Evidence that the thyrotropin-releasing hormone receptor and its ligand are recycled dissociated from each other

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We have examined the trafficking of the thyrotropin-releasing hormone receptor (TRHR) and its ligand, after TRHR-TRH internalization in rat pituitary GH₄C₁ cells. After rapid ligand-induced receptor sequestration, the cell surface receptor pool was replenished. Replenishment was insensitive to inhibition of protein synthesis and was dependent on the duration of internalization; therefore, the replenished receptors were not newly synthesized but recycled. The total amount of recycled receptors decreased with increasing internalization time, resulting in only partial replenishment of the cell-surface receptor pool after prolonged incubation with ligand. Thus, in addition to a receptor recycling pathway, a non-cycling route exists for TRHR sorting; this route became dominant with increasing internalization periods. TRHR entry into these pathways was not determined by the affinity of the receptor–ligand interaction, because the extent of receptor recycling was similar after TRH- and methyl-TRH (MeTRH)-induced internalization. Unlike results with the TRHR, the TRH recycling pool was not depleted by the non-cycling pathway. After multiple rounds of [³H]MeTRH internal-

ization, the amount of cell-associated radioactivity increased with increasing internalization time due to accumulation of the ligand or its metabolites in a non-cycling pathway, but the absolute amount of recycled ligand remained constant after short or long internalization times. The difference in the proportion of TRHR and MeTRH that were diverted into a non-cycling pathway indicated intracellular dissociation of the internalized TRHR–TRH complex. Dissociation of the internalized TRHR–TRH complex was dependent on the acidic pH in an intracellular compartment. Although extracellular acidic pH did not enhance cell-surface receptor–ligand (RL) dissociation, bafilomycin A1 inhibited both receptor and ligand recycling. We conclude that the TRHR–TRH system is unique among recycling receptors because, after RL sequestration, the TRHR–TRH complex becomes dissociated intracellularly via a bafilomycin A1-sensitive, acidic pH-dependent mechanism, and both the unoccupied TRHR and TRH recycle disassociated from each other.

INTRODUCTION

In many cell types, ligand binding induces cell-surface receptor redistribution, thereby regulating the number of receptors available for signal transduction. The pathways involved in receptor redistribution, which include receptor internalization as well as recycling and degradation, have been studied extensively for the low-density lipoprotein [1], transferrin [2] and epidermal growth-factor [3] receptors. Knowledge of the trafficking of G-protein-coupled receptors is most extensive for the adrenergic receptor system [4,5]. The internalized β -2 adrenergic receptor has been colocalized with the transferrin (Tf) receptor in endosomes of transfected 293 cells, suggesting that these two receptors are trafficked in the same internalization and recycling pathways [4].

The thyrotropin-releasing hormone receptor (TRHR) is a member of the G-protein-coupled receptor family [6,7], which signals through G_q/G₁₁ and phospholipase C [8,9]. Rat pituitary GH cells have served as a particularly useful model for studies on the TRHR signalling pathway [10,11]. TRHR distribution in these cells is regulated by ligand binding. TRH binding to the plasma-membrane TRHR induces rapid transformation of the receptor–ligand (RL) complex into an acid-resistant, internalized form ($t_{1/2}$ = 3 min) [12]. Following RL sequestration, the TRHR slowly becomes degraded [13]. Incubation with TRH for 48 h

results in a decrease in the total number of ligand-binding sites per cell to about 40 % of control values [13]. In addition, after RL sequestration, the plasma membrane receptor density becomes replenished [12]. However, it is not known whether the replenished receptors are recycled internalized receptors, newly synthesized receptors, or molecules from an intracellular storage pool. The finding that [³H]TRH binding is the same in intact and solubilized cells [14] does not exclude the existence of an intracellular pool of TRHRs that are able to bind ligand only upon insertion in the plasma membrane. In this paper, we present evidence for both a recycling pathway and a non-cycling/degradation pathway for the TRHR.

