Agonist-dependent up-regulation of thyrotrophin-releasing hormone receptor protein

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To study the effect of agonist on the TRH (thyrotrophin-releasing hormone) receptor protein, an epitope-tagged receptor was stably expressed in HEK-293 cells (human embryonic kidney 293 cells) and receptor levels were measured by immunoblotting. TRH caused a 5–25-fold increase in receptor protein during 48 h, which was half-maximal at 1 nM and was slowly reversible after hormone withdrawal. Chlordiazepoxide, an inverse agonist, had no effect. TRH up-regulation was mimicked by phorbol ester and blocked by the protein kinase C inhibitor GF109203X in combination with thapsigargin, which prevents a calcium response. TRH and phorbol ester increased the density of immunoreactive receptors localized at the cell surface and [³H]MeTRH (where MeTRH stands for [³H-methyl-His]TRH) binding. TRH also increased the concentration of a truncated, internalization-defective receptor. Analysis of cell lines stably expressing TRH receptors fused to the green fluorescent protein on a fluorescence-

activated cell sorter showed that TRH and phorbol ester caused 2.7- and 6.8-fold increases in fusion protein expression respectively. TRH receptor up-regulation was only partially accounted for by changes in receptor mRNA, which increased 1.7-fold. TRH caused a small increase in receptor concentration in the presence of cycloheximide, actinomycin D or MG132. In contrast with the results obtained with the TRH receptor, agonist decreased the concentration of stably expressed β_2 -adrenergic receptors. These results show that TRH increases receptor concentration by a complex mechanism that requires signal transduction but not receptor endocytosis.

Key words: G-protein-coupled receptor, protein kinase C, thyrotrophin-releasing hormone (TRH), thyrotrophin-releasing hormone receptor (TRHR).

INTRODUCTION

TRH (thyrotrophin-releasing hormone) receptor (TRHR) is a calcium-mobilizing G-protein-coupled receptor expressed in the anterior pituitary gland, where it regulates thyrotrophin and prolactin secretion, and in other tissues, where its function is less understood. In response to agonist binding, TRHR undergoes rapid and extensive internalization, which results from receptor phosphorylation, β -arrestin binding and targeting to clathrin-coated pits [1–4].

In pituitary cell lines expressing endogenous receptors, TRH binding leads to internalization of approx. 80 % of surface-binding sites, whereas TRH removal is followed within a few hours by the reappearance of most of the binding activity on the membrane [5]. However, recycling of the receptor may not be complete, since the sustained agonist occupation of receptor for 24–48 h results in a loss of 50–75 % of the receptor-binding sites, termed down-regulation [6,7]. One possible explanation for these findings is that a fraction of the internalized receptor enters a degradative pathway, leading to a gradual loss of receptor over time [8]. In addition, TRH decreases the transcription of the endogenous TRHR gene and destabilizes the TRHR mRNA, which would contribute to down-regulation [9–11].

TRHRs are found both on the plasma membrane and in intracellular pools [12]. Although TRH effects on surface TRH-binding sites have been documented, no information is available about how an agonist regulates the overall concentration of TRHR

protein. In the present study, we report that the TRHR protein concentration is increased in response to an agonist.

EXPERIMENTAL

Development of cell lines

Antibodies against many G-protein-coupled receptors, including TRHR, are not satisfactory for immunoprecipitation or immunoblotting. For this reason, we utilized cells expressing epitope-tagged receptors. HEK-293 cell lines (human embryonic kidney 293 cell lines), stably expressing either full-length or C-terminal-truncated forms of the rat TRHR tagged with two HA (haemagglutinin) epitopes at the N-terminus, have been described previously [4]. HEK-293 cells stably expressing GFP (green fluorescent protein)-tagged TRHRs or FLAG-tagged β_2 -adrenergic receptors were prepared by transfecting cells with the plasmids encoding receptors cloned in pcDNA3 using LIPOFECTAMINETM (Invitrogen) according to the manufacturer's instructions. The cells expressing receptors were selected in 0.5 mg/ml G418. The plasmid encoding FLAG-tagged β_2 -adrenergic receptor was provided by Dr Michel Bouvier (Universite de Montreal, Montreal, Canada). The plasmid encoding GFP-TRHR was obtained from Dr Karin Eidne (University of Western Australia, Perth, Australia). Cells were grown in DMEM (Dulbecco's modified Eagle's medium) or DMEM/F12 supplemented with 7.5 % (v/v) foetal bovine serum, in some

Abbreviations used: GFP, green fluorescent protein; HA, haemagglutinin; HEK-293 cells, human embryonic kidney 293 cells; TRH, thyrotrophin-releasing hormone; MeTRH, [³H-methyl-His]TRH; TRHR, TRH receptor.

