

# Carboxyl Tail Cysteine Mutants of the Thyrotropin-Releasing Hormone Receptor Type 1 Exhibit Constitutive Signaling: Role of Palmitoylation

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## ABSTRACT

We studied the role of carboxyl tail cysteine residues and their palmitoylation in constitutive signaling by the thyrotropin-releasing hormone (TRH) receptor type 1 (TRH-R1) in transfected mammalian cells and in *Xenopus laevis* oocytes. To study palmitoylation, we inserted a factor Xa cleavage site within the third extracellular loop of TRH-R1, added a carboxyl-terminal C9 immunotag and expressed the mutant receptor in Chinese hamster ovary cells. We identified TRH-R1-specific palmitoylation in the transmembrane helix-7/carboxyl-tail receptor fragment mainly at Cys-335 and Cys-337. In contrast to a mutant truncated at Cys-335 that was reported previously to be constitutively active, a receptor truncated at Lys-338 (K338Stop), which preserves Cys-335 and Cys-337, and C337Stop and

N336Stop, which preserve Cys-335, did not exhibit increased constitutive signaling. TRH-R1 mutants substituted singly by Gly or Ser at Cys-335 or Cys-337 did not exhibit constitutive signaling. By contrast, substitution of both cysteines (C335G/C337G or C335S/C337S) yielded TRH-R1 mutants that exhibited marked constitutive signaling in mammalian cells. In the oocyte, constitutive signaling by C335G/C337G resulted in homologous (of C335G/C337G) and heterologous (of M1 muscarinic receptor) desensitization. Because both Cys-335 and Cys-337 have to be substituted or deleted for constitutive signaling, we propose that a single palmitoylation site in the proximal carboxyl tail is sufficient to constrain TRH-R1 in an inactive conformation.

Seven transmembrane-spanning receptors (7TMRs or G protein-coupled receptors) form the largest family of molecules involved in the transduction of extracellular signals. All members of the family share structural homology consisting of an extracellular amino terminus; seven transmembrane hydrophobic helical segments that form three intracellular and three extracellular loops; and an intracellular carboxyl tail, which usually contains one or more Cys residues.

Palmitoylation is a reversible post-translational modification in which palmitate, a 16-carbon saturated fatty acid, is covalently attached via a thioester bond to Cys residues so as to promote interactions with membranes (for review, see Smotrys and Linder, 2004). Because a protein can undergo cycles of palmitoylation/depalmitoylation, palmitoylation has been suggested to be a regulatory mechanism for 7TMRs in

cells (Bouvier et al., 1995). Palmitoylation has been demonstrated for a number of 7TMRs, G proteins, 7TMR kinases, and regulators of G protein signaling (for review, see Qanbar and Bouvier, 2003).

Rhodopsin was the first 7TMR reported to be palmitoylated (Ovchinnikov et al., 1988). A number of subsequent studies have demonstrated that carboxyl tail cysteines, as in rhodopsin (Ovchinnikov et al., 1988), are palmitoylated and that this modification is important for 7TMR signaling (Qanbar and Bouvier, 2003). It has been proposed that palmitoylated carboxyl-tail Cys residues form a hydrophobic attachment to the cell membrane and thereby create a fourth intracellular loop (or eighth helix) in 7TMRs. The crystal structure of bovine rhodopsin (Palczewski et al., 2000; Okada and Palczewski, 2001) showed that the fourth loop exists and is an amphiphilic  $\alpha$  helix (helix 8) that is likely to be anchored to the plasma membrane. It has been postulated that this hypothetical loop may be important for receptor-G protein

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**ABBREVIATIONS:** 7TMR, seven transmembrane-spanning receptor; TRH, thyrotropin-releasing hormone; TRH-R1, thyrotropin-releasing hormone receptor type 1; HEK, human embryonic kidney; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; AP-1, activator protein-1; M1R, muscarinic receptor-1; CDE, chlordiazepoxide.

coupling in other 7TMRs (Krishna et al., 2002; Zhong et al., 2004).