To determine whether, after RL complex internalization, the TRHR remained associated with its ligand and was recycled as a RL complex, we compared the receptor and ligand sorting pathways. It is known that TRH follows both non-cycling and cycling pathways. For example, after incubation of GH₄C₁ cells with [³H]TRH for 43 h, about 80 % of the radioactivity became covalently associated with intracellular proteins [15]. Also, when GH₄C₁ cells containing internalized [³H]TRH were incubated at 37 °C in ligand-free medium, the intracellular radioactivity decreased by 60 % within 2 h [16]. The radioactivity released into the medium was intact [³H]TRH as shown by t.l.c. [16]. In addition, the recycled material had the binding-affinity charac-

Abbreviations used: HBSS/Ca²⁺, HEPES-buffered salt solution plus 0.5 mM Ca²⁺; MeTRH, 3-methyl-his-TRH; NTA, nitrilotriacetic acid; RL, receptor–ligand complex; Tf, transferrin; TRH, thyrotropin-releasing hormone; TRHR, thyrotropin-releasing hormone receptor.

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teristic of TRH for the TRHR and it could stimulate prolactin release from GH₃ cells [16]. The present study defines the relationship between the ligand recycling and non-cycling pathways. The difference in the proportions of TRH and TRHR sorted into the cycling and non-cycling pathways supports a model of intracellular dissociation of the internalized TRHR-TRH complex and subsequent recycling of the TRHR and TRH dissociated from each other. Dissociation of the internalized TRHR-TRH complex is dependent on the acidic endosomal pH.

EXPERIMENTAL

Materials

[³H]3-methyl-his-TRH ([³H]MeTRH) (62.8 Ci/mmol) and ⁵⁵FeCl₃ (1.75 mCi/mmol) were purchased from Du Pont-New England Nuclear Corp. (Boston, MA, U.S.A.). TRH and 3-methyl-his-TRH (MeTRH) were from Peninsula Laboratories Inc. (Belmont, CA, U.S.A.). Cycloheximide, chloroquine and human apo-transferrin were purchased from Sigma (St. Louis, MO, U.S.A.) and culture media were from Gibco BRL (Grand Island, NY, U.S.A.). Bafilomycin A1 was a gift from Dr. Timothy E. McGraw (Columbia University, NY, U.S.A.).

Methods

Cell culture

GH₄C₁ cells were grown at 37 °C in monolayer culture in Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum in a humidified atmosphere of 5% CO₂-95% air [17]. For receptor sequestration and recycling, as well as for ligand recycling experiments, 35 mm plastic culture dishes were seeded with equal aliquots of cells from a single donor culture and grown to late log phase before use.

Ligand-induced TRHR internalization

GH₄C₁ cells in 35 mm 6-well plates were incubated at 37 °C with 1 μM MeTRH diluted in Hepes-buffered salt solution containing 0.5 mM Ca²⁺(HBSS/Ca²⁺) for different periods of time (see Results section). The cells were then washed with ice-cold PBS and treated with ice-cold 0.2 M CH₃COOH/0.5 M NaCl, pH 2.5 (acid/salt wash), for 5 min [12]. After washing twice with cold PBS, [³H]MeTRH binding was performed at 4 °C (see below). Triplicate dishes were used in each experiment and the S.D. was calculated. The experiment was repeated three times.

Receptor replenishment

Following ligand-induced receptor sequestration for 30 or 60 min, GH₄C₁ cells were washed with ice-cold PBS and were then rewarmed to 37 °C in the absence of ligand for different periods of time (see Results section). Cell-surface receptor number was determined by [³H]MeTRH binding at 4 °C. Triplicates were performed in each experiment and the S.D.s were calculated. The experiment was repeated three times.

Ligand recycling

For a single round of internalization/recycling, GH₄C₁ cells were first incubated with 4 nM [³H]MeTRH in HBSS/Ca²⁺ for 60 min at 4 °C. After washing with ice-cold PBS, the radiolabelled ligand was internalized at 37 °C. For multiple rounds of internalization/recycling, [³H]MeTRH was present continuously

during binding and internalization at 37 °C. After ligand internalization for 5 min or 25 h, the cells were washed with ice-cold PBS and incubated in HBSS/Ca²⁺ buffer at 37 °C for different periods of time (see Results section). The medium was collected and the radioactivity was measured by scintillation counting or analysed by h.p.l.c. (see below). The cells were acid/salt washed and the radioactivity that remained cell-associated (acid/salt resistant) as well as the radioactivity in the wash (acid/salt sensitive) was measured. Triplicate dishes were used in each of the above experiments and the mean value and S.D. were calculated.

