# Rapid desensitization of the thyrotropin-releasing hormone receptor expressed in single human embryonal kidney 293 cells

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This study uses fluorescence microscopy combined with dynamic video imaging to examine the events associated with the rapid desensitization of the thyrotropin-releasing hormone receptor (TRH-R). In single non-pituitary human embryonic kidney 293 (HEK-293) cells, expressing either the rat or human TRH-Rs, TRH produced a rapid dose-dependent monophasic rise in $[Ca^{2+}]_i$. This $Ca^{2+}$ transient was completely abolished by pre-treatment of cells with the intracellular $Ca^{2+}$ antagonists thapsigargin or cyclopiazonic acid, but not EGTA, the voltage-operated $Ca^{2+}$ channel (VOCC) antagonist nifedipine or the second-messenger-operated $Ca^{2+}$ channel antagonist SK&F 96365. These results suggest that TRH causes the mobilization of $Ca^{2+}$ from thapsigargin/cyclopiazonic acid-sensitive intracellular $Ca^{2+}$ stores but not the influx of extracellular $Ca^{2+}$. HEK-293 cells also failed to respond to KCl or the slow $Ca^{2+}$-channel activator BAY K 8644, suggesting that they lack L-type VOCCs. Rat and human TRH-Rs are highly conserved except at the C-terminus where the sequence differs. The C-terminus is believed to be important in receptor desensitization. Despite differences in this region, rat and human TRH-Rs expressed in HEK-293 cells underwent rapid (within 1 min) desensitization. This desensitization was dose-dependent and did not involve receptor loss. Similarly the bradykinin receptor endogenous to HEK-293 cells also displays a rapid desensitization. We conclude that in TRH-R-expressing non-pituitary HEK-293 cells, TRH mobilizes intracellular $Ca^{2+}$ resulting in a monophasic $Ca^{2+}$ transient. The rat and human TRH-Rs as well as the endogenous bradykinin receptor also displayed rapid receptor desensitization.

## INTRODUCTION

Thyrotropin-releasing hormone (TRH) is a hypothalamic peptide which acts on the anterior pituitary gland to induce release of thyrotropin and prolactin. The TRH receptor (TRH-R) is a member of the G-protein-coupled seven-transmembrane-spanning family of receptors (GPCRs) [1–3]. TRH-R activation causes rapid stimulation of the phospholipase C (PLC) pathway via its $G_q/G_{11}$ G-protein, producing Ins(1,4,5)$P_3$ [4,5] and 1,2-diacylglycerol. Ins(1,4,5)$P_3$ causes a transient mobilization of $Ca^{2+}$ from internal [6] and possibly indirectly from external [7] sources, whereas diacylglycerol increases protein phosphorylation by activating protein kinase C [8]. Given that changes in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) are critical in the regulation of a variety of cellular processes, including hormone secretion, it is important to understand the mechanisms controlling these events in different cell types.

TRH-induced changes in $[Ca^{2+}]_i$ have been measured in pituitary and non-pituitary cell types expressing TRH-Rs [9–16]. These spectrofluorimetric studies have contributed to the analysis of TRH-induced changes in $[Ca^{2+}]_i$. Interpretation of these data can, however, be misleading, as these studies were often conducted in heterogeneous primary pituitary cultures and/or suspension cultures of established pituitary cell lines. It is generally believed that TRH causes a biphasic $Ca^{2+}$ response but the exact origins of the $Ca^{2+}$ pools involved are unclear. Indeed it has been suggested that the $Ca^{2+}$ pools mobilized after TRH-R activation are cell-type specific [13]. In pituitary cell types, the initial $Ca^{2+}$ spike predominantly involves the mobilization of Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores and the longer-lasting secondary plateau phase relies on the influx of extracellular $Ca^{2+}$. In contrast, when expressed in cells of non-pituitary origin, TRH induces a monophasic $Ca^{2+}$ response [13,16].

Receptor activation can lead not only to stimulation of intracellular events, for example the mobilization of $Ca^{2+}$, but also to desensitization of these responses. Receptor desensitization is potentially a physiologically important process, as it provides a means of regulating continuous receptor stimulation [17]. Chronic exposure of cells to TRH results in the internalization and subsequent loss of cell-surface TRH-Rs [18–20], a decrease in the levels of TRH-R mRNA [21,22] and a down-regulation of the $G_q$ and $G_{11}$ G-proteins [23]. In contrast, rapid receptor desensitization does not involve receptor loss but rather receptor phosphorylation. This phosphorylation results in an uncoupling of the receptor from its cognate G-protein and a loss of subsequent downstream events. Although rapid desensitization of a number of GPCRs has been demonstrated [6,17,24], it remains unclear whether or not the G-protein-coupled events of the TRH-R can be acutely desensitized, i.e. within the first few minutes of receptor activation.

The present study aims to eliminate the problems of cell heterogeneity by directly monitoring and characterizing TRH-induced changes in $[Ca^{2+}]_i$ in single cells of TRH-R-expressing clonal non-pituitary cell lines. We demonstrate that TRH causes a prompt dose-dependent monophasic rise in $[Ca^{2+}]_i$ in HEK-293 cells expressing TRH-Rs. By using a variety of intra- and extracellular $Ca^{2+}$ antagonists, we have also shown that this response does not involve the influx of extracellular $Ca^{2+}$ through either

Abbreviations used: TRH, thyrotropin-releasing hormone; TRH-R, TRH receptor; BK, bradykinin; BK-R, bradykinin receptor; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; VOCCs, voltage-operated $Ca^{2+}$ channels; SMOCCs, second-messenger-operated $Ca^{2+}$ channels; HEK-293, human embryonal kidney 293; TG, thapsigargin; CPZ cyclopiazonic acid; GPCRs, G-protein-coupled receptors; PLC, phospholipase C; GTP[S], guanosine 5′-[γ-thio]triphosphate; Thi, thienyl(alanine).

