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Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase

(hepatocyte/microsome/calcium pump)

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ABSTRACT **Thapsigargin, a tumor-promoting sesquiterpene lactone, discharges intracellular Ca^{2+} in rat hepatocytes, as it does in many vertebrate cell types. It appears to act intracellularly, as incubation of isolated rat liver microsomes with thapsigargin induces a rapid, dose-dependent release of stored Ca^{2+}. The thapsigargin-releasable pool of microsomal Ca^{2+} includes the pools sensitive to inositol 1,4,5-trisphosphate and GTP. Thapsigargin pretreatment of microsomes blocks subsequent loading with $^{45}Ca^{2+}$, suggesting that its target is the ATP-dependent Ca^{2+} pump of endoplasmic reticulum. This hypothesis is strongly supported by the demonstration that thapsigargin causes a rapid inhibition of the Ca^{2+}-activated ATPase activity of rat liver microsomes, with an identical dose dependence to that seen in whole cell or isolated microsome Ca^{2+} discharge. The inhibition of the endoplasmic reticulum isoform of the Ca^{2+}-ATPase is highly selective, as thapsigargin has little or no effect on the Ca^{2+}-ATPases of hepatocyte or erythrocyte plasma membrane or of cardiac or skeletal muscle sarcoplasmic reticulum. These results suggest that thapsigargin increases the concentration of cytosolic free Ca^{2+} in sensitive cells by an acute and highly specific arrest of the endoplasmic reticulum Ca^{2+} pump, followed by a rapid Ca^{2+} leak from at least two pharmacologically distinct Ca^{2+} stores. The implications of this mechanism of action for the application of thapsigargin in the analysis of Ca^{2+} homeostasis and possible forms of Ca^{2+} control are discussed.**

Tumor promoters have a common activity in stimulating the appearance of tumors in mouse skin following application of a subthreshold dose of a primary carcinogen. By far the greatest number of compounds identified by this test correspond to potent and highly selective stimulants of the signal-transduction enzyme protein kinase C. The identification of protein kinase C as a tumor promoter receptor suggests that activation of this enzyme may underlie the tumor promotion process, although direct evidence for this conclusion has not been reported. Recently, a second biochemical mechanism has been found for okadaic acid, a tumor promoter that has no action on protein kinase C (1). It inhibits several protein phosphatases, and it has been proposed that this inhibition mimics persistent phosphorylation through protein kinase C (1). This identification of a second biochemical action for a member of the class of tumor promoters calls attention to the importance of understanding the cellular mechanisms of action of other tumor promoters.

Thapsigargin (Fig. 1), a naturally occurring sesquiterpene lactone, promotes tumorigenesis in mouse skin (2) but does not activate protein kinase C (3, 4) or inhibit protein phosphatases. Thapsigargin induces acute responses in a large variety of cell types (4–14), but thapsigargin-induced cellular activation appears, in all cases, to be initiated by a single common event: a rapid and pronounced increase in the concentration of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) that occurs via a direct discharge of intracellular stored Ca^{2+} without hydrolysis of inositolphospholipids (4, 8, 12). These results imply that thapsigargin might act directly on a recognition site associated with the intracellular Ca^{2+} store (7, 15). Here, we provide evidence that thapsigargin increases $[Ca^{2+}]_i$ by specific and potent inhibition of the endoplasmic reticulum (ER) Ca^{2+}-ATPase.

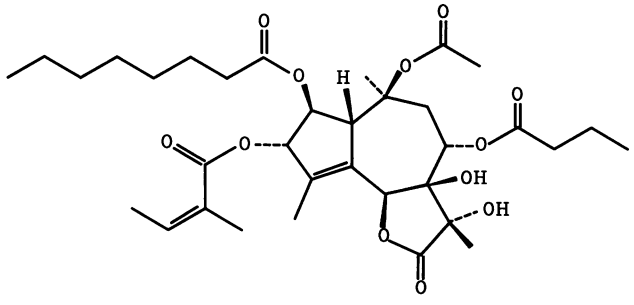

FIG. 1. Structure of thapsigargin.

