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ORIGINAL ARTICLE

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Capacitative Ca^{2+} entry contributes to the Ca^{2+} influx induced by thyrotropin-releasing hormone (TRH) in GH_3 pituitary cells

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Abstract Treatment of GH_3 cells with either hypothalamic peptide thyrotropin-releasing hormone (TRH), the endomembrane Ca^{2+}-ATPase inhibitor thapsigargin or the Ca^{2+} ionophore ionomycin mobilized, with different kinetics, essentially all of the Ca^{2+} pool from the intracellular Ca^{2+} stores. Any of the above-described treatments induced a sustained increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which was dependent on extracellular Ca^{2+} and was prevented by Ni^{2+} but not by dihydropyridines (DHPs), suggesting that it was due to capacitative Ca^{2+} entry via activation of a plasma membrane pathway which opened upon the emptying of the intracellular Ca^{2+} stores. The increase of the plasma membrane permeability to Ca^{2+} correlated negatively with the filling degree of the intracellular Ca^{2+} stores and was reversed by refilling of the stores. The mechanism of capacitative Ca^{2+} entry into GH_3 cells differed from similar mechanisms described in several types of blood cells in that the pathway was poorly permeable to Mn^{2+} and not sensitive to cytochrome P_{450} inhibitors. In GH_3 cells, TRH induced a transient $[Ca^{2+}]_i$ increase due to Ca^{2+} release from the stores (phase 1) followed by a sustained $[Ca^{2+}]_i$ increase due to Ca^{2+} entry (phase 2). At the single-cell level, phase 2 was composed of a DHP-insensitive sustained $[Ca^{2+}]_i$ increase, due to activation of capacitative Ca^{2+} entry, superimposed upon which DHP-sensitive $[Ca^{2+}]_i$ oscillations took place. The two components of the TRH-induced Ca^{2+} entry differed also in that $[Ca^{2+}]_i$ oscillations remained for several minutes after TRH removal, whereas the sustained $[Ca^{2+}]_i$ increase dropped quickly to prestimulatory levels, following the same time course as the refilling of the stores. The drop was prevented when the refilling was inhibited by thapsigargin. It is concluded that, even though the mechanisms of capacitative Ca^{2+} entry may show differences from cell to cell, it is also present and may contribute to the regulation of physiological functions in excitable cells such as GH_3. There, capacitative Ca^{2+} entry cooperates with voltage-gated Ca^{2+} channels to generate the $[Ca^{2+}]_i$ increase seen during phase 2 of TRH action. This contribution of capacitative Ca^{2+} entry may be relevant to the enhancement of prolactin secretion induced by TRH.

Key words Ca^{2+} influx (store-operated) · Capacitative Ca^{2+} entry · Intracellular Ca^{2+} stores · GH_3 pituitary cells · Thapsigargin · Thyrotropin-releasing hormone

Introduction

The hypothalamic peptide thyrotropin-releasing hormone (TRH) increases hypophyseal secretion of prolactin. A useful model system in which this process can be studied is the GH_3 cell line, derived from rat anterior pituitary, which secretes prolactin in response to TRH by a Ca^{2+}-dependent mechanism [9]. Both Ca^{2+} mobilization from the intracellular Ca^{2+} stores and Ca^{2+} entry from the extracellular medium are involved in stimulus-secretion coupling [32]. Ca^{2+} mobilization from the stores is responsible for an early and transient prolactin secretion (phase 1) while Ca^{2+} entry is needed for sustained secretion (phase 2). Ca^{2+} mobilization is mediated by inositol 1, 4, 5-trisphosphate production [10]. Phase 2 has been attributed to the increase of action potential firing frequency induced by TRH [5], which promotes Ca^{2+} entry through voltage-operated Ca^{2+} channels [18] and oscillations of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) [33]. However, blocking of voltage-operated Ca^{2+} channels by dihydropyridines

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(DHPs) only partially inhibits the increase of $[Ca^{2+}]_i$ [2, 16], suggesting that another mechanism could contribute to the TRH-induced Ca^{2+} influx. In agreement with this, it has been reported that about 50% of the prolactin secretion observed during phase 2 of stimulation by TRH is resistant to DHPs [2].

In non-excitable cells, agonists may also induce a biphasic change of $[Ca^{2+}]_i$ composed of a transient increase due to Ca^{2+} mobilization from the stores, followed by a sustained increase due to Ca^{2+} entry [32]. In many cases this Ca^{2+} entry takes place through a plasma membrane pathway which activates upon the emptying of the intracellular Ca^{2+} stores by the agonist ("capacitative Ca^{2+} entry", see [3, 19, 25 for reviews). The existence of capacitative Ca^{2+} entry has recently been reported also in some excitable neuroendocrine cells such as bovine chromaffin [26], PC12 [6] and GH_4C_1 pituitary cells [16]. However, GH_3 cells, closely related to GH_4C_1 cells, have been proposed to belong, together with NG115 401L neuroblastoma cells [13], to a restricted group showing no capacitative Ca^{2+} entry [34].

In the present paper we have studied the effects of emptying the intracellular Ca^{2+} stores on the entry of Ca^{2+} and Mn^{2+} (used as a Ca^{2+} surrogate for Ca^{2+} channels) in fura-2-loaded GH_3 cells. The Ca^{2+} stores were emptied by treatment either with the Ca^{2+} ionophore ionomycin [22], with the endomembrane Ca^{2+}- ATPase inhibitor thapsigargin [29] or with TRH. Our results are consistent with the existence of a capacitative Ca^{2+} entry pathway that activates reversibly upon emptying the intracellular Ca^{2+} stores. Ca^{2+} entry through capacitative Ca^{2+} entry contributes to the increase of $[Ca^{2+}]_i$ produced by stimulation by TRH.

Materials and methods

Two strains of GH_3 pituitary cells, kindly provided by Dr. C. Dieguez (Universidad de Santiago de Compostela, Spain) and by Dr. F. Barros (Universidad de Oviedo, Spain), were used; both behaved similarly. They were grown in RPMI 1640 medium supplemented with 15% horse serum and 2.5% fetal calf serum at 37°C and in an atmosphere of 95% air and 5% CO_2.

For fluorescence measurements, GH_3 cells were allowed to attach to poly-L-lysine-coated (0.01 mg/ml, 5 min) glass coverslips and were grown for 2–3 days. The cell-coated coverslips were washed with standard medium containing (in mM): NaCl,145; KCl,5; $MgCl_2$,1; $CaCl_2$,1; glucose,10; 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, Na^+ salt (sodium-HEPES),10; pH, 7.4, and were loaded with fura-2 by incubation with 5 μM fura-2/acetoxymethyl ester (fura-2/AM) at room temperature for about 1 h. $[Ca^{2+}]_i$ measurements were performed either on cell populations or at the single-cell level, as described below.

For $[Ca^{2+}]_i$ measurements and Mn^{2+} entry assays using cell populations, glass coverslips were introduced at a fixed angle (45°) into quartz cuvettes placed in the sample compartment of a spectrophotometer that allowed rapid (30–300 Hz) alternation of up to six different excitation wavelengths (Cairn Research Newnhan, Sittingbourne, Kent, UK). Temperature was 30°C. Emitted fluorescence was measured at >520 nm and integrated at every 1–s period. $[Ca^{2+}]_i$ was estimated from the ratio of the fluorescence values excited at 340 nm and at 380 nm [11]. Calibration was performed by comparison with fura-2 standards. Mn^{2+} entry was measured by the quenching of the fura-2 fluorescence excited at 360 nm, a wavelength which is not sensitive to changes in Ca^{2+} concentration [12]. This procedure has been described in detail elsewhere [8]. Perfusion with different media allowed us to stimulate and wash the same cells repetitively, in order to compare inhibition within the same cell sample and to document reversibility.

For single-cell measurements, the coverslips coated with fura-2-loaded cells were mounted under the microscope (Nikon Diaphot) in a chamber maintained at 36°C and epi-illuminated alternately at 340 nm and 380 nm. Light emitted at wavelengths longer than 520 nm was recorded by an extended ISIS-M camera (Photonic Science, Robertsbridge, East Sussex, UK) and analysed using an Applied Imaging Magical image processor (Sunderland, Tyne and Wear, UK). Four video frames at each wavelength were averaged by hardware, with an overall time resolution of about 3 s for each pair of images at alternate wavelengths. Ratios of data from consecutive frames obtained at 340 nm and 380 nm excitation were calculated pixel by pixel and $[Ca^{2+}]_i$ was estimated by comparison with fura-2 standards [31].

Quantification of $[Ca^{2+}]_i$ oscillations of the cells was performed using two parameters, the mean $[Ca^{2+}]_i$ increase and the oscillation index. The mean $[Ca^{2+}]_i$ increase was computed as the average of all the $[Ca^{2+}]_i$ values minus the "resting" $[Ca^{2+}]_i$ over the whole integration period. The "resting" $[Ca^{2+}]_i$ was arbitrarily chosen as the smallest $[Ca^{2+}]_i$ value in the whole trace. Thus, the mean $[Ca^{2+}]_i$ increase is equivalent to the mean area under the $[Ca^{2+}]_i$ peaks. The increase of this parameter reflects increases of $[Ca^{2+}]_i$, whether or not they are oscillatory. The oscillation index was computed as the average of all the differences (in absolute values) between each $[Ca^{2+}]_i$ value and the next, throughout the whole integration period. An increase of this parameter reflects an increase of oscillations and is largely independent of the actual $[Ca^{2+}]_i$ values.

Fura-2/AM was obtained from Molecular Probes, Eugene, Ore., USA. Nisoldipine was a generous gift from Bayer, Germany. Thapsigargin was obtained from Alomon Laboratories, Jerusalem, Israel. Other chemicals were obtained from Sigma, Madrid, Spain, or from Merck, Darmstadt, Germany.