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cases with 50 $\mu\text{g/ml}$ gentamicin and 2.5 $\mu\text{g/ml}$ amphotericin B, and experiments were performed in this media unless indicated otherwise.

Immunoprecipitation and immunoblotting

For immunoblotting, cells cultured in 35 mm dishes were rinsed twice with 2 ml of saline and lysed in 500 μl of ice-cold lysis buffer [150 mM NaCl/50 mM Tris, pH 8.0/1 % (w/v) Triton X-100/1 mM EDTA/1:1000 Protease Inhibitor Cocktail Set III (Calbiochem)]. Lysates were centrifuged for 10 min at 16 000 g in an Eppendorf microcentrifuge at 4 °C. The pellets from control and TRH-treated cultures did not contain significant amounts of immunoreactive TRHR. The supernatants were diluted directly in sample buffer or immunoprecipitated either with an anti-HA antibody (1:5000; Covance) before SDS/PAGE and immunoblotting for TRHR or with an anti-FLAG antibody (1:5000; Sigma) for β_2 -adrenergic receptors [4]. Either the immunoprecipitates or the lysates were run on SDS/polyacrylamide gels and blotted with an antibody directed to HA, FLAG or β -actin (Sigma). To compare the amounts of receptor by densitometry, samples from untreated and treated cultures were serially diluted, run on SDS/polyacrylamide gels and immunoblotted. Where histograms and graphs represent results from multiple experiments, films of blots from individual experiments were scanned and, after background subtraction, the average intensity of receptor bands was plotted in arbitrary units relative to the intensity of receptor in control lanes. Since the intensity of receptor bands in lanes from stimulated cells were often above the linear range, the TRH- and PMA-induced increases in receptor levels, measured by scanning blots from multiple experiments, were found to be generally lower than those obtained when samples from an individual experiment were serially diluted, run on Western blots and compared. The significance of differences between pairs was analysed by unpaired Student's *t* test.

Immunocytochemistry

For immunocytochemistry, cells grown on 25 mm coverslips were rinsed with PBS, fixed in 1 ml of 4 % (w/v) paraformaldehyde in PBS for 20 min at room temperature (20 °C) and incubated for 20 min in a blocking buffer, namely PBS containing 5 % (v/v) goat serum. A mouse monoclonal anti-HA antibody was added at a dilution of 1:1000 in the same buffer for 1–3 h and the coverslips were washed, incubated with TRITC (tetramethylrhodamine β -isothiocyanate)-conjugated anti-mouse secondary antibody (1:500; American Qualex, San Clemente, CA, U.S.A.) and then washed and mounted [13].

Real-time PCR analysis

Total RNA was isolated from cells in 100 mm dishes using RNeasy (Qiagen). Total RNA (10 μg) was digested with DNase, and cDNA was generated from 1.6 μg using the RETROScript kit (Ambion, Austin, TX, U.S.A.) with random primers. For control reactions, reverse transcriptase was omitted. Primers to the rat TRHR sequence were designed to amplify a 290 bp sequence corresponding to amino acids Leu¹⁶⁴ to Ser²⁶⁰. The primers were 5'-CTGGATCTCAATATCAGCACCTAC-3' and 5'-GGAA-GAAACGGTGCTGTTGAAACA-3'. Real-time analysis was performed using a plasmid encoding the rat TRHR (10 fg–100 pg) to establish a standard curve. PCRs amplifying the TRHR sequence contained 125 ng of primers, 5 μl of inactivated reverse transcription reaction and 25 μl of SYBRTaq Master Mix in

50 μl . PCR products were normalized to β -actin sequence amplifications from the same reverse transcription reaction using primers obtained from Biosource (Camarilla, CA, U.S.A.). Amplification of the reactions lacking reverse transcriptase served as controls for DNA contamination. Reactions were performed in a Bio-Rad iCycler after an initial 8 min 30 s denaturation step using 95° denaturation for 30 s, 45° annealing for 30 s and 72° extension for 30 s for 40 cycles. A melt curve was drawn at the end of the reaction to verify the absence of primer–primer dimers and non-specific PCR products. Changes in TRHR mRNA levels were determined by the $2^{-\Delta\Delta\text{CT}}$ method [14] using β -actin mRNA as a control.

