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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

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Thapsigargin stimulates an increase of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) in, and $^{45}Ca^{2+}$ efflux from, a clone of GH_4C_1 pituitary cells. This increase in $[Ca^{2+}]_c$ was followed by a lower sustained elevation of $[Ca^{2+}]_c$, which required the presence of extracellular Ca^{2+}, and was not inhibited by a Ca^{2+}-channel blocker, nimodipine. Thapsigargin had no effect on inositol phosphate generation. We used thyrotropin-releasing hormone (TRH) to mobilize Ca^{2+} from an InsP_3-sensitive store. Pretreatment with thapsigargin blocked the ability of TRH to cause a transient increase in both $[Ca^{2+}]_c$ and $^{45}Ca^{2+}$ efflux. The block of TRH-induced Ca^{2+} mobilization was not caused by a block at the receptor level, because TRH stimulation of InsP_3 was not affected by thapsigargin. Rundown of the TRH-releasable store by Ca^{2+}-induced Ca^{2+} release does not appear to account for the action of thapsigargin on the TRH-induced spike in $[Ca^{2+}]_c$, because BAY K 8644, which causes a sustained rise in $[Ca^{2+}]_c$, did not block Ca^{2+} release caused by TRH. In addition, caffeine, which releases Ca^{2+} from intracellular stores in other cell types, caused an increase in $[Ca^{2+}]_c$ in GH_4C_1 cells, but had no effect on a subsequent spike in $[Ca^{2+}]_c$ induced by TRH or thapsigargin. TRH caused a substantial decrease in the amount of intracellular Ca^{2+} released by thapsigargin. We conclude that in GH_4C_1 cells thapsigargin actively discharges an InsP_3-releasable pool of Ca^{2+} and that this mechanism alone causes the block of the TRH-induced increase in $[Ca^{2+}]_c$.

INTRODUCTION

Many hormones and neurotransmitters act upon their target cells to evoke an increase in Ins(1,4,5)P_3 which releases Ca^{2+} from an intracellular store (reviewed by Berridge & Michell, 1988). The role of Ca^{2+} from intracellular as well as extracellular sources in mediating biological actions is a subject of intense investigation, and therefore considerable interest was generated when a novel tumour promoter, thapsigargin, was demonstrated to release Ca^{2+} from intracellular stores in platelets (Thastrup *et al.*, 1987*a*,*b*) and a neuronal cell line (Jackson *et al.*, 1988), and to do so in the neuronal cell line without generation of inositol phosphates (Jackson *et al.*, 1988). Ionophores can release Ca^{2+} stores without generation of InsP_3 (Albert & Tashjian, 1986). Thapsigargin, however, is not a Ca^{2+} ionophore, because it cannot transfer $^{45}Ca^{2+}$ from an aqueous to an organic phase (Ali *et al.*, 1985), and therefore must exert its actions in a more specific manner. Thapsigargin treatment in these cells blocked subsequent release of intracellular Ca^{2+} by agents that stimulate InsP_3 formation, indicating that thapsigargin depleted the Ins(1,4,5)P_3-releasable Ca^{2+} stores. We investigated whether thapsigargin exerted similar effects on Ca^{2+} mobilization in a pituitary tumour cell line and whether Ca^{2+} mobilized by thapsigargin mediated some of the reagent's effects. By combining two techniques of radiolabelled $^{45}Ca^{2+}$ efflux and fluorescent Ca^{2+}-dye measurements in one perifusion system, we were able to study changes in $[Ca^{2+}]_c$ and $^{45}Ca^{2+}$ efflux simultaneously.

MATERIALS AND METHODS

Cell culture

GH_4C_1 cells, transfected with a plasmid containing the cDNA for proinsulin (B. J. Reaves, C. M. Van Itallie, H. H. Moore & P. S. Dannies, unpublished work), were maintained in DMEM (Gibco) supplemented with 15% (v/v) horse serum (Hyclone) at 37 °C in a humidified atmosphere of CO_2/air (1:19). Cells were harvested with 1 mg of Viokase (pancreatin from pig pancreas; Sigma grade VI)/ml, and used within 2–4 h. We used these cells because they made more prolactin than did the untransfected GH_4C_1 cells (B. J. Reaves, C. M. Van Itallie, H. H. Moore & P. S. Dannies, unpublished work).