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voltage- or second-messenger-operated $Ca^{2+}$ channels (VOCCs or SMOCCs), but rather the mobilization of $Ca^{2+}$ from thapsigargin/cyclopiazonic acid (TG/CPZ)-sensitive intracellular $Ca^{2+}$ stores. The structure of the TRH-R is highly homologous between rat [3], mouse [1] and human [25] except for the stretch of amino acids at the extreme end of the C-terminus. The C-termini of GPCRs are thought to be important in the events associated with receptor desensitization. We therefore compared acute desensitization events in cells expressing either rat or human TRH-Rs. Desensitization of the endogenously expressed bradykinin (BK) receptor (BK-R) was also examined. TRH-Rs from both species as well as the endogenous BK-R exhibited a rapid desensitization of the agonist-induced rises in $[Ca^{2+}]_i$.

## MATERIALS AND METHODS

### Materials

Tissue culture reagents, media, lipofectin and genetecin were supplied by Gibco, Paisley, Scotland, U.K. Glass coverslips (22 mm × 0.175 mm) were supplied by Arnold R. Horwell Ltd., West Hampstead, London, U.K. [3-Me-His$^2$]TRH and guanosine 5′-[γ-[$^{35}$S]thio]triphosphate ([$^{35}$S]GTP[S] 1245 Ci/mmol) were obtained from Dupont-NEN Products, Hertfordshire, U.K. All other drugs, including fura 2 penta-acetoxymethyl ester (fura 2/AM), were obtained from Calbiochem. The Bio-Rad protein assay kit was obtained from Bio-Rad, Richmond, CA, U.S.A.

### Tissue culture

Cell lines were maintained routinely in Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated foetal calf serum, glutamine (0.3 mg/ml), penicillin (100 units/ml), streptomycin (100 units/ml) and geneticin (800 mg/ml) and incubated at 37 °C in a humidified atmosphere of 5% (v/v) $CO_2$ in air.

### Cell lines expressing TRH-Rs

Using a stable transfection protocol [23], the full-length rat [3] or human [25] TRH-R was subcloned into the eukaryotic expression vectors pcDNA1 and pcDNA3 respectively and expressed in HEK-293 cells. Receptor-containing clones were identified using a functional total inositol phosphate assay [26] and verified with a TRH-R-binding assay. The cell lines expanded from these clones containing either the rat (293-E2 cell line) or the human (293-h10 cell line) TRH-Rs were then used for further study.

### Measurement of $[Ca^{2+}]_i$

Trypsin-treated single cells were plated on to sterilized glass coverslips. After 2 days, attached cells were washed (×2) with buffer A [127 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.5 mM $NaH_2PO_4$, 5 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 10 mM Hepes and 0.1% (w/v) BSA, pH 7.2]. Cells were loaded in this buffer with fura 2/AM (4 μM final concentration) for 30 min at 37 °C in a 5% (v/v) $CO_2$ humidified incubator. Unincorporated dye was removed by washing (×3) with buffer A. Coverslips were then transferred to a heated stage (37 °C) of an inverted Nikon Diaphot epifluorescence microscope with a ×40 oil immersion objective. Cells were incubated in a fixed volume of buffer A (1 ml). Drug solutions (5 ml) were added directly into the coverslip chamber and 1 ml volumes automatically obtained through suction.

Dynamic video imaging of changes in $[Ca^{2+}]_i$ was carried out in single cells using the MagiCal hardware and Tardis software provided by Applied Imaging (formerly Joyce Loebl Ltd.), Dukesway, Team Valley, Gateshead, Tyne and Weir, U.K. as previously described [27]. Fluorescent images were obtained by exposing cells to filtered 340 and 380 nm light alternated under computer control. The image viewed at a wavelength of 510 nm was focused on to the face of an intensified charge-coupled device camera (Photonic Sciences). Typically, eight images were averaged at each wavelength and a similar number were collected for background images which were subsequently subtracted on a pixel-by-pixel basis from the imaged samples. These images were held in memory for subsequent processing and analysis.

Fluorescence excitation shifts occur when fura 2 binds $Ca^{2+}$, i.e. the excitation efficiency increases at 340 nm and decreases at 380 nm. Ratios of values obtained at 340/380 nm therefore represent changes in $[Ca^{2+}]_i$. The 340/380 nm ratio was calculated on averaged video frames on a pixel-by-pixel basis, and this was proportional to $[Ca^{2+}]_i$.

During non-imaging periods in desensitization experiments, photobleaching of the fura 2 dye was minimized by inserting a low-percentage neutral-density filter between the light source and the filter wheel.

### Data analysis and presentation

Software-based image analysis allowed quantification of $[Ca^{2+}]_i$ in single cells versus time. ASCII files of these quantitative data were used to derive plots of the mean $[Ca^{2+}]_i$ versus time for single cells from different experiments. A minimum of ten cells was analysed from each experiment, and individual treatment regimes were carried out at least three times. Results are given as means ± S.E.M. Statistical analysis was performed using Student's *t* test.

### TRH-R-binding assay

Monolayer cultures were washed with PBS (×2) and harvested by scraping. The cells were resuspended in 20 mM Tris/HCl/2 mM $MgCl_2$, pH 7.4, and membranes prepared after homogenization and centrifugation at 20000 *g* for 30 min at 4 °C. The membrane pellet was resuspended in buffer B containing 40 mM Tris/HCl, pH 7.4, and 2 mM $MgCl_2$. Ligand-binding assays were carried out with [$^3$H][3-Me-His$^2$]TRH in buffer B (0.5 ml) and various concentrations of unlabelled peptide. After incubation on ice for 1 h, the membranes were filtered through Whatman GF-B filters and washed with buffer B (×3). All assays were performed in triplicate. Binding parameters were determined using Scatchard analysis. Protein concentrations were measured using a Bio-Rad protein assay kit with a BSA standard.

