Routes of Ca\textsuperscript{2+} Shuttling during Ca\textsuperscript{2+} Oscillations

**FOCUS ON THE ROLE OF MITOCHONDRIAL Ca\textsuperscript{2+} HANDLING AND CYTOSOLIC Ca\textsuperscript{2+} BUFFERS**

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**Background:** Ca\textsuperscript{2+} oscillations in mesothelial cells depend on Ca\textsuperscript{2+} influx. In some cell types, Ca\textsuperscript{2+} oscillations are strictly dependent on Ca\textsuperscript{2+} influx across the plasma membrane, whereas in others, oscillations also persist in the absence of Ca\textsuperscript{2+} influx. We observed that, in primary mesothelial cells, the plasmalemmal Ca\textsuperscript{2+} influx played a pivotal role. However, when the Ca\textsuperscript{2+} transport across the plasma membrane by the “lanthanum insulation method” was blocked prior to the induction of the serum-induced Ca\textsuperscript{2+} oscillations, mitochondrial Ca\textsuperscript{2+} transport was found to be able to substitute for the plasmalemmal Ca\textsuperscript{2+} exchange function, thus rendering the oscillations independent of extracellular Ca\textsuperscript{2+}. In a physiological situation, the Ca\textsuperscript{2+}-buffering capacity of mitochondria was found not to be essential for Ca\textsuperscript{2+} oscillations. Moreover, brief spontaneous Ca\textsuperscript{2+} changes were observed in the mitochondrial Ca\textsuperscript{2+} concentration without apparent changes in the cytosolic Ca\textsuperscript{2+} concentration, indicating the presence of a mitochondrial autonomous Ca\textsuperscript{2+} signaling mechanism. In the presence of calretinin, a Ca\textsuperscript{2+}-buffering protein, the amplitude of cytosolic spikes during oscillations was decreased, and the amount of Ca\textsuperscript{2+} ions taken up by mitochondria was reduced. Thus, the increased calretinin expression observed in mesothelioma cells and in certain colon cancer might be correlated to the increased resistance of these tumor cells to proapoptotic/pronecrotic signals. We identified and characterized (experimentally and by modeling) three Ca\textsuperscript{2+} shuttling pathways in primary mesothelial cells during Ca\textsuperscript{2+} oscillations: Ca\textsuperscript{2+} shuttled between (i) the endoplasmic reticulum (ER) and mitochondria, (ii) the ER and the extracellular space, and (iii) the ER and cytoplasmic Ca\textsuperscript{2+} buffers.

The calcium ion (Ca\textsuperscript{2+}) is a universal intracellular messenger that controls a diverse range of cellular processes including cell proliferation, apoptosis, fertilization, neurotransmitter release, and heartbeat among many others (1). Ca\textsuperscript{2+} pumps in the plasma membrane (plasma membrane Ca\textsuperscript{2+}-ATPase) and in endoplasmic reticulum (ER)\textsuperscript{2} membranes (SERCA) are responsible for the low cytosolic (c\textsubscript{cyt}) and nuclear free Ca\textsuperscript{2+} concentrations (c\textsubscript{nucl}) (50–100 nm) compared with the free Ca\textsuperscript{2+} concentrations in the extracellular space (1–2 mM) and the ER lumen (c\textsubscript{ER}) (100–500 μM). At rest, the free Ca\textsuperscript{2+} concentration in the mitochondrial matrix (c\textsubscript{mito}) is close to the resting c\textsubscript{cyt}, but it rises to 20–30 μM during stimulation, e.g. in motor nerve terminals in *Drosophila melanogaster* (2). Cell activation in a wide range of cell types results in Ca\textsuperscript{2+} oscillations and in transient waves of increased c\textsubscript{cyt} (3–6). These oscillations (or waves) are not restricted to c\textsubscript{cyt} but also c\textsubscript{nucl} (7), c\textsubscript{ER} (8), and c\textsubscript{mito} show Ca\textsuperscript{2+} oscillations (9). The spatial extent of the oscillatory Ca\textsuperscript{2+} signal is also important. (i) In astrocytes, the area of Ca\textsuperscript{2+} oscillations is sometimes restricted to only one protrusion regulating the release of gliotransmitters; i.e. different oscillatory frequencies can coexist at the same time within the same cell (10). (ii) In *Xenopus laevis* oocytes, regenerative spiral waves of release of free Ca\textsuperscript{2+} spread through the entire cell (11). (iii) Intercellular Ca\textsuperscript{2+} waves spreading via gap junctions occur in rat liver epithelial cells upon mechanical stimulation (12).

In cells maintained in vitro, serum starvation followed by readministration leads to intracellular Ca\textsuperscript{2+} signals, most often in the form of oscillations (13, 14). The precise mechanism(s) leading to these oscillations is poorly understood because serum contains a large number of known and as yet unidentified growth factors and mitogenic compounds, all potentially participating in this oscillatory activity (15). In Swiss 3T3 cells, serum-induced Ca\textsuperscript{2+} changes are essential but not sufficient to induce NF-κB activation and subsequent DNA synthesis (16). In some cell types, Ca\textsuperscript{2+} oscillations even persist in the absence of Ca\textsuperscript{2+} influx across the plasma membrane (3, 4), whereas in others, Ca\textsuperscript{2+} oscillations strictly depend on Ca\textsuperscript{2+} influx (5, 8).

**Results:** However, the “lanthanum insulation method” renders the oscillations independent of extracellular Ca\textsuperscript{2+}.

**Conclusion:** Multiple pathways of Ca\textsuperscript{2+} shuttling are operating simultaneously during Ca\textsuperscript{2+} oscillations.

**Significance:** Experimental and mathematical approaches shed light on the mechanism of Ca\textsuperscript{2+} oscillations.

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\*The abbreviations used are: ER, endoplasmic reticulum; SERCA, sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase; c\textsubscript{cyt}, cytosolic free Ca\textsuperscript{2+} concentration; c\textsubscript{nucl}, nuclear free Ca\textsuperscript{2+} concentration; c\textsubscript{ER}, free Ca\textsuperscript{2+} concentration in the ER lumen; c\textsubscript{mito}, free Ca\textsuperscript{2+} concentration in the mitochondrial matrix; MUC, mitochondrial calcium uniporter; CCCP, carbonyl cyanide m-chlorophenylhydrazone; prMC, primary mouse mesothelial cells; EBFP, enhanced blue fluorescent protein; InsP\textsubscript{3}, inositol triphosphate; ΔΨ\textsubscript{m}, mitochondrial membrane potential; BAFTA, 1,2-bis-(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid; AM, acetoxymethyl ester; CR, calretinin; qRT-PCR, quantitative RT-PCR; ROI, region of interest.

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Mitochondria influence cytosolic Ca\(^{2+}\) oscillations in at least two ways. First, mitochondria produce ATP, which is required for SERCA and plasma membrane Ca\(^{2+}\)-ATPase function, that results in Ca\(^{2+}\) extrusion and thus lowering of \(c_{\text{cyt}}\). Second, during \(c_{\text{cyt}}\) oscillations, \(c_{\text{mito}}\) also manifests oscillations, indicative of a role of mitochondria in shaping and/or modulating \(c_{\text{cyt}}\) oscillations (9). Ca\(^{2+}\) uptake into the mitochondria is determined by both the large negative voltage (−150 to −180 mV) across the inner membrane that results from the proton pumping by the respiratory chain and the Ca\(^{2+}\) concentration gradient between the cytoplasm and matrix (17). The mitochondrial calcium uniporter (MCU) is the key player responsible for the uptake of Ca\(^{2+}\) by mitochondria (18). The MCU has a rather low Ca\(^{2+}\) affinity and operates over a micromolar range of cytosolic Ca\(^{2+}\).

To address these questions, we performed lanthanum (La\(^{3+}\)) insulation experiments where both the Ca\(^{2+}\) influx and efflux across the plasma membrane are blocked (19). We hypothesized that under these experimental conditions mitochondria serving as a Ca\(^{2+}\) store/source might substitute for this function normally exerted by the extracellular space. Using a genetically encoded Ca\(^{2+}\) indicator targeted to the mitochondria, we managed to verify this assumption in vitro. In addition, we investigated the effects of the following compounds on \(c_{\text{cyt}}\) oscillations and mitochondrial Ca\(^{2+}\) handling: the proton uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), the mitochondrial Na\(^{+}\)/Ca\(^{2+}\) blocker CGP-37157, the mitochondrial MCU blocker Ru-360, and finally the “Ca\(^{2+}\)-buffering” protein calretinin. Based on the experimental findings, we built a mathematical model for Ca\(^{2+}\) oscillations taking into account the various processes implicated in these oscillations.

**Materials and Methods**

**Reagents**—Thapsigargin, LaCl\(_3\), and EGTA were purchased from Sigma-Aldrich, CGP-37157 and BAPTA-AM were obtained from Tocris Bioscience (Bristol, UK). Ru-360 was purchased from Calbiochem, and Rhodamine 123 was from Invitrogen. EGTA-AM and tetramethylrhodamine methyl ester were purchased from AAT Bioquest (Sunnyvale, CA). CGP-37157 was dissolved in pure ethanol as 100 mM stock solutions. Thapsigargin and Rhodamine 123 were dissolved as 100 mM stock solutions in DMSO. BAPTA-AM and EGTA-AM were dissolved as 30 mM stock solutions in DMSO. LaCl\(_3\) was dissolved in double distilled water. The final concentrations of the solvents were <0.1% in all experimental solutions. At these concentrations, the solvents did not modify the evoked Ca\(^{2+}\) signals in control experiments (data not shown).

**Isolation of Primary Mouse Mesothelial Cells**—Primary mouse mesothelial cells (pMC) were isolated from 4–6-month-old C57Bl/6j mice according to an established protocol (20) and as applied in our previous study (8). The pelleted cells enriched in mouse mesothelial cells were grown in DMEM/F-12 GlutaMAX medium supplemented with 15% FCS; 0.4 μg/ml hydrocortisone; 10 ng/ml epidermal growth factor; 1% insulin, transferrin, and selenium; 1 mM sodium pyruvate; 0.1 mM β-mercaptoethanol; 1% non-essential amino acids; 1% penicillin-streptomycin; and 2% Mycokill (PAA Laboratories, Pasching, Austria) (21). After a few days (>4 days in vitro), cultured cells showed the typical cobblestone-like morphology of mesothelial cells, and cell cultures maintained for ~60 days in vitro were used for the measurements.