Measurement of $[Ca^{2+}]_c$

This was essentially as described previously (Law *et al.*, 1989*a*,*b*). Cells were incubated in loading buffer (120 mM-NaCl, 4.8 mM-KCl, 20 mM-$NaHCO_3$, 1.0 mM-$MgSO_4$, 1.8 mM-$CaCl_2$, 5 mM-glucose, 1 mg of BSA/ml and 10 mM-Hepes adjusted to pH 7.4 with NaOH) containing 10 μM-indo-1/AM for 30 min at 37 °C. Loaded cells [(2–3) × 10^6] were trapped between two layers of Bio-Gel P2 in a quartz flow cell to align cells with incident light in a SLM 4800s spectrophotometer. Excitation wavelength was 350 nm. Cells were perifused at 30 °C with loading buffer plus 5% horse serum at a flow rate of 1.5 ml/min; medium was changed by a three-way valve. $[Ca^{2+}]_c$ was monitored by measuring the ratio of fluorescence emitted at 390 nm to that at 475 nm.

Measurement of $^{45}Ca^{2+}$ efflux

A pellet of indo-1-loaded cells was taken up in 40 μl of loading buffer to which no $CaCl_2$ had been added, and 20 μl of $^{45}Ca^{2+}$ (1.94 mCi/ml; Amersham) was added to this. Cells were then incubated at 37 °C for 1 h before being placed in the perifusion system as described above. Simultaneous changes in $[Ca^{2+}]_c$ and $^{45}Ca^{2+}$ efflux were measured with this perifusion system. $^{45}Ca^{2+}$ efflux was measured in fractions collected each 1 min. At the end

Abbreviations used: $[Ca^{2+}]_c$, cytoplasmic free Ca^{2+} concentration; TRH, thyrotropin-releasing hormone (thyroliberin).
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of an experiment, cells were lysed with 10 mM-NaOH to determine their total content of $^{45}Ca^{2+}$, which was used to calculate a fractional efflux value (radioactivity in fraction divided by total radioactivity in cells at that time). Radioactivity was measured by liquid-scintillation spectrometry with Ultrafluor (National Diagnostics) as scintillator.

Measurement of prolactin secretion

Fractions collected from the perifusion system were also analysed to determine the amount of prolactin secreted by these cells. Prolactin was measured by a radioimmunoassay by using PRL standard RP-2 from N.I.H. Iodinated prolactin was from NEN (Boston) and anti-prolactin antiserum from Arnel Products (New York, NY, U.S.A.).

Measurement of inositol phosphates

Analysis of inositol phosphates was carried out as previously described (Pachter *et al.*, 1988*b*). In brief, GH_4C_1 cells were plated at a density of 3×10^5 cells/35 mm dish, and 24 h later 8–10 μCi of *myo*-[2-^{3}H]inositol/ml was added for 48 h. Prelabelled dishes were washed twice with 1 ml of Ham's F10 medium, and 20 μl of this medium containing the test substance, or not, was added appropriately. Reactions were terminated by addition of 1 ml of ice-cold 10 % (w/v) $HClO_4$ containing 3 mM-EDTA and 1 mM-diethylenetriaminepenta-acetic acid. Solutions were neutralized with 6 M-KOH and centrifuged at low speed to remove salt. Inositol phosphates were separated by Dowex anion-exchange chromatography as described by Pachter *et al.* (1988*b*).

RESULTS

Addition of 1 μM-thapsigargin to GH_4C_1 cells loaded with the fluorescent Ca^{2+} indicator indo-1 produced an increase in $[Ca^{2+}]_c$ that took about 1 min to reach a peak value, after which the level declined to a new steady-state value that was still above the pre-treatment value. The elevation in $[Ca^{2+}]_c$ was always found, but the magnitude of the initial rise varied; we show two extreme cases in Figs. 1(*a*) and 5(*a*). We used 1 μM-thapsigargin in the experiments in this paper. A dose–response curve for thapsigargin stimulation of $[Ca^{2+}]_c$ in human lymphocytes showed that 0.1 μM is sufficient to cause a maximal response, suggesting that 1 μM is in excess of maximal (Scharff *et al.*, 1988). Thapsigargin alone had no effect on prolactin secretion from these cells (Fig. 1*b*). We used TRH to release an Ins(1,4,5)P_3-sensitive Ca^{2+} store as previously reported for rat pituitary tumour cells (Gershengorn *et al.*, 1984). Addition of 100 nM-TRH invariably caused a much higher initial peak in $[Ca^{2+}]_c$ than that seen with thapsigargin (Fig. 1*a*). This initial peak was followed by a much lower sustained level, still above the pre-treatment value. This biphasic change in $[Ca^{2+}]_c$ was sometimes composed of two distinct and well-separated phases, rather than two over-lapping responses (compare Figs. 1*a* and 5*a*). Within 2 min of addition of TRH, the prolactin secretion rate had reached a peak 5-fold above the initial rate, and this was followed by a gradual decline over the next 10 min to a rate 1.5-fold the initial one (Fig. 1*b*). Pretreatment of cells with thapsigargin decreased the peak stimulation of $[Ca^{2+}]_c$ by 80 % and decreased the transient peak of prolactin secretion by 50 %, but had little or no effect on stimulation of sustained responses to TRH (Fig. 1). Thapsigargin might interfere with the TRH-induced rise in $[Ca^{2+}]_c$ at a step before the release of Ca^{2+}, such as generation of InsP_3 or binding to the TRH receptor. We therefore tested the effect of thapsigargin, alone, and on TRH-stimulated changes of inositol phosphates. Fig. 2 shows that thapsigargin had no effect on InsP_1, InsP_2, InsP_3 or InsP_4, either under basal conditions or in the presence of TRH.

