Luminal Ca\textsuperscript{2+} Protects against Thapsigargin Inhibition in Neuronal Endoplasmic Reticulum*  

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Thapsigargin is a specific and potent inhibitor of sarcoplasmic or endoplasmic reticulum Ca\textsuperscript{2+}-ATPases. However, in whole rat brain microsomes, 1 \mu M thapsigargin had no significant effect on the 10-min time course of ATP-dependent Ca\textsuperscript{2+} uptake in the absence of the luminal Ca\textsuperscript{2+} chelator oxalate. In contrast, 50 \mu M oxalate resolved a thapsigargin-sensitive Ca\textsuperscript{2+} uptake rate (IC\textsubscript{50} = 1 \mu M thapsigargin) five times that of a thapsigargin-insensitive rate. This remaining ~20% of the total ATP-dependent accumulation was insensitive to thapsigargin (up to 10 \mu M), slightly less sensitive to vanadate inhibition, and unresponsive to 5 \mu M inositol 1,4,5-trisphosphate or 10 \mu M caffeine. Measuring both 12-min Ca\textsuperscript{2+} uptake and initial Ca\textsuperscript{2+} uptake rates, the apparent thapsigargin sensitivity increased as oxalate concentrations increased from 10 to 50 mM, corresponding to a range of luminal free Ca\textsuperscript{2+} concentrations of ~300 down to 60 nm. Addition of oxalate during steady-state \textsuperscript{45}Ca accumulation rapidly resolved the aforementioned thapsigargin sensitivity. These results strongly suggest that luminal Ca\textsuperscript{2+} may protect a large portion of neuronal endoplasmic reticulum Ca\textsuperscript{2+}-pumps against thapsigargin inhibition. Although high [Ca\textsuperscript{2+}] has been previously shown to protect against thapsigargin inhibition in several reticular membrane preparations, our results suggest that luminal Ca\textsuperscript{2+} alone is responsible for mitigating this effect in neurons.

The endoplasmic and sarcoplasmic reticula (ER\textsuperscript{1} and SR, respectively) actively accumulate Ca\textsuperscript{2+} via the sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) family of Ca\textsuperscript{2+} pumps. These pumps are encoded by at least three different genes, and alternative splicing creates a total of at least five isoforms (SERCA1a, SERCA1b, SERCA2a, SERCA2b, and SERCA3). The isoforms found in brain ER are SERCA2b and SERCA3, whereas the skeletal muscle isoforms are exclusively SERCA1 and SERCA2a (1–3). The isoforms appear to share overall structure-function similarities: all are thought to be asymmetrical transmembrane proteins with similar structure (4, 5) that can translocate two Ca\textsuperscript{2+} ions into the lumen by hydrolyzing one ATP molecule and forming an enzyme-phosphorylated (E–P) intermediate (6). Thapsigargin, a naturally occurring, tumor-promoting sesquiterpene lactone, has been shown to release Ca\textsuperscript{2+} from the ER by specifically inhibiting these Ca\textsuperscript{2+} pumps (7). Lytton et al. (8), using a COS expression system and cDNA clones for SERCA1a, -2a, -2b, and -3, demonstrated a stoichiometric, potent, and essentially irreversible inhibition of each of the SERCA isoforms by thapsigargin. Similarly, Campbell et al. (9) detected no difference in the Ca\textsuperscript{2+} affinities or inhibitor effects for avian subtypes 1, 2a, and 2b when expressed in COS cells. A protective effect of high [Ca\textsuperscript{2+}] against thapsigargin inhibition has been described for the skeletal muscle SERCA1 pump (10, 11) and a SERCA-type ATPase in ER microsomes of bovine adrenal chromaffin cells (12). Although most of the Ca\textsuperscript{2+} uptake into the ER and SR is likely due to the action of SERCAs, some recent evidence suggests the existence of thapsigargin-resistant ATP-dependent mechanisms capable of sequestering Ca\textsuperscript{2+} in some cell and microsome preparations (11, 12–19).

