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Effect of Cell Type on the Subcellular Localization of the Thyrotropin-Releasing Hormone Receptor

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SUMMARY

The localization of an epitope-tagged receptor for thyrotropin-releasing hormone (TRH) expressed in different cell contexts was studied with immunofluorescence microscopy. In pituitary lactotrophs, which normally express TRH receptors, and in AtT20 pituitary corticotrophs, TRH receptor immunoreactivity was primarily confined to the plasma membrane. In HEK 293 and COS7 cells, TRH receptors were predominantly intracellular. In transiently transfected COS7 cells, the TRH receptor colocalized with endoplasmic reticulum and Golgi markers. The pattern of TRH receptor immunofluorescence was the same over a wide range of receptor expression in transiently transfected COS7 cells, and all cell lines bound similar amounts of ^{3}H- and rhodamine-labeled TRH analogs, suggesting that cell-specific differences in TRH receptor localization were not simply the result of overexpression. In all cell contexts, TRH receptors on the plasma membrane underwent extensive ligand-driven endocytosis. Inhibitors of glycosylation did not alter the subcellular distribution of receptors. In HEK 293 cells expressing the transfected TRH receptor, protein synthesis inhibitors caused translocation of intracellular receptors to the cell surface, as shown by a marked increase in cell surface immunofluorescence and [^{3}H][N^3-*methyl*-His^2]TRH binding. These results demonstrate that the subcellular localization of the TRH receptor depends on the cell context in which it is expressed and that intracellular receptors are capable of translocation to the plasma membrane.

G protein-coupled receptors mediate the activity of a wide variety of hormones and neurotransmitters. Ligands for most members of the heptahelical receptor family are not membrane permeant, and receptors engaged in signal transduction are localized on the plasma membrane. Relatively few G protein-coupled receptors have been visualized with immunofluorescent staining, and most of those have been shown to be predominantly on the cell surface. Receptors localized primarily at the plasma membrane include the β_2, mouse α_2-10H, and α_{2A} adrenergic receptors (1, 2), as well as receptors for gastrin-releasing peptide (3), cholecystokinin (4, 5), and TRH (6). In contrast, the bulk of thrombin receptors in endothelial cells (7, 8) and mouse α_2-adrenergic-4H receptors (1) are found intracellularly in vesicular structures. It is not known whether the cellular distribution of a G protein-coupled receptor is determined only by intrinsic properties of the receptor or also influenced by the cellular milieu.

The pituitary TRHR is a calcium mobilizing receptor coupled via $G_{q/11}$ to phospholipase C. Signal transduction by TRH and regulation of the TRHR have been extensively characterized (9–11). Recently, we localized the TRHR and a fluorescently labeled TRH ligand in pituitary cells (6). We stably expressed an epitope-tagged TRHR in the GH_3 pituitary cell line and used immunofluorescence microscopy to show that the receptor is present on the plasma membrane before activation. The TRHR and labeled ligand undergo extensive temperature-dependent internalization after binding of an agonist. Fluorescently labeled TRH analog and transferrin colocalized in endocytic vesicles, and internalization was blocked by hypertonic sucrose, implying that the TRHR undergoes endocytosis via clathrin-coated pits.

TRH action has been studied in both pituitary lactotrophs and thyrotrophs, in which the receptor is expressed endogenously, and in heterologous cells after stable or transient transfection of wild-type or mutated TRHR cDNAs. Some aspects of TRH signaling, such as desensitization of the receptor and down-regulation of the receptor mRNA, have been reported to differ when the receptor is expressed in different cell types (9, 12–14). To determine whether differences in receptor localization could contribute to differences in TRHR regulation, we monitored the distribution of epitope-tagged TRHRs after transient or stable expression in several cell

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ABBREVIATIONS: MeTRH, [N^3-methyl-His^2]thyrotropin-releasing hormone; TRH, thyrotropin-releasing hormone; Rhod-TRH, rhodamine-labeled thyrotropin-releasing hormone analog; HEK, human embryonic kidney; TRHR, thyrotropin-releasing hormone receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

lines. The experiments also address the more general question of whether the subcellular localization of G protein-coupled receptors is cell type dependent. We report here that the distribution of the TRHR is strongly dependent on the cell in which it is expressed.

Materials and Methods

Rhod-TRH and the p-*myc*-FLAG-TRHR plasmid, which encodes mouse TRHR with a *myc* epitope (GGEQKLISEEDLE) inserted between residues Glu23 and Tyr24 in the amino-terminal portion and a FLAG epitope (DYKDDDDK) after amino acid Asp369 in the carboxyl-terminal tail in the pCDM8 vector, were prepared as previously described (6). Plasmid encoding a β_2-adrenergic receptor bearing an amino-terminal FLAG epitope was a gift from Dr. Brian Kobilka (Stanford University, Palo Alto, CA), and pRSV/Neo was from the American Type Culture Collection (Rockville, MD). M2 mouse monoclonal anti-FLAG antibody was from IBI (Rochester, NY). Rhodamine-labeled goat anti-mouse IgG was from Hyclone (Logan, UT). [^{3}H]MeTRH (82.5 Ci/mmol) was from DuPont-New England Nuclear (Boston, MA). *N*-Dodecylmaltoside was from Boehringer-Mannheim Biochemica (Mannheim, Germany). Monensin and TRH were from Calbiochem (La Jolla, CA), and protease and glycosylation inhibitors and wheat germ agglutinin-Sepharose from Sigma Chemical (St. Louis, MO). NBD hexanoic ceramide and $DiOC_6$ were from Molecular Probes (Eugene, OR).

Cell culture. Pituitary GHY cells, a subclone of the GH_3 line that does not express TRHRs (15), were grown in Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum. HEK 293 cells, monkey COS7 cells, and mouse pituitary corticotroph AtT20 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All cell lines were incubated at 37° in a humidified 5% CO_2/95% air atmosphere.

Transfection. Stable lines of GHY and HEK 293 cells were established by cotransfecting cells with p-*myc*-FLAG-TRHR, which uses a CMV promoter, and pRSV/Neo with lipofection and selecting with G-418 (0.25 mg/ml for GHY and 0.60 mg/ml for HEK 293). Clones were screened for expression of the FLAG epitope-tagged receptor by [^{3}H]MeTRH binding and immunofluorescent staining. Cells were transiently transfected with the p-*myc*-FLAG-TRHR plasmid by lipofection and DEAE-dextran methods.