**Plasmids and Lentiviral Infection**—For the generation of cell lines stably expressing the Ca\(^{2+}\) indicator proteins GCaMP3 (Addgene plasmid 22692 (22)) and mito-CAR-GECO1 (Addgene plasmid 46022 (23)), the lentiviral expression vector pLVTHM (Addgene plasmid 12247 (24)) was used. The GFP cassette in pLVTHM was replaced with cDNAs coding for the respective Ca\(^{2+}\) indicator proteins. Briefly, pGCaMP3 was produced in SCS110 dam− bacteria and digested with Afel and Xbal, and the fragment was inserted into the Pmel and SpeI sites of the backbone of pLVTHM to produce the final plasmid pLV-GCaMP3. The expression plasmid CMV-mito-CAR-GECO1 was used as template for the production of a DNA fragment coding for mito-CAR-GECO1. The required DNA fragment was synthesized by PCR using the following primers pairs: 5’-TAG CGT TTA AAC GGG CCC TC-3’ and 5’-GAG AAC TAG TTT ACT TCG CTG TCA TCA TTT GTA C-3’. The amplicon was digested with Pmel and SpeI and inserted into the unique sites of the plLVTHM vector to produce the final plLV-mito-CAR-GECO1 plasmid. Calretinin overexpression was achieved by the help of a lentiviral system. Briefly, the GFP cassette in pLVTHM was replaced with the human CALB2 cDNA coding for full-length calretinin using the previously described expression plasmid RSV-CALB2-neo (25) as template. The DNA fragment coding for full-length calretinin was synthesized by PCR using the primers Pmel-CALB2 (5’-AGT CGT TTA AAC ATG GCT GGC CCG CAG CAG CAG-3’) and SpeI-CALB2 (AGT CAC TAG TTT ACA TGG GGG GCT CGC TGC A-3’). The amplicon was digested with Pmel and SpeI and inserted into the unique sites of the plLVTHM vector to produce the final plLV-CALB2 plasmid. We also generated a lentivirus expressing calretinin (CR) tagged with the enhanced blue fluorescent protein (EBFP) allowing for the easy identification of cells overexpressing EBFP-CR. For this, the plLV-EBFP2-nuc plasmid (Addgene plasmid 36085) and CMV-CALB2-neo were used. The DNA fragment coding for full-length calretinin was synthesized by PCR using the primers Pmel-CALB2 (5’-AGT CGT TTA AAC ATG GCT GGC CCG CAG CAG CAG-3’) and SpeI-CALB2 (AGT CAC TAG TTT ACA TGG GGG GCT CGC TGC A-3’). The amplicon was digested with Pmel and SpeI and inserted into the unique sites of the plLVTHM vector to produce the final plLV-CALB2 plasmid. We also generated a lentivirus expressing calretinin (CR) tagged with the enhanced blue fluorescent protein (EBFP) allowing for the easy identification of cells overexpressing EBFP-CR. For this, the plLV-EBFP2-nuc plasmid (Addgene plasmid 36085) and CMV-CALB2-neo were used. The DNA fragment coding for full-length calretinin was synthesized by PCR using the primers Pmel-CALB2 (5’-AGT CGT TTA AAC ATG GCT GGC CCG CAG CAG CAG-3’) and SpeI-CALB2 (AGT CAC TAG TTT ACA TGG GGG GCT CGC TGC A-3’). The amplicon was digested with Pmel and SpeI and inserted into the unique sites of the plLVTHM vector to produce the final plLV-CALB2 plasmid.
Mission lentiviral transduction particles (Sigma-Aldrich) TRCN0000267404 and TRCN0000265169. Mission transduction particles directed toward human parvalbumin (TRCN0000056549) and non-infected cells served as controls. Infected cells were selected using 2 µg/ml puromycin dihydrochloride (Sigma-Aldrich) for 1 week. MCU transcript knockdown was verified by qRT-PCR analysis.

$qRT-PCR$—PrMC were seeded in 6-well plates, and RNA was extracted with 1 ml of PeqGold TriFast (PeqLab, Erlangen, Germany). cDNA synthesis (Quantitect Reverse Transcription kit, Qiagen, Hombrechtikon, Switzerland) and qRT-PCR (Rotor-Gene SYBR Green PCR kit, Qiagen) were performed following the manufacturers’ protocols. Primers were as follows: mUBC: forward, 5’-GGG CAC CGT GAA ACA ACT C-3’; reverse, 5’-ACC TCC AGG GTG ATG TTC CCA CCA-3’; mMCU: forward, 5’-CTC AGA TGG CGT TCG AGT CG-3’; reverse, 5’-GCC TCG CTG CAT CTT CAT GGC TGG C-3’.

$Calcium$ Imaging$—$prMC were isolated as described before (27) and grown on collagen-coated glass bottom 35-mm dishes (MatTek Corp., Ashland, MA). The buffer solution (Hepes-buffered saline) used for Ca$^{2+}$ imaging experiments contained 120 mM NaCl; 5.4 mM KCl, 0.8 mM Mg$^{2+}$Cl, 20 mM Hepes, 1 mM CaCl$_2$, and 10 mM glucose, pH 7.4 (adjusted by NaOH). In the low Ca$^{2+}$ solution, CaCl$_2$ was replaced with an equimolar concentration of NaCl. The drugs (thapsigargin, FCS, and EGTA) were added to the solutions and remained in the solution until the end of the experiments. In some experiments, cells were pretreated either with 250 µM GGP-37157 or with 10 µM Ru-360 for 30 min at 37 °C. Cells were loaded either with 30 µM BAPTA-AM or 30 µM EGTA-AM for 15 min at 37 °C. We used a DMI6000 inverted confocal microscope integrated to a Leica TCS-SP5 work station to examine fluorescence signals indirectly, reporting $c_{cyt}$ or $c_{mito}$. The following excitation wavelengths were used to illuminate the fluorophores: 488 nm for GCaMP3 and 561 nm for mito-CAR-GECO1. Fluorescence emissions were recorded with a 20 X objective and bandpass filters of 505–550 nm for Rhodamine 123. The distribution of Rhodamine 123 between the mitochondrial matrix and cytosol is proportional to the mitochondrial membrane potential. As the mitochondrial network is distributed within the entire cytoplasmic space, circle-shaped ROIs were randomly assigned to the cytoplasmic region for the fluorescence intensity measurements. The signal intensity is proportional to the amount of Rhodamine 123 dye incorporated by mitochondria in this ROI. For the normalization and thus the measurement of Rhodamine 123 released by mitochondria, an ROI within the nuclear region not containing mitochondria was selected, and the fluorescence intensity in this ROI was determined. The relative (rel.) $\Delta \Psi$ was calculated according to the following equation.

$$\Delta \psi_{rel} = \frac{F_{mito}}{F_{nuc}}$$  
(Eq. 1)

where $F_{mito}$ and $F_{nuc}$ are the fluorescence intensity of Rhodamine 123 in the mitochondrial and nuclear regions, respectively. The mitochondrial membrane potential was additionally measured with tetramethylrhodamine methyl ester. For these measurements, cells were preincubated with 50 nm tetramethylrhodamine methyl ester for 30 min.

$Estimation$ of the Intracellular Calretinin Concentration by Western Blot Analysis$—$Protein samples were isolated from cultured prMC. Cells were grown in 25-cm$^2$ flasks and harvested at near confluence. Total proteins were extracted with ice-cold radioimmunoprecipitation assay buffer. Serial dilutions of protein extracts (50, 5, 0.5, and 0.005 µg) from each cell culture sample as well as 40 ng of purified human recombinant calretinin were loaded onto SDS-polyacrylamide gels (12.5%). After separation, proteins were transferred onto nitrocellulose membranes (Bio-Rad) and incubated overnight at 4 °C with the calretinin-specific antibody CR7699/4 (Swant, Marly, Switzerland) at a dilution of 1:10,000. Rabbit secondary antibody linked to horsedash peroxidase (Sigma-Aldrich) was diluted at 1:10,000, and membranes were incubated for 4 h. For the detection, the chemiluminescent reagent Luminata Classico Forte (EMD Millipore Corp., Billerica, MA) was used. Chemiluminescent and normal illumination digital images were recorded on a system from Cell Biosciences (Santa Clara, CA). Area densities of calretinin bands were measured with ImageJ software. From the density curves, the cell protein concentration corresponding to 40 ng of calretinin was determined. This allowed determination of calretinin or more precisely that of the fusion protein EBFP-calretinin content in µg/mg of total protein. Based on previous estimation of a protein concentration of about 0.2 g/ml (28) in mammalian cells, the intracellular concentration of EBFP-calretinin was estimated.
Frequency Determination and Amplitude Scan—Computerized peak recognition for frequency and amplitude analyses was realized via the Microsoft Excel 2010 environment as described before (8); normalized recordings from >30 oscillating prMC were evaluated. The oscillation frequency as well as the average amplitude was determined for three time windows: 1–5, 5–9, and 9–13 min after serum administration.

Mathematical Simulation—To build the mathematical model, we considered four compartments: the extracellular space, cytoplasm, mitochondrial matrix, and ER lumen (Fig. 1). A fifth element placed within the cytoplasm in some simulations was the presence of a Ca\(^{2+}\) buffer. Membrane junctions between the ER and the plasma membrane ensured that the functional unit components (Ca\(^{2+}\) channels and pumps) are concentrated spatially in a very small space (29). Similarly close contacts were also assumed to exist between mitochondria and ER (30). One oscillatory unit represents an inositol trisphosphate receptor (InsP\(_3\)) assumed to exist between mitochondria and ER (30). One oscillatory unit represents an inositol trisphosphate receptor (InsP\(_3\)) cluster and its surrounding. We presumed that changes in \(c_{\text{cyt}}(t)\) and \(c_{\text{mito}}\) of the entire cell were similar to that of individual units, i.e. spatially homogenous. In our view, this simplification is acceptable because the oscillations are slow and the cell size is small. In this case, the spatial diffusion of Ca\(^{2+}\) rapidly equilibrates the putative spatial differences and thus synchronizes the functions of individual functional units (31). In a cell with a 10-μm diameter, the diffusion is estimated to equilibrate spatial heterogeneity in \(c_{\text{cyt}}\) in less than 0.1 s (32). However, because Ca\(^{2+}\) waves not only depend on Ca\(^{2+}\) diffusion but also on the action of Ca\(^{2+}\) pumps and channels, the Ca\(^{2+}\) wave is ~10 times slower (33). Although our model is a minimal deterministic point model and cannot produce the stochastic and spatial phenomena of the Ca\(^{2+}\) oscillations, it is a useful tool to illuminate the observed characteristics of the mitochondrial Ca\(^{2+}\) handling. Our aim was to build the most simple model still able to produce the experimentally observed phenomena.

