Kinetic analysis of the internalization and recycling of [^{3}H]TRH and C-terminal truncations of the long isoform of the rat thyrotropin-releasing hormone receptor-1

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The C-terminal tail of the long splice variant of the rat thyrotropin-releasing hormone (TRH) receptor-1 (TRHR-1L) comprises around 93 amino acids. A series of C-terminal truncations was constructed and expressed transiently in HEK-293 cells. The extent of steady-state internalization of these in response to [^{3}H]TRH was dependent upon the degree of truncation. Little effect was produced by deletion of the C-terminal to 50 amino acids, although there was a substantial decrease in the extent of internalization by deletion to 45–46 amino acids. The rate of internalization of TRHR-1L in response to ligand was substantially decreased by the acid-wash procedures often used in the analysis of cellular distribution of receptors with peptide ligands, and thus an alternative procedure using a Mes-containing buffer was employed in the present study. Apart from a truncation anticipated to eliminate post-translational acylation of the receptor, which altered both the association and dissociation rates of [^{3}H]TRH, the kinetics of ligand binding were unaffected by C-terminal truncation. Equally, the rate of recycling to the plasma membrane of internalized receptors was unaffected by C-terminal truncation. Although the extent of internalization of the full-length receptor was impaired by pre-exposure of cells to TRH, this was not true of C-terminal truncation mutants, which displayed limited steady-state internalization ratios. A mutant with a substantial C-terminal deletion also displayed decreased functional desensitization compared with the full-length receptor.

Key words: desensitization, internalization, receptors.

INTRODUCTION

Thyrotropin-releasing hormone (TRH) is a hypothalamic, post-translationally protected tripeptide intimately involved in controlling the production of thyrotropin and prolactin from the anterior pituitary [1]. In the rat, TRH functions by binding to a small group of molecularly defined G-protein-coupled receptors (GPCRs) [2–6] encoded by two distinct genes. Alternate splicing of the rat TRH receptor (TRHR)-1 gene allows the production of distinct long (TRHR-1L) and short isoforms of the GPCR, which differ in sequence only in the C-terminal domain. Both of these splice variants selectively interact with G_q and/or G_{11} to cause activation of the phosphoinositidase, phospholipase Cβ1, hydrolysis of PtdIns(4,5)P_2 and elevation of intracellular Ca^{2+} levels [7–10]. This is also true for TRHRs from other species, the most extensively studied of which has been the single form from the mouse [11–13].

As a structurally stabilized small peptide with high affinity for its GPCRs, TRH is an ideal ligand with which to explore aspects of the internalization and recycling of a GPCR with a peptide ligand. Indeed, in studies on the mouse TRHR, both fluorescently and radiolabelled forms of TRH have been used to investigate these processes [13–15]. For many GPCRs, the C-terminal tail is an important element in the regulation of their desensitization and endocytosis [16–18]. Indeed, truncation of the C-terminal tail of many GPCRs, including the mouse TRHR [13,19], has been shown to interfere with these processes. One issue in these studies, although it is rarely examined directly, is, because analysis of the kinetics of recycling of GPCRs to the cell surface relies on the dissociation of the ligand into the extracellular medium, the binding characteristics of the ligand to a GPCR should be unaltered by mutagenesis of the GPCR.

On examination of the sequence of the C-terminal tail of the rat TRHR-1L, a number of structural motifs which might contribute to the processes of GPCR internalization and recycling were recognized [16,20,21]. Therefore, in the present work, a series of C-terminal deletions of the rat TRHR-1L have been generated, which eliminate or would be anticipated to compromise the roles of these motifs. After expression of these deletions in HEK-293 cells, the extent and kinetics of internalization and recycling of the wild-type receptor and the truncation mutants were explored.

MATERIALS AND METHODS

Materials

All materials for tissue culture were supplied by Life Technologies Inc. (Paisley, Scotland, U.K.) or Sigma. [^{3}H]TRH (specific radioactivity 74 Ci/mmol) was from NEN Life Science Products. Oligonucleotides were purchased from Oswel (Southampton, U.K.).

Construction of the rat vesicular stomatitis virus (VSV)–TRHR-1L and C-terminally truncated forms

Production and subcloning of the VSV-tagged TRHR-1L has been described previously [22]. Briefly, the coding sequence of the rat TRHR-1L [4] was modified by PCR amplification. Using the N-terminal primer 5′-AAAGCTAGCGCCACCATGTAC-ACCGATATAGAGATGAACAGGCTGGGAAAGGAGAA-TGAAACCGTCAGTGAACTGAAC-3′, an *Nhe*I restriction site and the VSV epitope (YTDIEMNRLGK) were introduced

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; GPCR, G-protein-coupled receptor; TRH, thyrotropin-releasing hormone; TRHR, TRH receptor; TRHR-1L, long splice variant of TRHR-1; TRH_i, internal TRH; TRH_s, surface TRH; VSV, vesicular stomatitis virus.

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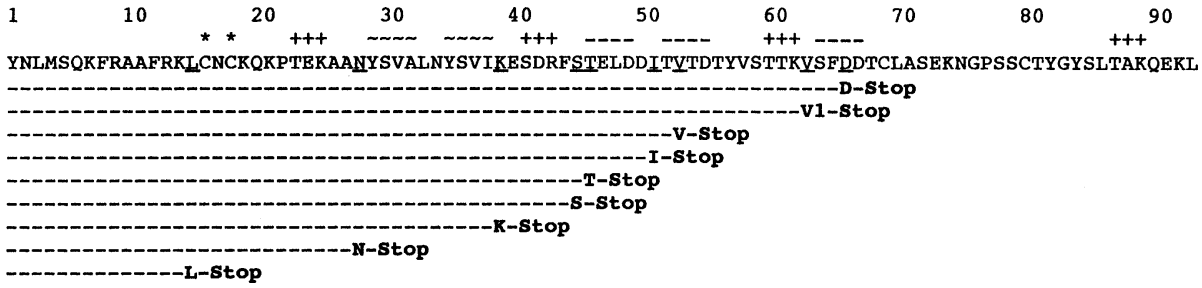

