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Abstract

To study the role of calreticulin in Ca(2+) homeostasis and apoptosis, we generated cells inducible for full-length or truncated calreticulin and measured Ca(2+) signals within the cytosol, the endoplasmic reticulum (ER), and mitochondria with "cameleon" indicators. Induction of calreticulin increased the free Ca(2+) concentration within the ER lumen, [Ca(2+)](ER), from 306 +/- 31 to 595 +/- 53 microm, and doubled the rate of ER refilling. [Ca(2+)](ER) remained elevated in the presence of thapsigargin, an inhibitor of SERCA-type Ca(2+) ATPases. Under these conditions, store-operated Ca(2+) influx appeared inhibited but could be reactivated by decreasing [Ca(2+)](ER) with the low affinity Ca(2+) chelator N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine. In contrast, [Ca(2+)](ER) decreased much faster during stimulation with carbachol. The larger ER release was associated with a larger cytosolic Ca(2+) response and, surprisingly, with a shorter mitochondrial Ca(2+) response. The reduced mitochondrial signal was not associated with visible morphological alterations of mitochondria or with disruption of the contacts between [...]
Calreticulin Differentially Modulates Calcium Uptake and Release in the Endoplasmic Reticulum and Mitochondria*

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To study the role of calreticulin in Ca\(^{2+}\) homeostasis and apoptosis, we generated cells inducible for full-length or truncated calreticulin and measured Ca\(^{2+}\) signals within the cytosol, the endoplasmic reticulum (ER), and mitochondria with “cameleon” indicators. Induction of calreticulin increased the free Ca\(^{2+}\) concentration within the ER lumen, [Ca\(^{2+}\)]\(_{\text{ER}}\), from 306 ± 31 to 595 ± 53 μM, and doubled the rate of ER refilling. [Ca\(^{2+}\)]\(_{\text{ER}}\) remained elevated in the presence of thapsigargin, an inhibitor of SERCA-type Ca\(^{2+}\) ATPases. Under these conditions, store-operated Ca\(^{2+}\) influx appeared inhibited but could be reactivated by decreasing [Ca\(^{2+}\)]\(_{\text{ER}}\) with the low affinity Ca\(^{2+}\) chelator N,N,N\(^{-}\),N\(^{-}\),N\(^{-}\)-tetakis(2-pyridylmethyl)ethylenediamine. In contrast, [Ca\(^{2+}\)]\(_{\text{ER}}\) decreased much faster during stimulation with carbachol. The larger ER release was associated with a larger cytosolic Ca\(^{2+}\) response and, surprisingly, with a shorter mitochondrial Ca\(^{2+}\) response. The reduced mitochondrial signal was not associated with visible morphological alterations of mitochondria or with disruption of the contacts between mitochondria and the ER but correlated with a reduced mitochondrial membrane potential. Altered ER and mitochondrial Ca\(^{2+}\) responses were also observed in cells expressing an N-truncated calreticulin but not in cells overexpressing calnexin, a P-domain containing chaperone, indicating that the effects were mediated by the unique C-domain of calreticulin. In conclusion, calreticulin overexpression increases Ca\(^{2+}\) fluxes across the ER but decreases mitochondrial Ca\(^{2+}\) and membrane potential. The increased Ca\(^{2+}\) turnover between the two organelles might damage mitochondria, accounting for the increased susceptibility of cells expressing high levels of calreticulin to apoptotic stimuli.

Ca\(^{2+}\) signals control key biological functions, including fertilization, development, cardiac contraction, and secretion of neurotransmitters and hormones (1). At the cellular level, Ca\(^{2+}\) can be either a life and death signal, as changes in cytosolic free Ca\(^{2+}\) concentration can control cell growth and proliferation or induce apoptosis, the programmed cell death (2). These diverging effects reflect the precise spatial and temporal encoding of Ca\(^{2+}\) signals, which depends largely on the controlled release of Ca\(^{2+}\) from intracellular organelles. The main intracellular Ca\(^{2+}\) store is the endoplasmic reticulum (ER), but mitochondria also take up and release Ca\(^{2+}\) very efficiently and are often strategically located close to Ca\(^{2+}\) sources (see Refs. 3–6 and reviewed in Ref. 7). This intimate connection allows mitochondria to shape Ca\(^{2+}\) signals (8) by modulating the release of Ca\(^{2+}\) from the ER (9) and the influx of Ca\(^{2+}\) across the plasma membrane (10), or by providing a local source of Ca\(^{2+}\) for ER refilling (11). To achieve such precise control over Ca\(^{2+}\) fluxes, the ER and mitochondria are equipped with a variety of Ca\(^{2+}\) transport and storage proteins and exert a tight control of the Ca\(^{2+}\) concentration within their lumen. Ca\(^{2+}\) fluxes across the ER membrane are stringently dependent on the free Ca\(^{2+}\) concentration within the ER, [Ca\(^{2+}\)]\(_{\text{ER}}\), as Ca\(^{2+}\)-allosterically modulates the activity of the InsP\(_3\) receptor, the main Ca\(^{2+}\)-release channel of the ER. In addition, changes in [Ca\(^{2+}\)]\(_{\text{ER}}\) regulate the Ca\(^{2+}\) permeability of store-operated channels (SOC) at the plasma membrane (12). The mechanism of this “capacitative” coupling is still elusive and has been proposed to involve the diffusion of a soluble messenger (13), direct interaction between InsP\(_3\) receptors and SOC channels (14), or a secretion-like docking mechanism (15).

In addition to these Ca\(^{2+}\) signaling functions, the Ca\(^{2+}\) concentration within the ER lumen and the mitochondrial matrix also affects many functions of these organelles. The activity of several ER resident chaperone proteins is modulated by changes in [Ca\(^{2+}\)]\(_{\text{ER}}\), which thereby indirectly regulates the processing, sorting, and secretion of cargo proteins (16). In mitochondria, Ca\(^{2+}\) directly controls the activity of several dehydrogenases, thereby coupling the cell metabolism to the Ca\(^{2+}\) signal (17, 18). The mitochondrial “decoding” of Ca\(^{2+}\) signals allows cells to quickly respond to an increased energy demand but can be turned into a death signal during concomitant exposure to apoptotic stimuli (reviewed in Ref. 19). In the presence of ceramide, even physiological Ca\(^{2+}\) responses of

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The abbreviations used are: ER, endoplasmic reticulum; Doc, doxycycline; InsP\(_3\), inositol 1,4,5-trisphosphate; SOC, store-operated Ca\(^{2+}\) influx; SERCA, sarco/endoplasmic reticulum Ca\(^{2+}\) transport ATPase; [Ca\(^{2+}\)]\(_{\text{ER}}\), [Ca\(^{2+}\)]\(_{\text{cyt}}\), and [Ca\(^{2+}\)]\(_{\text{mito}}\), cytosolic, ER, and mitochondrial free Ca\(^{2+}\) concentration, respectively; CCh, carbachol; YC, yellow cameleon; DeRed, Red fluorescent protein from Discosoma sp.; TPEN, N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine; Tg, thapsigargin; Tet, tetracycline; HA, hemagglutinin; TMRM, tetramethylrhodamine methyl ester; CRT, calreticulin; HEDTA, N-(2-hydroxyethyl)ethylenediamine-N',N'-triacetic acid.

