Desensitization of Thyrotropin-releasing Hormone Receptor-mediated Responses Involves Multiple Steps*

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Desensitization and recovery of the inositol 1,4,5-trisphosphate (IP₃) and intracellular free calcium concentration ([Ca²⁺]i) responses to thyrotropin-releasing hormone (TRH) were measured in HEK293 cells stably expressing the G protein-coupled TRH receptor. TRH caused a large, rapid, and transient increase in IP₃ and a biphasic increase in [Ca²⁺]i. Desensitization of the TRH response was measured by exposing cells to TRH, washing, and then incubating the cells in hormone-free medium before reintroducing TRH and measuring IP₃ and [Ca²⁺]i, and intracellular Ca²⁺ pool size. When cells were incubated with 1 μM TRH for 10 s or 10 min and reexposed to TRH, there was almost no IP₃ or [Ca²⁺]i increase. The IP₃ response recovered first, followed by the [Ca²⁺]i response. The ionomycin-releasable intracellular Ca²⁺ pool was almost completely depleted by TRH, and pool refilling was slow. Thrombin, endothelin, and carbachol, when combined, stimulated large increases in IP₃ and [Ca²⁺]i, but did not block the IP₃ or [Ca²⁺]i responses to TRH measured 10 min later. In contrast, cells exposed to TRH first responded to combined agonists with a nearly normal increase in IP₃, but no rise in [Ca²⁺]i. Thus, the IP₃ response to TRH displays homologous desensitization, whereas the [Ca²⁺]i response displays heterologous desensitization because depletion of intracellular Ca²⁺ pools prevents responses to other hormones.

The Ca²⁺-mobilizing pathway for G protein-coupled receptors involves multiple steps (1). Agonist binding causes the activation of G protein, resulting in the stimulation of phospholipase Cβ and increased hydrolysis of phosphatidylinositol (4,5)bisphosphate, producing inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol. IP₃ binds to its receptor, a Ca²⁺ channel on the endoplasmic reticulum membrane, releasing Ca²⁺ from the lumen of the endoplasmic reticulum and increasing the intracellular free Ca²⁺ concentration ([Ca²⁺]i).

Thyrotropin-releasing hormone (TRH) acts on receptors in lactotrophs and thyrotrophs of the anterior pituitary that are coupled to Gq/11 to cause a biphasic increase in [Ca²⁺]i (2). The initial transient [Ca²⁺]i spike, which is primarily due to release of intracellular Ca²⁺, is followed by a sustained [Ca²⁺]i elevation that results from increased influx through L-type Ca²⁺ channels and capacitative Ca²⁺ influx. The initial [Ca²⁺]i spike is terminated as IP₃ concentrations decline, intracellular Ca²⁺ stores become exhausted, and cytoplasmic Ca²⁺ is resquestered and pumped from the cell.

Desensitization is defined as a decline in the response to an agonist over time or a decline in the cell to a subsequent agonist exposure. There are conflicting reports about whether the TRH response undergoes either form of desensitization in pituitary cells or following expression of the receptor in other cell types (3–9). The contradictory findings may be a consequence of differences in the methods used to assess the TRH response. In some cases, IP₃ mass has been measured at intervals after TRH has been given (5, 8, 9), whereas in others (6, 7), the rate of total inositol phosphate accumulation has been measured at different times after TRH has been administered to metabolically labeled cells. In other reports (2–4, 10, 11), only the [Ca²⁺]i response has been followed. There is little information about the IP₃ response to repetitive applications of TRH, although it is well documented that the [Ca²⁺]i response to high doses of TRH requires 5–20 min to recover (2). It is unclear whether the refractory period results from desensitization of the receptor or exhaustion of calcium pools.

In this study, we have carried out a detailed analysis of desensitization of the TRH response. To establish the molecular basis for desensitization, we have monitored IP₃ mass, [Ca²⁺]i, the size of the intracellular Ca²⁺ pool, and the responsiveness to other Ca²⁺-mobilizing hormones in the continued presence of TRH and following the withdrawal and re-administration of TRH. We show that the TRH response undergoes profound desensitization over time and that the receptor is desensitized and unable to stimulate IP₃ production after TRH is withdrawn. The rate-limiting step in the recovery of the [Ca²⁺]i response to TRH is refilling of the intracellular Ca²⁺ pools.