Staining and fluorescence microscopy. Immunocytochemistry was performed as described previously (6). Briefly, cells plated on coverslips were treated as described in the text, fixed, and permeabilized with 0.2% Nonidet P-40. The cells were stained at room temperature for 60 min with 1 μg/ml of M2 antibody against the FLAG epitope, washed, and then stained for 20 min with a 1:100 dilution of rhodamine-labeled goat anti-mouse IgG. Stained coverslips were washed and mounted with polyvinyl alcohol on glass slides. No staining was observed when the same protocol was applied to cells that had been transfected with the wild-type receptor or to untransfected cells, and no staining was observed in cells expressing the epitope-tagged receptor after staining with an irrelevant monoclonal antibody at the same concentration. Cells that stained with antibody to the FLAG epitope did not stain well with monoclonal antibody against the *myc* epitope, suggesting that the extracellular *myc* epitope is sterically hindered, perhaps because of glycosylation, or that the anti-*myc* antibody binds less strongly under the conditions used. We were unable to compare the localization of the epitope-tagged versus the native TRHRs because no antibody to the native receptor is available, but the presence of epitope tags in two positions does not alter the subcellular localization of adrenergic receptors (1). Only the epitope-tagged TRHR was studied in all experiments unless stated otherwise.

Staining of live cells with Rhod-TRH was carried out as previously described (6). Cells were incubated with Rhod-TRH at ~170 nM in HEPES-buffered Hanks' balanced salt solution at either 0° or 37° and washed before visualization. As shown previously for GHY cells (6), only a dim and diffuse background fluorescence was obtained when receptor-negative GHY, HEK, or COS cells were stained with Rhod-TRH. Staining was reduced to this background level if cells were pretreated with TRH or incubated simultaneously with Rhod-TRH and an excess of TRH.

Sequential staining of endoplasmic reticulum or Golgi apparatus and intracellular TRHR was achieved by staining live cells with DiOC6 (16) or NBD-C6-ceramide (17), followed by immunofluorescent staining of the epitope-tagged TRHR. Cells were incubated with 2.5 μg/ml $DiOC_6$ in Hanks' balanced salt solution at room temperature for 10 sec or with 10 μM NBD-C_6-ceramide in F10 medium containing 1 mg/ml bovine serum albumin at 37° for 10 min. Images were obtained using fluorescein filters. The cells were then immunofluorescently stained for the FLAG epitope on the microscopic stage using rhodamine-labeled second antibody, and images were obtained using rhodamine filters. Residual dye from live staining was minimal after the second staining procedure and did not appreciably bleed through the rhodamine filter.

In all conventional fluorescence microscopy, a Nikon inverted fluorescence microscope equipped with a 150-W xenon lamp, a Nikon 40× objective, and selective filters was used. Digital images were taken with a Cohu CCD 4910 camera in conjunction with a Colorado video integrator unit. This unit has gain and integration controls that adjust the sensitivity of the system. Digital data were processed and stored with Metamorph software (Universal Imaging, Media, PA). Confocal microscopy was performed on an ACAS 570 (Meridian Instruments) using an argon laser with excitation at 514 nm and emission at 575 nm as previously reported (6). Cells shown are representative of the majority of cells observed in multiple experiments. When only one calibration bar is shown, it refers to all panels.

Binding assays. [^{3}H]MeTRH binding and internalization were analyzed as previously described (18). Cells were incubated with [^{3}H]MeTRH in serum-free F10 medium at 37° or 0° for 1 hr, rinsed three times with 0.15 M NaCl, washed briefly with ice-cold acid/salt solution (0.5 M NaCl, 0.2 M acetic acid, pH 2.5), and then lysed with 0.1 N NaOH. Radioactivity removed with the acid/salt wash was presumed to be on the cell surface. Nonspecific binding, which was determined in parallel dishes containing a 1000-fold excess of unlabeled TRH, was < 5% of total and has been subtracted. TRHR was solubilized with either digitonin or *N*-dodecylmaltoside, as noted in the text, in Tris-Mg buffer (20 mM Tris, 2 mM $MgCl_2$, pH 7.4) with protease inhibitors (0.3 mg/ml EDTA, 35 μg/ml phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin) at 0° for 30 min. Solubilized receptor was incubated at 4° overnight with either 10 nM [^{3}H]MeTRH or, for nonspecific binding, 10 nM [^{3}H]MeTRH plus 10 μM TRH. Bound [^{3}H]MeTRH was assayed by filtration through Whatman GF/A filters that had been soaked in 0.3% polyethyleneimine (19). To confirm that tunicamycin effectively inhibited glycosylation, three 100-mm dishes of stably transfected GHY cells were incubated with and three dishes were incubated without 10 μg/ml tunicamycin for 18 hr; 5 nM [^{3}H]MeTRH was added for the last hour to label receptors. Cells were washed and receptors were solubilized with 0.1% *N*-dodecylmaltoside. The solution containing solubilized receptors was incubated with 0.5 mg of wheat germ agglutinin-Sepharose for 2 hr at 4°, the reaction mixture was diluted in Tris-Mg buffer and centrifuged, and the resin was washed two additional times at 4°. The combined supernatant fractions were filtered through polyethyleneimine-soaked filters to determine the amount of [^{3}H]MeTRH associated with unbound receptor, and the wheat germ agglutinin-Sepharose was counted to determine the amount of [^{3}H]MeTRH bound to glycosylated receptor.

Results

Effect of cell type on receptor localization. To determine whether the distribution of the TRHR is affected by cell context, we expressed an epitope-tagged TRHR in several

different cell lines and studied its localization by indirect immunofluorescence microscopy. The epitope-tagged receptor was stably transfected into the pituitary GHY line, which does not express an endogenous TRHR (15), and into HEK 293 cells. The receptor was transiently transfected into GHY cells, AtT20 pituitary corticotroph cells, HEK 293, and COS7 simian kidney cells. The Ca^{2+} response to 1 μM TRH was determined in Fura-2-loaded GHY and HEK 293 cells expressing similar densities of either the epitope-tagged or wild-type mouse TRHR and found to be of similar shape and amplitude (data not shown). Because the FLAG epitope was at the intracellular carboxyl terminus, cells were permeabilized before staining, allowing visualization of intracellular as well as cell surface TRHR.