Thyrotropin-releasing hormone (TRH), a tripeptide, is widely distributed in the nervous system as well as in extra-neural tissues. The peptide is synthesized in the hypothalamus and transported by the portal vascular system to the anterior pituitary, where it binds to the 7TMR TRH receptor (TRH-R1) (Gershengorn and Osman, 2001) on thyrotropic and lactotropic cells to promote secretion of thyrotropin (thyroid-stimulating hormone) and prolactin, respectively. TRH-R1 signaling is transduced primarily by the Gq family of G proteins, resulting in activation of phosphoinositide-specific phospholipase C and elevation of intracellular  $[Ca^{2+}]$  (Gershengorn and Osman, 1996).

We have previously demonstrated that truncation of TRH-R1 at Cys-335 (C335Stop), which results in a mutant in which Cys-335, Cys-337, and the remainder of the carboxyl tail are deleted, produces a receptor with markedly increased constitutive signaling (Matus-Leibovitch et al., 1995). The present work was undertaken to assess the role of Cys-335 and Cys-337 in constitutive signaling because changes in 7TMRs that lead to constitutive signaling are important for our understanding of the process of receptor activation. In this report, we show that Cys-335 and Cys-337 are palmitoylated and that only mutations that lack both residues exhibit pronounced constitutive activity.

## Materials and Methods

**Tissue Culture.** HEK293 and CHO cells were cultured in Dulbecco's modified Eagle's medium and Ham's F-12, respectively, supplemented with 10% fetal calf serum and antibiotics (100 U/ml penicillin and 100  $\mu$ g/ml streptomycin). The cells were maintained at 37°C in 5% CO<sub>2</sub>.

**Oocyte Isolation and Maintenance.** Mature *Xenopus laevis* female frogs were purchased from the South African *Xenopus* Facility and maintained as described previously (Oron et al., 1987). Stage V oocytes were isolated, defolliculated, and maintained as described previously (Oron et al., 1987). For expression studies, oocytes were injected with 0.5 to 1.0 ng of desired transcript(s) and assayed 48 to 74 h later.

**Mutagenesis.** All DNA procedures were performed as described by Sambrook et al. (1989). A factor Xa site (IEGR) within the third extracellular loop of the mouse TRH-R1 was generated through sense primer (CTC TCC AGC CCT TTC CAG ATA GAA GGA CGG AAT TGG TTC TTG CTC TTT) and antisense primer (AAA GAG CAA GAA CCA ATT CCG TCC TTC TAT CTG GAA AGG GCT GGA GAG) by standard polymerase chain reaction protocols, using the overlap extension technique. A C9 tag, a nine-amino acid sequence of the rhodopsin carboxyl tail (TETSQVAPA), allows detection by the anti-rhodopsin 1D4 monoclonal antibody and was added on the carboxyl terminus of TRH-R1 (Huang et al., 2005). The C9 tag was added through antisense primer (CGG CTC TAG ATT AAG CTG GAG CTA CTT GAG ATG TTT CAG TAC CAC CGT TCT CAG AGG CCA AGC A). All other point mutations were performed by using QuikChange II site-directed mutagenesis kits from Stratagene (La Jolla, CA). All mutants were verified by double-stranded dideoxy DNA sequencing of the final plasmid. For oocyte expression experiments, the desired complementary RNAs were transcribed in vitro as described previously (Gershengorn et al., 1990).

**Receptor Binding.** For equilibrium binding experiments, cells were incubated at 37°C for 1 h with [<sup>3</sup>H]methyl-His-TRH (MeTRH; 0.1–20 nM) in Hanks' balanced salt solution, pH 7.4, as described previously (Narayanan et al., 1992). Equilibrium  $K_d$  values were derived from saturation experiments for which curves were fitted by

nonlinear regression analysis and drawn with the PRISM program (GraphPad Software Inc., San Diego, CA).

