# Mobilization of $Ca^{2+}$ by thapsigargin and 2,5-di-(t-butyl)-1,4-benzohydroquinone in permeabilized insulin-secreting RINm5F cells: evidence for separate uptake and release compartments in inositol 1,4,5-trisphosphate-sensitive $Ca^{2+}$ pool

Md. Shahidul ISLAM and Per-Olof BERGGREN*

The Rolf Luft Center for Diabetes Research, Department of Edocrinology, Karolinska Institute, Box 60 500, Karolinska Hospital, S-104 01 Stockholm, Sweden

We characterized and directly compared the $Ca^{2+}$-releasing actions of two inhibitors of endoplasmic-reticulum (ER) $Ca^{2+}$-ATPase, thapsigargin and 2,5-di-(t-butyl)-1,4-benzohydroquinone (tBuBHQ), in electropermeabilized insulin-secreting RINm5F cells. Ambient free calcium concentration ($[Ca^{2+}]$) was monitored by $Ca^{2+}$-selective mini-electrodes. After ATP-dependent $Ca^{2+}$ uptake, thapsigargin and tBuBHQ released $Ca^{2+}$ with an $EC_{50}$ of $\sim 37$ nM and $\sim 2\ \mu$M respectively. Both agents mobilized $Ca^{2+}$ predominantly from the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool, and in this respect thapsigargin was more specific than tBuBHQ. The total increase in $[Ca^{2+}]$ obtained with thapsigargin and Ins(1,4,5)$P_3$ was, on the average, only 7% greater than that with Ins(1,4,5)$P_3$ alone. In contrast, the total increase in $[Ca^{2+}]$ obtained with tBuBHQ and Ins(1,4,5)$P_3$ was 33% greater than that obtained with only Ins$P_3$ ($P < 0.05$). Although $Ca^{2+}$ was rapidly mobilized by thapsigargin and tBuBHQ, complete depletion of the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool was difficult to achieve. After the release by thapsigargin or tBuBHQ, Ins(1,4,5)$P_3$ induced additional $Ca^{2+}$ release. The additional Ins(1,4,5)$P_3$-induced $Ca^{2+}$ release was not altered by supramaximal concentrations of thapsigargin and tBuBHQ, or by Bafilomycin $A_1$, an inhibitor of V-type ATPases, but was decreased by prolonged treatment with the ER $Ca^{2+}$-ATPase inhibitors. These results suggest the existence of distinct uptake and release compartments within the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool. When treated with the inhibitors, the two compartments became distinguishable on the basis of their $Ca^{2+}$ permeability. Apparently, thapsigargin and tBuBHQ readily mobilized $Ca^{2+}$ from the uptake compartment, whereas $Ca^{2+}$ from the release compartment could be mobilized only very slowly, in the absence of Ins(1,4,5)$P_3$.

## INTRODUCTION

The cytosolic free calcium concentration ($[Ca^{2+}]_i$) plays a key role in the regulation of diverse cellular processes, including the stimulus–secretion coupling in insulin-secreting cells [1]. Agonists can raise $[Ca^{2+}]_i$ either by stimulating influx of $Ca^{2+}$ through voltage-gated or receptor-operated $Ca^{2+}$ channels in the plasma membrane or by releasing $Ca^{2+}$ from intracellular pools. Intracellular non-mitochondrial $Ca^{2+}$ pools appear to be associated with the endoplasmic reticulum (ER), but may be distinct from ER in the form of a specialized $Ca^{2+}$-storing organelle [2,3]. Ins(1,4,5)$P_3$, formed after stimulation of surface receptors, releases $Ca^{2+}$ from specialized intracellular stores that possess receptors for the trisphosphate. In many cells, including insulin-secreting cells, part of the intracellular $Ca^{2+}$ pools is insensitive to Ins(1,4,5)$P_3$ [4,5]. The Ins(1,4,5)$P_3$-insensitive $Ca^{2+}$ pools may contain a caffeine-sensitive pool, responsible for $Ca^{2+}$-induced $Ca^{2+}$ release [6,7]. Both the Ins(1,4,5)$P_3$- and $Ca^{2+}$-induced $Ca^{2+}$ release may be involved in the generation of complex patterns of $Ca^{2+}$ signalling, such as $Ca^{2+}$ oscillations and propagating $Ca^{2+}$ waves [8].

$Ca^{2+}$ is sequestered into ER by an ATP-dependent $Ca^{2+}$ pump, embedded in the ER membrane (ER $Ca^{2+}$-ATPase). It has been suggested that functionally different $Ca^{2+}$ pools may contain different isoforms of the ER $Ca^{2+}$-ATPase [9] or may utilize different uptake mechanisms [10]. Inhibition of ER $Ca^{2+}$-ATPase leads to the release of $Ca^{2+}$, through unknown pathways. Thapsigargin is a highly potent and selective agent for inhibition of the sarcoplasmic- and endoplasmic-reticulum $Ca^{2+}$-ATPase family (SERCA) [11,12]. A more recent addition to the list of ER $Ca^{2+}$-ATPase inhibitors is 2,5-di-(t-butyl)-1,4-benzohydroquinone (tBuBHQ) [13]. Effects of tBuBHQ have been reported in fewer studies and some aspects of actions of the two inhibitors have been compared in different cells [14–17]. Both thapsigargin and tBuBHQ are being increasingly used as tools for defining intracellular $Ca^{2+}$ stores as well as for studying $Ca^{2+}$ fluxes and $Ca^{2+}$-dependent processes [15–25]. However, it appears that the intracellular $Ca^{2+}$ pools mobilized by these agents vary in different cell types. Thapsigargin, for instance, releases $Ca^{2+}$ from Ins(1,4,5)$P_3$-sensitive pool in many cells [15,21,26], whereas in others it affects both the Ins(1,4,5)$P_3$-sensitive and -insensitive pools [11,27]. The clonal cell line RINm5F, established from a rat insulinoma, is a useful experimental model of insulin-secreting cells [28,29]. In the present study, we characterized and directly compared thapsigargin- and tBuBHQ-induced $Ca^{2+}$ release in electropermeabilized RINm5F cells. Our study identifies the thapsigargin- and tBuBHQ-sensitive $Ca^{2+}$ pools and their relationship to the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool in these cells.