MATERIALS AND METHODS

Measurements of $[Ca^{2+}]_i$. Hepatocytes were prepared by perfusion of collagenase (type I; Sigma) (16) through livers from fed 250-g male rats and were kept in a Krebs–Henseleit buffer (119 mM NaCl/4.7 mM KCl/1.1 mM KH_2PO_4/1.2 mM $MgSO_4$/25 mM $NaHCO_3$, buffered with CO_2 to pH 7.4) containing 1.0 mM Ca^{2+}. The cells were stored at 3.0×10^6 per ml and continuously gassed with O_2/CO_2, 19:1 (vol/vol). After 5 min of incubation at 37°C, 10 μM indo-1 acetoxymethyl ester (Molecular Probes) was added and the incubation continued for a further 30 min. After centrifugation at 50 $\times$ *g* for 2 min, the indo-1-loaded hepatocytes were resuspended at 3.0×10^6 per ml in Krebs–Henseleit buffer including 1.0 mM $CaCl_2$ or 1.0 mM EGTA. The cell suspension was stored on ice until use. Indo-1 fluorescence was measured at 37°C in a Perkin–Elmer LS-3B fluorescence spectrophotometer with monochromator settings of 355 nm (excitation) and 400 nm (emission). $[Ca^{2+}]_i$ was determined as described by

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Abbreviations: $[Ca^{2+}]_i$, concentration of cytosolic free Ca^{2+}; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; Ins-P_3, inositol 1,4,5-trisphosphate.

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Grynkiewicz *et al.* (17), using 250 nM as the apparent dissociation constant for Ca^{2+} and indo-1. Maximum (F_{max}) and minimum (F_{min}) fluorescence values were obtained by addition of digitonin (25 μg/ml) and further addition of 10 mM EGTA, respectively. In calculation of $[Ca^{2+}]_i$, we corrected for extracellular indo-1.

Ca^{2+} Efflux and Uptake by Liver Microsomes and Plasma Membrane Vesicles. Ca^{2+}-sensitive electrode membranes containing the neutral ionophore ETH1001 were prepared as described by Clapper and Lee (18). The microsomal fraction sedimenting at 35,000 × *g* for 20 min was prepared from livers of fed 250-g male rats as described by Dawson and Irvine (19). Protein concentration was determined by the method of Lowry *et al.* (20), using bovine serum albumin as standard. Ca^{2+} uptake and release were routinely measured with a Ca^{2+}-sensitive electrode as described (21), with the exception that oligomycin and ruthenium red were omitted from the assay medium (22). The assay medium consisted of 150 mM sucrose, 50 mM KCl, 10 mM Hepes/KOH (pH 7.0), 5% (wt/vol) poly(ethylene glycol), 1 mM dithiothreitol, 2 mM $MgCl_2$, 5 mM ATP, 90 μg of creatine kinase, and 5 mM phosphocreatine in a volume of 2.5 ml. Uptake was started by the addition of microsomes (1.8 mg/ml). Experiments were carried out at 30°C.

$^{45}Ca^{2+}$ uptake and efflux were measured in the microsomal fraction by a filtration method (23). The incubation mixture contained, in a total volume of 0.5 ml, 100 mM KCl, 20 mM Hepes/KOH (pH 7.0), 10 mM $MgCl_2$, 50 μM $CaCl_2$, 200 μM EGTA, and 7.4 KBq of $^{45}Ca^{2+}$. Microsomes (0.5 mg of protein) were added and, after equilibration for 3 min at 37°C, Ca^{2+} uptake was started by the addition of 2.5 mM ATP. Samples (50 μl) were removed at the indicated times, vacuum-filtered on 0.2-μm filters (Sartorius) and washed with two 1-ml aliquots of 100 mM KCl/10 mM Hepes/KOH, pH 7.0, at 0°C. $^{45}Ca^{2+}$ retained on the filter was measured with an LKB 1214 RackBeta liquid scintillation counter.

Plasma membrane vesicles were prepared from rat liver as described (24). Ca^{2+} uptake and release were measured with a Ca^{2+}-sensitive electrode as described for liver microsomes, with the exception that the reaction medium contained, in a volume of 1 ml, 100 mM KCl, 20 mM Hepes/KOH (pH 7.0), 5% poly(ethylene glycol), 2.5 mM dithiothreitol, 4 mM $MgCl_2$, 100 μg of creatine kinase, 10 mM phosphocreatine, 1.25 mM ATP, and 1 mg (protein) of rat liver plasma membrane vesicles.