The distribution of the TRHR depended on the cell type in which it was expressed (Fig. 1). In stably transfected GHY cells, fluorescence was confined to the plasma membrane, as previously reported for stably transfected GH_3 cells, which express endogenous wild-type as well as epitope-tagged receptors (6); GHY is a subclone of GH_3. Exposure of GHY cells to TRH at 37° caused a redistribution of the epitope-tagged TRHR to intracellular vesicles (Fig. 1).

Receptor localization was very different in HEK 293 cells stably expressing the epitope-tagged TRHR (Fig. 1). Plasma membrane fluorescence was visible in the transfected HEK 293 cells, but intracellular staining was always more intense. The staining was specific because HEK 293 cells that were stably expressing a comparable level of wild-type rather than epitope-tagged mouse TRHR gave only faint background fluorescence. Confocal microscopy confirmed that the majority of the immunofluorescence in HEK 293 cells was intracellular, although plasma membrane receptors were visible (Fig. 2). After incubation with TRH at 37°, receptors appeared in prominent intracellular vesicles, and surface staining was no longer detectable (Fig. 2). The intensity of staining varied considerably from cell to cell for both GHY and HEK 293 cells, but for both cell lines, the pattern of receptor staining seemed to be the same in lightly and heavily stained cells.

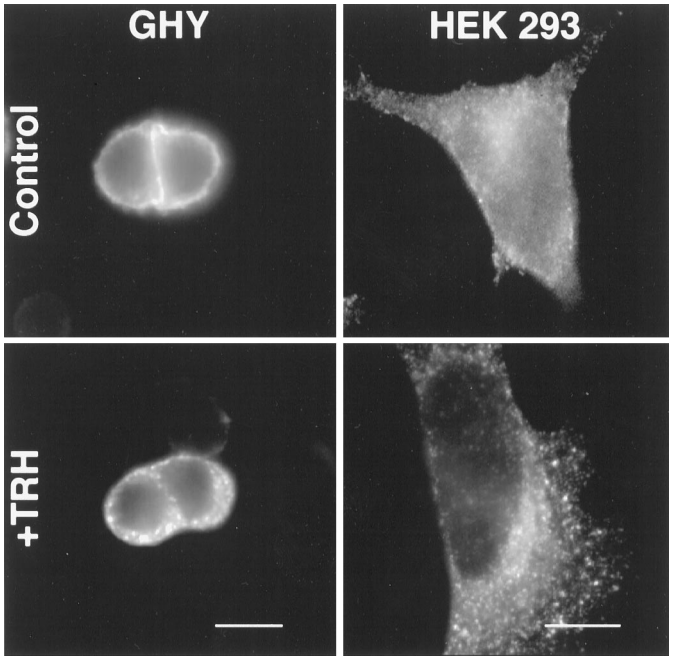

Fig. 1. Immunolocalization of TRHRs in stably transfected cells. Stably transfected (*left*) GHY and (*right*) HEK 293 cells were stained for TRHR immunofluorescence (*top*) before or (*bottom*) after incubation with 100 nM TRH at 37° for 1 hr. *Calibration bars*, 10 μm.

The plasma membrane receptors on transfected HEK 293 cells could be readily visualized with a ligand rather than a receptor probe. Staining with Rhod-TRH was confined to the plasma membrane after binding at 0° (Fig. 3). The fluorescence pattern in HEK 293 cells was different with the Rhod-TRH and the receptor probes because the ligand probe labels only receptors on the cell surface. When HEK 293 cells were incubated with Rhod-TRH for 10 min at 37°, intracellular vesicles were evident, showing that the ligand was internalized in a temperature-dependent manner (Fig. 3).

When transiently expressed in two pituitary cell lines, GHY and AtT20 (Fig. 4), the epitope-tagged TRHR was localized on the plasma membrane in a pattern similar to that seen in stably transfected pituitary GHY (Fig. 1) and GH_3 (6) cells. A different receptor distribution was observed when two nonpituitary cell lines, HEK 293 and COS7, were transiently transfected with the receptor. Both cell surface and intracellular receptor fluorescence was visible after transient transfection of HEK 293 cells (Fig. 4). In transiently transfected COS7 cells, most receptor was intracellular in a reticular pattern, and the intracellular receptor closely colocalized with markers for the endoplasmic reticulum and the Golgi apparatus (Fig. 5). In HEK 293 cells, the intracellular receptor was more diffusely distributed and was not obviously present in the endoplasmic reticulum; receptor appeared to colocalize with the Golgi in some but not all cells (data not shown). In COS7 cells, plasma membrane fluorescence was not easily visible, and incubation with TRH at 37° did not cause an obvious change in receptor distribution in COS7 cells. The same pattern of TRHR immunofluorescence was obtained in COS7 cells that had been transfected by lipofection or DEAE-dextran procedures, and the same pattern was obtained regardless of transfection efficiency. Although hardly visible in immunofluorescent staining, there was functional TRHR on the plasma membrane of COS7 cells. This could be visualized by staining with the ligand probe, Rhod-TRH, at 0° (Fig. 3). Again, the staining patterns were markedly different using the ligand and receptor probes because Rhod-TRH labels only the small fraction of receptors on the plasma membrane. In COS7 cells, Rhod-TRH internalized after ligand binding at 37° (Fig. 3).

We also expressed an epitope-tagged β_2-adrenergic receptor in GH_3 and COS7 cells using identical transient transfection protocols. β_2-Adrenergic receptor immunofluorescence was mostly on the surface of GH_3 cells (Fig. 6). We observed a plasma membrane localization of the β_2-adrenergic receptor in approximately half of the stained COS7 cells, such as those shown in the Fig. 6, and a reticular distribution in most of the remainder. These results are in general agreement with those of von Zastrow *et al.* (1), who found a plasma membrane distribution of the β_2-adrenergic receptor in 75% of transiently transfected COS7 cells. Localization of the β_2-adrenergic receptor was clearly different from localization of the TRHR in the same cells in that we never observed a COS7 cell with a predominantly plasma membrane localization of the TRHR.

