Characterization and modeling of Ca\(^{2+}\) oscillations in mouse primary mesothelial cells

László Peczé, Beat Schwaller

Anatomy, Department of Medicine, University of Fribourg, Route Albert-Gockel 1, CH-1700 Fribourg, Switzerland

1. Introduction

Mesoderm-derived mesothelial cells cover the body's serous cavities (pleural, pericardial and peritoneal) and internal organs. They have a flattened, simple squamous epithelial morphology possessing microvilli and cilia on their luminal surface and express both mesenchymal and epithelial cell intermediate filaments. Under normal physiological conditions, the mesothelium is a slowly renewing tissue with 0.16–0.5% of cells undergoing mitosis, i.e. most cells are in a quiescent (G0) state [1]. Mesothelial cells secrete glycosaminoglycans, proteoglycans and phospholipids forming the lubricant that reduces the friction between organs and serosal surfaces. The cells are also involved in transport across serosal cavities, antigen presentation, inflammation and tissue repair, coagulation and fibrinolysis [1,2].

Only few studies have investigated Ca\(^{2+}\) signals in primary mesothelial cells (prMC). Such transient Ca\(^{2+}\) signals were observed after acute acidification [3], histamine administration [4], serum re-administration [4], ryanodine treatment [5], platelet-derived growth factor administration [6] and K\(^{+}\)-induced membrane depolarization [5]. However, Ca\(^{2+}\) signals in the form of oscillations have not been reported previously.

Brief changes in the cytosolic and intra-organellar Ca\(^{2+}\) concentration serve as specific signals for various physiological processes. In mesothelial cells lining the surface of internal organs and the walls of body cavities, a re-entry in the cell cycle (G0-G1 transition) evoked by serum re-administration induces long-lasting Ca\(^{2+}\) oscillations with a slowly decreasing frequency. Individual mesothelial cells show a wide range of different oscillatory patterns within a single, supposedly homogenous cell population. Changes in the cytoplasmic Ca\(^{2+}\) concentration (c\(_{cyt}\)) show baseline oscillatory patterns i.e., discrete Ca\(^{2+}\) transients starting from a constant basal c\(_{cyt}\) level. The ER Ca\(^{2+}\) concentration (c\(_{ER}\)) displays a sawtooth wave at a semi-depleted ER state; the minimum level is reached just briefly after the maximal value for c\(_{cyt}\). These oscillations depend on plasmalemmal Ca\(^{2+}\) influx and on the inositol trisphosphate concentration [InsP\(_3\)]; the Ca\(^{2+}\) influx is a crucial determinant of the oscillation frequency. Partial blocking of SERCA pumps modifies the oscillation frequency in both directions, i.e. increasing it in some cells and lowering it in others. Current mathematical models for Ca\(^{2+}\) oscillations mostly fail to reproduce two experimentally observed phenomena: the broad range of interspike intervals and constant basal c\(_{cyt}\) levels between two Ca\(^{2+}\) spikes. Here we developed a new model based on – and fitted to – Ca\(^{2+}\) recordings of c\(_{cyt}\) and c\(_{ER}\) recorded in primary mouse mesothelial cells. The model allowed for explaining many features of experimentally observed Ca\(^{2+}\) oscillations. We consider this model to be suitable to simulate various types of InsP\(_3\) receptor-based baseline Ca\(^{2+}\) oscillations.