To determine if the rise in $[Ca^{2+}]_c$ depended on intracellular Ca^{2+}, we investigated the effect of thapsigargin under conditions in which Ca^{2+} entry was substantially decreased. Because the 5 % serum used in the medium contains Ca^{2+}, we lowered the extracellular Ca^{2+} concentration by omitting the addition of 1.8 mM-$CaCl_2$ and titrated the medium with EGTA until opening the voltage-sensitive Ca^{2+} channels with 56 mM-KCl barely increased $[Ca^{2+}]_c$ (Fig. 3*a*). We reasoned that this titration end-point would minimize the loss of Ca^{2+} from intracellular stores. When cells were switched to this medium with 0.2 mM-EGTA, $[Ca^{2+}]_c$ declined for a few minutes and then stabilized at a new lower level (Fig. 3*a*). When the concentration of EGTA was then increased to 2 mM, a further decrease in $[Ca^{2+}]_c$ was detected (results not shown), so the lower limit of detection of $[Ca^{2+}]_c$ was below the level achieved in 0.2 mM-EGTA. In low-Ca^{2+}-containing medium, thapsigargin or TRH caused only a spike in $[Ca^{2+}]_c$, indicating that the initial rise in Ca^{2+} came from an intracellular source, but that any sustained rise requires extracellular Ca^{2+}. Thapsigargin considerably decreased the size of the Ca^{2+} spike associated with TRH (Fig. 3*a*) and almost completely blocked the TRH-induced spike in prolactin secretion

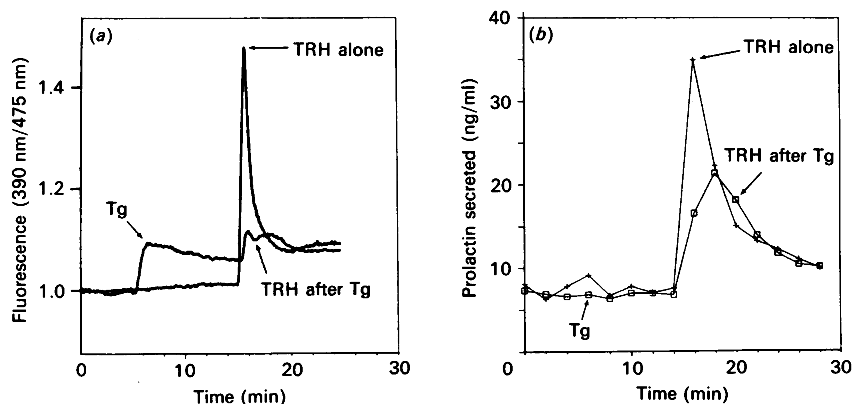

Fig. 1. Pretreatment of cells with thapsigargin inhibits TRH-induced changes in $[Ca^{2+}]_c$ and prolactin secretion

Time courses are shown for simultaneous changes in $[Ca^{2+}]_c$ (*a*) and prolactin secretion (*b*) from cells challenged with 1 μM-thapsigargin (Tg) and/or 100 nM-TRH. Tg was added at 5 min and TRH at 15 min. All Figures shown are representative traces for experiments that have been repeated at least twice.