EXPERIMENTAL PROCEDURES

Materials—Hanks’ balanced salt solution (HBSS) was purchased from Life Technologies, Inc. Thrombin, carbachol, endothelin, trichlorehane, and tricystamine were purchased from Sigma. [H]TRH, [H]N-3,4-methyl-His²³TRH, and kits for the detection of IP₃ were from NEN Life Science Products. Fura-2/AM and BAPTA were from Molecular Probes, Inc. (Eugene, OR). TRH and ionomycin from Calbiochem, cyclosporin from Sandoz Pharmaceuticals (East Hanover, NJ), and (N³methyl-His³)TRH from Bachem (Philadelphia, PA).

Cell Culture—A HEK293 cell line stably expressing the wild-type mouse TRH receptor (301 cells) has been described previously (12). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum as monolayer cultures at 37 °C in a humidified 95% air and 5% CO₂ environment.

Single Cell Ca²⁺ Imaging—Ca²⁺ imaging was carried out essentially

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The abbreviations used are: IP₃, inositol 1,4,5-trisphosphate; [Ca²⁺]i, intracellular free Ca²⁺ concentration; TRH, thyrotropin-releasing hormone; HBSS, Hanks’ balanced salt solution; BAPTA, 1,2-bis[2-aminophenoxy]ethane-N,N,N′,N′-tetraacetic acid.

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as described by Nelson and Hinkle (13). All Ca\textsuperscript{2+} measurements were performed on cells in HBSS buffered to pH 7.4 with 15 mM HEPES. Cells plated on coverslips were loaded with 4 \mu M Fura-2/AM, 0.1% bovine serum albumin, and 1 \mu g/ml cyclosporin A in HBSS at room temperature for 50–60 min. The coverslip was washed and put into a Sykes-Moore chamber on a Nikon inverted microscope on a heated stage at 37°C. The chamber was perfused with medium at 37°C, and 340/380 nm fluorescence ratios were acquired every 1200 ms. The length of [Ca\textsuperscript{2+}] imaging experiments was limited by gradual leaking of the Fura-2; similar difficulties were encountered with Fura-2/PE (data not shown).

**Ca\textsuperscript{2+} Pool Size Determination—**To estimate the size of the intracellular Ca\textsuperscript{2+} pool, 1.5 mM BAPTA was added to the medium to chelate extracellular Ca\textsuperscript{2+}. 30–60 s before 500 nM ionomycin was added. The increase in [Ca\textsuperscript{2+}] stimulated by ionomycin was used as the measure of pool size. Ionomycin (500 nM) completely eliminated the TRH-induced increase in [Ca\textsuperscript{2+}], measured 2.5 min later, indicating that the IP\textsubscript{3}-releasable pool was completely empty. Conversely, pretreatment of cells with 1 \mu M TRH for 10 min almost eliminated subsequent ionomycin-induced Ca\textsuperscript{2+} release. Ionomycin did not increase [Ca\textsuperscript{2+}], in cells that had been pretreated with thapsigargin, whether extracellular Ca\textsuperscript{2+} was present or not, indicating that extracellular Ca\textsuperscript{2+} entry was minimal.

**Radioreceptor Assay of IP\textsubscript{3}—**Cells plated in 35-mm dishes were rinsed twice with HBSS and incubated at 37°C with or without hormone as described below. At the end of the treatment, the medium was aspirated; 0.8 ml of 20% ice-cold trichloroacetic acid was added; and the dish was put on ice immediately. Cells were scraped off the dish, transferred to an Eppendorf tube, and pelleted at 12,000 \texttimes g for 50–60 min. The coverslip was washed and put into a Sykes-Moore chamber on a Nikon inverted microscope on a heated stage at 37°C. The chamber was perfused with medium at 37°C, and 340/380 nm fluorescence ratios were acquired every 1200 ms. The length of [Ca\textsuperscript{2+}] imaging experiments was limited by gradual leaking of the Fura-2; similar difficulties were encountered with Fura-2/PE (data not shown).