Receptor density in different cell lines. Despite the striking differences in the apparent density and cellular distribution of the epitope-tagged TRHR in the various cell

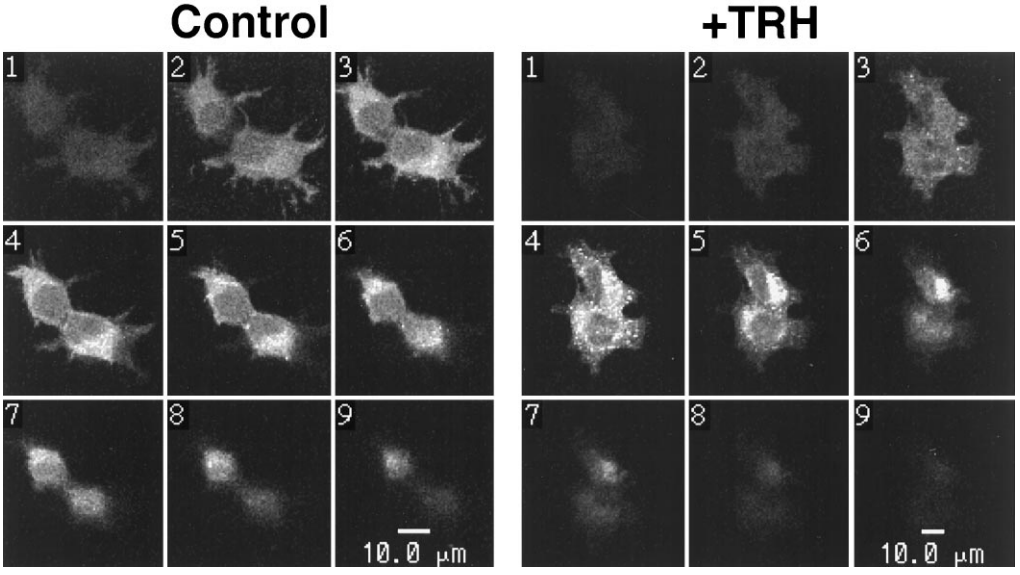

Fig. 2. Confocal microscopic localization of the TRHR in HEK 293 cells. Stably transfected HEK 293 cells were stained for TRHR immunofluorescence and examined with confocal microscopy. Optical sections (1 μm thick) are from the bottom (*1*) to the top (*9*) of cells after (*left*) no treatment or (*right*) incubation with 100 nM TRH for 20 min at 37° before staining.

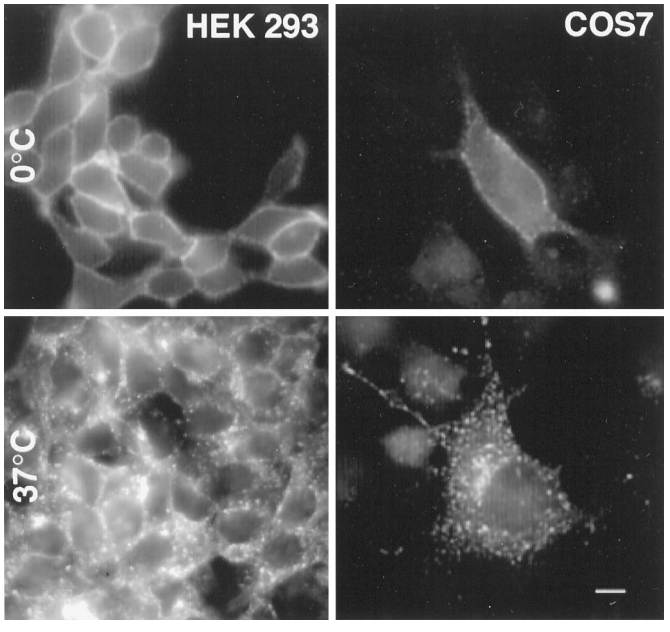

Fig. 3. Staining with a fluorescent analog of TRH (Rhod-TRH). Stably transfected HEK 293 cells (*left*) and transiently transfected COS7 cells (*right*) expressing TRHRs were incubated with Rhod-TRH as described in Materials and Methods. *Top*, cells stained with Rhod-TRH for 1 hr at 0°. *Bottom*, cells stained for 1 hr at 0° and then warmed to 37° for 10 min (HEK 293) or 20 min (Cos7). *Calibration bars,* 10 μm.

types, the lines bound similar amounts of the potent TRH agonist [^{3}H]MeTRH (Table 1). HEK 293 and COS7 cells are much larger than GHY cells (Fig. 4, *calibration bars*). The stably transfected HEK 293 cells expressed more [^{3}H]MeTRH binding sites/cell than GHY cells but bound similar amounts per milligram of cell protein, whereas the transiently transfected COS7 cells bound approximately three times more per milligram of cell protein. The affinities of the epitope-tagged TRHRs for [^{3}H]MeTRH were measured by Scatchard analysis. Measured K_d values were similar in different cell contexts: 1.4 nM (GHY), 4.7 nM (stably transfected HEK 293 cells), and 1.3 nM (transiently transfected COS7 cells). Values in the three transfected lines were all within a factor of 2 of the reported K_d value of the native TRHR in GH_3 cells, 2–3 nM for [^{3}H]MeTRH (10).

Internalization in different cell lines. Endocytosis of receptor-bound [^{3}H]MeTRH was measured biochemically by the formation of an acid/salt-resistant complex at 37°. [^{3}H]MeTRH was internalized to a similar extent in all cell types (Table 1), and internalization was temperature dependent in all cell lines. The amounts [^{3}H]MeTRH internalized after a 1-hr incubation at 4° were 37%, 25%, and 14% of the amounts internalized at 37° for GHY, HEK 293, and COS7 cells, respectively. The half-time for internalization of [^{3}H]MeTRH was 3–5 min at 37° in both GHY and HEK 293 cells. Temperature-dependent internalization of Rhod-TRH was easily visible by 5 min and extensive at 10 min, as shown for HEK 293 cells in Fig. 3.