Figure 1 C-terminal truncation mutants of the rat TRHR-1L

The sequence of the predicted C-terminal tail (amino acids 320–412) of rat TRHR-1L is shown. This was derived by analysis of the amino acid sequence from the SwissProt database, accession number Q01717. A series of truncated forms of this receptor was produced by mutagenic insertion of appropriate stop codons (see Methods for details). The names given to the mutants reflect the identity of the last amino acid of the truncated protein. *, potential site for post-translational acylation; ~, potential clathrin/AP2 binding site; +, potential site for protein-kinase-C-mediated phosphorylation; —, potential site for phosphorylation by casein kinase II.

adjacent to the sequence of codon 2 of the TRHR-1L. Using the C-terminal primer 5′-GCTATCTAGAGTCAAAGCTTCTCC-TGTTTGGCAGTCAAA-3′, an *Xba*I restriction site following the stop codon was introduced. A *Hin*dIII restriction site was introduced in parallel by the same primer just in front of the stop codon. This *Hin*dIII restriction site changed the last four nucleotides of the TRHR-1L coding sequence from 5′-AATA-3′ to 5′-GCTT-3′, and thus altered the last amino acid from isoleucine to leucine. The amplified fragment of VSV–TRHR1-L, digested with *Nhe*I and *Xba*I, was ligated into the pcDNA3.1(+) expression vector (Invitrogen) digested with *Nhe*I and *Xba*I. The functionality of VSV–TRHR-1L was characterized by ligand binding experiments and agonist-mediated inositol phosphate production [22]. The spectrum of C-terminally truncated forms (Figure 1) of the engineered VSV–TRHR-1L cDNA was produced by a PCR strategy with the same N-terminal primer as above and new C-terminal primers incorporating a TGA stop codon followed by new restriction sites. The identity of the restriction sites in the C-terminal primers differed to allow the individual truncated mutants to be distinguished according to their length and C-terminal restriction sites. The C-terminal restriction sites used in the present study were (the one-letter symbol represents the last amino acid of the truncated C-terminus; see Figure 1): D-Stop, *Bam*HI; V1-Stop, *Kpn*I; V-Stop, *Apa*I; I-Stop, *Xba*I; T-Stop, *Eco*RI; S-Stop, *Bam*HI; K-Stop, *Kpn*I; N-Stop, *Hin*dIII; L-Stop, *Xba*I. (Full sequences of the C-terminal primers are available from G. M.) After PCR amplification and cleavage by appropriate restriction enzymes truncated mutants were ligated into pcDNA3.1(+) expression vector and were fully sequenced before their expression and analysis.

Transient transfection of HEK-293 cells

HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.584 g/l L-glutamine and 10% (v/v) newborn-calf serum at 37 °C. Cells were seeded to 60–80% confluence on 60-mm or 100-mm dishes the day before transfection. Transfection was performed using LipofectAMINE reagent (Life Technologies Inc.) according to the manufacturer's instructions. One day after transfection, the cells were apportioned to 12-well cell-culture plates treated with poly-D-lysine in the ratio: one 60-mm dish of cells to 6 wells or one 100-mm dish of cells to 18 wells. If the number of samples required for a set of experiments exceeded these numbers, and more than one dish of cells was transfected, then the cells were mixed together before seeding to produce the required number of wells with similar populations of transfected cells per well. Experiments were performed one day after seeding.

Steady-state internal [^{3}H]TRH$_i$/surface [^{3}H]TRH$_s$ ratio

On the day of the experiment, DMEM was removed from the wells and 400 μl of Hepes-buffered DMEM/cycloheximide (serum-free DMEM/20 mM Hepes, pH 7.2/100 μM cycloheximide) was added per well and cells were incubated at 37 °C. After 40 min, 100 μl of 250 nM [^{3}H]TRH (50 nM [^{3}H]TRH and 200 nM TRH) in Hepes-buffered DMEM/cycloheximide was added to produce 50 nM [^{3}H]TRH (final concentration) and incubation was continued for 1 h at 37 °C. Plates were placed on ice, cooled for 10 min and washed three times with ice-cold 0.15 M NaCl. The membrane-bound radioligand was then immediately stripped by treatment with 0.8 ml of 0.2 M acetic acid/0.5 M NaCl (pH 2.6) for 4 min followed by 0.8 ml of 0.15 M NaCl wash. Both fractions were collected to estimate the surface-associated [^{3}H]TRH. The internalized (non-acid treated) radioligand was estimated after solubilization of the cells with 1% (w/v) SDS/1% (v/v) Triton X-100/10 mM Tris/HCl, pH 8.0. Non-specific binding and internalization were determined in parallel samples in the presence of 10 μM non-radioactive TRH. After subtraction of non-specific binding, results were expressed as the ratio $[TRH]_i/[TRH]_s$ based on: $[TRH]_s k_i = [TRH]_i k_e$ or $[TRH]_i/[TRH]_s = k_i/k_e$ [23], where $[TRH]_i$ and $[TRH]_s$ are internal and surface [^{3}H]TRH, respectively, and k_i and k_e are the internalization and exocytic rate constants. All measurements were performed in triplicate on three to six independent transfections (see Figure 3). In no experiment did the sum of specifically membrane-bound or internalized [^{3}H]TRH exceed 4% of the total radioligand added. Total specifically-bound radioligand typically accounted for 85–95% of total bound radioligand. In some experiments, the time course of attainment of $[TRH]_i/[TRH]_s$ ratios were assessed. In these experiments, five time points at 37 °C were investigated and membrane-bound [^{3}H]TRH in naive cells was determined by incubation of non-treated cells in 50 nM [^{3}H]TRH for 40 min, on ice. Further analysis and estimation of $[TRH]_i/[TRH]_s$ ratios were performed as above.

Binding experiments

Binding studies were performed directly on the cell monolayers. Transfected cells in 12-well cell-culture plates were cooled on ice

for 10 min. The DMEM was changed for 500 μl of Hepes/DMEM (serum-free DMEM/20 mM Hepes, pH 7.2) and the binding of 10 nM [^{3}H]TRH was assayed in the presence of various concentrations of non-radioactive TRH. After 1 h, the wells were briefly washed three times with ice-cold Hepes/DMEM, the cells were solubilized in 1 % (w/v) SDS/Triton X-100 solution and the associated radioactivity was estimated by scintillation spectroscopy. The K_d was calculated according to DeBlasi et al. [24].