**TRH Receptor Desensitization—**The 301 cell line expresses \sim 200,000 TRH receptors/cell, with an apparent \( K_D \) of 10 nM (12). [Ca\textsuperscript{2+}], was monitored in single 301 cells loaded with Fura-2. TRH stimulation produced a biphasic increase in [Ca\textsuperscript{2+}] (Fig. 1), with an early transient peak and a later maintained phase. The initial [Ca\textsuperscript{2+}] spike was abolished by thapsigargin treatment, indicating that the Ca\textsuperscript{2+} came from an intracellular pool, and the sustained [Ca\textsuperscript{2+}] increase quickly subsided after the addition of the extracellular Ca\textsuperscript{2+} chelator BAPTA, indicating that it depended on the influx of extracellular Ca\textsuperscript{2+}. The amplitude of the [Ca\textsuperscript{2+}] spike depended on TRH concentration, reaching an apparent maximum at 1 nM (Fig. 1 and Table I). The [Ca\textsuperscript{2+}] response occurred earlier and more synchronously, and the upstroke of the response was steeper at higher doses of TRH (Table I).

The intracellular concentration of IP\textsubscript{3} was measured in 301 cells at different times after the addition of TRH. The TRH-stimulated increase in IP\textsubscript{3} was also biphasic and strongly concentration-dependent (Fig. 2). The peak concentration of IP\textsubscript{3} occurred within 10 s at TRH concentrations of 10 nM or higher, and IP\textsubscript{3} increased \sim 15-fold at 1 \mu M TRH. In contrast to the [Ca\textsuperscript{2+}] response, the IP\textsubscript{3} response increased with TRH concentrations between 10 and 1000 nM, the highest dose tested. The IP\textsubscript{3} concentration dropped rapidly within 1 min of TRH addition, but remained at more than twice the basal level for at least 10 min. At 1 nM, TRH caused no more than a 70% increase in the overall IP\textsubscript{3} concentration at times from 2 s to 10 min, and this increase was not highly significant (\( p \geq 0.07 \)), even though the peak [Ca\textsuperscript{2+}] response was essentially maximal under these conditions. The concentration dependence of the [Ca\textsuperscript{2+}] and IP\textsubscript{3} responses is shown in Fig. 3.

**Desensitization and Recovery of the IP\textsubscript{3} Response to TRH—**Desensitization of the TRH response was measured by exposing cells to TRH, washing to remove free hormone, and then

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**TABLE I**

Concentration dependence of TRH-mediated [Ca\textsuperscript{2+}], responses

| TRH Concentration (nM) | Peak Increase in [Ca\textsuperscript{2+}] (340/380 nm ratio) | Lag (sec) | Rate of Increase (sec\textsuperscript{-1}) |
|------------------------|---------------------------------------------------------------|----------|-----------------------------------------|
| 0.1 nM                 | 1.92 ± 0.13                                                   | 10.4 nM  | 0.41 ± 0.2                                 |
| 1 nM                   | 5.39 ± 0.54                                                   | 5.5 nM   | 0.13 ± 0.7                                 |
| 10 nM                  | 4.13 ± 0.32                                                   | 1.9 nM   | 0.06 ± 0.2                                 |
| 1000 nM                | 5.02 ± 0.37                                                   | —        | —                                         |

\( ^{a} \) Responses complete within time resolution of the system.

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**FIG. 1** Typical single cell [Ca\textsuperscript{2+}], responses of 301 cells to TRH. Fura-2-loaded cells were bathed in HBSS at 37°C, and 340/380 nm fluorescence ratios were recorded every 1200 ms. The following additions were made at the times shown: 1 nM TRH (a), 1 \mu M TRH and then 1.5 mM BAPTA (b), and 1 \mu M thapsigargin (Tgn) and then 1 \mu M TRH (c).

**FIG. 2** IP\textsubscript{3} responses to TRH. Dishes of 301 cells were incubated in HBSS at 37°C with 1 nM (L), 10 nM (O), 100 nM (C), or 1 \mu M (triangle) TRH for the indicated times, and IP\textsubscript{3} was measured by radioreceptor assay. Points show the average of duplicate values; although errors are not shown for clarity, errors averaged 10%.
incubating the cells in hormone-free medium for various times before reintroducing TRH and measuring IP₃, [Ca²⁺], and the intracellular Ca²⁺ pool size. Cells were first incubated with 1 μM TRH for 10 s or 10 min or with 1 nM TRH for 10 min. The amplitude of the initial [Ca²⁺]i spike was the same in all three protocols, but the ability of the cell to respond to a subsequent challenge with TRH depended on both the dose and the duration of the first exposure. When cells were incubated with 1 μM TRH for either 10 s or 10 min, washed, and immediately challenged again with TRH, there was almost no IP₃ response (Fig. 4). Cells gradually recovered the ability to respond to TRH and gave a full IP₃ response by 40 min. The t₀½ for recovery was ~5 min after an initial 10-s incubation with TRH versus ~10 min after an initial 10-min incubation. Since the Kd for TRH is 10 nM, 1 nM TRH occupies only ~10% of receptors at equilibrium. Nonetheless, incubation with 1 nM TRH for 10 min (Fig. 4) significantly desensitized the IP₃ response to a subsequent challenge with 1 μM TRH. Immediately after withdrawal of 1 nM TRH, 1 μM TRH increased IP₃ to only 63% of the IP₃ level reached in naive cells.