Radioligand binding

After ligand-induced TRHR internalization and different times of recycling, GH₄C₁ cells were chilled on ice and washed with ice-cold PBS (pH 7.4). The cells were then incubated with 4 nM [³H]MeTRH in HBSS/Ca²⁺ (pH 7.2) at 4 °C for 60 min, at which time equilibrium binding was reached. Non-specific binding was determined by the addition of a 250-fold molar excess of unlabelled MeTRH. The binding medium was aspirated and the cells were washed three times by dipping the dishes into a large volume of ice-cold 0.15 M NaCl solution [10]. The washed cells were solubilized with 0.1 M NaOH and the radioactivity was measured by scintillation counting. All treatment groups included one dish, treated the same way as the others in the group, for the measurement of protein concentration for each time-point. Cell protein was determined by the Pierce BCA method, using bovine albumin as standard.

H.p.l.c. analysis

The recycling medium was freeze dried and resuspended in water at a final concentration of radioactivity of 30000 c.p.m./ml and an aliquot was injected into a C₁₈, 5 μm, 4.6 mm × 25 cm column, at room temperature. The solvents used were A = 0.05 M NaH₂PO₄ and B = CH₃CN. A linear gradient from 0 to 100% of B was developed over 40 min. The flow rate was 1.0 ml/min. Authentic [³H]MeTRH was used as standard.

Fluorescence microscopy

GH₄C₁ cells were plated on poly-L-lysine-coated glass cover slips and washed twice with HBSS/Ca²⁺ before treatment with bafilomycin A1 (100 nM) for 1 h at 37 °C. Acridine Orange (5 μg/ml) was then added to the incubation medium for 10 min at 37 °C to determine alkalization of acidified intracellular compartments. The cells were then washed four times with HBSS/Ca²⁺ alone or HBSS/Ca²⁺ containing bafilomycin A1. The coverslips were examined using a confocal laser scanning microscope (Sarastro 2000, Molecular Dynamics Inc., Sunnyvale, CA, U.S.A.).

⁵⁵Fe-Tf uptake

⁵⁵FeCl₃ was mixed with 6.3 mM nitrilotriacetic acid (NTA) to final concentrations of 200 and 400 μM, respectively, in 0.1 M HCl. The final volume was adjusted to 250 μl with 20 mM Hepes/Tris (pH 6.0). The solution was titrated to pH 7.0 with 10 M NaOH and mixed with PBS, 0.5 M NaHCO₃ and 400 μM dialysed human apotransferrin (apo-Tf) to obtain final concentrations of 150 μM ⁵⁵FeNTA, 10 mM NaHCO₃ and 50 μM apo-Tf. The mixture was then incubated in a rotating tube at room temperature for 4 h. The ⁵⁵Fe-Tf complex was isolated by gel filtration. The sample was counted and the A₂₈₀ was measured to

calculate the concentration of ^{55}Fe and Tf respectively. GH_4C_1 cells were incubated at 37°C for 8 h in serum-free F10 medium containing 250 nM ^{55}Fe in the form of the ^{55}Fe -Tf complex in the presence or absence of 500 nM bafilomycin A1. At the end of the incubation period the cells were washed twice with ice-cold PBS, solubilized in 0.1 M NaOH and the radioactivity was measured by scintillation counting.

RESULTS AND DISCUSSION

Evidence for receptor recycling and non-cycling pathways

MeTRH binding to TRHRs induced rapid cell-surface TRHR sequestration with a $t_{1/2}$ of about 10 min (Figure 1). The decrease in plasma-membrane ligand-binding sites represented a decrease in receptor number and not a change in receptor affinity, as determined by Scatchard analysis (results not shown). Following internalization, the cell-surface TRHR number became replenished. After ligand-induced receptor internalization for 60 min, the cells were washed with cold buffer and incubated at 37°C in

Figure 1 Time-course of MeTRH-induced TRHR sequestration

GH_4C_1 cells were incubated with MeTRH ($1\ \mu\text{M}$) at 37°C for the times indicated. The cells were then acid/salt washed at 4°C for 5 min and the cell-surface receptor number was determined by binding $[^3\text{H}]\text{MeTRH}$ at 4°C for 60 min. Each point represents the mean of triplicate dishes and the error bars give the S.D.