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free calcium concentration; $[Ca^{2+}]$, ambient free calcium concentration; ER, endoplasmic reticulum; tBuBHQ, 2,5-di-(t-butyl)-1,4-benzohydroquinone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; SERCA, sarcoplasmic- and endoplasmic-reticulum $Ca^{2+}$-ATPase.

* To whom correspondence should be addressed.

## MATERIALS AND METHODS

### Culture and permeabilization of cells

Clonal insulin-secreting RINm5F cells were maintained in culture in RPMI 1640 medium supplemented with 10% foetal-calf serum, penicillin (100 i.u./ml) and streptomycin (100 $\mu$g/ml), in a humidified incubator at 37 °C under an atmosphere of 5% $CO_2$ in air. Cells were detached from culture flasks with trypsin/EDTA, washed twice with RPMI 1640 medium and twice with a cold buffer with no added $Ca^{2+}$, containing 110 mM KCl, 10 mM NaCl, 2 mM $KH_2PO_4$, 1 mM $MgCl_2$, 0.5 mg/ml BSA and 25 mM Hepes (pH 7.0 adjusted with KOH). The contaminating $Ca^{2+}$ concentration in this buffer was 1–2 $\mu$M. The electropermeabilization apparatus consisted of a Plexiglass chamber with platinum electrodes placed 0.5 cm apart, a capacitor and a switch designed to discharge the capacitor in a single event [30]. For permeabilization the capacitor was charged to the required voltage and then discharged through the cell suspension in the permeabilization chamber. With six pulses of 3.2 kV/cm, more than 95% of the cells became permeable, as verified by Trypan Blue uptake. After permeabilization, the cell suspension was centrifuged and the pellet kept on ice until use. Cells remained permeabilized over the period of the experiment (about 2 h). Rat hepatocytes were isolated by collagenase digestion as described previously [31] and were electropermeabilized by the same method that was used for RINm5F cells.

### Measurements of the ambient free $Ca^{2+}$ concentration [$Ca^{2+}$]

$Ca^{2+}$-sensitive mini-electrodes were constructed and calibrated, with some modifications of the method originally described by Tsien and Rink [32]. We used borosilicate capillary tubing, 5 cm long and 0.6 mm inner diameter, with 'omega dot' for rapid filling. The tubing was cleaned, heat-polished and silicone-treated [33]. The membrane solution for $Ca^{2+}$ electrode was prepared by dissolving premixed Calcium Cocktail I and approx. 30% (w/v) poly(vinyl chloride) in tetrahydrofuran, to obtain a thin liquid of appropriate consistency [32]. The tip of the tubing was filled by briefly dipping into the membrane solution. The reference electrode, made from similar tubing, was conventionally pulled into capillary pipettes and was filled with 1 M KCl. Electrode responses were recorded by a purpose-built high-impedance electrometer.

Cells were added to a Plexiglass chamber, containing 52 $\mu$l of incubation buffer, with continuous stirring. The incubation buffer was the same as the washing buffer, supplemented with 2 mM Mg-ATP and an ATP-regenerating system, consisting of 10 mM phosphocreatine and 20 units of creatine kinase/ml. In addition, the incubation buffer contained mitochondrial inhibitors, consisting of 0.4 $\mu$M antimycin and 2 $\mu$g of oligomycin/ml. Additions were made from 100–200-times-concentrated stock solutions, and the increase in final volume after each addition never exceeded 1%. Thapsigargin, tBuBHQ, $Ca^{2+}$ ionophore A23187 and the $Ca^{2+}$-channel-blocking agent manoalide were dissolved in dimethyl sulphoxide.

All experiments were done at room temperature. None of the substances used in the study interfered with electrode function.

### Materials

RPMI 1640 medium, penicillin, streptomycin, trypsin/EDTA and foetal-calf serum were from either Flow Laboratories (Irvine, Scotland) or Northumbria Biologicals (Cramlington, Northumberland, U.K.). Thapsigargin was purchased from GIBCO BRL (Gaithersburg, MD, U.S.A.) and tBuBHQ from EGA-Chemie (Steinheim, Germany). Ins(1,4,5)$P_3$, GTP and heparin were from Sigma (St. Louis, MO, U.S.A.). Manoalide was provided by Allergan (Irvine, CA, U.S.A.). Calcium Cocktail I, containing neutral carrier ETH 1001, was from Fluka (Buchs, Switzerland). Capillary tubing was from Federick Haer (Brunswick, ME, U.S.A.), and A23187 from Calbiochem (La Jolla, CA, U.S.A.). Bafilomycin $A_1$ was purchased from Dr. K. Altendorf, Osnabrück, Germany. Other chemicals were from Sigma and Merck.