**Metabolic Labeling and Immunoprecipitation of CHO Cells.** Cells lines expressing TRH-R1 or mutant receptors were grown in 150-mm dishes in Ham's F-12 medium supplemented with 10% FCS, 400  $\mu$ g/ml G418 (Geneticin), and 1% penicillin/streptomycin. At confluence, cells were labeled with 200  $\mu$ Ci/ml [<sup>3</sup>H]palmitic acid in F-12 medium for the time periods indicated. After labeling, cells were washed once with ice-cold factor Xa digestion buffer A (50 mM Tris, pH 8.0, 100 mM NaCl, and 6 mM CaCl<sub>2</sub>) and then incubated at 4°C for 48 h in buffer A supplied with 1  $\mu$ g/ml factor Xa. The amount of cleaved receptor fragment was found to reach a plateau by 48 h, and this digestion time was used for all studies. Cells were lysed in 1.5 ml of buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM CaCl<sub>2</sub>, 5 mM KCl, 5 mM MgCl<sub>2</sub>, 4 mM EDTA, and 20% glycerol) supplemented with Complete protease inhibitor tablets (1 tablet/25 ml; Roche Diagnostics, Indianapolis, IN). A small amount of insoluble material was separated from the clear supernatant by centrifugation at 15,000g for 10 min at 4°C. Each supernatant was incubated with agitation at 4°C overnight with 20  $\mu$ l of agarose beads conjugated with 1D4 antibody. Beads were washed with 2 ml of ice-cold buffer B three times and then incubated at 4°C for 1 h in 60  $\mu$ l of 0.5 mM C9 peptide. In some experiments, eluted samples were separated into two equal parts (27  $\mu$ l). One part was treated with 3  $\mu$ l of 1 M Tris-HCl, pH 7.5, and the other part treated with 3  $\mu$ l of 10 M hydroxylamine, pH 7.5, and incubated at room temperature for 1 h. Radiolabeled samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 8 to 16% acrylamide gradient gels and visualized by fluorography using Hyperfilm-MP films (Amersham Biosciences Inc., Piscataway, NJ). Films were exposed for 10 days at –80°C. The amount of receptor fragment in 5  $\mu$ l of each sample was estimated by Western blotting using the 1D4 antibody.

**Assay of Luciferase Activity.** Signaling was measured using the PathDetect AP-1 *cis*-reporting system as described previously (Sun and Gershengorn, 2002). HEK293 cells were transfected with plasmids encoding AP-1 luciferase cDNA and wild-type or mutant TRH-R1 cDNA using the FuGENE 6 transfection protocol provided by the manufacturer. After 24 h, cells were washed once with 1 ml of serum-free medium and 200  $\mu$ l of serum-free medium containing the required amount of TRH was added. Six hours later, cells were washed with phosphate-buffered saline and lysed with 0.2 ml of lysis buffer (25 mM Gly-Gly, pH 7.8, 15 mM MgSO<sub>4</sub>, 4 mM EGTA, 1 mM dithiothreitol, and 1% Triton X-100). Cell lysates (0.025 ml) were combined automatically with 0.125 ml of reaction buffer (25 mM Gly-Gly, pH 7.8, 15 mM MgSO<sub>4</sub>, 4 mM EGTA, 1 mM dithiothreitol, 15 mM KH<sub>2</sub>PO<sub>4</sub>, and 2 mM ATP) and 0.025 ml of luciferin (0.4 mM) in reaction buffer, and the luminescence was measured for 10 s in a TR717 microplate luminometer (Tropix, Bedford, MA). The levels of luciferase activity detected by this assay reflect the activation of signaling by TRH (Sun and Gershengorn, 2002).

**Assays in Oocytes.** Responses mediated by TRH-R1, its mutants, or rat cholinergic-muscarinic M1 receptor (M1R) were measured by two-electrode voltage clamp (Axoclamp; Axon Instruments Inc., Union City, CA) at  $V_H = -80$  mV, as described previously (Oron et al., 1987). Each experiment was performed on a number of oocytes ( $n$ ) of several different donor frogs ( $N$ ).

**Data Analyses.** Statistical significance was determined using Student's  $t$  test with a probability criterion of  $P < 0.05$ .