### Binding of [$^{35}$S]GTP[S] to membranes

[$^{35}$S]GTP[S] binding to membranes prepared from 293-E2 cells was measured as previously described [28]. Briefly, reconstituted 293-E2 membranes were incubated in a final assay volume of 250 μl in buffer C [50 mM Tris/HCl, 2 mM EDTA, 10 mM $MgCl_2$, 2 mM dithiothreitol, 200 mM NaCl, 0.4 units/ml adenosine deaminase, 1% (v/v) BSA] containing [$^{35}$S]GTP[S] (0.2 nM) and GDP (10 μM) at 25 °C for 45 min. Samples were then filtered under vacuum through Whatman GF-B filters presoaked with buffer D (50 mM Tris/HCl, pH 7.4/5 mM $MgCl_2$). Filters were washed (×2) with 4 ml of buffer D, digested with formic acid and counted in Emulsifier SAFE scintillation fluid. Non-specific binding was determined in the presence of 10 μM unlabelled GTP[S].

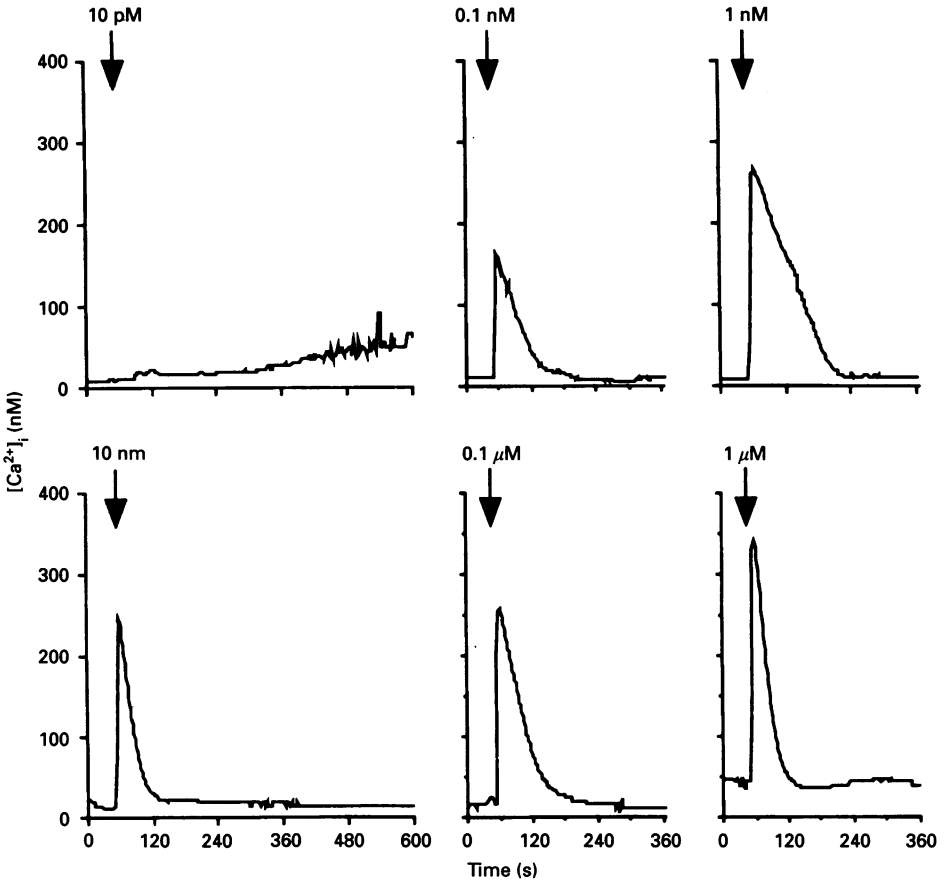


**Figure 1 Effect of various TRH concentrations (10 pM–1 μM) on $[Ca^{2+}]_i$ in 293-E2 cells**

Graphs represent an average plot of $[Ca^{2+}]_i$ measurements versus time (s) in a minimum of ten cells from representative experiments. TRH added at $t = 50$ s produced a monophasic rise in $[Ca^{2+}]_i$. $P < 0.001$, compared with control response ($n = 10$).

## RESULTS

### Effect of TRH on $[Ca^{2+}]_i$ in HEK-293 cells expressing the rat TRH-R (293-E2 cells)

Figure 1 shows examples of averaged traces from single experiments consisting of at least ten individual 293-E2 cells illustrating TRH-induced changes in $[Ca^{2+}]_i$. The application of various concentrations of TRH (10 pM–1 μM) to 293-E2 cells produced dose-dependent changes in $[Ca^{2+}]_i$. TRH produced a rapid transient monophasic increase in $[Ca^{2+}]_i$, and maximal responses were obtained with TRH concentrations of 10 nM and higher. Within 10 s of the application of 1 μM TRH, $[Ca^{2+}]_i$ had risen from $49.4 \pm 2.7$ nM at $t = 50$ s to $348.7 \pm 25.6$ nM at $t = 57$ s. The maximal concentration of 1 μM TRH was used in subsequent experiments. $[Ca^{2+}]_i$ oscillations were also often observed after the exposure of cells to concentrations of TRH ranging from 0.1 nM to 1 μM (Figure 2).

The origin of the $Ca^{2+}$ mobilized by TRH was investigated using a variety of intra- and extra-cellular $Ca^{2+}$ antagonists.

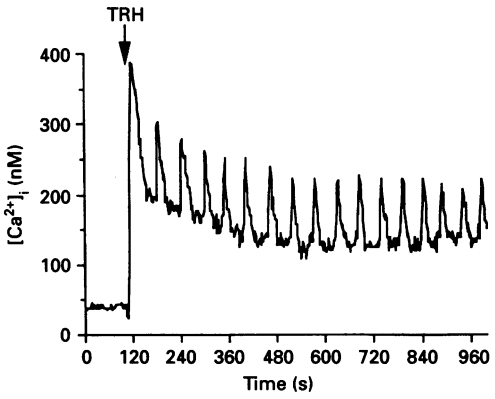


**Figure 2 TRH-induced $[Ca^{2+}]_i$ oscillations in 293-E2 cells**

This trace illustrates a typical example of a single TRH-treated (1 μM; $t = 108$ s) oscillating cell.