**Dissociation of TRH from the TRH Receptor**—The ability of the cell to respond to a second challenge with TRH may be limited by how fast bound TRH dissociates from receptors following the first exposure to agonist. To measure ligand dissociation rates, cells were incubated with 1 μM [³H]TRH for either 10 s or 10 min and washed, and specifically bound [³H]TRH was followed over time (Fig. 5, left panel). The amount of [³H]TRH bound was the same after 10 s or 10 min of incubation, indicating that receptors were essentially saturated in both protocols. However, [³H]TRH dissociated faster from receptors after the 10-s incubation than after 10 min. An additional assay was used to measure the number of unoccupied receptors on the cell surface at various times after cells had been incubated with TRH and then washed (Fig. 5, right panel). Again, TRH receptors became available more rapidly when the initial incubation was brief. These data agree with previous findings in pituitary cells (14) and reflect the fact that the TRH-receptor complex internalizes extensively in 10 min in 301 cells.

**Desensitization and Recovery of the TRH Receptor-mediated Increase in [Ca²⁺]i**—Incubation of 301 cells with 1 μM TRH for 10 min completely abolished the [Ca²⁺]i response to a second exposure to 1 μM TRH (Fig. 6). The [Ca²⁺]i response recovered partially after 5 min, but remained at only 25% of the control response at 25 min, even though the IP₃ response was almost fully recovered at this point. When cells were initially exposed to 1 μM TRH for 10 s, the ability of the cells to mount an [Ca²⁺]i response to 1 μM TRH was half of the control 2 min after washing and nearly 100% after 10 min (Fig. 6). Preincubation with 1 nM TRH for 10 min also prevented an [Ca²⁺]i response to 1 μM TRH immediately after washing (Fig. 6), even though the IP₃ response was quite large. The [Ca²⁺]i response recovered to ~70% of the control response by 10 min.

**Emptying and Refilling of Intracellular Ca²⁺ Pools**—The size of the intracellular Ca²⁺ pool was estimated by adding BAPTA to chelate extracellular Ca²⁺ and then adding 500 nM ionomycin to dump intracellular Ca²⁺ stores. The intracellular Ca²⁺ pool was almost completely depleted by 1 μM TRH within 1 min (Figs. 7 and 8). The pool was also fully depleted by 1 nM TRH, but in this case, only after 10 min (Fig. 7). Ca²⁺ pools refilled slowly, such that they were just 25% replenished after 10–25 min in cells that had been exposed to TRH for 10 min (Fig. 8). Refilling was faster and more complete in cells that had been exposed to 1 μM TRH for only 10 s (Fig. 8). Interestingly, the [Ca²⁺]i response to TRH recovered before Ca²⁺ pools had refilled, indicating that partially full stores were adequate for a maximal response.

Because dye leakage became a problem in long experiments, we also measured Ca²⁺ pool sizes after loading with Fura-2/AM during the refilling period. In this protocol, Ca²⁺ pools seemed to recover fully within 1 h after cells had been incubated with 1 μM TRH for 10 min and washed. Ca²⁺ pool sizes, determined as the increase in 340/380 nm fluorescence ratios caused by

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**Fig. 3.** Concentration dependence of TRH-mediated increases in [Ca²⁺]i and IP₃ in 301 cells. The peak increases in [Ca²⁺]i and IP₃ measured in experiments like those shown in Figs. 1 and 2, are shown. [Ca²⁺]i values represent the means ± S.E. of 16–20 cells from a single experiment and are representative of the results of 2–10 experiments. Errors in the IP₃ measurements were within symbol size.

