated without or with PNGaseF to deglycosylate receptors and then run on SDS-PAGE for standard immunoblotting with anti-HA antibody (*left panels*) or phosphorimaging (*right panels*). *B*, cells were treated as in *A*, except that U73122 or vehicle was present during the last 30 min of the incubation with [^{32}P]orthophosphate and during TRH stimulation. *C*, HEK293 cells were co-transfected with HA-tagged TRH receptors and wild type (*wt GRK*) or dominant negative (*dn GRK*) GRK2 or empty plasmid (*none*), as described for Fig. 6. In experiments shown in *panels B* and *C*, receptors were deglycosylated before SDS-PAGE; deglycosylation was incomplete, and a glycosylated monomer band can be seen. The *dark circles* show major ^{32}P-labeled bands corresponding to receptor monomer and dimer.

proven useful for immunocytochemistry (9) because the antibodies were not sufficiently sensitive in immunoblotting. Instead, we developed a pituitary cell model by transfecting GHFT pituitary cells with epitope-tagged TRH receptors. GHFT cells are pre-lactotrophs immortalized by the targeted expression of T antigen (45). Before transfection, the GHFT cells displayed little specific binding of [^{3}H]MeTRH and no calcium response to TRH (Fig. 9, *A* and *C*). After stable transfection with HA-tagged TRH receptors, GHFT cells expressed TRH receptors at an average density of ~ 0.03 pmol/mg protein (Fig. 9*C*, Table I) and responded to TRH with a clear increase in intracellular calcium (Fig. 9*A*). The HA-tagged TRH receptors were localized on the cell surface (Fig. 9*B*).

HA-tagged TRH receptors isolated from stably transfected pituitary GHFT cells ran similarly to receptors isolated from stably transfected HEK293 cells (Fig. 10*A*). TRH caused an

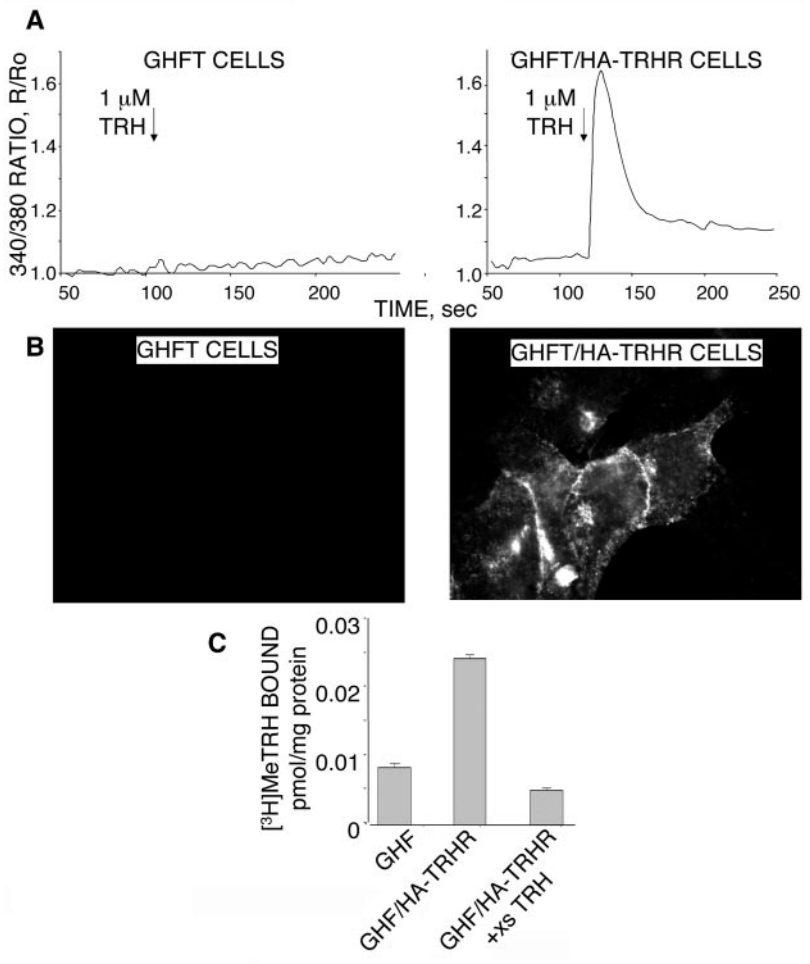

FIG. 9. **Characterization of GHFT pituitary cell model.** *A*, GHFT or a clonal line transfected with an HA-tagged TRH receptor, GHFT/HA-TRHR cells, were loaded with fura2, and calcium responses were measured as described in the legend to Fig. 4. *Traces* show averaged results from 25–35 individual cells. *B*, GHFT or GHFT/HA-TRHR cells were fixed and stained with antibody against the HA epitope. *C*, specific binding of [^{3}H]MeTRH to parental GHFT cells or GHFT/HA-TRHR cells was measured. The mean and S.E. of triplicate determinations are shown.