### Statistical analysis

Statistical significance was judged by Student's $t$ test for unpaired data.

## RESULTS

After addition of electropermeabilized RINm5F cells (final concn. $3.9 \times 10^7$ cells/ml) to the incubation buffer, containing ATP, an ATP-regenerating system and mitochondrial inhibitors, ambient [$Ca^{2+}$] was decreased from $6.43 \pm 0.25$ $\mu$M (mean ± S.E.M., $n = 45$) to a steady-state concentration of $530 \pm 0.06$ nM (mean ± S.E.M., $n = 45$). Addition of thapsigargin or tBuBHQ rapidly released $Ca^{2+}$. The rate of increase in [$Ca^{2+}$] and the steady-state [$Ca^{2+}$] reached after additions of thapsigargin or tBuBHQ, were dependent on the concentration of the two substances. The relationship between the concentration of thapsigargin or tBuBHQ and the magnitude of $Ca^{2+}$ release is shown in Figure 1. The half-maximally effective concentration of thapsigargin was ~37 nM and the maximally effective concentration ~156 nM. For tBuBHQ, the half-maximally effective concentration and the maximally effective concentration were

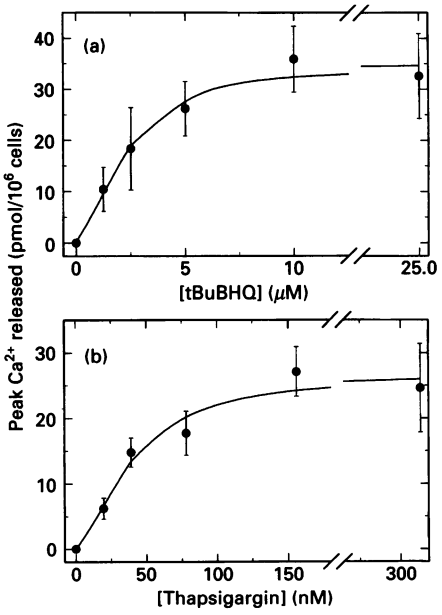


**Figure 1 Concentration-response curves of (a) tBuBHQ- and (b) thapsigargin-induced $Ca^{2+}$ release in permeabilized RINm5F cells**

Electropermeabilized RINm5F cells were incubated in an intracellular-like buffer containing ATP, an ATP-regenerating system and mitochondrial blockers. Changes in medium [$Ca^{2+}$] were measured by $Ca^{2+}$-sensitive mini-electrodes. Amounts of $Ca^{2+}$ released were estimated from the increase in medium [$Ca^{2+}$]. Values are means ± S.E.M. of at least three different experiments for each concentration of thapsigargin or tBuBHQ.

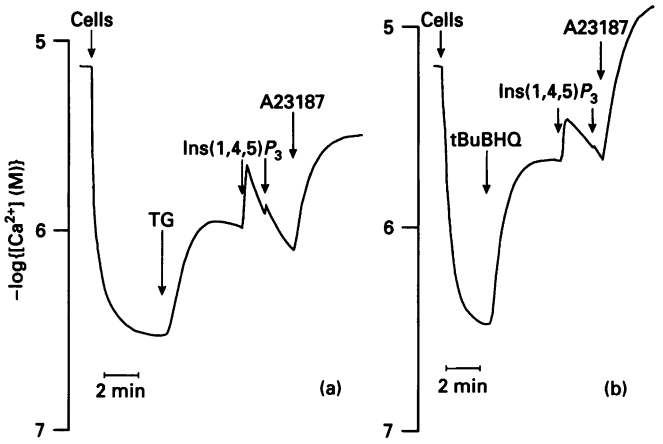


**Figure 2 Effect of Ins(1,4,5)$P_3$ on the release of $Ca^{2+}$ after maximal $Ca^{2+}$ release by thapsigargin and tBuBHQ**

Conditions for the experiments are as mentioned in the legend to Figure 1. After addition of thapsigargin (TG; 5 $\mu$M) (**a**) or tBuBHQ (25 $\mu$M) (**b**), [$Ca^{2+}$] increased to a maximum. Ins(1,4,5)$P_3$ released additional $Ca^{2+}$. Other additions, as indicated by arrows, are A23187 (2 $\mu$M). The traces are representative of at least three different experiments.

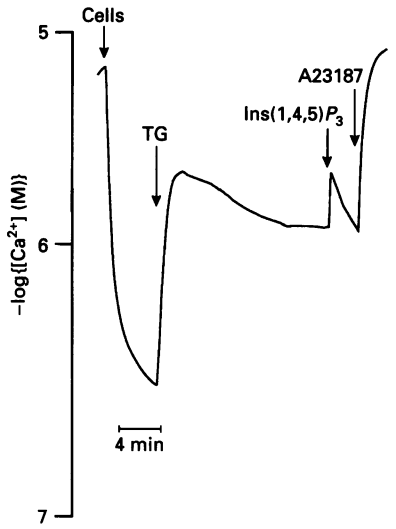


**Figure 3 $Ca^{2+}$ release by Ins(1,4,5)$P_3$ after prolonged treatment with thapsigargin**

For details of incubation and [$Ca^{2+}$] measurements, see the legend to Figure 1. Ins(1,4,5)$P_3$ (5 $\mu$M) was added 16 min after addition of thapsigargin (TG; 5 $\mu$M). Note change in the time scale in this Figure. The trace is representative of at least three different experiments.