**Fig. 4.** Desensitization and recovery of IP₃ responses to TRH in 301 cells. To determine the maximal TRH response of naive cells in each experiment, control dishes were collected for the measurement of the basal IP₃, [Ca²⁺]i or peak TRH-stimulated IP₃, level, which was measured 10 s after the addition of 1 μM TRH. To determine the IP₃ response of cells that had previously been exposed to TRH, cells were first incubated with 1 μM TRH for 10 min, 1 nM TRH for 10 min, or 1 μM TRH for 10 s. Dishes were then washed and incubated in medium without hormone for 0–90 min, when the responses to a second TRH challenge were measured by incubating cells with either no hormone (□) or 1 μM TRH (○) for 10 s and quantitating IP₃. Values shown are the means ± range of duplicate dishes. Where not visible, errors fell within symbol size.

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R. Yu and P. M. Hinkle, unpublished observations.
ionomycin, were 2.6 ± 0.2 (n = 26) in control cells and 2.8 ± 0.2 (n = 56) and 3.2 ± 0.7 (n = 24) 1 and 2 h after removal of TRH, respectively.

**Heterologous Effects on [Ca^{2+}]_{i} and IP_{3} Responses**—The results described above imply that following TRH treatment, 301 cells should not be able to increase [Ca^{2+}]_{i} in response to any other agonist until the Ca^{2+} stores are replenished. Thrombin, endothelin, and carbachol all increase [Ca^{2+}]_{i} in 301 cells, although none of these agonists gives a response as large as TRH (data not shown). When combined into an agonist mixture, thrombin, endothelin, and carbachol stimulated large increases in IP_{3} and [Ca^{2+}]_{i} (Fig. 9 and Table II). The IP_{3} response to the combined agonists occurred as quickly as the IP_{3} response to TRH, but the peak was lower (7.5-fold versus 15-fold), and the IP_{3} level fell to baseline in 40 s, whereas it remained elevated for at least 10 min in the continued presence of TRH (Fig. 2 and Table II). The peak increase in [Ca^{2+}]_{i}, stimulated by the mixture was indistinguishable from that stimulated by TRH (Fig. 9 and Table II).

As predicted, when cells were first exposed to 1 μM TRH for 10 min and then to the mixture, the combined agonists did not increase [Ca^{2+}]_{i}, substantially, although they caused an IP_{3} increase that was close to that in untreated cells (Fig. 9 and Table II). These results support the idea that Ca^{2+} pool depletion, not impaired IP_{3} generation, prevents [Ca^{2+}]_{i} responses to other hormones. The converse was not true. When cells were preincubated with the mixture for 10 min, they could still respond to TRH with a normal elevation of [Ca^{2+}]_{i}, even though the Ca^{2+} pool was substantially reduced by the agonist mixture (Fig. 9 and Table II). These results again suggest that a full [Ca^{2+}]_{i} response requires only a partly full intracellular Ca^{2+} pool. The IP_{3} response to TRH, administered after the combined agonists, was close to that of control cells (Table II).

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**Fig. 5. Kinetics of TRH dissociation and receptor recycling.** Left panel, 301 cells were incubated with 980 nM TRH plus 20 nM [3H]TRH at 37 °C for either 10 s (○) or 10 min (□). Cells were then washed and incubated in TRH-free buffer for 0–90 min. Cell-associated radioactivity was measured and compared with the value at 0 min. Right panel, 301 cells were incubated with 1 μM unlabeled TRH at 37 °C for 10 s (○) or 10 min (□). Cells were then washed and incubated in TRH-free buffer for 0–90 min. At the end of this period, cells were incubated with 980 nM TRH and 20 nM [3H]TRH at 0 °C for 60 min to measure surface receptors. Cell-associated radioactivity was measured and compared with the value of cells that had not been treated with TRH. Values shown are the means ± range of duplicate dishes in one of two determinations.

**Fig. 6. Desensitization and recovery of the [Ca^{2+}]_{i} response to TRH in 301 cells.** Fura-2-loaded cells were incubated at 37 °C as follows: with 1 μM TRH for 10 min, with 1 nM TRH for 10 min, or with 1 μM TRH for 10 s. [Ca^{2+}]_{i} was recorded before (□) and immediately after (○) TRH addition. At intervals from 0 to 25 min, [Ca^{2+}]_{i} was measured before (□) and after (○) the addition of 1 μM TRH. Values shown are the means ± S.E. of 16–20 cells in one experiment and are representative of multiple experiments; TRH responses are the peak values. Where not visible, errors fell within symbol size. The earliest time when it was possible to measure the [Ca^{2+}]_{i} response to re-addition of TRH was limited by the time required to wash out either 1 nM or 1 μM TRH.