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mitochondria to InSp$_3$-generating agonists are sufficient to induce apoptosis, possibly via Ca$^{2+}$-dependent opening of the permeability transition pore (20). The Ca$^{2+}$ content of the ER also affects the cell sensitivity to apoptotic stimuli. A decreased [Ca$^{2+}$]$_{ER}$ was observed in cells overexpressing the antia apoptotic protein Bcl-2 (21, 22), and a variety of conditions that decreased [Ca$^{2+}$]$_{ER}$ has been shown to protect cells from ceramide-induced cell death (23). The opposite effect was observed in cells overexpressing the Ca$^{2+}$-ATPases (SERCA2b) or the ER-resident Ca$^{2+}$-binding chaperone calreticulin, which increased the Ca$^{2+}$ content of the ER (23–25). Conversely, cells lacking the calreticulin had a decreased ER Ca$^{2+}$ content and were more resistant to apoptotic stimuli (26). Calreticulin-deficient cells, however, had normal [Ca$^{2+}$]$_{ER}$ levels, suggesting that the ability of calreticulin to modulate the cell sensitivity to apoptotic stimuli might be linked to changes in the total Ca$^{2+}$ content of the ER rather than to changes in [Ca$^{2+}$]$_{ER}$.

Calreticulin is a 46-kDa Ca$^{2+}$-binding chaperone that interacts in a Ca$^{2+}$-dependent fashion with several ER resident proteins, with unfolded glycoproteins, and with Ca$^{2+}$-transporters at the ER membrane (27, 28). Calreticulin is composed of three structural and functional domains as follows: a highly conserved N-terminal domain, involved in chaperone function and in the interactions with other ER chaperones; a proline-rich P-domain, which shares significant amino acid sequence identity with calnexin, calmegin, and CALNUC and is involved in the chaperone function of calreticulin; and a C-terminal domain that binds Ca$^{2+}$ ions with low affinity and high capacity (29). The Ca$^{2+}$-binding C-domain has been postulated to be the “Ca$^{2+}$ sensor” that regulates calreticulin interactions with other proteins (25, 29). Because of the central role of the ER in Ca$^{2+}$ signaling, both the chaperoning functions of calreticulin as well as its interactions with ER Ca$^{2+}$-transporters can interfere with Ca$^{2+}$ signals. For example, calreticulin inhibits repetitive Ca$^{2+}$ waves by interacting selectively with distinct isoforms of SERCA2 (30, 31). On the other hand, conflicting results have been reported regarding the role of calreticulin in the modulation of store-operated Ca$^{2+}$ influx (SOC). Stable up-regulation of calreticulin in HEK-293 cells inhibits thapsigargin-induced Ca$^{2+}$ or Mn$^{2+}$ influx (32), whereas transient expression in RBL-1 cells only delays the activation of the I$_{CRAC}$ current, to an extent that correlated with the extent of store depletion (33). Similarly, in Xenopus oocytes overexpressing calreticulin and stimulated with InSp$_3$-generating agonists, SOC inhibition correlated with increased [Ca$^{2+}$]$_{ER}$ levels as expected from the capacitative mechanism (34).

Because of the plethoric effects of the protein and the different expression system used, the role of calreticulin in Ca$^{2+}$ signaling remains controversial. To clarify the role of calreticulin in Ca$^{2+}$ homeostasis and in apoptosis, we generated cell lines inducible for either the full-length calreticulin, an N-truncated version lacking the chaperoning N-domain, or its chaperone homologue calnexin. The effects of a controlled increase in protein levels on cytosolic, ER, and mitochondrial Ca$^{2+}$ signals were measured using genetically encoded Ca$^{2+}$-sensitive “cameleon” indicators. The bright fluorescence and molecular targeting of the probes allowed precise quantification of the changes in free [Ca$^{2+}$] occurring within the different cell compartments at different times after the induction of protein expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dubelco’s modified Eagle’s culture medium, fetal calf serum, penicillin, streptomycin, and gentamicin were obtained from Invitrogen. Thapsigargin, nigericin, monensin, ATP, and HEPES were purchased from Sigma. Ionomycin was obtained from Calbiochem. Hygromycin B, doxycycline, EGTA, and HEDTA were from Fluka (Buchs, Switzerland). JC-1 and TMRM were from Molecular Probes (Eugene, OR). Transfet transfection reagent was purchased from Promega (Madison, WI). All other chemicals were of analytic grade and were obtained from Fluka or Sigma. The “Ca$^{2+}$” medium contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 10 mM glucose, and 20 mM Heps, pH 7.4. For the “Ca$^{2+}$-free medium” CaCl$_2$ was omitted, and 0.5 mM EGTA was included. Drugs were dissolved in dimethyl sulfoxide (Me$_2$SO) or ethanol and diluted in the recording medium on the day of use, at a final solvent concentration <0.1%.

**Constructs**—Plasmids YC2.1, YC2.2, and YC4.2p were kindly provided by Dr. R. Y. Tsien. Plasmid YC2 was and YC4.2p were generated as described previously (11). cDNA encoding full-length or truncated (P + C-domain HA-tagged) rabbit calreticulin and canine calnexin were subcloned into the pTRE plasmid to generate pTRE-CRT, pTRE-P + C and pTRE-CNX expression vectors, respectively. These vectors were used to generate Tet-On inducible cell lines. Plasmid DNAs were purified using a Qiagen column by the Maxi-prep purification protocol recommended by the manufacturer.

**Generation of the Tet-On Cell Lines**—The Tet-On cell lines were generated by co-transfecting pTRE-CRT, pTRE-P + C, or pTRE-CNX with pTRE-Hyg at a ratio 2:1 into HEK-293 cells (HeLa cells) by the Ca$^{2+}$–phosphate protocol. Transfected cells were selected for growth in the presence of 200 µg of hygromycin B/mL of culture medium. Single colonies of the hygromycin B-resistant cells were tested for doxycycline (Dox)-dependent expression of calreticulin, P + C-domain, and calnexin by Western blotting with anti-calreticulin, anti-HA, and anti-calnexin antibodies. Three cell lines with the highest inducible expression of calreticulin, P + C-domain, and calnexin were selected for this study.