FACS analysis

Control HEK-293 cells or HEK-293 cells stably expressing TRHR–GFP were either treated with 10 nM PMA or 1 μM TRH or left untreated overnight. The next day, cells from 10 cm dishes or a 75 cm² flask were washed twice with 10 ml of Hanks balanced salt solution containing 15 mM Hepes, and then pelleted in a IEC tabletop centrifuge on setting 3 for 5 min. Cells were resuspended in the same buffer and used to analyse fluorescence using a BD FACSCalibur System (BD Biosciences). The GFP was excited at 488 nm and its emission was measured at 530 nm.

Fluorimetric analysis

Control HEK-293 cells or HEK-293 cells stably expressing TRHR–GFP were treated with drugs for 18–20 h. For each point, cells on a 6 cm dish were rinsed twice with balanced salt solution and suspended in 2 ml of Hanks balanced salt solution containing 15 mM Hepes (pH 7.4), and placed in a cuvette and subjected to stirring. Fluorescence was measured on a PerkinElmer LS5 fluorimeter with excitation and emission at 480 and 515 nm respectively, using slit widths of 5 and 10 nm respectively. The fluorescence obtained with HEK-293 cells was approx. 25 % of that measured in an equivalent concentration of cells expressing TRHR–GFP, and has been subtracted.

RESULTS

In all the experiments described in this paper, TRHRs were expressed at 0.5–2 pmol/mg of protein, similar to the expression levels of endogenous receptors, namely 1.1 pmol/mg of protein [15]. A TRHR tagged with an N-terminal HA epitope was stably expressed in HEK-293 cells. To determine whether ligand binding altered the concentration of receptors, we incubated HA–TRHR HEK-293 cells for 18 h with either TRH or the inverse agonist chlordiazepoxide and we followed the receptor concentration by immunoblotting with anti-HA antibodies. As shown in previous studies, the TRHR runs in broad monomer and dimer bands typical of glycosylated G-protein-coupled receptors and undergoes an up-shift due to phosphorylation in response to TRH [4] (Figure 1A). After 18 h, TRH caused a large increase in the level of receptor protein, whereas chlordiazepoxide had little effect. Other experiments showed that 100 μM chlordiazepoxide was capable of blocking the induction of receptor protein by 100 nM TRH (results not shown). The amount of receptor protein did not increase significantly until TRH had been present for approx. 8 h and continued to increase for 48 h (Figure 1B). The increase in receptor protein, measured after an 18 h incubation, was half-maximal at 1 nM and maximal at 100 nM TRH, consistent with the receptor K_d of 10 nM (Figure 1C). Titration of the samples

Figure 1 Effects of TRH agonist and antagonist on TRHR concentration

HA-TRHR HEK-293 cells were incubated as described, lysed, and 20 μ l of the sample was subjected to SDS/PAGE and immunoblotted using anti-HA antibody. Total protein concentrations were not affected by the treatments. **(A)** TRH (1 μ M) or 100 μ M chlordiazepoxide (CDE) for 1 or 18 h; **(B)** TRH (1 μ M) for 0–48 h; **(C)** TRH (0–1 μ M TRH) for 18 h; **(D)** cells were incubated for 18 h with or without 1 μ M TRH. On the following day, one set of dishes was harvested immediately (0 h). The remaining dishes were washed and incubated in TRH-free medium for the indicated time periods before collection. Results were similar for a washout experiment where TRH was added at different time points and cells were harvested simultaneously. In the graphs below the blots in **(A, C and D)**, results are expressed as the means $\pm$ range of the intensity of receptor bands for two independent experiments, whereas the graph in **(B)** shows individual points from two experiments. Results are normalized to receptor levels in the far left lanes. Increases due to TRH were significant ($P < 0.01$).

indicated that the total receptor protein increased by 10–25-fold in the experiments shown in Figures 1(A–C). To study the reversibility of this agonist effect, we incubated cells for 18 h with a maximally effective concentration of TRH and then withdrew the hormone and measured receptor protein levels at intervals (Figure 1D). Receptor concentrations did not decrease substantially for more than a day and remained above those of untreated cells as late as 48 h after TRH washout.

To ascertain whether this TRH response occurred in other cell lines, we tested a different clone of stably transfected HEK-293 cells, a pool of stably transfected HEK-293 cells and a pool of stably transfected Chinese-hamster ovary cells, all expressing the full-length TRHR. In each case, TRH increased the amount of TRHR detected in Western blots; results with the pool are shown

in Figure 2(A). The protein kinase C activator PMA also increased the amount of receptor monomer and dimer. These differences were not due to the movement of receptors to or from a detergent-insoluble compartment, since detergent supernates contained essentially all of the immunoreactive receptor in all cases. TRH sometimes increased the receptor protein in transiently transfected cells, but the changes were small and inconsistent.

