Calreticulin differentially modulates calcium uptake and release in the endoplasmic reticulum and mitochondria

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SUMMARY

To study the role of calreticulin in Ca\textsuperscript{2+} homeostasis and apoptosis, we generated cells inducible for full-length or truncated calreticulin and measured Ca\textsuperscript{2+} signals within the cytosol, the ER, and mitochondria with “cameleon” indicators. Induction of calreticulin increased the free Ca\textsuperscript{2+} concentration within the ER lumen, \([\text{Ca}^{2+}]_{\text{ER}}\), from 306±31 to 595±53 μM, and doubled the rate of ER refilling. \([\text{Ca}^{2+}]_{\text{ER}}\) remained elevated in the presence of thapsigargin, an inhibitor of SERCA-type Ca\textsuperscript{2+} ATPases. In these conditions, store-operated Ca\textsuperscript{2+} influx appeared inhibited but could be reactivated by decreasing \([\text{Ca}^{2+}]_{\text{ER}}\) with the low-affinity Ca\textsuperscript{2+} chelator TPEN. In contrast, \([\text{Ca}^{2+}]_{\text{ER}}\) decreased much faster during stimulation with carbachol. The larger ER release was associated with a larger cytosolic Ca\textsuperscript{2+} response, but surprisingly, with a shorter mitochondrial Ca\textsuperscript{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\textsuperscript{2+} responses were also observed in cells expressing a 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\textsuperscript{2+} fluxes across the ER, but decreases mitochondrial Ca\textsuperscript{2+} and membrane potential. The increased Ca\textsuperscript{2+} turnover between the two organelles might damage mitochondria, accounting for the increased susceptibility of cells expressing high levels of calreticulin to apoptotic stimuli.
INTRODUCTION

Ca²⁺ signals control key biological functions, ranging from fertilization, development, cardiac contraction, and secretion of neurotransmitters and hormones (1). At the cellular level, Ca²⁺ can be either a life and death signal, as changes in cytosolic free Ca²⁺ 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 Ca²⁺ signals, which depends largely on the controlled release of Ca²⁺ from intracellular organelles. The main intracellular Ca²⁺ store is the endoplasmic reticulum (ER), but mitochondria also take up and release Ca²⁺ very efficiently and are often strategically located close to Ca²⁺ sources (3-6), reviewed in (7). This intimate connection allows mitochondria to shape Ca²⁺ signals (8), by modulating the release of Ca²⁺ from the ER (9) and the influx of Ca²⁺ across the plasma membrane (10), or by providing a local source of Ca²⁺ for ER refilling (11). To achieve such as precise control over Ca²⁺ fluxes, the ER and mitochondria are equipped with a variety of Ca²⁺ transport and storage proteins, and exert a tight control of the Ca²⁺ concentration within their lumen. Ca²⁺ fluxes across the ER membrane are stringently dependent on the free Ca²⁺ concentration within the ER, [Ca²⁺]_{ER}, as Ca²⁺ allosterically modulates the activity of the InsP₃R, the main Ca²⁺-release channel of the ER. In addition, changes in [Ca²⁺]_{ER} regulate the Ca²⁺ 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 InsP₃ receptors and SOC channels (14), or a secretion-like docking mechanism (15).

In addition to these Ca²⁺ signaling functions, the Ca²⁺ 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 [Ca²⁺]_{ER}, which thereby indirectly regulates the processing, sorting, and secretion of cargo proteins (16). In mitochondria, Ca²⁺ directly controls the activity of several dehydrogenases, thereby coupling the cell metabolism to the Ca²⁺ signal (17,18). The mitochondrial “decoding” of Ca²⁺ 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 (19)). In the presence of...
ceramide, even physiological Ca\textsuperscript{2+} responses of mitochondria to InsP\textsubscript{3}-generating agonists are sufficient to induce apoptosis, possibly via Ca\textsuperscript{2+}-dependent opening of the permeability transition pore (20). The Ca\textsuperscript{2+} content of the ER also affects the cell sensitivity to apoptotic stimuli. A decreased [Ca\textsuperscript{2+}]\textsubscript{ER} was observed in cells overexpressing the antiapoptotic protein Bcl-2 (21,22), and a variety of conditions that decreased [Ca\textsuperscript{2+}]\textsubscript{ER} has been shown to protect cells from ceramide-induced cell death (23). The opposite effect was observed in cells overexpressing the Ca\textsuperscript{2+}-ATPases (SERCA2b) or the ER-resident Ca\textsuperscript{2+}-binding chaperone calreticulin, which increased the Ca\textsuperscript{2+} content of the ER (23-25). Conversely, cells lacking the calreticulin had a decreased ER Ca\textsuperscript{2+} content and were more resistant to apoptotic stimuli (26). Calreticulin-deficient cells, however, had normal [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+} content of the ER rather than to changes in [Ca\textsuperscript{2+}]\textsubscript{ER}.