**Cell Culture**—HEK-293 or Tet-On cell lines were grown in Dubelco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, 2 mM-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin and were maintained in a humidified incubator at 37 °C in the presence of 95% air. Cells were subcultured at 200,000 cells per coverslip. With HEK-293 at 60% of confluency, the cells were transiently transfected with cDNAs encoding the yellow cameleon probes. Cells were imaged 3–5 days after transfection. Stable HEK-293 transfecants were grown in the presence of geneticin (100 µg/ml) for 3 weeks, and 20 clones were expanded for each condition and tested for expression of the probes. 2 µg of Dox/ml was used to induce into the culture medium to induce expression of calreticulin, its P + C-domain, or calnexin in Tet-On cell lines.

**Immunoblotting and Immunocytochemistry**—Western blot analysis with the use of goat anti-calreticulin, anti-HA, and rabbit anti-calnexin antibodies was carried out as described (25). For indirect immunofluorescence of calreticulin expressing HEK Tet-On cells were plated on coverslips pretreated with polylysine and cultured in the presence or absence of 2 µg of Dox/ml for 72 h. Cells were washed 3 times with PBS, fixed with 3.7% paraformaldehyde for 20 min, and permeabilized with 0.3% Triton X-100 for 20 min. Calreticulin was detected by incubation with a goat anti-calreticulin antibody followed by staining with a rabbit polyclonal antibody conjugated to Texas Red (Jackson Immuno-Research).

**[Ca$^{2+}$]$_{i}$ Measurements**—Cells plated on 25-mm coverslips were superfused at 37 °C in a thermostatic chamber (Harvard Apparatus, Holliston, MA) equipped with gravity feed inlets and vacuum outlet for solution changes. Dual-emission ratio imaging of [Ca$^{2+}$] with cameleon probes was performed as described previously (11). Cameleon fluorescence from cells was imaged on a Axiosvert S100 TV using a 100×, 1.3 NA oil-immersion objective (Carl Zeiss AG, Feldbach, Switzerland). Cells were excited by the 430 ± 10 nm line from a monochromator (DeltaRam, Photon Technology International Inc., Monmouth Junction, New Jersey) through a 450DRLP dichroic mirror. Fluorescence emission from the cameleons was imaged using a cooled, 16-bits CCD back-illuminated transfer MicroMax camera (Princeton Instruments, Roper Scientific, Trenton, NJ) at two emission wavelengths, using a filter wheel (Ludi Electronic Products, Hawthorn, NY) to alternately change the two emission filters (475DF15 and 535DF25, Omega Optical, Brattleboro, VT). Image acquisition and analysis was performed with the Metamorph/Metafluor 4.1.2 software (Universal Imaging, West Chester, PA). Changes in fluorescence ratio, $R = (fluorescence intensity at 535 nm − background intensity at 535 nm)/fluorescence intensity at 475 nm − background intensity at 475 nm), were calibrated in [Ca$^{2+}$] using Equation 1.

\[
[Ca^{2+}] = K_d (R_{Rmax} - R_{Rmin} - R)^{1/n} \tag{Eq. 1}
\]

where $R_{Rmax}$ and $R_{Rmin}$ are the ratios obtained, respectively, in the absence of Ca$^{2+}$ and at saturating Ca$^{2+}$. $K_d$ is the apparent dissociation constant, and $n$ is the Hill coefficient of the Ca$^{2+}$-calibrations curves.
obtained in situ for each camelion. For better three-dimensional rendering wide field or confocal image stacks were deconvoluted after acquisition on a Silicon Graphics Octane work station using the Huygens 2 software, and shadow projections were constructed using the Imaris software (Bitplane AG, Zurich, Switzerland).

RESULTS

To generate cells inducible for calreticulin, we stably transfected HEK-293 cells with a rabbit calreticulin cDNA construct driven by the tetracycline promoter (Tet-ON). The activation of calreticulin gene transcription by doxycycline (Dox), added to the culture medium, was confirmed by immunoblotting with a goat polyclonal CRT antibody (Fig. 1A). Quantification of the immunoblot indicated that the cellular calreticulin content increased by 2.5-fold within 24 h and remained at this level up to 5 days in culture. The induction was specific for calreticulin, as addition of Dox had no effect on the expression of other ER luminal chaperones such as ERP57 or Bip (not shown). An immunostaining with a calreticulin-specific antibody confirmed that protein expression was much stronger in Dox-induced cells and still displayed the reticular pattern typical of the ER (Fig. 1B, left). No immunoreactivity was observed in the cytosol or at the plasma membrane, confirming that, after induction, calreticulin remained localized within the ER lumen. The ER structure was not noticeably altered, because Dox induction did not affect the intracellular distribution of the ER-targeted Ca²⁺ indicator YC4ER (Fig. 1B, right). This indicated that the increase in calreticulin did not interfere with the import, ER retention, or folding efficiency of the GFP-based chaperone family members revealed that the induction of calreticulin markedly increased Ca²⁺ concentration within the ER lumen. The ER structure was not noticeably altered, because Dox induction did not affect the intracellular distribution of the ER-targeted Ca²⁺ indicator YC4ER (Fig. 1B, right). This indicated that the increase in calreticulin did not interfere with the import, ER retention, or folding efficiency of the GFP-based indicator. Moreover, the Ca²⁺ affinity of both the ER-targeted probe YC4ER and of the cytosolic probe YC2, measured in situ in cells permeabilized with ionomycin or digitonin, was not affected by the increased expression of calreticulin (Fig. 1C). Thus, Dox induction increased the amount of calreticulin within the ER lumen in a controlled manner, without interfering with the targeting specificity or Ca²⁺ dependence of the calmeleon Ca²⁺ indicators.