When cells transit from a quiescent state (G0) and re-enter the cell cycle during tissue remodeling and repair [7], this transition is generally associated with Ca\(^{2+}\) signals, most often in the form of oscillations [8,9]. Depending on the shape and length of these oscillations, different types can be distinguished including sinusoidal and baseline spiking oscillations. The baseline spiking oscillations represent discrete Ca\(^{2+}\) transients starting from a constant basal c\(_{cyt}\) level. Sinusoidal oscillation is a term for a continuous fluctuation in the cytoplasmic Ca\(^{2+}\) concentration (c\(_{cyt}\)) starting from a c\(_{cyt}\) value that is higher than the resting c\(_{cyt}\). Most probably, sinusoidal oscillations are the result of high-frequency overlapping baseline spiking oscillations [10]. The resting c\(_{cyt}\) level represents c\(_{cyt}\) before the administration of an inducer of oscillations. The basal c\(_{cyt}\) denotes a constant c\(_{cyt}\) level observable during the interspike period; the resting and the basal c\(_{cyt}\) may be different, most often basal c\(_{cyt}\) is lower than resting c\(_{cyt}\) in mesothelial cells. A G0-G1 cell cycle transition with the associated Ca\(^{2+}\) transients can be mimicked in vitro by serum deprivation followed by serum re-administration. Serum contains a plethora of known and as-yet-unidentified growth factors and mitogenic compounds [11]. These factors promote the cell to pass from G0 through G1 to the DNA synthesis (S) phase. The stimulation of DNA synthesis usually requires the simultaneous action of at least two growth factors or mitogens. In Swiss 3T3 fibroblasts, the epidermal growth factor (EGF) and insulin are the best-characterized combination...
Studies revealed that the separate administration of either platelet-derived growth factor, vasopressin, prostat glandulin, bombesin or EGF evokes Ca\(^{2+}\) transients and also induces inositol trisphosphate (InsP\(_3\)) production [13,14]. In Swiss 3T3 cells, serum-induced Ca\(^{2+}\) increases are essential, but not sufficient to induce NF-κB activation and subsequent DNA synthesis [15]. Hormone receptors, for example EGF receptor, activate phospholipase C [16] that causes the hydrolysis of phosphatidylinositol bisphosphate to InsP\(_3\) and diacylglycerol (DAG) [17]. The molecular identity of the ion channels mediating hormone-induced Ca\(^{2+}\) entry in mesothelial cells is still unknown. Most probably transient receptor potential canonical (TRPC) channels are involved, because these channels are activated by DAG [18]. On the other hand, a drop in the Ca\(^{2+}\) concentration in the lumen of the ER (cER) after hormonal stimulation activates store-operated Ca\(^{2+}\) channels (SOC). STIM molecules function as Ca\(^{2+}\) sensors in the ER membrane, interact with and activate plasmalemmal Orai Ca\(^{2+}\) channels [19]. Both InsP\(_3\) production and Ca\(^{2+}\) influx play an essential role in hormone-induced Ca\(^{2+}\) oscillations examined in many non-excitable cell types, for instance, in microvascular endothelial cells [20], in astrocytes [21] and in pre-adipocytes [22].

However, despite the effort made to understand the mechanism of Ca\(^{2+}\) oscillations, there is still a lot of debate and controversy. First of all, there is no mathematical model, which can recapitulate the various patterns of Ca\(^{2+}\) oscillations observable even in a homogenous population of cells growing in cell culture. Here, we investigated Ca\(^{2+}\) oscillations in mouse peritoneal mesothelial cells (MPMC) and partially blocking the function of sarcoendoplasmic reticulum Ca\(^{2+}\)-ATPases (SERCA)

2. Material and methods

2.1. Reagents

Thapsigargin (THAPS), carbonoxeno, N-methyl-D-glucamine and EGTA were purchased from Sigma Aldrich (St. Louis, MO, USA). Ionomycin (IONO), SKF 96365 hydrochloride, ryanodine, ML-9, U73122, BAPTA-AM were obtained from Tocris Bioscience (Bristol, UK) and Ru360 from Calbiochem (San Diego, CA).

2.2. Primary mesothelial cell isolation

Mesothelial cells were isolated from 4–6 months old C57BL/6j mice (C57) according to an established protocol [23]. Briefly, mice were sacrificed by cervical dislocation. The abdominal wall was exposed by incision into the fur and removal of it. The peritoneal cavities were washed with injecting approximately 50 ml of PBS (Sigma, St. Louis) via a 25G needle using a peristaltic pump and a second 21G needle to allow exit of the PBS solution until it was clear, i.e. devoid of mobile and poorly attached cells. Residual PBS was aspirated with a syringe and the peritoneal cavity was filled with 5 ml of 0.25% Trypsin/EDTA solution (Sigma Aldrich). The body temperature of mouse corpses was measured after 4 h and 72 h, filtered, aliquoted and frozen at −80 °C [29].