Figure 2 Time-course of replenishment of cell-surface TRHRs after induction of RL internalization by MeTRH

GH_4C_1 cells were incubated with MeTRH ($1\ \mu\text{M}$) at 37°C for 60 min. The receptor number that remained on the cell surface after this internalization period (zero time-point of recycling) was lower than the receptor number after 60 min of internalization in Figure 1; however, this represents the usual variation from experiment to experiment. After washing at 4°C with PBS, cultures were warmed to 37°C in the absence of ligand for the times indicated. Cell-surface receptor number was determined by $[^3\text{H}]\text{MeTRH}$ binding at 4°C for 60 min. Total cell-surface-specific binding before internalization was 24528 ± 2523 c.p.m./mg protein. Each point gives the mean value and the error bars give the S.D. of triplicate dishes. Points without error bars indicate that the S.D. was within the symbol size. The kinetics of receptor replenishment were essentially the same when internalization was induced with 4 nM MeTRH, the concentration used for ligand recycling studies.

Table 1 Lack of requirement for protein synthesis for replenishment of cell surface TRHRs

The protocol used for this experiment was described in the legend to Figure 2. MeTRH ($1\ \mu\text{M}$) was used to induce TRHR internalization for 30 min at 37°C . Cell surface receptor replenishment was measured after 0 or 60 min of incubation at 37°C in the absence or presence of $1\ \mu\text{g}/\text{ml}$ cycloheximide. This concentration of cycloheximide inhibited protein synthesis in GH_4C_1 cells by more than 95% in < 10 min. Results are presented as mean values $\pm$ S.D. Total cell-surface-specific $[^3\text{H}]\text{MeTRH}$ binding before internalization was 28457 ± 342 c.p.m./mg protein.

Replenishment time (min)	$[^3\text{H}]\text{MeTRH}$ bound (c.p.m./mg protein)	
	Control	Cycloheximide
0	1655 ± 287	2345 ± 338
60	24290 ± 155	23100 ± 225

Table 2 Relationship of replenishment of cell surface TRHRs to the duration of ligand-induced receptor internalization

GH_4C_1 cells were incubated with MeTRH ($1\ \mu\text{M}$) at 37°C for either 30 or 60 min to induce receptor internalization. The cells were then washed with PBS at 4°C and reincubated at 37°C for 0 min or 60 min. After the replenishment period, they were washed again and binding with 4 nM $[^3\text{H}]\text{MeTRH}$ was performed at 4°C for 60 min. Results are presented as mean values $\pm$ S.D. Total cell-surface-specific binding before receptor internalization was 20975 ± 2230 c.p.m./mg protein.

Replenishment time (min)	$[^3\text{H}]\text{MeTRH}$ bound (c.p.m./mg protein)	
	Internalization time (min)	
	30	60
0	111 ± 287	110 ± 50
60	8110 ± 155	3871 ± 1431

the absence of ligand for different times to allow for cell surface receptor replenishment. Cell-surface receptors were measured by $[^3\text{H}]\text{MeTRH}$ binding at 4°C . The receptor replenishment process was linear for the first 2 h (Figure 2). In a separate experiment, where receptor replenishment was measured after 60, 120 and 180 min, replenishment reached a plateau between 120 and 180 min.

At least three possible modes of receptor replenishment need to be considered: newly synthesized TRHRs or TRHRs from an intracellular storage pool may be inserted into the plasma membrane, and alternatively, internalized receptors are recycled. The insensitivity of the replenishment process to cycloheximide indicated that the replenished receptors were not newly synthesized and that the replenishment process did not depend on ongoing protein synthesis (Table 1).