Effect of Calreticulin Induction on ER [Ca²⁺] Homeostasis—To assess whether the sustained increase in calreticulin levels interfered with ER Ca²⁺ homeostasis, we measured the changes in the free Ca²⁺ concentration within the ER lumen, [Ca²⁺]ER, using the low affinity ER-targeted ratiometric “camleleon” indicator YC4ER (K_D = 290 μM (11)). YC4ER measurements revealed that the induction of calreticulin markedly increased the resting [Ca²⁺]ER levels (Fig. 2) with the basal [Ca²⁺]ER values averaging 306 ± 31 μM in the absence and 595 ± 53 μM 2 h after Dox-dependent induction of calreticulin expression. The increase could not be attributed to a specific ER region, as higher [Ca²⁺]ER levels were observed throughout the ER network in the ratio images (Fig. 2A). Thus, the 2.5-fold increase in calreticulin levels caused, after 3 days of induction, a doubling in the free Ca²⁺ concentration within the ER lumen. The doubling in resting [Ca²⁺]ER could reflect either an increased Ca²⁺ pumping activity, or a decrease in the passive Ca²⁺ permeability, or “leak” of the ER. To distinguish between these possibilities, we studied the effect of the SERCA inhibitor thapsigargin (Tg) on calreticulin-dependent changes in free ER Ca²⁺. Tg induced a slow decrease in [Ca²⁺]ER in both control and calreticulin-induced cells (Fig. 2B). A linear fit of the initial [Ca²⁺]ER decay revealed that the kinetics of Ca²⁺ release were nearly identical (∆[Ca²⁺] = −5.2 ± 0.3 versus −6.0 ± 0.3 μM/s), despite the higher [Ca²⁺]ER in the calreticulin overexpressers. Consequently, calreticulin-overexpressing cells retained a higher [Ca²⁺]ER level throughout the course of Tg stimulation. A further decline was observed upon addition of the ionophore ionomycin (Fig. 2, B and D), indicating that the ER Ca²⁺ store was not fully depleted by Tg. Thus, a block of SERCA ATPases unmasked a nearly identical passive Ca²⁺ permeability in the ER, regardless of the increase in calreticulin levels.

In contrast, upon stimulation with the InsP₃-generating agonist carbachol (CCh), [Ca²⁺]ER decreased much faster in CRT-induced cells, and similar depleted levels were achieved within 100 s of agonist stimulation (Fig. 2, C and D). The faster kinetics of Ca²⁺ release (∆[Ca²⁺] = −4.5 ± 0.6 versus −11.4 ±...
increased the agonist-induced ER Ca\textsuperscript{2+} rest. However, induction of calreticulin expression markedly reduced Ca\textsuperscript{2+} entry across the plasma membrane and the ER Ca\textsuperscript{2+} stores. Compared with previous studies using fura-2 (32), the differences between control and calreticulin overexpresser cells were striking, reflecting the better adequacy of the Y2 probe to quantify [Ca\textsuperscript{2+}]ER changes in the micromolar range. Subsequent addition of Ca\textsuperscript{2+} to assess the activity of store-operated Ca\textsuperscript{2+} channels at the plasma membrane revealed that, as previously reported (32), Ca\textsuperscript{2+} influx was severely blunted in Tg-stimulated calreticulin-overexpressing cells (Fig. 3). This decreased influx correlated well with the increased [Ca\textsuperscript{2+}]ER levels measured with YC4ER (Fig. 2) and indicated that, consistent with the capacitative hypothesis, the activity of SOC channels is determined by changes in [Ca\textsuperscript{2+}]ER levels. Accordingly, Ca\textsuperscript{2+} influx was similar in control and Dox-induced cells stimulated with CCh, which had comparable [Ca\textsuperscript{2+}]ER levels (Figs. 2 and 3). However, in this case the activity of SOC channels could not be readily inferred from the changes in [Ca\textsuperscript{2+}]cyt, because of the concomitant ER Ca\textsuperscript{2+} pumping activity. Although Ca\textsuperscript{2+} re-addition produced similar [Ca\textsuperscript{2+}]cyt changes, larger [Ca\textsuperscript{2+}]ER increases were observed in calreticulin-overexpressing cells, indicating that substantially more Ca\textsuperscript{2+} was taken up by the ER (Fig. 2A). This suggested that the net flux of Ca\textsuperscript{2+} ions across the plasma membrane was, in fact, larger in calreticulin-induced cells but that the Ca\textsuperscript{2+} entering the cell was rapidly taken up by the ER. Thus, the increased [Ca\textsuperscript{2+}]ER levels observed in the presence of Tg correlated with decreased SOC activity. In contrast, SOC activity was high in calreticulin overexpresser cells stimulated with CCh but did not translate into a larger cytosolic Ca\textsuperscript{2+} signal because of the high concomitant ER Ca\textsuperscript{2+} pumping activity.

**Time Course of the CRT Effects on ER and Cytosolic [Ca\textsuperscript{2+}]**—

1.4 $\mu$M/min) suggested that calreticulin overexpression increased the InsP\textsubscript{3}-stimulated Ca\textsuperscript{2+} permeability of the ER. Importantly, re-addition of Ca\textsuperscript{2+} to the external medium resulted in a rapid increase of the [Ca\textsuperscript{2+}]ER in calreticulin-overexpressing cells (Fig. 2C). The recovery rates were 1.9-fold higher in calreticulin overexpressers than in control, non-induced cells, at any given [Ca\textsuperscript{2+}]ER (Fig. 2C, inset). Because this assay measures the net flow of Ca\textsuperscript{2+} from the external space to the ER, this indicates that both the influx of Ca\textsuperscript{2+} across the plasma membrane and the ER Ca\textsuperscript{2+} pumping activity were increased in cells expressing high levels of calreticulin. In the absence of agonist stimulation, the increased rates of ER refill were not balanced by a parallel increase in the endogenous ER Ca\textsuperscript{2+} permeability, resulting in higher [Ca\textsuperscript{2+}]ER levels at rest. However, induction of calreticulin expression markedly increased the agonist-induced ER Ca\textsuperscript{2+} permeability, and therefore, upon stimulation, more Ca\textsuperscript{2+} was released from the ER lumen.

**Effect of CRT Induction on Cytosolic Ca\textsuperscript{2+} Signals**—To assess how these changes in ER luminal Ca\textsuperscript{2+} homeostasis influenced Ca\textsuperscript{2+} signals in the cytosol, we monitored changes in cytoplasmic Ca\textsuperscript{2+}, [Ca\textsuperscript{2+}]cyt, with the cytosolic YC2 probe ($K_D = 1.24 \mu$M). Ca\textsuperscript{2+} release from ER stores was measured in the absence of external Ca\textsuperscript{2+}, and Ca\textsuperscript{2+} influx was subsequently measured by re-adding Ca\textsuperscript{2+} to the external medium. Fig. 3 shows that both CCh and Tg elicited a much larger increase in [Ca\textsuperscript{2+}]cyt in calreticulin overexpressing cells, indicating that substantially more Ca\textsuperscript{2+} was released from the intracellular Ca\textsuperscript{2+} stores. Compared with previous studies using fura-2 (32),
expression and remained elevated thereafter. In contrast, Ca\textsuperscript{2+} increased 24 h after Dox-dependent induction of calreticulin as a determinant of SOC activity, we acutely modulated [Ca\textsuperscript{2+}] of external Ca\textsuperscript{2+}, which was re-added when indicated. B, average [Ca\textsuperscript{2+}]\textsubscript{cyt} responses elicited by Tg in control (cont) and calreticulin-induced cells. C, [Ca\textsuperscript{2+}]\textsubscript{cyt} responses elicited by CCh. B, average [Ca\textsuperscript{2+}]\textsubscript{cyt} responses measured with CCh. Data are mean ± S.E. of the indicated number of experiments (*, p < 0.02; **, p < 0.0001).