Properties of intracellular receptors. To determine whether the intracellular receptors in HEK 293 cells could bind ligand, we measured [^{3}H]MeTRH binding after homogenization and detergent solubilization of receptors. Homogenization did not uncover new [^{3}H]MeTRH binding sites, indicating that the intracellular receptors were inactive or oriented so they did not have access to [^{3}H]MeTRH (Table 2). Furthermore, no additional [^{3}H]MeTRH binding activity could be exposed after solubilization with detergents (Table 2). These data suggest that most of the intracellular receptors are not able to bind ligand.

In transiently transfected COS7 cells, the intensity of the immunofluorescence of the epitope-tagged TRHR varied enormously among cells. An example is shown in Fig. 7, in which a cell (*top left*) stained much more brightly than two other cells (*top right*), even though microscopy and photography were identical. When the sensitivity of the system was increased, the fluorescence of the two cells (*top right*) became visible (*bottom*). The same reticular pattern of receptor immunofluorescence was seen in the dimmest and the brightest cells.

Effects of glycosylation inhibitors. One potential reason was much of the epitope-tagged TRHR protein failed to localize to the plasma membrane is that the receptor had not undergone normal glycosylation in the HEK 293 and COS7 cells. We tested this possibility indirectly by incubating with

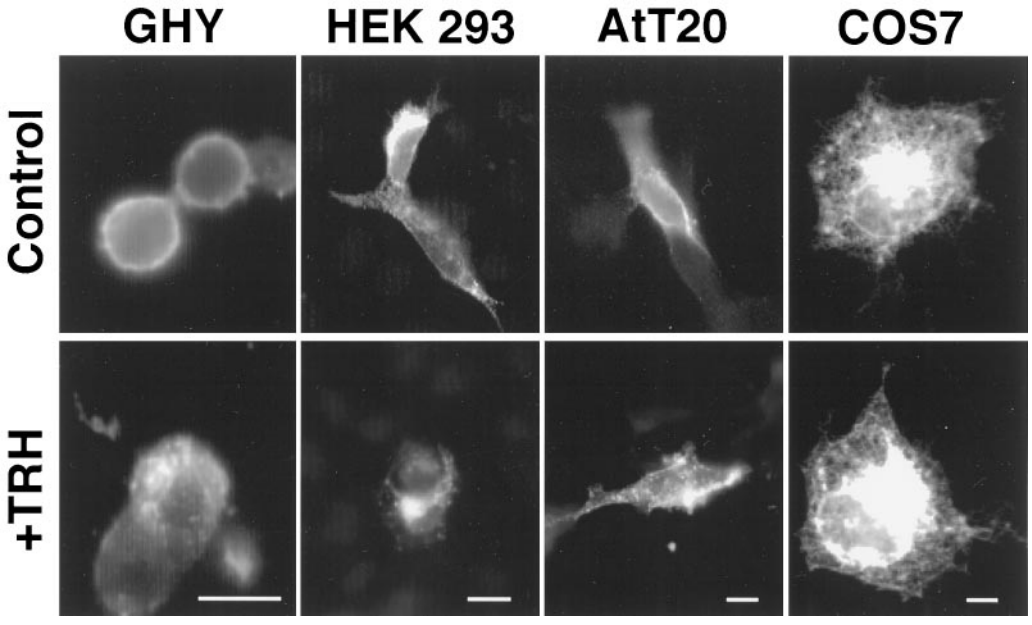

Fig. 4. Immunolocalization of TRHRs in transiently transfected cells. GHY, HEK 293, AtT20, and COS7 cells were transiently transfected with cDNA encoding the epitope-tagged TRHR. Cells were stained 24–48 hr later (*top*) before or (*bottom*) after incubation with 100 nM TRH for 30–60 min at 37°. *Calibration bars*, 10 μm.

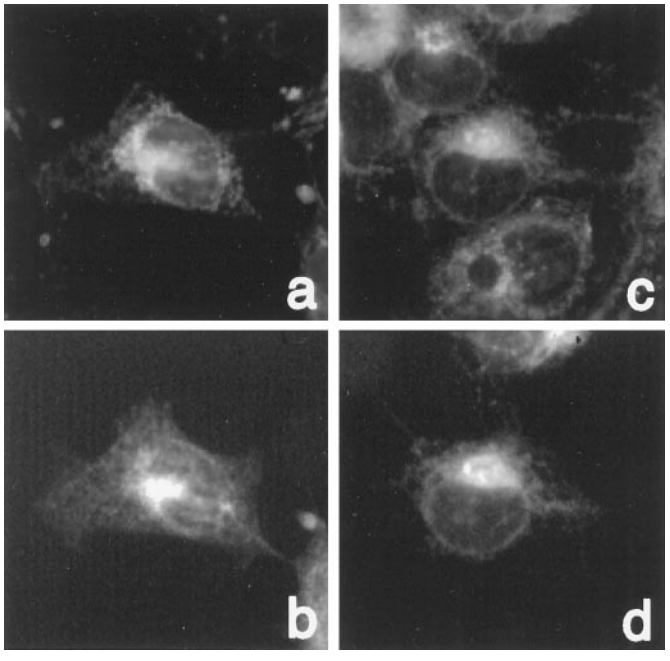

Fig. 5. Colocalization of endoplasmic reticulum or the Golgi apparatus and TRHR in COS7 cells. COS7 cells that had been transiently transfected with cDNA encoding the epitope-tagged TRHR were sequentially stained for (a) endoplasmic reticulum and then (b) TRHR or (c) the Golgi apparatus and then (d) TRHR as described in Materials and Methods. a/b and c/d show the same fields. The cells in the center of a–d expressed TRHR. Note that most cells stained for the endoplasmic reticulum and Golgi did not stain for TRHR, showing that retention and bleed-through of residual dyes from the first staining were minimal.

tunicamycin, a glycosylation inhibitor, GH cells that expressed epitope-tagged TRHRs (Fig. 8). Tunicamycin did not affect the normal plasma membrane distribution of receptors, suggesting that glycosylation is not necessary for transport of TRHRs to the plasma membrane. To confirm that tunicamycin effectively inhibited glycosylation, we measured the effect of tunicamycin on the ability of the TRHR to adhere to wheat germ agglutinin-Sepharose, which normally binds the *N*-glycosylated receptor (19), as described in Materials and Methods. Tunicamycin treatment reduced the amount of receptor-bound [^{3}H]MeTRH absorbed to wheat germ agglutinin from 7400 ± 909 to 2600 ± 147 cpm ($p = 0.01$) and reduced the amount not absorbed by the lectin from 3705 ± 513 to 2522 ± 238 cpm ($p = 0.10$). Monensin (10 μM), which interferes with transfer through the Golgi stacks and also inhibits glycosylation, did not alter the apparent localization of the epitope-tagged TRHR (data not shown).