In this study, we were interested in characterizing neuronal ER Ca\textsuperscript{2+} accumulation both in terms of thapsigargin sensitivity and the way by which high [Ca\textsuperscript{2+}] might protect against thapsigargin inhibition. We employed \textsuperscript{45}Ca flux studies to characterize both thapsigargin-sensitive and -resistant ATP-dependent Ca\textsuperscript{2+} uptake processes by their kinetics, vanadate sensitivities, and responsiveness to inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and caffeine. Most important, we found that lowering the luminal free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{l}) relieved a protection against thapsigargin inhibition. This study provides new insights into the nature of neuronal ER Ca\textsuperscript{2+} pumps as well as new information about the protective effect of high [Ca\textsuperscript{2+}] which until now has largely been attributed to the binding of extramicrosomal, or cytosolic, free Ca\textsuperscript{2+} to the E1 conformation of the pump (10, 11).

**EXPERIMENTAL PROCEDURES**

**Microsomal Isolation**—Whole rat brain microsomes were isolated as described previously (20). The final microsomal pellet was resuspended in Buffer A, a Ca\textsuperscript{2+}-free buffered solution (pH 7.4) containing 150 mM KCl, 1.4 mM MgCl\textsubscript{2}, 20 mM HEPES, and 2 mM KH\textsubscript{2}PO\textsubscript{4}. The total protein content of microsomal suspensions was determined by the Bradford method (21). A typical test volume (300 \mu l) of radioactive Buffer B (see below) resulted in a final microsomal protein concentration of ~0.1 mg/ml.

**\textsuperscript{45}Ca Technique**—Microsomal suspensions were preincubated at 37 °C for 12–14 min. An experiment was initiated by the addition of 1 ml of warmed Buffer B to a 300-\mu l volume of microsomes. Buffer B (pH 7.4) contained tracer \textsuperscript{45}Ca, 150 mM KCl, 1.4 mM MgCl\textsubscript{2}, 20 mM HEPES, 2 mM KH\textsubscript{2}PO\textsubscript{4}, 0.13 mM EGTA, and 0.117 mM CaCl\textsubscript{2} (to give a final free [Ca\textsuperscript{2+}] of 0.3–0.4 \mu M, as measured with mini-electrodes), \textsuperscript{3}H-mg-ATP (to a final concentration of 3 mM), \textsuperscript{3}H-mg-ATP (to a final concentration of 3 mM), 0.01 mM digitonin, and 50 mM oxalate (addition of oxalate gave a free [Ca\textsuperscript{2+}] of 0.1–0.2 \mu M, as measured with mini-electrodes). Microsomes accumulated \textsuperscript{45}Ca for various time intervals. An experiment was terminated by the addition of 3 ml of ice-cold wash solution (pH 7.4) containing 150 mM KCl, 1.4 mM MgCl\textsubscript{2}, 20 mM HEPES, and 2 mM KH\textsubscript{2}PO\textsubscript{4}. The sample was then passed over a Whatman GF/B glass-fiber filter over a vacuum and washed three times with 5 ml of the same ice-cold buffer solution. ATP-dependent Ca\textsuperscript{2+} uptake was determined by subtracting \textsuperscript{44}Ca accumulation in the absence of...
MG-ATP from 45Ca accumulation in the presence of Mg-ATP. Unless otherwise noted, data are means ± S.E. and are expressed as μmol or nmol of Ca2+/g of protein.

In experiments where oxalate and/or digitonin are indicated, microsomes were preincubated in Buffer A along with 50 mM oxalate and/or 10 μM digitonin. For the experiment shown in Fig. 5, however, oxalate was added 5 min after the addition of isotope (time 0). Where thapsigargin is indicated, it was added at the beginning of preincubation at a concentration of 1 μM; however, for preloading experiments, 1 μM thapsigargin was added to the preincubation mixture 2 min prior to time 0. Any other drugs were added at time 0 (in Buffer B) unless stated otherwise.

Ca2+ Preloading Technique—Some microsomes were loaded with Ca2+ prior to time 0 by preincubating in Buffer A, 50 μM Mg-ATP, and 50 μM Ca2+ without EGTA. To measure uptake in the presence of ATP, Buffer B was added at time 0, adjusting final concentrations to 3 mM Mg-ATP, 90 μM CaCl2, and 100 μM EGTA. To measure uptake in the absence of ATP, 1 ml of Buffer B alone was added at time 0 so as to dilute the [Mg-ATP] down to 10 μM, a concentration that in our preparation did not significantly increase 45Ca accumulation as compared with that measured in the absence of ATP (data not shown). Therefore, for preloaded microsomes, the ATP-dependent uptake (see Fig. 3) represents uptake in the presence of 3 mM ATP minus uptake in the presence of 10 μM ATP.