To better assess the effects of high expression of calreticulin on Ca\textsuperscript{2+} handling, we measured the [Ca\textsuperscript{2+}]\textsubscript{ER} and [Ca\textsuperscript{2+}]\textsubscript{cyt} responses at different times following the induction of protein expression. Fig. 4A shows that the resting [Ca\textsuperscript{2+}]\textsubscript{ER} levels were increased 24 h after Dox-dependent induction of calreticulin expression and remained elevated thereafter. In contrast, Ca\textsuperscript{2+} influx, taken as the peak [Ca\textsuperscript{2+}]\textsubscript{cyt} upon Ca\textsuperscript{2+} re-addition to Tg-treated cells, was inhibited only 3 days after the induction with Dox (Fig. 4B, circles). The amount of releasable Ca\textsuperscript{2+} followed a similar delayed time course; the peak of Tg-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} release was only marginally increased 24 h post-induction and became significantly increased only 2 or 3 days after Dox induction of calreticulin expression (Fig. 4B, squares). The strong correlation between SOC activation and the total stored Ca\textsuperscript{2+} likely reflected the higher residual [Ca\textsuperscript{2+}]\textsubscript{ER} levels achieved at the end of the Tg stimulation in calreticulin-overexpressing cells. Although an ~5-min stimulation with Tg is routinely used to deplete Ca\textsuperscript{2+} stores, YC4\textsubscript{ER} measurements indicated that [Ca\textsuperscript{2+}]\textsubscript{ER} did not reach fully depleted levels within the first 5 min in cells induced to express calreticulin for 3 days (Fig. 2). Thus, induction of calreticulin expression had two temporally distinct effects on Ca\textsuperscript{2+} homeostasis as follows: acute induction caused an immediate increase in [Ca\textsuperscript{2+}]\textsubscript{ER}, whereas more sustained expression of high levels of calreticulin was required to increase the total amount of stored Ca\textsuperscript{2+} and to inhibit store-operated Ca\textsuperscript{2+} influx.

To show that the [Ca\textsuperscript{2+}]\textsubscript{ER} levels were indeed the prime determinant of SOC activity, we acutely modulated [Ca\textsuperscript{2+}]\textsubscript{ER} using the low affinity Ca\textsuperscript{2+} chelator N,N,N’,N’-tetrakis(2-pyrindinylmethyl)-ethylenediamine (TPEN). This cell membrane-permeant Ca\textsuperscript{2+} chelator has a $K_D$ that matches the free Ca\textsuperscript{2+} levels in the ER lumen, providing an excellent tool to clamp [Ca\textsuperscript{2+}]\textsubscript{ER} without affecting the [Ca\textsuperscript{2+}]\textsubscript{cyt} responses (35). Fig. 5 shows that addition of TPEN produced a rapid decrease in [Ca\textsuperscript{2+}]\textsubscript{ER} but had only minor effect on [Ca\textsuperscript{2+}]\textsubscript{cyt}. The effects of TPEN were reversible (not shown). The chelator did not prevent the [Ca\textsuperscript{2+}]\textsubscript{ER} changes induced by Tg. Therefore, it was possible to artificially impose normal resting and depleted [Ca\textsuperscript{2+}]\textsubscript{ER} levels in calreticulin-overexpressing cells (Fig. 5A). Cytosolic Ca\textsuperscript{2+} measurements revealed that a robust Ca\textsuperscript{2+} influx could be elicited with Tg in the presence of the ER luminal Ca\textsuperscript{2+} chelator, as expected from the capacitative mechanism (Fig. 5B). This indicates that SOC channels were fully functional in calreticulin overexpresser cells when [Ca\textsuperscript{2+}]\textsubscript{ER} was artificially clamped to the level found in non-induced cells. Therefore, the high expression of calreticulin had no effect per se on the activity of SOC channels, which is determined primarily by the [Ca\textsuperscript{2+}]\textsubscript{ER} level in the ER lumen.

Effect of Calreticulin Induction on Mitochondrial Ca\textsuperscript{2+} Signals—In addition to communicating with the plasma membrane, the ER is also involved in a cross-talk with mitochondria, which are strategically located close to the sites of Ca\textsuperscript{2+} release and can capture part of the Ca\textsuperscript{2+} released by the ER (6). To assess whether the increased [Ca\textsuperscript{2+}]\textsubscript{ER} levels and InsP\textsubscript{3}-induced ER permeability also affected mitochondria, we measured Ca\textsuperscript{2+} changes within the mitochondrial matrix, [Ca\textsuperscript{2+}]\textsubscript{mit}. Two mitochondrial probes of different affinities were used as follows: the high affinity YC2\textsubscript{mit} probe ($K_D = 1.24 \textbf{\mu M}$), to allow accurate measurements within the low micromolar range, and the low affinity YC4.1\textsubscript{mit} probe ($K_D = 105 \textbf{\mu M}$), to better resolve the high levels achieved during peak [Ca\textsuperscript{2+}]\textsubscript{mit} responses (11). The basal [Ca\textsuperscript{2+}]\textsubscript{mit} levels reported by the YC2\textsubscript{mit} probe were not affected by the induction of calreticulin. Surprisingly, however, YC4.1\textsubscript{mit} measurement revealed that the [Ca\textsuperscript{2+}]\textsubscript{mit} responses were blunted in calreticulin-induced cells (Fig. 6A). The peak [Ca\textsuperscript{2+}]\textsubscript{mit} levels measured with YC4.1\textsubscript{mit} were close to