Results

Intracellular Ca^{2+} pools in GH_3 cells

The relative Ca^{2+} content of the intracellular Ca^{2+} pools of GH_3 cells was estimated by mobilizing the stored Ca^{2+} into the cytosol in cells perfused with Ca^{2+}-free medium to prevent Ca^{2+} entry. Under these conditions the size of the transient $[Ca^{2+}]_i$ increase is a good index of the relative degree of filling of the stores [22]. Mobilizing agents tested included the Ca^{2+} ionophore ionomycin, the endomembrane ATPase inhibitor thapsigargin and TRH. Ionomycin mobilized the whole stored Ca^{2+} pool, as shown by the fact that none of the other two agents was able to produce a further $[Ca^{2+}]_i$ peak when added after ionomycin (Fig. 1A, E). TRH produced a substantial release, as indicated by the fact that the ionomycin-induced $[Ca^{2+}]_i$ peak was reduced by 89 ± 3% (mean ± SEM; $n = 5$) in cells first treated with TRH (Fig. 1B, compare to Fig. 1A). Treatment with 500 nM thapsigargin for 5 min produced an even more extensive mobilization of Ca^{2+} from the stores, the ionomycin-induced $[Ca^{2+}]_i$ peak being barely detectable after treatment with

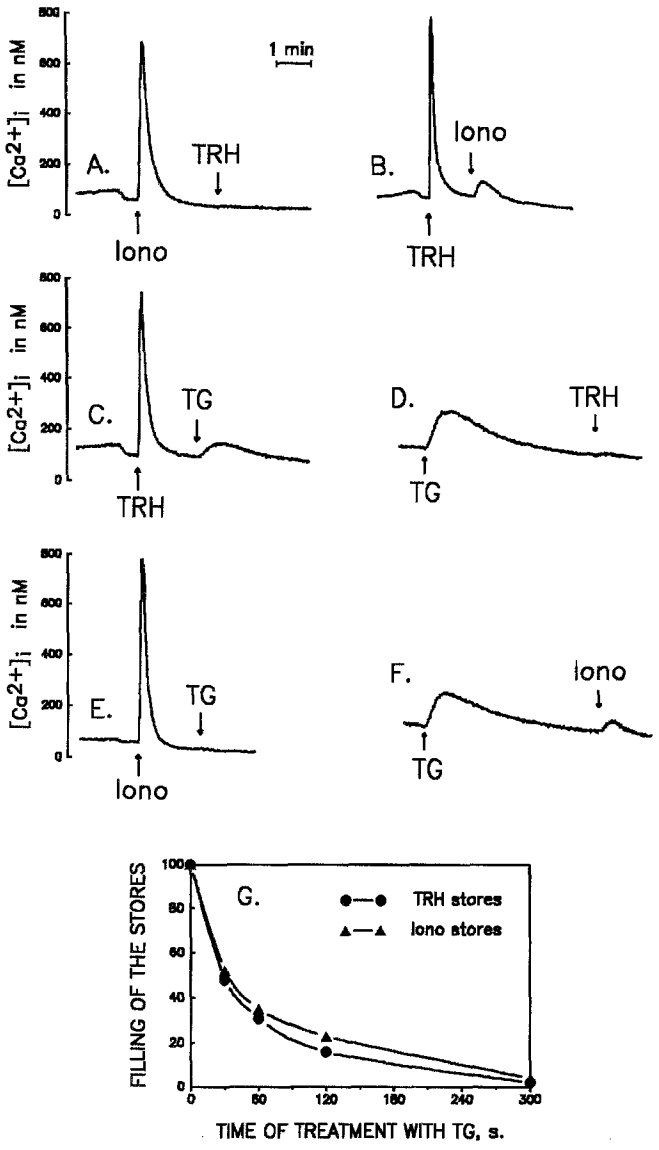


Fig. 1A–G Intracellular Ca^{2+} pools in GH_3 cells. The size and overlapping of the different intracellular Ca^{2+} pools were assayed by sequential perfusion with different Ca^{2+} mobilizers in Ca^{2+}-free medium. (*Iono* Ionomycin, *TRH* thyrotropin releasing hormone, *TG* thapsigargin). The concentrations used were: iono, 100 nM; TRH, 100 nM; TG, 500 nM. Perfusion with the Ca^{2+}-free (containing 500 μM EGTA) medium was started 30 s before adding the Ca^{2+} mobilizer. **G** The correlation between the degree of filling of the intracellular Ca^{2+} stores and the time of treatment with TG is shown in experiments performed with the same cell batch. Filling was quantified as the height of the ionomycin-or the TRH-induced $[Ca^{2+}]_i$ peak (in Ca^{2+}-free medium) and is expressed as a percentage of the control, not treated with TG. In similar experiments with different cell batches the degree of filling was (mean ± SEM) 34 ± 6% after 1-min treatment with TG (n = 3) and 4 ± 3% after 5-min treatment with TG (n = 6)

thapsigargin (Fig. 1F). Treatment with thapsigargin also prevented the TRH-induced $[Ca^{2+}]_i$ peak Fig. 1D). Treatment with TRH substantially decreased, but did not completely abolish, the subsequent thapsigargin-induced $[Ca^{2+}]_i$ peak (Fig. 1C, compare to Fig. 1D). These results indicate that the three Ca^{2+} mobilizers tested, ionomycin, thapsigargin and TRH, are able to act essentially on the same Ca^{2+} pool.

Ca^{2+} mobilization by ionomycin and TRH produced a sharp $[Ca^{2+}]_i$ peak, suggesting a fast release from the stores to the cytosol followed by extrusion to the extracellular medium. Thapsigargin, in contrast, produced a much more sluggish $[Ca^{2+}]_i$ rise which was sustained for longer periods, suggesting a slower release. Figure 1G illustrates the time course of the emptying of intracellular Ca^{2+} stores by thapsigargin. The relative degree of filling after treatment for different time periods was estimated from the size of the $[Ca^{2+}]_i$ peaks induced, in Ca^{2+}-free medium, by either ionomycin (triangles) or TRH (circles). The half-emptying time was about 30 s, full emptying requiring about 5 min. This relatively slow emptying is consistent with passive leak from the stores to the cytosol unopposed by active pumping, which is blocked by thapsigargin, in contrast to an accelerated leak, as would be the case for ionomycin and TRH. In cells first treated with thapsigargin, and then incubated in Ca^{2+}-containing medium for up to 30 min, addition of ionomycin in Ca^{2+}-free medium did not produce any $[Ca^{2+}]_i$ peak, indicating that the effect of thapsigargin is irreversible and the stores are unable to refill (results not shown).

Emptying of the intracellular Ca^{2+} stores increases Ca^{2+} entry

Treatment with thapsigargin in Ca^{2+}-containing medium resulted in a sustained $[Ca^{2+}]_i$ increase, which was due to Ca^{2+} influx since it was reversibly prevented by Ca^{2+} removal or by Ni^{2+} addition (Fig. 2A). Treatment with thapsigargin either in Ca^{2+}-free medium (Fig. 2B) or in Ca^{2+}-containing medium to which Ni^{2+} had also been added to prevent Ca^{2+} entry (Fig. 2C), resulted in a transient increase of $[Ca^{2+}]_i$, which returned to or decreased below the resting level within 2–3 min. This reflects release of Ca^{2+} from the stores to the cytosol followed by extrusion to the extracellular medium. When the medium perfusing cells treated in this manner was switched to Ca^{2+}-containing medium (with no Ni^{2+}), $[Ca^{2+}]_i$ increased quickly to levels well above those found before treatment with thapsigargin (Fig. 2, B, C). These $[Ca^{2+}]_i$ overshoots reflect increased Ca^{2+} entry through the plasma membrane. Note that, in cells with filled Ca^{2+} stores (before treatment with thapsigargin), switching perfusion from Ca^{2+}-free to Ca^{2+}-containing medium had much smaller effects on $[Ca^{2+}]_i$ (Fig. 2B, first "Ca0" pulse). The modest, but noticeable, increase of $[Ca^{2+}]_i$ observed in control cells upon switching from Ca^{2+}-free to Ca^{2+}-containing medium represented mainly the averaged result of spontaneous oscillations of $[Ca^{2+}]_i$, which only happen in Ca^{2+}-containing medium, as revealed by study at the single-cell level (see below). Treatment of the cells with *tert*butyl-benzohydroquinone (BHQ),

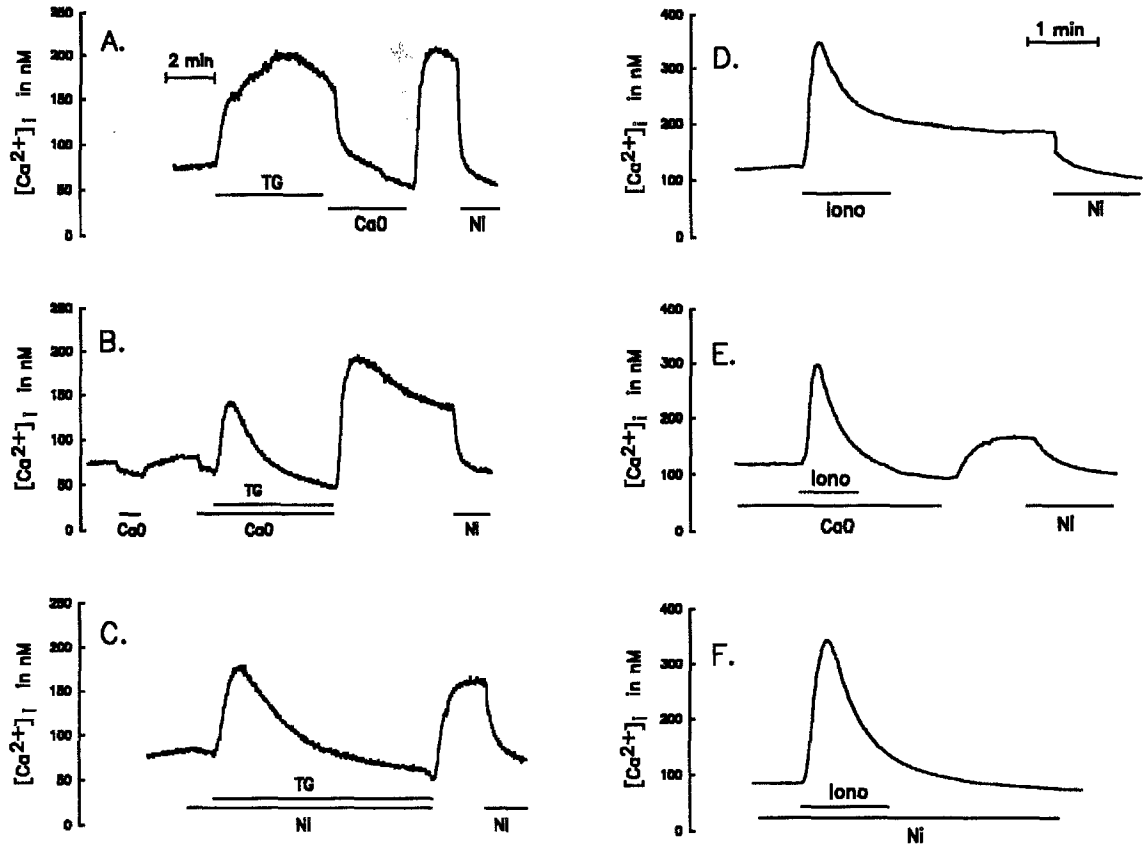


Fig. 2A–F Thapsigargin and ionomycin are able to induce an increase of Ca^{2+} entry in GH_3 cells. Perfusion was changed from standard incubation medium (with 1 mM Ca^{2+}) to media containing either no Ca^{2+} (0.5 mM EGTA; *Ca0*), 0.5 μM thapsigargin (*TG*), 5 mM Ni^{2+} (*Ni*) or 100 nM ionomycin (*Iono*), as indicated. Each *trace* is representative of 3–7 similar experiments