In addition, we stably expressed a truncated TRHR (Δ 335–412 HA-TRHR) in HEK-293 cells and measured the receptor concentration after 18 h exposure to TRH, phorbol ester or no drug. This receptor mutant, which lacks two putative palmitoylation sites and the distal region of the cytoplasmic tail, signals normally but does not undergo ligand-dependent endocytosis [3]. TRH significantly increased receptor concentration, and PMA

Figure 2 Up-regulation of wild-type and C-truncated TRHRs

(A) Pools of HEK-293 cells stably expressing wild-type HA-TRHR or (B) a clone of HEK-293 cells stably expressing $\Delta 335$ –412 HA-TRHR were incubated with vehicle, 10 nM PMA or 1 μ M TRH for 18 h. Lysates from $\Delta 335$ –412 HA-TRHR cells were immunoprecipitated with an anti-HA antibody. Either the resulting immunoprecipitate or lysates from HA-TRHR HEK-293 cells were immunoblotted with an anti-HA antibody. The histogram shows the intensity of receptor bands from 12 (PMA) or 20 (TRH) experiments (A) or the mean and range from duplicate experiments (B). Increases due to TRH and PMA were significant at $P < 0.01$ in (A) and the increase due to TRH was significant at $P < 0.05$ in (B).

produced an even larger effect (Figure 2B). The finding that TRH increases the amount of $\Delta 335$ –412 HA-TRHR protein proves that receptor endocytosis is not required.

TRHR couples via $G_{q/11}$ with phospholipase C; TRH stimulates the production of inositol triphosphate, which releases intracellular calcium, and diacylglycerol, which activates protein kinase C. To probe the mechanism of the TRH-dependent increase in receptor protein concentration, we exposed cells to drugs that activate protein kinase C (the phorbol ester PMA) or increase intracellular calcium (the ionophore ionomycin) to mimic hormone action. PMA increased TRHR protein as effectively as TRH, whereas ionomycin did nothing by itself and did not alter the TRH response (Figures 2 and 3A). The drug U73122 partially blocks the activation of phospholipase C by TRH, and it partially inhibited the TRH response. GF109203X, a protein kinase C inhibitor, and thapsigargin, an inhibitor of the calcium pump that maintains intracellular calcium stores, both decreased the TRH effect. The combination of GF109203X and thapsigargin, which should block both arms of the TRH signalling pathway, completely abrogated the ability of TRH to increase the receptor protein concentration. These results are corroborated by binding studies that revealed changes in radioligand binding similar to changes in receptor protein (Figure 3B). Since [3 H]MeTRH (where MeTRH stands for [N^3 -methyl-His]TRH) is membrane-impermeable, these differences reflect an increase in receptor at the plasma membrane. TRH was washed out before the addition of [3 H]MeTRH to allow internalized receptors to cycle to the cell surface. Nonetheless, the increases in ligand binding were always smaller than the increases in total receptor protein. To explore further whether surface receptors increased, we immunostained cells from control, TRH- and PMA-treated cells without permeabilization (Figure 4). As shown by the more intense

Figure 3 Role of signal transduction

HA-TRHR HEK-293 cells were incubated for 18 h with combinations of the following drugs: 1 μ M TRH, 10 nM PMA, 100 nM ionomycin (iono), 10 μ M U73122, 5 μ M GF109203X and 100 nM thapsigargin (Thaps). Inhibitors were added 1 h before TRH. (A) The next day, cell lysates were prepared and immunoblotting was performed with an anti-HA antibody. The histogram below the blot represents the means $\pm$ S.E.M. for three independent experiments. Increases due to TRH were significant in cells incubated with no drug ($P < 0.01$), ionomycin and U73122 ($P < 0.05$). (B) Parallel dishes were rinsed three times, incubated for 1 h in TRH-free media and then incubated for 1 h at 37 $^{\circ}$ C with 5 nM [3 H]MeTRH with or without excess unlabelled hormone to measure specific binding [7]. Results are expressed as means $\pm$ range.