The dependence of the surface receptor replenishment process on the duration of internalization, distinguished between the two other replenishment mechanisms and supported the concept of a TRHR recycling pathway. We compared the level of TRHR replenishment at 60 min, after two different times of TRH-induced TRHR internalization. After 30 min of internalization, plasma-membrane receptors were replenished to 40% of the original density, but after 60 min of internalization the surface receptor density reached only 20% of the original plasma-membrane TRHR level (Table 2). One possible explanation for this result is that receptors from an intracellular storage pool

Table 3 TRHR replenishment after internalization induced by MeTRH or TRH

GH₄C₁ cells were incubated with 4 nM MeTRH or 20 nM TRH at 37 °C for 60 min. After washing at 4 °C, cultures were warmed to 37 °C in the absence of ligand for 0 or 60 min. Cell-surface receptor number was estimated by [³H]MeTRH binding at 4 °C for 60 min. Total cell-surface binding before internalization was 16210 ± 3145 c.p.m./mg protein. Results are presented as mean values ± S.D.

Replenishment time (min)	[³ H]MeTRH bound (c.p.m./mg protein)	
	MeTRH	TRH
0	2785 ± 738	3445 ± 1060
60	11255 ± 299	8944 ± 1185

were being inserted into the plasma membrane simultaneously with receptor sequestration and that this pool was depleted during prolonged internalization. This possibility is unlikely because, since ligand recycling is much slower (see below) than RL sequestration [12], simultaneous receptor sequestration and replenishment would result in progressive accumulation of intracellular ligand. However, intracellular ligand accumulation reaches a maximum within 6 min [12]. Therefore, the dependence of surface receptor replenishment on the duration of internalization can be best explained by the recycling of internalized receptors and the time-dependent diversion of some of these TRHRs into a non-cycling pathway during each round of internalization. After prolonged periods of multiple internalization rounds, the non-cycling pathway becomes dominant. Endosomes may be the sorting compartment between the TRHR recycling and non-cycling pathways [18,19].

Because dissociation of the internalized RL complex controls the recycling of certain receptors [20,21], we determined the effect of decreasing the rate of RL dissociation on TRHR recycling. We measured receptor replenishment after the internalization of two TRH ligands with different affinity for the TRHR. MeTRH is a superagonist with a K_d of 3 nM, while TRH has a K_d of 25 nM [22]. There was no statistically significant difference in TRHR recycling after internalization of the two types of RL complexes (Table 3). We conclude that the affinity of ligand binding in the RL complex does not appear to alter TRHR trafficking into the recycling versus the noncycling pathway. However, this conclusion is based on the assumption that the affinities of the TRHR-TRH and TRHR-MeTRH complexes are similar in the endosomal compartment and on the plasma membrane.

Ligand recycling pathway

To determine whether, after RL internalization, the TRHR was recycled associated with its ligand, we characterized the ligand recycling pathway and compared it with that of the TRHR. [³H]MeTRH was bound to receptors at 4 °C and the cells were then washed. After internalization at 37 °C for 30 min, the cells were washed again and reincubated in the absence of ligand at 37 °C for different times. Ligand recycling was measured during the second 37 °C incubation in medium alone, and in medium containing cycloheximide or excess unlabelled ligand. The medium was removed and counted for radioactivity and the cells were acid/salt washed to release surface-bound [³H]MeTRH. The amount of cell-surface-associated radioactivity decreased only slightly (between 400 and 600 c.p.m./mg protein) (Figure 3b). The amount of radioactivity released into the medium

Figure 3 Kinetics of [³H]MeTRH recycling after a single round of RL internalization

[³H]MeTRH (4 nM) was bound to GH₄C₁ cells for 60 min at 4 °C (18690 ± 6083 c.p.m./mg protein were specifically bound to receptors). After washing with PBS, the cells were incubated in HBSS/Ca²⁺ buffer at 37 °C for 30 min to allow RL internalization. The cells were then washed with ice-cold PBS and were returned to 37 °C for the times indicated to allow for exocytosis of the internalized ligand. The cycling buffers were HBSS/Ca²⁺ alone (■), HBSS/Ca²⁺ supplemented with 1 μM MeTRH (●) or HBSS/Ca²⁺ with 1 μM MeTRH plus 1 μg/ml cycloheximide (▲). The medium radioactivity was determined and the cells were acid/salt washed for 5 min at 4 °C. The radioactivity in the acid/salt wash and in the cells was then measured. Each point represents the mean value and the error bars give the S.D. for triplicate dishes. Points without error bars indicate that the S.D. was within the symbol size. For details of (a) (b) and (c) see text.