2.3. Plasmids and lentiviral infection

For the generation of primary mesothelial cell lines stably expressing the Ca\(^{2+}\) indicator proteins, GCaMP3 (Addgene plasmid 22692 [25]), GEM-GECO1 (Addgene Plasmid 32442 [26]) and D1ER (Addgene 36325 [27]), the lentiviral expression vector pLVTHM (Addgene plasmid 12247 [28]) was used. The GFP cassette in pLVTHM was replaced with cDNAs coding for the respective Ca\(^{2+}\) indicator proteins. Briefly, pCAMP3 was grown in SC510 dam− bacteria, digested with Afill and Xbal and the fragment was inserted into the compatible pMEL and SpeI sites of the backbone of pLVTHM to produce the final pLV-GCaMP3. The pCMV-GEM-GECO1 was digested with BamHI, filled with Klenow enzyme, and then digested with XbaI. The cDNA insert was ligated into pLVTHM using the compatible Pmel and SpeI sites. The plasmid pcDNA-D1ER was grown in SC511 dam− bacteria, digested with HindIII, filled with Klenow enzyme, digested with XbaI and ligated into the compatible pMEL and SpeI sites of pLVTHM. All lentiviral plasmids were verified by restriction enzyme digestion. Lentivirus was produced by the calcium phosphate transfection method using HEK293T cells and three plasmids: one of the expression plasmids (pLV-GCaMP3, pLV-GEMGECO1, pLV-D1ER), the envelope plasmid (pMD2G/VSVG Addgene plasmid 12259) and the packaging plasmid (pCMV-ΔR8.91, a kind gift from Prof. D. Trono (EPFL, Lausanne)). Viral supernatants were collected after 48 h and 72 h, filtered, aliquoted and frozen at −80 °C.

2.4. Calcium imaging

Mesothelial cells were grown on collagen-coated glass bottom 35 mm dishes (MatTek Corp., Ashland, MA). The buffer solution (DBPS) used for Ca\(^{2+}\) imaging experiments contained (in mM): NaCl 138, Na2HPO4 8, CaCl2 2, MgCl2 0.5, KCl 2.7, KH2PO4 1.6, and pH 7.4. In the low Ca\(^{2+}\) solution, CaCl2 was replaced with an equimolar concentration of NaCl. In the low Na+ solution, NaCl and Na2HPO4 were replaced with an equimolar concentration of Na-methyl-D-glucamine. The drugs (THAPS, FCS, IONO, Pyr3, SKF 96365, ryanodine, ML-9) were added to the above-mentioned solutions and remained in the solution until the end of the experiments. In some experiments, cells were pre-treated either with 250 μM carbonoxenol or with 10 μM Ru360 for 30 min at 37 °C.

We used an inverted confocal microscope DMI6000 integrated to a Leica TCS-SP5 workstation to examine fluorescence signals indirectly reporting cyt or cER. The following excitation wavelengths were used to illuminate the fluorophores: 488 nm for GCaMP3, 404 nm for GEM-GEO1 and 455 nm for D1ER. Fluorescence emissions were recorded with a 20× objective and bandpass filters of 505–550 nm for GCaMP3, 425–490 nm simultaneously with 520–575 nm for GEM-GEO1 and 460–508 nm simultaneously with 515–565 nm for D1ER. Fluorescence images for cyt or cER measurements were collected every 3 s. Circular-shaped regions of interest (ROI) were placed inside the cytoplasmic area of cells. The fluorescence values were calculated after background subtraction (fluorescence intensity values were normalized in each circular region). The values were normalized in each circular region. The fluorescence images for cyt or cER measurements were collected every 3 s. Circular-shaped regions of interest (ROI) were placed inside the cytoplasmic area of cells. The fluorescence values were calculated after background subtraction (fluorescence intensity values were normalized in each circular region). The values were normalized in each circular region.

The absolute cyt was calculated using the fluorescent Ca\(^{2+}\) indicator GEM-GEO1. GEM-GECO1 ratios were converted to Ca\(^{2+}\) concentrations using the equation:

\[
\text{c}_{\text{cyt}} = \left( \frac{K_d}{R_{\text{max}}} \right)^{1/H} \left( \frac{R}{R_{\text{max}} - R} \right) \left( R_{\text{max}} - R \right)
\]

where \(K_d\) is the apparent dissociation constant (\(K_d = 340 \text{ nM}\)) and \(H\) is the Hill coefficient (\(H = 2.94\)) [26]; \(R_{\text{max}}\) is the maximal fluorescence
ratio obtained after ionomycin (30 μM) treatment ([Ca\textsuperscript{2+}], \approx 1 mM).  
\( R_{\text{min}} \) is the experimental minimal fluorescence ratio obtained during ionomycin (10 μM) treatment in an extracellular solution containing 50 mM EGTA.