Calreticulin is a 46-kDa Ca\textsuperscript{2+}-binding chaperone that interacts in a Ca\textsuperscript{2+}-dependent fashion with several ER resident proteins, with unfolded glycoproteins, and with Ca\textsuperscript{2+} transporters at the ER membrane (27,28). Calreticulin is composed of three structural and functional domains: 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\textsuperscript{2+} ions with low affinity and high capacity (29). The Ca\textsuperscript{2+}-binding C-domain has been postulated to be the “Ca\textsuperscript{2+}-sensor” that regulates calreticulin interactions with other proteins (25,29). Because of the central role of the ER in Ca\textsuperscript{2+} signaling, both the chaperoning functions of calreticulin as well as its interactions with ER Ca\textsuperscript{2+} transporters can interfere with Ca\textsuperscript{2+} signals. For example, calreticulin inhibits repetitive Ca\textsuperscript{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\textsuperscript{2+} influx (SOC). Stable upregulation of calreticulin in HEK-293 cells inhibits thapsigargin-induced Ca\textsuperscript{2+} or Mn\textsuperscript{2+} influx (32), whereas transient expression in RBL-1 cells only delays the activation of the I\textsubscript{CRAC} current, to an extent that correlated with the extent of store depletion.
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, a 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 - Dulbecco's modified Eagle's culture medium, fetal calf serum, penicillin, streptomycin, geneticin were obtained from Gibco (Paisley, Scotland). Thapsigargin, nigericin, monensin, ATP, and HEPES were purchased from Sigma (St. Louis, MO). Ionomycin was obtained from Calbiochem (Juro, Switzerland). Hygromycin B, Doxycyclin, EGTA, and HEEDTA were from Fluka (Buchs, Switzerland). JC-1 and TMRM were from Molecular Probes (Eugene, OR). Transfast transfection reagent was purchased from Promega (Catalys AG, Switzerland). All other chemicals were of analytic grade and were obtained from Fluka or Sigma. The "Ca\textsuperscript{2+} medium" contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2}, 10 mM glucose and 20 mM Hepes, pH 7.4. For the "Ca\textsuperscript{2+} free medium" CaCl\textsubscript{2} was omitted and 0.5 mM EGTA was included. Drugs were dissolved in dimethylsulfoxide (DMSO) or ethanol and diluted in the recording medium on the day of use, at a final solvent concentration <0.1%.

Constructs - Plasmids YC2, YC2.1, and YC4\textsubscript{ER} were kindly provided by Dr. R.Y. Tsien. Plasmid YC2\textsubscript{mit} and YC4.1\textsubscript{mit} were generated as previously described (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 pTK-Hyg at a ratio 20:1 into HEK293 cells (HeLa cells) by the Ca\textsuperscript{2+}-phosphate protocol. Transfected cells were selected for growth in the presence of 200 µg hygromycin B/ml culture medium. Single colonies of the hygromycin B resistant cells were tested for doxycycline (Dox)-dependent expression of
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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 Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 2mM L-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, and were maintained in a humidified incubator at 37°C in the presence of 5% CO₂ / 95% air. Cells (~200,000) were plated on 25 mm glass coverslips. HEK-293 at 60% of confluence, cells were transiently transfected with cDNAs encoding the yellow cameleons probes. Cells were imaged 3 to 5 days after transfection. Stable HEK-293 transfectants 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 Dox/ml was added 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 pre-treated with polylsine 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 anti-goat antibody conjugated to Texas-Red (Jackson Immunoresearch).

[Ca²⁺] 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 [Ca²⁺] with cameleons probes was performed as previously described (11). Cameleon fluorescence from
cells was imaged on a Axiovert S100 TV using a 100X, 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, NJ) through a 455DRLP dichroic mirror. Fluorescence emission from the cameleons was imaged using a cooled, 16 bits CCD back-illuminated frame transfer MicroMax camera (Princeton Instruments, Ropper Scientific, Trenton N.J) at two emission wavelengths, using a filterwheel (Ludl Electronic Products, Hawthorn, N.Y) to alternatively 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 \( R = (\text{fluorescence intensity at } 535 \text{ nm} - \text{background intensity at } 535 \text{ nm})/(\text{fluorescence intensity at } 475 \text{ nm} - \text{background intensity at } 475 \text{ nm}) \) were calibrated in \([\text{Ca}^{2+}]\) using the equation:

\[
[\text{Ca}^{2+}] = K'_d \left( \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right)^{1/n},
\]

where \( R_{\text{max}} \) and \( R_{\text{min}} \) are the ratios obtained respectively in the absence of \( \text{Ca}^{2+} \) and at saturating \( \text{Ca}^{2+} \). \( K'_d \) is the apparent dissociation constant and \( n \) is the Hill coefficient of the \( \text{Ca}^{2+} \) calibrations curves obtained \textit{in situ} for each cameleon.

For better 3D rendering widefield or confocal image stacks were deconvoluted after acquisition on a Silicon Graphics Octane workstation 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 tetracyclin promoter (Tet-ON). The activation of calreticulin gene transcription by doxycyclin (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 24h, and remained at this level for 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, since Dox induction did not affect the intracellular distribution of the ER-targeted Ca$^{2+}$ 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 Ca$^{2+}$ affinity of both the ER-targeted probe YC4ER and of the cytosolic probe YC2, measured in situ in cells permeabilized with ionomycin or digitonin, were 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 Ca$^{2+}$ dependency of the cameleon Ca$^{2+}$ indicators.