RESULTS

Oxalate is a Ca2+-precipitating anion that accumulates in mitochondrial and ER-type vesicles, maintaining very low [Ca2+]i, by acting as a high-capacity Ca2+ buffer (22, 23). In the absence of oxalate, rat brain microsomes accumulate 45Ca in an extramicrosomal free Ca2+- and ATP-dependent manner, taking up Ca2+ rapidly during the first 1–2 min and reaching a steady-state after ~3 min (20, 24). In our preparation, most of this ATP-dependent uptake would presumably be due to the pumping action of SERCAs, which should be inhibited by thapsigargin. However, as illustrated in Fig. 1A, preincubation with and continued exposure to 1 μM thapsigargin (a maximal concentration) had no significant effect on the 10-min time course of ATP-dependent 45Ca uptake. In contrast, at a similar [Ca2+]i of ~0.3 μM, the addition of 50 mM oxalate significantly increased the magnitude of total ATP-dependent uptake and resolved a large thapsigargin sensitivity (Fig. 1B). In the presence of oxalate, these two apparently distinct ATP-dependent uptake processes had significantly different kinetics: the thapsigargin-sensitive component (slope = 41 ± 12 nmol/g·min·s−1) took up Ca2+ approximately five times faster than the thapsigargin-insensitive component (slope = 8 ± 2 nmol/g·min·s−1). In the presence of 50 mM oxalate, ~80% of the total ATP-dependent 45Ca uptake was potently and specifically inhibited by thapsigargin, having an IC50 of ~1 mM and being maximally inhibited in the range of 10–100 mM (Fig. 1C). The thapsigargin-insensitive portion of the uptake was resistant to inhibition up to 10 μM (data not shown).

To ensure that the apparent lack of thapsigargin sensitivity in the absence of oxalate was not due to an increased lipophilic association of the drug with membranes, we compared the effects of thapsigargin in microsomal suspensions that were either more dilute or more concentrated than typically used. The differential thapsigargin sensitivity, observed in the presence versus absence of oxalate, was unaffected by either halving (~0.05 mg/ml) or doubling (~0.2 mg/ml) the final microsomal protein concentration (data not shown). Regardless of the presence of oxalate, a 10 μM concentration of the Ca2+ ionophore A23187 released >90% of the actively accumulated Ca2+, indicating that membrane vesicles were responsible for our measured 45Ca uptake. Addition of a 100 mM concentration of the mitochondrial uncoupler carbonyl cyanide p-trifluoroxymethylenylhydrazone had no effect on measured uptake, indicating that none of our ATP-dependent Ca2+ accumulation could be attributed to mitochondrial contamination. Both the thapsigargin-sensitive and -insensitive processes were detected in the presence of 10 μM digitonin, a detergent known to selectively permeabilize plasma membrane vesicles at the concentration used (23). Although digitonin caused a small consistent decrease of 10–20% in the total ATP-dependent 45Ca uptake, the proportional effect of thapsigargin, with or without oxalate, remained unaltered by digitonin. Because our preparation may have been slightly contaminated with plasma membrane vesicles, all further experiments were carried out in the presence of 10 μM digitonin, unless otherwise stated.

To further investigate whether these thapsigargin-sensitive
Ca$^{2+}$ thapsigargin. Because preloading required 50 
pendent initial uptake rates in the absence or presence of 
which the profile of thapsigargin sensitivity changed. There-
ent uptake, regardless of the presence of thapsigargin.