$\sim 2\ \mu$M and $\sim 10\ \mu$M respectively. $Ca^{2+}$ release by higher concentrations of the inhibitors (up to 10 $\mu$M thapsigargin and 100 $\mu$M tBuBHQ) was not different from the release obtained by the maximally effective concentrations of these substances. To ensure complete inhibition of the ER $Ca^{2+}$-ATPase, in subsequent experiments we used thapsigargin and tBuBHQ at final concentrations of 5 $\mu$M and 25 $\mu$M respectively. Addition of tBuBHQ, after maximal $Ca^{2+}$ release by thapsigargin, or vice versa, did not cause any further increase in [$Ca^{2+}$] (results not shown). Half of the $Ca^{2+}$ release by maximally effective concentrations of thapsigargin and tBuBHQ was complete in about 1.5 min. At 2–4 min after addition of thapsigargin or tBuBHQ, [$Ca^{2+}$] reached its

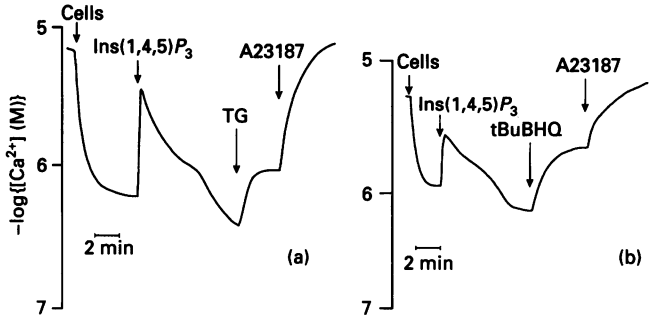


**Figure 4 Effect of thapsigargin and tBuBHQ, after Ins(1,4,5)$P_3$-induced release and subsequent re-uptake of $Ca^{2+}$**

For details of incubation and [$Ca^{2+}$] measurements, see the legend to Figure 1. At points indicated by the arrows Ins(1,4,5)$P_3$ (5 $\mu$M) and either thapsigargin (TG; 5 $\mu$M) (**a**) or tBuBHQ (25 $\mu$M) (**b**) was added. The traces are typical of at least three different experiments.

maximum and maintained a plateau for 1–2 min. [$Ca^{2+}$] then slowly declined to a slightly lower steady-state level. As shown in Figs. 2(a) and 2(b), [$Ca^{2+}$] increased to its maximum after addition of thapsigargin or tBuBHQ. Addition of Ins(1,4,5)$P_3$ at this stage caused rapid release of further $Ca^{2+}$. The uptake phase of Ins(1,4,5)$P_3$-induced $Ca^{2+}$ release was not affected by the presence of the inhibitors. The additional $Ca^{2+}$ release by Ins(1,4,5)$P_3$, after release by thapsigargin or tBuBHQ, was modest [estimated to be about 15% of the $Ca^{2+}$ released by Ins(1,4,5)$P_3$ in the absence of thapsigargin or tBuBHQ], but was seen in all experiments. The increase in [$Ca^{2+}$] was not due to any inadvertent contamination of Ins(1,4,5)$P_3$ solution by $Ca^{2+}$, since addition of a second pulse of Ins(1,4,5)$P_3$ was ineffective. Furthermore, the $Ca^{2+}$ release by Ins(1,4,5)$P_3$ was completely blocked by heparin (results not shown). The additional Ins(1,4,5)$P_3$-induced $Ca^{2+}$ release was not decreased by 10 $\mu$M thapsigargin or 100 $\mu$M tBuBHQ and was not altered by GTP (up to 50 $\mu$M), by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or by a specific inhibitor of V-type ATPases, Bafilomycin $A_1$ (10 $\mu$M) (results not shown). When added 10–18 min after thapsigargin, Ins(1,4,5)$P_3$ still released $Ca^{2+}$, although its magnitude was decreased (Figure 3). Similar results were seen with tBuBHQ (results not shown). GTP (final concn. 10–50 $\mu$M), in the presence of 3% (w/v) polyethylene glycol [34], neither released $Ca^{2+}$ by itself, nor increased Ins(1,4,5)$P_3$-induced $Ca^{2+}$ release (results not shown). In RINm5F cells, as in many other cells, $Ca^{2+}$ released by Ins(1,4,5)$P_3$ is taken up, back into the Ins(1,4,5)$P_3$-sensitive pool ([35]; M. S. Islam and P.-O. Berggren, unpublished work). In Figures 4(a) and 4(b), thapsigargin or tBuBHQ was added after Ins(1,4,5)$P_3$-induced release and subsequent re-uptake of $Ca^{2+}$. In these experiments, maximum [$Ca^{2+}$] obtained with thapsigargin or tBuBHQ was always less than that obtained with Ins(1,4,5)$P_3$.