![Fig. 3. Effect of calreticulin induction on cytosolic Ca\textsuperscript{2+} signals. A, [Ca\textsuperscript{2+}]\textsubscript{cyt} changes induced by Tg. Cells were stimulated in the absence of external Ca\textsuperscript{2+}, which was re-added when indicated. B, average [Ca\textsuperscript{2+}]\textsubscript{cyt} responses elicited by Tg in control (cont) and calreticulin-induced cells. C, [Ca\textsuperscript{2+}]\textsubscript{cyt} responses elicited by CCh. B, average [Ca\textsuperscript{2+}]\textsubscript{cyt} responses measured with CCh. Data are mean ± S.E. of the indicated number of experiments (*, p < 0.02; **, p < 0.0001).](image-url)
in mitochondrial membrane potential, $\Delta \psi_m$, which determines the driving force for $\text{Ca}^{2+}$, also directly impact on $[\text{Ca}^{2+}]_{\text{mit}}$. To distinguish between these two possibilities, we measured $\Delta \psi_m$ and assessed the morphology of mitochondria as well as their interactions with the ER. To assess the morphology of mitochondria without relying on the extent of their negative membrane potential, we used the genetically targeted indicator DsRedmit. Fig. 7A shows that the staining pattern of DsRedmit was not markedly altered in calreticulin-induced cells. Upon Dox induction, mitochondria retained their "worm-like" appearance and did not appear swollen or condensed (Fig. 7A). Although a variety of mitochondria morphologies was observed both in control and Dox-induced cells, no systematic alterations could be observed in association with the induction of calreticulin expression. More importantly, the overlap between the mitochondrial and the ER signal was similar in control and calreticulin cells, as assessed by co-labeling cells with YC4ER and Mitotracker Red (Fig. 7B). In both conditions, mitochondria appeared embedded into the ER, suggesting that the induction of calreticulin did not disrupt the interactions between the ER and mitochondria. Thus, although the resolution of the confocal microscope did not allow us to resolve the contact points between the ER and mitochondria, the structural integrity as well as the relationship between the two organelles appeared to be preserved.

We next measured the mitochondrial membrane potential, $\Delta \psi_m$, using the rhodamine-based dye TMRM, which accumulates into polarized mitochondria. The $\Delta \psi_m$-driven accumulation of TMRM into mitochondria was quantified as the ratio of the mitochondrial over cytosolic fluorescence intensity (38). The TMRM ratio was significantly lower in Dox-induced cells (Fig. 7C, left panel), indicating that $\Delta \psi_m$ was reduced by long term overexpression of calreticulin. The decrease in $\Delta \psi_m$ was not due to TMRM photoactivation and subsequent local generation of reactive oxygen species (ROS) (39), as determined by time lapse imaging. The TMRM ratio was already lower in Dox-induced cells illuminated for the first time, and did not change subsequently over the 20 min recording period (data not shown). The decrease in $\Delta \psi_m$ was confirmed by measurements with JC-1, a potentiometric dye that forms red-emitting aggregates at negative $\Delta \psi_m$ (38). As shown in Fig. 7C, the proportion of red-emitting JC-1 aggregates was markedly reduced in Dox-induced cells (Fig. 7C, right panel). Thus, the abnormal $[\text{Ca}^{2+}]_{\text{mit}}$ response of calreticulin-overexpressing cells correlated with a decreased mitochondrial membrane potential, with no visible alteration in the mitochondrial architecture.

**Role of the $\text{Ca}^{2+}$-Binding C-domain of Calreticulin**—Calreticulin is a multifunctional protein, and different regions of the protein perform different functions (29). For example, the N- and C-domains of calreticulin are involved in chaperone function, whereas the C-domain of the proteins plays a role of $\text{Ca}^{2+}$ storage and "$\text{Ca}^{2+}$ sensing" in the ER lumen (29). To identify the region of calreticulin involved in $\text{Ca}^{2+}$-and organelle homeostasis, we generated Tet-ON cells inducible for a truncated calreticulin, encoding the P + C-domain, which contains a critical $\text{Ca}^{2+}$-binding region in calreticulin. Dox-induced expression of the P + C-domain was at similar levels as the wild-type protein, as assessed by immunofluorescence and Western blotting (not shown). Fig. 8 shows that the P + C-domain mimicked the effects of the full-length calreticulin. The Dox-induced cells overexpressing the P + C-domain had a higher resting $[\text{Ca}^{2+}]_{\text{mit}}$ increased residual $[\text{Ca}^{2+}]_{\text{mit}}$ levels after Tg stimulation, and a lower $[\text{Ca}^{2+}]_{\text{mit}}$ signal (Fig. 8, C and D). In addition the reduction of TMRM fluorescence was also measured in P + C-induced cells (Fig. 8E). This suggested that the $\text{Ca}^{2+}$ sensing and $\text{Ca}^{2+}$ storage C-domain of calreticulin...
was responsible for the deleterious effects. Despite repeated attempts we were unable to generate cells overexpressing either the N- or C-domain alone. However, it is unlikely that the chaperone P-domains of calreticulin play a role because Dox-inducible expression of ER chaperone calnexin, which contains a similar P-domain, did not reproduce the effect on [Ca$^{2+}$]$_{ER}$ (Fig. 8B). In summary, these data suggest that the low affinity, high capacity Ca$^{2+}$-binding C-domain, rather than the chaperone interacting regions of calreticulin, mediate the effects on [Ca$^{2+}$]$_{ER}$ leading to modulation of SOC and mitochondrial Ca$^{2+}$ homeostasis.

**DISCUSSION**

In this study we report that differential expression of calreticulin in the lumen of ER affects the Ca$^{2+}$ homeostasis of distinct cellular compartments. Altered Ca$^{2+}$ signals were observed in the ER, in the cytosol, at the plasma membrane, and in the mitochondria. The most predominant effects of increased expression of calreticulin occurred at the level of ER, where the protein resides. Consistent with all previous studies (23, 32, 34, 40), we found that calreticulin overexpression increased the total amount of Ca$^{2+}$ stored in the ER, an effect that occurred within days after the induction of protein expression. In addition, we found that the increased expression of calreticulin has a significant effect on the free intraluminal ER Ca$^{2+}$. The free Ca$^{2+}$ concentration within the ER lumen, [Ca$^{2+}$]$_{ER}$, nearly doubled within 24 h of induction of calreticulin expression and remained at these elevated levels for several days. This is in contrast to an earlier report where oocyte [Ca$^{2+}$]$_{ER}$ levels were either not affected (34) or slightly decreased (31) when calreticulin was overexpressed. Although different expression systems were used, these diverging effects of calreticulin relate to cellular systems expressing the same SERCA isoform. In this study, increased [Ca$^{2+}$]$_{ER}$ levels and Ca$^{2+}$ pumping activity were observed in calreticulin-overexpressing HEK-293 cells, which contain predominantly the SERCA2b isoform (Fig. 2). In contrast, in oocytes co-injected with calreticulin and the SERCA2b expression vectors, decreased [Ca$^{2+}$]$_{ER}$ and Ca$^{2+}$ pumping activities were observed (31). In both cases, Ca$^{2+}$ pumping activity directly correlated with the [Ca$^{2+}$]$_{ER}$ levels, consistent with recent results (24) showing that overexpression of SERCA2b increases [Ca$^{2+}$]$_{ER}$ by 25% in Chinese hamster ovary cells. In the present study, calreticulin levels were increased by 2.5-fold, Ca$^{2+}$ pumping activity by 1.9-fold, and [Ca$^{2+}$]$_{ER}$ by 1.8-fold. This excellent correlation reflected the imbalance between the increased Ca$^{2+}$ pumping activity and the endogenous Ca$^{2+}$ permeability of the ER, which was unaffected by calreticulin.