Ca\(^{2+}\) transports across the plasma membrane were defined as \(J_{\text{IN}}\) and \(J_{\text{EFF}}\) and the transports across the ER membrane were termed \(J_{\text{SERCA}}\) and \(J_{\text{EREFF}}\), respectively. \(J_{\text{IN}}\) includes Ca\(^{2+}\) channels in the plasma membrane, e.g. voltage-gated Ca\(^{2+}\) channels, transient receptor potential channels, store-operated channels, P2X purinoreceptors, hyperpolarization-activated cyclic nucleotide-gated channels, etc. The \(J_{\text{EFF}}\) represents the pumps involved in Ca\(^{2+}\) extrusion, plasma membrane Ca\(^{2+}\)-ATPases and Na\(^+\)/Ca\(^{2+}\) exchangers. The SERCA pumps transport Ca\(^{2+}\) from the cytoplasm to the ER, whereas the \(J_{\text{EREFF}}\) represents the ER channels involved in emptying the ER,ryanodine receptor and InsP\(_3\)R. The function of the mitochondrial exchangers (\(J_{\text{MEXC}}\)) and the mitochondrial calcium uniporter (\(J_{\text{MCU}}\)) are responsible for the Ca\(^{2+}\) transport across the mitochondrial inner membrane (see Fig. 1).

We denote by \(c_{\text{cyt}}\) the Ca\(^{2+}\) concentration (in nm) in the cytosol and by \(c_{\text{ER}}\) that in the lumen of the ER. Mitochondrial matrix free concentration is denoted by \(c_{\text{mito}}\). The equations for the model are as follows.

\[
\frac{dc_{\text{cyt}}}{dt} = J_{\text{IN}} - J_{\text{EFF}} - J_{\text{SERCA}} + J_{\text{EREFF}} + J_{\text{ERLEAK}} - J_{\text{MCU}} + J_{\text{MEXC}}
\]  
(Eq. 2)

where \(J_{\text{IN}}\) is the flux of Ca\(^{2+}\) ions entering the cell, \(J_{\text{EFF}}\) is the Ca\(^{2+}\) flux pumped out of the cell, \(J_{\text{SERCA}}\) denotes the Ca\(^{2+}\) flux pumped from the cytosol to ER, \(J_{\text{EREFF}}\) is the flux of Ca\(^{2+}\) passing from the ER to the cytosol, \(J_{\text{MCU}}\) denotes the function of MCU, \(J_{\text{MEXC}}\) displays the function of mitochondrial Ca\(^{2+}\) exchangers (mitochondrial Na\(^+\)/Ca\(^{2+}\) and H\(^+\)/Ca\(^{2+}\) exchangers), and finally \(J_{\text{ERLEAK}}\) represents a small flux of Ca\(^{2+}\) diffusing from the ER to the cytosol (all values in nm/s).

The constant \(\gamma\) is the ratio between the changes in \(c_{\text{cyt}}\) and \(c_{\text{ER}}\) caused by the same quantity of Ca\(^{2+}\) ions transported through the ER membrane. This value is derived from the difference in the effective volume of the ER lumen and the cytoplasm and from the different fraction of free and protein-bound Ca\(^{2+}\) in these compartments (34). The value of the \(\gamma\) parameter was estimated experimentally (8).

The quantity of Ca\(^{2+}\) pumped out of the cell through the plasma membrane increases as a function of the Ca\(^{2+}\) concentration in the cytosol. The Na\(^+\)/Ca\(^{2+}\) exchangers have low Ca\(^{2+}\) affinity but high capacity for Ca\(^{2+}\) transport, whereas the plasma membrane Ca\(^{2+}\)-ATPases have a high Ca\(^{2+}\) affinity but a low transport capacity. Although the individual components of extrusion systems are usually modeled by Hill equations (35), the overall flux can be simulated by a simple linear equation (36).


**Ca^2+ Shuttling during Ca^2+ Oscillations in Mesothelial Cells**

Based on the experimental results of Herrington et al. (37).

\[
J_{\text{eff}} = \begin{cases} 
0, & r_1c_{\text{cyt}} - r_2 = 0 \\
0, & r_1c_{\text{cyt}} - r_2, r_1c_{\text{cyt}} - r_2 > 0 
\end{cases} \quad \text{(Eq. 5)}
\]

where \(r_1\) and \(r_2\) are two positive constants.

SERCA pumps the Ca^2+ ions from the cytosol to the ER. The quantity of the transported Ca^2+ ions depends on \(c_{\text{cyt}}\) levels. We assume a linear relationship because the ER influx is also composed of different SERCA pumps with different \(K_d\) values (38). Nevertheless, our model can also work when \(J_{\text{SERCA}}\) is simulated with the conventional Hill equations.

\[
J_{\text{SERCA}} = \begin{cases} 
0, & r_1c_{\text{cyt}} - r_2 = 0 \\
r_1c_{\text{cyt}} - r_2, r_1c_{\text{cyt}} - r_2 > 0 
\end{cases} \quad \text{(Eq. 6)}
\]

where \(r_1\) and \(r_2\) are two positive constants.

Ca^2+ ions are released from the ER to the cytosol through InsP_3R and ryanodine receptor. Because we found experimentally that ryanodine receptor does not play a role in serum-induced oscillations in mesothelial cells (8) similarly to other non-excitable cells (39), we focused on InsP_3R. In our model, InsP_3R is influenced both by \(c_{\text{cyt}}\) and \(c_{\text{ER}}\) but without an allosteric regulation between the two. InsP_3R has Ca^2+ binding sites not only on the cytoplasmic side but also on the luminal side (40). Experimental data show that an increase in inositol trisphosphate (InsP_3) concentration causes a significant Ca^2+ release from the ER in the absence of cytosolic Ca^2+ (\(c_{\text{cyt}} = 0\)) (41). Moreover, the effects of luminal Ca^2+ do not affect the cytosolic binding sites (42, 43). Therefore we modeled InsP_3R function as the sum of two individual contributions.

\[
J_{\text{EREFF}} = J_{\text{cystdep}} + J_{\text{EREdep}} \quad \text{(Eq. 7)}
\]

where

\[
J_{\text{cystdep}} = r_{\text{cystdep}} \exp\left(-\frac{(\log(c_{\text{cyt}}) - \mu)^2}{\alpha^2}\right) \quad \text{(Eq. 8)}
\]

and

\[
J_{\text{EREdep}} = r_{\text{EREdep}} \log(c_{\text{ER}}) - r_2 \quad \text{(Eq. 9)}
\]

with positive constants \(\alpha\) and \(r_1\).

We introduced the dependence of InsP_3R on the [InsP_3], which has an influence both on \(J_{\text{cystdep}}\) and on \(J_{\text{EREdep}}\). According to the experimental data from several studies (44–46), elevating \(c_{\text{IP3}}\) mainly changes the mean and the maximum (\(\mu\) and \(r_{\text{cystdep}}\)) of the bell-shaped curve of \(c_{\text{cyt}}\) dependence. Nevertheless, based on the experimental data presented (47, 48), elevating \(c_{\text{IP3}}\) also has an effect on the loading of the ER. Increased \(c_{\text{IP3}}\) reduces the amount of the stored Ca^2+ ions. We simulated this effect by changing the \(r_{\text{cystdep}}\) parameter.

\[
\mu = \mu_{\text{min}} + \left(\mu_{\text{max}} - \mu_{\text{min}}\right)\frac{K_b}{K_b + c_{\text{IP3}}} \quad \text{(Eq. 10)}
\]

\[
r_{\text{cystdep}} = r_{\text{cystmin}} + \left(r_{\text{cystmax}} - r_{\text{cystmin}}\right)\frac{K_b}{K_b + c_{\text{IP3}}} \quad \text{(Eq. 11)}
\]

where \(K_b\) is the half-saturation constant of InsP_3R for InsP_3 and \(c_{\text{IP3}}\) represents the InsP_3R sensitivity to the inositol trisphosphate molecule, which was taken as equal to inositol trisphosphate concentration in \(\mu\). \(\mu_{\text{max}}, \mu_{\text{min}}, r_{\text{cystmin}}, r_{\text{cystmax}}, r_{\text{cystmin}}\), and \(r_{\text{cystmax}}\) are positive constants. The parameter \(J_{\text{EREpeak}}\) accounts for a Ca^2+ flux from the ER to the cytoplasm independently of known Ca^2+ channels, and this parameter is assumed to represent a small constant value (49).

\[
J_{\text{EREpeak}} = \beta \quad \text{(Eq. 13)}
\]

where \(\beta\) is the ratio between the changes in \(c_{\text{cyt}}\) and \(c_{\text{ER}}\) caused by the same quantity of Ca^2+ ions transported through the mitochondrial inner membrane. This value is derived from the difference in the effective volume of the mitochondrial matrix and the cytoplasm in the effective fraction of free and protein-bound Ca^2+ in these compartments.