up-shift in the mobility of the monomeric form, indicative of phosphorylation within 1 min of TRH addition and increased the apparent dimer/monomer ratio slightly from 0.25 to 0.35. When GHFT cells were transiently co-transfected with HA- and FLAG-tagged receptors, the receptors co-precipitated if cells expressed both receptors but not if cells were transfected with either HA- or FLAG-tagged receptors and mixed before lysis and immunoprecipitation (Fig. 10*B*). Receptor oligomers did not dissociate during immunoisolation, because only multimers, but not monomers, were detected in lanes representing a HA immunoprecipitate/FLAG blot and vice versa. Receptors tagged with an amino-terminal HA epitope ran in broad bands overlapping the immunoglobulin heavy chain and above 120 kDa, as expected for glycosylated monomers and dimers, whereas receptors tagged with a FLAG epitope ran at sizes predicted for nonglycosylated monomers and dimers. Based on densitometry of results from three experiments, 10.4 ± 4.0% of FLAG-tagged and 25.5 ± 1.1% of HA-tagged TRH receptors were co-precipitated with the opposite antibody, the same fractions seen with HEK293 cells.

DISCUSSION

We have provided definitive biochemical evidence that type 1 rat TRH receptors exist both as monomers and dimers in non-stimulated cells and that TRH enriches the dimeric species in a dose- and time-dependent manner. Receptors tagged with HA and FLAG epitopes were co-precipitated only when they were expressed in the same cell, and both intact and truncated TRH receptors ran at the appropriate sizes for monomer and homodimer before and after deglycosylation. Our findings complement studies showing that bioluminescence resonance energy transfer occurs between TRH receptor pairs and is increased by TRH (43). TRH receptors probably dimerize during biosynthesis, because even receptors that were not properly transported to the plasma membrane ran as dimers on SDS-PAGE.[2] Dimerization may be important for correct folding and processing of TRH receptors and other GPCRs; in fact, it has been suggested that receptors serve as chaperone proteins to each other when they are delivered from the endoplasmic reticulum to the plasma membrane (59, 60). TRH receptor dimers must also be present on the plasma membrane, because dimers were found after surface-labeling of unstimulated cells. TRH either increases the stability of preexisting dimers or increases the proportion of receptors in dimers.

In previous work (43, 58), dimerization of TRH receptors was observed in transiently transfected COS cells, where receptor densities were likely to have been much higher than those found in pituitary cells. In the present study, the observed receptor dimerization/oligomerization did not result from receptor overexpression because the HEK293 cell lines expressed receptors at densities typical of the concentration of endogenous receptors in rat pituitary GH3 cells (55), and pituitary GHFT cells expressed receptors at much lower levels. After lysis of cells expressing HA- and FLAG-tagged receptors, between 10 and 30% of receptors could be immunoprecipitated with the opposite antibody. The fraction of receptors in multimers in the cell must be substantial, because tagged receptors would be present as HA/HA and FLAG/FLAG dimers as well as

[2] C-C. Zhu, and P. M. Hinkle, unpublished data.