### Involvement of extracellular $Ca^{2+}$ in the TRH-induced $[Ca^{2+}]_i$ response in 293-E2 cells

Pretreatment of 293-E2 cells with $Ca^{2+}$-free buffer A containing the $Ca^{2+}$ chelator EGTA (2 mM; 6 min) had no effect on the $[Ca^{2+}]_i$ response to TRH (Figure 3a). Similar treatment of cells with the L-type VOCC blocker, nifedipine (1 μM), did not alter the response to TRH (Figure 3b). After the application of the SMOCC blocker, SK&F 96365 (1 μM), 293-E2 cells responded normally to TRH (Figure 3c). Collectively these data suggest

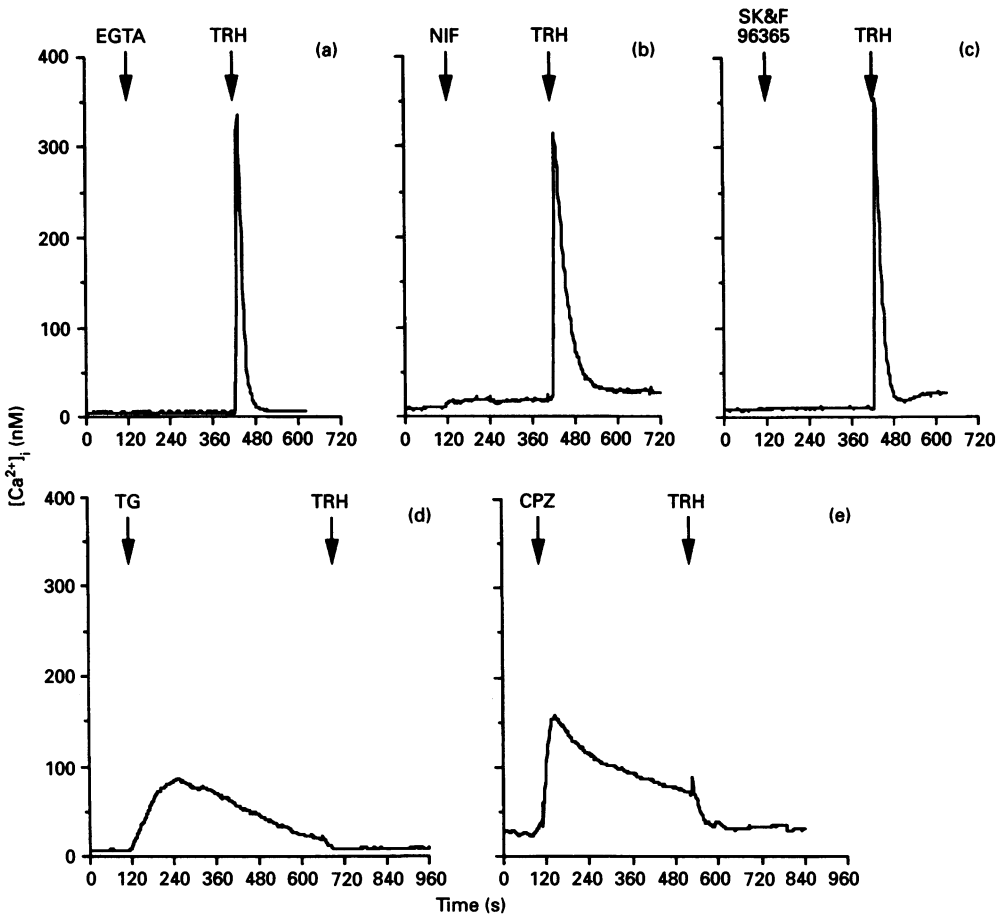


**Figure 3 Effect of (a) EGTA, (b) nifedipine, (c) SK&F 96365, (d) TG and (e) CPZ pretreatment on the TRH-induced $[Ca^{2+}]_i$ response in 293-E2 cells**

(**a**) Cells were preincubated for 6 min in $Ca^{2+}$-free buffer A with 2 mM EGTA, and TRH (1 $\mu$M; $t = 420$ s) was added. (**b**) 293-E2 cells were pretreated with nifedipine (NIF; 1 $\mu$M; $t = 108$ s), and TRH (1 $\mu$M) was subsequently added at $t = 420$ s. (**c**) 293-E2 cells were pretreated with SK&F 96365 (1 $\mu$M; $t = 10$ s), and TRH (1 $\mu$M; $t = 420$ s) was then added. (**d**) 293-E2 cells were treated with TG (1 $\mu$M; $t = 108$ s), and TRH (1 $\mu$M; $t = 688$ s) was then added. (**e**) 293-E2 cells were pretreated with CPZ (1 $\mu$M; $t = 108$ s), and TRH (1 $\mu$M; $t = 533$ s) was then added.

that mobilization of extracellular $Ca^{2+}$ is not involved in the TRH-induced $Ca^{2+}$ response. Neither did $[Ca^{2+}]_i$ increase after treatment of cells with the L-type VOCC activator BAY K8644 or depolarization with KCl (2–50 mM; results not shown). Although $[Ca^{2+}]_i$ appeared not to return to prestimulated values after the application of TRH to either nifedipine- or SK&F 96365-pretreated cells, these values were in fact not significantly higher than basal prestimulated $[Ca^{2+}]$.

### Involvement of intracellular $Ca^{2+}$ in the TRH-induced $Ca^{2+}$ response in 293-E2 cells

The importance of intracellular $Ca^{2+}$ mobilization in the TRH-induced $Ca^{2+}$ response was investigated using the structurally unrelated ATPase inhibitors TG and CPZ. Both compounds are thought to deplete Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores in the endoplasmic reticulum. Both compounds (1 $\mu$M) produced a slow but substantial increase in $[Ca^{2+}]_i$ (Figures 3d and 3e). Although the subsequent addition of TRH to these cells produced a temporary fall in $[Ca^{2+}]_i$, it did not, however, produce the expected rise in $[Ca^{2+}]_i$ (Figures 3d and 3e). These results imply that, in 293-E2 cells, the mobilization of $Ca^{2+}$ from intracellular pools is responsible for the TRH-induced $Ca^{2+}$ response. The slight decrease in $[Ca^{2+}]_i$ after the application of TRH to TG- or CPZ-pretreated cells is consistent with the idea that receptor stimulation can lead to activation of $Ca^{2+}$ pumping from cells, an effect masked under normal circumstances [29]. Alternatively, dye compartmentalization, which can occur at 37 °C, may also explain this observation.