There is a fast Ca^2+ influx into the mitochondria matrix if \(c_{\text{cyt}}\) reaches a certain value. This fast influx is attributable to the function of MCU. We used Hill equations with a very high Hill coefficient as was done in the work of Marhl et al. (50). For simplicity, we did not take into account the changes in mitochondrial transmembrane potential and in mitochondrial volume during the Ca^2+ oscillations in line with Marhl et al. (50), but we have to consider it during a protonophore treatment. The passage of calcium ions through the MCU requires the large membrane potential difference generated by the action of the electron transport chain (51).

\[
J_{\text{MCU}} = \Delta\Psi \times r_0 \times v_{\text{MCU,max}} \frac{c_{\text{cyt}}}{K_{d,\text{MCU}} + c_{\text{cyt}}} \quad \text{(Eq. 14)}
\]

where \(r_0\) and \(v_{\text{MCU,max}}\) are positive constants, \(K_{d,\text{MCU}}\) is the dissociation constant of MCU for Ca^2+ ions, and \(H\) is the Hill coefficient. In our model, \(J_{\text{MCU}}\) has a constant basal activity. That ensures that mitochondria can store a small amount of Ca^2+ ions, which are released into the cytoplasm immediately after the collapse of the mitochondrial membrane potential. Higher \(\Delta\Psi\) means increased Ca^2+ uptake but slower mitochondrial Ca^2+ release.

To simulate the function of mitochondrial exchangers (Na+/Ca^2+ and H+/Ca^2+ exchangers), we consider that both will transport Ca^2+ ions with a low velocity when there is a concentration gradient between the two sides of the mitochondrial inner membrane. For the simplicity, we neglected the changes in sodium and proton concentrations during the Ca^2+ oscillations. Depending on the calcium concentration gradient, the exchangers can work in both directions.

\[
J_{\text{MEXC}} = \left(\frac{c_{\text{mito}}}{p \times c_{\text{cyt}}} - 1\right) \times (\Delta\Psi \times r_{\text{m1}} - r_{\text{m2}}) \quad \text{(Eq. 15)}
\]

where \(r_{\text{m1}}\) and \(r_{\text{m2}}\) are positive constants.

The Ca^2+ influx across the plasma membrane is composed of passive leakage and the agonist-activated fluxes: the capacitive
(store-operated channel-dependent) and the non-capacitive (arachidonate- or diacylglycerol-regulated) Ca\(^{2+}\) influx (52). We simulated the changes in \(J_{\text{IN}}\) starting from the beginning of the administration of serum \((t_1)\) using the following equations.

\[
J_{\text{IN}} = 0.1 \text{nm}/s \text{ if } t < t_1 (\text{only passive leakage}) \quad (\text{Eq. 16})
\]

\[
J_{\text{IN}} = \frac{r_{\text{IN,MAX}}}{K_{\text{IN}}} (t - t_1) \text{ if } t_1 \leq t \leq t_2 \quad (\text{Eq. 17})
\]

\[
J_{\text{IN}} = \left( r_{\text{IN,MAX}} - r_{\text{IN,Min}} \right) \times \exp\left( - K_{\text{IN}} (t - t_2) \right) + r_{\text{IN,Min}} \text{ if } t > t_2 \quad (\text{Eq. 18})
\]

We simulated the changes in \(c_{\text{IP3}}\) from the beginning of the administration of serum \((t_1)\) with the following equations. The resting \(c_{\text{IP3}}\) was set to 15 nm (53).

\[
c_{\text{IP3}} = 0.015 \text{ if } t < t_1 \quad (\text{Eq. 19})
\]

\[
c_{\text{IP3}} = \frac{c_{\text{IP3,MAX}}}{K_{\text{IP3}} + (t - t_1)} \text{ if } t \geq t_1 \quad (\text{Eq. 20})
\]

To simulate the effect of calretinin, we neglected its fast kinetics. Because this protein is considered as a fast Ca\(^{2+}\) buffer (54), calretinin reaches the Ca\(^{2+}\) steady state in a few milliseconds, which is much faster than our observed Ca\(^{2+}\) changes lasting for a few seconds. The fast kinetics of calretinin plays an important role at the mouth of voltage-gated Ca\(^{2+}\) channels in excitable cells (55) where fast and large changes in Ca\(^{2+}\) concentrations are expected.

\[
\frac{dc_{\text{CR}}}{dt} = J_{\text{IN}} - J_{\text{EFF}} - J_{\text{SERCA}} + J_{\text{ERF}} + J_{\text{ERL}} - J_{\text{MCU}} + J_{\text{MEXC}} + c_{\text{CR}} \nu
\]

(Eq. 21)

where \(c_{\text{CR}}\) is the concentration of calretinin in the cytoplasm and \(\nu\) is the average number of the Ca\(^{2+}\) binding sites of calretinin occupied by Ca\(^{2+}\). Calretinin has four high affinity Ca\(^{2+}\) binding sites and one low affinity binding site. The binding kinetics of the Ca\(^{2+}\) binding sites were simulated with Hill equations.

\[
\nu = 4 \times \frac{c_{\text{H}}^{h}}{K_{h}^{h}} + \frac{c_{\text{d}}^{h}}{K_{d}^{h}} + \frac{c_{\text{H}}^{d}}{K_{d}^{d}} + \frac{c_{\text{cyt}}^{h}}{K_{h}^{cyt}} + \frac{c_{\text{cyt}}^{d}}{K_{d}^{cyt}}
\]

(Eq. 22)

where \(K_{h}^{h}\) is the dissociation constant for the high affinity Ca\(^{2+}\) binding sites, \(K_{d}^{h}\) is the dissociation constant for low affinity Ca\(^{2+}\) binding site, and \(h\) is the Hill coefficient for the high affinity binding sites. Among the high affinity Ca\(^{2+}\) binding sites, there is a positive cooperativity \((h > 1)\). The values for the parameters came from the study of Faas et al. (54).

The values of each parameter are listed in Table 1. The initial values of parameters are derived either from our experiments in primary mesothelial cells or from fitting to experimental data previously reported in the above mentioned articles. The presented values are the result of the sequential fitting of the initial values to our \textit{in situ} recordings. All computations of the model were implemented in the Microsoft Excel 2010 environment. The model system was discretized with a temporal resolution of 0.1 s (supplemental Excel document). There were no significant differences in the solution of the differential equations if we increased the temporal resolution (not shown). For visualization, Prism5 (GraphPad Software, Inc.) software was used.

### Results

**Characterizing Ca\(^{2+}\) Fluctuations in Mitochondria of Primary Mouse Mesothelial Cells**—In the absence of serum, prMC did not show Ca\(^{2+}\) oscillations as reported before (8). However, in a small fraction of cells (2–3%), isolated arrhythmic mitochondrial increases in \(c_{\text{mito}}\) were present without detectable changes in \(c_{\text{cyt}}\) (Fig. 2A). The addition of 1% FCS to the cell culture medium containing prMC that were grown in the absence of serum for 24 h resulted in a sudden rise of \(c_{\text{cyt}}\) lasting, on average, for \(~40\) s followed by Ca\(^{2+}\) oscillations (Fig. 2, B–D). The percentage of prMC responding to serum readministration with Ca\(^{2+}\) oscillations was in the order of 70%. Non-oscillatory cells showed only an initial single Ca\(^{2+}\) transient or a so-called peak-plateau response (53). A wide range of different oscillatory patterns in \(c_{\text{cyt}}\) was present in a supposedly homogenous population of mesothelial cells. Most cells displayed long period (> 10 min) baseline spiking oscillations with various frequencies of one spike per 3 min (Fig. 2B) up to 10 per min (Fig. 2D); also maximal spike amplitudes varied between individual cells. The baseline spiking oscillations represent discrete Ca\(^{2+}\) transients starting from a constant basal \(c_{\text{cyt}}\) level (Fig. 2, B and C). Sinusoidal oscillation is a term for a continuous fluctuation.

### TABLE 1

Parameters used for the modeling

| Equation to determine | Parameter name | Value |
|-----------------------|----------------|-------|
| \(\gamma\)            |                | 450   |
| \(\rho\)              |                | 4     |
| \(r_{t_1}\)           |                | 0.17/s|
| \(r_{t_2}\)           |                | 18.8 nm/s |
| \(r_{t_3}\)           |                | 0.27/s |
| \(t_1\)               |                | 22 nm/s |
| \(t_2\)               |                | 1300/s |
| \(t_3\)               |                | 2.1 nm/s |
| \(t_4\)               |                | 2.2 nm/s |
| \(K_{c_{\text{IP3}}}\) |                | 2.2 nm/s |
| \(c_{\text{IP3,MAX}}\) |                | 6352 nm/s |
| \(c_{\text{IP3,MIN}}\) |                | 7042 nm/s |
| \(c_{\text{ERF}}\)    |                | 2.5 nm/s |
| \(c_{\text{ERL}}\)    |                | 260 mm |
| \(c_{\text{MCU}}\)    |                | 110 mm |
| \(c_{\text{MEXC}}\)   |                | 180 mm |
| \(c_{\text{IP3}}\)    |                | 208 nm |
| \(c_{\text{IP3,MIN}}\) |                | 7.4 |
| \(c_{\text{IP3,MAX}}\) |                | 0.005555 mV |
| \(c_{\text{IP3,MIN}}\) |                | 5 nm/s |
| \(c_{\text{IP3,MAX}}\) |                | 208 nm |
| \(c_{\text{IP3,MIN}}\) |                | 7.4 |
| \(c_{\text{IP3,MAX}}\) |                | 0.01 s |
| \(c_{\text{IP3,MIN}}\) |                | 2 s |
| \(c_{\text{IP3,MAX}}\) |                | 1.8 \(\mu\) |
| \(c_{\text{IP3,MIN}}\) |                | 0.1 \(\mu\) |

*Alternatively, the \(J_{\text{EFF}}\) and \(J_{\text{SERCA}}\) can be simulated conventionally with a Hill equation with the following parameters: \(J_{\text{MAX}}\), 260 and 170 nm/s; \(K_{a}\), 460 and 480 nm; and Hill coefficients, 3.5 and 2.4, respectively.*
Ca\(^{2+}\) Shuttling during Ca\(^{2+}\) Oscillations in Mesothelial Cells