(Figure 3a) increased at approximately the same rate as intracellular radioactivity decreased (Figure 3c). The average $t_{1/2}$ for these two processes in three independent experiments was calculated to be 60 min and was unaffected by cycloheximide. Repetition of this experiment three times revealed that the decrease in medium radioactivity in the 120 min sample from cells incubated in HBSS/Ca²⁺ (Figure 3a) was not a reproducible finding. The recycled radioactive material in the medium was analysed by reverse-phase h.p.l.c. and was found to comigrate with authentic [³H]MeTRH (results not shown). Thus, the internalized ligand was recycled in an intact form.

The conditions of this experiment were designed to characterize ligand recycling after a single round of internalization/recycling. For example, we determined that no ligand recycling took place during the first internalization period at 37 °C because the amount of radioactivity associated with the cells after binding at 4 °C did not decrease at the end of the internalization incubation (Figure 3c and legend). However, this result did not exclude the possibility that the recycled ligand remained bound to the

Figure 4 Relationship of [³H]MeTRH recycling to the duration of internalization

GH₄C₁ cells were incubated with 4 nM [³H]MeTRH at 37 °C for the times indicated. The cells were washed with ice-cold PBS and rewarmed to 37 °C for 60 min. Medium radioactivity was measured (■). The cells were acid/salt washed for 5 min at 4 °C and the acid wash-resistant, cell associated radioactivity (▨) was counted. Panels (a) and (b) represent two experiments. Each bar represents the mean value and the error bars give the S.D. of triplicate dishes.

receptor throughout recycling and that the same RL complex was reinternalized two or more times. We eliminated this possibility because a large excess of MeTRH in the extracellular medium did not affect the rate of ligand recycling (Figure 3). If the RL complex was recycled to the cell surface intact, the large excess of unlabelled MeTRH in the medium would have displaced the [³H]MeTRH in the RL complex. This was not observed. Thus, the internalized RL complex must have dissociated after a single round of internalization; RL dissociation may have occurred in an intracellular compartment or at the plasma membrane. A comparison between the ligand and receptor sorting pathways was necessary to distinguish between these two possibilities.

Comparison between the ligand and receptor recycling and non-cycling pathways

Because the amount of recycled TRHRs decreased with increasing internalization time, probably due to the diversion of TRHRs into a non-cycling pathway, we investigated whether the amount of recycled MeTRH was similarly affected by a ligand non-cycling pathway. TRH is degraded by GH₄C₁ cells with *t*_{1/2} of about 20 h [15], indicating that a slow non-cycling route exists which directs internalized ligand to a degradation pathway. However, under the conditions of a single round of internalization/recycling, essentially 100% of the internalized ligand was cycled back to the cell surface within 180 min. Thus, only a small amount of ligand, that cannot be easily measured, appears to be trafficked along the non-cycling route after one round of internalization. In order for the ligand to gain access to the non-cycling pathway we studied MeTRH recycling after multiple rounds of internalization. For these experiments, [³H]MeTRH binding and internalization took place during an initial incubation at 37 °C for different periods of time, and ligand recycling was measured as in Figure 3. The amount of intact recycled [³H]MeTRH (determined by reverse-phase h.p.l.c.) remained constant after 5, 15, 30 and 60 min of multiple internalization rounds (Figure 4a). The lack of effect of increasing internalization time on ligand recycling was not because short internalization times may not have allowed enough internalized ligand to accumulate in the non-cycling pathway, because the

Figure 5 Kinetics of [³H]MeTRH cycling after 5 min and after 25 h of binding and internalization

GH₄C₁ cells were incubated with 4 nM [³H]MeTRH at 37 °C for 5 min (■) or 25 h (●). The protocol described in Figure 4 was followed except that the amounts of acid-wash-sensitive (cell surface) radioactivity are also presented. Each point represents the mean value and the error bars give the S.D. of triplicate dishes. Points without error bars indicate that the S.D. was within the symbol size.