Because the thapsigargin-sensitive and -insensitive uptake 
processes were equally affected by Ca$^{2+}$ preloading, we then 
 focused our attention on an alternative hypothesis: that oxalate 
was capable of “transforming” apparently thapsigargin-insen-
pumps into thapsigargin-sensitive ones, presumably by 
removing a protective effect of Ca$^{2+}$. If, in fact, luminal calcium 
ions were protecting thapsigargin-susceptible pumps against 
thapsigargin inhibition, there should be a range of [Ca$^{2+}$], 
in which the profile of thapsigargin sensitivity changed. Therefore, 
as illustrated in Fig. 4, we measured ATP-dependent 45Ca 
accumulation as well as ATP-dependent initial uptake rates in 
the presence and absence of thapsigargin at various oxalate 
concentrations. The relative contribution of the thapsigargin-
sensitive and -insensitive uptakes changed most over the range 
of 10–50 mM oxalate. This sensitivity was unaffected by oxalate 
concentrations of 0.01–1.0 mM, and concentrations greater than 
50 mM had no additional effect. Both the magnitude of total 
ATP-dependent uptake (Fig. 4A) and the apparent thapsigar-
gargin sensitivity, in terms of 12-min accumulation (Fig. 4B) 
as well as initial Ca$^{2+}$ uptake rates (Fig. 4C), increased with 
increasing oxalate concentrations. These data, in combination 
with the results of Fig. 3, were not consistent with our initial 
 hypothesis, but did support the second hypothesis, that in-
creasing [Ca$^{2+}$], could protect against thapsigargin inhibition. 
To illustrate this point (Fig. 4B), we calculated an estimated 
maximal [Ca$^{2+}$], by dividing the solubility product for Ca$^{2+}$ 
and oxalate ($-3 \times 10^{-9} \text{ M}^2$) by each oxalate concentra-
tion. To ensure that it was in fact only luminal Ca$^{2+}$, and not extramicro-
osomal Ca$^{2+}$, that was mediating this protective effect, we ad-
justed the total Ca$^{2+}$ concentration in the presence of 100 
mM EGTA and 50 mM oxalate to give a measured [Ca$^{2+}$], identical 
to that measured in the absence of oxalate, 0.3 mM. The results 
shown in Fig. 4D confirmed that luminal Ca$^{2+}$ was responsible 
for the protective effect: the apparent thapsigargin-insensitive 
uptake as a percent of the total ATP-dependent uptake was 
$121 \pm 25\%$ in the absence of oxalate, but was $31 \pm 5\%$ and $37 \pm 
7\%$ in the presence of 50 mM oxalate for 0.1 and 0.3 mM free 
Ca$^{2+}$, respectively. To determine if this protective effect of 
Ca$^{2+}$ was reversible, microsomes were allowed to accumulate 
and -insensitive uptake processes were in fact distinct, we 
compared their vanadate, IP$_3$, and caffeine sensitivities. Both 
processes were susceptible to vanadate inhibition, but differed 
slightly in their sensitivities (Fig. 2A): vanadate inhibited the 
thapsigargin-sensitive accumulation with an IC$_{50}$ of 16 ± 1 
M, whereas the IC$_{50}$ for the thapsigargin-insensitive accumulation 
was 30 ± 6 M. Additionally, as illustrated in Fig. 2B, only 
the thapsigargin-sensitive accumulation was affected by either 5 
mM IP$_3$ or 10 mM caffeine. After 10 min, IP$_3$ decreased the total 
thapsigargin-sensitive ATP-dependent uptake from 20 ± 1 
to 10 ± 1 mol/g, and caffeine decreased it to 13 ± 1 mol/g.

We were primarily interested in understanding why thapsi-
gargin could have such a profound inhibitory effect in the 
presence of oxalate, but not in its absence (Fig. 1). To account 
for this behavior, we assumed that oxalate, by maintaining an 
extraordinarily low free [Ca$^{2+}$] inside the microsomes, was removing 
any inhibitory effect of rising [Ca$^{2+}$] on pump activity, a phe-
omenon we (20) and others (22) had previously documented. 
The constant uptake rate in Fig. 1B could be explained by two 
effects of oxalate: relief of any [Ca$^{2+}$]-dependent inhibition of 
the pump and at the same time elimination of the normal leak 
of Ca$^{2+}$ out of the store (due to “trapping” of Ca$^{2+}$ in the 
lumen). Our initial hypothesis, then, was that the thapsigar-
sensitive portion of the ATP-dependent uptake was more 
responsive to feedback inhibition by increasing [Ca$^{2+}$], than 
was the thapsigargin-insensitive component. If true, the thap-
sigargin-sensitive pump activity in the absence of oxalate 
would be depressed early in the time course by increasing 
[Ca$^{2+}$]; after several minutes, the total ATP-dependent uptake 
would be largely due to the activity of the thapsigargin-insen-
sitive mechanism, and this could account for the observation 
that 1 mM thapsigargin had little or no effect on the prolonged 
time course of uptake (Fig. 1A). To directly test this hypothesis, 
we preloaded microsomes with Ca$^{2+}$ and measured ATP-de-
pendent initial uptake rates in the absence or presence of 
thapsigargin. Because preloading required 50 mM unchelated 
Ca$^{2+}$ (see “Experimental Procedures”), digitonin was elimi-
nated from these experiments due to adverse interactions with 
high [Ca$^{2+}$]. As shown in Fig. 3, preloading microsomes re-
sulted in a 40–50% decrease in the initial rate of ATP-depend-
45Ca for 5 min, reaching steady state, in the absence of oxalate and either with or without thapsigargin; at 5 min, the microsomal suspensions were injected with either 50 mM oxalate or an equal volume of Buffer A (see “Experimental Procedures”). As illustrated in Fig. 5, the addition of oxalate almost immediately transformed the 45Ca accumulation into a linear function and resolved thapsigargin-sensitive (−30 nmol g−1 s−1) and thapsigargin-insensitive (−8 nmol g−1 s−1) uptake rates similar to those measured when microsomes had been preincubated with 50 mM oxalate (Fig. 1B). Therefore, the protection against thapsigargin due to luminal Ca2+ was reversible.