**Materials.** [<sup>3</sup>H][methyl-His]TRH ([<sup>3</sup>H]MeTRH) and [9,10-<sup>3</sup>H(N)]-palmitic acid were purchased from PerkinElmer Life Science and Analytical Products (Boston, MA). [myo-<sup>3</sup>H]Inositol was obtained from Amersham Biosciences Inc. Restriction endonucleases, Vent DNA polymerase, and deoxynucleotides were obtained from New England Biolabs (Beverly, MA). The mammalian expression vector pcDNA3.1 was from Invitrogen (Carlsbad, CA). The C9 peptide was synthesized by Synpep (Dublin, CA). FuGENE 6 transfection reagent was purchased from Roche Diagnostics (Indianapolis, IN). CHO cell line and 1D4 antibody were from American Type Culture

Collection (Manassas, VA). Ham's F-12 medium, DMEM, and fetal bovine serum were purchased from Cellgro, Mediatech (Herndon, VA). Bovine factor Xa and SuperSignal West Pico chemiluminescent substrate were purchased from Pierce Chemical (Rockford, IL). ND96 and NDE media composition was described previously (Oron et al., 1987). Chlordiazepoxide (CDE) and TRH were from Sigma (Rehovot, Israel). Acetylcholine and collagenase type IV were from Sigma.

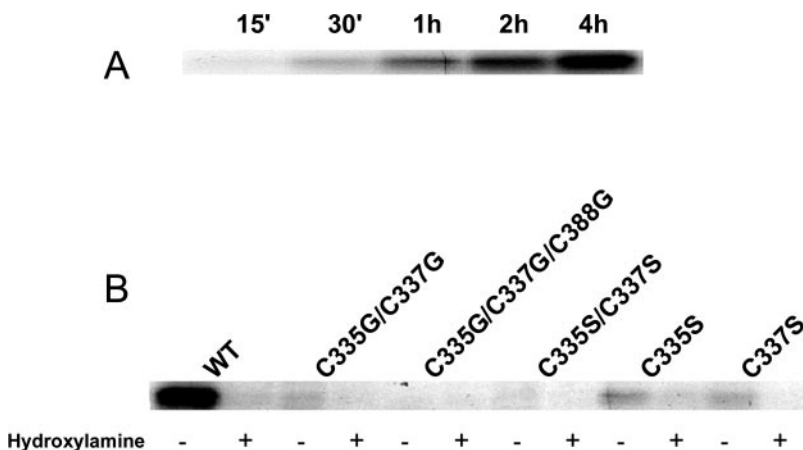
## Results

**TRH-R1 Is Palmitoylated on Cys-335 and Cys-337.** To monitor palmitoylation, a factor Xa proteolytic site was inserted between amino acid residues 297 and 299 within the third extracellular loop of TRH-R1. The mutant receptor was further modified by extending the carboxyl tail to include the C9 peptide. This allowed immunoprecipitation of a receptor fragment comprised of transmembrane helix 7/carboxyl tail after digestion of cell surface receptors by factor Xa. This approach allowed us to monitor cell surface receptors only. The transmembrane helix 7/carboxyl-tail fragment did not aggregate upon solubilization as did the intact receptor; aggregation interfered with isolation. The modified receptor exhibited similar affinities for TRH and EC<sub>50</sub> values for TRH-stimulated accumulation of inositol phosphate second messengers (not shown).

For palmitoylation studies, cells transfected with the modified TRH-R1 were labeled with [<sup>3</sup>H]palmitate. Receptors on intact cells were digested with factor Xa for 48 h at 4°C. The digested fragments were immunoprecipitated on 1D4 beads and separated on SDS-PAGE gels. The band intensity of autoradiograms on the gels corresponded to the degree of palmitoylation. Incorporation of [<sup>3</sup>H]palmitate increased with time of labeling (Fig. 1A). There was no effect of TRH on the rate of [<sup>3</sup>H]palmitate incorporation (not shown). Substitution of all Cys residues in the carboxyl tail (C335G/C337G/C388G) abolished palmitoylation. Substitution of either Cys-335 or Cys-337 yielded TRH-R1 mutants that exhibited palmitoylation that was much less than 50% of TRH-R1 (Fig. 1B). Double substitution of Cys-335 and Cys-337 resulted in little incorporation of radioactive palmitate, suggesting that Cys-388 was weakly palmitoylated (Fig. 1B). Palmitoylation occurred via a thioester bond because the palmitate was cleaved by hydroxylamine. These results identified Cys-335 and Cys-337 as the preferred sites of palmitoylation in the carboxyl tail of TRH-R1 under the conditions used for these experiments.