**Fig. 7. Depletion of intracellular Ca^{2+} pools by TRH.** 301 cells were loaded with Fura-2 and treated with 1 nM (○) or 1 μM (□) TRH for 0–10 min. The intracellular Ca^{2+} pool size was quantified as described under "Experimental Procedures" and is expressed as a percentage of the value in control untreated cells. Values are the means ± S.E. of 30 cells in one of two similar experiments.
DISCUSSION

In this report, we have demonstrated that the TRH response undergoes profound desensitization. Restoration of the [Ca\(^{2+}\)]\(_i\) response to TRH involves multiple steps, including ligand dissociation, recovery of receptor 1G protein 1 phospholipase C coupling, and intracellular Ca\(^{2+}\) pool replenishment. The characteristics of the IP\(_3\) response measured in 301 cells expressing the TRH receptor closely resemble those reported previously for pituitary cells (3, 16, 17), and the activity of phospholipase C has been reported to increase with TRH doses up to 1–10 \(\mu M\) in isolated membranes (18). Rapid kinetic studies have shown that the [Ca\(^{2+}\)]\(_i\) peak precedes the IP\(_3\) peak (19). These findings all indicate that a very small increase in the average IP\(_3\) concentration is sufficient to trigger a large increase in [Ca\(^{2+}\)]\(_i\). There are a number of possible explanations. Single cell Ca\(^{2+}\) imaging has shown that there is a highly variable delay between the addition of a low dose of TRH and the onset of the [Ca\(^{2+}\)]\(_i\) rise (Table I) (2, 10). If there is a similar asynchrony in the TRH-mediated increase in IP\(_3\), then the average IP\(_3\) value will seriously underestimate the amplitude of a short-lived increase in IP\(_3\) in individual cells. High concentrations of TRH stimulate a rapid and highly synchronous increase in [Ca\(^{2+}\)]\(_i\), (Table I) (2, 13). Imaging studies have provided strong evidence for spatial heterogeneity in intracellular Ca\(^{2+}\) release (20), and biochemical evidence supports the existence of heterogeneous Ca\(^{2+}\) stores in pituitary cells responsive to TRH (21). The small amount of IP\(_3\) generated by low concentrations of TRH may release Ca\(^{2+}\) from stores near the plasma membrane, and the resultant increase in [Ca\(^{2+}\)]\(_i\) may sensitize IP\(_3\) receptors (1, 20) or otherwise contribute to...
Ca\(^{2+}\) release as it spreads through the cell.

At the other extreme of the dose-response relationship, very high concentrations of TRH produced much higher levels of IP\(_3\) than necessary for a maximal [Ca\(^{2+}\)] spike. Very high levels of IP\(_3\) are likely to alter the kinetics of pool refilling, even though they do not increase the size of the initial [Ca\(^{2+}\)] spike. In addition, the peak Ca\(^{2+}\) concentration in the vicinity of the secretory granules may increase as the agonist dose is raised, even though the average Ca\(^{2+}\) concentration reported by fluorescent indicators does not.

Desensitization is defined as a diminishing response in the continued presence of an agonist or a diminished response to a subsequent exposure to an agonist. In this study, we documented both of these forms of desensitization. In the continued presence of TRH, IP\(_3\) increased 15-fold and then fell rapidly to a plateau 1.5–3 times the basal level. This IP\(_3\) response is typical of many receptors coupled to G\(_q\) (22–25). Gershengorn and co-workers found that the rate of total inositol phosphate production, measured over 30 min, decreases with time of exposure to TRH in pituitary cells (7) and in several other cell types (6), but not in HEK293 cells (6). The reason for the discrepancy is not known, but it may be because the transient IP\(_3\) response to TRH was obscured in measurements done over a 30-min period or because the density of receptors was much greater when they were introduced by adenovirus-mediated gene transfer (6) rather than stable transfection, as in our work.