**FIGURE 2.** Ca\(^{2+}\) oscillations in \(c_{\text{c}}\), \(c_{\text{ER}}\), and \(c_{\text{mito}}\) in prMC. A, a representative recording shows spontaneous mitochondrial Ca\(^{2+}\) transients (red trace) in a resting cell without changes in \(c_{\text{c}}\) (green trace). B–D, single cell fluorescence recordings derived from time lapse videos show the simultaneous changes in \(c_{\text{c}}\) (green traces) and \(c_{\text{mito}}\) (red traces) after serum readministration. Despite the different oscillatory frequencies (increasing from B to D) in \(c_{\text{c}}\), the traces for \(c_{\text{mito}}\) were very similar in all three recordings: a fast initial rise followed by a quasiexponential decay with varying kinetics. The small (basal) spikes in \(c_{\text{c}}\) coincide with the peak in \(c_{\text{mito}}\), which is slightly delayed in comparison with the peak in \(c_{\text{c}}\). An experimental recording (black dashed line) in prMC showing low frequency oscillations was selected for the fitting. The model shows the changes in \(c_{\text{c}}\) (green trace), \(c_{\text{mito}}\) (red trace), and \(c_{\text{ER}}\) (blue trace). The changes in \(c_{\text{mito}}\) consist of an initial rise after FCS administration followed by a slow return to basal levels. Each Ca\(^{2+}\) spike in \(c_{\text{mito}}\) results in a small hump in \(c_{\text{c}}\) reaching its relative maximum with a small delay compared with the maximum in \(c_{\text{c}}\). Original recordings for \(c_{\text{c}}\) in prMC were previously presented (8); they show sawtooth-like waves at a semidepleted ER state during Ca\(^{2+}\) oscillations as shown in our model here (blue trace).

in \(c_{\text{c}}\) starting from a \(c_{\text{c}}\) value that is higher than the resting (basal) \(c_{\text{c}}\) (Fig. 2D). Most probably, sinusoidal oscillations are the result of high frequency overlapping baseline spiking oscillations (56). In prMC maintained in cell culture medium for longer periods (>10 passages), the percentage of the cells showing sinusoidal oscillations was increased as exemplified in Fig. 2D. However, the percentage of cells showing oscillatory activity was rather low at higher passages (~20~40% at passages >10). The average frequency of the baseline spiking oscillations was found to be 15, 13, and 13 mHz in the following time segments: 1–5, 5–9, and 9–13 min after serum administration, respectively. The average amplitude of spikes (fold increase in GCaMP3 fluorescence intensity) was found to be 2.66, 2.53, and 2.53 in the above mentioned time segments. By using two Ca\(^{2+}\) indicators targeted to either the mitochondrial matrix (mito-CAR-GECO1) or the cytoplasm (GCaMP3), we simultaneously monitored changes in \(c_{\text{mito}}\) and \(c_{\text{c}}\), respectively. The initial serum-induced rise in \(c_{\text{c}}\) was paralleled by a rapid rise in \(c_{\text{mito}}\) that reached the peak value within 30 s after the addition of 1% FCS (Fig. 2, B–D); from then on, \(c_{\text{mito}}\) decreased continuously until it reached its initial basal value, generally within the time span of 15 min monitored in most experiments. High frequency oscillations in \(c_{\text{c}}\) resulted in continuous elevation in \(c_{\text{mito}}\) (Fig. 2D). The rate of decay in \(c_{\text{mito}}\) was also rather variable between cells, and oscillations in \(c_{\text{c}}\) did not stop when \(c_{\text{mito}}\) had reached its basal levels. In some cells during the decreasing phase in \(c_{\text{mito}}\) small fluctuations (short rises in \(c_{\text{mito}}\)) coincided with the \(c_{\text{c}}\) spikes but with a small delay (e.g. shown in Fig. 2B, inset). For the modeling, we took into account our previous results where basal and maximal \(c_{\text{c}}\) values during Ca\(^{2+}\) spikes in prMC were found to be 100 and 200–300 nM, respectively (8). Similarly, the values for the resting \(c_{\text{ER}}\) were taken as 150–250 \(\mu\text{M}\), and the values after serum readministration were taken as 100–150 \(\mu\text{M}\) (8). The pattern of \(c_{\text{ER}}\) changes is best described as a sawtooth wave (8). These data were incorporated to build the mathematical model where Ca\(^{2+}\) concentrations in all compartments (\(c_{\text{c}}, c_{\text{mito}}, \) and \(c_{\text{ER}}\)) were calculated and fitted to one \(c_{\text{c}}\) recording (Fig. 2E). The model accurately recapitulated the...
experimental findings, in particular with respect to $c_{\text{mito}}$, which had not been modeled in our previous study (8). The pattern in $c_{\text{mito}}$ is best described as a sudden rise after serum readministration followed by a rather smooth decay phase with small humps (increases in $c_{\text{mito}}$) as the result of the oscillatory Ca$^{2+}$ spikes.

**Modulation of Mitochondrial Ca$^{2+}$ Transport Mechanisms Affects Ca$^{2+}$ Oscillations**—In the next series of experiments and modeling simulations, we investigated how altering mitochondrial function, mostly in relation to Ca$^{2+}$ handling, affects $c_{\text{cyst}}$ oscillations. The simulation showed that inhibition of the mitochondrial Ca$^{2+}$ release ($J_{\text{MEXC}}$) or mitochondrial Ca$^{2+}$ uptake ($J_{\text{MCU}}$) during Ca$^{2+}$ oscillations decreased the oscillation frequency (Fig. 3, A and B). The experimental verification of our predictions was hampered by the absence of pharmacologically mitochondrially targeted compounds that immediately reach the mitochondrial inner membrane when added to the recording solution. Briefly, after serum readministration, the addition of CGP-37157 (50 μM), a nonspecific blocker of the mitochondrial Na$^{+}$/Ca$^{2+}$ exchanger, had no effect on the patterns of Ca$^{2+}$ oscillations in either $c_{\text{cyst}}$ or $c_{\text{mito}}$ (data not shown). However, some cells pretreated with CGP-37157 for 30 min displayed stairlike increases in $c_{\text{mito}}$ (Fig. 3C), an effect predicted from our model (see Fig. 3A, right part ($c_{\text{mito}}$)). Ruthenium compounds, e.g. ruthenium red and Ru-360, are potent and effective blockers of MCU in isolated mitochondria, but their usefulness for intact cells is limited by their poor membrane permeability and selectivity (57). Pretreatment of cells with 10 μM Ru-360 reduced the average oscillation frequency (approximately a 30% decrease during each time segment) and the initial mitochondrial Ca$^{2+}$ uptake (Fig. 3D). However, it is currently still unclear whether in intact cells Ru-360 acts uniquely by the inhibition of the mitochondrial Ca$^{2+}$ uptake or additionally by the inhibition of the extracellular Ca$^{2+}$ influx. Therefore, we also used a molecular approach, i.e. down-regulation of MCU by shRNA to decrease the mitochondrial Ca$^{2+}$ uptake. The down-regulation of MCU mRNA levels by 60–90% as determined by qRT-PCR resulted in a 40–60% decrease in the initial mitochondrial Ca$^{2+}$ uptake (Fig. 3E). This led to an ~20% reduction in the $c_{\text{cyst}}$ oscillation frequency calculated by frequency scan analysis (Fig. 3F) in line with the predictions from our model (Fig. 3B). In all time windows (bins of 3 s), the oscillation frequency was lower in prMC where MCU had been down-regulated. Thus, both approaches (Ru-360 and shMCU)
underscore the importance of mitochondria in Ca\(^{2+}\) oscillations.

CCCP is an inhibitor of oxidative phosphorylation by acting as a protonophore; i.e., it allows H\(^+\) to cross the inner mitochondrial membrane, resulting in the collapse of \(\Delta \Psi\). During Ca\(^{2+}\) oscillations, \(\Delta \Psi\) was slightly increased (more negative), but it collapsed immediately after CCCP (100 \(\mu\)M) treatment (Fig. 4A). The collapse of the membrane potential after addition of 10 or 100 \(\mu\)M CCCP was also confirmed by using the tetramethylrhodamine methyl ester indicator dye (data not shown).

When applied during Ca\(^{2+}\) oscillations resulting from serum readministration, CCCP blocked Ca\(^{2+}\) oscillations at 100 \(\mu\)M but not at 10 \(\mu\)M (data not shown). An immediate drop in \(c_{\text{mito}}\) was observed after CCCP treatment (Fig. 4B) followed by a continuous elevation in \(c_{\text{mito}}\) in some cells (~20%) but not in others (Fig. 4, A and C). In a few cases (~5% of cells), administration of ATP (1 \(\mu\)M) partially reverted the CCCP-induced oscillation stop (Fig. 4C). However, addition of ATP to the recording solution in the absence of serum was equally able to evoke Ca\(^{2+}\) oscillations in some cells (data not shown). The reason for this