Ca^{2+}-ATPase Activity. The ATPase activity of the rat liver microsomal fraction was determined by P_i release from ATP (25). The incubation mixture contained, in a final volume of 1 ml, 100 mM KCl, 5 mM $MgCl_2$, 20 mM Hepes/KOH (pH 7.0), 1 μg of oligomycin, 1 μg of calcium ionophore A23187, 50 μM $CaCl_2$ (only for $+Ca^{2+}$ medium), 1 mM EGTA (only for $-Ca^{2+}$ medium), and 2 mg of protein. The hydrolysis was started by the addition of 1 mM ATP. After incubation at 37°C for 10 min, the reaction was stopped by the addition of 0.5 ml of 5% (wt/vol) trichloroacetic acid. The resultant precipitate was spun down in a bench centrifuge, and 1 ml of the supernatant was removed. P_i in the supernatant was determined by the method of Fiske and Subbarow (26).

The activity of the hepatocyte plasma membrane Ca^{2+}-ATPase was not assayed, because this activity is so low when compared to the background of Mg^{2+}-stimulated ATPase that meaningful data cannot be obtained (24).

The preparation of rat cardiac sarcoplasmic reticulum (SR) was as detailed by Sunida *et al.* (27). Rat skeletal muscle SR was prepared according to Saito *et al.* (28). Erythrocyte membrane "ghosts" were prepared as described by Jarrett and Penniston (29), with the exception that EDTA was not present in any of the buffers. ATPase activity was measured as described for the liver microsomes with the exception that the erythrocyte ghosts were incubated for 30 min at 37°C in the presence of 0.1 mM ouabain. Protein concentrations used were as follows: cardiac SR, 0.25 mg/ml; skeletal muscle SR, 0.1 mg/ml; and erythrocyte ghosts, 0.6 mg/ml.

RESULTS

Thapsigargin-Induced $[Ca^{2+}]_i$ Increase. Isolated rat hepatocytes displayed a rapid and dose-dependent increase in $[Ca^{2+}]_i$ upon thapsigargin stimulation (Fig. 2), with an ED_{50} value of around 80 nM. The thapsigargin-induced $[Ca^{2+}]_i$ rise was slower than that elicited by hormonal stimulation, the maximum level not being attained until after 40–60 sec. In the absence of extracellular Ca^{2+}, thapsigargin produced a transient increase in $[Ca^{2+}]_i$; in the presence of extracellular Ca^{2+} (1 mM), thapsigargin produced a sustained increase in $[Ca^{2+}]_i$ (Fig. 2). This pattern of events is found in a diverse range of cells (see Table 2) and is very comparable to that described for platelets (7), which proceeds by a direct discharge of intracellular sequestered Ca^{2+}. These results demonstrate that hepatocytes can be regarded as a representative cell type in which the analysis of thapsigargin's mechanism may be undertaken.

Thapsigargin Discharges Microsomal Ca^{2+}. Addition of thapsigargin to Ca^{2+}-loaded microsomal membrane vesicles led to a rapid and complete release of the sequestered Ca^{2+} (Fig. 3), as measured with Ca^{2+}-sensitive electrodes. Similarly, thapsigargin released essentially all the $^{45}Ca^{2+}$ whose uptake was ATP-dependent (Fig. 4). The effectiveness of thapsigargin to empty the microsomal Ca^{2+} pool was further supported by the observation that it abolished the Ca^{2+}-releasing ability of both GTP (20 μM) and Ins-P_3 (1 μM) (Fig. 3 *A* and *C*). The amount of thapsigargin-releasable Ca^{2+} was decreased by pretreatment with either GTP or Ins-P_3 (Fig. 3 *B* and *D*), which indicates that the pools releasable by these compounds are included in the thapsigargin-sensitive pool. In contrast to GTP, both thapsigargin and Ins-P_3 induced an immediate Ca^{2+} release. However, the initial rate of Ca^{2+} release for Ins-P_3 was found to be much faster than that for thapsigargin. Pretreatment with either GTP or Ins-P_3 did not change the Ca^{2+}-release rate for thapsigargin (2.7 and 2.9 nmol/min per mg of protein). The inability of GTP to increase the size of the thapsigargin-sensitive pool suggests that the effect of thapsigargin, unlike that of Ins-P_3 (22, 23), is independent of a membrane fusion or communication be-