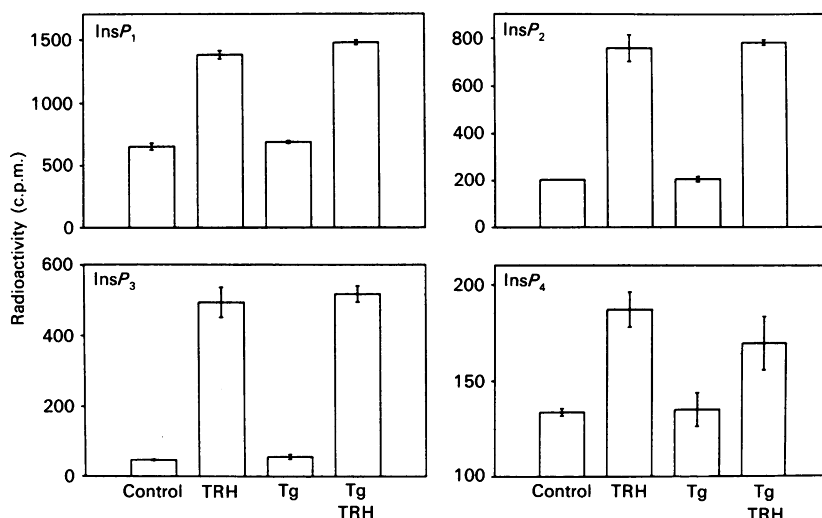

Fig. 2. Thapsigargin has no effect on generation of inositol phosphates

Cells were treated with either medium alone (control) or 1 μM-thapsigargin (Tg) for 12 min, and followed by addition of 100 nM-TRH, or medium alone, for 20 s. At the end of the experiment inositol phosphates were separated by Dowex chromatography. Total amounts (c.p.m.) of $InsP_1$, $InsP_2$, $InsP_3$ and $InsP_4$ are shown. Results are expressed as means of duplicates (bars indicate range) in a typical experiment.

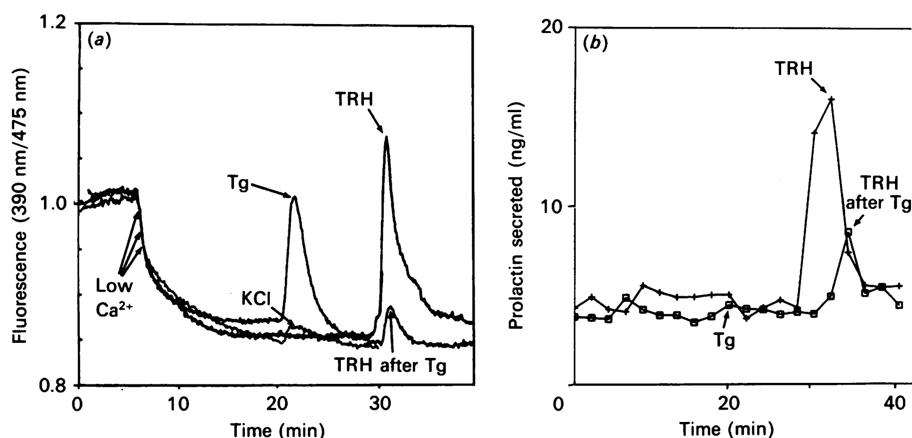

Fig. 3. Thapsigargin releases Ca^{2+} from an intracellular store and blocks a spike in $[Ca^{2+}]_c$ and prolactin secretion caused by TRH

Three perifusion columns were switched to a low-Ca^{2+}-containing medium at 5 min; 15 min later, 1 μM-thapsigargin (Tg) was added to one column, and 50 μM-KCl to another. At 30 min the remaining unchanged column received 100 nM-TRH, and so did the one perifused with Tg. Changes in $[Ca^{2+}]_c$ are shown for all three runs in (*a*). Changes in prolactin secretion for columns which had received Tg and/or TRH are shown in (*b*).

(Fig. 3*b*). The remaining increase in Ca^{2+} caused by TRH in the presence of thapsigargin may be because 1 μM is not a maximum dose of thapsigargin, or because there is a small store of internal Ca^{2+} susceptible to TRH and not to thapsigargin.

The requirement for extracellular Ca^{2+} may occur because thapsigargin stimulates Ca^{2+} entry, as indicated by Mn^{2+} quenching of the fura-2 signal in lymphocytes (Scharff *et al.*, 1988). Nimodipine, a Ca^{2+}-channel blocker, inhibited thapsigargin-induced platelet aggregation and 5-hydroxytryptamine release at a high concentration of 300 μM (Thapstrup *et al.*, 1987*a*). We tested 1 μM-nimodipine, a concentration sufficient to decrease substantially the Ca^{2+} entry stimulated by a maximally effective dose of BAY K 8644 (J. A. Pachter, G. J. Law & P. S. Dannies, unpublished work), and to lower $[Ca^{2+}]_c$ (Fig. 4). The pattern of change of $[Ca^{2+}]_c$ in the presence of nimodipine was similar to that in the absence of the Ca^{2+}-channel blocker (cf. Figs. 1*a* and 4); thapsigargin still caused an extended rise in $[Ca^{2+}]_c$ and still prevented the TRH-induced Ca^{2+} spike. Dihydropyridine-sensitive Ca^{2+} channels therefore do not appear to be involved in the action of thapsigargin or TRH in pituitary cells. The sustained rise in $[Ca^{2+}]_c$ induced by TRH is not increased in the presence of thapsigargin (Figs. 1*a* and 4), indicating that these two agents may modulate Ca^{2+} entry by a similar mechanism, which is maximally activated by TRH alone.