The absolute \( c_{\text{ER}} \) was calculated using the FRET-based Ca\textsuperscript{2+} indicator D1ER. FRET ratios were converted to Ca\textsuperscript{2+} concentrations using the equation:

\[
\frac{c_{\text{ER}}}{K_0} = (F - F_{\text{min}})/(F_{\text{max}} - F) \text{M}^{-1} \text{H}^{-1} 
\]

where \( K_0 \) is the low affinity dissociation constant (\( K_0 = 200 \text{μM} \)), \( H \) is the Hill coefficient (\( H = 1.67 \)), \( F \) is the YFP/CFP ratio, \( F_{\text{max}} \) is the minimal ratio after the treatment of ionomycin (1 μM) and EGTA (50 mM). The D1ER Ca\textsuperscript{2+} indicator has biphasic Ca\textsuperscript{2+}-binding kinetics with \( K_0 \) values of 0.81 and 69 μM, and Hill coefficients of 1.18 and 1.67, respectively based on in vitro calibration data [27]. Based on the assumption that \( c_{\text{ER}} \) is not expected to fall below 1 μM, we ignored the high affinity \( K_H \) of D1ER, in line with previous studies, e.g. [30]. Furthermore, we chose to use the in vivo measured value for the low affinity \( K_0 \) [30], which is three-fold higher than the one calculated in vitro [27]. The noise was reduced using a rolling average method with a window size of 3. The LAS-AF (Leica) and Prism5 (GraphPad Software, Inc., San Diego, CA) software were used for data analysis.

2.5. Frequency and amplitude scan

Computerized peak recognition for frequency and amplitude scans was achieved by using the Microsoft Excel 2010 environment. Normalized recordings from 100 oscillating GCaMP3-expressing prMC were evaluated. A peak was recognized and the number 1 was assigned, if the respective relative fluorescence value was higher than that of the neighboring values. In order to avoid the peaks produced by noise, this value needed to be higher than the average of the surrounding 10 values. In all other cases, the number 0 was assigned. This method allowed us to separate the real Ca\textsuperscript{2+} spikes from small peaks derived from noise. Summing up the assigned numbers during a time interval (30 s) allowed for measuring the number of spikes and calculating the frequency scan. To perform the amplitude scans, the relative fluorescence values were assigned to each spike in a certain time interval (30 s). Then, the average peak amplitude was calculated.

2.6. Building up the model

To build up the mathematical model, we considered only three compartments: the extracellular space, the cytoplasm and the ER lumen. Endoplasmic reticulum–plasma membrane junctions ensure that the functional unit components (Ca\textsuperscript{2+} channels and pumps) are concentrated spatially in a very small space [31]. We assume that changes in \( c_{\text{cyt}} \) or \( c_{\text{ER}} \) of the entire cell are similar to that of individual units, i.e. spatially homogenous. In our view, this simplification is acceptable, since the oscillations are slow and the cell size is small. In this case the spatial diffusion of Ca\textsuperscript{2+} will equilibrate the spatial differences and thus synchronizes the functions of individual functional units [32]. 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 [33]. However, because the Ca\textsuperscript{2+} wave not only depends on Ca\textsuperscript{2+} diffusion, but also on the action of Ca\textsuperscript{2+} pumps and channels the Ca\textsuperscript{2+} wave is approximately 10 times slower [34]. At higher frequencies the synchronization between the individual oscillatory units is lost producing overlapping waves and finally irregular Ca\textsuperscript{2+} waves in the cell [35].