The increased Ca$^{2+}$ pumping activity, however, was not mediated by SERCA isoforms, as inferred from the effects of thapsigargin. Thapsigargin, added at concentrations that fully inhibit SERCA, unmasked a nearly identical passive ER Ca$^{2+}$ permeability in control and calreticulin overexpressers (Fig. 2B). Because at steady state the Ca$^{2+}$ pumping activity is equal to the ER Ca$^{2+}$ leak, this indicates that, under resting conditions, the activity of SERCA was not altered in the calreticulin overexpressers. Thus, thapsigargin-insensitive Ca$^{2+}$ pumps mediate the increased ER refilling observed during Ca$^{2+}$ re-addition to Ca$^{2+}$-depleted cells (Fig. 2C). A likely candidate is the Pmr1 family of Ca$^{2+}$ transport ATPases, which has been shown recently (41) to be expressed and functional in mammalian cell lines. The thapsigargin-insensitive Pmr1 pump is localized mainly to the Golgi complex, but a substantial fraction...
is present and functional in the ER. The Pmr1 store had a reduced Ca$^{2+}$ leak and weak InsP$_3$ responses, and COS-7 cells overexpressing the Pmr1 pump had delayed Ca$^{2+}$ influx (42). It is tempting to speculate that calreticulin, by interacting with the Golgi-targeted Pmr1 pump, might promote its retention in the ER, thereby accounting for the increased Ca$^{2+}$ pumping activity observed in calreticulin overexpressers. In any case, the existing evidence strongly suggests that calreticulin interacts differentially with distinct Ca$^{2+}$ pump isoforms and modulates the rates of Ca$^{2+}$ uptake into the ER, thereby directly altering [Ca$^{2+}$]$_{ER}$. The physiological relevance of these interactions is not clear, but a decreased [Ca$^{2+}$]$_{ER}$ has been shown to activate the transcription of the calreticulin gene (43). Therefore, an increase in calreticulin level in the ER would rapidly restore normal [Ca$^{2+}$]$_{ER}$ levels, thereby abrogating its transcriptional activation. Consistent with such a feedback mechanism, the [Ca$^{2+}$]$_{ER}$ increase was the first perturbation observed upon the induction of calreticulin.

In addition to increasing the total and free Ca$^{2+}$ of the ER, calreticulin also increased the rates of agonist-induced Ca$^{2+}$ release. Increased release was observed over a wide range of [Ca$^{2+}$]$_{ER}$, indicating that it did not simply reflect the increased driving force for Ca$^{2+}$ but increased fluxes through InsP$_3$-gated channels. This was unexpected, because it was reported recently (24) that the rates of ATP-induced Ca$^{2+}$ release were decreased in cells with increased [Ca$^{2+}$]$_{ER}$ due to overexpression of SERCA. This effect was attributed to the Ca$^{2+}$-dependent inhibition of InsP$_3$-gated channels. Because in our calreticulin-induced cells the InsP$_3$ channels were also exposed to higher amounts of Ca$^{2+}$ ions, both on the ER and on the cytosolic side, the increased release might reflect a direct action of calreticulin on InsP$_3$-gated Ca$^{2+}$ channels.

Because of the increased ER Ca$^{2+}$ load and the increased driving force for Ca$^{2+}$, more Ca$^{2+}$ was released into the cytosol during stimulation with agonists and/or thapsigargin, and store-operated Ca$^{2+}$ influx was reduced when measured with the Ca$^{2+}$ re-addition protocol (Fig. 3). However, analysis of the cytosolic and ER responses at different times after induction indicated that calreticulin levels had no direct effects on store-operated Ca$^{2+}$ influx. Decreased SOC activity was only observed in cells induced to express CRT for 3 days and correlated with an increase in total stored Ca$^{2+}$, rather than with the resting [Ca$^{2+}$]$_{ER}$ levels (Fig. 4). In previous studies, decreased Ca$^{2+}$ influx was observed in stable calreticulin overexpressers (32) but not in cells transiently transfected with calreticulin (33). Our observations reconcile these apparently discrepant findings and caution against the Ca$^{2+}$ re-addition protocol to assess store-operated Ca$^{2+}$ influx, because 1) the degree of
store depletion cannot be readily estimated from the cytosolic Ca\(^{2+}\) responses, and 2) the concomitant activity of SERCA greatly affects the dynamics of the [Ca\(^{2+}\)]\(_{cyt}\) signal, precluding accurate estimates of the influx component.

The effects of calreticulin extended beyond the ER and affected another organelle, the mitochondria. However, the larger release of Ca\(^{2+}\) from the ER was not associated with an equally larger Ca\(^{2+}\) accumulation in mitochondria but with a reduced signal as [Ca\(^{2+}\)]\(_{mit}\) rapidly returned to basal levels despite the presence of InsP\(_3\)-generating agonists (Fig. 6). The abnormal [Ca\(^{2+}\)]\(_{mit}\) response did not reflect structural damage, because the shapes and numbers of mitochondria as well as their relationship to the ER appeared normal by confocal microscopy, but was associated with a mitochondrial depolarization (Fig. 7). The depolarization, by reducing the driving force for Ca\(^{2+}\), is expected to reduce mitochondrial Ca\(^{2+}\) uptake and might thus account for the blunted [Ca\(^{2+}\)]\(_{mit}\) response. In addition, the activity of the mitochondrial Ca\(^{2+}\) uniporter might be further inhibited by the high Ca\(^{2+}\) concentrations found at the ER/mitochondria microdomain. Prolonged exposures to high Ca\(^{2+}\) concentrations might desensitize the uniporter, as exposures to low Ca\(^{2+}\) concentrations are needed to reset the uniporter into rapid uptake mode, its most efficient mode of Ca\(^{2+}\) uptake (44). Furthermore, the mitochondria Ca\(^{2+}\) uptake sites have been shown to be already close to saturation during physiological stimulations (6, 37), suggesting that exposure of mitochondria to higher Ca\(^{2+}\) microdomains might not translate into higher [Ca\(^{2+}\)]\(_{mit}\) responses.