Effects of protein synthesis inhibitors. As shown in Fig. 9, cycloheximide caused a marked redistribution of receptor immunofluorescence from the intracellular space to the plasma membrane in transfected HEK 293 cells. This redistribution required ~24 hr and was observed at cycloheximide concentrations of ≥2 μg/ml. Puromycin, which is another protein synthesis inhibitor, caused a similar redistribution of receptors to the plasma membrane. Incubation of pituitary cells with cycloheximide did not cause a detectable redistribution of TRHRs (data not shown).

Cycloheximide increased the amount of [^{3}H]MeTRH binding to intact HEK 293 cells (Fig. 10), indicating that the receptors that seemed to have been translocated to the plasma membrane by immunocytochemistry were able to bind ligand. The effects of cycloheximide were maximal at ~5 μg/ml, at which protein synthesis, measured as the incorporation of [^{3}H]Leu into acid-insoluble material, was inhibited by > 95%, and the translocation of receptors reached a maximum at 48–72 hr. Cycloheximide caused an absolute increase in the number of functional receptors even in cultures in which cell division was almost completely blocked. Cycloheximide and puromycin both significantly increased the amount [^{3}H]MeTRH bound/mg of cell protein in HEK 293 cells (Fig. 10). Cycloheximide did not substantially increase [^{3}H]MeTRH binding to pituitary GHY cells expressing the epitope-tagged receptor (Table 3). This finding, like the immunolocalization studies, indicates that GHY cells do not have a large intracellular receptor pool.

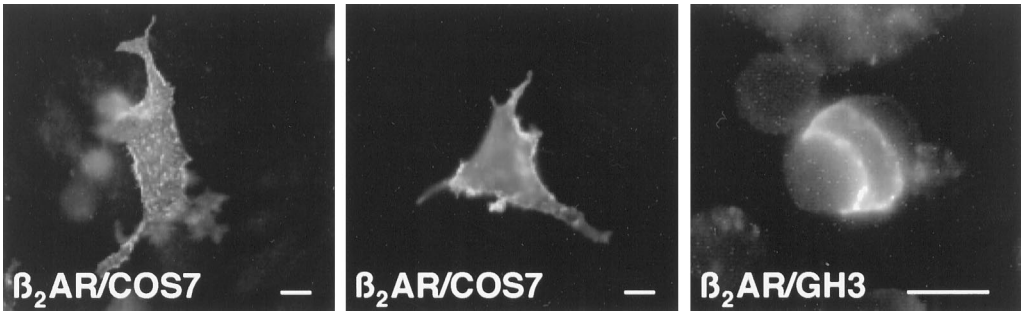

Fig. 6. Localization of the β_2-adrenergic receptor in COS7 and GH_3 cells. COS7 (*left* and *center*) or GH_3 (*right*) cells were transiently transfected with a β_2-adrenergic receptor containing an amino-terminal FLAG epitope. Cells were then fixed and stained for the FLAG epitope either (*middle*) without permeabilization or (*left and right*) after detergent permeabilization.

TABLE 1

Density of TRH receptors in different cell lines

Specific binding of 2.5 nM [^{3}H]MeTRH to pituitary GHY, HEK 293, and COS7 cells expressing an epitope-tagged TRH receptor was measured after a 1-hr incubation at 37°. The fraction of specifically bound hormone internalized was measured with an acid/salt as described in Materials and Methods. Before transfection, none of the lines showed detectable calcium responses to TRH or specific binding of [^{3}H]MeTRH. Nearly 100% of stably transfected GHY and HEK 293 cells and ~15% of COS7 cells expressed TRH receptors, based on the fraction of fura-2-loaded single cells responding to TRH with a rise in intracellular [Ca^{2+}]. The results shown for COS7 cells are corrected for the fact that only 15% of the cells expressed TRH receptors. Value are mean ± range or standard error of two or three dishes in a representative experiment.

Cell line	[^{3}H]MeTRH bound		[^{3}H]MeTRH internalized
	pmol/mg of protein	*sites/cell*	*% of total bound*
GHY (stable)	0.316 ± 0.014	51,300	94.3 ± 1.6
HEK 293 (stable)	0.472 ± 0.008	195,000	92.1 ± 0.3
COS7 (transient)	1.09 ± 0.053	299,000	80.2 ± 2.1

TABLE 2

Cell surface versus total TRH receptors

Specific binding of [^{3}H]MeTRH was measured using GH_3 cells or stably transfected HEK293 cells. In experiment 1, cells from a single culture were suspended in Hanks' balanced salt solution, pelleted, and resuspended in either Hanks' balanced salt solution (intact cells) or homogenized with 25 strokes of a Dounce homogenizer in 20 mM Tris Cl and 2 mM $MgCl_2$, pH 7.6 (cell homogenates), and binding of 5 nM [^{3}H]MeTRH was measured after 1 hr at 0°. In experiment 2, receptors were solubilized with detergents, and specific binding of [^{3}H]MeTRH to solubilized receptors was measured after 18 hr at 0° as described in Materials and Methods. Results are expressed as a percentage of the amount of [^{3}H]MeTRH binding to cell homogenates, which was measured in the same experiment. Values are the mean ± standard error of triplicate determinations.

Conditions	[^{3}H]MeTRH bound	
	GH_3 cells	HEK 293 cells
	fmol/mg of protein	
Experiment 1		
Intact cells	100 ± 6	968 ± 25
Cell homogenates	82 ± 8	1044 ± 38
	%	
Experiment 2		
Cell homogenates	≡100	≡100
1% Digitonin solubilized	46[a]	51.1 ± 7.3
0.1% *N*-Dodecylmaltoside solubilized	47.7 ± 12.9	22.0 ± 3.2

[a] Ref. 19.