**Constitutive Signaling by TRH-R1 Mutants Modified in the Cys-335/Lys-338 Domain.** We reported previously that the TRH-R1 truncation mutant C335Stop lacking the palmitoylation sites and the remainder of the carboxyl tail exhibited pronounced constitutive signaling (Matus-Leibovitch et al., 1995). Because C335Stop lacks the two preferred palmitoylation sites, we studied the effects of altering Cys-335, Asn-336, and Cys-337. All mutant receptors exhibited indistinguishable binding affinities (Table 1). When both Cys-335 and Cys-337 were substituted by Gly or Ser, the resulting TRH-R1 mutants exhibited pronounced constitutive signaling in HEK293 cells (Fig. 2). C335G/C337G and C335S/C337S, however, exhibited TRH-stimulated signaling that was indistinguishable from TRH-R1, even though their levels of expression were 2- to 3-fold lower than TRH-R1 (Fig. 3). C337Stop and N336Stop truncation mutants (not shown), and mutants in which Cys-335 or Cys-337 were singly substituted by either Gly or Ser, did not signal constitutively and exhibited no change in TRH-stimulated activity (Fig. 3). Likewise, C388G exhibited no increase in basal signaling and TRH-stimulated activity resembled that of TRH-R1.

We have previously used *X. laevis* oocytes to study TRH-R1 signaling (Oron et al., 1987). In oocytes, activation of TRH-R1 and other 7TMRs, such as the M1 muscarinic receptor, leads to Ca<sup>2+</sup> mobilization that causes gating of chloride channels, which can be monitored electrophysiologically. TRH-R1 evokes large responses in oocytes when TRH binds. Because TRH-R1 exhibits low basal signaling activity, it is not desensitized in oocytes and activation of TRH-R1 is inhibited by pretreatment with the inverse agonist CDE, acting as an antagonist (Joels and Drummond, 1989). It is difficult to measure constitutive signaling by 7TMRs in oocytes directly because constitutively active receptors are desensitized. In mammalian cells (Heinflink et al., 1995) and in oocytes (Grimberg et al., 1999), desensitized responses by constitutively signaling TRH-R1 mutant receptors can be resensitized by pretreatment with CDE. Thus, although basal signaling by constitutively active 7TMRs is not directly measurable in oocytes, it can be inferred when desensitized responses are resensitized by inverse agonists. To confirm that prevention of palmitoylation of Cys-335 and Cys-337 leads to constitutive signaling activity, we monitored responses in oocytes expressing mutant receptors. In this series of experiments, we used K338Stop as reference (Matus-Leibovitch et al., 1995) to engineer different mutants of TRH-R1



**Fig. 1.** Palmitoylation of Cys-335, Cys-337, and Cys-388 of TRH-R1 in mammalian cells. A, time course of incorporation of [<sup>3</sup>H]palmitate. CHO cells were labeled with [<sup>3</sup>H]palmitate, and the transmembrane helix 7/carboxyl-tail fragments of the receptors containing C9 epitope tags were isolated by adsorption to beads conjugated with 1D4 antibody as described under *Materials and Methods*. B, effects of substitution of Cys-335, Cys-337, or Cys-388 on [<sup>3</sup>H]palmitate incorporation. Receptors were TRH-R1 (WT), C335G/C337G, C335G/C337G/C388G, C335S/C337S, C335S, and C337S. Cells were incubated with [<sup>3</sup>H]palmitate for 4 h, and aliquots of factor Xa-digested cell lysates were analyzed directly (–) or after exposure to 1 M hydroxylamine (+) for 1 h at room temperature to cleave thioester bonds. These experiments are representative of two or more similar experiments.