Effect of calreticulin induction on ER [Ca$^{2+}$] homeostasis. To assess whether the sustained increase in calreticulin levels interfered with ER Ca$^{2+}$ homeostasis, we measured the changes in the free Ca$^{2+}$ concentration within the ER lumen, [Ca$^{2+}$]ER, using the low-affinity ER-targeted ratiometric “cameleon” indicator YC4ER (KD=290 µM, ref (11)). YC4ER measurements revealed that the induction of calreticulin markedly increased the resting [Ca$^{2+}$]ER levels (Fig. 2), the basal [Ca$^{2+}$]ER values averaging 306±31 µM in the absence and 595±53 µM 72h after Dox-dependent induction of calreticulin expression. The increase could
not be attributed to a specific ER region, as higher \([Ca^{2+}]_{ER}\) 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 \(Ca^{2+}\) concentration within the ER lumen.

The doubling in resting \([Ca^{2+}]_{ER}\) could reflect either an increased \(Ca^{2+}\) pumping activity, or a decrease in the passive \(Ca^{2+}\) 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 \(Ca^{2+}\). Tg induced a slow decrease in \([Ca^{2+}]_{ER}\) in both control and calreticulin-induced cells (Fig. 2B). A linear fit of the initial \([Ca^{2+}]_{ER}\) decay revealed that the kinetics of \(Ca^{2+}\) release were nearly identical (\(\Delta Ca^{2+} = -5.2\pm0.3\) vs. -6.0±0.3 \(\mu M/s\)), despite the higher \([Ca^{2+}]_{ER}\) in the calreticulin overexpressers. Consequently, calreticulin overexpressing cells retained a higher \([Ca^{2+}]_{ER}\) level throughout the course of Tg stimulation. A further decline was observed upon addition of the ionophore ionomycin (Fig. 2B and 2D), indicating that the ER \(Ca^{2+}\) store was not fully depleted by Tg. Thus, block of SERCA ATPases unmasked a nearly identical passive \(Ca^{2+}\) permeability in the ER, regardless of the increase in calreticulin levels.

In contrast, upon stimulation with the InsP3-generating agonist carbachol (CCh), \([Ca^{2+}]_{ER}\) decreased much faster in CRT-induced cells, and similar depleted levels were achieved within 100s of agonist stimulation (Fig. 2C and 2D). The faster kinetics of \(Ca^{2+}\) release (\(\Delta Ca^{2+} = -4.5\pm0.6\) vs. -11.4±1.4 \(\mu M/min\)) suggested that calreticulin overexpression increased the InsP3-stimulated \(Ca^{2+}\) permeability of the ER. Importantly, re-addition of \(Ca^{2+}\) to the external medium resulted in a rapid increase of the \([Ca^{2+}]_{ER}\) 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 \([Ca^{2+}]_{ER}\) (Fig.2C inset). Because this assay measures the net flow of \(Ca^{2+}\) from the external space to the ER, this indicates that both the influx of \(Ca^{2+}\) across the plasma membrane and the ER \(Ca^{2+}\) pumping activity were increased in cells expressing high levels of calreticulin. In the absence of agonist stimulation, the increased rates of ER refilling were not balanced by a parallel increase in the endogenous ER \(Ca^{2+}\) permeability, resulting in higher \([Ca^{2+}]_{ER}\) levels at rest. However, induction of calreticulin
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expression markedly increased the agonist-induced ER Ca\(^{2+}\) permeability, and therefore, upon stimulation, more Ca\(^{2+}\) was released from the ER lumen.