Figure 4 Immunolocalization of TRHRs

HA-TRHR HEK-293 cells were incubated for 18 h with no drug, 1 μ M TRH or 10 nM PMA, fixed, and stained with an anti-HA antibody without permeabilization. The images were captured under the same conditions and processed in the same manner.

staining in the middle and right panels, TRH and PMA both increased the density of receptors at the plasma membrane.

To investigate the specificity of the TRH effect on receptors, we prepared a stable cell line expressing a TRHR fused to GFP at its cytoplasmic C-terminal tail and analysed GFP fluorescence using a fluorimeter to estimate receptor concentration. In agreement with results obtained by immunoblotting, PMA increased the

Figure 5 Effects of TRH agonist and antagonist on cells expressing GFP-tagged TRHRs

Either untransfected HEK-293 cells or cells stably expressing TRHR-GFP were treated for 18 h with either no drug, 10 nM PMA, 100 μ M chlordiazepoxide (CDE) or 100 μ M chlordiazepoxide + 100 nM TRH. The cells were suspended and GFP fluorescence was analysed with a PerkinElmer LS5 fluorimeter as described in the Experimental section. Results are expressed as the means $\pm$ S.E.M. for triplicate determinations. Increases due to TRH and PMA alone were significant ($P < 0.01$). The experiment was repeated three times with similar results.

Figure 6 FACS analysis of cells expressing GFP-tagged TRHRs

Either untransfected HEK-293 cells or cells stably expressing TRHR-GFP were treated for 18 h with no drug, 10 nM PMA or 1 μ M TRH and the fluorescence of individual cells was analysed by FACS. n = number of cells analysed.

receptor concentration, as did TRH (Figure 5). The inverse agonist chlordiazepoxide completely blocked the ability of 100 nM TRH to increase the receptor protein. In other experiments, addition of 10 μ M TRH partially reversed the effect of chlordiazepoxide (results not shown).

The effect of TRH and PMA on the fluorescence of cells expressing a GFP-tagged TRHR was also investigated using FACS after 18 h exposure to no drug, PMA or TRH. As shown in Figure 6, activation of TRHR and protein kinase C caused 2.7- and 6.8-fold increases in the average fluorescence intensity of cells, consistent with the large increases in receptor seen in immunoblots. There was negligible fluorescence from cells not expressing the GFP-tagged receptor in the FACS experiments, and relative changes were larger than those observed by fluorimetry.

We used real-time PCR to quantify the effect of TRH treatment on the concentration of receptor mRNA. Cells were incubated with or without TRH for 18 h and RNA was isolated and reverse-transcribed; the cDNA was used for real-time PCR. TRH did not alter β -actin mRNA, but caused a 1.71 ± 0.05 -fold increase ($n = 4$) in TRHR mRNA, normalized to β -actin (Figure 7). TRH increased the total receptor by 6-fold in cell lysates from the same experiment, but did not alter the concentration of β -actin protein. The effect of TRH on receptor message concentrations

Figure 7 Real-time PCR analysis of TRHR mRNA levels

HA-TRHR HEK-293 or control HEK-293 cells were incubated with or without 1 μ M TRH. The next day, RNA was isolated and treated with DNase, and cDNA was generated. (A) Relative fluorescence of the TRHR PCR product versus cycle number in a real-time PCR using TRHR primers. Representative single reactions are plotted ($n = 4$). (B) Immunoblots using anti-HA and anti- β -actin antibodies.

was between 1.4- and 2.4-fold in three independent experiments, whereas the effect on protein concentration was considerably larger (Figure 7B).

The ability of TRH to increase the receptor protein was largely inhibited by blocking protein synthesis (with cycloheximide or anisomycin), RNA synthesis (with actinomycin D) or proteasome activity (with MG132; Figure 8).

To assess the specificity of the effects seen with TRHRs, we prepared HEK-293 cells stably expressing a FLAG-tagged β_2 -adrenergic receptor in the same vector as that used to develop TRHR cell lines. In agreement with previously published results [16], 18 h treatment with the agonist isoprenaline caused a loss of receptor, whereas 18 h exposure to 10 nM PMA caused a large increase in receptor protein. Neither isoprenaline nor PMA had an effect on β -actin (Figure 9).

DISCUSSION

These experiments show an unexpected and large up-regulation of TRHR protein in response to agonist. The up-regulation does not occur in response to antagonist and depends on signal transduction. Up-regulation of TRHRs is primarily due to the activation of protein kinase C, based on the ability of phorbol esters to mimic the TRH effect and a protein kinase C inhibitor to prevent it. Since TRH increased receptor concentrations in several clonal lines, a pool, two different cell types and cells stably transfected with either the full-length or a truncated receptor or a GFP-tagged receptor, it is highly unlikely that the response resulted from a particular chromosomal integration site.