### Involvement of G-protein coupling in the TRH-induced intracellular $Ca^{2+}$ response

Suramin sodium has previously been shown to uncouple G-proteins from their receptors. The effect of this compound on $G_q/G_{11}$-linked TRH $Ca^{2+}$ mobilization was examined (Figure 4a). Pretreatment of cells with suramin sodium (10 $\mu$M) significantly reduced the $Ca^{2+}$ response to TRH. $[Ca^{2+}]_i$ rose from $29.4 \pm 8.6$ nM to $155.2 \pm 14.9$ nM at $t = 422$ s compared with a control response of $329.7 \pm 25.6$ nM. Despite a dose-dependent reduction in total binding, neither TRH-R affinity or number were altered by treatment with suramin sodium (1–100 $\mu$M; results not shown). Suramin did, however, abolish both TRH- and [3-Me-His$^2$]TRH-stimulated [$^{35}$S]GTP[S] binding in membranes prepared from 293-E2 cells (Figure 4b).

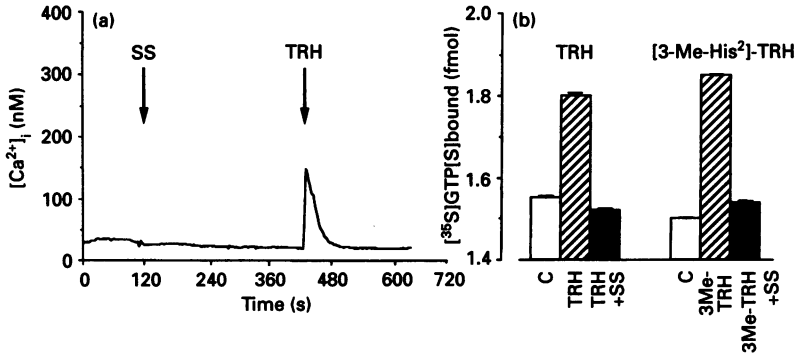


**Figure 4 Effect of suramin sodium pretreatment on (a) TRH-induced $Ca^{2+}$ response in 293-E2 cells and (b) TRH- and [3Me-His²]TRH-stimulated [³⁵S]GTP[S] binding in cell membranes prepared from 293-E2 cells**

(**a**) Suramin sodium (SS) pretreatment (10 $\mu$M; $t = 108$ s) significantly reduced the TRH-induced $[Ca^{2+}]_i$ response (1 $\mu$M; $t = 422$ s); $P = 0.02$, compared with control response ($n = 5$). (**b**) SS (32 $\mu$M) treatment abolished both TRH (1 $\mu$M)- and [3-Me-His²]TRH (1 $\mu$M)-stimulated [³⁵S]GTP[S] binding in cell membranes prepared from 293-E2 cells. Results are representative of those obtained in three separate experiments.

## Desensitization of the TRH- and BK-induced intracellular $Ca^{2+}$ response

Exposure of 293-E2 cells to a 1 min pulse of TRH caused the expected transient increase in $[Ca^{2+}]_i$ (Figure 5a). Subsequent exposure of cells to further 1 min pulses of TRH at various intervals ($t = 1000$, 2400 and 3600 s; Figure 5a) failed to elicit any further response. Similar results were obtained when cells were continuously exposed to TRH (results not shown). This lack of response appeared not to be related to cell viability. Using the same experimental design, cells that initially showed no response to buffer subsequently responded normally to TRH (Figure 5b). This acute desensitization could not be explained by changes in receptor number ($B_{max.}$ 13.5 pM/mg of protein) or affinity ($K_d$ 4 nM) after TRH exposure, as these parameters were unaltered in control buffer-treated cells compared with cells treated with TRH. The desensitization of the TRH-induced $Ca^{2+}$ response also appears to be a dose-related phenomenon. Low-dose TRH pretreatment (0.1 nM; Table 1) partially desensitized the $Ca^{2+}$ response to submaximal concentrations of TRH (0.1 nM–0.1 $\mu$M), whereas the $Ca^{2+}$ response to 1 $\mu$M TRH was unaltered. In contrast, pretreatment with higher concentrations of TRH (1 nM, 5 nM, 0.1 $\mu$M) completely desensitized cells (results not shown).

Treatment of 293-E2 cells with BK (1 $\mu$M; Figure 6a) caused $[Ca^{2+}]_i$ to rise from $8.5 \pm 1.3$ at $t = 47$ s to $145 \pm 17$ nM at $t = 59$ s. This response was monophasic in nature and resembled the low-dose TRH responses observed in this cell line. Pretreatment of cells with a potent BK-R antagonist (sodium adamantanecetyl-D-Arg-[Hyp³,Thi⁵,⁸,D-Phe⁷]bradykinin) abolished the BK- but not the TRH-induced mobilization of intracellular $Ca^{2+}$ (results not shown). As the characteristics of desensitization, i.e. the rapidity, extent and duration of desensitization, appear to be receptor-specific, we also examined desensitization of the BK-induced $[Ca^{2+}]_i$ response in 293-E2 cells. After initial treatment with BK (1 $\mu$M; $t = 47$ s; Figure 6a), cells subsequently failed to respond to BK (1 $\mu$M; $t = 2195$ s) but continued to respond to TRH (1 $\mu$M; $t = 2500$ s; Figure 6a). So, although the BK-induced $Ca^{2+}$ response was smaller than that induced by TRH, BK-R desensitization nevertheless occurred. These results further imply that $Ca^{2+}$ depletion is not primarily responsible for the desensitization phenomenon observed in the present experiments.