Dissociation of membrane-bound [^{3}H]TRH

Transfected cells on 12-well cell-culture plates were placed on ice for 10 min. The DMEM medium in each well was replaced with 500 μl of 10 nM [^{3}H]TRH in ice-cold Hepes/DMEM. Cells were incubated for 1 h, on ice, to achieve steady-state binding of [^{3}H]TRH. The wells were washed briefly twice with ice-cold Hepes/DMEM and 1 ml of ice-cold Hepes/DMEM was added to allow dissociation of membrane-bound radioligand. The medium was withdrawn after 5, 10, 15, 30 or 50 min, the cell monolayers were solubilized and radioactivity was estimated. After subtraction of non-specific binding, the results were expressed as d.p.m./dish for each time point.

Stripping of membrane-bound ligand for recycling experiments using a Mes-based buffer

Cells expressing the wild-type TRHR-1L were exposed to [^{3}H]TRH (50 nM) for 1 h at 37 °C, and the 12-well cell-culture plates were then placed on ice and cooled for 10 min. Cells were washed twice with 0.8 ml of Hepes/DMEM, twice with 0.8 ml of Mes-based DMEM (serum-free DMEM/20 mM Mes/0.3 M NaCl, pH 5), each for 3 min, and twice with 0.8 ml of Hepes/DMEM. Finally 1 ml of Hepes-buffered DMEM/cycloheximide without TRH or with 50 nM or 50 μM TRH was added and the cells were incubated for various times at 37 °C.

Radioligand recycling

Cells were incubated with [^{3}H]TRH for 1 h at 37 °C to achieve a steady-state intracellular pool. The membrane-bound radioligand was stripped using the Mes-based method (see above). The12-well cell-culture dishes were warmed to 37 °C, the medium was withdrawn at various time points and retained for measurement of the exocytosed radioligand. At the end of the experiments the cell monolayers in each well were solubilized with 1 % SDS/Triton X-100 solution (internalized [^{3}H]TRH remaining inside the cells). The radioactivity was measured in both fractions (external medium and solubilized cells) at each time point. After subtraction of non-specific binding, the results were expressed as a percentage of total [^{3}H]TRH in the external medium at each time point.

Receptor recycling

After incubation for 1 h with the appropriate concentrations of non-radioactive TRH, cell monolayers were stripped as described for the radioligand recycling protocol, except that no TRH was added to the 1 ml of Hepes-buffered DMEM/cycloheximide in which recycling was monitored. The 12-well cell-culture dishes were warmed to 37 °C for various times and were then cooled on ice. The medium was removed and the cell monolayer in each well was incubated with 500 μl of 10 nM [^{3}H]TRH in ice-cold Hepes/DMEM for 1 h. The monolayers were quickly washed three times with ice-cold Hepes/DMEM and cell-associated [^{3}H]TRH was measured after solubilization. The results were expressed as a percentage of [^{3}H]TRH bound to naive cells after subtraction of non-specific binding.

Inositol phosphate production

Transfected cells on 12-well cell-culture dishes were labelled with *myo*-[^{3}H]inositol (1 μCi/ml) in inositol-free DMEM supplemented with 2 % (v/v) dialysed newborn-calf serum and 1 % (w/v) glutamate for 24 h. On the day of the experiment, cells were washed with Krebs/Ringer/Hepes buffer [130 mM NaCl/5 mM KCl/1.2 mM $MgSO_4$/1.2 mM $CaCl_2$/20 mM Hepes/1.2 mM Na_2PO_4/10 mM glucose (pH 7.4) containing 0.1 % (w/v) BSA] and incubated for 10 min with 600 μl Krebs/Ringer/Hepes. Cells were stimulated for various times by the addition of 600 μl Krebs/Ringer/Hepes containing 100 nM TRH supplemented with 30 mM LiCl. It was found that the addition of 30 mM LiCl without 100 nM TRH over similar time periods did not significantly alter basal accumulation of [^{3}H]inositol phosphates (results not shown). At the end of the incubation period the reactions were terminated by cooling the dishes on ice and aspiration of the Krebs/Ringer/Hepes buffer. Cells were lysed with 0.75 ml of 20 mM formic acid, for 30 min, on ice. Samples were centrifuged at 14000 ***g*** for 3 min. The supernatants were loaded on to Dowex 1 X-8-200 followed by the immediate addition of 3 ml of 50 mM NH_4OH (to release [^{3}H]inositol). The columns were then washed with 4 ml of 40 mM ammonium formate followed by 5 ml of 2 M ammonium formate (to release [^{3}H]inositol phosphates). In parallel, the level of receptor expression was estimated by measuring [^{3}H]TRH specific binding per dish. Total [^{3}H]inositol phosphate production was calculated as: [^{3}H]inositol phosphates/[^{3}H]inositol phosphates + [^{3}H]inositol $\times$ 1000. As Gershengorn et al. [25] have indicated that the maximal size of the TRH-responsive pool of phosphoinositides is directly related to the number of TRH receptors, data are presented as total inositol phosphate (IP) production divided by specific [^{3}H]TRH binding, i.e. IP/(d.p.m./dish) $\times$ 1000.

RESULTS

A series of nine truncations of the rat TRHR-1L was generated which resulted in the C-terminal tail of this GPCR being shortened from 93 amino acids to a minimum of 15 (Figure 1). Following transient expression of the full-length rat TRHR-1L in HEK-293 cells, redistribution of the specific binding of [^{3}H]TRH (50 nM) approximated steady state after incubation at 37 °C for 1 h (Figure 2). Both the L-Stop and T-Stop mutants were transiently expressed and the extent and kinetics of changes in [^{3}H]TRH_i/[^{3}H]TRH_s specific binding ratios were measured at 37 °C. The results showed different distribution ratios for the three forms in response to TRH at each pre-steady-state time point (Figure 2). Direct measurement of the decrease in cell-surface levels of these forms of TRHR-1L allowed assessment of both the extent of internalization of each GPCR construct and the half-times required to produce this internalization (Figure 2, inset). Analysis of these data using a two-compartment model showed the rate of endocytosis of the T-Stop mutant to be only 36 % of that of the full-length TRHR-1L (Table 1). The full-length rat TRHR-1L and each of the nine C-terminal truncations were then transiently expressed in HEK-293 cells. [^{3}H]TRH (50 nM) was added and the [^{3}H]TRH_i/[^{3}H]TRH_s binding ratios were measured after incubation for 1 h at 37 °C (Figure 3). Although levels of the individual truncated forms of rat TRHR-1L varied in expression by up to 3-fold in different transfections this did not influence the [^{3}H]TRH_i/[^{3}H]TRH_s ratio, indicating the receptor internalization components were not saturated by