This mechanism might account for the preserved amplitude of the peak [Ca\(^{2+}\)]\(_{mit}\) response in calreticulin overexpressers, despite the larger release of Ca\(^{2+}\) from the ER. The increased ER Ca\(^{2+}\) pumping activity of calreticulin overexpressers (Fig. 2) might further contribute to the abnormal [Ca\(^{2+}\)]\(_{mit}\) response, by dissipating more efficiently the local Ca\(^{2+}\) microdomain surrounding mitochondria. Because of its slow kinetics, the increased ER Ca\(^{2+}\) pumping is not likely to affect the peak [Ca\(^{2+}\)]\(_{mit}\) but might contribute to the faster decay of the [Ca\(^{2+}\)]\(_{mit}\) response by removing more efficiently the Ca\(^{2+}\) released by mitochondria (11). Thus, several mechanisms might account for the abnormal [Ca\(^{2+}\)]\(_{mit}\) response observed in calreticulin-induced cells, including a decrease in mitochondrial membrane potential, an inhibition of the Ca\(^{2+}\) uniporter, together with an increased Ca\(^{2+}\) uptake and release from the ER. A causal link between the increased ER Ca\(^{2+}\) release and mitochondrial depolarization might even be postulated, as mitochondria are likely to be damaged by the chronic exposure to high Ca\(^{2+}\) concentrations.

These perturbations of Ca\(^{2+}\) homeostasis are unlikely due to the chaperone function of calreticulin, as impaired ER and mitochondrial Ca\(^{2+}\) responses were observed in cells induced to express a truncated calreticulin lacking the chaperone N-domain of the protein (Fig. 8). Most importantly, the overexpression of calnexin, an ER chaperone similar to calreticulin and containing a chaperoning P-domain, did not affect cytosolic or ER Ca\(^{2+}\) homeostasis. This indicates that the effects do not require either the N- or the P-domain but are mediated by the unique C-domain of calreticulin. Thus, alterations in Ca\(^{2+}\) sensing, rather that in chaperone activity, are responsible for the increased Ca\(^{2+}\) pumping and release activity, which lead to higher Ca\(^{2+}\) turnover between the ER and mitochondria. These findings have important physiological implications because different levels of calreticulin are expressed in different tissues (28). Furthermore, expression of the protein is up-regulated under the conditions of stress and starvation (28). In the immune system, the CRT gene is activated in stimulated cytotoxic T-cells (45) where it may play a role in a Ca\(^{2+}\)-dependent...
signaling and/or cytotoxic T-cell killing. In many cancer cells, including prostate cancer, calreticulin expression is increased or up-regulated by different steroids (46, 47). Expression of calreticulin is also differentially regulated during development (48). Because the Ca\textsuperscript{2+} signals of multiple cellular compartments are differentially modulated by calreticulin, changes in calreticulin expression levels might define specific patterns of cellular Ca\textsuperscript{2+} responses in these cell types. By allowing the ER to take up, store, and release more Ca\textsuperscript{2+}, an increase in calreticulin might “arm” the cellular Ca\textsuperscript{2+} signaling machinery, thereby allowing previously “silent” cells to generate Ca\textsuperscript{2+} signals. In the long run, however, an increase in calreticulin appears to be deleterious for the cell, despite the reduction in signaling and/or cytotoxic T-cell killing. In many cancer cells, including prostate cancer, calreticulin expression is increased or up-regulated by different steroids (46, 47). Expression of calreticulin is also differentially regulated during development (48). Because the Ca\textsuperscript{2+} signals of multiple cellular compartments are differentially modulated by calreticulin, changes in calreticulin expression levels might define specific patterns of cellular Ca\textsuperscript{2+} responses in these cell types. By allowing the ER to take up, store, and release more Ca\textsuperscript{2+}, an increase in calreticulin might “arm” the cellular Ca\textsuperscript{2+} signaling machinery, thereby allowing previously “silent” cells to generate Ca\textsuperscript{2+} signals. In the long run, however, an increase in calreticulin appears to be deleterious for the cell, despite the reduction in signaling and/or cytotoxic T-cell killing. In many cancer cells, including prostate cancer, calreticulin expression is increased or up-regulated by different steroids (46, 47). Expression of calreticulin is also differentially regulated during development (48). Because the Ca\textsuperscript{2+} signals of multiple cellular compartments are differentially modulated by calreticulin, changes in calreticulin expression levels might define specific patterns of cellular Ca\textsuperscript{2+} responses in these cell types. By allowing the ER to take up, store, and release more Ca\textsuperscript{2+}, an increase in calreticulin might “arm” the cellular Ca\textsuperscript{2+} signaling machinery, thereby allowing previously “silent” cells to generate Ca\textsuperscript{2+} signals. In the long run, however, an increase in calreticulin appears to be deleterious for the cell, despite the reduction in signaling and/or cytotoxic T-cell killing. In many cancer cells, including prostate cancer, calreticulin expression is increased or up-regulated by different steroids (46, 47). Expression of calreticulin is also differentially regulated during development (48). Because the Ca\textsuperscript{2+} signals of multiple cellular compartments are differentially modulated by calreticulin, changes in calreticulin expression levels might define specific patterns of cellular Ca\textsuperscript{2+} responses in these cell types. By allowing the ER to take up, store, and release more Ca\textsuperscript{2+}, an increase in calreticulin might “arm” the cellular Ca\textsuperscript{2+} signaling machinery, thereby allowing previously “silent” cells to generate Ca\textsuperscript{2+} signals. In the long run, however, an increase in calreticulin appears to be deleterious for the cell, despite the reduction in signaling and/or cytotoxic T-cell killing. In many cancer cells, including prostate cancer, calreticulin expression is increased or up-regulated by different steroids (46, 47). Expression of calreticulin is also differentially regulated during development (48). Because the Ca\textsuperscript{2+} signals of multiple cellular compartments are differentially modulated by calreticulin, changes in calreticulin expression levels might define specific patterns of cellular Ca\textsuperscript{2+} responses in these cell types. By allowing the ER to take up, store, and release more Ca\textsuperscript{2+}, an increase in calreticulin might “arm” the cellular Ca\textsuperscript{2+} signaling machinery, thereby allowing previously “silent” cells to generate Ca\textsuperscript{2+} signals. In the long run, however, an increase in calreticulin appears to be deleterious for the cell, despite the reduction in