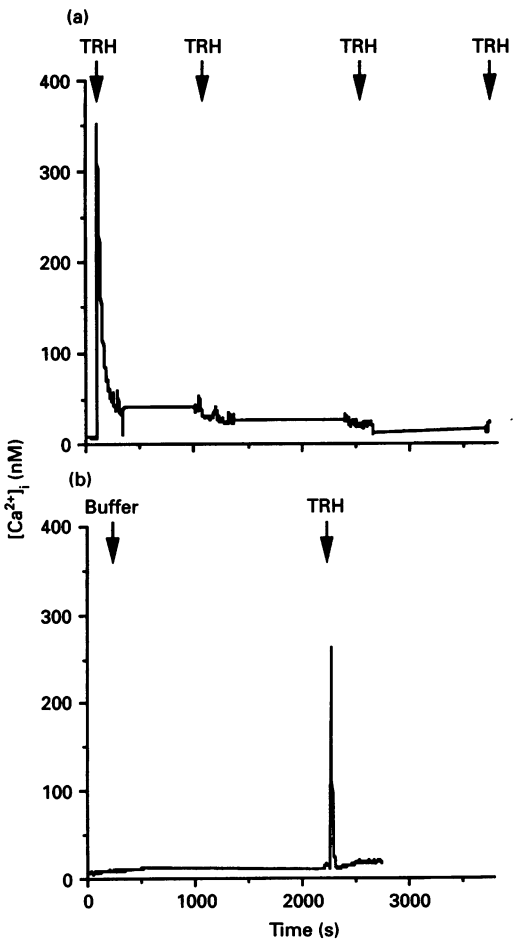


**Figure 5 Effect of (a) several pulses of TRH and (b) buffer A and TRH on $[Ca^{2+}]_i$ in 293-E2 cells**

(**a**) 293-E2 cells initially challenged with TRH (1 $\mu$M; $t = 98$ s) responded with an increase in $[Ca^{2+}]_i$ but failed to respond to subsequent regular TRH challenges (1 $\mu$M; $t = 1000$, 2500 and 3600 s). (**b**) Application of buffer A (5 ml; $t = 100$ s) had no effect whereas TRH (1 $\mu$M; $t = 2250$ s) produced the expected increase in $[Ca^{2+}]_i$.

When cells were pretreated with TRH (1 $\mu$M; $t = 47$ s; Figure 6b), no intracellular $Ca^{2+}$ mobilization was observed after the application of either BK (1 $\mu$M; $t = 2230$ s) or TRH (1 $\mu$M; $t = 2430$ s). Similar results were observed in 293-h20 cells expressing the human TRH-R (Figures 6c and 6d), suggesting that this pattern of desensitization is not species-specific. Again this desensitization could not be accounted for by a reduction in receptor number ($B_{max.}$ 0.52 pM/mg of protein; $K_d$ 4 nM). In 293-E2 cells this effect was dose-dependent, as pretreatment of cells with lower TRH concentrations (0.1 nM; $t = 110$ s; Figure 6e) only partially desensitized the $[Ca^{2+}]_i$ response to BK (1 $\mu$M; $t = 2125$ s). These cells were then treated with $Ca^{2+}$-free buffer A containing EGTA (2 mM; 5 min). The subsequent addition of 1 $\mu$M ionomycin ($Ca^{2+}$-free salt) produced a rapid rise in $[Ca^{2+}]_i$, suggesting that intracellular $Ca^{2+}$ stores had not been depleted.

**Table 1 Desensitization of the TRH-induced $Ca^{2+}$ response in 293-E2 cells after pretreatment with low-dose TRH**

293-E2 cells initially treated with TRH (0.1 nM) were subsequently challenged with various concentrations of TRH (0.1 nM–1 $\mu$M). Compared with control responses, low-dose TRH pretreatment desensitized subsequent $[Ca^{2+}]_i$ responses to low-dose but not to high-dose TRH treatment; $^*P < 0.05$, $^{**}P < 0.001$ ($n = 3$).

| [TRH] | Control $[Ca^{2+}]_i$ response in 293-E2 cells (nM) | $[Ca^{2+}]_i$ response in 293-E2 cells after pretreatment with 0.1 nM TRH (nM) |
|---|---|---|
| 0.1 nM | 178±6 | 103±3* |
| 10 nM | 265±121 | 141±50* |
| 0.1 $\mu$M | 242±293 | 139±42** |
| 1 $\mu$M | 352±144 | 292±89 |

## DISCUSSION

Time-related changes in $[Ca^{2+}]_i$ in single HEK-293 cells expressing either the rat or human TRH-Rs were characterized. Although HEK-293 cells do not normally express TRH-R, this cell line has been commonly used for the expression of a variety of recently cloned GPCRs [30,31]. The application of TRH produced a prompt dose-dependent monophasic increase in $[Ca^{2+}]_i$ in all imaged TRH-R-expressing cells. As the influx of extracellular $Ca^{2+}$ through either SMOCCs or L-type VOCCs were not involved in this TRH-induced $Ca^{2+}$ response, the involvement of intracellular $Ca^{2+}$ was examined. Treatment of 293-E2 cells with either of the $Ca^{2+}$-ATPase inhibitors TG and CPZ resulted in a transient but pronounced rise in $[Ca^{2+}]_i$. The subsequent addition of TRH failed to provoke a $Ca^{2+}$ response, suggesting that Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores normally released during TRH-R activation are absent and that TRH-induced $Ca^{2+}$ mobilization is dependent on these intracellular stores. These data are in accord with the findings of a recent study that showed that, in cell lines lacking L-type VOCC activity (for example HeLa or C6 cells), TRH similarly generated a monophasic $Ca^{2+}$ response which was also blocked by TG [13].