In experiments shown in Figures 5(a) and 5(b), the Ins(1,4,5)$P_3$-sensitive pool was emptied by a supramaximal dose (20 $\mu$M) of Ins(2,4,5)$P_3$, a poorly metabolizable analogue of Ins(1,4,5)$P_3$ [36]. We used Ins(2,4,5)$P_3$ in an attempt to achieve a sustained rise in [$Ca^{2+}$]. However, in these cells, $Ca^{2+}$ released by Ins(2,4,5)$P_3$ did not maintain a steady state for long. The released $Ca^{2+}$ was slowly (in about 25 min) taken up into thapsigargin-insensitive pools (results not shown). When thapsigargin or tBuBHQ was added after maximal $Ca^{2+}$ release by Ins(2,4,5)$P_3$, no increase in [$Ca^{2+}$] was seen, although exchangeable $Ca^{2+}$ was still present in vesicular pools, as evidenced by release in response to A23187.

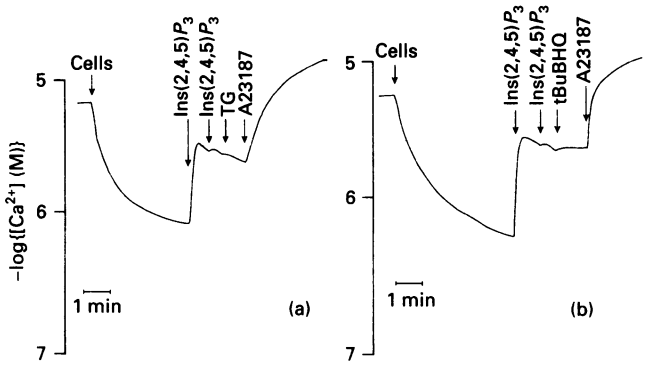


**Figure 5 Effect of thapsigargin and tBuBHQ on the release of $Ca^{2+}$, when added after depletion of the Ins(1,4,5)$P_3$-sensitive pool**

For details of incubation and $[Ca^{2+}]$ measurements see the legend to Figure 1. After $Ca^{2+}$ uptake, the Ins(1,4,5)$P_3$-sensitive pool was depleted by 20 $\mu$M Ins(2,4,5)$P_3$. No further $Ca^{2+}$ release was seen after thapsigargin (TG; 5 $\mu$M) (**a**) or tBuBHQ (25 $\mu$M) (**b**), whereas $Ca^{2+}$ was still releasable by A23187. The traces are representative of at least three different experiments.

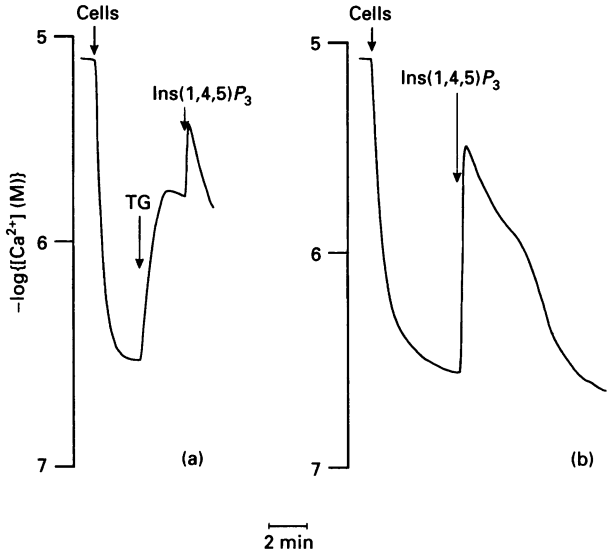


**Figure 6 Comparison of $Ca^{2+}$ release by thapsigargin and Ins(1,4,5)$P_3$ with that released by Ins(1,4,5)$P_3$ alone**

For details of incubation and $[Ca^{2+}]$ measurements, see the legend to Figure 1. (**a**) After $Ca^{2+}$ release by thapsigargin (TG; 5 $\mu$M) reached its maximum, Ins(1,4,5)$P_3$ (5 $\mu$M) was added. (**b**) Control experiment done under identical experimental conditions using the same cell preparation and the same electrodes. Ins(1,4,5)$P_3$ was added as indicated by arrow. Total increase in $[Ca^{2+}]$ obtained by thapsigargin and Ins(1,4,5)$P_3$ in (**a**) was compared with the increase in $[Ca^{2+}]$ obtained by Ins(1,4,5)$P_3$ alone in (**b**). The traces are representative of experiments that have been repeated at least three times.