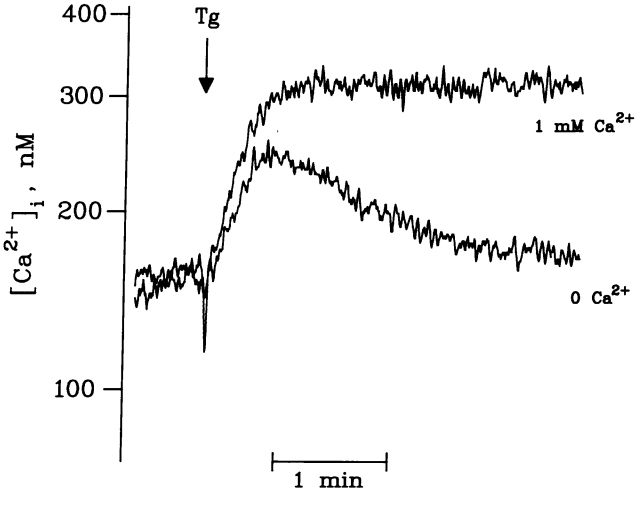

FIG. 2. Thapsigargin (Tg, 170 nM) increases $[Ca^{2+}]_i$ of indo-1-loaded rat hepatocytes. The traces reflect the calibrated change in $[Ca^{2+}]_i$ from the fluorescence of indo-1-loaded hepatocytes in the presence (1 mM $CaCl_2$) or absence (1 mM EGTA) of extracellular Ca^{2+} at 37°C. Each trace is representative of at least three separate experiments.

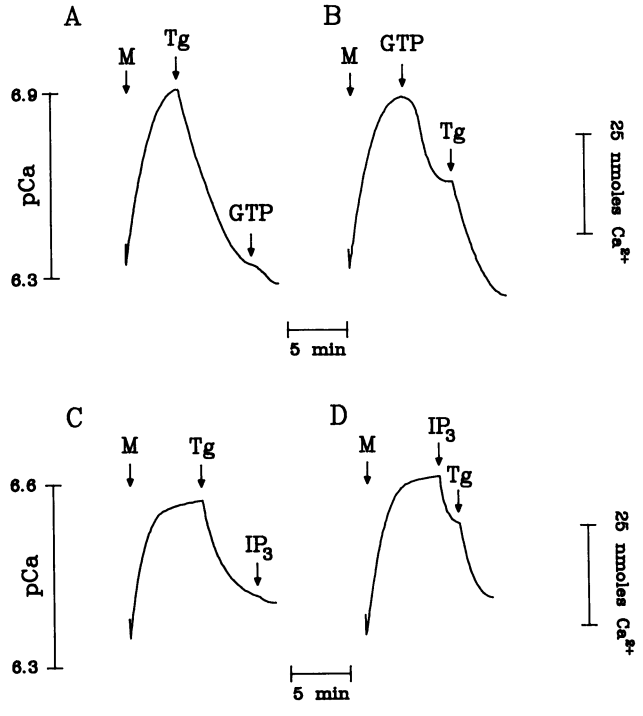

FIG. 3. Effect of thapsigargin (Tg, 170 nM) on GTP- and inositol 1,4,5-trisphosphate (Ins-P_3)-stimulated Ca^{2+} efflux from rat liver microsomes. (*A* and *B*) GTP (30 μM) was added as shown. (*C* and *D*) GTP (30 μM) was present in the initial incubation medium prior to the addition of microsomes (M), and Ins-P_3 (IP_3, 1 μM) was added as shown. Experiments were carried out at 30°C. Measurements were made with Ca^{2+}-sensitive electrodes.

tween different Ca^{2+} pools. These experiments establish that the mechanism of thapsigargin is distinct from those of Ins-P_3 and GTP.