FIGURE 4. Effect of the proton uncoupler CCCP on Ca\(^{2+}\) oscillations. A, serum addition to prMC led to a rapid rise in \(c_{\text{mito}}\) (red trace) followed by a gradual decay. Addition of CCCP (100 \(\mu\)M) resulted in a rapid collapse of the membrane potential as evidenced by measuring Rhodamine 123 fluorescence signals (green trace). As the result of CCCP application, \(c_{\text{mito}}\) immediately returned to basal levels because the slightly elevated \(c_{\text{mito}}\) level (compared with the \(c_{\text{cyt}}\) level) could not be maintained when the driving force for mitochondrial Ca\(^{2+}\) uptake was eliminated. B, the role of the membrane potential and ATP treatment was investigated in greater detail, and one representative recording for each experimental condition is depicted. B, the addition of CCCP (100 \(\mu\)M) at \(t = 9\) min blocked the serum-induced cytosolic Ca\(^{2+}\) oscillations. CCCP treatment led to a rapid fall in \(c_{\text{mito}}\) in some cells, a slow increase in \(c_{\text{mito}}\) occurred afterward (B), whereas in others, \(c_{\text{mito}}\) remained low (not shown). C, administration of ATP (1 \(\mu\)M) reestablished the CCCP-inhibited Ca\(^{2+}\) oscillations in some cells. D, addition of CCCP (100 \(\mu\)M) prior to serum readministration lowered the resting \(c_{\text{mito}}\) (also reflected by the simultaneous small increase in \(c_{\text{cyt}}\)). However, from then onward, \(c_{\text{cyt}}\) dropped to levels lower then the basal \(c_{\text{cyt}}\) before CCCP treatment. The serum readministration at \(t = 8\) min evoked only a single Ca\(^{2+}\) transient. E, at a lower CCCP concentration (10 \(\mu\)M), addition of 1% FCS at \(t = 6\) min resulted in elevations in \(c_{\text{cyt}}\) and \(c_{\text{mito}}\) followed by a few oscillations in \(c_{\text{mito}}\). The small amount of Ca\(^{2+}\) ions taken up by mitochondria (red trace) during Ca\(^{2+}\) spikes (green trace) was released back to the cytosol almost immediately. F, in this simulation experiment, the mitochondrial membrane potential was switched off (\(\Delta \Psi = 0\)) at \(t = 3\) min; this resulted in a slight increase in \(c_{\text{cyt}}\) and a decrease in \(c_{\text{mito}}\) to values lower than basal \(c_{\text{cyt}}\) and \(c_{\text{mito}}\) as observed experimentally in D and E. Serum readministration evoked oscillatory activity in \(c_{\text{cyt}}\) and \(c_{\text{mito}}\). After the CCCP-induced collapse in \(\Delta \Psi\), ER Ca\(^{2+}\) release by thapsigargin (5 \(\mu\)M) resulted in mitochondrial Ca\(^{2+}\) uptake (red trace) independent of \(\Delta \Psi\). A representative recording displays the simultaneous changes in \(c_{\text{mito}}\) (red) and \(c_{\text{cyt}}\) (green). H, during FCS-induced Ca\(^{2+}\) oscillations, ATP levels in prMC were increased but dropped quickly after CCCP administration. The panel shows the mean ± S.D. of three independent experiments. Error bars represent S.D.
effect is currently unknown; ATP might act on receptors on the surface of prMC but was also shown to cross the plasma membrane and to have an impact from the intracellular side (58). Application of CCCP before serum administration led to an immediate fall in the basal \( c_{\text{mito}} \) reaching a new plateau 1–2 min later; the fall in \( c_{\text{mito}} \) was accompanied by a visible small rise in \( c_{\text{cyt}} \) in ~20% of cells, indicative of a release of mitochondrial \( Ca^{2+} \) to the cytosolic compartment. In addition, CCCP also decreased the basal level in \( c_{\text{cyt}} \) (both at 100 and 10 \( \mu \text{M} \)), signifying that also the plasma membrane potential was affected. Serum readministration following CCCP (100 \( \mu \text{M} \)) treatment was still able to briefly elevate both \( c_{\text{cyt}} \) and \( c_{\text{mito}} \) but the increase in \( c_{\text{mito}} \) was smaller compared with cells not treated with CCCP; moreover, \( c_{\text{mito}} \) returned quickly to the level reached after CCCP addition, i.e. not to basal levels before treatment (Fig. 4D). Serum readministration after treatment with the lower CCCP concentration (10 \( \mu \text{M} \)) evoked low amplitude \( Ca^{2+} \) oscillations, and the mitochondrial \( Ca^{2+} \) rise during a cytosolic \( Ca^{2+} \) spike was small, and \( c_{\text{mito}} \) immediately returned to levels before serum administration but to lower levels than the basal \( c_{\text{mito}} \) before CCCP administration (Fig. 4E). The model also correctly predicted that the collapse in \( \Delta \Psi \) (at \( t = 3 \) min) resulted in a lower \( c_{\text{mito}} \). Serum administration (modeled as increasing \( J_{\text{IN}} \) and \( J_{\text{ER}} \)) led to an increase in \( c_{\text{cyt}} \) and \( c_{\text{mito}} \) followed by oscillations in \( c_{\text{cyt}} \) and \( c_{\text{mito}} \) (Fig. 4, compare F with the experimental recording shown in E). To provide more evidence for the presence of \( \Delta \Psi \)-independent mitochondrial \( Ca^{2+} \) uptake as shown in Fig. 4D, we induced \( Ca^{2+} \) release from the ER by thapsigargin after CCCP administration (Fig. 4G). We observed a rise not only as expected in \( c_{\text{cyt}} \) but in parallel also in \( c_{\text{mito}} \), confirming the existence of a \( \Delta \Psi \)-independent mitochondrial \( Ca^{2+} \) uptake. Addition of 1% FCS also resulted in another increase in the intracellular ATP concentration that lasted during the entire period of \( Ca^{2+} \) oscillations. Shortly after the collapse of \( \Delta \Psi \) induced by CCCP, an immediate fall in ATP levels was observed (Fig. 4H). Overall, our findings indicate that the oscillation stop induced by the protonophore CCCP is not exclusively the result of the decreased mitochondrial \( Ca^{2+} \) uptake but also mediated via CCCP-induced changes in plasmalemmal \( Ca^{2+} \) influx and decreased ATP production.

The Role of \( Ca^{2+} \) Influx on \( Ca^{2+} \) Oscillations and on Mitochondrial \( Ca^{2+} \) Handling—A decrease in extracellular [\( Ca^{2+} \)] by the addition of 0.25 \( \text{mM} \) EGTA to the extracellular solution resulted in a reduction in the oscillation frequency (Fig. 5A). In this condition, i.e. when \( c_{\text{cyt}} \) oscillations were not blocked completely, the amplitude of the \( Ca^{2+} \) signals was not affected, and the pattern of mitochondrial \( Ca^{2+} \) release/uptake was not affected (Fig. 5A). This could be accurately modeled in our simulation (Fig. 5B). When oscillations were induced by the addition of 1% FCS to the \( Ca^{2+} \)-containing recording solutions (\([Ca^{2+}]_{o} \approx 1 \) \( \text{mM} \)), decreasing \([Ca^{2+}]_{o} \) to \( <1 \) \( \mu \text{M} \) by the addition of 10 \( \mu \text{M} \) EGTA at \( t = 9 \) min resulted in an immediate stop of the oscillations, indicating the necessity of \( Ca^{2+} \) influx for the oscillations in \( c_{\text{cyt}} \) (Fig. 5C). Removal of the extracellular \( Ca^{2+} \) had no visible effect on the decay curve of \( c_{\text{mito}} \), and basal levels were reached at the end of the observation period (15 min). When the serum readministration was carried out in the “\( Ca^{2+} \)-free” condition, most prMC did not show any response in \( c_{\text{cyt}} \). In ~5% of prMC, an initial small rise in \( c_{\text{cyt}} \) was observed but without signs of \( Ca^{2+} \) oscillations in support of the hypothesis that extracellular \( Ca^{2+} \) is essential for the sustained oscillations (Fig. 5E). Interestingly, different results were obtained in the \( Ca^{2+} \)-free condition when both \( Ca^{2+} \) influx and efflux across the plasma membrane were blocked by the addition of 1 \( \text{mM} \) La\(^{3+}\), the so-called lanthanum insulation (19, 59), prior to the serum readministration. After serum addition, an immediate rise in \( c_{\text{cyt}} \) and \( c_{\text{mito}} \) was detected; although \( c_{\text{cyt}} \) decayed to basal levels within the next 2 min, \( c_{\text{mito}} \) remained elevated and did not show the typical decay curve as seen e.g. in Fig. 5A, C, and E. Moreover, long lasting but slow oscillations in \( c_{\text{cyt}} \) were observable (Fig. 5G), and at each cytosolic \( Ca^{2+} \) spike, a corresponding spike in \( c_{\text{mito}} \) occurred. This indicates that during the \( La^{3+} \) insulation a considerable amount of \( Ca^{2+} \) ions released from the ER, leading to the transient increase in \( c_{\text{cyt}} \), is taken up by mitochondria as evidenced by the mitochondrial \( Ca^{2+} \) spikes (Fig. 5G). Thus, blocking the \( Ca^{2+} \) efflux across the plasma membrane leads to a shutting off of the \( Ca^{2+} \) ions between the ER and mitochondria, leading to these slow oscillations. Of note, the mitochondria remain in a rather \( Ca^{2+} \)-loaded state because \( Ca^{2+} \) cannot be transported out of the cell. We estimate that the mitochondrial \( Ca^{2+} \) uptake and release velocities likely determine the frequency of \( Ca^{2+} \) oscillations. \( La^{3+} \)-induced blocking of the \( Ca^{2+} \) transport across the plasma membrane at a time point when serum-induced \( Ca^{2+} \) oscillations were ongoing led to a complete block of the oscillations (Fig. 5, I and K). In some cases, \( La^{3+} \) treatment caused a final longer lasting \( Ca^{2+} \) spike (Fig. 5K), whereas in other prMC, \( La^{3+} \) completely blocked any further spikes (Fig. 5F). In all cases, the mathematical model could truthfully recapitulate the experimental findings by changing the parameters \( J_{\text{IN}} \) and \( J_{\text{ER}} \) at different time points (Fig. 5, D, F, H, J, and L). Of note during \( La^{3+} \) insulations, the width (duration) of \( Ca^{2+} \) spikes was wider (longer) both \( in \text{vivo} \) and \( in \text{silico} \). Moreover, the \( La^{3+} \)-evoked oscillation block in the presence of extracellular \( Ca^{2+} \) ([\( Ca^{2+} \])_{o} \approx 1 \text{mM} \) is, according to our model, mostly due to the decreased levels of \( Ca^{2+} \) ions present in the different cell compartments; i.e. the sum of \( c_{\text{cyt}} \) + \( c_{\text{ER}} \) + \( c_{\text{mito}} \) is smaller than the sum prior to agonist administration.