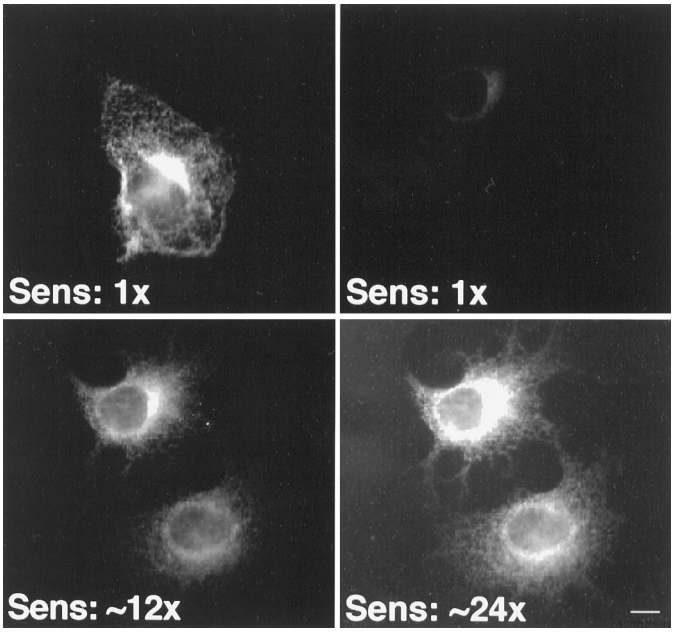

Fig. 7. Immunolocalization in cells expressing different levels of receptors. TRHRs were immunofluorescently stained in transiently transfected COS7 cells. The four images were taken from the same coverslip, and the imaging system was set identically except that the sensitivity (*Sens*) was increased as shown by adjusting the gain and integration controls. The intensity of an image increases approximately linearly with sensitivity settings. *Top right* and *bottom*, same cells. *Calibration bar*, 10 μm.

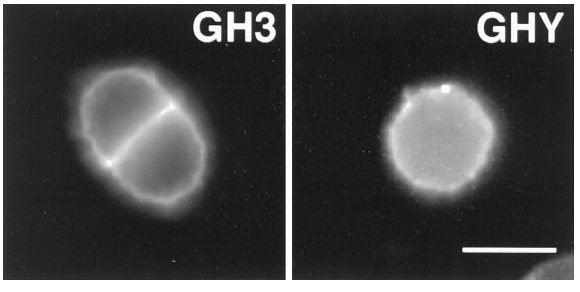

Fig. 8. TRHR immunolocalization after inhibition of glycosylation. Stably transfected (*left*) GH3 and (*right*) GHY cells were treated with 10 μg/ml tunicamycin for 18 hr before staining. *Calibration bars*, 10 μm.

Discussion

The findings reported here show that the distribution of a single G protein-coupled receptor can differ dramatically in different cell types. The TRHR was confined to the plasma membrane in two pituitary lines, GHY and AtT20, but was predominantly intracellular in two nonpituitary lines, HEK 293 and COS7. Cell type-dependent differences in receptor localization have been demonstrated previously for thrombin receptors (7, 8). There is a sizable intracellular pool of thrombin receptors in human umbilical vein endothelial cells, which express an endogenous receptor, or in transfected Rat1 cells, and these vesicular receptors cycle to the membrane to replenish the plasma membrane receptors depleted by thrombin activation. In megakaryoblastic cells, however, there does not seem to be a significant intracellular pool of thrombin receptors. For most G protein-coupled receptors, subcellular localization has not been found to differ with cell

Fig. 9. Effect of protein synthesis inhibitors on TRHR localization. Stably transfected HEK 293 cells were incubated for 72 hr with (*left*) no drug, (*middle*) 2 μg/ml cycloheximide, or (*right*) 500 ng/ml puromycin. Cells were then stained for TRHR immunofluorescence.

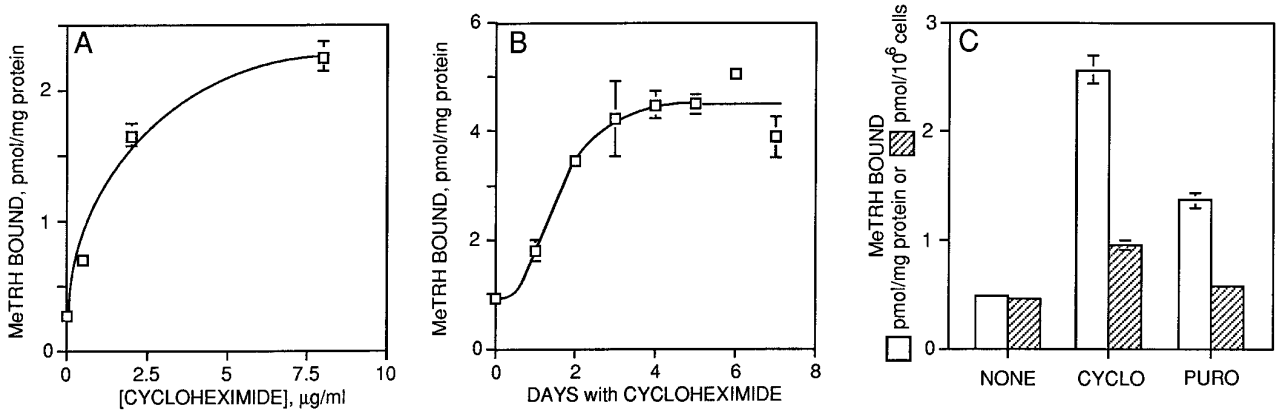

Fig. 10. Effect of protein synthesis inhibitors on the density of cell surface TRHRs. Stably transfected HEK 293 cells were incubated with protein synthesis inhibitors as follows. A, 72 hr with cycloheximide at the concentrations shown. B, 2 μg/ml cycloheximide for the times shown. C, 72 hr with (*NONE*) no drug, (*CYCLO*) 2 μg/ml cycloheximide, or (*PURO*) 500 ng/ml puromycin. Cell surface receptor was quantified by incubating cells with 5 nM [^{3}H]MeTRH for 1 hr at 0° and measuring specific binding, which is expressed as binding/mg of cell protein (A and B and *open bars* in C) or binding/cell (*hatched bars* in C). Where not visible, error bars were within symbol size.