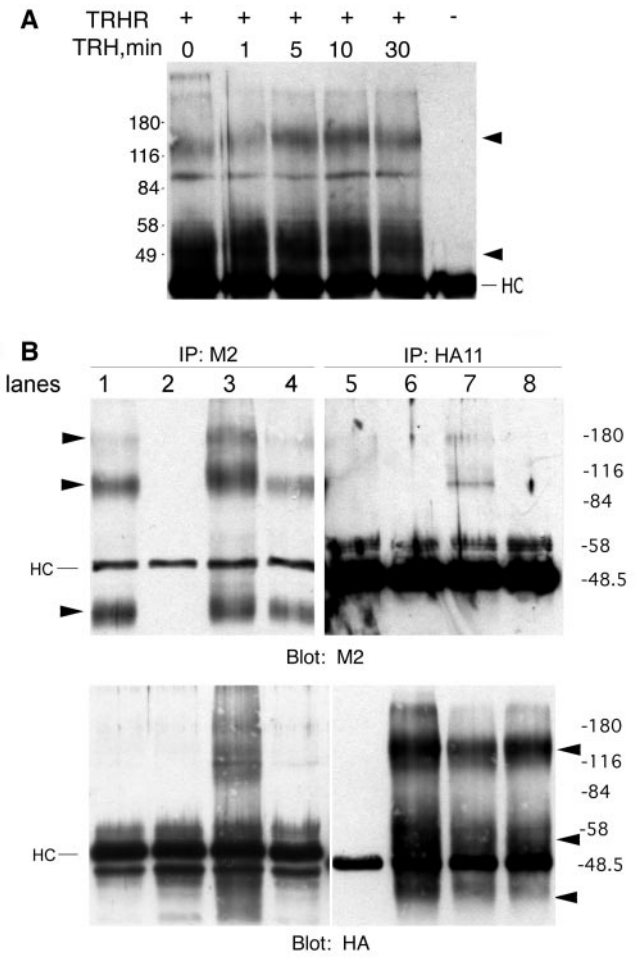

FIG. 10. **Co-precipitation of HA- and FLAG-TRH receptors in pituitary cells.** *A*, GHFT/HA-TRHR cells were stimulated with 1 μM TRH for 0, 1, 5, 10, or 30 min. Receptors were immunopurified as described under "Experimental Procedures," and samples were run on SDS-PAGE and blotted with antibody against the HA epitope. The *farthest right lane* shows the lysate of non-transfected GHFT cells. *HC*, heavy chain. *B*, GHFT cells were transiently transfected with plasmids encoding HA- or FLAG-tagged TRH receptors. Lysates were then immunoprecipitated (*IP*) and immunoblotted with antibodies to HA or FLAG, as described in the legend to Fig. 2. *Lane 1*, FLAG; *lane 2*, HA; *lane 3*, FLAG + HA; *lane 4*, MIX. When the same antibody was used for immunoprecipitation and immunoblotting, shorter exposure times were used. The heavy chains of M2 and HA11 antibodies run slightly differently. *Arrowheads* denote TRH receptors.

FLAG/HA complexes, and some breakdown of oligomers might have occurred during immunoprecipitation.

Dimerization of GPCRs has been reported to occur by a variety of mechanisms (13, 16). Covalent disulfide bond formation is involved in dimerization of the Ca^{2+}-sensing receptor (61), the κ-opioid receptor (62), and the muscarinic m3 receptor (21). Non-covalent hydrophobic interactions appear to be critical for dimerization of other GPCRs including the β_2-adrenergic receptor (18) and the dopamine D_2 receptor (30). For various GPCRs, dimerization has been reported to depend on the amino terminus (31, 61, 63), cytoplasmic carboxyl terminus (22), or membrane-spanning regions (18, 30, 64). More than one type of interaction is required to maintain dimeric forms of the somatostatin (40) and metabotropic glutamate receptors (33, 34). The TRH receptor has a disulfide bond between Cys-98 and Cys-179 in the 1st and 2nd extracellular loops (1), but neither strong reducing agents nor reduction and carboxymethylation caused dissociation of oligomerized TRH receptors into monomers, indicating that intermolecular disulfide bonds are probably not solely responsible for dimerization. Like the δ-opioid receptor (27) and others (19, 40, 42), TRH receptors were still capable of forming dimers/oligomers after deletion of the carboxyl-terminal tail. In addition, TRH receptor dimerization did not depend on glycosylation, because receptor dimers were just as abundant when receptors were isolated from stably transfected cells, where receptors were heavily glycosylated, and transiently transfected cells, where receptors were minimally glycosylated. These findings all suggest that strong hydrophobic interactions contribute to the stability of TRH receptor dimers.

Ligand stimulation can increase (7, 17, 18, 20, 31, 32, 65), decrease (27, 38), or have no effect on GPCR dimerization and oligomerization (15, 21, 60). TRH treatment of intact cells increased the relative abundance of TRH receptor multimers detected biochemically in this study, and TRH increased bioluminescence resonance energy transfer between receptors in intact cells (43). TRH is a membrane-impermeant peptide and would not be expected to act on any intracellular receptor. Because TRH increased apparent dimerization in cells treated with U73122, which blocked receptor activation of phospholipase C (Fig. 4), TRH effects on receptor dimerization/oligomerization must not require phospholipase C-coupled signaling or resultant increases in intracellular calcium and downstream kinase activity.