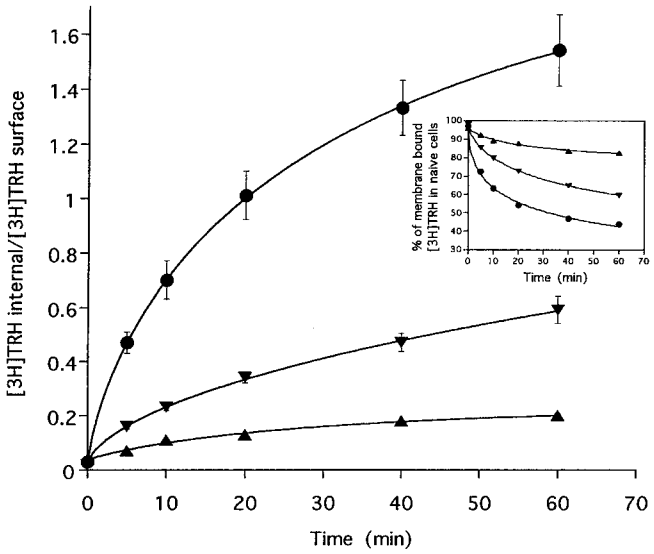

Figure 2 Kinetics of internalization of forms of the TRHR-1L

Wild-type TRHR-1L (●), T-Stop (▼) and L-Stop (▲) truncations were expressed transiently in HEK-293 cells. [^{3}H]TRH (50 nM) was added and the ratios of the specific binding of internalized and cell-surface [^{3}H]TRH were measured at various time points. Values shown are means ± S.E. obtained from three independent experiments performed in duplicate. Where not apparent, the errors fall within the size of the symbols. Insert: levels of cell surface [^{3}H]TRH-specific binding were measured by the acid-wash procedure, as described in the Materials and methods section, and the percentage of membrane-bound [^{3}H]TRH is given as a function of time.

Table 1 Apparent affinity for TRH and rate constants of endocytosis and recycling of wild-type TRHR-1L and some C-terminally truncated mutants

Values were calculated using a two-compartment model as described in [21,36]. Data (means ± S.D.) are derived from between three and seven separate experiments for each construct, and each experiment was performed in duplicate.

Receptor	K_d TRH (nM)	Endocytosis rate constant (min^{-1})	Recycling rate constant (min^{-1})
TRHR-1L	11.5 ± 1.9	0.071 ± 0.009	0.051 ± 0.005
T-Stop	12.2 ± 3.8	0.026 ± 0.002	0.048 ± 0.004
N-Stop	11.1 ± 5.1	0.018 ± 0.004	0.044 ± 0.004

the levels of expression achieved. Furthermore, expression levels did not correlate with the degree of C-terminal truncation. Truncations which resulted in the C-terminal tail being at least 50 amino acids in length had no effect on the TRH-induced distribution ratio of the TRHR-1L forms (Figure 3). By contrast, more extreme truncations resulted in a substantial reduction in the [^{3}H]TRH$_i$/[^{3}H]TRH$_s$ ratio. The most dramatic decline was produced by truncation to 45 or 46 amino acids in the C-terminal tail (S-Stop, T-Stop). Further truncation resulted in a further decrease in distribution ratios so that little internalization could be monitored in some of the most truncated mutants (Figure 3).

Ligand binding characteristics

Assessment of internalized versus cell surface [^{3}H]TRH-specific binding ratios can only provide a useful monitor of the relative cellular distribution of the TRHR-1L mutants if the binding characteristics of [^{3}H]TRH in the mutants and wild type are equivalent. To evaluate this the capacity of various concentrations of TRH to compete for the specific binding of [^{3}H]TRH was determined for the wild type and for T-Stop, N-Stop and L-Stop mutants. The IC_{50} for TRH was not significantly different in these cells and allowed estimation of K_d for TRH (Table 1). The rate of dissociation of [^{3}H]TRH from full-length TRHR-1L and the T-Stop and N-Stop mutants in the presence of 50 μM non-radioactive TRH was very similar (Figure 4). However, dissociation of [^{3}H]TRH from the L-Stop mutant was not discernable under these conditions (Figure 4). The L-Stop mutant was therefore not used in the analyses of the role of the C-terminal tail in recycling of the TRHR-1L to the plasma membrane.

Effectiveness of removal of cell-surface [^{3}H]TRH

The acid-wash procedure used in the present study was deliberately designed as a stringent treatment to effectively remove ligand from the cell surface receptor. This treatment removed 96% of the [^{3}H]TRH specifically bound at the cell surface following expression of full-length TRHR-1L. The effectiveness was similar with the individual truncation constructs (results not shown). However, recycling of TRHR-1L and its ligand required analysis of the rate of their reappearance on the cell surface following initial stripping of TRH. It was of concern that the acid-wash procedure would be too harsh and might modulate the subsequent internalization and recycling processes, and an effective Mes-buffer wash was developed to strip [^{3}H]TRH specifically bound to the exterior of cells expressing TRHR-1L. The capacity of cells to internalize [^{3}H]TRH over 1 h at 37 °C after stripping membrane-bound ligand with the acid-wash and Mes-buffer procedures was compared. Internalization of [^{3}H]TRH was distinctly less (approx. 50% of maximum) after the acid wash and the total amount of specific binding of [^{3}H]TRH to the acid-washed cells was only 70% of that in cells treated with the Mes buffer (results not shown). Detailed studies on rates of recycling and exocytosis were therefore performed using the Mes-buffer wash.