Effect of CRT induction on cytosolic Ca\(^{2+}\) signals. To assess how these changes in ER luminal Ca\(^{2+}\) homeostasis influenced Ca\(^{2+}\) signals in the cytosol, we monitored changes in cytoplasmic Ca\(^{2+}\), [Ca\(^{2+}\)]\(_{cyt}\), with the cytosolic YC2 probe (K\(_D\)=1.24µM). Ca\(^{2+}\) release from ER stores was measured in the absence of external Ca\(^{2+}\), and Ca\(^{2+}\) influx was subsequently measured by re-adding Ca\(^{2+}\) to the external medium. Figure 3 shows that both CCh and Tg elicited a much larger increase in [Ca\(^{2+}\)]\(_{cyt}\) in calreticulin overexpressing cells, indicating that substantially more Ca\(^{2+}\) was released from the intracellular Ca\(^{2+}\) stores. Compared to previous studies using fura-2 (32), the differences between control and calreticulin overexpresser cells were striking, reflecting the better adequacy of the YC2 probe to quantify [Ca\(^{2+}\)]\(_{cyt}\) changes in the micromolar range. Subsequent addition of Ca\(^{2+}\) to assess the activity of store-operated Ca\(^{2+}\) channels at the plasma membrane revealed that, as previously reported (32), Ca\(^{2+}\) influx was severely blunted in Tg-stimulated calreticulin overexpressing cells (Fig. 3). This decreased influx correlated well with the increased [Ca\(^{2+}\)]\(_{ER}\) levels measured with YC4\(_{ER}\) (Fig. 2) and indicated that, consistent with the capacitative hypothesis, the activity of SOC channels is determined by changes in [Ca\(^{2+}\)]\(_{ER}\) levels. Accordingly, Ca\(^{2+}\) influx was similar in control and Dox-induced cells stimulated with CCh, which had comparable [Ca\(^{2+}\)]\(_{ER}\) levels (Figs. 2 and 3). However, in this case the activity of SOC channels could not be readily inferred from the changes in [Ca\(^{2+}\)]\(_{cyt}\), because of the concomitant ER Ca\(^{2+}\) pumping activity. While Ca\(^{2+}\) re-addition produced similar [Ca\(^{2+}\)]\(_{cyt}\) changes, larger [Ca\(^{2+}\)]\(_{ER}\) increases were observed in calreticulin overexpressing cells, indicating that substantially more Ca\(^{2+}\) was taken up by the ER (Fig. 2A). This suggested that the net flux of Ca\(^{2+}\) ions across the plasma membrane was, in fact, larger in calreticulin-induced cells, but that the Ca\(^{2+}\) entering the cell was rapidly taken up by the ER. Thus, the increased [Ca\(^{2+}\)]\(_{ER}\) 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\(^{2+}\) signal because of the high concomitant ER Ca\(^{2+}\) pumping activity.
Time-course of the CRT effects on ER and cytosolic \([Ca^{2+}]\). To better assess the effects of high expression of calreticulin on \(Ca^{2+}\) handling, we measured the \([Ca^{2+}]_{ER}\) and \([Ca^{2+}]_{cyt}\) responses at different times following the induction of protein expression. Figure 4A shows that the resting \([Ca^{2+}]_{ER}\) levels were increased 24 hours after Dox-dependent induction of calreticulin expression, and remained elevated thereafter. In contrast, \(Ca^{2+}\) influx, taken as the peak \([Ca^{2+}]_{cyt}\) upon \(Ca^{2+}\) re-addition to Tg-treated cells, was inhibited only 3 days after the induction with Dox (Fig. 4B, circles). The amount of releasable \(Ca^{2+}\) followed a similar delayed time-course: the peak of Tg-induced \([Ca^{2+}]_{cyt}\) release was only marginally increased 24 hours 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 \(Ca^{2+}\) likely reflected the higher residual \([Ca^{2+}]_{ER}\) levels achieved at the end of the Tg stimulation in calreticulin overexpressing cells. Although a ~5 min. stimulation with Tg is routinely used to deplete \(Ca^{2+}\) stores, YC4\(_{ER}\) measurements indicated that \([Ca^{2+}]_{ER}\) did not reach fully depleted levels within the first 5 minutes in cells induced to express calreticulin for 3 days (Fig. 2). Thus, induction of calreticulin expression had two temporally distinct effects on \(Ca^{2+}\) homeostasis: acute induction caused an immediate increase in \([Ca^{2+}]_{ER}\), whereas more sustained expression of high levels of calreticulin was required to increase the total amount of stored \(Ca^{2+}\) and to inhibit store-operated \(Ca^{2+}\) influx.

To show that the \([Ca^{2+}]_{ER}\) levels were indeed the prime determinant of SOC activity, we acutely modulated \([Ca^{2+}]_{ER}\) using the low-affinity \(Ca^{2+}\) chelator N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN). This cell membrane-permeant \(Ca^{2+}\) chelator has a \(K_D\) that matches the free \(Ca^{2+}\) levels in the ER lumen, providing an excellent tool to clamp \([Ca^{2+}]_{ER}\) without affecting the \([Ca^{2+}]_{cyt}\) responses (35). Figure 5 shows that addition of TPEN produced a rapid decrease in \([Ca^{2+}]_{ER}\), but had only minor effect on \([Ca^{2+}]_{cyt}\). The effects of TPEN were reversible (not shown). The chelator did not prevent the \([Ca^{2+}]_{ER}\) changes induced by Tg. Therefore, it was possible to artificially impose normal resting and depleted \([Ca^{2+}]_{ER}\) levels in calreticulin overexpressing cells (Fig. 5A). Cytosolic \(Ca^{2+}\) measurements revealed that a robust \(Ca^{2+}\) influx could be elicited with Tg in the presence of the ER luminal \(Ca^{2+}\) chelator, as expected from the capacitative mechanism (Fig. 5B). This indicates that SOC
channels were fully functional in calreticulin overexpresser cells when [Ca\(_{\text{ER}}^2+\)] 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\(_{\text{ER}}^2+\)] level in the ER lumen.