Physiological concentrations of $Ca^{2+}$-mobilizing hormones

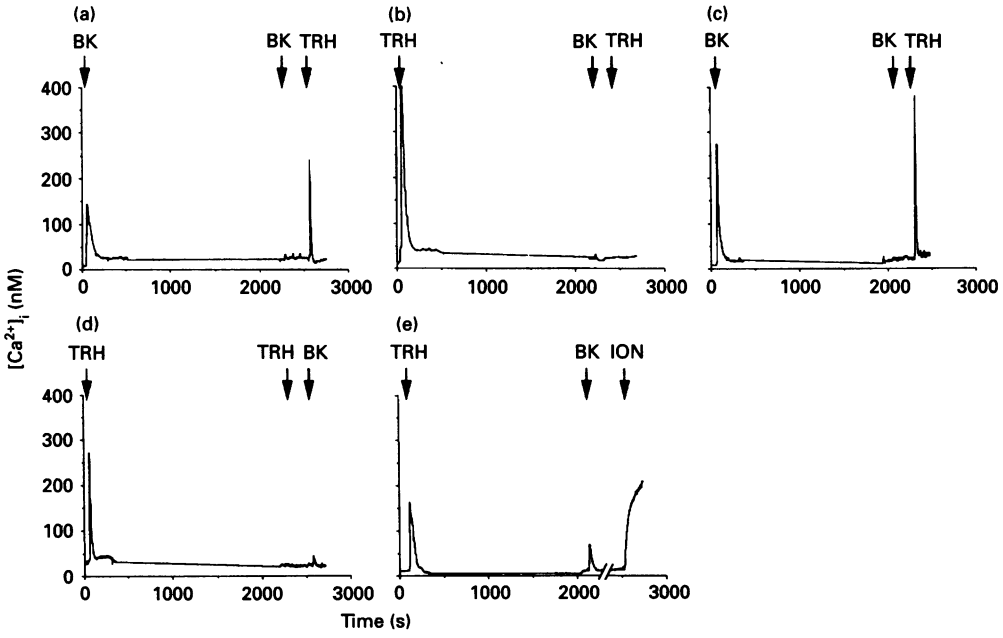


**Figure 6 Effect of BK on $[Ca^{2+}]_i$ response**

(**a**) Effect of BK pretreatment on $[Ca^{2+}]_i$ response to subsequent BK and TRH challenge in 293-E2 cells. Although the application of BK (1 $\mu$M; $t = 47$ s) produced a transient rise in $[Ca^{2+}]_i$, it also completely desensitized the cells to the effect of BK (1 $\mu$M; $t = 2195$ s) but only partially desensitized cells to the effects of TRH (1 $\mu$M; $t = 2500$ s). (**b**) TRH pretreatment (1 $\mu$M; $t = 47$ s) abolished $[Ca^{2+}]_i$ responses to both BK (1 $\mu$M; $t = 2230$ s) and TRH (1 $\mu$M; $t = 2430$ s) in 293-E2 cells. (**c**) Effect of BK pretreatment on $[Ca^{2+}]_i$ response to subsequent BK and TRH challenge in 293-h20 cells expressing the human TRH-R. Although the application of TRH (1 $\mu$M; $t = 47$ s) produced a transient rise in $[Ca^{2+}]_i$, it markedly desensitized cells to the effect of BK (1 $\mu$M; $t = 2080$ s) but not TRH (1 $\mu$M; $t = 2263$ s). (**d**) Effect of TRH pretreatment on $[Ca^{2+}]_i$ responses to TRH and BK in 293-h20 cells. TRH pretreatment (1 $\mu$M; $t = 47$ s) abolished subsequent effects of TRH (1 $\mu$M; $t = 2286$ s) and BK (1 $\mu$M; $t = 2523$ s) on$[Ca^{2+}]_i$. (**e**) Low-dose TRH pretreatment (0.1 nM; $t = 110$ s) partially desensitized the $[Ca^{2+}]_i$ response to BK (1 $\mu$M; $t = 2125$ s). $Ca^{2+}$-free buffer A containing EGTA (2 mM; 5 min) was then added, followed by 1 $\mu$M ionomycin (ION).

have been shown to produce repetitive and periodic $Ca^{2+}$ spikes/oscillations. These oscillations are believed to play an important role in cellular signal-transduction processes [15,16]. In the present study, TRH-induced $Ca^{2+}$ oscillations were occasionally observed. As these oscillations were an apparently random occurrence in only a small proportion of the cells imaged, we were unable to investigate the source of $Ca^{2+}$ involved in these responses. Similar observations have, however, been demonstrated in HeLaR cells transfected with the mouse TRH-R [13]. These responses were independent of extracellular $Ca^{2+}$ and more likely arose from $Ca^{2+}$ release and reuptake at intracellular $Ca^{2+}$ storage pools. Indeed the $Ca^{2+}$ oscillations/spikes observed in both this and the present study are characteristic of those produced by the cytoplasmic oscillator [32] which are typically insensitive to extracellular $Ca^{2+}$.

TRH-R belongs to a family of hormone and neurotransmitter receptors whose actions are mediated via the activation of G-proteins. Suramin sodium has previously been shown to antagonize the interaction between receptors and the G-proteins that regulate adenylate cyclase [33,34] or PLC activity [35,36]. It is believed to intercalate directly with the receptor–G-protein complex thereby forming an $\alpha$-helix which in some way uncouples the receptor from its G-protein. In the present study the partial inhibition by suramin of the TRH-induced rise in $[Ca^{2+}]_i$ could reflect an interaction of the compound with either the TRH-R or its associated G-protein(s). Owing to the lack of a specific TRH-R antagonist, the effects of suramin on TRH binding and TRH-stimulated [$^{35}$S]GTP[S] binding were investigated instead. The dose-dependent inhibition of both TRH and [3-Me-His$^2$]TRH-induced [$^{35}$S]GTP[S] binding in 293-E2 cells by suramin sodium without altering either TRH-R affinity or number suggests that suramin acts directly at the site of receptor–G-protein coupling to prevent any interaction between these two proteins. Similar data have been reported for the polyanionic compound L-451,167 in CHO cells expressing the $\alpha_2$-adrenoceptor [34].