Receptor and [^{3}H]TRH recycling

To monitor recycling of receptors to the plasma membrane, HEK-293 cells expressing full-length rat TRHR-1L were treated with 50 nM TRH for 1 h at 37 °C to produce near steady-state internalization. The cells were then stripped with Mes-buffer wash and the extent of recycling to the plasma membrane was assessed at 37 °C for various times up to 2 h. Recycling of internalized TRHR-1L to the cell surface reached 85–90% over this time period. The rate of recycling of internalized T-Stop and N-Stop mutations was similar to that of the wild-type receptor (Table 1) but, as the steady-state level of internalization of these mutants was lower than for the full-length receptor (Figure 3), near maximum levels of cell surface T-Stop and N-Stop receptors occurred more rapidly (results not shown). However, it was not possible to recover 100% of the cell surface binding observed in TRH-untreated cells. After internalization in response to 50 nM [^{3}H]TRH, the kinetics of recycling of [^{3}H]TRH and the possibility of re-internalization of [^{3}H]TRH still bound to the receptor were thus explored. After washing with Mes, fresh medium was added to the cells and the rate and extent of release of [^{3}H]TRH into the extracellular medium was monitored in the absence of TRH or with the addition of 50 nM or 50 μM TRH to compete with [^{3}H]TRH for binding to the recycled receptor. Both concentrations of TRH increased the rate of [^{3}H]TRH release to the medium to the same degree (Figure 5). Assuming that, in the presence of 50 μM TRH, all of the [^{3}H]TRH recycled to the cell

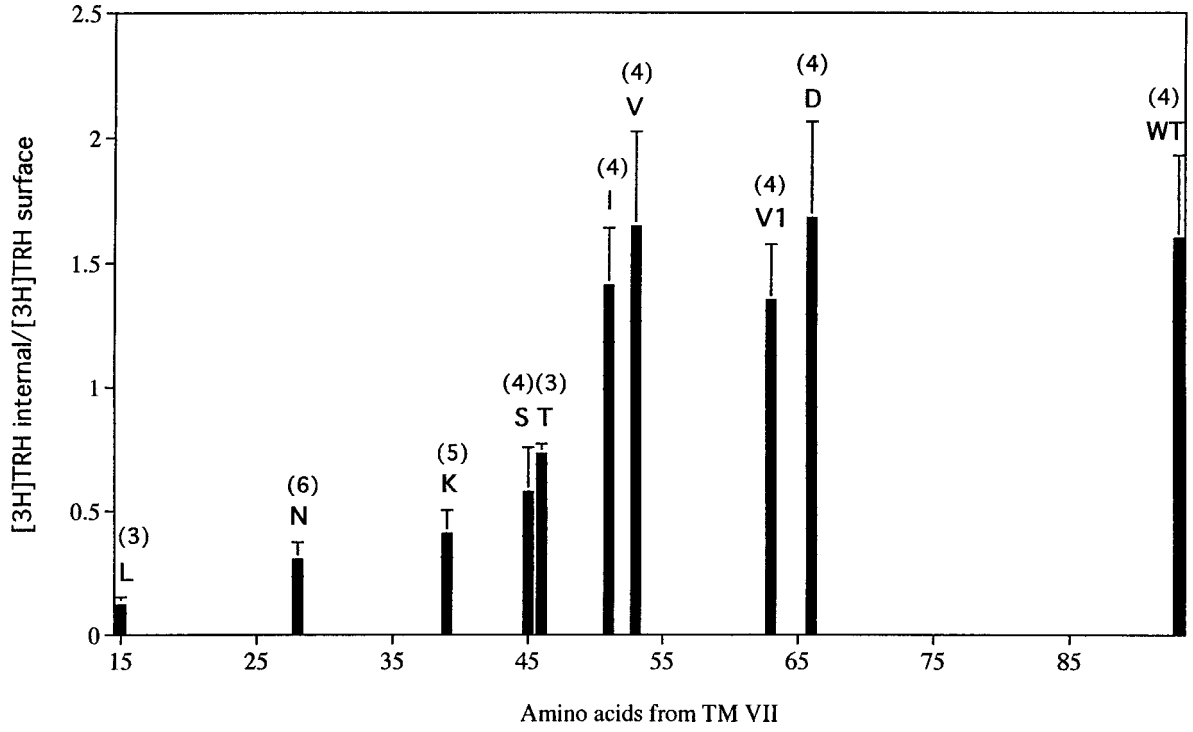

Figure 3 Degree of TRH-induced internalization of truncated forms of the rat TRHR-1L is determined by the length of the C-terminal tail

$[^3H]TRH_i/[^3H]TRH_s$ ratios were measured following transient expression of both wild-type rat TRHR-1L (WT) and the C-terminal truncations described in Figure 1. These ratios were determined as shown in the Materials and methods section following treatment of cells with 50 nM [^{3}H]TRH for 1 h at 37 °C. Data are the means ± S.E.M. of 3–6 experiments performed on separately transfected cells (the numbers of experiments are given in parentheses above the bars). Expression levels monitored by the total specific binding of [^{3}H]TRH varied 3-fold between different transfections and the individual GPCR constructs, however, neither of these two parameters influenced the measured $[^3H]TRH_i/[^3H]TRH_s$ ratios. TM VII, transmembrane region 7.

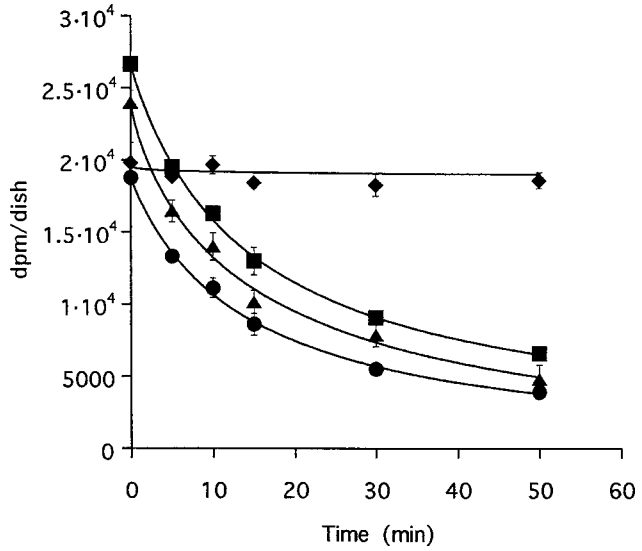

Figure 4 Dissociation of [^{3}H]TRH from TRHR-1L truncation mutants

Specific binding of [^{3}H]TRH (10 nM) to intact cells expressing the wild-type TRHR-1L (■), or T-Stop (▲), N-Stop (●) or L-Stop (◆) truncation mutants of this receptor was achieved by incubation at 4 °C for 1 h. The medium was then replaced with fresh medium containing 50 μM non-radioactive TRH. The levels of [^{3}H]TRH associated with the receptors were then assessed at various time points after removal of the fresh medium and solubilization of the cells. Values are means ± S.D. of duplicate assays from a representative of three independent experiments.