**Effect of calreticulin induction on mitochondrial Ca\(^{2+}\) 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\(^{2+}\) release, and can capture part of the Ca\(^{2+}\) released by the ER (6). To assess whether the increased [Ca\(_{\text{ER}}^2+\)] levels and InsP\(_3\)-induced ER permeability also affected mitochondria, we measured Ca\(^{2+}\) changes within the mitochondrial matrix, [Ca\(_{\text{mit}}^2+\)]. Two mitochondrial probes of different affinities were used: the high-affinity YC2\(_{\text{mit}}\) probe (K\(_D\)=1.24 µM), to allow accurate measurements within the low micromolar range, and the low-affinity YC4.1\(_{\text{mit}}\) probe (K\(_D\)=105 µM), to better resolve the high levels achieved during peak [Ca\(_{\text{mit}}^2+\)] responses (11). The basal [Ca\(_{\text{mit}}^2+\)] levels reported by the YC2\(_{\text{mit}}\) probe were not affected by the induction of calreticulin. Surprisingly however, YC4.1\(_{\text{mit}}\) measurement revealed that the [Ca\(_{\text{mit}}^2+\)] responses were blunted in calreticulin-induced cells (Fig. 6A). The peak [Ca\(_{\text{mit}}^2+\)] levels measured with YC4.1\(_{\text{mit}}\) were close to 100 µM, consistent with earlier findings in chromaffin and HeLa cells (11,36) and were only slightly reduced in calreticulin cells (120±16 (n=10) vs. 77±12 µM, (n=9) p=0.05). However, a marked difference in the decay kinetics of the [Ca\(_{\text{mit}}^2+\)] signal was observed, as [Ca\(_{\text{mit}}^2+\)] returned much faster to basal levels despite the continuous presence of the InsP\(_3\)-generating agonist (Fig. 6B). This suggested that mitochondria were still able to take up the Ca\(^{2+}\) released by the ER, but that Ca\(^{2+}\) extrusion from mitochondria was facilitated. As a result, the average [Ca\(_{\text{mit}}^2+\)] level measured during CCh application was significantly reduced in calreticulin induced cells (Fig. 6B). This finding was unexpected, as calreticulin induction increased both the amount of releasable Ca\(^{2+}\), the driving force for ER-to-cytosol Ca\(^{2+}\) release, and the InsP\(_3\)-induced Ca\(^{2+}\) permeability of the ER (Figs. 2 and 3). The shorter duration of the [Ca\(_{\text{mit}}^2+\)] signal suggested that the ability of mitochondria to retain Ca\(^{2+}\) loads was impaired.

**Effects of calreticulin on mitochondria morphology and membrane potential.** The abnormal mitochondrial response of calreticulin cells suggested that calreticulin induction might cause
structural or functional damages to mitochondria. Because mitochondria are tightly coupled to Ca\(^{2+}\) release sites at the ER membrane (6,37), subtle change in the architecture of the mitochondrial network might be sufficient to cause dramatic effects on [Ca\(^{2+}\)]\(_{\text{mit}}\) signals. On the other hand, changes in mitochondrial membrane potential, \(\Delta \psi_m\), which determines the driving force for Ca\(^{2+}\), also directly impact on [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 DsRed\(_{\text{mit}}\). Figure 7A shows that the staining pattern of DsRed\(_{\text{mit}}\) 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 were 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 YC4\(_{\text{ER}}\) 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 minutes 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 $[Ca^{2+}]_{mit}$ response of calreticulin-overexpressing cells correlated with a decreased mitochondrial membrane potential, with no visible alteration in the mitochondrial architecture.