Some controversy surrounds the desensitization of the G-protein-coupled events of the TRH-R. Whereas some groups have shown that TRH can maintain second-messenger production at a constant level for periods of up to 1 h [20,37], others have shown a marked reduction in the rate of second-messenger production within minutes of exposure to the peptide [4,38,39]. The methodology employed to measure the events of receptor desensitization, experimental design, data interpretation and varying cell types may all contribute to these conflicting results. To assess desensitization more directly at the level of the single cell, we examined the effects of TRH pretreatment on the TRH-induced $Ca^{2+}$ response in 293-E2 cells. After either a 1 min pulse or continuous exposure of cells to a high concentration of TRH, 293-E2 cells at first responded with the expected monophasic rise in $[Ca^{2+}]_i$. Subsequent exposure of these cells to TRH at various intervals for a period of up to 1 h failed to promote $Ca^{2+}$ mobilization. Desensitization of the TRH-induced $Ca^{2+}$ response was dose-dependent, as low-dose TRH pretreatment reduced, whereas high-dose pretreatment completely abolished, $Ca^{2+}$ responses to subsequent TRH treatment. Although these data suggest that TRH-Rs expressed in HEK-293 cells undergo rapid desensitization, this desensitization could be attributed to the depletion of internal $Ca^{2+}$ stores. We therefore challenged TRH-treated cells with ionomycin in buffer containing EGTA. Under these conditions ionomycin, but not TRH, was able to mobilize intracellular $Ca^{2+}$. These results indicate that intracellular $Ca^{2+}$ stores have not been totally depleted. It should also be noted that fura-2-labelled cells, which showed no initial response to buffer, were capable of responding normally to TRH for up to 1 h. This demonstrates that neither dye-quenching nor reduced cell viability could account for the TRH-induced desensitization observed in the present study.

Although in general the rat, human and mouse TRH-Rs show high sequence homology, considerable sequence variation exists at the C-terminus of these receptors [25]. Compared with the human TRH-R, the long form of the rat TRH-R has an extended terminus containing an extra 13 amino acids including an additional potential phosphorylation site. The terminal region of a variety of receptors including TRH-R have been associated with receptor–G-protein-coupled events and receptor desensitization [40]. We therefore examined whether the truncated C-terminus of human TRH-R altered the ability of the receptor to desensitize. Despite sequence differences, the TRH-induced $Ca^{2+}$ response in 293-h20 cells expressing human TRH-R was similarly desensitized. Interestingly, in a recent study by Pedersen et al. [39], desensitization of mouse TRH-R was shown to be cell-type-specific.

Although the internalization and eventual loss of TRH-Rs are associated with long-term exposure to TRH [18–20], the rapid desensitization observed in the present study cannot be accounted for by receptor loss, as receptor number and affinity were unaltered after the acute exposure of cells to TRH. A possible explanation for this rapid desensitization is receptor phosphorylation. The occupation of the receptor with its agonist is believed to induce receptor phosphorylation at multiple sites resulting in an uncoupling of the receptor from its cognate G-protein. This is certainly the case for the widely studied $\beta$-adrenoceptor [41]. Agonist-dependent phosphorylation of the PLC-linked muscarinic $M_3$ [42], substance P [43] and cholecystokinin [44] receptors has also been demonstrated. The time course measured for this receptor phosphorylation is indeed rapid enough to account for the acute desensitization of the TRH-induced $Ca^{2+}$ response.

Although rapid receptor desensitization appears to be a feature common to PLC-linked receptors, the level of desensitization has been reported to vary from receptor to receptor [45,46]. BK-Rs are expressed in normal rat kidney fibroblasts [47] and in the kidney-derived HEK-293 cells. In 293-E2 and 293-h10 cells, BK produced a transient monophasic rise in $[Ca^{2+}]_i$. This appears to be a receptor-specific effect, as a potent BK-R antagonist abolished the BK- but not the TRH-induced mobilization of intracellular $Ca^{2+}$. We subsequently carried out a series of experiments comparing the level and patterns of desensitization of the endogenously expressed BK-R and exogenously expressed TRH-R in 293-E2 cells. High-dose TRH treatment of cells resulted in desensitization of both the TRH- and BK-induced $Ca^{2+}$ response. In contrast, high concentrations of BK desensitized the BK- but not the TRH-induced $Ca^{2+}$ response. Therefore despite similar intracellular signalling pathway, it initially appears that these two receptors have different desensitization mechanisms. However, it is also possible that this might simply reflect differences in the efficiency of receptor–G-protein coupling. It has been suggested that there is a regulatory negative feedback loop between cytosolic $Ca^{2+}$ and PLC activity [48]. The mobilization of intracellular $Ca^{2+}$ could therefore influence rapid desensitization by virtue of its ability to feedback negatively on PLC activity. Perhaps the extensive $Ca^{2+}$ mobilization induced by TRH results in a marked negative regulatory effect on PLC activity thereby desensitizing the responses to both TRH and BK. Compared with TRH, BK is less effective in mobilizing intracellular $Ca^{2+}$ and may therefore have a smaller negative regulatory effect on PLC activity such that the subsequent exposure to a more vigorous stimulus, i.e. TRH, is still capable of mobilizing $Ca^{2+}$. It has been proposed that within any given cell there is a limited pool of G-proteins

shared by a variety of endogenous receptors. It is possible that TRH-R stimulation and subsequent receptor uncoupling limits the G-protein pool available to couple to other endogenously expressed receptors, in this case the BK-R.

The problems often associated with the measurement of agonist-induced changes in $[Ca^{2+}]_i$ in heterogeneous populations of cells were eliminated in the present study by combining dynamic video imaging with fluorescence microscopy to measure acute time-related changes in $[Ca^{2+}]_i$ in single HEK-293 cells expressing either the rat or human TRH-Rs. These cells are non-pituitary in origin and apparently lack L-type VOCC activity. TRH produces a prompt dose-dependent rise in $[Ca^{2+}]_i$ in these cells, an effect solely dependent on the mobilization of TG/CPZ-sensitive intracellular $Ca^{2+}$ stores. We have also clearly demonstrated a rapid desensitization of the rat and human TRH-Rs as well as the endogeneously expressed BK-R.

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