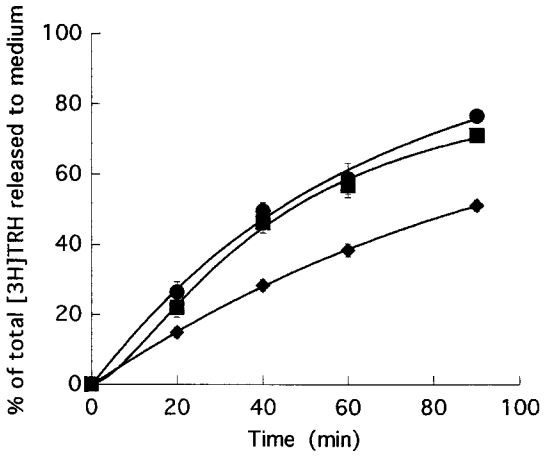

Figure 5 Analysis of the re-internalization of [^{3}H]TRH–TRHR-1L complexes, after recycling to the cell surface

Cells expressing wild-type TRHR-1L were exposed to [^{3}H]TRH (50 nM) for 1 h at 37 °C. They were subsequently washed with the Mes-based buffer and fresh medium containing no TRH (◆), 50 nM TRH (■) or 50 μM (●) TRH. [^{3}H]TRH released into the medium was then collected at various times and measured concurrently with that of solubilized cells. The values represent the percentage of total specifically-bound [^{3}H]TRH released into the extracellular medium at each time point. The results (means ± S. E.) are from three independent experiments each performed in duplicate.

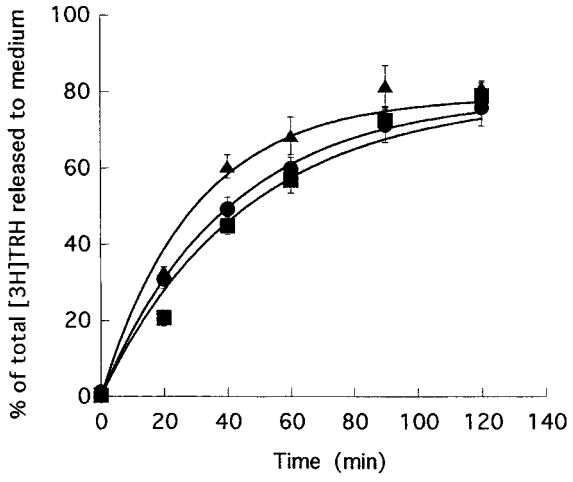

Figure 6 Kinetics of exocytosis of internalized [^{3}H]TRH from TRHR-1L truncation mutants

HEK-293 cells expressing the wild-type TRHR-1L (■), or the T-Stop (▲) or N-Stop (●) mutants were exposed to [^{3}H]TRH (50 nM) for 1 h at 37 °C. After washing with Mes-based buffer, to strip [^{3}H]TRH from cell-surface receptors, TRH (50 nM) was added and the release of [^{3}H]TRH into the extracellular medium and the [^{3}H]TRH remaining in solubilized cells was measured at each time point. Full-length TRHR-1L and the T-Stop and N-Stop mutants allowed exocytosis and release of [^{3}H]TRH to the medium with similar kinetics. Data were fitted to the exponential equation:

$r = m_r\,(1 - e^{-k})$

where r = % of ligand recycled, m_r = maximum extent of recycling and k = rate rate constant for recycling (see Table 1), as in [36]. Results (means ± S. E.) were obtained from three independent experiments performed in duplicate.

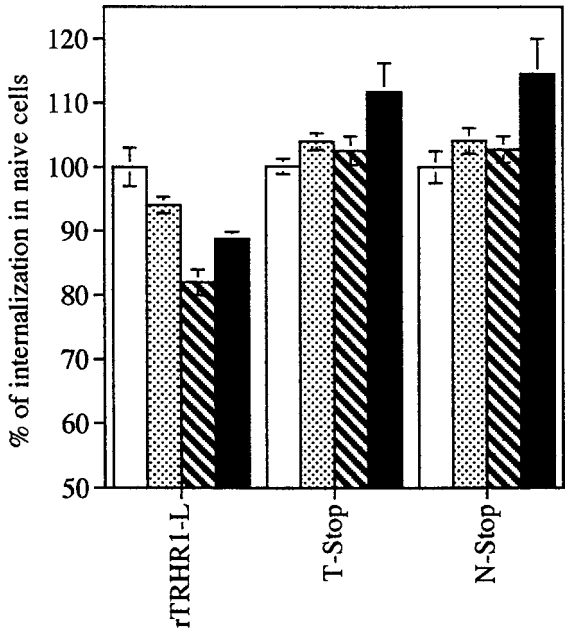

Figure 7 Internalization of the wild-type TRHR-1L, but not the T-Stop and N-Stop truncation mutants, is altered by pre-exposure to a high concentration of TRH

HEK-293 cells, transfected to transiently express either full-length TRHR-1L or the T-Stop or N-Stop mutants of this receptor, were exposed to TRH (1 μM) for 0 (open bars), 5 (black bars) or 60 (hatched bars) min at 37 °C or for 20 min on ice (dotted bars). Cell-surface-bound TRH of treated and untreated cells was removed using the Mes-buffer wash. The cells were then exposed to [^{3}H]TRH (50 nM) for 1 h at 37 °C. Internalization ratios were then measured using the acid-wash procedure. Internalization of [^{3}H]TRH bound to either the T-Stop or N-Stop mutants was unaffected by the pretreatment. By contrast, it was decreased by approx. 20% in the full-length TRHR-1L by pre-incubation at 37 °C but was unaffected by pre-incubation on ice. The results are the means ± S.E. of three independent experiments performed in duplicate.

surface bound to the receptor was replaced so that re-internalization of the receptor was now with unlabelled TRH, this is consistent with a half-time of 41 ± 11 min for recycling of the receptor. Furthermore, as the release of [^{3}H]TRH to the medium was three times slower (119 ± 38 min) in the absence of externally added TRH (Figure 5), then a substantial degree of re-internalization of the rat TRHR-1L with bound and/or rebound [^{3}H]TRH was clearly occurring in the absence of added external TRH.