In principle, IP\(_3\) concentrations could rise and then fall for several reasons. 1) Metabolism of IP\(_3\) may be accelerated. 2) Substrate (phosphatidylinositol (4,5)bisphosphate) may be depleted. 3) Downstream kinases may turn off phospholipase C. 4) The receptor/G protein/phospholipase signal pathway may become uncoupled. Since TRH causes a biphasic increase in diacylglycerol as well as IP\(_3\) and a burst followed by a gradual increase in total inositol phosphates (16), the activity of phospholipase C must change over time, not the metabolism of IP\(_3\). The concentration of phosphatidylinositol (4,5)bisphosphate declines after TRH is added, but quickly recovers (26), making substrate exhaustion unlikely. Downstream kinases do not turn off phospholipase C generally because TRH caused little reduction in the IP\(_3\) response to other G\(_q\)-coupled receptors. Activation of protein kinase C with phorbol esters could not increase [Ca\(^{2+}\)] because the intracellular Ca\(^{2+}\) pool eventually recovered fully. The consequence of depleted Ca\(^{2+}\) pools was heterologous desensitization of the [Ca\(^{2+}\)] response. Although other agonists were able to evoke a nearly normal increase in IP\(_3\) after TRH, they could not increase [Ca\(^{2+}\)] because the intracellular Ca\(^{2+}\) pool was empty.

Hormone dissociation is a necessary first step before a previously occupied receptor can be activated again. Ligand dissociation is not likely to be the only factor in desensitization of the IP\(_3\) response, however. There was a sizable plasma membrane receptor pool available for activation shortly after a 10-s exposure to 1 \(\mu\)M TRH, but TRH could not stimulate phospholipase C activity effectively. A cell-surface receptor pool of similar size was present 25 min after exposure to 1 \(\mu\)M TRH for 10 min, and in this case, TRH could generate a nearly maximal IP\(_3\) response.

Desensitization of the [Ca\(^{2+}\)] response to agonists has been documented in many other studies (22–25). Anderson et al. (4) expressed rat and human TRH receptors in HEK293 cells and found that the [Ca\(^{2+}\)] response mediated by the receptors was desensitized by high concentrations of TRH and did not recover at all, even in the face of normal Ca\(^{2+}\) pool refilling. The difference between previous results and ours may be due to the much higher concentration of TRH receptors in the transfected HEK293 cells used by Anderson et al. (4). In GH-cells and primary pituitary cell cultures, pool refilling does not seem to occur until TRH is withdrawn and then requires as long as 20 min (2).

Our study highlights the importance of regulation of the Ca\(^{2+}\) pool as a means of desensitizing receptor-mediated [Ca\(^{2+}\)] responses. Pool replenishment was the rate-limiting step in recovery of the TRH response in 301 cells. The other agonists tested here did not deplete Ca\(^{2+}\) pools as thoroughly as TRH, even though the IP\(_3\) responses were large. One of the reasons that TRH depletes the Ca\(^{2+}\) pool so thoroughly is that it stimulates efflux of Ca\(^{2+}\) from the cytoplasm, apparently by

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**Fig. 10. Recovery of the TRH response in 301 cells.** Changes in IP\(_3\), [Ca\(^{2+}\)], and the intracellular Ca\(^{2+}\) pool are shown schematically. The dotted line depicts the initial response to TRH. Arrows denote responses to single applications of TRH given at 10, 20, 30, or 40 min after washing out the TRH. The experiment could not be extended unless cells were loaded with Fura-2 during the recovery period. When this was done, Ca\(^{2+}\) pools were found to refill in ~1 h.
activating a plasma membrane Ca\(^{2+}\) pump (29). Nonetheless, all methods used to quantitate Ca\(^{2+}\) pool sizes are indirect. If TRH treatment changes cytoplasmic Ca\(^{2+}\) buffering capacity or Ca\(^{2+}\) reuptake, then the increase in [Ca\(^{2+}\)]\(_i\), caused by ionomycin, used here to estimate pool size, might be altered (30), as might any localized release of Ca\(^{2+}\) from specialized domains on the endoplasmic reticulum membrane (31, 32).

In summary, we have shown that the signal transduction pathway to TRH becomes profoundly desensitized at multiple levels. In 301 cells, the IP\(_3\) response to TRH undergoes homologous desensitization, but the [Ca\(^{2+}\)]\(_i\) response undergoes heterologous desensitization because the TRH-treated cell cannot respond to any Ca\(^{2+}\)-mobilizing agonist until pool refilling has occurred. Additional study is needed to identify the molecular mechanisms responsible for these complex levels of desensitization.