another inhibitor of endomembrane Ca^{2+}-ATPases [14], had the same effects as thapsigargin upon both the emptying of the intracellular Ca^{2+} stores and the increase of Ca^{2+} entry (results not shown).

Treatment of GH_3 cells suspended in Ca^{2+}-containing medium with low concentrations of the Ca^{2+} ionophore ionomycin elicited a biphasic $[Ca^{2+}]_i$ increase composed of a transient peak followed by a sustained increase (Fig. 2D). Addition of Ni^{2+}, which does not interfere with Ca^{2+} transport by ionomycin [22], decreased $[Ca^{2+}]_i$ to or below the resting level, before addition of ionomycin (Fig. 2D). Note that the $[Ca^{2+}]_i$ scale in the right panels of Fig. 2 is more expanded than in the left panels, so that the late $[Ca^{2+}]_i$ increase was about the same as the observed after treatment with thapsigargin (Fig. 2A). The early $[Ca^{2+}]_i$ peak induced by ionomycin was not prevented by Ca^{2+} removal (Fig. 2E) nor by addition of Ni^{2+} (Fig. 2F), indicating that it was due to Ca^{2+} release from the intracellular stores. In contrast the late and sustained $[Ca^{2+}]_i$ increase was not observed in Ca^{2+}-free medium (Fig. 2E) nor in Ca^{2+}-containing medium to which Ni^{2+} had been added (Fig. 2F), suggesting that it was due to increased Ca^{2+} entry. Switching the medium perfusing the cells treated in such a manner to Ca^{2+}-containing medium produced a $[Ca^{2+}]_i$ overshoot (Fig. 2E, end of "Ca0" pulse) which, again, could be inhibited by Ni^{2+}.

The increased Ca^{2+} entry observed after ionomycin treatment is not mediated through any residual ionophore that may have remained associated with the plasma membrane. The following observations support this view:

(1) the increase of $[Ca^{2+}]_i$ after Ca^{2+} store depletion with ionomycin is blocked by Ni^{2+}, which does not block Ca^{2+} transport through ionomycin-sensitive pathways [22];

(2) addition of 100 nM ionomycin to cells treated with thapsigargin and suspended in Ca^{2+}-containing medium did not produce a further increase of $[Ca^{2+}]_i$ (results not shown);

(3) ionomycin treatment did not increase the $[Ca^{2+}]_i$ overshoots obtained after treatment with thapsigargin (see below, Fig. 7A).

This lack of a noticeable effect of ionomycin at the plasma membrane level may seem rather surprising. However, we have shown in other cell kinds that the Ca^{2+}-permeabilizing effect of low concentrations of ionomycin and other Ca^{2+} ionophores is much more

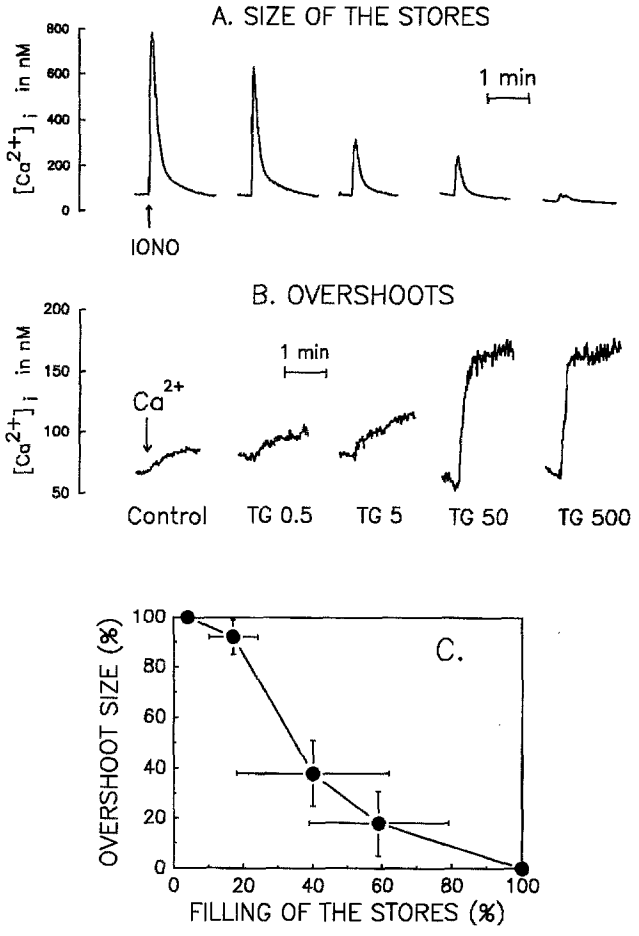


Fig. 3A–C Correlation between the degree of filling of the intracellular Ca^{2+} stores and the size of the $[Ca^{2+}]_i$ overshoots. GH_3 cells were treated in Ca^{2+}-free medium with the indicated concentrations of thapsigargin (*TG*, in nM) for 5 min. Then the degree of filling of the intracellular Ca^{2+} stores was estimated from the size of the $[Ca^{2+}]_i$ peaks induced by ionomycin (100 nM) in Ca^{2+}-free medium (**A**). **B** In parallel samples the size of the $[Ca^{2+}]_i$ overshoots upon switching from Ca^{2+}-free to Ca^{2+}-containing (1 mM) medium was measured. **C** The correlation between the degree of filling of the stores, expressed as a percentage of the control, not treated with thapsigargin, and the size of the $[Ca^{2+}]_i$ overshoots, expressed as a percentage of the maximum, obtained after treatment with 500 nM thapsigargin. Each value is the mean of three determinations with different cell batches. *Bars* represent SEM

evident at the endomembrane than at the plasma membrane level, so that the Ca^{2+} stores can be efficiently emptied with no significant increase of the ionophore-mediated Ca^{2+} entry through the plasma membrane [22]. This may be due to a preferential distribution of the ionophore in the endomembranes, to a more favourable surface-to-volume ratio conditioning a larger flux in the organelles, or to the existence of a large $[Ca^{2+}]_i$ gradient between the stores and the cytosol, which can be collapsed easily by even slight ionophore action. Therefore, results in Fig. 2 suggest that depletion of the intracellular Ca^{2+} stores by treatment with low concentrations of ionomycin increases the plasma membrane permeability to Ca^{2+}. The effects of thapsigargin and ionomycin upon Ca^{2+} entry were not additive (results not shown), suggesting that both manoeuvers activate the same Ca^{2+} entry mechanism, namely capacitative Ca^{2+} entry.

Depletion of the Ca^{2+} stores can be graded by using different concentrations of thapsigargin. Figure 3A documents the Ca^{2+} contents of the stores, measured as the size of the $[Ca^{2+}]_i$ peaks elicited by ionomycin in Ca^{2+}-free medium, after treatment with different thapsigargin concentrations for 5 min. The half-maximal effect was obtained at less than 5 nM thapsigargin. Figure 3B shows that the $[Ca^{2+}]_i$ overshoots, used as a measure of Ca^{2+} influx, increased gradually with depletion of the Ca^{2+} stores. Figure 3C summarizes the negative correlation between the size of the Ca^{2+} overshoot and the filling degree of the stores obtained in three similar experiments with different cell batches. Half-maximal entry was obtained at about 40% filling of the stores, a value very similar to that reported previously in other cell types [23].

Effect of depletion of the Ca^{2+} stores on permeability to Mn^{2+}

In several cell types the capacitative Ca^{2+} entry pathway allows passage of Mn^{2+} [3, 22]. Mn^{2+} entry can be demonstrated by the quenching of fura-2 fluorescence [12]. Changes of $[Ca^{2+}]_i$ and Mn^{2+} entry can be followed simultaneously and independently by measuring the fluorescence (F) excited from fura-2 at three different wavelengths, 340 nm, 360 nm and 380 nm [8, 22]. Since F_{360} is insensitive to Ca^{2+}, but quenched by Mn^{2+}, its decrease reflects only Mn^{2+} entry. On the other hand, since F_{340} and F_{380} are similarly quenched by Mn^{2+}, the ratio F_{340}/F_{380} continues to be a valid measurement of $[Ca^{2+}]_i$, even in the presence of Mn^{2+} [8]. In the experiment shown in Fig. 4, Mn^{2+} was added to the incubation medium (containing also 1 mM Ca^{2+}) and then the cells were treated with thapsigargin. As before (see Fig. 2), thapsigargin induced a $[Ca^{2+}]_i$ increase, seen as the rise of F_{340}/F_{380} (upper trace in Fig. 4). The lower trace shows the simultaneous recording of the quenching of F_{360}. Before addition of thapsigargin, the decrease of F_{360} was slow. Upon addition of thapsigargin, the rate of F_{360} quenching increased after a lag period of about 1 min (compare with the dotted line, showing the rate before application of thapsigargin), indicating that there was a delayed acceleration of Mn^{2+} entry. Note that the $[Ca^{2+}]_i$ increase (upper trace) did not show such a lag. We interpret these results to mean that the first part of the $[Ca^{2+}]_i$ increase produced by thapsigargin was due to release of Ca^{2+} from the stores and that Mn^{2+} entry only started once they had been emptied. The $[Ca^{2+}]_i$ trace does show a further increase about 2 min after addition of thapsigargin, coincident with the maximal rate of the F_{360} decrease, which may also reflect increased Ca^{2+} entry.