Following expression of full-length rat TRHR-1L and both T-Stop and N-Stop truncation mutants, the release of [^{3}H]TRH into the extracellular medium was measured at different times following a 1 h incubation with 50 nM [^{3}H]TRH. Re-internalization of the forms of TRHR-1L with bound [^{3}H]TRH was restricted by including 50 nM TRH in the medium (see above). Although the extent of internalization of the three constructs during the 1 h incubation with [^{3}H]TRH was different, as predicted by the data of Figures 2 and 3, the kinetics of exocytosis of [^{3}H]TRH by the three constructs (and thus the recycling rates of these forms of the receptor) were very similar (Figure 6 and Table 1). These data also indicate that the recycling/exocytotic pathways of the cells were not saturated by the levels of receptor internalization achieved. Given that the extent of steady-state internalization of T-Stop and N-Stop truncation mutants was substantially lower than that of the wild-type receptor (Figure 3) but that their kinetics of TRH association and dissociation are very similar, then the similarity of their recycling rates must also indicate than the C-terminal tail of the TRHR-1L is not an active regulator of the recycling processes (Table 1).

Receptor desensitization

Both mouse and rat TRH receptors have been shown to rapidly desensitize the generation of inositol phosphates and/or Ca^{2+} mobilization in response to treatment with TRH [8,13,21,26]. To assess the effects of pretreatment of cells with TRH on the capacity of internalized TRHR-1L to subsequently recycle to the plasma membrane, cells expressing the wild-type TRHR-1L were treated for up to 1 h with 50 nM [^{3}H]TRH. The cells were washed with Mes buffer and left for 1 h at 37 °C in the presence of 50 nM extracellular TRH. The release of [^{3}H]TRH into the extracellular medium over this time period reflected a measure of the recycling of TRHR-1L (results not shown). The fraction of [^{3}H]TRH recycled and released at the cell surface was unaffected by the period of initial exposure to [^{3}H]TRH, with 70–75% being recovered over a 1 h period (results not shown). Cells expressing the full-length TRHR-1L were also exposed to 1 μM TRH for various times at 37 °C or were maintained on ice. Following membrane stripping, the cells were exposed to 50 nM [^{3}H]TRH for 1 h and the ratios of [^{3}H]TRH_i/[^{3}H]TRH_s specific binding was measured. The extent of internalization of [^{3}H]TRH was decreased by prior exposure to TRH at 37 °C for as little as 5 min, with only 80% of the maximum internalization being achieved after 1 h of preincubation with non-radioactive TRH (Figure 7). Exposure to TRH on ice, to inhibit internalization of the receptor, did not modify the extent of subsequent internalization of [^{3}H]TRH (Figure 7). By contrast, the subsequent extent of internalization of both T-Stop and N-Stop mutants was

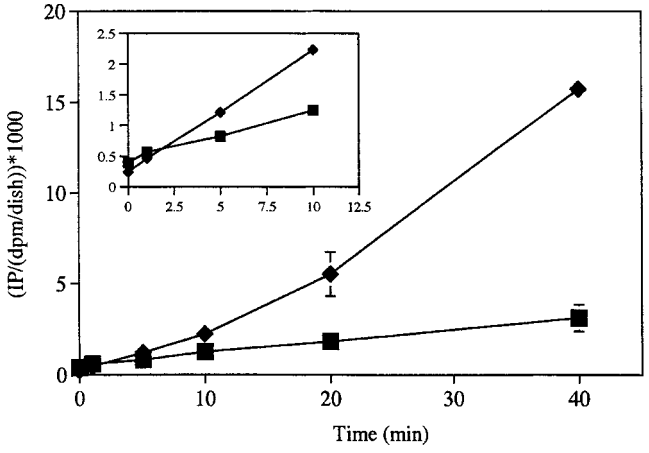

Figure 8 Comparison of desensitization of wild-type and N-Stop forms of TRHR-1L

HEK-293 cells, transfected to transiently express wild-type TRHR-1L (■) or the N-Stop truncation (◆) were treated with 50 nM TRH and production of total [^{3}H]inositol phosphates (IP) was measured at times up to 40 min, as described in the Materials and methods section. The results are the means ± S.D. of two experiments performed in duplicate. Inset: values obtained over the initial 10 min period of incubation.

unaffected by pre-exposure to TRH for up to 1 h (Figure 7), again indicating a central role for the C-terminal tail of TRHR-1 in this process. To assess the relative functional desensitization of the wild-type and N-stop forms of the TRHR-1, TRH stimulation of [^{3}H]inositol phosphate production was measured in cells prelabelled with *myo*-[^{3}H]inositol in the presence of LiCl (15 mM) over time. A linear increase in [^{3}H]inositol phosphate generation was observed for the N-Stop variant over the first 10 min, whereas the full-length TRHR-1L displayed a reduced and biphasic response over this time period (Figure 8).

DISCUSSION

Analysis of receptor internalization and recycling to the plasma membrane frequently indicates key roles for clathrin-coated pits in these processes [16,27,28]. This indicates the likely contribution of constitutively recycling endosomes [27]. As a central role for ligand-stimulated receptor internalization appears to be to allow separation of the fate of the receptor between resensitization, often by removal of phosphate groups from serine and threonine residues in its C-terminal tail, and its trafficking towards lysosomes for degradation, these mechanisms have been actively studied for some time [16,27–29]. Construction of green-fluorescent-protein-tagged forms of receptors has recently allowed a novel, non-invasive means of observing aspects of these processes in real time [20,30–33]. Furthermore, a series of ELISA-based approaches may be used if an antibody to extracellular elements of a receptor or to an introduced epitope tag is available, and the disappearance and reappearance of receptor at the cell surface is all that is to be monitored [34]. However, such studies have yet to provide detailed quantitative analysis of receptor processing or to address issues of ligand dissociation from receptor and possible re-internalization of a receptor following recycling from the cell interior to the surface [35,36]. Many of the approaches available for such detailed study rely on the detection of binding of high-affinity radiolabelled agonist ligands to, and their release from, receptors. This is generally most appropriate for peptide ligands, as these often have high affinity for and are known to be co-internalized along with the receptor [36]. In these studies, methods are required to separate the cell-surface-associated ligand from the internalized ligand. This is usually performed by an acid-wash procedure, which increases the dissociation of ligand from receptor at low temperature to prevent further endocytosis and recycling of the receptor–ligand complex [23]. Specifically-bound ligand remaining after this type of wash procedure is assumed to be intracellular ligand–receptor complex.