Ca\textsuperscript{2+} transport across the plasma membrane was defined as IN and EFF, and the transports across the ER membrane were termed SERCA and ERLEAK, respectively. IN includes Ca\textsuperscript{2+} channels in the plasma membrane: e.g. voltage-gated Ca\textsuperscript{2+} channels (VGCC), transient receptor potential channels (TRP channels), SOC (store-operated channels), P2X purinoreceptors, hyperpolarization-activated cyclic nucleotide-gated channels (HCN channels), etc. The EFF represents the pumps involved in Ca\textsuperscript{2+} extrusion: PMCA (plasma membrane Ca\textsuperscript{2+} ATPases) and NCXs (Na\textsuperscript{+}–Ca\textsuperscript{2+} exchangers). The SERCA pumps transport Ca\textsuperscript{2+} from the cytoplasm to the ER, while the ERLEAK represents the ER channels involved in emptying the ER: RyR (ryanodine receptors) and InsP\textsubscript{3}Rs (inositol trisphosphate receptors) (Fig. 1).

We denote by \( c_{\text{cyt}} \) the Ca\textsuperscript{2+} concentration (in nM) in the cytosol and by \( c_{\text{ER}} \) the one in the lumen of the ER. The equations for the model are:

\[
\frac{dc_{\text{cyt}}}{dt} = J_{\text{IN}} - J_{\text{EFF}} - J_{\text{SERCA}} + J_{\text{EREFF}} + J_{\text{ERLEAK}} 
\]

\[
\frac{dc_{\text{ER}}}{dt} = \gamma (J_{\text{SERCA}} - J_{\text{EFF}} - J_{\text{ERLEAK}}) 
\]

where \( J_{\text{IN}} \) is the amount of Ca\textsuperscript{2+} entering the cell, \( J_{\text{SERCA}} \) is the Ca\textsuperscript{2+} quantity pumped out of the cell, \( J_{\text{EREFF}} \), denotes the amount of Ca\textsuperscript{2+} pumped from the cytosol to ER, \( J_{\text{ERLEAK}} \), the flux of Ca\textsuperscript{2+} passing from the ER to the cytosol and finally \( J_{\text{ERLEAK}} \), represents a small flux of Ca\textsuperscript{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\textsuperscript{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 from the different fraction of free and protein-bound Ca\textsuperscript{2+} in these compartments:

\[
\gamma = V_{\text{cyt}} \cdot f_{\text{ER}} / V_{\text{ER}} \cdot f_{\text{cyt}} 
\]

where, \( V_{\text{cyt}} \) is the effective volume of the cytoplasm, \( V_{\text{ER}} \) is the effective volume of the ER, \( f_{\text{ER}} \) and \( f_{\text{cyt}} \), are the fractions of free Ca\textsuperscript{2+} in cytoplasm and ER [36]. The value of the \( \gamma \) parameter was estimated experimentally. The Ca\textsuperscript{2+} peak in the cytoplasm evoked by ionomycin is mainly due to the depletion of ER stores. Ionomycin depletes approximately 80–90% of the ER Ca\textsuperscript{2+}. This causes an elevation in \( c_{\text{ER}} \). Thus, the \( \gamma \) parameter can be estimated according to the following equation:

\[
\gamma = \left( c_{\text{ERb}} - c_{\text{ERa}} \right) / \left( c_{\text{cytm}} - c_{\text{cytb}} \right) 
\]

where \( c_{\text{ERa}} \) is the \( c_{\text{ER}} \) before ionomycin treatment, \( c_{\text{ERb}} \) is the \( c_{\text{ER}} \) after ionomycin treatment, \( c_{\text{cytm}} \) is the maximal \( c_{\text{cyt}} \) after ionomycin treatment, \( c_{\text{cytb}} \) is the \( c_{\text{cyt}} \) before ionomycin treatment (all values in nM). However, the measured \( c_{\text{cytm}} \) is influenced by the Ca\textsuperscript{2+} extrusion system (EFF) resulting in a slight overestimation of the \( \gamma \) value.