Effect of the Intracellular Buffer Calretinin on \( Ca^{2+} \) Oscillations—Based on previous findings that human mesothelioma \( in \text{vivo} \), mesothelioma cells \( in \text{vitro} \), and reactive mesothelial cells express calretinin (60, 61), we hypothesized that prMC also might express this protein and that its presence might affect the \( Ca^{2+} \) oscillations. However, calretinin protein expression levels in prMC were found to be below the detection limit of our Western blot analysis (8), i.e. lower than ~100 \( \text{nm} \) and thus unlikely to affect the \( Ca^{2+} \) oscillations as the result of the \( Ca^{2+} \)-buffering capacity of calretinin. In support of this assumption, oscillation patterns (frequency, amplitude, and duration) in prMC from either wild type or calretinin knockout (CR/––) mice were indistinguishable (data not shown). However, to mimic the situation of calretinin-expressing reactive mesothelial cells and to investigate the putative role of calretinin in those cells, we overexpressed a fusion protein consisting of EBFP separated from full-length calretinin by a small linker peptide by infection of prMC with the appropriate lenti-
virus. We estimated in a semiquantitative way by Western blot analyses the expression levels of EBFP-calretinin. The expression level was found to be \( \sim 75 \) pg of EBFP-calretinin/\( \mu \)g of total protein, leading to an estimated upper concentration of 250 \( \mu \)M calretinin. The EBFP tag on calretinin served as a marker for the distinction of the two populations with or without calretinin.
Our model simulations incorporating calretinin with the protein, prMC were infected with the lentivirus LV-EBFP2-X, restriction to cells showing faint blue fluorescence, i.e. low EBFP-calretinin expression levels. In the oscillating EBFP-calretinin, the Ca\(^{2+}\) spike amplitudes were smaller, and the half-width of Ca\(^{2+}\) spikes (duration) was increased (Fig. 6B). The largest effect caused by EBFP-calretinin was the reduction of the amplitude of the first Ca\(^{2+}\) spike after serum readministration (Fig. 6C); on average it was half the size compared with the situation without calretinin. Likely as the consequence of the reduction in c\(_{\text{cyt}}\) and in c\(_{\text{mito}}\) also was clearly diminished (Fig. 6D). The frequency of oscillation slightly decreased (~10–20% reduction in each time segment).

Our model simulations incorporating calretinin with the known Ca\(^{2+}\) binding characteristics showed similar modifications: a decrease both in the amplitudes of c\(_{\text{cyt}}\) spikes and in the amount of mitochondrial Ca\(^{2+}\) uptake (Fig. 6E). In our model, an increase in calretinin concentration resulted in an increase of the oscillation frequency, a prediction not supported by our experimental findings. One reason may be that calretinin, in addition to its buffering capacity, might act as a Ca\(^{2+}\) sensor in prMC. We had previously shown that calretinin is able to directly modify the activity of a Ca\(^{2+}\) channel (60), and direct targets for calretinin implicated in Ca\(^{2+}\) transportation might also be present in prMC. As a control to exclude that observed effects were mediated by the EBFP part of the fusion protein, prMC were infected with the lentivirus LV-EBFP2-X leading to the expression of EBFP only. No differences in the Ca\(^{2+}\) oscillations patterns were observed between cells expressing EBFP and non-infected control cells (data not shown). Based on the fact that Ca\(^{2+}\) oscillations in EBFP-calretinin-expressing cells were limited to those with faint fluorescence, we reasoned that the concentration in these cells was ~10-fold (e.g. 25 \(\mu\)M) lower than the global concentration (250 \(\mu\)M) estimated from Western blot analyses. Thus, we tested whether the commonly used synthetic Ca\(^{2+}\) chelators BAPTA and EGTA, which have different properties (e.g. \(K_{\text{d}}\) on-rate constant (\(k_{\text{on}}\)), and diffusion coefficient (\(D\)) than calretinin, were able to recapitulate the effects of calretinin. In prMC loaded with BAPTA-AM (30 \(\mu\)M), serum readministration evoked a slow and prolonged \(c_{\text{cyt}}\) elevation paralleled by a minute increase in \(c_{\text{mito}}\) (Fig. 6F). Most importantly, the initial rise in \(c_{\text{cyt}}\) as also seen in EBFP-calretinin-expressing prMC (Fig. 6C) was completely abolished. In contrast, after EGTA-AM loading serum, readministration induced a short rise in \(c_{\text{cyt}}\) and \(c_{\text{mito}}\) (Fig. 6E) followed by a rapid return to essentially baseline levels. No Ca\(^{2+}\) oscillations were observed in both cases. This further indicates that the properties of calretinin are clearly distinct from those of either BAPTA or EGTA.

**Discussion**

Characteristics of mitochondrial Ca\(^{2+}\) transport have not been examined in detail in most cell types. The main reason why we know relatively little about mitochondrial Ca\(^{2+}\) handling is because the molecular identity of the channels involved in mitochondrial transport have only recently been discovered (18, 62, 63), and specifically targeted, pH- and \(\Delta\Psi\)-insensitive Ca\(^{2+}\) indicators are only currently available (23). Nevertheless, there are few models for Ca\(^{2+}\) oscillations where the function of mitochondrial Ca\(^{2+}\) uptake has been taken into account (64).

Our experiments affirm previous data that mitochondria, even at the resting state, are able to transport and store Ca\(^{2+}\) ions (65). The fast release of the stored Ca\(^{2+}\) from the mitochondria due to the decrease/collapse of the membrane potential indicates that the strongly negative \(\Delta\Psi\) ensures a constant Ca\(^{2+}\) uptake into the mitochondria. This uptake is in a steady-state equilibrium with the constant Ca\(^{2+}\) efflux mediated by the mitochondrial exchangers (66), and the efflux is an electrogenic process (67). The electrochemical proton gradient across the inner mitochondrial is used to remove the excess Ca\(^{2+}\) ions (68). Our recordings show that this basal steady-state mitochondrial Ca\(^{2+}\) concentration can fluctuate, showing “spontaneous” mitochondrial Ca\(^{2+}\) spikes. Most probably this is mediated by an endogenous MCU activator that has not been identified at the molecular level yet. Ca\(^{2+}\) transients in \(c_{\text{cyt}}\) were previously reported to evoke an increase in \(c_{\text{mito}}\) activating both cytoplasmic (19) and mitochondrial enzymes (23). Thus, Ca\(^{2+}\) transients observed selectively in \(c_{\text{mito}}\) in some prMC (Fig. 2A) might allow for the autonomous activation of mitochondrial enzymes. The Ca\(^{2+}\) ions causing the mitochondrial spike are likely to originate from the cytosolic compartment; however, our results indicate that the amount of Ca\(^{2+}\) ions responsible for the increase in \(c_{\text{mito}}\) was not sufficient to be detected as a decrease in \(c_{\text{cyt}}\). Alternatively, at basal conditions, the equilibrium level of \(c_{\text{cyt}}\) might be regulated by a rather rapid constant exchange of Ca\(^{2+}\) ions among the cytosol, the extracellular space, and/or the ER compartment.

The Ca\(^{2+}\) oscillation models usually differ in how they simulate the functions of InsP\(_3\)R, the channel that transports Ca\(^{2+}\) ions from the ER to the cytosol. The \(c_{\text{cyt}}/[\text{InsP}_3\text{R}]\) models (for a review, see Ref. 69) postulate that the InsP\(_3\)R has a binding site.

![FIGURE 5: Modulation of Ca\(^{2+}\) transport across the plasma membrane: representative experimental findings (left panels) and model simulations (right panels). A, a decrease in external Ca\(^{2+}\) from 1 to ~0.75 mM by administration of 0.25 mM EGTA results in a decreased oscillation frequency. B, the model was able to recapitulate the effect of reduction of Ca\(^{2+}\) influx on Ca\(^{2+}\) oscillations. The parameter \(r_{\text{inj}}\), representing \(J_{\text{inj}}\) was reduced from 0.85 to 0.65 mM/s at \(t = 9\) min. C, removal of extracellular Ca\(^{2+}\) by the addition of EGTA (10 mM) at \(t = 9\) min resulted in one final Ca\(^{2+}\) spike before cessation of oscillations. D, the model correctly predicted an immediate stop in Ca\(^{2+}\) oscillations when \(J_{\text{inj}}\) was decreased to zero. E, in the absence of extracellular Ca\(^{2+}\) (\(J_{\text{ex}}< 1 \mu\)M), prMC did not show Ca\(^{2+}\) oscillations. In a few cells, a single Ca\(^{2+}\) transient was visible. F, model traces for \(c_{\text{cyt}}\) (green) and \(c_{\text{mito}}\) (red) in zero Ca\(^{2+}\) influx (\(J_{\text{inj}} = 0\) confirmed the experimental findings shown in E. G, addition of lanthanum chloride (La\(^{3+}\) insul.) prior to serum administration rendered Ca\(^{2+}\) oscillations independent of extracellular Ca\(^{2+}\) ions. Note that (i) \(c_{\text{mito}}\) remained elevated during the entire period (no slow decay phase), (ii) the frequency of Ca\(^{2+}\) oscillations was lower than in control conditions (e.g. as shown in the initial period in A or C), and (iii) the half-width of Ca\(^{2+}\) transients representing the duration of a Ca\(^{2+}\) spike was increased. H, the model confirmed that mitochondria were able to substitute for the role of the extracellular Ca\(^{2+}\) reservoir during Ca\(^{2+}\) oscillations. In line with the experimental findings, the half-width of Ca\(^{2+}\) spikes was increased, and \(c_{\text{mito}}\) remained elevated. I, La\(^{3+}\) insul. induced oscillations after serum administration (at \(t = 8–10\) min) blocked the Ca\(^{2+}\) oscillations either with (K) or without (\(J\)) a final large Ca\(^{2+}\) transient. J-L, the model was able to recapitulate both phenomena: it revealed that \(c_{\text{total}}\) i.e. the total amount of Ca\(^{2+}\) ions in the cell \((c_{\text{mito}} + c_{\text{cyt}} + c_{\text{eql}})\), determined the response to La\(^{3+}\) insul.
for InsP₃, an activating binding site for Ca²⁺, and an inhibiting binding site for Ca²⁺. In these models, all binding sites are localized on the cytoplasmic side, and the function of InsP₃ does not depend on cER. Binding of Ca²⁺ to the activating site and of InsP₃ to the inhibiting site opens the channel, whereas Ca²⁺ binding to the inhibiting site closes the InP₃R. Moreover, the binding of Ca²⁺ to the inhibiting site occurs rather slowly and with a lower affinity as compared with the activating site, subsequently resulting in oscillations in cCyt. In these models, the InsP₃ concentration uniquely determines the oscillation frequency (70). In the “store loading” models (also called “cCyt/cER” models), the function of InsP₃R depends not only on cCyt but also on cER. In these models, the Ca²⁺ influx across the plasma membrane plays a critical role in determining