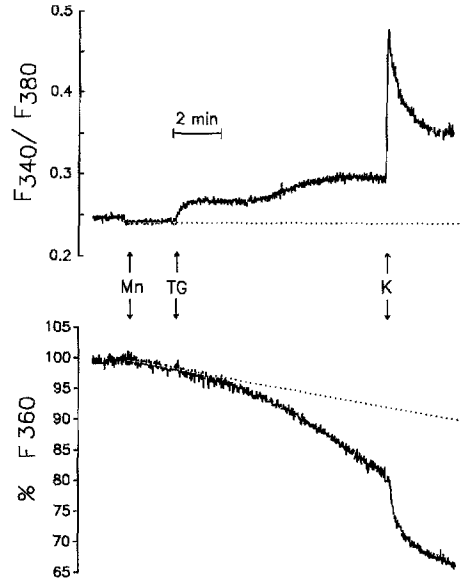


Fig. 4 Effects of thapsigargin on the entry of Mn^{2+} into GH_3 cells. The entry of Mn^{2+} was estimated from the quenching of the fura-2 fluorescence excited at 360 nm (*lower trace*; see Materials and methods). The *upper panel* illustrates the changes in $[Ca^{2+}]_i$, shown here as the ratio of the fluorescences excited at 340 nm and 380 nm, which were measured simultaneously. The perfusion medium contained 1 mM Ca^{2+}. At the *arrows* perfusion was changed to medium containing Mn^{2+} (50 μM), or Mn^{2+} plus thapsigargin (*TG*, 0.1 μM) or high K^+ (70 mM, replacing an equimolar amount of Na^+). This experiment is representative of 3 similar ones

The thapsigargin-induced Mn^{2+} entry was usually sluggish and experimental conditions (relative concentrations of Ca^{2+} and Mn^{2+} and state of the cells) should be carefully optimized in order to obtain clear results. These difficulties were not found in other cell preparations, for which massive entry of Mn^{2+} could be demonstrated after depletion of the intracellular Ca^{2+} stores [3, 22]. The entry of Mn^{2+} through voltage-operated Ca^{2+} channels could also be easily demonstrated in GH_3 cells [31]. This is illustrated in the last part of the experiment of Fig. 4, where depolarization of the cells with a high K^+ concentration produced a sudden increase of $[Ca^{2+}]_i$ (upper trace) coincident with a large acceleration of Mn^{2+} entry (lower trace).

Effects of DHPs and cytochrome P_{450} inhibitors on the thapsigargin-induced Ca^{2+} entry

In order to test whether voltage-operated Ca^{2+} channels could contribute to the Ca^{2+} entry induced by empying the Ca^{2+} stores we tested the effects of nisoldipine, an inhibitor of voltage-operated Ca^{2+} channels [30]. Nisoldipine, which fully blocked the increase of $[Ca^{2+}]_i$ induced by depolarization with a high K^+ concentration ([31] see below), had only a minor effect on the $[Ca^{2+}]_i$ overshoot obtained by adding Ca^{2+} to GH_3 cells first treated with thapsigargin (Fig. 5A). In five similar experiments, nisoldipine inhibited by only 16 ± 6% (mean ± SEM) the $[Ca^{2+}]_i$ overshoots in thapsigargin-treated GH_3 cells. Similar results were obtained in ionomycin-treated cells (results not shown).

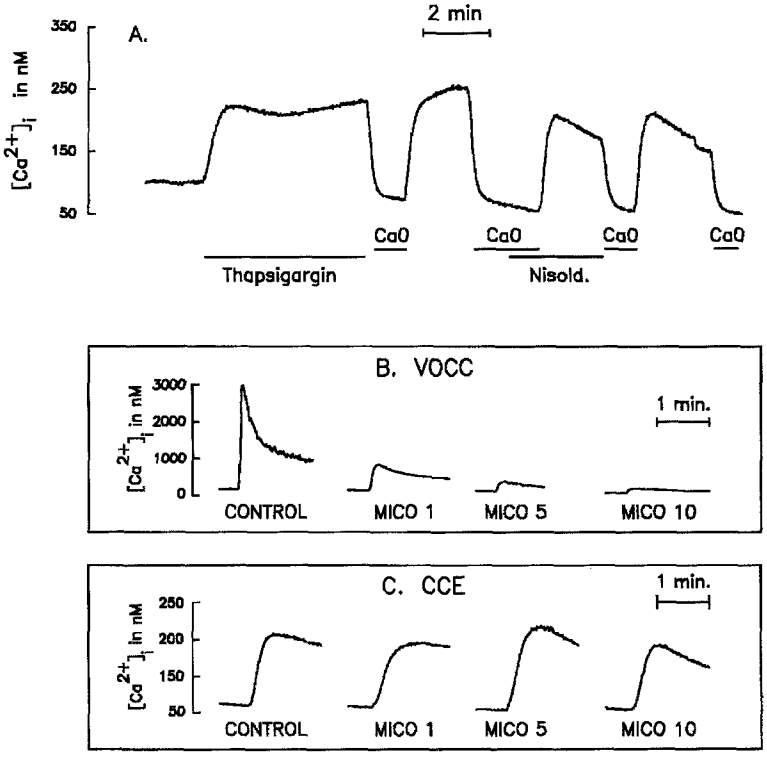


Fig. 5A–C Effects of nisoldipine and miconazole on the $[Ca^{2+}]_i$ overshoots observed after treatment with thapsigargin and on Ca^{2+} entry through voltage-gated Ca^{2+} channels (*VOCC*). The concentration of nisoldipine in part **A** was 1 μM. **B** Depolarization was induced by perfusion with 70 mM K^+ in the absence (control) or in the presence of several concentrations of miconazole (1, 5 and 10 μM). **C** Miconazole was added to thapsigargin-treated cells incubated in Ca^{2+}-free medium and, 2 min later, the cells were perfused with a solution containing the same concentration of miconazole and 1 mM Ca^{2+} to induce the $[Ca^{2+}]_i$ overshoot. **A** Representative of six similar experiments. **B, C** Representative of 2–5 similar experiments

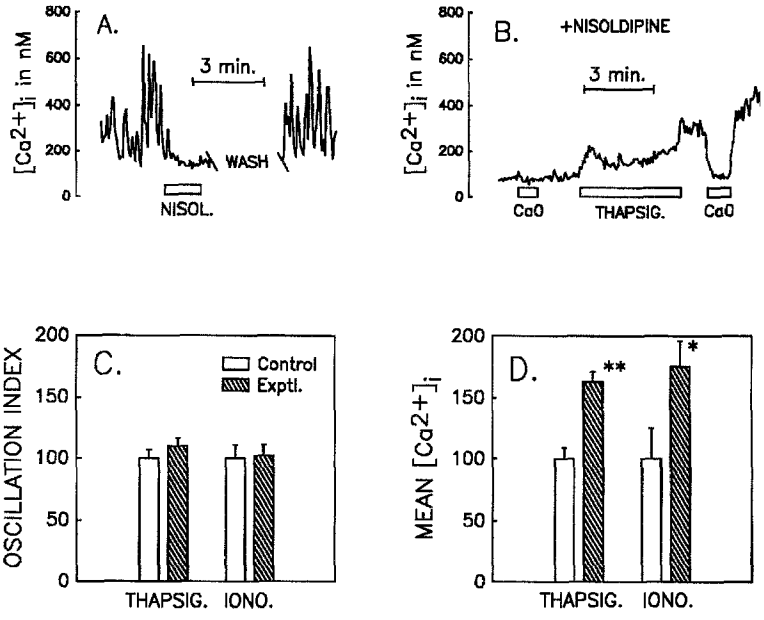


Fig. 6A–D Effects of thapsigargin and ionomycin on $[Ca^{2+}]_i$ oscillations in single GH_3 cells. **A** The effect of 0.5 μM nisoldipine on spontaneous $[Ca^{2+}]_i$ oscillations. *Wash* represents a 20-min period to washout nisoldipine. **B** The effect of 0.3 μM thapsigargin in the presence of 0.5 μM nisoldipine in a representative cell. The cells were perfused with medium containing 1 mM Ca^{2+} which was then changed to medium containing either thapsigargin (0.3 μM), or no Ca^{2+} (0.5 mM EGTA, *Ca0*), as indicated. **C, D** Average values of the oscillation indexes and the mean $[Ca^{2+}]_i$ increases (see Meterials and methods) before (*open boxes*) and after (*dashed boxes*) thapsigargin (0.3 μM; $n = 100$ cells) or ionomycin (100 nM; $n = 68$ cells). Integration periods of 3 min were used for calculations. Values are expresssed as a percentage of the controls. *Bars* represent SEM. $^{*}P < 0.05$, $^{**}P < 0.001$ (Student's *t*-test)

We have reported recently that cytochrome P_{450} inhibitors are able to block the entry of Ca^{2+} and Mn^{2+} induced by emptying the intracellular Ca^{2+} stores in several non-excitable cell types [3]. In GH_3 cells cytochrome P_{450} inhibitors also block the entry of Ca^{2+} through voltage-operated Ca^{2+} channels [31]. Cytochrome P_{450} inhibitors had little effect on thapsigargin-induced Ca^{2+} entry into GH_3 cells. Figure 5 compares the effects of several concentrations of miconazole, a cytochrome P_{450} inhibitor, on the increases of $[Ca^{2+}]_i$ induced either by depolarization with a high K^+ concentration (B) or by adding Ca^{2+} to thapsigargin-treated cells (C). It is clear that, whereas Ca^{2+} entry through voltage-operated Ca^{2+} channels was effectively antagonized by miconazole, entry through the capacitative Ca^{2+} entry pathway was little affected. Similar results were obtained with other cytochrome P_{450} inhibitors (econazole and nordihydroguaiaretic acid, results not shown).