Fig. 1. Schematic model of cellular compartments implicated in Ca\textsuperscript{2+} oscillations in mouse primary mesothelial cells. The plasma membrane contains components responsible for Ca\textsuperscript{2+} influx (IN) and efflux (EFF). The relevant intracellular Ca\textsuperscript{2+} release (EREFF) and uptake (SERCA) systems are localized in the ER membranes. Values of Ca\textsuperscript{2+} concentrations in the extracellular space, the cytoplasm and in the ER lumen are given. A small constant leak (ERLEAK) occurs independently of Ca\textsuperscript{2+} channels.
The quantity of Ca\(^{2+}\) pumped out the cell through the plasma membrane increases as a function of the Ca\(^{2+}\) concentration in the cytosol. Although the individual components (NCXs and PMCs) of extrusion systems are usually modeled by Hill equations [37], the overall flux can be simulated by a simple linear equation [38] based on the experimental results of Herrington et al. [39]. The advantage of the linear flux simulation is that it does not have a saturation concentration. The different \(K_d\) values of individual components (PMCs and NCXs) ensure that the extrusion flux will never reach its saturation point in the range of biologically relevant values of \(c_{cyt}\).

\[
J_{EF} = \left\{ \begin{array}{ll}
0 & r_1 \cdot c_{cyt} - r_2 \leq 0 \\
r_1 \cdot c_{cyt} - r_2, r_1 \cdot c_{cyt} - r_2 > 0
\end{array} \right.
\] (7)

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

SERCA pumps the Ca\(^{2+}\) ions from the cytosol to ER. The quantity of the transported Ca\(^{2+}\) ions depends on \(c_{cyt}\) levels. We assume a linear relationship, because the ER influx is also composed of different SERCA pumps with different \(K_d\) values [40]. Nevertheless, our model can also work when EFF or/and SERCA fluxes were simulated with the conventional Hill equations. The rather limited number of experimental data [41] shows that SERCA function depends not only on \(c_{cyt}\), but also on \(c_{ER}\). This is likely the case for a resting state, when the \(c_{ER}\) is high, however during the Ca\(^{2+}\) oscillations the ER is in a semi-depleted state. Because in this semi-depleted state \(c_{ER}\) has essentially no or only a minimal influence on SERCA activity [41], the SERCA dependence on \(c_{ER}\) was not implemented into the model.

\[
J_{SERCA} = \left\{ \begin{array}{ll}
0 & r_1 \cdot c_{cyt} - r_2 \leq 0 \\
r_1 \cdot c_{cyt} - r_2, r_1 \cdot c_{cyt} - r_2 > 0
\end{array} \right.
\] (8)

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

Ca\(^{2+}\) ions are released from the ER to the cytosol through InsP3R and RyR. Because we found experimentally that RyR does not play a role in serum-induced oscillations in mesothelial cells similarly to other non-excitable cells [42], we focused on InsP3R. In our model, InsP3Rs are influenced both by \(c_{cyt}\) and by \(c_{ER}\), however without an allosteric regulation between the two. InsP3Rs have Ca\(^{2+}\) binding sites not only on the cytoplasmic side, but also on the luminal side [43]. Experimental data show that an increase in [InsP3] causes a significant Ca\(^{2+}\) release from the ER in the absence of cytosolic Ca\(^{2+}\) ([\(c_{cyt}\) = 0] [44]. Moreover, the effects of luminal Ca\(^{2+}\) do not affect the cytosolic binding sites [45,46]. Therefore we modeled InsP3R function as the sum of two individual contributions:

\[
J_{ERFLUX} = J_{CYTYP} + J_{ERFLUX} - J_{CYTYP} + J_{ERFLUX} > 0
\] (9)

where

\[
J_{CYTYP} = r_{1_{max}} \exp(-\left(\log(c_{cyt}) - \mu\right)^2/\alpha^2)
\]

\[
J_{ERFLUX} = r_{2_1} \log(c_{ER}) - r_{2_2}
\]

with positive constants \(\alpha\) and \(r_{2_1}\).

We introduced the dependence of InsP3R on the InsP3 concentration ([InsP3]), which has an influence both on \(J_{CYTYP}\) and on \(J_{ERFLUX}\). According to the experimental data from several studies [47–49], elevating [InsP3] mainly changes the mean and the maximum (\(\mu\), \(r_{1_{max}}\)) of the bell-shaped curve of \(c_{cyt}\) dependence. Nevertheless, based on the experimental data presented in [50,51], elevating [InsP3] also has an effect on the loading of the ER. Increased [InsP3] reduces the amount of the stored Ca\(^{2+}\) ions. We simulated this effect by changing the \(r_{2_2}\) parameter:

\[
\mu = \mu_{min} + (\mu_{max} - \mu_{min}) \frac{K_d}{K_d + [InsP3]}
\] (12)

\[
r_{1_{max}} = r_{1_{min}} + (r_{1_{min}} - r_{1_{max}}) \frac{K_d}{K_d + [InsP3]}
\] (13)

\[
r_{2_2} = r_{2_{min}} + (r_{2_{min}} - r_{2_{max}}) \frac{K_d}{K_d + [InsP3]}
\] (14)

where \(K_d\) is the half-saturation constant of InsP3R for InsP3 and [\(c_{cyt}\)]. The advantage of the linear flux simulation is that it does not have a saturation concentration. The different \(K_d\) values of individual components (PMCs and NCXs) ensure that the extrusion flux will never reach its saturation point in the range of biologically relevant values of \(c_{cyt}\).

\[
J_{ERLEAK} = \beta
\] (15)

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 in situ recordings.

The Ca\(^{2+}\) influx across the plasma membrane is composed of a passive leakage and the agonist-activated fluxes: the capacitive (SOC-dependent) and the non-capacitive (arachidonate or DAG regulated) Ca\(^{2+}\) influx [53]. We simulated the changes in \(J_{ln}\) starting from the beginning of the administration of serum (\(t_1\)) using the following equations:

\[
J_{ln} = 0.1 \frac{nM}{s}, \text{ if } t < t_1 \text{ (only passive leakage)}
\] (18)

\[
J_{ln} = r_{ln_{max}} \frac{(t_1 - t)}{K_{B_{1,1}} + (t_1 - t) t_1 \text{, if } t_1 \leq t \leq t_2}
\] (19)

\[
J_{ln} = (r_{ln_{max}} - r_{ln_{min}}) \cdot \exp \left( -K_{IN_{2}}(t - t_2) \right) + r_{ln_{min}}, \text{ if } t > t_2.
\] (20)

\[\text{Table 1 Parameters used for the modeling.}\]

<table>
<thead>
<tr>
<th>Equation to determine</th>
<th>Parameter name</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(J_{ERFLUX}) (Eq. 7)</td>
<td>(\gamma)</td>
<td>450</td>
</tr>
<tr>
<td>(J_{ERFLUX}) (Eq. 8)</td>
<td>(r_{1_1})</td>
<td>0.17/s</td>
</tr>
<tr>
<td>(J_{ERFLUX}) (Eq. 9)</td>
<td>(\mu)</td>
<td>18.8 nM/s</td>
</tr>
<tr>
<td>(J_{ERFLUX}) (Eq. 12)</td>
<td>(K_d)</td>
<td>2.4 nM</td>
</tr>
<tr>
<td>(J_{ERFLUX}) (Eq. 13)</td>
<td>(r_{1_{min}})</td>
<td>22 nM/s</td>
</tr>
<tr>
<td>(J_{ERFLUX}) (Eq. 14)</td>
<td>(K_{fl})</td>
<td>300 s</td>
</tr>
<tr>
<td>(J_{ERFLUX}) (Eq. 15)</td>
<td>(\beta)</td>
<td>2.5 nM/s</td>
</tr>
</tbody>
</table>

\(r_{1_1}\) and \(r_{2_2}\) are positive constants. The parameter \(K_d\) represents the inositol-trisphosphate concentration in \(\mu\). \(\mu_{max}, r_{1_{max}}, r_{2_{min}}\) and \(r_{2_{max}}\) are positive constants. The parameter \(\beta\) represents the 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 [52].

Alternatively, \(J_{ERFLUX}\) and \(J_{ERLEAK}\) can be simulated conventionally with a Hill equation with the following parameters: \(V_{max} \approx 260 \text{ nM/s} \) and \(170 \text{ nM/s}, K_d \approx 460 \text{ nM} \) and 480 \text{ nM}, and Hill coefficients of 3.5 and 2.4, respectively.
We simulated the changes in [InsP3] from the beginning of the administration of serum (11) with the following equations. The resting [InsP3] was set to 15 nM [54]:

\[ [\text{InsP3}]_0 = 0.015, \text{if } t < t_1 \]  
\[ [\text{InsP3}]_0 = \frac{(t-t_1)}{K_{\text{IP}3} + (t-t_1)}, \text{if } t \geq t_1 \]  

The values for the above parameters are reported in the figure legends for each simulation.