In addition to triggering phosphoinositide turnover, TRH causes its receptor to associate with β-arrestin, migrate to coated pits, and subsequently internalize (66–69). It is plausible that agonist binding promotes receptor oligomerization by causing the concentration of receptors in coated pits or other membrane regions. Consistent with this idea, TRH did not increase dimerization of carboxyl-terminally truncated receptors that do not bind to β-arrestin or cluster on the membrane (49). Kroeger *et al.* (43) find that energy transfer between TRH receptors was not blocked by a dominant negative dynamin, which would be expected to prevent conversion of coated pits to coated vesicles but not to block clustering of receptors on the membrane. Alternatively, TRH-stimulated receptor dimerization or dimer stabilization may result from agonist-induced phosphorylation or conformational changes that position the receptor in an orientation that favors the interaction between neighboring receptors.

In response to ligand binding, GPCRs are usually phosphorylated by G protein-coupled receptor kinases and often by kinases activated by downstream signals, such as protein kinase A and protein kinase C. TRH receptors undergo agonist-induced internalization through the clathrin- and dynamin-mediated endocytotic pathway (49, 66, 67, 69), presumably initiated by receptor phosphorylation and the subsequent interaction of phosphorylated receptors with β-arrestin, which targets receptors to clathrin-coated pits for endocytosis (68). Hanyaloglu *et al.* (58) present intriguing data suggesting that phosphorylation of the TRH receptor by CKII is important for TRH-stimulated recruitment of β-arrestin. They showed that CKII phosphorylates TRH-activated receptors and that removal of all three CKII sites in the cytoplasmic tail reduces TRH-induced receptor phosphorylation, β-arrestin recruitment, receptor internalization, and desensitization (58). They also found that inhibition of CKII with apigenin inhibits TRH-induced, β-arrestin-dependent internalization. Although there are other potential phosphorylation sites in TRH receptors, these findings suggest that phosphorylation by CKII is critical.

We have provided direct evidence that TRH stimulates phosphorylation of its receptor. TRH treatment rapidly increased incorporation of ^{32}P into receptor monomers and multimers and caused a shift in receptor mobility on gels, which was prevented by an inverse agonist and reversed by phosphatase

treatment. Removal of residues 335–412, which include the three CKII sites, abolished the TRH-induced upshift, showing that some phosphorylation either takes place at sites in the cytoplasmic tail or depends on this region of the receptor. TRH clearly promoted phosphorylation of both monomers and dimers in our experiments, whereas phosphorylation was only evident in bands running at the molecular weight of oligomers in previous studies (44, 58). The differences may be the result of the cell lines used (HEK293 and pituitary GHFT cells in this study and COS cells in previous ones), differences in receptor density, or differences in conditions used for lysis, sample preparation, and SDS-PAGE. A question that remains open is whether phosphorylation promotes receptor dimerization or preexisting dimers become phosphorylated in response to agonist.

In an earlier report (57) we showed that TRH can stimulate β-arrestin translocation and receptor internalization when the receptor is expressed in $F_{q/11}$ cells, which lack the α subunits of G_q and G_{11}, the cognate G proteins for the TRH receptor. Here we report that TRH can stimulate receptor phosphorylation without activating phospholipase C. These results all indicate that signaling is not required for TRH-stimulated receptor phosphorylation. This is the expected result for phosphorylation by a GRK (GPCR kinase) but is surprising for phosphorylation by CKII unless CKII can distinguish between activated and non-activated TRH receptors. This interesting possibility remains to be tested directly.

In summary, TRH stimulates oligomerization and phosphorylation of the TRH receptor when the receptor is expressed in heterologous cells or in a pituitary cell context. Neither response requires signal transduction. It is clear that receptor phosphorylation is involved in modulating receptor functions, but the physiological relevance of TRH receptor dimerization remains to be defined.

Acknowledgments—We thank John Puskas and Caroline Perkowski for excellent technical assistance and Thomas Graves and Jeffrey Smith for many helpful discussions.

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J. Biol. Chem. 2002, 277:28228-28237.