**Role of the Ca$^{2+}$ binding C-domain of calreticulin.** Calreticulin is a multifunctional protein and different region of the protein perform different function (29). For example the N+P-domain of calreticulin are involved in chaperone function, whereas the C-domain of the proteins plays a role of Ca$^{2+}$ storage and “Ca$^{2+}$ sensing” in the ER lumen (29). To identify the region of calreticulin involved in Ca$^{2+}$ and organelle homeostasis, we generated Tet-ON cells inducible for a truncated calreticulin, encoding the P+C-domain, which contains a critical 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). Figure 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 $[Ca^{2+}]_{ER}$, increased residual $[Ca^{2+}]_{ER}$ levels after Tg stimulation and a lower $[Ca^{2+}]_{mit}$ signal (Fig. 8C, D). In addition the reduction of TMRM fluorescence was also measured in P+C induced cells (Fig. 8E). This suggested that the “Ca$^{2+}$-sensing” and 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 plays 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 that 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, \([\text{Ca}^{2+}]_{\text{ER}}\), nearly doubled within 24h of induction of calreticulin expression and remained at these elevated levels for several days. This is in contrast to earlier report where in oocytes \([\text{Ca}^{2+}]_{\text{ER}}\) 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 \([\text{Ca}^{2+}]_{\text{ER}}\) levels and Ca\(^{2+}\) pumping activity were observed in calreticulin overexpressing HEK-293 cells, which contain predominantly the SERCA 2b isoform (Fig. 2). In contrast, in oocytes co-injected with calreticulin and the SERCA2b expression vectors, decreased \([\text{Ca}^{2+}]_{\text{ER}}\) and Ca\(^{2+}\) pumping activity were observed (31). In both cases, Ca\(^{2+}\) pumping activity directly correlated with the \([\text{Ca}^{2+}]_{\text{ER}}\) levels, consistent with recent results showing that overexpression of SERCA2b increases \([\text{Ca}^{2+}]_{\text{ER}}\) by 25\% in CHO cells (24). In the present study, calreticulin levels were increased by 2.5 fold, Ca\(^{2+}\) pumping activity by 1.9 fold, and \([\text{Ca}^{2+}]_{\text{ER}}\) 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 recently been shown to be expressed and functional in mammalian cell lines (41). 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 modulate 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 feed-back 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 though InsP\(_3\)-gated channels. This was unexpected, because it was reported recently that the rates of ATP-induced Ca\(^{2+}\) release were decreased in cells with increased [Ca\(^{2+}\)]\(_{ER}\) due to overexpression of SERCA (24). 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\textsuperscript{2+} influx was reduced when measured with the Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+}, rather than with the resting [Ca\textsuperscript{2+}]\textsubscript{ER} levels (Fig. 4). In previous studies, decreased Ca\textsuperscript{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\textsuperscript{2+} re-addition protocol to assess store-operated Ca\textsuperscript{2+} influx, because 1) the degree of store depletion cannot be readily estimated from the cytosolic Ca\textsuperscript{2+} responses, and 2) the concomitant activity of SERCA greatly affects the dynamics of the [Ca\textsuperscript{2+}]\textsubscript{cyst} 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\textsuperscript{2+} from the ER was not associated with an equally larger Ca\textsuperscript{2+} accumulation in mitochondria, but with a reduced signal as [Ca\textsuperscript{2+}]\textsubscript{mit} rapidly returned to basal levels despite the presence of InsP\textsubscript{3}-generating agonists (Fig. 6). The abnormal [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}, is expected to reduce mitochondrial Ca\textsuperscript{2+} uptake and might thus account for the blunted [Ca\textsuperscript{2+}]\textsubscript{mit} response. In addition, the activity of the mitochondrial Ca\textsuperscript{2+} uniporter might be further inhibited by the high Ca\textsuperscript{2+} concentrations found at the ER/mitochondria microdomain. Prolonged exposures to high Ca\textsuperscript{2+} concentrations might desensitize the uniporter, as exposures to low Ca\textsuperscript{2+} concentrations are needed to reset the uniporter into rapid uptake mode, its most efficient mode of Ca\textsuperscript{2+} uptake (44). Furthermore, the mitochondria Ca\textsuperscript{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\textsuperscript{2+} microdomains might not translate into higher [Ca\textsuperscript{2+}]\textsubscript{mit} responses.
This mechanism might account for the preserved amplitude of the peak $[\text{Ca}^{2+}]_{\text{mit}}$ response in calreticulin overexpressers, despite the larger release of $\text{Ca}^{2+}$ from the ER. The increased ER $\text{Ca}^{2+}$ pumping activity of calreticulin overexpressers (Fig. 2) might further contribute to the abnormal $[\text{Ca}^{2+}]_{\text{mit}}$ response, by dissipating more efficiently the local $\text{Ca}^{2+}$ microdomain surrounding mitochondria. Because of its slow kinetics, the increased ER $\text{Ca}^{2+}$ pumping is not likely to affect the peak $[\text{Ca}^{2+}]_{\text{mit}}$, but might contribute to the faster decay of the $[\text{Ca}^{2+}]_{\text{mit}}$ response by removing more efficiently the $\text{Ca}^{2+}$ released by mitochondria (11). Thus, several mechanisms might account for the abnormal $[\text{Ca}^{2+}]_{\text{mit}}$ response observed in calreticulin-induced cells, including a decrease in mitochondrial membrane potential, an inhibition of the $\text{Ca}^{2+}$ uniporter, together with an increased $\text{Ca}^{2+}$ uptake and release from the ER. A causal link between the increased ER $\text{Ca}^{2+}$ release and mitochondrial depolarization might even be postulated, as mitochondria are likely to be damaged by the chronic exposure to high $\text{Ca}^{2+}$ concentrations.

These perturbations of $\text{Ca}^{2+}$ homeostasis are unlikely due to the chaperone function of calreticulin, as impaired ER and mitochondrial $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ homeostasis. This indicates that the effects do require neither the N- nor the P-domain, but are mediated by the unique C-domain of calreticulin. Thus, alterations in “$\text{Ca}^{2+}$-sensing”, rather than in chaperone activity, are responsible for the increased $\text{Ca}^{2+}$ pumping and release activity, which lead to higher $\text{Ca}^{2+}$ turnover between the ER and mitochondria. These findings have important physiological implications because different levels of calreticulin are expressed in different tissue (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 $\text{Ca}^{2+}$-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 $\text{Ca}^{2+}$ signals of multiples...
cellular compartments are differentially modulated by calreticulin, changes in calreticulin expression levels might define specific pattern of cellular Ca\(^{2+}\) responses in these cell types. By allowing the ER to take up, store, and release more Ca\(^{2+}\), an increase in calreticulin might “arm” the cellular Ca\(^{2+}\) signaling machinery, thereby allowing previously “silent” cells to generate Ca\(^{2+}\) signals. In the long run however, an increase in calreticulin appears to be deleterious for the cell, despite the reduction in Ca\(^{2+}\) influx that compensates for the increased Ca\(^{2+}\) release from the ER. The high Ca\(^{2+}\) microdomains around mitochondria, which might in the short term increase mitochondrial metabolism, might in the long run damage and depolarize mitochondria. This defective signaling might account for the increased susceptibility of cells expressing high levels of calreticulin to apoptotic stimuli.
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FOOTNOTES