Thapsigargin could decrease the TRH-induced $[Ca^{2+}]_c$ peak by stimulating efflux of the Ca^{2+} released by TRH. To assess this possibility, we loaded the cells with $^{45}Ca^{2+}$ to monitor the amount of Ca^{2+} being released from the cells. Simultaneous changes in $[Ca^{2+}]_c$ and $^{45}Ca^{2+}$ efflux were then determined. In the experiment shown in Fig. 5, thapsigargin gave a large peak Ca^{2+} response, and this effect coincided with a 160 % increase in the fractional rate of $^{45}Ca^{2+}$ efflux. TRH was found to cause a similar

increase in $^{45}Ca^{2+}$ efflux to that produced by thapsigargin alone. Thapsigargin inhibited the TRH-induced increase in $[Ca^{2+}]_c$, and also inhibited TRH-induced release of $^{45}Ca^{2+}$.

These results indicate that thapsigargin prevented TRH from discharging an internal store of Ca^{2+}. If thapsigargin prevented TRH-induced Ca^{2+} release by discharging the same internal store, then TRH should decrease the thapsigargin-induced Ca^{2+} release. To test this possibility, we perifused cells in low-Ca^{2+}-containing medium to measure release dependent on intracellular Ca^{2+}, gave a maximally effective dose of TRH (1 μM), and then challenged with thapsigargin. Data in Fig. 6 are consistent with the idea that there is a substantial overlap of these two stores of Ca^{2+}, although thapsigargin appears to release proportionally more of the TRH store than TRH does of the thapsigargin store (cf. Figs. 3*a* and 6).

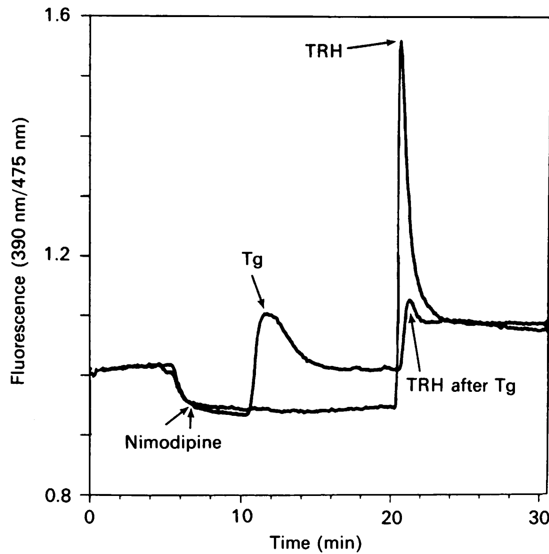

Fig. 4. Nimodipine does not block thapsigargin- and/or TRH-induced changes in $[Ca^{2+}]_c$

A time course for changes in $[Ca^{2+}]_c$ is shown: 1 μM-nimodipine was added at 5 min, 1 μM-thapsigargin (Tg) at 10 min and 100 nM-TRH at 20 min.

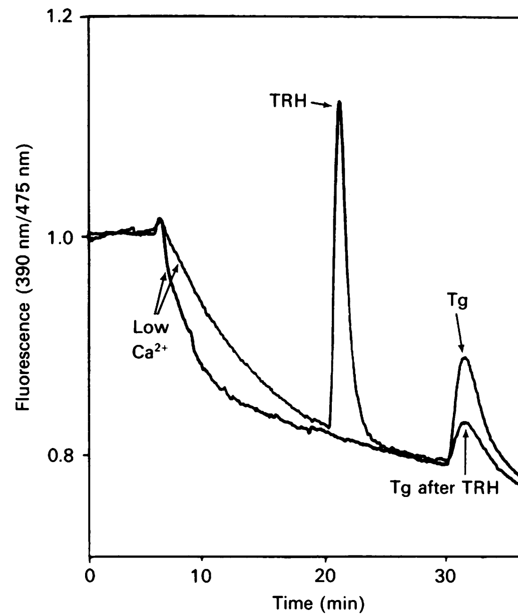

Fig. 6. Release of intracellular Ca^{2+} by thapsigargin is decreased by pretreatment of cells with TRH

Cells were switched to a low-Ca^{2+}-containing medium at 5 min. Additions of 1 μM-TRH (20 min) and 1 μM-thapsigargin (Tg) (30 min) were made as indicated, and representative changes in $[Ca^{2+}]_c$ are shown.