Given the importance of the C-terminal tail in agonist-induced desensitization and internalization of many receptors [16], it is perhaps not surprising that C-terminally truncated forms of receptors may display poor internalization characteristics [17–19]. The extreme case is the gonadotrophin-releasing hormone receptor, which, at least in mammalian species [21], lacks a C-terminal tail and displays slow kinetics of agonist-induced internalization [21]. By contrast, forms of the receptor from species such as catfish have a C-terminal tail and internalize effectively in response to agonist when expressed in heterologous systems [21]. Previous studies on the mouse TRH receptor have demonstrated that severe truncation of the C-terminal tail (Cys^{335} → Stop) severely compromises steady-state internalization and that less severe truncation can also significantly reduce the extent of steady-state internalization [19]. For the mouse receptor, Nussenzveig et al. [19] have suggested a key role for the phenylalanine residue equivalent to that which lies immediately upstream of the S-Stop and T-Stop mutants generated in the present study. As both of these truncation mutants display significantly decreased degrees of internalization (Figure 3), either this phenylalanine residue is not the key amino acid or an extended motif incorporating this amino acid is required. In the mouse receptor, truncation well beyond this phenylalanine residue was required to reduce ligand-stimulated internalization to 50 % of that of the wild type. In the present study, this was achieved with truncations downstream of the equivalent phenylalanine residue. As the degree of internalization was decreased from essentially that of the wild type in the I-stop mutant to approx. 50 % in the T-stop mutant, and only four amino acid residues separate these sites (Figure 1), we have narrowed identification of the key elements for ligand-induced internalization substantially. However, it has been indicated that a form of the mouse Cys^{335} → Stop TRHR undergoes constitutive internalization and recycling [15]. In studies such as this, it is important to know if the modification made to a receptor alters the ligand-binding characteristics of the mutant. If it does, then data obtained using radiolabelled ligands are unlikely to be easy to analyse.

In the present work, the rat TRHR-1L [2–4] was used as the starting point to explore the effects of different degrees of C-terminal truncation on steady-state internalization of the receptors in response to TRH. We also analysed the potential effects of different extents of C-terminal truncation on ligand-association and -dissociation characteristics, the rate and extent of receptor recycling from endosomes to the cell surface and the possibility of re-internalization of the receptor following recycling, because of either the slow rate of dissociation of [^{3}H]TRH or its rebinding under conditions in which external TRH was removed. We were also concerned that the acid-wash procedure generally used to remove surface-bound ligand might compromise the capacity of treated cells to subsequently re-internalize receptor. This proved to be the case and we were compelled to adopt a different wash strategy to overcome this problem.

Analysis of the rate of recycling of receptor to the cell surface (Table 1) following agonist-induced internalization requires the means to both monitor its appearance and assess if the receptor is then subsequently re-internalized as part of a dynamic cycle.

To do so, the rate and extent of release of pre-internalized [^{3}H]TRH into the extracellular medium was measured. When cell-surface-bound [^{3}H]TRH was removed after a pre-internalization period and medium was replaced without added TRH, release of [^{3}H]TRH into the medium could be monitored over time. Although this might be assumed to provide a direct measure of the rate of recycling, it did not. When the same experiments were performed with addition of either 50 nM or 50 μM unlabelled TRH to the extracellular medium, the measured rate of release of [^{3}H]TRH was three times faster than in the absence of unlabelled TRH. The most obvious interpretation of these data is that, in the presence of unlabelled TRH, receptors which are recycled to the cell surface and from which [^{3}H]TRH is dissociated before a new round of internalization is initiated will then preferentially bind unlabelled TRH, because of the relative concentrations of [^{3}H]TRH and unlabelled TRH, and internalize again as a TRH–TRHR-1L complex. In the absence of unlabelled TRH, dissociated [^{3}H]TRH may rebind to the receptor and thus initiate re-internalization. The kinetics of release of [^{3}H]TRH in the presence of a sufficiently high concentration of unlabelled TRH thus provides a minimum estimate for the rate of recycling of the TRHR-1L, as receptors which are recycled to the cell surface and then re-internalized without dissociation of bound [^{3}H]TRH are not monitored. The unlabelled TRH does not inherently 'displace' [^{3}H]TRH from receptors recycled to the cell surface but effectively competes with it for rebinding if dissociation of [^{3}H]TRH occurs during the time frame that the receptor is present at the cell surface.

Comparison of the rate of recycling of the wild-type and truncation mutant receptors indicated that it was similar (Table 1), except for the L-Stop mutant (results not shown). However, the altered dissociation characteristics of TRH from the L-Stop mutant means that the recycling of this construct cannot be usefully analysed based on assays which require dissociation of the [^{3}H]TRH into the extracellular medium. The estimated rate constant for endocytosis of TRHR-1L was lower in these studies than reported by Heding et al. [21], although the rate constant for recycling was very similar. It is unclear if this simply reflects differences due to transient versus stable expression of the receptor, as both studies were performed with HEK-293 cells.

It was also of considerable interest to note that the extent of recycling of TRHR-1L to the plasma membrane was unaffected by the period of pretreatment of the cells with TRH, whereas the extent of endocytosis was reduced by up to 20 % by pre-exposure of the cells to TRH for as little as 5 min. Once internalized, it was anticipated that the constitutive recycling pathway should allow exocytosis of the receptor at an unaltered rate, however, the decreased extent of internalization was unexpected. This seemed to be a genuine effect, as it was not observed with truncated forms of the TRHR-1L and thus indicates a potential role for desensitization and alteration in the structure of the C-terminal tail of TRHR-1L in the kinetics of the internalization process. To begin to explore this, a series of desensitization assays were performed, which allowed comparison of the capacity and kinetics of the full-length receptor and N-Stop mutant to generate [^{3}H]inositol phosphates in LiCl-treated cells (Figure 8). This confirmed that the full-length receptor desensitized more readily than the truncated mutant. The ability to analyse the rate and the mechanistic details of internalization and recycling of a receptor by monitoring the cellular distribution of its ligand adds significantly to such studies. However, as the present studies demonstrate clearly, care must be taken to analyse differences in the distribution of the ligand separately from the receptor before quantitative analyses can be applied appropriately.

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