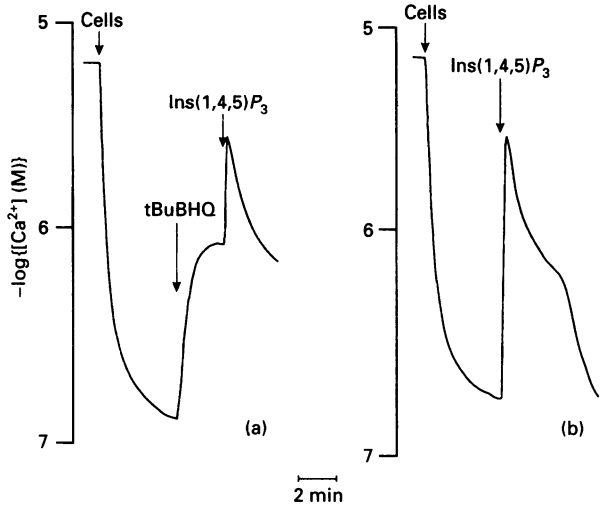


**Figure 7 Comparison of $Ca^{2+}$ release by tBuBHQ and Ins(1,4,5)$P_3$ with that released by Ins(1,4,5)$P_3$ alone**

For details of incubation and $[Ca^{2+}]$ measurements, see the legend to Figure 1. (**a**) After $Ca^{2+}$ release by tBuBHQ (25 $\mu$M) reached its maximum, Ins(1,4,5)$P_3$ (5 $\mu$M) was added. (**b**) Control experiments done under identical experimental conditions, using the same cell preparation and same electrodes. Ins(1,4,5)$P_3$ (5 $\mu$M) was added as indicated by arrow. Increase in total $[Ca^{2+}]$ obtained by tBuBHQ and Ins(1,4,5)$P_3$ in (**a**) was compared with the increase in $[Ca^{2+}]$ obtained by Ins(1,4,5)$P_3$ alone in (**b**). The traces are representative of experiments that have been repeated at least three times.

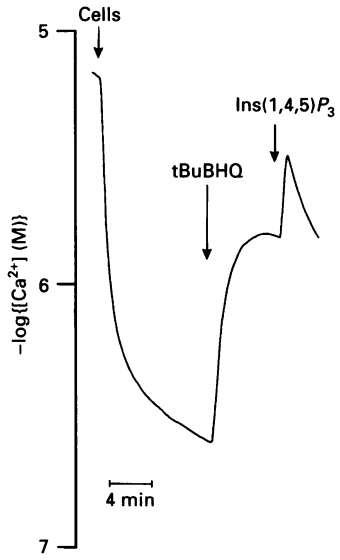


**Figure 8 Manoalide did not inhibit $Ca^{2+}$ release by tBuBHQ or Ins(1,4,5)$P_3$**

For details of incubation and $[Ca^{2+}]$ measurements see legend to Figure 1. Under these experimental conditions, the buffer also contained manoalide (6 $\mu$M final concn.). At 10 min after onset of $Ca^{2+}$ uptake, tBuBHQ (25 $\mu$M) was added. Ins(1,4,5)$P_3$ was added at the point indicated. The trace is representative of three similar experiments.

Finally, an estimate of $Ca^{2+}$ release by thapsigargin and tBuBHQ from the Ins(1,4,5)$P_3$-insensitive pools was made by another approach. If thapsigargin and tBuBHQ release $Ca^{2+}$ from the Ins(1,4,5)$P_3$-insensitive pools, the magnitude of $Ca^{2+}$ release by either of these agents plus Ins(1,4,5)$P_3$ must be greater than that by Ins(1,4,5)$P_3$ alone. We compared the total change in $[Ca^{2+}]$ by thapsigargin or tBuBHQ plus Ins(1,4,5)$P_3$ with that obtained by Ins(1,4,5)$P_3$ alone, in a series of carefully controlled experiments. The control experiments were done with the same cell preparation and the same electrodes. As shown in Figure 6(a), after maximal increase in $[Ca^{2+}]$ by thapsigargin, addition of Ins(1,4,5)$P_3$ caused a further increase in $[Ca^{2+}]$. The total increase in $[Ca^{2+}]$ thus obtained by thapsigargin and Ins(1,4,5)$P_3$ was on average about 7% greater than that obtained by Ins(1,4,5)$P_3$ alone in control experiments (cf. Figure 6b). This difference was

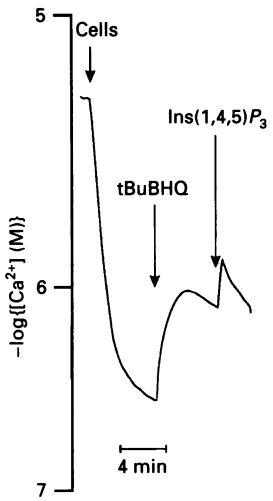


**Figure 9 $Ca^{2+}$ release by tBuBHQ and Ins(1,4,5)$P_3$ from electropermeabilized rat hepatocytes**

For details of incubation and [$Ca^{2+}$] measurements, see the legend to Figure 1. Addition of tBuBHQ (25 $\mu$M) caused release of $Ca^{2+}$. Further $Ca^{2+}$ was released by Ins(1,4,5)$P_3$ (5 $\mu$M). The trace is representative of three similar experiments.

not statistically significant. In similar experiments with tBuBHQ, the total increase in [$Ca^{2+}$] obtained by tBuBHQ and Ins(1,4,5)$P_3$ was about 33% greater than that by Ins(1,4,5)$P_3$ alone ($P < 0.05$) (Figures 7a and 7b). $Ca^{2+}$ release by thapsigargin or tBuBHQ was not blocked by heparin. A marine product, manoalide, has been reported to inhibit $Ca^{2+}$ release induced by agonists or by tBuBHQ [37,38]. Manoalide (up to 6 $\mu$M, for 10 min) [38] did not block $Ca^{2+}$ release by the inhibitors or by Ins(1,4,5)$P_3$ (Figure 8). Figure 9 shows experiments done with electropermeabilized rat hepatocytes, where again Ins(1,4,5)$P_3$ released additional $Ca^{2+}$ when added after $Ca^{2+}$ release by tBuBHQ.