REFERENCES
1. Berridge, M. J. (1993) Nature 361, 315–325
2. Hinkle, P. M., Nelson, E. J., and Ashworth, R. (1996) Trends Endocrinol. Metab. 7, 370–374
3. Gershengorn, M. C. (1986) Annu. Rev. Physiol. 48, 515–526
4. Anderson, L., Alexander, C. L., Facenda, E., and Edube, K. A. (1995) Biochem. J. 311, 385–392
5. Drummond, A. H., Bushfield, M., and Macphee, C. H. (1994) Mol. Pharmacol. 25, 201–208
6. Falk-Pedersen, E., Heinflink, M., Alvia, M., Nussenzeig, D. R., and Gershengorn, M. C. (1994) Mol. Pharmacol. 45, 684–690
7. Perlman, J. H., and Gershengorn, M. C. (1991) Endocrinology 129, 2679–2686
8. Torjesen, P. A., Bjoro, T., Ostberg, B. C., and Haug, E. (1988) Mol. Cell. Endocrinol. 56, 107–114
9. Imai, A., and Gershengorn, M. C. (1985) J. Biol. Chem. 260, 10536–10540
10. Akerman, S. N., Zorec, R., Cheek, T. R., Moreton, R. B., Berridge, M. J., and Mason, W. T. (1991) Endocrinology 129, 475–488
11. Villalobos, C., and Garcia-Sancho, J. (1995) Pflugers Arch. Eur. J. Physiol. 430, 925–935
12. Shupnik, M. A., Week, J., and Hinkle, P. M. (1996) Mol. Endocrinol. 10, 90–99
13. Nelson, E. J., and Hinkle, P. M. (1994) Endocrinology 135, 1084–1092
14. Hinkle, P. M., and Kinsella, P. A. (1982) J. Biol. Chem. 257, 5462–5470
15. Ramsdell, J. S., and Tashjian, A. H., Jr. (1986) J. Biol. Chem. 261, 5301–5306
16. Drummond, A. H. (1986) J. Exp. Biol. 124, 337–358
17. Gershengorn, M. C., Heinflink, M., Nussenzeig, D. R., Hinkle, P. M., and Falk-Pedersen, E. (1994) J. Biol. Chem. 269, 6779–6783
18. Martin, T. F., Lucas, D. O., Bajjalieh, S. M., and Kowalchyk, J. A. (1986) J. Biol. Chem. 261, 2918–2927
19. Tashjian, A. H., Jr., Heslop, J. P., and Berridge, M. J. (1987) Biochem. J. 243, 305–308
20. Berridge, M. J., and Dupont, G. (1994) Curr. Opin. Cell Biol. 6, 267–274
21. Koshibayama, H., and Tashjian, A. H., Jr. (1991) Biochem. Biophys. Res. Commun. 177, 551–558
22. Bohn, S. K., Grady, E. F., and Bunnett, N. W. (1997) Biochem. J. 322, 1–18
23. Fisher, S. K. (1995) Eur. J. Pharmacol. 288, 231–250
24. Lehue, M. J. (1993) Biochem. Biophys. Acta 1179, 171–188
25. Wojcikiewicz, R. J., Tobin, A. B., and Nahorski, S. R. (1993) Trends Pharmacol. Sci. 14, 279–285
26. Rebecchi, M. J., and Gershengorn, M. C. (1983) Biochim. Biophys. Acta 716, 287–294
27. Berstein, G., Blank, J. L., Jhon, D. Y., Exton, J. H., Rice, S. G., and Ross, E. M. (1992) Cell 70, 411–418
28. Freedman, N. J., and Lefkowitz, R. J. (1996) Recent Prog. Horm. Res. 51, 319–351
29. Nelson, E. J., and Hinkle, P. M. (1994) J. Biol. Chem. 269, 30854–30860
30. Wagner, K. A., Yacono, P. W., Golan, D. E., and Tashjian, A. H., Jr. (1993) Biochem. J. 292, 175–182
31. Bootman, M. D., and Berridge, M. J. (1995) Cell 83, 675–678
32. Parker, I., and Yao, Y. (1995) Ciba Found. Symp. 188, 50–60