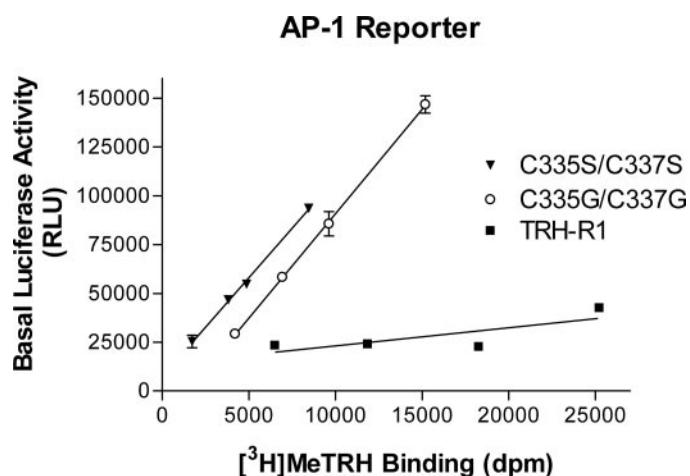


in the Cys335-Lys338 domain. K338Stop expressed in oocytes was indistinguishable from TRH-R1 displaying the same amplitude of response to TRH and similar  $EC_{50}$  values (not shown). Activation of C335G/C337G/K338Stop exhibited

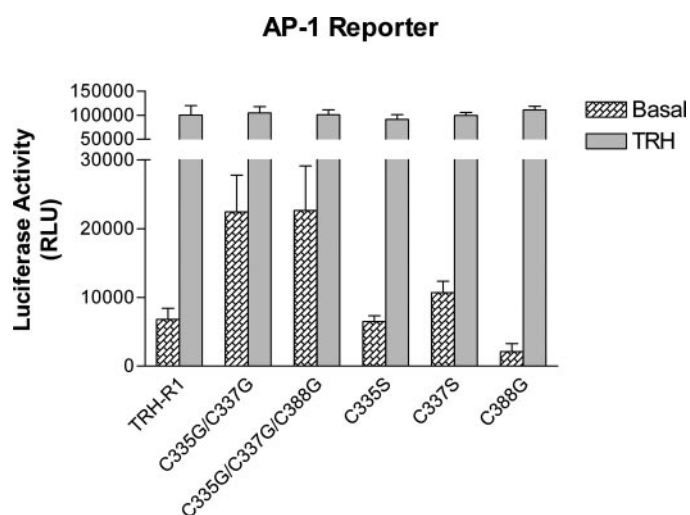
TABLE 1

Binding affinities ( $K_d$ ) and levels ( $B_{max}$ ) of receptors

| Receptor          | $K_d$         | $B_{max}$    |
|-------------------|---------------|--------------|
|                   | nM            | dpm          |
| TRH-R1            | 3.6 (2.9–4.3) | 13,600 ± 380 |
| C335S             | 4.5 (3.9–5.1) | 6800 ± 190   |
| C337S             | 3.2 (2.3–4.1) | 3700 ± 90    |
| C388S             | 4.6 (3.8–5.3) | 9300 ± 210   |
| C335S/C337S       | 2.8 (2.3–3.4) | 11,400 ± 170 |
| C335G/C337G       | 3.6 (2.7–4.5) | 16,700 ± 320 |
| C335S/C337S/C388S | 4.1 (3.3–4.8) | 22,100 ± 570 |



**Fig. 2.** Constitutive signaling by C335G/C337G and C335S/C337S in mammalian cells. HEK293 cells were transfected with various amounts of receptor-expressing plasmids, and signaling in the absence of TRH was measured using the AP-1 luciferase reporter system. The slopes of the lines characterizing the relationship between signaling (basal luciferase activity) and receptor expression ( $[^3H]$ MeTRH binding) for C335G/C337G and C335S/C337S were significantly greater than for TRH-R1. The data points represent triplicate samples in three experiments (mean  $\pm$  S.E.).