Inhibitory Effect of Thapsigargin on Ca^{2+}-ATPase Activity. The ATP-driven accumulation of $^{45}Ca^{2+}$ into liver microsomes was completely blocked by thapsigargin pretreatment (Fig. 4), suggesting that the effect of thapsigargin may be consequent to inhibition of Ca^{2+} uptake, rather than due

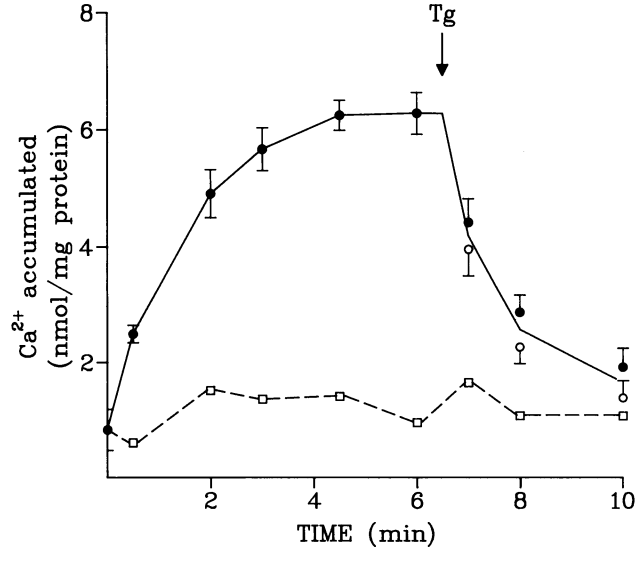

FIG. 4. Effect of thapsigargin on uptake and efflux of $^{45}Ca^{2+}$ by rat liver microsomes. ●, Thapsigargin (Tg, 68 nM) was added at t = 6.5 min; ○, hexokinase/glucose (11.2 units per ml/4.5 mM) mixture was added at t = 6.5 min; □, thapsigargin (68 nM) was added at t = 0 min. Experiments were performed at 37°C. Data points represent the mean of three experiments, and the bars show SD.

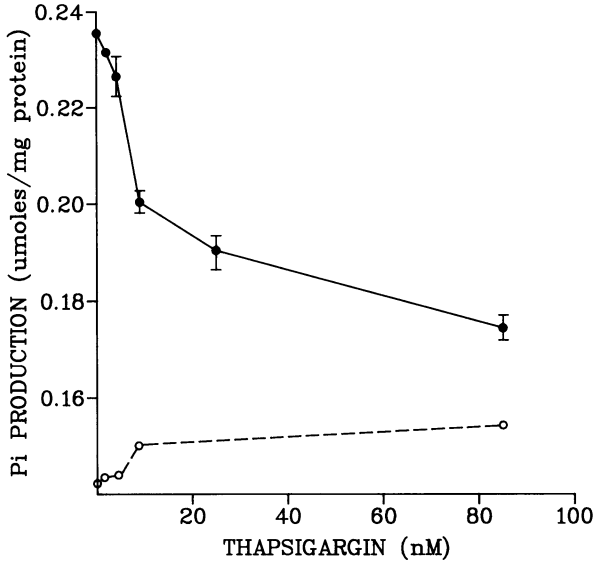

FIG. 5. Inhibition of the microsomal Ca^{2+}-ATPase by thapsigargin in the presence (●) or absence (○) of Ca^{2+}. Experiments were performed at 37°C. Data are the means ± SD of four experiments.

to direct stimulation of an efflux process. Support for this possibility was provided by hexokinase/glucose treatment (to remove ATP), which also induced Ca^{2+} release, with kinetics and maximum effect very similar to those produced by thapsigargin (Fig. 4). Direct measurement of enzymatic ATP hydrolysis revealed that thapsigargin inhibited the microsomal Ca^{2+}-ATPase with an IC_{50} value of ≈30 nM (Fig. 5). This inhibition was specific for Ca^{2+}-stimulated ATPase activity, as basal Mg^{2+}-ATPase in this membrane fraction was unaffected (Fig. 5). Further, the inhibitory effect was restricted to the microsomal form of the enzyme, as even high concentrations of thapsigargin did not produce any significant Ca^{2+} efflux from Ca^{2+}-loaded plasma membrane vesicles (Fig. 6). Similar experiments (data not shown) using both Ca^{2+}-electrode and $^{45}Ca^{2+}$-accumulation measurements failed to demonstrate any effect of thapsigargin on the initial rate of Ca^{2+} uptake or, in agreement with the data in Fig. 6, Ca^{2+} efflux.