The abbreviations used: Dox, Doxycyclin; InsP₃, inositol 1,4,5-trisphosphate; InsP₃R, inositol 1,4,5-trisphosphate receptor; SOC, store-operated Ca²⁺ influx, SERCA, sarco(endo)plasmic reticulum Ca²⁺ transport ATPase, [Ca²⁺]cyt, [Ca²⁺]ER, and [Ca²⁺]mit, cytosolic, ER, and mitochondria free Ca²⁺ concentration, respectively; CCh, carbachol, YC, yellow cameleon; DsRed, Red fluorescent protein from Discosoma sp.; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid, TPEN, N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine; Tg, thapsigargin.
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FIGURE LEGENDS

**Figure 1:** Time-course and specificity of calreticulin induction by Doxycyclin. A. Western blot of HEK-293 cells stably transfected with the Tet-ON CRT construct. Cells were treated with Doxycyclin 2 μM for the indicated time. B. Effects of Doxycyclin induction on calreticulin immunostaining and on YC4ER fluorescence. Tet-ON cells were cultured for 3 days in the absence (top) or presence (bottom) of Doxycyclin 2 μM. The cells were then fixed and stained with a goat anti-CRT antibody, and observed by confocal microscopy using identical acquisition settings (left). YC4ER fluorescence was assessed in live cells by confocal microscopy (right). Images are shadow projections of 6 adjacent, 400 nm wide z sections deconvoluted with the iterative constrained Tikhonov-Miller restoration algorithm. The induced calreticulin retained its reticular staining pattern and did not affect the subcellular distribution of YC4ER. C. In situ Ca^{2+} calibration curves of cells expressing YC2CYT and YC4ER, cultured in the absence (control, black circles) or presence of Doxycyclin for 3 days (Dox, green circles). The induction of calreticulin had no effect on the Ca^{2+} affinity of the probes. Size bar 5 μm.

**Figure 2:** Effect of calreticulin induction on ER [Ca^{2+}] homeostasis. A. YC4ER emission ratio images (535/475 nm) of control and calreticulin-induced cells, measured before and after stimulation with the SERCA inhibitor thapsigargin (Tg). B. [Ca^{2+}]ER changes in control and Dox treated cells in response to Tg (1 μM) followed by ionomycin to maximally deplete ER stores. The spatially averaged 535/475 ratio values were converted into [Ca^{2+}] using the calibration curve of Fig 1. C. [Ca^{2+}]ER changes induced by 100 μM Carbachol (CCh). Stimuli were applied in the absence of external Ca^{2+}, and Ca^{2+} was re-added when indicated. Inset: Rate of ER Ca^{2+} pumping, measured at different [Ca^{2+}]ER values in control (o) and Dox induced cells (•). Data are pooled from 5 and 4 independent experiments. D. Average [Ca^{2+}]ER values measured in control and Dox treated cells under resting and depleted conditions. Data are mean ±S.E.M (* p<0.01). The number of experiments for each condition is indicated. Size bar 5 μm.
**Figure 3:** Effect of calreticulin induction on cytosolic Ca\(^{2+}\) signals. A. [Ca\(^{2+}\)\(_{\text{cyt}}\)] changes induced by Tg. Cells were stimulated in the absence of external Ca\(^{2+}\), which was re-added when indicated. B. Average [Ca\(^{2+}\)\(_{\text{cyt}}\)] responses elicited by Tg in control and calreticulin -induced cells. C. [Ca\(^{2+}\)\(_{\text{cyt}}\)] responses elicited by CCh. D. Average [Ca\(^{2+}\)\(_{\text{cyt}}\)] responses measured with CCh. Data are mean ±S.E.M of the indicated number of experiment (* p<0.02 and ** p<0.0001).

**Figure 4:** Time-course of calreticulin effects on ER and cytosolic [Ca\(^{2+}\)]. A. [Ca\(^{2+}\)\(_{\text{cyt}}\)] responses measured at different times after Dox induction. B. The average [Ca\(^{2+}\)\(_{\text{cyt}}\)] response elicited by Tg in Ca\(^{2+}\)-free medium (release, squares) or during subsequent readdition of Ca\(^{2+}\) (influx, circles) are plotted against the duration of calreticulin induction. C. Corresponding [Ca\(^{2+}\)\(_{\text{ER}}\)] levels measured at different times during Dox induction. Data are mean ±S.E.M (* p< 0.02 and ** p<0.001).