**Fig. 3.** Constitutive (basal) and TRH-stimulated signaling of carboxyl-tail Cys substitution mutants in mammalian cells. Basal and TRH (1  $\mu$ M)-stimulated luciferase activity in HEK293 cells expressing TRH-R1, C335G/C337G, C335G/C337G/C388G, C335S, C337S, or C388G. The bars represent the results of triplicate samples (mean  $\pm$  S.D.) in one of three experiments.

a markedly lowered (desensitized) response that was only 12% of that evoked by K338Stop (Table 2). To confirm that the lowered response exhibited by C335G/C337G/K338Stop was caused by its constitutive signaling, we assayed responses after 120-min pretreatment with 20  $\mu$ M CDE (Table 2). CDE caused more than 2-fold increase in the responsiveness of C335G/C337G/K338Stop, thereby confirming that it was desensitized as a result of constitutive signaling.

We also assessed constitutive signaling of C335G/C337G/K338Stop by testing for heterologous desensitization of coexpressed muscarinic M1 receptors. We described previously heterologous desensitization caused by a constitutively active 7TMR, such as a viral 7TMR (Lupu-Meiri et al., 2001), as a sensitive reporter of constitutive signaling in oocytes. Coexpression of C335G/C337G/K338Stop and the muscarinic M1 receptor caused more than 40% desensitization of M1 receptors (Table 2). In view of the homologous and heterologous desensitization exhibited by C335G/C337G/K338Stop, but not by K338Stop, the constitutive signaling of C335Stop can now be ascribed to the truncation of these two neighboring cysteine residues.

## Discussion

Two approaches have been applied to study palmitoylation of 7TMRs. Mass spectroscopy has been used to identify the sites of and quantify the stoichiometry of palmitoylation of only a limited number of 7TMRs, including rhodopsin (Ovchinnikov et al., 1988; Papac et al., 1992), endothelin receptors A and B (Stannard et al., 2003), and bradykinin B<sub>2</sub> receptor (Soskic et al., 1999). In most studies of 7TMR palmitoylation, labeling with  $[^3H]$ palmitate coupled with site-specific mutagenesis has been used (Qanbar and Bouvier, 2003). This second approach provides no insight regarding the stoichiometry of palmitoylation but is easier to use, principally because it does not require large amounts of receptor protein for analysis, unlike mass spectroscopy, and it offers the advantage of study of the turnover of palmitoylation and thereby its regulation. Neither of these approaches, however, allows for distinction between palmitoylation of mature receptors on the cell surface versus that of immature or intracellular receptors. We developed a modified method using  $[^3H]$ palmitate labeling to analyze palmitoylation of cell surface TRH-R1 specifically. By inserting a factor Xa proteolytic cleavage site in the third extracellular loop and cleaving cell surface receptors in intact cells, we were able to monitor palmitoylation of TRH-R1 on the cell surface. This approach had the added advantage of examination of only a fragment

TABLE 2

Homologous and heterologous desensitization caused by constitutive signaling of C335G/C337G/K338Stop in *X. laevis* oocytes

Some oocytes were pretreated for 2 h with 50  $\mu$ M CDE. Values are presented as mean  $\pm$  S.E. *n* indicates number of oocytes; *N* indicates number of donor frogs.

| Receptor                              | Response       | <i>n</i> | <i>N</i> |
|---------------------------------------|----------------|----------|----------|
| nA                                    |                |          |          |
| Responses to 10 $\mu$ M TRH           |                |          |          |
| K338Stop                              | 2145 $\pm$ 130 | 159      | 17       |
| C335G/C337G/K338Stop                  | 252 $\pm$ 44   | 156      | 17       |
| C335G/C337G/K338Stop + CDE            | 601 $\pm$ 131  | 38       | 4        |
| Responses to 10 $\mu$ M acetylcholine |                |          |          |
| M1R                                   | 2140 $\pm$ 442 | 59       | 7        |
| M1R + C335G/C337G/K338Stop            | 1240 $\pm$ 64  | 58       | 7        |