Independent evidence about the lack of an effect of emptying the Ca^{2+} stores on voltage-operated Ca^{2+} channels was obtained from measurements of $[Ca^{2+}]_i$ in single cells. GH_3 cells display spontaneous $[Ca^{2+}]_i$ oscillations which are due to action potential firing [27]. DHPs reversibly inhibited the spontaneous $[Ca^{2+}]_i$ oscillations (Fig. 6A), but not the $[Ca^{2+}]_i$ increases induced by thapsigargin nor the $[Ca^{2+}]_i$ overshoots in thapsigargin-treated cells (Fig. 6B). Analysis of the effects of thapsigargin in single cells showed that oscillations were little modified, but that they took place superimposed upon a higher "resting" $[Ca^{2+}]_i$ between oscillations. Figure 6C, D summarizes the results in all the cells analysed, data being expressed as mean $[Ca^{2+}]_i$ increases and oscillation indexes (see Materials and methods).

Thapsigargin did not modify the oscillation index (Fig. 6C), which purely reflects Ca^{2+} oscillations, but increased the mean $[Ca^{2+}]_i$ increase (Fig. 6D) which depends on both oscillations and sustained increases of $[Ca^{2+}]_i$. Emptying the Ca^{2+} stores with ionomycin had similar results (Fig. 6C, D). These results reinforce the conclusion that the Ca^{2+} entry pathway activated by treatment with thapsigargin in GH_3 cells is distinct from voltage-operated Ca^{2+} channels.

Refilling of the intracellular Ca^{2+} stores

The $[Ca^{2+}]_i$ overshoots obtained upon addition of Ca^{2+} to thapsigargin-treated cells were rather reproducible (Fig. 7A). This is consistent with the irreversibility of

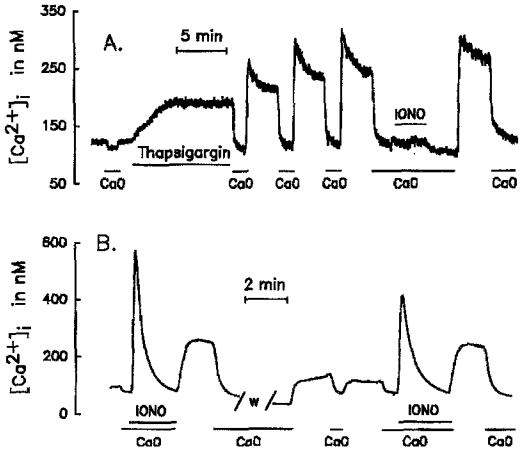


Fig. 7A, B Comparison of repetitive $[Ca^{2+}]_i$ overshoots following treatment with either thapsigargin or ionomycin. Perfusion of cells was switched from a Ca^{2+}-containing medium to Ca^{2+}-free medium before and after treatment with either thapsigargin (0.5 μM; **A**) or ionomycin (100 nM; **B**). The ionomycin was washed after the first overshoot by perfusion with Ca^{2+}-free medium containing serum albumin 2% (w). The traces are representative of 10 (**A**) and 4 (**B**) similar experiments

the effect of thapsigargin on the endomembrane Ca^{2+} -ATPase activity [29] and see above). Addition of ionomycin in Ca^{2+}-free medium confirmed that the stores remained empty (Fig. 7A) and did not modify the size of the subsequent $[Ca^{2+}]_i$ overshoot. When the Ca^{2+} stores were emptied with ionomycin instead of with thapsigargin, alternate perfusion with Ca^{2+}-free and Ca^{2+}-containing media produced a large $[Ca^{2+}]_i$ overshoot the first time, which rapidly adapted in successive pulses (Fig. 7B). This may be due to refilling of the Ca^{2+} stores during perfusion with Ca^{2+}-containing medium and the consequent deactivation of capacitative Ca^{2+}entry. In fact, the last ionomycin pulse in Fig. 7B (in Ca^{2+}-free medium) produced a large $[Ca^{2+}]_i$ peak, suggesting that the stores had refilled to a large extent. In the experiments shown in Fig. 8, the intracellular Ca^{2+} stores were emptied by treatment with 100 nM ionomycin for 1 min in Ca^{2+}-free medium. Then ionomycin was removed by washing with Ca^{2+}-free, albumin-containing medium for 10 min. A new addition of ionomycin at this stage did not produce a $[Ca^{2+}]_i$ peak, indicating that the Ca^{2+} stores were completely empty (results not shown). Incubation of these cells in standard Ca^{2+}-containing medium resulted in an increase of $[Ca^{2+}]_i$ (Fig. 8A) and, 3 min later, the intracellular Ca^{2+} stores had refilled completely. This is shown by the fact that the $[Ca^{2+}]_i$ peak produced by a new ionomycin addition (in Ca^{2+}-free medium) was of the same size as the control, before any ionomycin treatment (compare the first and last "iono" peaks in Fig. 8A). Both the increase of $[Ca^{2+}]_i$ upon Ca^{2+} readdition and the refilling of the stores were partially prevented by nisoldipine (Fig. 8B) and completely abolished by Ni^{2+} (Fig. 8C). Addition of either nisoldipine or Ni^{2+} 30 s before ionomycin had no effect on the initial $[Ca^{2+}]_i$ peak induced by the ionophore. These results indicate that Ca^{2+} entry from the external medium (either through the capacitative Ca^{2+} entry pathway or through voltage-operated Ca^{2+}channels) and the subsequent increase in $[Ca^{2+}]_i$ are required for store refilling. This is consistent with previous results in non-excitable cells [24].

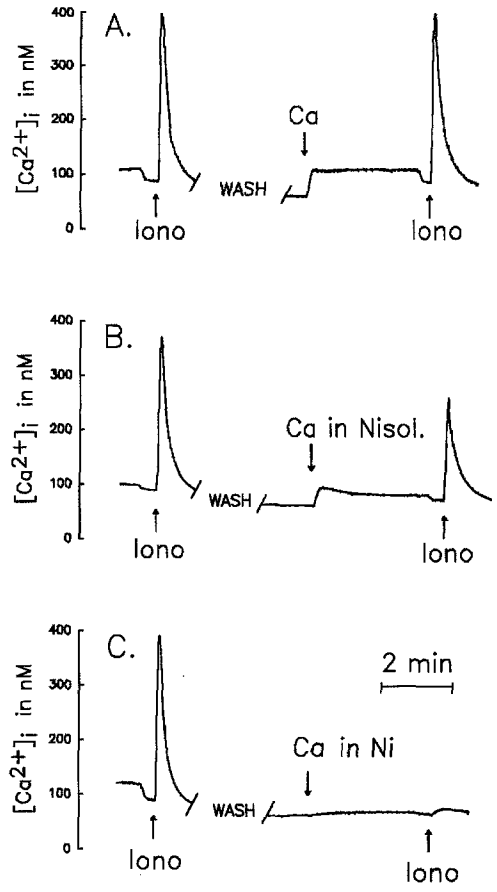


Fig. 8A–C Refilling of the intracellular Ca^{2+} stores is dependent on the $[Ca^{2+}]_i$ increase. (**A**) Cells were treated with 100 nM ionomycin in Ca^{2+}-free medium to empty the intracellular Ca^{2+} stores (first $[Ca^{2+}]_i$ peak). Then the ionophore was washed out by perfusion with Ca^{2+}-free medium containing 2% albumin for 5 min and external Ca^{2+} was added to allow refilling of the stores. Finally, the filling level of the stores was assessed by a second addition of ionomycin (100 nM). **B, C** The protocol was the same, except that external Ca^{2+} was added together with 1 μM nisoldipine (**B**) or 5 mM Ni^{2+} (**C**)

Characterization of the Ca^{2+} signal in response to TRH

Figure 9A compares the average changes in $[Ca^{2+}]_i$ produced by TRH in a population of GH_3 cells in the presence and in the absence of extracellular Ca^{2+}. In the absence of Ca^{2+} TRH produced a sharp $[Ca^{2+}]_i$ peak due to Ca^{2+} release from the intracellular Ca^{2+} stores (phase 1). Then $[Ca^{2+}]_i$ returned to or below the resting level observed before TRH addition within 2–5 min (Fig. 9A; "-Ca" trace). When external Ca^{2+} was present ("CONT." trace) the sharp $[Ca^{2+}]_i$ increase was followed by a maintained plateau (phase 2) which is the expression of Ca^{2+} entry from the extracellular medium. On close inspection of phase 2 two components could be distinguished (Fig. 9A). Immediately after the sharp $[Ca^{2+}]_i$ peak, there was a shoulder, sometimes showing a secondary $[Ca^{2+}]_i$ maximum, which decreased slowly to a steady level within 2–4 min (phase 2a). This steady level, well above the resting $[Ca^{2+}]_i$ before TRH addition, was usually maintained for 15–30 min (phase 2b).