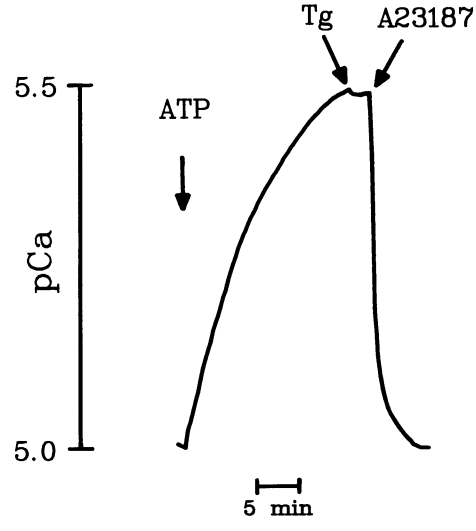

FIG. 6. Lack of effect of thapsigargin on Ca^{2+} efflux from rat liver plasma membrane vesicles. Ca^{2+} uptake was started by the addition of 1.25 mM ATP, and thapsigargin (Tg, 340 nM) and A23187 (1 μM) were added as shown. Experiment was carried out at 30°C.

Table 1. Effect of thapsigargin (170 nM) on Ca^{2+}-ATPases of liver ER, cardiac muscle SR, skeletal muscle SR, and erythrocyte plasma membrane

Membrane preparation	Inhibition, %
Rat liver ER	100
Rat cardiac muscle SR	62 ± 5
Rat skeletal muscle SR	0
Human erythrocyte ghosts	0

Typically, the values for Ca^{2+}-stimulated ATPase activity measured in the absence of thapsigargin were as follows: erythrocyte ghosts, 0.29 μmol/30 min; cardiac muscle SR, 0.16 μmol/10 min; rat liver microsomes, 0.26 μmol/10 min; rat skeletal muscle SR, 0.21 μmol/10 min. Experiments were performed at 37°C. Inhibition values represent means ± SEM (n = 3).

The microsomal Ca^{2+}-ATPase was uniquely sensitive to thapsigargin; the Ca^{2+}-ATPases of skeletal muscle SR and of erythrocyte plasma membrane were insensitive, and cardiac SR Ca^{2+}-ATPase was ≈10-fold less sensitive (Table 1). Consequently, the inhibition of the ER Ca^{2+}-ATPase by thapsigargin must involve a specific domain that is structurally distinct from Ca^{2+}-ATPases of SR and of plasma membrane.

DISCUSSION

Increasingly, thapsigargin is used as a probe for intracellular Ca^{2+}-storage and -release processes (Table 2). The general characteristics of the cellular thapsigargin Ca^{2+} response are (*i*) a $[Ca^{2+}]_i$ rise that is slower than that elicited by hormonal stimulation, (*ii*) no concomitant breakdown of inositolphospholipids with the production of inositol phosphates, and (*iii*) an initiation of $[Ca^{2+}]_i$ rise by release of intracellular sequestered Ca^{2+}. In general the initial intracellular discharge leads directly to a Ca^{2+} influx, in keeping with prediction from the capacitative model of Ca^{2+} entry (30); however, in a limited number of examples such as the NG115-401L neuroblastoma cell line, thapsigargin releases intracellular Ca^{2+} without an accompanying influx, suggesting that receptor-coupled production of a code signal may be required, as noted earlier (4, 15). In comparison, hormonally induced increases in $[Ca^{2+}]_i$ are found to be transient rather than sustained, even in the presence of extracellular Ca^{2+}. The reasons for the diverse shapes of thapsigargin and hormone Ca^{2+} signals are probably to be found in differences in the rate of Ca^{2+} release from intracellular stores and in the $[Ca^{2+}]_i$ oscillation and desensitization phenomena, which are generally associated with hormonal responses.

In this study we have shown that thapsigargin discharges stored Ca^{2+} from the ER by a direct interaction. Comparative studies with Ins-P_3 and GTP indicated that thapsigargin acts through a different mechanism, because thapsigargin-induced Ca^{2+} release encompassed both of the stores sensitive to these stimulants. Furthermore, thapsigargin does not displace ^{3}H-labeled Ins-P_3 from specific binding sites in any cell so far examined, including membranes from cerebral cortex or cerebellum (K. Ansari, P. H. Andersen, and O.T., unpublished results) or from NG115-401L cells (K. Peggs and M.R.H., unpublished results).