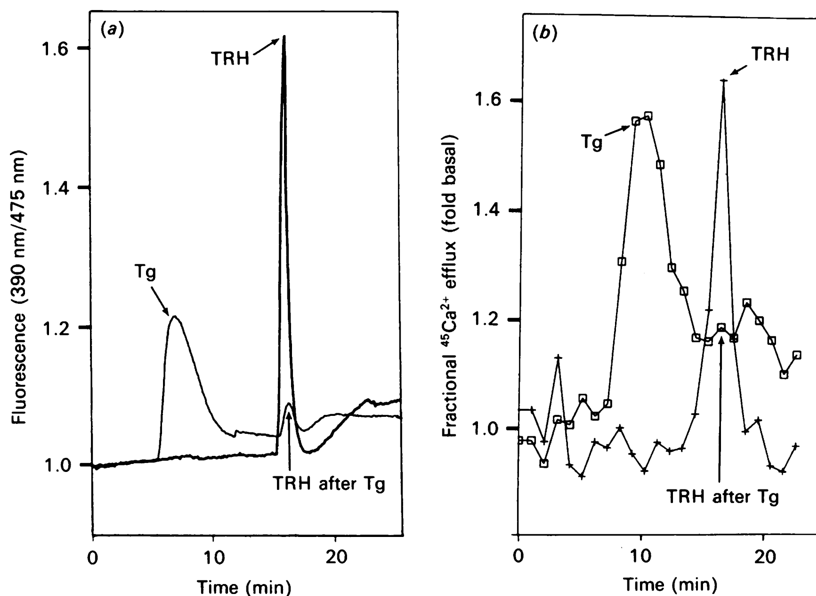

Fig. 5. Thapsigargin discharges Ca^{2+} ions from an InsP_3-sensitive Ca^{2+} store

Time courses are shown for concurrent changes in $[Ca^{2+}]_c$ and $^{45}Ca^{2+}$ efflux from perifused cells; 1 μM-thapsigargin (Tg) was added at 5 min and/or 100 nM-TRH at 15 min. Changes for $[Ca^{2+}]_c$ are shown in (*a*) and those for $^{45}Ca^{2+}$ efflux in (*b*). The peak $^{45}Ca^{2+}$ value for each run was approx. 3.9% (±0.7; range of two values) of total radioactivity in the cells at the time of measurement.

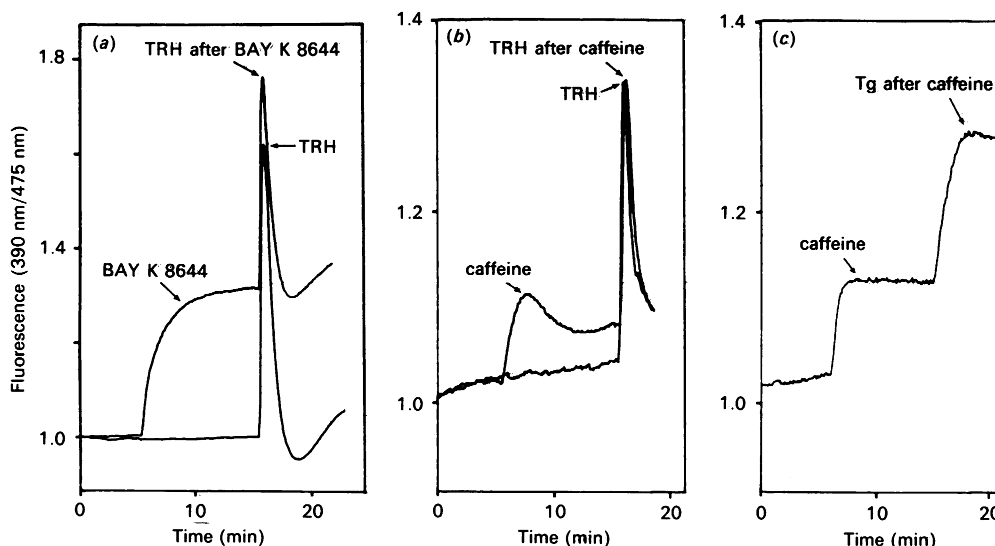

Fig. 7. An increase in $[Ca^{2+}]_c$ by BAY K 8644 or caffeine does not discharge an InsP_3-sensitive Ca^{2+} store

(*a*) Changes in $[Ca^{2+}]_c$ for additions of 1 μM-BAY K 8644 at 5 min and/or 100 nM-TRH at 15 min. (*b*) Changes in $[Ca^{2+}]_c$ for 10 mM-caffeine given at 5 min and/or 100 nM-TRH at 15 min. (*c*) Changes in $[Ca^{2+}]_c$ for 10 mM-caffeine added at 5 min and 1 μM-thapsigargin (Tg) at 15 min. Similar results were found in a total of 11 separate experiments.