**FIGURE 6.** The effect of increased mobile cytosolic Ca²⁺-buffering capacity resulting from ectopic EBFP-calretinin expression on Ca²⁺ oscillations. A, fluorescence image of cultured prMC expressing the fusion protein EBFP-calretinin (blue). The faint green and red fluorescence signals represent the basal fluorescence intensities of GCaMP3 (cytosolic Ca²⁺ indicator) and mito-CAR-GECO1 (mitochondrial Ca²⁺ indicator), respectively. The faintly fluorescent non-blue cells are control cells not expressing EBFP-calretinin. The scale bar represents 250 μm. B, single cell fluorescence intensity recordings from time lapse videos show a representative Ca²⁺ response in cCyt and cMito of cells expressing EBFP-calretinin. Oscillations were only observed in cells with faint blue fluorescence, i.e., relatively low EBFP-calretinin expression levels. Note the relatively small signal in cCyt as compared with control cells (e.g., in Figs. 2C and 4B). C, Ca²⁺ oscillations were monitored in a mixed population of mito-CAR-GECO1/GCAMP3 prMC (green trace) and EBFP-calretinin/mito-CAR-GECO1/GCAMP3 prMC (black trace). Averages of cCyt values of 10 randomly selected oscillatory prMC each were plotted; data are mean ± S.D. Serum-induced maximal amplitudes in cCyt were considerably lower in EBFP-calretinin-expressing prMC, data are mean ± S.D. from eight cells each. Only a few cells (<1%) showed a second Ca²⁺ transient, but oscillations in cCyt were completely blocked by 30 μM EGTA. F and G, averages of cCyt and cMito values of 10 randomly selected prMC were plotted; data are mean ± S.D. Asterisks indicate significance at p < 0.05. Error bars represent S.D. CTR, control.
the oscillation frequency (8, 71, 72). At a constant [InoP]3, the duration of the interspike period is determined by the velocity of cellular Ca2+ replenishment, which is manifested as a continuous ER loading together with a constant basal $c_{\text{cyt}}$. The experimentally observable sawtooth wave oscillations in $c_{\text{ER}}$ during the cytoplasmic baseline spiking oscillations are an important argument in favor of the store loading theory (8). However, the store loading-based models cannot cope with the fact that in some cells the Ca2+ oscillations do not depend on Ca2+ influx across the plasma membrane. Our experiments and modeling studies revealed that the incorporation of mitochondria as an additional Ca2+ source/store in the store loading-based models considerably augments the quality of the simulations. That is, the modeling predictions are more congruent with the experimental findings, which allows for a better mechanistic understanding. The mitochondrial Ca2+ transport enables the store loading-based models also to display Ca2+ oscillation in the absence of extracellular Ca2+.

The simulation of the La3+ insulation was previously endeavored by Sneyd et al. (73). Although their model does not contain mitochondria and moreover $c_{\text{cyt}}$ is continuously decreasing during the oscillations, their model reveals important aspects of the Ca2+ oscillations, namely their dependence on the total Ca2+ load of the cell. In their model, the cell has a high resting Ca2+; upon agonist stimulation, the activation of plasma membrane Ca2+-ATPases causes a net loss of Ca2+ from the cells even though the Ca2+ influx is augmented after stimulation (73). A similar phenomenon is also observed in our model; the total cellular Ca2+ content ($c_{\text{cyt}} + c_{\text{ER}} + c_{\text{mito}}$) determines the response to the La3+ insulation; blocking of the Ca2+ influx and efflux results in an oscillation stop that can either occur after a final Ca2+ spike or directly after La3+ addition, i.e. without a change in $c_{\text{cyt}}$. In contrast to the previous model (73), basal $c_{\text{cyt}}$ levels during the interspike phase of the oscillations remain constant. This is in line with the experiments carried out by us and others (74).

Shuttling of Ca2+ ions between the ER and mitochondria was experimentally demonstrated in the study of Ishii et al. (9). They reported that in HeLa cells the cycles of ER/mitochondrion shuttling are repeated until $c_{\text{mito}}$ has reached the basal level prior to the stimulation. In our study with prMC, we observed Ca2+ oscillation even (i) when $c_{\text{mito}}$ had reached its basal levels or (ii) if $c_{\text{mito}}$ had been considerably lowered by CCCP administration. One has to keep in mind that CCCP also results in the collapse of the plasma membrane potential (75), which subsequently reduces the plasmalemmal Ca2+ influx (76). Thus, one reason for the CCCP-evoked stop in oscillations might be a disturbed Ca2+ influx. Moreover, the CCCP-mediated drop in ATP production likely leading to an impairment of the ER Ca2+ transport might also contribute to the oscillation arrest (77); i.e. the effects of protonophores are not exclusively attributed to the reduced mitochondrial Ca2+ uptake as was proposed in earlier studies (9). When CCCP was administered before serum, it caused a Ca2+ transient due to the mitochondrial release, which was followed by a period of lower resting $c_{\text{cyt}}$. A lower $c_{\text{cyt}}$ is a sign of the reduced Ca2+ influx (resting plasmalemmal Ca2+ leakage). There was a similar decrease in resting $c_{\text{cyt}}$ when the extracellular free Ca2+ was chelated by EGTA (data not shown).

The Ca2+ influx across the plasma membrane is important to sustain the Ca2+ oscillations in prMC (78) but not in HeLa cells (9). The different dependence of these cell types on extracellular Ca2+ for the oscillations might be the result of differences in the contribution/importance of the various Ca2+ shuttling pathways between ER and mitochondria on the one hand and between ER and the extracellular space on the other. Our results indicate that plasmalemmal Ca2+ extrusion systems and mitochondrial Ca2+ uptake channels compete for the Ca2+ ions released from the ER. We hypothesize that in some cells, such as prMC and HEK cells (74), the shuttling between the extracellular space and the ER dominates over the shuttling between mitochondria and the ER. However, in HeLa cells and hepatocytes, the ER/mitochondrion shuttling prevails. This might explain why Ca2+ oscillations in some cells are strongly dependent on extracellular Ca2+ ions but not in others.

Another often neglected aspect about “Ca2+ shuttling” pathways is the contribution of cytoplasmic Ca2+ buffers present at rather high concentrations in the cytosol of some cell types. They are expected to modulate the Ca2+ shuttling among all compartments, extracellular space, ER, and mitochondria, as well as to transiently affect $c_{\text{cyt}}$ (Fig. 7). A strong interdependence between cytoplasmic Ca2+ buffers and mitochondria has
been demonstrated before. The expression levels of parvalbumin, a Ca\(^{2+}\)-buffering protein with slow binding kinetics, and the mitochondrial volume in fast twitch muscle cells and in parvalbumin-expressing neurons are inversely regulated (Ref. 79, and for more details, see Ref. 80). In our study, we observed that overexpression of calretinin modifies Ca\(^{2+}\) signals and associated oscillations. It reduces the amount of Ca\(^{2+}\) ions shuttling both between the ER and mitochondria and between the ER and the cytoplasm. Our model predicts that at calretinin concentrations >1 \(\mu\)M Ca\(^{2+}\) oscillations should be blocked in prMC. This is in apparent contradiction with the experimental results where oscillations still existed in EBFP-calretinin-expressing cells likely expressing levels higher than 1 \(\mu\)M (Fig. 6). However, in our modeling, the Ca\(^{2+}\) microdomain was not considered, and Ca\(^{2+}\) binding characteristics of calretinin (e.g. \(K_d\) and \(k_{\text{on}}\)) might be different in the cytosol of prMC than the parameters determined \(in\ vitro\) (34). Furthermore, adaptation/compensation mechanisms might be induced in prMC overexpressing calretinin that would still allow for the generation of Ca\(^{2+}\) oscillations.

Of relevance, calretinin reduced the mitochondrial Ca\(^{2+}\) uptake and Ca\(^{2+}\) accumulation in human malignant mesothelioma, mostly of the epithelioid type, calretinin is overexpressed (81). This might cause changes, e.g. a delay or blocking of apoptotic/necrotic processes (78, 82). Thus, the increased calretinin expression in mesothelioma cells and moreover in certain colon cancer (83) and derived cell lines (84) might be correlated or causally linked to the increased resistance of these tumor cells to the apoptotic/necrotic signals either occurring in healthy physiological conditions or resulting from treatment with chemotherapy drugs such as oxaliplatin or 5-fluorouracil (85). In support, colon cancer cells resistant to aurora kinase inhibitors are characterized by higher calretinin expression levels (86). Moreover, down-regulation of calretinin by lentiviral infection induces apoptosis in mesothelioma cell lines \(in\ vitro\) via an intrinsic mitochondrion-mediated pathway (87). Also down-regulation of calretinin in colon cancer cells is associated with cell growth arrest and increased apoptosis (88).

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Ca²⁺ Shuttling during Ca²⁺ Oscillations in Mesothelial Cells

Ca²⁺ shuttling during Ca²⁺ oscillations in mesothelial cells is a critical process for maintaining cell homeostasis. This process involves the coordinated movement of calcium ions between cellular compartments, such as the endoplasmic reticulum (ER) and the cytosol, to regulate various cellular functions. Understanding the mechanisms behind this process is essential for the study of cellular physiology and the development of therapeutic strategies for diseases associated with calcium dysregulation.

Key points:
1. **Ca²⁺ Oscillations**: Ca²⁺ oscillations are periodic increases and decreases in intracellular Ca²⁺ concentration that are crucial for cellular functions such as muscle contraction, neurotransmitter release, and gene expression.
2. **Ca²⁺ Shuttling**: Ca²⁺ shuttling refers to the movement of calcium ions between different cellular compartments, primarily between the ER and the cytosol, to maintain dynamic Ca²⁺ oscillations.
3. **Signaling Pathways**: The signaling pathways that control Ca²⁺ shuttling are complex and involve various regulators such as inositol 1,4,5-trisphosphate (InsP³) receptors and the sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase (SERCA).
4. **Mitochondria and Ca²⁺**: Mitochondria play a significant role in Ca²⁺ regulation, serving as a calcium buffer and contributing to the oscillatory profile of Ca²⁺.
5. **Calcium-Dependent Proteins**: Calcium-dependent proteins, such as calretinin and calbindin, help in the efficient shuttling of calcium ions.

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