Addition of 5 mM Ni^{+} at any time during phase 2 brought $[Ca^{2+}]_i$ to the same values as those observed in the absence of external Ca^{2+} within a few seconds (results not shown). Activation of a plasma membrane Ca^{2+} entry pathway by TRH treatment was confirmed using Mn^{2+} as a surrogate for Ca^{2+}. In the experiment of Fig. 9B and C Mn^{2+} (50 μM) was added to the extracellular medium (which also contained 1 mM Ca^{2+}) and changes in $[Ca^{2+}]_i$ (ratio F_{340}/F_{380}, panel B) and Mn^{2+} entry (quenching of F_{360}, panel C) were measured simultaneously (see Materials and methods). Upon

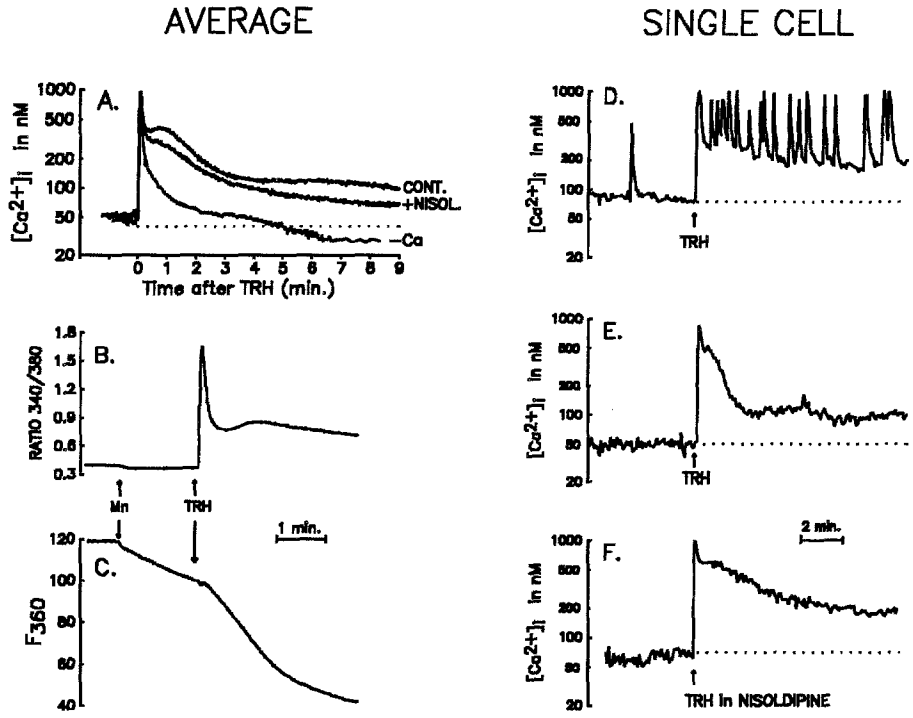
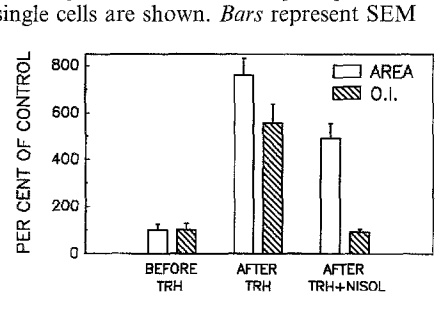


Fig. 9A–F Effects of TRH on $[Ca^{2+}]_i$ and on Mn^{2+} entry into GH_3 cells. **A** The effects of TRH (100 nM) on a population of GH_3 cells in control medium (*CONT.*), in the presence of 1 μM nisoldipine (+ *NISOL*) or in the absence of extracellular Ca^{2+} (*-Ca*). This experiment is representative of 5 similar ones. **B** Changes in the ratio of the fluorescences excited at 340 nm and 380 nm, used here as a index of $[Ca^{2+}]_i$. **C** The fluorescence excited at 360 nm was measured simultaneously and used as a index of Mn^{2+} entry. Mn^{2+} (50 μM) and TRH (100 nM) were added as shown. **D–F** Typical traces obtained in oscillating (**D**) and non-oscillating (**E**) single cells. **F** The cells had been treated with 1 μM nisoldipine

addition of TRH there was a $[Ca^{2+}]_i$ peak followed by a sustained plateau (Fig. 9B, compare with Fig. 9A). On the other hand, TRH induced, after a short lag of about 15 s, an increase in the rate of F_{360} quenching (Fig. 9C), which demonstrates an acceleration of Mn^{2+} entry.

Measurements of $[Ca^{2+}]_i$ at the single-cell level allowed identification of the same two phases after TRH stimulation. Figure 9D shows the most common pattern of response. Many of the cells showed spontaneous $[Ca^{2+}]_i$ oscillations not synchronized among different cells (see first $[Ca^{2+}]_i$ peak in trace D). Upon TRH addition there was an early $[Ca^{2+}]_i$ peak (phase 1), which was synchronized in all cells. This was followed by asynchronous $[Ca^{2+}]_i$ oscillations, which were more frequent than the spontaneous ones and continued until the end of the experiment (phase 2). These $[Ca^{2+}]_i$ oscillations usually arose from a $[Ca^{2+}]_i$ plateau well above the resting level before TRH addition (Fig. 9D). In a fraction of cells showing no $[Ca^{2+}]_i$ oscillations, TRH addition produced an early synchronized $[Ca^{2+}]_i$ peak followed by a sustained plateau (Fig. 9E). Statistical analysis of phase 2 (integration period chosen to be the last 4 min of a 6-min period of TRH stimulation) in 342 single cells from 7 different batches showed a significant increase of both the mean $[Ca^{2+}]_i$ increase (160 ± 5 vs 54 ± 3 nM in the control, before TRH; mean ± SEM) and the oscillation index (53 ± 2 vs 19 ± 2 nM/3 s). From the above results, it seems clear that two different components may contribute to the $[Ca^{2+}]_i$ increase seen during phase 2: (1) a sustained $[Ca^{2+}]_i$ increase, and (2) an increase in the frequency of $[Ca^{2+}]_i$ oscillations. The sustained $[Ca^{2+}]_i$ increase tended to be more prominent soon after TRH addition and then to decrease with time, whereas the $[Ca^{2+}]_i$ oscillations, once they appeared, remained increased for several minutes. This may be responsible for the transition between phases 2a and 2b seen in the averaged $[Ca^{2+}]_i$ signal. The statistical analysis of the single-cell results was consistent with this interpretation. The oscillation index, a parameter which quantifies purely $[Ca^{2+}]_i$ oscillations, increased after treatment with, TRH but the increase was similar during either phase 2a or 2b (56 ± 2 and 49 ± 2 nM /3 s compared to 29 ± 2 nM/3 s before TRH treatment; mean ± SEM, n = 342; phase 2a defined as 2–4 min after TRH addition, phase 2b as 4–6 min). The mean $[Ca^{2+}]_i$ increase, which is sensitive to both $[Ca^{2+}]_i$ oscillations and sustained $[Ca^{2+}]_i$ changes, was larger during phase 2a than during phase 2b (193 ± 7 vs 128 ± 4 nM, compared to 54 ± 3 nM before TRH). Inhibition by DHPs tended to be larger during phase 2b than during phase 2a (see below).

Fig. 10 Effects of nisoldipine (1 μM) on the $[Ca^{2+}]_i$ response to TRH (100 nM). Average effects on the mean $[Ca^{2+}]_i$ and oscillation index of 27–33 single cells are shown. *Bars* represent SEM



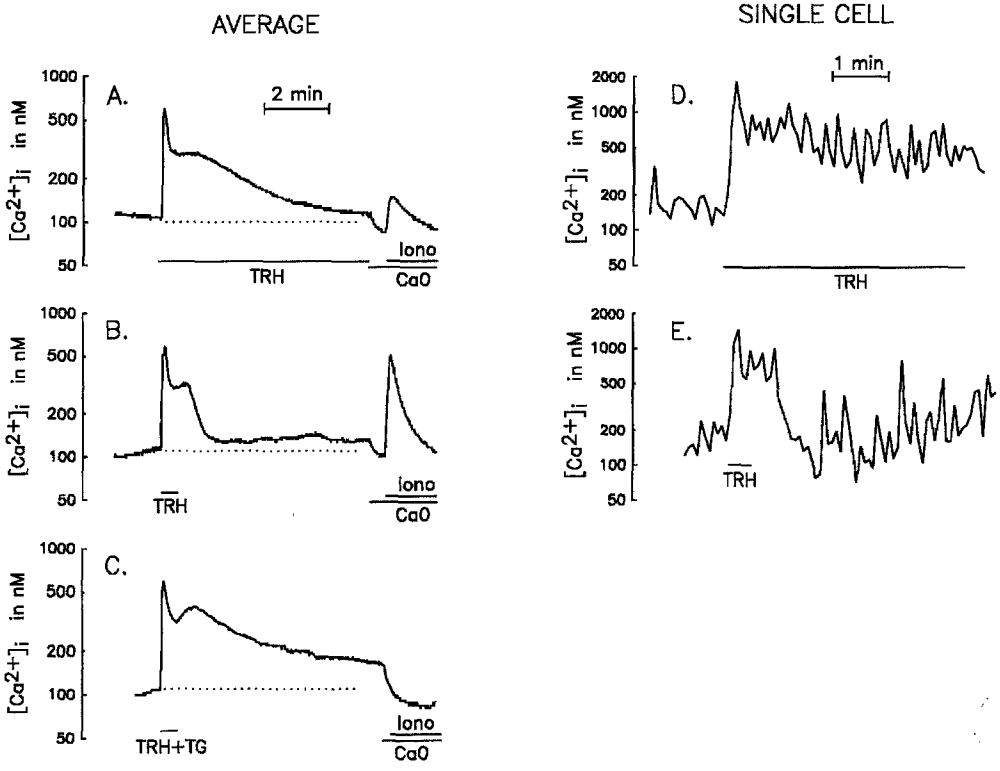


Fig. 11A–E Comparison of the effects of long and short treatments with TRH on $[Ca^{2+}]_i$ and on the Ca^{2+} contents of the intracellular Ca^{2+} stores. Except when indicated otherwise, the incubation medium contained 1 mM Ca^{2+}. The concentrations of TRH and ionomycin were both 100 nM. Thapsigargin (*TG*) was 300 nM

$[Ca^{2+}]_i$ oscillations but not sustained Ca^{2+} entry are blocked by DHPs

DHPs blocked $[Ca^{2+}]_i$ oscillations, either those that were spontaneous (see above) or induced by TRH. The effect of nisoldipine on the average $[Ca^{2+}]_i$ increase induced by TRH in a population of GH_3 cells is shown without comment in Fig. 9A ("+NISOL" trace). Phase 1 was not substantially modified. Phase 2 was inhibited but not abolished by nisoldipine (compare to trace "-Ca"). Percentage inhibition varied with the cell batch (mean ± SEM, 33 ± 8% in four experiments, measured 2 min after TRH addition), but in all cases it was incomplete. Figure 9F shows the results in a representative single cell. DHPs abolished $[Ca^{2+}]_i$ oscillations, but not the sustained $[Ca^{2+}]_i$ increase. Statistical analysis of the results in 33 single cells (compared to 27 DHP-untreated cells of the same batch) is summarized in Fig. 10. DHPs prevented completely the increase of the oscillation index seen during phase 2, but inhibited only partially the mean $[Ca^{2+}]_i$ increase. These results reinforce the idea that two different components, one sensitive and the other insensitive to DHPs, contribute to the increase of $[Ca^{2+}]_i$ during phase 2 of stimulation by TRH.