Increases in $[Ca^{2+}]_c$ have been demonstrated to cause further increases in $[Ca^{2+}]_c$ in some muscle and neuronal cells (Fabiato, 1985; Lipscombe *et al.*, 1988), in a process known as Ca^{2+}-induced Ca^{2+} release. Thapsigargin could directly deplete the TRH-releasable stores, or it could indirectly deplete them by mobilizing Ca^{2+} from other sources that would trigger release from the TRH-releasable stores. To determine if Ca^{2+}-induced Ca^{2+} release were the cause of thapsigargin inhibition of TRH-induced Ca^{2+} release, we increased $[Ca^{2+}]_c$ in two other ways. We used BAY K 8644, which increases Ca^{2+} influx through voltage-dependent Ca^{2+} channels in GH_4C_1 pituitary cells (Enyeart *et al.*, 1987), and caffeine, which in muscle and nerve cells mobilizes Ca^{2+} from internal stores (Endo, 1975; Lipscombe *et al.*, 1988). BAY K 8644 (1 μM) consistently elevated $[Ca^{2+}]_c$ higher than the effect of thapsigargin, but did not stop TRH from stimulating $[Ca^{2+}]_c$ to a level at least as high as that caused by TRH alone (Fig. 7*a*). We found that 10 mM-caffeine caused an increase in $[Ca^{2+}]_c$ that was similar to that seen with thapsigargin alone, but, unlike thapsigargin, caffeine pretreatment did not change the response of $[Ca^{2+}]_c$ to TRH (Fig. 7*b*). The same dose of caffeine did not block the increase in $[Ca^{2+}]_c$ caused by addition of 1 μM-thapsigargin (Fig. 7*c*).

DISCUSSION

In this paper we used a perifusion system to measure changes in $[Ca^{2+}]_c$ in intact cells. $[Ca^{2+}]_c$ is subject to regulation by Ca^{2+} channels and Ca^{2+} pump/exchangers at the level of both plasma and internal membranes, and this makes it difficult to assign a change in $[Ca^{2+}]_c$ to any specific site in an intact cell. Intact cells, however, do retain all the vital components, which may act in concert to regulate $[Ca^{2+}]_c$ and are useful to investigate the overall effect of drugs. We have been able to extend the usefulness of the $[Ca^{2+}]_c$ measurements by simultaneously measuring hormone release or $^{45}Ca^{2+}$ efflux. As far as we know, these data presented are the first analysis of simultaneous changes in $[Ca^{2+}]_c$ and $^{45}Ca^{2+}$ efflux, as well as a biologically relevant output event, secretion.

We found that thapsigargin caused an increase in $[Ca^{2+}]_c$ in GH_4C_1 cells that did not trigger prolactin release. Certain increases in $[Ca^{2+}]_c$ alone appear to be capable of causing prolactin release, because high KCl concentrations (Law *et al.*, 1989*b*) and BAY K 8644 (Enyeart *et al.*, 1987; Pachter *et al.*, 1988*a*) trigger this response, but ionomycin does not release prolactin from GH_4C_1 cells, although it does cause a spike of $[Ca^{2+}]_c$ (Albert & Tashjian, 1986). The reason for differences among Ca^{2+}-elevating agents has not been identified. It is, however, thought-provoking that both KCl and BAY K 8644 initially mobilize Ca^{2+} from extracellular sources by opening plasma-membrane Ca^{2+} channels, whereas ionomycin and thapsigargin elevate $[Ca^{2+}]_c$, by both intracellular discharge and extracellular Ca^{2+} entry. The difference may be related to the spatial localization or kinetics of the $[Ca^{2+}]_c$ rise. Alternatively, voltage-dependent channel activation may have some other, as yet undefined, consequences that are essential for secretion. Measurement of changes in $[Ca^{2+}]_c$ in single cells may clarify differences.