## DISCUSSION

We defined the intracellular $Ca^{2+}$ pools that are sensitive to thapsigargin and tBuBHQ, and studied their relationship to the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool in insulin-secreting cells. This is especially interesting, since mobilization of $Ca^{2+}$ from the Ins(1,4,5)$P_3$-sensitive pool is part of the mechanisms that regulate $Ca^{2+}$ oscillations in pancreatic $\beta$-cells [39]. Although the two ER $Ca^{2+}$-ATPase inhibitors are used to identify intracellular $Ca^{2+}$ pools in many cells, direct comparison between these two agents in permeabilized cells has not been reported. In permeabilized RINm5F cells, the two inhibitors released $Ca^{2+}$ from intracellular non-mitochondrial $Ca^{2+}$ pools in a concentration-dependent manner. The half-maximally effective concentrations of tBuBHQ and thapsigargin were comparable with values reported in other cell types [11,18,22,31,37,40]. Thapsigargin and tBuBHQ released $Ca^{2+}$ from the Ins(1,4,5)$P_3$-sensitive pool. In experiments where the Ins(1,4,5)$P_3$-sensitive pool was depleted by a supramaximal dose of the Ins$P_3$, no further net release of $Ca^{2+}$ was seen after addition of the inhibitors. When thapsigargin and Ins(1,4,5)$P_3$ were used together, the total increase in [$Ca^{2+}$] was not significantly greater than that released by Ins(1,4,5)$P_3$ alone. Thus, in RINm5F cells, as in many other cell types, the thapsigargin-sensitive and Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pools are largely coincident [21,26,41]. On the other hand, the total increase in [$Ca^{2+}$] by tBuBHQ plus Ins(1,4,5)$P_3$ was significantly greater than that caused by Ins(1,4,5)$P_3$ alone. Thus, although tBuBHQ was claimed to release specifically from the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool [31], the present study, together with others, indicate that it may also release from part of the Ins(1,4,5)$P_3$-insensitive pools [22,24,37]. The reason for such a difference in action between the two inhibitors is not clear. Recent reports indicate that tBuBHQ has other actions apart from inhibition of ER $Ca^{2+}$-ATPase [15,17,23]. It remains a possibility that tBuBHQ may also interfere with the cytosolic $Ca^{2+}$-binding sites.

The maximal increase in [$Ca^{2+}$] obtained by thapsigargin or tBuBHQ was slightly smaller than that obtained by the maximally effective concentration of Ins(1,4,5)$P_3$. This is in contrast with studies where thapsigargin releases substantially more $Ca^{2+}$ than that released by Ins(1,4,5)$P_3$, implying a complete inclusion of the Ins(1,4,5)$P_3$-sensitive pool within the thapsigargin-sensitive pool [11,19,20,42,43]. One explanation for such differences may be variation in size of the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool, which is often cell-specific [42,44] and may also be GTP-mediated [45,46]. It is also noteworthy that, in experiments where microsomes are used instead of cells, conspicuously less $Ca^{2+}$ is released by Ins(1,4,5)$P_3$ than by thapsigargin [11,20,42]. In microsomes of bovine adrenal glomerulosa cells, $Ca^{2+}$ release by thapsigargin is larger than that by Ins(1,4,5)$P_3$, whereas in intact cells of the same type the reverse is true [20]. This may be due to the fact that Ins(1,4,5)$P_3$ releases less $Ca^{2+}$ from microsomes than from cells, because of the decrease in size of the Ins(1,4,5)$P_3$-releasable pool during preparation of microsomes [47,48].

Although a major part of the $Ca^{2+}$ in the Ins(1,4,5)$P_3$-sensitive pool was rapidly mobilized by thapsigargin and tBuBHQ, complete depletion of the Ins(1,4,5)$P_3$-sensitive pool was difficult to achieve. When added even as long as 15–18 min after thapsigargin or tBuBHQ, Ins(1,4,5)$P_3$ still released further $Ca^{2+}$. This is in accordance with studies which show that agonists or Ins(1,4,5)$P_3$ release additional $Ca^{2+}$ after $Ca^{2+}$ release from intracellular stores by thapsigargin [20,21,26,49–51]. Other studies, however, demonstrate total depletion of the Ins(1,4,5)$P_3$-sensitive pool by thapsigargin or tBuBHQ, within a short time [15,16,18,22,31,52]. It is possible that the buffering action of $Ca^{2+}$ indicators [31] or EGTA [18,52], used in some of these studies, might have masked the small additional Ins(1,4,5)$P_3$-induced $Ca^{2+}$ release. Most of these studies used the Fura-2 method to measure [$Ca^{2+}$]. In this technique, after $Ca^{2+}$ release by the inhibitors, [$Ca^{2+}$] often reaches near the saturation point of the indicator. Any additional release of $Ca^{2+}$ by Ins(1,4,5)$P_3$ is difficult to appreciate. This is also because of non-linearity of fluorescence in this range, and noise. In this respect, our method of measuring $Ca^{2+}$ by electrodes has distinct advantage. Moreover, for permeabilization these studies used detergents, a method which is difficult to control and may result in partial permeabilization of $Ca^{2+}$-storing vesicles [43,53,54]. Electropermeabilization is unlikely to cause leakiness of the $Ca^{2+}$-storing vesicles, since this technique is clean and it creates holes selectively in the plasma membrane [53]. In experiments with hepatocytes which were electropermeabilized, there was also additional release of $Ca^{2+}$ by Ins(1,4,5)$P_3$ when it was added after maximal $Ca^{2+}$ release by tBuBHQ. This is in contrast with results obtained in saponin-permeabilized hepatocytes [31], suggesting that the cause of some differences in the pattern of $Ca^{2+}$ release may be experimental rather than biological.