The continued presence of TRH is required for sustained Ca^{2+} entry but not for $[Ca^{2+}]_i$ oscillations

Results in Fig. 11 further advance more evidence for the dual nature of the TRH-induced $[Ca^{2+}]_i$ entry. When TRH (but not Ca^{2+}) was removed from the perfusion medium soon after the first $[Ca^{2+}]_i$ peak, $[Ca^{2+}]_i$ dropped suddenly (Fig. 11B, compare to Fig. 11A). Single-cell studies (traces on the right hand side) revealed that short (20 s) TRH pulses produced an increase of $[Ca^{2+}]_i$ oscillations which was maintained for several minutes, but did not produce the sustained (non-oscillatory) $[Ca^{2+}]_i$ increase (Fig. 11E, compare to Fig. 11D). The values for the oscillation indexes before and 3 min after a 20-s treatment with TRH were (mean ± SEM) were 12 ± 1 and 32 ± 4, respectively (n = 85; $P < 0.001$, Student's t-test). DHPs completely prevented the increase of $[Ca^{2+}]_i$ during phase 2 in this type of experiments (results not shown). The above-described results indicate that the continued presence of TRH is required for the maintained Ca^{2+} entry of phase 2, but not for Ca^{2+} oscillations. This is consistent with the observation that TRH-induced electrical activity remains increased for several minutes after TRH removal [4].

Ionomycin (in Ca^{2+}-free medium) was added at the end of the traces shown in Figs. 11A, B in order to estimate the Ca^{2+} content of the intracellular stores. Note that, in the continuous presence of TRH, the Ca^{2+} stores remained almost empty (Fig. 11A), whereas they refilled to a large extent after removal of TRH (Fig. 11B). In Fig. 11C thapsigargin was added during the short pulse together with TRH in order to prevent store refilling. The second phase of the response to TRH was enlarged to values similar to or above those obtained by continued stimulation by TRH.

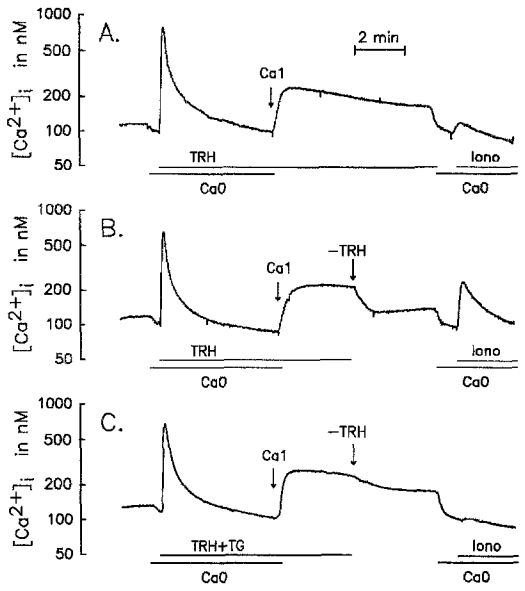


Fig. 12A–C $[Ca^{2+}]_i$ overshoots following treatment with TRH. Averaged signals of the whole cell populations are shown. The effects of TRH removal (-TRH) and the size of the Ca^{2+} stores at the end of the experiment (*Iono*) are also illustrated. Except when indicated otherwise, the incubation medium contained 1 mM Ca^{2+}. The concentrations of TRH and ionomycin were both 100 nM. Thapsigargin was used at 300 nM

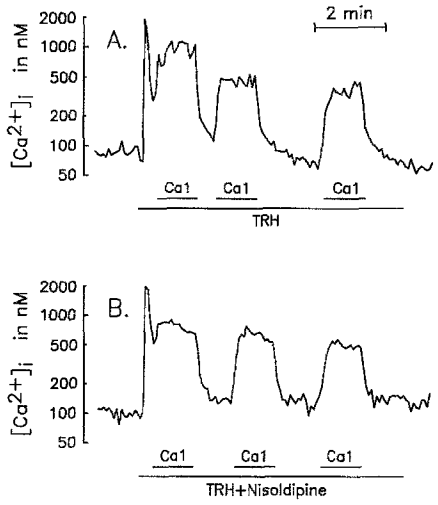


Fig. 13 A, B Effects of nisoldipine on the $[Ca^{2+}]_i$ overshoots in GH_3 cells treated with TRH. Averaged signals of the whole cell populations are shown. Except when indicated otherwise, the incubation medium contained no Ca^{2+}. Concentrations of TRH and nisoldipine were 100 nM and 0.5 μM, respectively

Addition of ionomycin at the end of the experiment confirmed that the intracellular Ca^{2+} stores remained empty. Thus, the sustained Ca^{2+} entry observed during phase 2 seems to be associated in all cases with empty Ca^{2+} stores.

Emptying and refilling of the intracellular Ca^{2+} stores after treatment with TRH: correlation with Ca^{2+} overshoots

Modifications of the plasma membrane permeability to Ca^{2+} can also be estimated from the size of the $[Ca^{2+}]_i$ overshoots obtained upon switching the perfusion from a Ca^{2+}-free to a Ca^{2+}-containing medium. Figure 12A shows that addition of Ca^{2+} to cells which had been treated with TRH in Ca^{2+}-free medium produced a large $[Ca^{2+}]_i$ overshoot. Addition of ionomycin in Ca^{2+}-free medium at this stage showed that the intracellular Ca^{2+} stores were almost empty. Figure 12B shows that removal of TRH at the top of a $[Ca^{2+}]_i$ overshoot caused a rapid decrease of $[Ca^{2+}]_i$ to about 50% of the previous level. This was accompanied by partial refilling of the stores (late "iono" pulse in Fig. 12B). These results suggest that, upon removal of TRH, the Ca^{2+} stores refill and Ca^{2+} entry decreases. If the experiment of Fig. 12B was repeated in the presence of thapsigargin, the $[Ca^{2+}]_i$ decrease upon TRH removal was much smaller and the Ca^{2+} stores did not refill (Fig. 12C). Therefore it seems that, as shown above for cells whose stores had been depleted by ionomycin, a high permeability to Ca^{2+} is maintained while the Ca^{2+} stores are empty, but it drops quickly when they refill upon removal of TRH.

The time course of store refilling after emptying with either ionomycin or TRH was studied in some detail. Upon removal of ionomycin (by albumin wash in Ca^{2+}-free medium) refilling proceeded steadily to reach 100% within about 5 min ($t_{½}$ = 2 min). Following treatment with TRH the stores refilled quickly ($t_{½}$ = 0.5 min) to about 50% of their initial content. Refilling to 100% was much slower ($t_{½}$ > 10 min). These results suggest that TRH may also have a long-lasting effect, perhaps mediated through protein phosphorylation, on the refilling mechanism of a fraction of the intracellular Ca^{2+} stores. Alternatively, the store-mobilizing effect of TRH may remain partly activated for some time after removal of TRH.

The $[Ca^{2+}]_i$ overshoots observed after treatment with TRH were little modified by inhibitors of voltage-operated Ca^{2+} channels. This is documented in Fig. 13, where the overshoots obtained in TRH-treated cells were similar in the absence (A) and in the presence (B) of 0.5 μM nisoldipine.

Discussion

We report here that emptying the intracellular Ca^{2+} stores of GH_3 cells, either by blocking Ca^{2+} accumulation into them with thapsigargin or BHQ, or by increasing the leak with ionomycin, induces a Ca^{2+} entry through the plasma membrane. This Ca^{2+} entry does not take place through voltage-operated Ca^{2+} channels, since it is not sensitive to DHPs which fully abolished the high-K^+-induced $[Ca^{2+}]_i$ increase.

Because the above-mentioned treatments do not induce inositolphosphate production [1, 16] the observed Ca^{2+} influx cannot be mediated by these messengers. Ca^{2+} influx correlated negatively with the degree of filling of the stores (Fig. 3C) and refilling restored a low permeability to Ca^{2+} (Fig. 7B). These properties are similar to the capacitative Ca^{2+} entry which arises by activation of a plasma membrane pathway upon emptying the intracellular Ca^{2+} stores of many non-excitable cells [3, 25]. Reports on the existence of this mechanism are scarce in excitable cells, where the "noise" of residual Ca^{2+} influx though volatge-operated Ca^{2+} channels may make it harder to demonstrate. Capacitative Ca^{2+} entry remained elusive in chromaffin cells [28], but was finally demonstrated [26]. Thapsigargin-induced Ca^{2+} entry could not be documented in a recent report with GH_3 cells [34], but this may have been due to Ca^{2+} buffering by quin2, which was the dye used in that work. Finally, the biphasic $[Ca^{2+}]_i$ increase reported previously in GH_3 cells treated with low concentrations of ionomycin [1] can now be reinterpreted in light of the results shown in this paper. The late $[Ca^{2+}]_i$ increase observed was attributed to Ca^{2+} entry due to the ionophore effect of ionomycin at the plasma membrane level [1]. However, we show here that this late Ca^{2+} entry remains after washing off the ionophore and is prevented by Ni^{2+}, which does not interfere with the ionophore effect of ionomycin [22]. Even more relevant, the Ca^{2+} overshoots induced by thapsigargin are not increased by low concentrations of ionomycin, suggesting that the same mechanism, namely capacitative Ca^{2+} entry, is activated by both treatments.

Capacitative Ca^{2+} entry of GH_3 cells shows some differences from the similar mechanisms described previously in several types of blood cells. In these, the capacitative Ca^{2+} entry pathway was permeable to Mn^{2+}, and cytochrome P_{450} inhibitors were effective blockers [3, 22]. In GH_3 cells the entry of Mn^{2+} through the capacitative Ca^{2+} entry pathway seems much more sluggish than the entry of Ca^{2+} (Fig. 4) and cytochrome P_{450} antagonists have little or no inhibitory effect (Fig. 5). Poor Mn^{2+} entry through the capacitative Ca^{2+} entry pathway has also been reported in parotid [20, 21] and lachrymal acinar cells [15], in chromaffin cells [26] and in hepatocytes [17]. We also find little effect of cytochrome P_{450} inhibitors in hepatocytes (unpublished results). These differences are puzzling and open the question as to whether several kinds of capacitative Ca^{2+} entry may exist and be distributed differently among different cell kinds.