DISCUSSION

Several studies have documented a protective effect of Ca2+ against thapsigargin inhibition. For example, Sagara et al. (10) showed a protection of skeletal muscle SERCA1 phosphorylation against thapsigargin inhibition when the enzyme was preincubated with Ca2+. In studies on neurally derived chromaffin cells of the adrenal medulla, Caspersen and Treiman (12) found that preincubation of microsomes with thapsigargin in the absence of Ca2+ resulted in a complete inhibition of Ca2+-pump E−P formation. But when the microsomes were preincubated with thapsigargin and 0.6 mM free Ca2+, a differential sensitivity to thapsigargin emerged, and this sensitivity varied among the fractions of these ER membranes on an isopycnic sucrose gradient. This work suggests that Ca2+ may have a protective effect against thapsigargin inhibition in neurally derived cells.

In neuronal ER vesicles, we observed an oxalate concentration dependence of thapsigargin sensitivity, consistent with a gradual removal of Ca2+ protection. Our data contribute new information about the nature of this protective effect, namely, that [Ca2+]i mediates this protection from thapsigargin inhibition. We eliminated any contributing effect of [Ca2+]o by adjusting the total [Ca2+] in the presence of 50 mM oxalate to give a [Ca2+]i identical to that in the absence of oxalate, 0.3 μM, as measured with Ca2+ mini-electrodes (Fig. 4D). Indeed, we found that the proportional effect of thapsigargin in the presence of oxalate and 100 μM EGTA with either 90 μM Ca2+ (0.1 μM [Ca2+]i) or 95 μM total Ca2+ (0.3 μM [Ca2+]i) was the same. We propose that the bulk of the ATP-dependent 45Ca uptake observed over 10 min in the absence of oxalate (Fig. 1A) is largely attributable to the activity of typical thapsigargin-sus-
acceptable SERCA pumps. The fact that thapsigargin had no apparent effect on this time course would be understandable if the isolated microsomes had a sufficiently high [Ca\(^{2+}\)], (prior to exposure to radioactive buffer) to elicit the protective behavior against thapsigargin inhibition. If this were in fact true, one would expect, in the absence of thapsigargin, the rate of ATP-dependent uptake in the presence of oxalate to be similar to the initial rate of uptake in the absence of oxalate. Comparing Fig. 1A (without oxalate) with Fig. 1B (with oxalate), the uptake rates are indeed similar: the initial uptake rate in the absence of oxalate was 70 ± 26 nmol·g\(^{-1}\)·s\(^{-1}\), and the uptake rate in 50 mM oxalate was 49 ± 6 nmol·g\(^{-1}\)·s\(^{-1}\). Measuring only early time points, to arrive at better estimates of initial uptake rates, revealed only small differences (Fig. 4C): 33 ± 3 nmol·g\(^{-1}\)·s\(^{-1}\) in the absence of oxalate and 50 ± 16 nmol·g\(^{-1}\)·s\(^{-1}\) in 50 mM oxalate. This slight increase in the initial rate in the presence of oxalate would be expected since lowering luminal free [Ca\(^{2+}\)] eliminates the normal negative feedback on pump activity (20, 22). In the absence of oxalate, 1 μM thapsigargin slightly decreased the initial rate of ATP-dependent 45Ca uptake from 33 ± 3 to 22 ± 3 nmol·g\(^{-1}\)·s\(^{-1}\) (Fig. 4C). This slight effect of thapsigargin is probably due to sufficiently low luminal free Ca\(^{2+}\) in a small portion of the microsomes, prior to time 0, such that thapsigargin is capable of binding to and inhibiting a minimal fraction of the thapsigargin-susceptible pumps. Because thapsigargin seems to affect a relatively small percentage of these pumps in the absence of oxalate, a decrease in the measured ATP-dependent uptake was detectable only at early time points, providing a plausible explanation for the apparent lack of thapsigargin sensitivity at later times (Fig. 1A). The noticeable difference in the apparent thapsigargin-insensitive uptake rate in the absence versus presence of 50 mM oxalate (Figs. 1, 3, and 4) further supports our second hypothesis and bolsters the notion that most of the uptake observed in the absence of oxalate is likely due to the action of thapsigargin-susceptible pumps.