We considered the possibility that the additional $Ca^{2+}$ release by Ins(1,4,5)$P_3$, after thapsigargin- or tBuBHQ-induced $Ca^{2+}$ release, was from a different Ins(1,4,5)$P_3$-sensitive pool. Such an Ins(1,4,5)$P_3$-sensitive pool might have $Ca^{2+}$-uptake mechanisms insensitive to these inhibitors and might even be structurally

different from ER [55]. Nicotera et al. [56] demonstrated a tBuBHQ-insensitive Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool in rat liver cell nuclei (but see ref. [57]). Some isoforms of the SERCA family, with low sensitivity to the inhibitors, could mediate such uptake [58,59]. However, it is now established that thapsigargin inhibits completely and with equal potency all the known isoforms of the SERCA family [12]. Moreover, if the RINm5F cells possess some novel isoform of SERCA, it appears unlikely that it will not be inhibitable by two highly potent and structurally different inhibitors. Alternatively, the putative thapsigargin-insensitive Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool may utilize $H^+$-dependent $Ca^{2+}$ uptake [10]. However, in our study the thapsigargin-insensitive Ins(1,4,5)$P_3$ response was not decreased by the protonophore CCCP or a potent and specific inhibitor of V-type ATPases, Bafilomycin $A_1$ [60]. Although none of these results can rule out the existence of a different Ins(1,4,5)$P_3$-sensitive pool, insensitive to thapsigargin or tBuBHQ, in RINm5F cells, this does not appear very likely.

The present results fit best to a model where the Ins(1,4,5)$P_3$-sensitive pool is viewed to have distinct 'uptake' and 'release' compartments, as has been proposed by others [48,61,62]. When treated with thapsigargin or tBuBHQ, the two compartments can be distinguished on the basis of their permeability to $Ca^{2+}$. According to our model, the release compartment is impermeable to $Ca^{2+}$, in the absence of Ins(1,4,5)$P_3$. When treated with thapsigargin or tBuBHQ, the uptake compartment shows a high rate of leakage. Since thapsigargin interacts only with the ER $Ca^{2+}$-ATPase, the latter molecule is also most likely to be the structure that mediates the thapsigargin-induced $Ca^{2+}$ leak. From the structural model of SERCA and from studies in sarcoplasmic reticulum, it appears that the ER $Ca^{2+}$-ATPase molecule may contain a channel that mediates passive $Ca^{2+}$ efflux [63,64]. Thus the basis of high permeability may be the presence of the $Ca^{2+}$-ATPase molecule, which by definition is predominantly located on the uptake compartment. Thapsigargin and tBuBHQ release predominantly from this compartment. The release compartment is impermeable and can be mobilized only very slowly in the absence of Ins(1,4,5)$P_3$.

In the present study the evidence for the presence of uptake and release compartments within the Ins(1,4,5)$P_3$-sensitive pool is different from that obtained in earlier studies [48,61,62] and goes further in defining permeability of these compartments and action of the inhibitors. It is unlikely that our results are solely due to some artifact of permeabilization, as has been suggested by Menniti et al. [61]. Indeed, Brüne and Ullrich [62] demonstrated compartmentalization within the Ins(1,4,5)$P_3$-sensitive pool also in intact cells. The physiological significance of these findings is unclear. However, it is interesting, since the two compartments and their communicating pathway may offer additional sites for regulatory control. For instance, in permeabilized RINm5F cells, $[Ca^{2+}]_i$ oscillations linked to glycolytic oscillations may be a phenomenon of the uptake compartment [65].

To summarize, our results show that thapsigargin and tBuBHQ released $Ca^{2+}$ mostly from the Ins(1,4,5)$P_3$-sensitive pool in permeabilized RINm5F cells. Thapsigargin was more specific in mobilizing $Ca^{2+}$ from this pool than was tBuBHQ. Although the two inhibitors mobilized a large part of the $Ca^{2+}$ from the Ins(1,4,5)$P_3$-sensitive pool, they did not readily deplete this pool. Additional $Ca^{2+}$ was released by Ins(1,4,5)$P_3$, when added long after continued release by thapsigargin or tBuBHQ. This pattern of $Ca^{2+}$ release from the Ins(1,4,5)$P_3$-sensitive pool may indicate the presence of distinct uptake and release compartments, which differ in terms of $Ca^{2+}$ permeability when treated with the inhibitors.

We are grateful to Dr. Robin F. Irvine for a generous supply of Ins(2,4,5)$P_3$ and to Allergan for supplying manoalide. Financial support was obtained from the Swedish Medical Research Council (04x-09890 and 19x-00034), the Bank of Sweden Tercentenary Foundation, the Swedish Diabetes Association, and Nordic Insulin Foundation, the Swedish Hoechst Diabetes Research Foundation, Magnus Bergvalls Foundation, NOVO Industry, Farmitalia Carlo-Erba, Ulf Widengrens Memorial Foundation and Funds of the Karolinska Institute. We thank Dr. George E. N. Kass for helpful discussions. M.S.I. is the recipient of a Swedish Institute Guest Researcher Scholarship.

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Received 19 November 1992/22 January 1993; accepted 29 January 1992