Figure 6 Lack of effect of low extracellular pH on the dissociation of cell surface TRHR-TRH complexes

[³H]MeTRH (4 nM) was bound to GH₄C₁ cells at 4 °C for 60 min at pH 7.2. (a) The cells were then incubated at 4 °C with HBSS/Ca²⁺ at pH 7.2 (■) or pH 6.0 (●) for the times indicated and the radioactivity released into the medium was measured. (b) After [³H]MeTRH binding, the cells were incubated at 4 °C with Mes/Ca²⁺ at the indicated pH values for 60 min. The radioactivity released into the medium (■) and remaining cell-associated (●) was measured. Each point represents the mean value of triplicate dishes and the error bars give the S.D. Points without error bars indicate that the S.D. was within the symbol.

extent and rate of ligand recycling (determined by h.p.l.c.) remained constant even after 25 h of internalization compared with 5 min of internalization (Figure 4b and Figure 5).

We expected that a significant amount of the internalized

Figure 7 Alkalization of intracellular compartments by bafilomycin A1

GH₄C₁ cells were incubated in HBSS/Ca²⁺ buffer alone (panel A) or in HBSS/Ca²⁺ containing 100 nM bafilomycin A1 (panel B) for 1 h at 37 °C. They were subsequently incubated with Acridine Orange (5 μg/ml) for 10 min, washed four times with HBSS/Ca²⁺ or HBSS/Ca²⁺ plus bafilomycin A1, and visualized by fluorescence confocal light microscopy. The punctate fluorescent pattern, representing acidified compartments in untreated cells (arrowheads in panel A), were not seen in bafilomycin A1-treated cells (panel B). The bar (lower right) designates 10 μm.

ligand would proceed into degradation compartments after 25 h of internalization, rather than being in the endosomal/sorting compartments, which was the case after 5 min of internalization. Because more radioactivity accumulated intracellularly after 25 h as compared with 5 min of internalization (Figure 4b), a portion of the internalized MeTRH was indeed directed to a non-cycling pathway after the long internalization period. Ligand entry into the non-cycling pathway must have been slow, because the intracellular radioactivity increased only 3-fold between 1 h and 25 h of internalization and because there were no changes in the amounts of intracellular radioactivity between 5 min and 60 min of internalization. The fate of the ligand accumulating in the non-cycling pathway was not studied, but it is unlikely that it is intact MeTRH. It has been shown that 80% of the radioactivity from [³H]TRH bound to GH₃ cells for 43 h is covalently associated with cellular proteins [15].

We conclude that the amount of recycled [³H]MeTRH was not dependent on the duration of internalization. Thus, the non-cycling pathway diverts different proportions of TRHR and TRH away from the recycling pathway. These results give indirect evidence that the internalized TRHR–TRH complex dissociates intracellularly and that the TRHR and TRH subsequently recycle dissociated from each other. This finding appears to be unique for the TRHR–TRH trafficking pathway. In most systems where ligand recycling has been studied, the intact internalized RL complex is recycled [20,21,23–25].

Table 4 Inhibition of TRHR and MeTRH recycling by bafilomycin A1

The protocols for ligand and receptor recycling described in the legends for Figures 2 and 3 were followed except that 500 nM bafilomycin A1 was included during the internalization and recycling incubation periods. The amounts of recycled TRHR and [³H]MeTRH after 120 min of recycling are given. For the ⁵⁵Fe uptake experiment, GH₄C₁ cells were incubated with 250 nM ⁵⁵Fe in the form of ⁵⁵Fe–Tf for 8 h at 37 °C in the presence or absence of 500 nM bafilomycin A1. Results are presented as mean values ± S.D.

Treatment	Receptor recycling	Ligand recycling	⁵⁵ Fe uptake*
	[³ H]MeTRH bound (c.p.m./mg protein)	Medium radioactivity (c.p.m./mg protein)	Medium radioactivity (c.p.m./mg protein)
Control	12263 ± 1490	5964 ± 996	176232 ± 14452
Bafilomycin A1	8450 ± 992	3624 ± 670	63265 ± 1228

* The background ⁵⁵Fe uptake at 4 °C was 1800 c.p.m./mg protein. The ⁵⁵Fe uptake experiment was done for 8 h compared with 2 h for the TRHR/TRH recycling experiments because, due to the low Tf receptor number in GH₄C₁ cells, there was insufficient uptake at 2 h.