**Figure 5:** Effects of TPEN on ER and cytosolic [Ca\(^{2+}\)]. A. Effect of the cell permeant low affinity Ca\(^{2+}\) chelator TPEN on [Ca\(^{2+}\)\(_{\text{ER}}\), measured in the absence of external Ca\(^{2+}\) in Dox induced cells. B. Averaged [Ca\(^{2+}\)\(_{\text{ER}}\)] levels measured before and after the addition of TPEN, which reduced the free ER Ca\(^{2+}\) by 2.2 x. C. Effect of TPEN on the Tg-induced [Ca\(^{2+}\)\(_{\text{cyt}}\)] responses. D. Averaged [Ca\(^{2+}\)\(_{\text{cyt}}\)] responses in control cells and in calreticulin -induced cells treated with TPEN. Data are mean ±S.E.M (* p<0.05).

**Figure 6:** Effect of calreticulin induction on mitochondrial Ca\(^{2+}\) signals. A. [Ca\(^{2+}\)\(_{\text{mit}}\)] changes measured with the low affinity YC4\(_{\text{mit}}\) probe, whose K\(_D\) matches the peak [Ca\(^{2+}\)\(_{\text{mit}}\)] values. B. Mean [Ca\(^{2+}\)\(_{\text{mit}}\)] levels recorded during the time of CCh application (~120 sec). Data are mean ±S.E.M (* p<0.01).

**Figure 7:** Effect of calreticulin induction on mitochondria morphology and membrane potential. A. Staining patterns of a mitochondrial-targeted DsRed\(_{\text{mit}}\) (kindly provided by Dr.
T. Pozzan, Padova). Images are shadow projections of z stacks of confocal images deconvoluted and processed using the Huygens and Imaris softwares. B. YC4ER-expressing cells were co-labeled with mitotracker red to assess the interaction between the ER and mitochondria. Images were acquired and processed as in A. C. TMRM (left) and JC-1 (right) measurements of the mitochondrial membrane potential. $\Delta \psi_m$ is expressed as the ratio of the mitochondrial over cytosolic TMRM fluorescence (left) or as the percentage of the JC-1 stained area containing red fluorescent aggregates (right). Data are mean ±S.E.M (*p<0.005). Size bar 5 µm.

**Figure 8:** Effect of calreticulin domains. A. Schematic representation of calreticulin and calnexin domains. B. Average $[\text{Ca}^{2+}]_{\text{ER}}$ values in cells induced to express the P-domain containing chaperone calnexin. $[\text{Ca}^{2+}]_{\text{ER}}$ was measured in the absence of external Ca$^{2+}$, before and 5’ after application of Tg. C. $[\text{Ca}^{2+}]_{\text{ER}}$ values in cells induced to express the P+C-domain of calreticulin (P+C-domain). D. $[\text{Ca}^{2+}]_{\text{mit}}$ responses measured with the YC4.1mit probe in P+C-domain Tet-ON cells. The average values recorded during the CCh application are shown. E. TMRM fluorescence intensity ratio between mitochondria and the cytosol, measured in control and P+C-domain-induced cells. Data are mean ±S.E.M (* p<0.05 and ** p<0.005).
[Image A] CRT protein expression over time with Dox treatment.

[Image B] CRT and YC4ER fluorescence images with and without Dox treatment.

[Image C] Graph showing the emission ratio (% max) vs. Ca²⁺ concentration (log[M]) for control and Dox 72 h treatments.
A

[Ca^{2+}]_{\text{mit}} (\mu M)

CCh 100 \mu M

YC4.1_{\text{mit}}

cont

Dox

50 s

B

averaged [Ca^{2+}]_{\text{mit}} (\mu M)

(10)

(9)

CCh

Control

Dox
A
CALRETICULIN

NH$_2$
Signal sequence
unique N-domain
P-domain
C-domain

---KDEL--COO$	ext{^\text{-}}$
ER retrieval signal

lectin-like chaperoning

NH$_2$
CALNEXIN

P-domain homology
TM
COO$	ext{^\text{-}}$

B

\begin{align*}
\text{[Ca}^{2+}]_{\text{ER}} & \text{ (M)} \\
\text{basal} & \text{Control} \\
(7) & (7) \\
\text{Tg 1 \mu M} & \text{CNX} \\
(7) & (7) \\
\end{align*}

C

\begin{align*}
\text{[Ca}^{2+}]_{\text{ER}} & \text{ (M)} \\
\text{basal} & \text{Control} \\
(16) & \text{P+C} \\
\text{Tg 1 \mu M} & \text{Control} \\
(16) & \text{P+C} \\
\end{align*}

D

\begin{align*}
\text{averaged [Ca}^{2+}]_{\text{mit}} & \text{ (M)} \\
\text{CCh 100 \mu M} & \text{Control} \\
(9) & \text{P+C} \\
\end{align*}

E

\begin{align*}
\text{TMRM ratio} & \text{ (F_mito/F_cytol)} \\
\text{Control} & \text{P+C} \\
(75) & (81) \\
\end{align*}
Calreticulin differentially modulates calcium uptake and release in the endoplasmic reticulum and mitochondria
Serge Arnaudeau, Maud Frieden, Kim Nakamura, Cyril Castelbou, Marek Michalak and Nicolas Demaurex

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