The increase in TRHR protein caused by TRH appears to involve a number of mechanisms. (i) TRH causes a small but reproducible increase in mRNA for the TRHR, suggesting either an increase in transcription or message stabilization. TRH destabilizes the endogenous TRHR mRNA and, in most but not all settings, destabilizes the message after transfection [9,10,17–20]. These effects depend on AU-rich sequences in the 3'-untranslated region, which was not present in the plasmid used in our studies [17]. (ii) Since TRH caused no up-regulation in the presence of the proteasome inhibitor MG132, TRH may increase the receptor protein partially by decreasing the degradation of the receptor that normally takes place via the proteasome. We have shown previously that newly synthesized TRHR undergoes some ubiquitination and proteasomal degradation in the endoplasmic

Figure 8 Effects of protein, RNA synthesis and proteasome inhibitors

HA-TRHR HEK-293 cells were incubated for 18 h with or without 100 nM TRH in the presence of 10 μ g/ml cycloheximide (Chx), 1 μ g/ml actinomycin D (ActD) or MG132 (1 h with 1 μ M and 18 h with 50 nM). Cells were used for immunoblotting with (A) anti-HA or (B) anti- β -actin antibody. The inhibitors reduced the total cell protein and β -actin and blunted the up-regulation of receptor protein normally caused by TRH. (C) The histogram represents the means $\pm$ S.E.M. of receptor intensity of blots for three independent experiments. The increase due to TRH was significant ($P < 0.01$) only in untreated cells.

reticulum [12]. It is possible to envision a mechanism in which TRH or protein kinase C activation facilitates folding or post-translational modification of the receptor. Alternatively, MG132 may have affected other proteins or pathways required for up-regulation. (iii) TRH may increase the amount of newly synthesized receptor, since its effects are greatly diminished by protein and RNA synthesis inhibitors. Alternatively, the mechanism(s) responsible for receptor up-regulation may depend on a labile protein.

Previous studies reported a decrease in TRHR mRNA with prolonged agonist exposure, which is supposed to depend on regulatory elements in the 3'-untranslated region of the mRNA that were not present in the receptor construct used in our experiments [9–11]. In pituitary tumour cell lines, TRH causes a decrease in TRH binding sites at the plasma membrane, indicating that the competing influences of TRH on receptor mRNA and receptor protein result in a net decrease in surface receptors. Additional work will be required to understand how hormonal activation of the TRHR affects receptor concentrations in more physiological settings.

In addition to TRHR, a few other G-protein-coupled receptors, including the SST1 (somatostatin receptor 1) [21,22], dopamine DA_{2Long} receptor [23], dopamine DA₃, angiotensin II [24], GnRH (gonadotropin-releasing hormone) [25] and 5HT_{2A} receptors, are up-regulated by agonists. In most cases, effects on the density

Figure 9 Effects of agonist and protein kinase C activation on β_2 -adrenergic receptor protein

HEK-293 cells stably expressing FLAG-tagged β_2 -adrenergic receptors were incubated for 18 h with either vehicle, 100 μ M isoprenaline (Iso) or 10 nM PMA. Lysates from 6 cm dishes were immunoprecipitated (IP) and immunoblotted with (A) anti-FLAG or (B) anti- β -actin antibody as described in Figure 1. (C) The histogram shows the means $\pm$ S.E.M. of receptor intensities for four experiments; one point is omitted in which PMA caused a 14-fold increase in intensity.

of binding sites but not receptor protein have been documented. An agonist probably causes stabilization of the SST1 receptor and synthesis of GnRH, 5HT_{2A} and DA_{2Long} receptors. In contrast, most G-protein-coupled receptors undergo homologous down-regulation [8], and there are numerous examples of receptors whose concentrations decrease rapidly after agonist exposure in heterologous expression systems similar to the one used here [16]. In some cases, this down-regulation occurs when the agonist-occupied receptor undergoes endocytosis and sorting to lysosomes. In others, down-regulation occurs even though receptors either do not internalize or internalize and recycle without degradation.

The results of the present study indicate that regulation of TRHRs is more complex than previously appreciated. Not only is the density of surface-binding sites controlled by agonist, but also the concentration of receptor protein. The pool of receptors potentially available for signalling appears to expand significantly in response to the agonist. Future studies will be necessary to appreciate the physiological significance of this TRH-mediated, signal-dependent regulation.

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