Paradoxically, in GH_3 cells, the other main Ca^{2+} entry mechanism, voltage operated Ca^{2+} channels, allows entry of Mn^{2+} and is sensitive to cytochrome P_{450} inhibitors [31].

We document here that Ca^{2+} entry through a capacitative Ca^{2+} entry pathway correlates with the increase of $[Ca^{2+}]_i$ seen during phase 2 of TRH action. The entry of Ca^{2+} (and Mn^{2+}) induced by TRH is composed of a sustained DHP-insensitive component and DHP-sensitive $[Ca^{2+}]_i$ oscillations. The latter would reflect the contribution of voltage-operted Ca^{2+} channels, the firing frequency of which is known to be increased by TRH [5]. This effect is due to inhibition, by phosphorylation, of an inwardly rectifying K^+ channel, thus causing membrane depolarization and increased action potential firing rate [4, 7]. The DHP-insensitive component of Ca^{2+} entry is due to activation of capacitative Ca^{2+} entry by emptying of the Ca^{2+} stores induced by TRH, which is able to release about 90% of the ionomycin-sensitive stored Ca^{2+} pool (Fig. 1). Both Ca^{2+} entry components differ also in their kinetics of disappearance after TRH removal. $[Ca^{2+}]_i$ oscillations remain for some minutes after TRH removal. This is not unexpected as they arise ultimately from the TRH-induced phosphorylation of plasma membrane K^+ channels [4, 7]. In contrast, the sustained DHP-insensitive Ca^{2+} entry disappears shortly after TRH removal. This is due to inactivation of capacitative Ca^{2+} entry owing to rapid refilling of the intracellular Ca^{2+} stores (Figs. 11, 12). The observation that sustained Ca^{2+} entry remains after TRH removal if store refilling is prevented by thapsigargin reinforces this view.

In summary, the present results indicate that capacitative Ca^{2+} entry, which is responsible for the sustained $[Ca^{2+}]_i$ increase induced by agonists in many non-excitable secretory cells [25], may contribute to a similar function in excitable cells in close cooperation with voltage-gated Ca^{2+} channels. This view explains why DHPs, which abolish action potentials in GH_3 cells, are unable to prevent completely the TRH-induced Ca^{2+} entry. Cooperation of both mechanisms enlarges the $[Ca^{2+}]_i$ reached at the top of the oscillations and hence, presumably, the final physiological output of stimulation by TRH. The sustained $[Ca^{2+}]_i$ increase may also sensitize the release of secretory vesicles by the $[Ca^{2+}]_i$ oscillations. Further research will be required to gain a better understanding of the reasons for such a dual pathway.

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References

1. Albert PR, Tashjian AH Jr (1984) Iomomycin acts as an ionophore to release TRH-regulated Ca^{2+} stores from GH_4C_1 cells. J Biol Chem. 259:5827–5832
2. Albert PR, Tashjian AH Jr (1984) Relationship of thyrotropin-releasing hormone-induced spike and plateau phases in cytosolic free Ca^{2+} concentrations to hormone secretion. Selective blockade using iomomycin and nifedipine J Biol Chem 259: 15350–15363
3. Alvarez J, Montero M, García-Sancho J (1992) Cytochrome P_{450} may regulate plasma membrane Ca^{2+} permeability according to

the filling state of the intracellular Ca^{2+} stores. FASEB J 6: 786–792
4. Barros F, Villalobos C, García-Sancho J, Camino D, Peña P (1994) The role of the inwardly rectifying K^+ current in resting potential and thyrotropin-releasing hormone-induced changes in cell excitability of GH_3 rat anterior pituitary cells. Pflügers Arch 426:221–230
5. Bjoro T, Sand O, Ostberg BC, Gordeladze JO, Torjesen P, Gautvik KM, Haug E (1990) The mechanisms by which vasoactive intestinal peptide (VIP) and thyrotropin-releasing hormone (TRH) stimulate prolactin release from the pituitary cells. Biosci Rep 10:189–199
6. Clementi E, Scheer H, Zacchetti D, Fasolato C, Pozzan T, Meldolesi J (1992) Receptor-activated Ca^{2+} influx. Two independently regulated mechanisms of influx stimulation coexist in neurosecretory PC12 cells. J Biol Chem 267:2164–2172
7. Delgado LM, Peña P, Camino D, Barros F (1992) Okadaic acid and calyculin a enhance the effect of thyrotropin-releasing hormone on GH_3 rat anterior pituitary cells excitability. FEBS Lett 311:41–45
8. García-Sancho J, Alonso MT, Sánchez A (1989) Receptor-operated calcium channels in human platelets. Biochem Soc Trans 17:980–982
9. Gershengorn MC (1986) Mechanisms of thyrotropin-releasing hormone stimulation of pituitary hormone secretion. Annu Rev Physiol 48:515–526
10. Gershengorn MC, Geras E, Purrello VS, Rebecchi MJ (1984) Inositol trisphosphate mediates thyrotropin-releasing hormone mobilization of non-mitochondrial calcium in rat mammotropic pituitary cells. J Biol Chem 259:10675–10681
11. Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450
12. Hallam TH, Rink TJ (1985) Agonists stimulate divalent cation channels in the plasma membrane of human platelets. FEBS Lett 186:175–179
13. Jackson TR, Patterson SI, Thastrup O, Hanley MR (1988) A novel tumour promoter, thapsigargin, transiently increases cytoplasmic free Ca^{2+} without generation of inositol phosphates in NG115-401L neural cells. Biochem J 253:81–86
14. Kass GEN, Llopis J, Chow SC, Duddy SK, Orrenius S (1990) Receptor-operated calcium influx in rat hepatocytes. Identification and characterization using manganese. J Biol Chem 265:17486–17492
15. Kwan C, Putney, JW Jr (1990) Uptake and intracellular sequestration of divalent cations in resting and methacholine-stimulated mouse lacrimal acinar cells. Dissociation by Sr^{2+} and Ba^{2+} of agonist-stimulated divalent cation entry from the refilling of the agonist-sensitive intracellular pool. J Biol Chem 265: 678–680.
16. Law GH, Pachter JA, Thastrup O, Hanley MR, Dannies PS (1990) Thapsigargin, but not caffeine, blocks the ability of thyrotropin-releasing hormone to release Ca^{2+} from an intracellular store in GH_4C_1 pituitary cells. Biochem J 267:359–364
17. Llopis J, Kass GEN, Gahm A, Orrenius S (1992) Evidence for two pathways of receptor-mediated Ca^{2+} entry in hepatocytes. Biochem J 284:243–247
18. Matteson DR, Armstrong CM (1986) Properties of two types of calcium channels in clonal pituitary cells. J Gen Physiol 87: 161–183
19. Meldolesi J, Clementi E, Fasolato C, Zacchetti D, Pozzan T (1991) Calcium influx following receptor activation. Trends Pharmacol Sci 12:289–292
20. Merrit JE, Hallam TJ (1988) Platelets and parotid acinar cells have different mechanisms for agonist-stimulated divalent cation entry. J Biol Chem 263:6161–6164
21. Mertz LM, Baum BJ, Ambudkar IS (1990) Refill status of the agonist-sensitive Ca^{2+} pool regulates Mn^{2+} influx into parotid acini. J Biol Chem 265:15010–15014
22. Montero M, Alvarez J, García-Sancho J (1991) Agonist-induced Ca^{2+} influx in human neutrophils is secondary to the emptying of intracellular Ca^{2+} stores. Biochem J 277: 73–79
23. Montero M, Alvarez J, García-Sancho J (1992) Control of plasma membrane Ca^{2+} entry by the intracellular Ca^{2+} stores. Kinetic evidence for a short-lived mediator. Biochem J 288: 519–525
24. Montero M, Alonso-torre SR, Alvarez J, García-Sancho J (1993) The pathway for refilling intracellular Ca^{2+} stores passes through the cytosol in human leukemia cells. Pflügers Arch 424: 465–469
25. Putney JW Jr, Bird G St. J (1993) The inositol phosphate-calcium signalling system in nonexcitable cells. Endocrine Rev 14:610–631
26. Robinson IM, Cheek TR, Burgoyne RD (1992) Ca^{2+} influx induced by the Ca^{2+}-ATPase inhibitors 2, 5-di-(t-butyl)-1,4-benzohydroquinone and thapsigargin in bovine adrenal chromaffin cells. Biochem J 288:457–463
27. Schlegel W, Winiger BP, Mollard P, Vacher P, Wuarin F, Zahnd GR, Wollheim CB, Dufy B (1987) Oscillations of cytosolic Ca^{2+} in pituitary cells due to action potentials. Nature 329:719–721
28. Stauderman KA, Pruss RM (1989) Dissociation of Ca^{2+} entry and Ca^{2+} mobilization responses to angiotensin II in bovine adrenal chromaffin cells. J Biol Chem 264:18349–18355
29. Thastrup O (1990) Role of Ca^{2+}-ATPases in regulation of cellular Ca^{2+} signalling as studied with the selective microsomal Ca^{2+}-ATPase inhibitor thapsigargin. Agents Actions 29:9–15
30. Tsien RW, Lipscombe D, Madison DW, Bley KR, Fox AP (1988) Multiple types of neuronal calcium channels and their selective modulations. Trends Neurosci 11:431–438
31. Villalobos C, Fonteriz R, López, MG, García AG, García-Sancho J (1992) Inhibition of voltage-gated Ca^{2+} entry into GH_3 and chromaffin cells by imidazole antimycotics and other cytochrome P_{450} blockers. FASEB J 6:2742–2747
32. Williamson JR, Monck JR (1989) Hormone effects on cellular Ca^{2+} fluxes. Annu Rev Physiol 51:107–124
33. Winiger BP, Schlegel W (1988) Rapid transient elevations of cytosolic calcium triggered by thyrotropin-releasing hormone in individual cells of the pituitary line GH_3B_6. Biochem J 255: 161–167
34. Wong WL, Brostrom MA, Kuznetsov G, Gmitter-Yellen D, Brostrom CO (1993) Inhibition of protein synthesis and early protein processing by thapsigargin in cultured cells. Biochem J 289:71–79