TABLE 3

Effect of protein synthesis inhibitors on cell surface TRH receptors

GHY and HEK 293 cells stably expressing epitope-tagged TRH receptors were incubated with or without 2 μg/ml cycloheximide for 72 hr. Specific binding of 5 nM [^{3}H]MeTRH was measured after a 1-hr incubation at 0°. Values are mean ± range of duplicates in a representative experiment.

Cell line	[^{3}]MeTRH bound	
	Control	Cycloheximide
	pmol/mg of protein	
GHY (stable)	0.150 ± 0.025	0.161 ± 0.020
HEK 293 (stable)	0.484 ± 0.017	2.558 ± 0.209

context. Gastrin-releasing peptide, cholecystokinin receptors, and several classes of adrenergic receptors display the same subcellular distribution when expressed in a variety of cell contexts (1–5). It is not possible to predict which receptors will be localized aberrantly in a heterologous cell and which will not.

The molecular basis for the cell type dependence of receptor localization remains uncertain. Because the FLAG epitope is at the carboxyl terminus of the epitope-tagged TRHR, only completely synthesized receptors were visualized with immunocytochemistry. One possible explanation for the cell type dependence of receptor localization is that the levels of expression in COS7 or HEK 293 cells were so high that some aspect of post-translational processing or trafficking was saturated so receptors were retained in the endoplasmic reticulum; in this model, blocking synthesis of other proteins might facilitate transport of the long-lived TRHR to the plasma membrane. However, simple overexpression is unlikely to be the entire explanation for two reasons. First, cycloheximide was more effective than puromycin in allowing receptors to move to the plasma membrane, although the drugs were equally effective at blocking protein synthesis. Second, the pattern of localization was the same in COS7 cells expressing low as well as high levels of total receptor, based on fluorescence intensity.

The TRHR has two potential *N*-glycosylation sites at its amino terminus (20) and is normally glycosylated (19). It was recently reported that deletion of residues 2–22 of the TRHR, which removes the two amino-terminal glycosylation sites, does not prevent receptor expression or binding (21). In the current study, inhibition of glycosylation did not cause any detectable trapping of TRHRs intracellularly in pituitary cells. These results both indicate that glycosylation is not essential for plasma membrane localization. It is not known whether the TRHR is post-translationally modified in any other manner (e.g., by palmitoylation or phosphorylation) or whether such modifications influence receptor localization.

In addition to demonstrating cell type dependence of the localization of the TRHR, our results support previous studies showing that different G protein-coupled receptors can have different localizations in the same cell. von Zastrow *et al.* (1) showed that different subtypes of the α_2-adrenergic receptor differed from one another in their subcellular distribution, as well as in their response to agonist. The same differences were found when these adrenergic receptors were transfected into either COS7 or HEK 293 cells. When transiently expressed in COS cells, the β_2-adrenergic receptor was clearly localized on the cell surface in half of the cells,

but the TRHR was not predominantly plasma membrane in any cell. Because the TRHRs and β_2-adrenergic receptors were tagged with the same epitope and the intensity of the immunofluorescence was similar in cells expressing the two types of receptors, it is likely that the differences in localization result from intrinsic differences in receptor structures. The importance of receptor structure to subcellular localization has also been highlighted through the study of naturally occurring mutations in a number of membrane proteins. Certain mutations in rhodopsin (22) block normal trafficking to the plasma membrane and result in the accumulation of receptor in the endoplasmic reticulum, accounting for some hereditary forms of retinitis pigmentosa.

All of the available data for the TRHR indicate that receptors that do reach the plasma membrane are functional. [^{3}H]MeTRH binds with comparable affinity to receptors expressed in a variety of cell types, and agonist binding drives a temperature-dependent internalization of both the ligand and the receptor in all cell lines (6, 9, 23, 24). Clathrin-dependent pathways are used in different cell types.[1] Similar findings have been reported for the hCG/LH receptor, which internalizes identically in Leydig cells, where it is expressed naturally, and after transfection into L-cells (25). It is not possible, however, to generalize the conclusion that receptors will internalize identically in different cell contexts. The cholecystokinin receptor that occurs naturally in pancreatic acinar cells does not undergo agonist-dependent internalization, but the cholecystokinin receptor expressed at higher densities in CHO cells is internalized via a classic clathrin-dependent mechanism when activated (4, 5).

A number of aspects of TRHR expression and regulation have been reported to depend on the context in which the receptor is expressed (9, 12–14). The cell type dependence of receptor localization shown here may help explain some of these findings. In pituitary cells, the level of TRHR mRNA is regulated by steroid, thyroid, and peptide hormones, as well as by drugs that activate second messenger pathways, including cAMP analogs and phorbol esters (for a review, see Ref. 9). Both the rate of transcription of the TRHR gene and the stability of the TRHR message are controlled. Our results suggest that all of the receptor synthesized in pituitary cells will be transported to the plasma membrane; therefore, it is likely that any change in the level of the message and the rate of receptor synthesis will result in a change in the number of functional receptors. In contrast, only a fraction of TRHRs synthesized in transfected HEK 293 or COS7 cells will appear at the plasma membrane, and changes in receptor mRNA levels in these cells are likely to have little effect on the density of functional receptors at the plasma membrane. These differences may contribute to the cell type dependence of the effects of TRH, cAMP, and phorbol esters on TRHR mRNA. Any agent that affects the trafficking of receptors from the endoplasmic reticulum to the plasma membrane could also alter the number of functional TRHRs.

It is often convenient to express receptors in heterologous lines such as COS and HEK 293 that are easy to transfect and grow. The current results show that the possibility of aberrant receptor localization needs to be considered when interpreting data obtained in such systems. For example, it is difficult to determine experimentally whether a mutated receptor is not expressed or is expressed but does not bind ligand. Western blots would not be useful for distinguishing between these possibilities for TRHRs in cells like COS7 and HEK 293 because most receptors are not at the membrane. It is also well established that the repertoire of G proteins and effectors may differ with cell type and can influence signal transduction. Additional experimentation will be required to establish the molecular basis for the localization of G protein-coupled receptors.

[1] R. Yu and P. M. Hinkle, unpublished observations.

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