The various SERCA-type pumps are thought to share a common mechanism of pump cycling (6), involving a conformational change from E1 (with two cytosolic facing, high-affinity Ca\(^{2+}\)-binding sites) to E2-P (with two luminal facing, low-affinity Ca\(^{2+}\)-binding sites) following cytosolic Ca\(^{2+}\) binding and ATP-dependent phosphorylation; E2-P then loses two Ca\(^{2+}\) ions to the lumen. In skeletal muscle SR, thapsigargin is thought to preferentially bind the E2 conformation of the pump, thereby shifting the E1-E2 equilibrium toward E2 (10, 25–28). Sagara et al. (10, 26) suggested that thapsigargin preferentially binds the enzyme conformation that exists in the absence of Ca\(^{2+}\), thereby forming a very stable “dead-end complex.” The protective effect of high [Ca\(^{2+}\)] against thapsigargin inhibition might be explained by a Ca\(^{2+}\)-induced shift in the E1-E2 equilibrium to the E1 (Ca\(^{2+}\)-bound) conformation of the pump (10, 11). In skeletal muscle, the protective effect of Ca\(^{2+}\) was lost after very short periods (1 s) of pump activity (10). In contrast, in ER from chromaffin cells, there was no change in the degree of thapsigargin-sensitive E–P formation for each membrane fraction, even when measuring pump phosphorylation up to 120 s (12). These diverging observations may be due to differences in the SERCA pump subtypes that exist in neurally derived ER and in skeletal muscle SR. Our results suggest that the majority of Ca\(^{2+}\)-pumping activity observed in neuronal ER is attributable to thapsigargin-sensitive SERCA subtypes that are susceptible to luminal Ca\(^{2+}\) protection against thapsigargin inhibition. In our system, it seems unlikely that this protection would be due to a cytosolic Ca\(^{2+}\)-induced shift in E1-E2 equilibrium toward the E1 conformation, as suggested for the SR. Assuming thapsigargin favors the E2 conformation of the pump, an alternative explanation is that increasing [Ca\(^{2+}\)](i), which slows pump activity, leads to pumps that exist in the E2 conformation longer; more Ca\(^{2+}\) ions in the lumen may result in E2-P pump conformations that have Ca\(^{2+}\)-bound for longer periods of time, and thapsigargin may have a reduced affinity for or be incapable of inactivating this Ca\(^{2+}\)-bound E2-P conformation of the pump.

In most whole cell preparations, as well as in many isolated microsome systems, thapsigargin treatment results in a significant release of accumulated Ca\(^{2+}\), even in the absence of Ca\(^{2+}\)-precipitating anions like oxalate. In our preparation of whole rat brain microsomes, however, we saw little or no effect of thapsigargin (at maximal doses) in the absence of oxalate. In E1-E2 equilibrium toward the E1 (Ca\(^{2+}\)-bound) conformation of the pump (10, 11). In skeletal muscle, the protective effect of Ca\(^{2+}\) was lost after very short periods (1 s) of pump activity (10). In contrast, in ER from chromaffin cells, there was no change in the degree of thapsigargin-sensitive E–P formation for each membrane fraction, even when measuring pump phosphorylation up to 120 s (12). These diverging observations may be due to differences in the SERCA pump subtypes that exist in neurally derived ER and in skeletal muscle SR. Our results suggest that the majority of Ca\(^{2+}\)-pumping activity observed in neuronal ER is attributable to thapsigargin-sensitive SERCA subtypes that are susceptible to luminal Ca\(^{2+}\) protection against thapsigargin inhibition. In our system, it seems unlikely that this protection would be due to a cytosolic Ca\(^{2+}\)-induced shift in E1-E2 equilibrium toward the E1 conformation, as suggested for the SR. Assuming thapsigargin favors the E2 conformation of the pump, an alternative explanation is that increasing [Ca\(^{2+}\)](i), which slows pump activity, leads to pumps that exist in the E2 conformation longer; more Ca\(^{2+}\) ions in the lumen may result in E2-P pump conformations that have Ca\(^{2+}\)-bound for longer periods of time, and thapsigargin may have a reduced affinity for or be incapable of inactivating this Ca\(^{2+}\)-bound E2-P conformation of the pump.