Mechanism of RL dissociation

It has been found that low pH in an intracellular compartment causes dissociation of several internalized RL complexes and affects receptor and ligand recycling [20,26]. To determine whether dissociation of the TRHR–ligand complex was sensitive to pH we first measured the rate of dissociation of cell-surface-bound [³H]MeTRH in buffers of pH 7.2 and pH 6.0 at 4 °C (Figure 6a). The rate of cell-surface RL dissociation did not differ between the two pH values. We then examined the effect of a more extensive range of extracellular pH on cell-surface RL dissociation and found that dissociation at pHs between 5.0 and 6.5 was similar to that at pH 7.0 (Figure 6b).

However, extracellular acidic pH does not necessarily mimic the acidic environment in intracellular compartments. Therefore, we determined the effect of intracellular compartment alkalization by the specific vacuolar-type ATPase inhibitor bafilomycin A1 [27,28] on MeTRH and TRHR recycling. To determine whether bafilomycin A1 exerted its alkalizing action in GH₄C₁ cells, the cells were stained with Acridine Orange, a permeable weak base which accumulates in acidified compartments. The high concentration of Acridine Orange in acidic compartments causes a fluorescence shift from green to orange. In control GH₄C₁ cells, the acidified compartments appeared as punctate fluorescent structures (Figure 7A). Incubation with 100 nM bafilomycin A1 at 37 °C for 1 h abolished the punctate fluorescent pattern, indicating that alkalization of acidic intracellular compartments had occurred (Figure 7B). Furthermore, compartment alkalization by bafilomycin A1 inhibited pH-dependent endosomal functions in GH₄C₁ cells. Although 100 nM bafilomycin A1 was sufficient to determine compartment alkalization by confocal microscopy, 500 nM of this agent was necessary for inhibition of pH-dependent endosomal function. Incubation of the cells with 500 nM bafilomycin A1 inhibited the pH-dependent ⁵⁵Fe–Tf uptake via the Tf receptor internalization pathway [29] by 60% (Table 4). This inhibition of pH-dependent endosomal function was correlated with a decrease in TRHR and MeTRH recycling by bafilomycin A1 (Table 4). Chloroquine, another alkalizing agent, had similar effects on the recycling processes (results not shown). We conclude that, similar to other recycling receptors [30–32], dissociation of the internalized TRHR–TRH complex depends, at least in part, on the acidic environment in endosomes.

CONCLUSION

In summary, we propose a new model for the intracellular trafficking of the TRHR and TRH (Figure 8). Ligand binding induces the sequestration of the TRHR–TRH complex. The RL

Figure 8 A new working model for TRHR and TRH trafficking

TRH binding to the plasma-membrane TRHR induces internalization of the RL complex, which becomes dissociated in an intracellular compartment of unknown identity (A) by a mechanism dependent on acidic pH. The dissociated receptor and ligand are sorted into a recycling pathway (B) and a non-cycling pathway (C). The non-cycling pathway depletes the recycling pathway of TRHR faster than TRH. Newly internalized TRH enters the recycling pool through ligand-induced resequestration of the recycling receptors (D). More quantitative studies using antibodies to the TRHR will be necessary to elucidate these processes fully. Antibodies to the TRHR are not yet available.

complex is internalized into an intracellular compartment (the endosome is a likely candidate) where it dissociates by a mechanism that is dependent on acidic pH. The dissociated TRHR and TRH are subsequently sorted into recycling and non-cycling pathways. The non-cycling pathway depletes the recycling pool of receptor more rapidly than it accumulates ligand. The slow ligand-non-cycling pathway together with the rapid ligand-reinternalization pathway result in sustaining a constant amount of ligand in the recycling pool, while the amount of recycling TRHRs decreases. Antibodies to the TRHR will be necessary to verify our model and to elucidate the biochemical mechanisms involved in TRHR recycling. TRHR recycling may be an important resensitization mechanism, similar to that implicated for the β -2-adrenergic receptor [33], because a decrease in TRHR number correlates well with the functional attenuation of TRH-induced prolactin release [34].

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