Table 2. Survey of thapsigargin-sensitive cells

Cell type	Ref(s).	Sustained Ca^{2+} influx	ED_{50}, nM
Human platelets	7, 8	Yes	≈60
Mouse neuroblastoma cell line NG115-401L	3	No	≈20
Human lymphocytes	9	Yes	1–10
Rat peritoneal macrophages	10	Yes	<15
Bovine adrenal chromaffin cells	11	Yes	—
Rat parotid acinar cells	12	Yes	—
Human neutrophils	13	Yes	≈10
Human colon adenocarcinoma cell line HCA-7	14	Yes	—
Rat hepatocyes	This work	Yes	≈80

In all cases, cells responded with an initial increase in $[Ca^{2+}]_i$.

The finding of a highly potent inhibition of the Ca^{2+}-activated microsomal ATPase, the presumptive Ca^{2+} pump, indicates that the molecular target for thapsigargin is, unexpectedly, the Ca^{2+}-accumulation system rather than the efflux system. A similar dose dependency of the thapsigargin-induced Ca^{2+} release and pump inhibition, together with the ability of ATP depletion (hexokinase/glucose) to elicit an identical release, provides strong evidence for identification of the ER Ca^{2+}-ATPase as the site of thapsigargin action. Whether thapsigargin causes the Ca^{2+} pump itself to act as a direct pathway for Ca^{2+} release, or whether a constitutive Ca^{2+} leak, situated at a separate molecular site in the ER membrane, is responsible for the Ca^{2+} discharge observed, is presently unknown.

A striking observation is that, while thapsigargin is a very potent inhibitor of ER Ca^{2+}-ATPase, it is very much less effective on cardiac SR Ca^{2+}-ATPase and apparently ineffective against the enzymes from skeletal muscle SR and plasma membrane. This suggests that the Ca^{2+}-ATPase of ER differs significantly from the other isoforms. It has recently been shown (31) that the ER Ca^{2+}-ATPase is immunologically similar to the cardiac enzyme and differs from the skeletal muscle SR enzyme. However, it is significant that the cloning of multiple molecular forms of the Ca^{2+}-ATPase has revealed that the predominant form, likely to correspond to the ER Ca^{2+}-ATPase (31), has an extended C-terminal tail that may include another transmembrane domain, when compared to the cardiac SR Ca^{2+}-ATPase. Lytton and MacLennan (31) have suggested that the extended C terminus might provide a site for potential physiological regulation. Clearly, this would be the principal site to investigate for thapsigargin interaction. Therefore, thapsigargin may provide a powerful pharmacological tool for examining hypothetical interactions at this putative regulatory-factor site.

The physiological significance of these results is unclear, but modulation of pump activity could provide a means of tuning the intensity or duration of $[Ca^{2+}]_i$ elevation following intracellular Ca^{2+} discharge or extracellular Ca^{2+} influx. In this context, there is increasing evidence for both positive and negative control of different Ca^{2+}-ATPases. For example, the plasma membrane Ca^{2+} pump has been shown to be sensitive to cGMP-dependent protein kinase (32), Ca^{2+}/calmodulin (33), and the hormone glucagon (34); the cardiac SR Ca^{2+}-ATPase is regulated by phospholamban, which is a substrate for multiple signal-transduction kinases (35); and skeletal muscle SR Ca^{2+}-ATPase has been proposed to be directly responsible for Ca^{2+}-dependent Ca^{2+} efflux from SR (36), a situation directly analogous to the ER Ca^{2+}-ATPase acting as an induced Ca^{2+} leak channel.

The short- and long-term activities of thapsigargin—Ca^{2+} mobilization and tumor promotion, respectively—may be causally related. Certainly, the identification of the ER Ca^{2+}-ATPase as the molecular site of thapsigargin action raises the important possibility that genetic lesions or oncogene regulation of this Ca^{2+} pump should be considered as a candidate factor in multistage carcinogenesis.

In conclusion, thapsigargin provides a molecular probe of Ca^{2+} homeostasis mechanisms that can be used at low doses in intact living cells. In this regard, it complements the phorbol diesters for activation of protein kinase C. Improved understanding of the interaction of thapsigargin with the Ca^{2+}-ATPase may provide unexpected dividends in insight

into the role of Ca^{2+}-pump regulation in intracellular Ca^{2+} storage/release and initiation or maintenance of $[Ca^{2+}]_i$ oscillations.

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