in the absence of thapsigargin inhibition, even prior to initiation of ATP-dependent 45Ca uptake. It is conceivable, during an isolation procedure that involves homogenization and thus disruption of ER membranes, that endogenous luminal Ca\(^{2+}\) buffers (which in living cells may subserve the function of oxalate) are lost or that microsomes accumulate and store small amounts of Ca\(^{2+}\) during the isolation protocol (the results of Fig. 4 suggest that very low levels of luminal free Ca\(^{2+}\) could mediate protection against thapsigargin). In fact, it is possible that oxalate is relieving a protection against thapsigargin inhibition in a number of microsomal studies reported in the literature. Particularly in
isolated subcellular membrane preparations, oxalate is often automatically included when measuring thapsigargin sensitivity of $^{45}$Ca uptake (8, 9, 14), and the same effect of thapsigargin is observed in the absence of oxalate is unclear.

Several groups have documented thapsigargin-resistant ATP-dependent uptake in a variety of cell and membrane preparations, including PC12 cells (13–15), dog brain microsomes (11), saponin-permeabilized DDT-MF-2 smooth muscle cells (16), permeabilized cell and microscope preparations of rat pituitary GH$_2$C$_1$ cells (17), cultured arterial myocytes (18), and DC-3F Chinese hamster lung cells (19). Kijima et al. (11) found that in dog brain microsomes, $\sim 70\%$ of their Ca$^{2+}$-loading activity was inhibited by thapsigargin, and only the thapsigargin-sensitive portion of this activity was responsive to 10 $\mu$M IP$_3$ (which released $\sim 27\%$ of the total preloaded Ca$^{2+}$). Our observations using rat brain microsomes were similar: $\sim 80\%$ of the oxalate- and ATP-dependent Ca$^{2+}$ uptake was thapsigargin-sensitive; 5 $\mu$M IP$_3$ released $\sim 40\%$ of the total ATP-dependent $^{45}$Ca accumulation; and only the thapsigargin-sensitive portion of this uptake was responsive to IP$_3$. In contrast to other studies that found a caffeine or ryanodine sensitivity associated with the thapsigargin-resistant Ca$^{2+}$ pools (17, 18), we observed no effect of 10 mM caffeine on the thapsigargin-insensitive pool. Caffeine did, however, cause a 37% reduction in the thapsigargin-sensitive $^{45}$Ca accumulation. Our results suggest that the thapsigargin-sensitive and -insensitive uptake mechanisms are functionally segregated. Poulsen et al. (29) have also demonstrated a thapsigargin-sensitive Ca$^{2+}$ pool in adrenal chro-
maffin cells responsive to both IP$_3$ and caffeine. In two of the aforementioned studies (16, 19), no differential sensitivity to vanadate was observed for these two uptake processes. In contrast, we observed a small but significant difference in the uptake kinetics of this thapsigargin-insensitive process are much slower than those of the thapsigargin-sensitive pump. This study also suggests that in neural cells, the thapsigargin-insensitive pool may be functionally segregated from both the IP$_3$ and ryanodine receptor Ca$^{2+}$ release channels. Finally, our results demonstrate for the first time that the ability of high [Ca$^{2+}$] to protect some neuronal ER Ca$^{2+}$ pumps against thapsigargin inhibition is conferred by luminal free Ca$^{2+}$ and cannot be explained by an effect of cytosolic Ca$^{2+}$. These new data suggest that either the mechanism by which Ca$^{2+}$ can protect against thapsigargin inhibition is different for the SERCA subtypes that exist in neuronal ER as compared with skeletal muscle SR or that a generalized model for Ca$^{2+}$ protection should be re-evaluated in terms of possible luminal Ca$^{2+}$ effects.

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