of the receptor containing the 7th transmembrane helix and carboxyl tail that is more easily purified and analyzed by SDS-PAGE than is the intact receptor (Fig. 1). Using this method, we found that Cys-335 and Cys-337 in the carboxyl tail of cell surface TRH-R1 were palmitoylated primarily and that Cys-388 was palmitoylated to a lesser extent under the conditions used for these experiments. We noted that native TRH-R1 seemed to be more than 2 or 3 times more heavily palmitoylated than C335S or C337S. This was not caused by lower levels of expression of the mutated receptors on the cell surface because maximal binding of [<sup>3</sup>H]MeTRH to intact cells did not correlate with [<sup>3</sup>H]palmitate incorporation (Table 1). For example, the expression levels of C335G/C337G and C335S/C337S were not different from TRH-R1 but had much lower [<sup>3</sup>H]palmitate incorporation. Although we have not determined the reason for the lower than expected [<sup>3</sup>H]palmitoylation of, for example, C335S and C337S, several explanations are possible. It may be that the native sequence of the carboxyl tail with both Cys residues represents a better substrate for the fatty acyltransferase(s), and, therefore, the rate of palmitoylation may be lower in C335S and C337S than in TRH-R1. A more interesting explanation is that the rate of depalmitoylation is increased in C335S and C337S compared with TRH-R1 because one palmitate in C335S and C337S forms a weaker intercalation into the cell surface membrane than two in TRH-R1 and allows increased accessibility to fatty acid deacylases. In this regard, it is noteworthy that two cysteines are often found in the proximal aspect of carboxyl tails of 7TMRs (Qanbar and Bouvier, 2003) and that dual palmitoylation may increase the stability of the intercalation and thereby of the fourth intracellular loop (or eighth helix).

We then used site-specific mutagenesis to inquire whether absence of palmitoylation would affect signaling by TRH-R1. Substitution of Cys-388 that prevents palmitoylation had no effect on signaling. This was similar to findings with the 5-hydroxytryptamine(4a) receptor in which palmitoylation of a cysteine residue in the distal aspect of the carboxyl tail (in addition to two more proximal cysteine residues) was observed and substitution of the distal cysteine to generate an acylation-deficient mutant at that site did not affect signaling (Ponimaskin et al., 2002). Substitution of the more proximal residues of TRH-R1, Cys-335 and Cys-337, individually had no effect on signaling, whereas substitution of both Cys-335 and Cys-337 caused marked increases in constitutive signaling activities but had no effect on TRH-stimulated signaling. These observations were similar to findings with 5-hydroxytryptamine(4a) receptor also (Ponimaskin et al., 2002). By contrast, mutation of one or two cysteine residues in similar positions within the proximal carboxyl tails of some other 7TMRs led to uncoupling of signaling (Qanbar and Bouvier, 2003). For example, an acylation-deficient mutant of  $\beta_2$ -adrenergic receptor does not couple to Gs (O'Dowd et al., 1989), and an acylation-deficient mutant of M2 muscarinic receptor coupled less well than native receptor to G<sub>i2</sub> and G<sub>o</sub> (Hayashi and Haga, 1997). However, an acylation-deficient mutant of  $\alpha_{2A}$ -adrenergic receptor coupled effectively to G protein (Kennedy and Limbird, 1993), and acylation-deficient mutants of other 7TMRs did not exhibit constitutive signaling. Thus, it seems that inhibition of palmitoylation affects basal and agonist-stimulated G protein coupling differently in different 7TMRs.

In summary, TRH-R1 mutant receptors in which both cysteine residues in the proximal aspect of the carboxyl tail were substituted to prevent palmitoylation exhibited marked constitutive signaling but similar TRH-stimulated signaling as TRH-R1. We conclude that palmitoylation is not necessary for G protein coupling by the TRH/TRH-R1 complex but is necessary to constrain TRH-R1 in an inactive state in the absence of ligand.

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