We found that thapsigargin induced a biphasic $[Ca^{2+}]_c$ response in GH_4C_1 cells: an initial $[Ca^{2+}]_c$ peak that was followed by a decline to a sustained elevated level. A similar pattern of events is observed in a variety of cells such as platelets, lymphocytes, adrenal chromaffin cells and hepatocytes (reviewed by Hanley *et al.*, 1988). An exception to the pattern is the neuroblastoma cell line NG115–401L, which only shows a transient Ca^{2+} signal (Jackson *et al.*, 1988). The sustained elevation, in cell types that have it, is dependent on the presence of extracellular Ca^{2+}. The sustained rise seen at the population level may reflect a series of spikes when examined at the single-cell level, as reported for TRH (Winiger & Schlegel, 1988). Mn^{2+} quenching of the fura-2 signal indicates that Ca^{2+} entry is responsible for the sustained rise (Thastrup *et al.*, 1989). Nimodipine preferentially blocks L-type, voltage-sensitive, Ca^{2+} channels in GH_4C_1 cells (McCarthy & Cohen, 1987), but does not block an increase in $[Ca^{2+}]_c$ caused by thapsigargin, or stop thapsigargin's ability to block a spike in $[Ca^{2+}]_c$ caused by TRH. This indicates that the Ca^{2+} mobilized by thapsigargin does not pass through L-type channels. Both TRH and thapsigargin cause a sustained rise in $[Ca^{2+}]_c$ that depends on extracellular Ca^{2+} and that is not blocked by nimodipine. In addition, dopamine lowered $[Ca^{2+}]_c$ in normal pituitary cells, but did not prevent the extended rise in $[Ca^{2+}]_c$ caused by TRH (Law

et al., 1988). The sustained rise in Ca^{2+} could come from a direct entry of extracellular Ca^{2+} into the cytosol via nimodipine-insensitive Ca^{2+} channels, or via intracellular stores that require a supply of extracellular Ca^{2+} to maintain them. The cause of the extended rise in $[Ca^{2+}]_c$ is unknown at present; the data indicate, however, that a change in Ins(1,4,5)P_3 formation is not necessary for the extended rise.

We demonstrated that thapsigargin blocks the ability of TRH to cause a spike in $[Ca^{2+}]_c$ without changing the stimulation of inositol phosphates produced by TRH. This finding is similar to the effect of thapsigargin on bradykinin action in NG115-401L cells (Jackson *et al.*, 1988), and indicates that thapsigargin does not act at the level of ligand–receptor interaction. We also showed that thapsigargin most probably does not cause Ca^{2+}-induced Ca^{2+} depletion of TRH-releasable stores, because the sustained elevation of $[Ca^{2+}]_c$ produced by BAY K 8644 did not mimic the above action of thapsigargin on TRH (Fig. 7*a*, and Pachter *et al.*, 1988*a*). In addition, caffeine, considered to release Ca^{2+} from an intracellular store regulated by a mechanism of Ca^{2+}-induced Ca^{2+} release (see Lipscombe *et al.*, 1988), elevated $[Ca^{2+}]_c$ in GH_4C_1 cells without affecting release of Ca^{2+} induced by TRH. Caffeine appears to release intracellular Ca^{2+} from a store separate from that used by agents which stimulate InsP_3 accumulation (Kanaide *et al.*, 1987; Thayer *et al.*, 1988). Depleting the caffeine-sensitive store did not affect mobilization of $[Ca^{2+}]_c$ induced by TRH or thapsigargin. This is further evidence that thapsigargin is directly affecting the Ins(1,4,5)P_3-sensitive Ca^{2+} store. In addition to Ca^{2+} re-sequestration, Ca^{2+} efflux could rapidly dissipate a Ca^{2+} signal released from an intracellular store. Our data, however, indicate that thapsigargin has a direct effect on Ca^{2+} in an Ins(1,4,5)P_3-sensitive store, because thapsigargin simultaneously blocks increases both in $[Ca^{2+}]_c$ and in $^{45}Ca^{2+}$ efflux associated with the addition of TRH.

Thapsigargin may discharge the Ins(1,4,5)P_3-sensitive storage pool of Ca^{2+} by directly opening a pore by which Ca^{2+} is released. Recent evident in liver microsomes suggests that thapsigargin acts by an inhibition of uptake of Ca^{2+} by the endoplasmic-reticulum Ca^{2+} pump (ER-Ca^{2+}-ATPase), rather than a direct stimulation of efflux from intracellular stores (Thastrup *et al.*, 1989). It is apparent that, if inhibition of the ER-Ca^{2+}-ATPase alone accounts for the initial rise in $[Ca^{2+}]_c$ provoked by thapsigargin in intact cells, the Ins(1,4,5)P_3-sensitive pool must have a pronounced passive leak, or the pump arrest must be coupled to the induction of a leak.

In conclusion, thapsigargin induces a rise in cytosolic Ca^{2+} which subsequently impairs the ability of TRH to cause a $[Ca^{2+}]_c$ transient. This effect of thapsigargin occurs in the absence of detectable alterations of inositol phosphates, and is not reproduced by caffeine or agents that regulate Ca^{2+} entry via voltage-sensitive Ca^{2+} channels. Caffeine mobilizes cytosolic Ca^{2+} from a different source from that by thapsigargin in GH_4C_1 pituitary cells.

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