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 (Supplementary Excel document). There were no significant differences in the solution of the differential equations, if we increased the temporal resolution (not shown). For the visualization, Prism5 (GraphPad Software, Inc., San Diego, CA) software was used.

3. Results

3.1. Characterization of serum-induced Ca\(^{2+}\) oscillation in mesothelial cells

Primary mouse mesothelial cells (prMC) maintained in the absence of serum in the medium for 24 h responded to the addition of 10% FCS to the cell culture medium with a sudden rise of c\(_{\text{ER}}\) lasting for approximately 40 s, followed by Ca\(^{2+}\) oscillations (Fig. 2A and Supplementary movie) in approximately 70% of cells. The Ca\(^{2+}\) responses in individual mesothelial cells showed a wide range of different oscillatory patterns that were elicited in a presumably homogenous population of mesothelial cells. Most cells displayed long-period (>10 min) baseline spiking oscillations, while some cells initially showed few short-period sinusoidal, i.e. overlapping baseline oscillations before changing to baseline spiking oscillations. In prMC cultured for longer periods (>10 passages) the duration of the sinusoidal oscillations was increased, however, the frequency of cells showing baseline spiking oscillations was strongly reduced. The frequency and the amplitude scans from oscillating prMC revealed that the amplitude didn’t change in the time period from 2 to 13 min after serum re-administration, while the oscillation frequency decreased during this time period (Fig. 2B). During oscillations the Ca\(^{2+}\) spike amplitudes reached c\(_{\text{ER}}\) values ranging from 140 to 250 nM as measured with the GEM-GECON indicator; resting c\(_{\text{ER}}\) was between 80–120 nM and basal c\(_{\text{ER}}\) during the baseline oscillations was usually slightly lower (Fig. 3), calculated to be in the order of 80–100 nM. Using a FRET-based Ca\(^{2+}\) indicator D1ER, the resting c\(_{\text{ER}}\) was found to be in the order of 150–250 nM and serum re-administration caused a reduction in c\(_{\text{ER}}\) to 100–150 nM. The pattern of c\(_{\text{ER}}\) changes is best described as a sawtooth wave (Fig. 2C).

Ca\(^{2+}\) waves propagating from one cell to the next possibly via gap junctions were sometimes observed; the presence of connexin 43 in mesothelial cells allowing for the transfer of Ca\(^{2+}\) ions or other low-molecular weight molecules (e.g. InsP3) between connected cells has been reported before [55]. Since Ca\(^{2+}\) oscillations persisted in cell cultures pre-treated with carbenoxolone, a gap junction blocker [56] (data not shown), we considered Ca\(^{2+}\) and/or InsP3 fluxes through gap junctions not essential for oscillations and we didn’t implement these mechanisms in our model. Similarly we did not consider the function of mitochondria as putative Ca\(^{2+}\) stores or sources in our model, since pretreatment of the cells with Ru360, a cell-permeable mitochondrial Ca\(^{2+}\) uptake blocker [57], did not inhibit Ca\(^{2+}\) oscillations (data not shown).

With the aim to investigating the role of RyRs in mesothelial cell Ca\(^{2+}\) oscillations, cells were exposed to a high concentration of ryanodine (50 μM); ryanodine fully closes RyRs at micromolar concentrations [58]. In most cells ryanodine did not affect serum-induced Ca\(^{2+}\) oscillations (Fig. 2D), but in some cases transiently and slightly reduced the frequency of oscillations together with a short-lasting lowering of basal c\(_{\text{ER}}\). Thus, RyRs appeared to be minimally implicated in Ca\(^{2+}\) oscillations in prMC. Preventing InsP3 production by the phospholipase C inhibitor U-73122, the serum-induced oscillations in prMC were blocked indicating that these oscillation are essentially based on elevated [InsP3] (Fig. 2E). Lower serum concentration resulted in a decreased oscillation frequency. The duration of oscillations was shorter and c\(_{\text{ER}}\) recovered faster from its semi-depleted ER state (Fig. 2F).

Based on previous findings that human mesothelioma, mesothelial cells in vitro and reactive mesothelial cells express calretinin [59, 60], an ER-hand with complex binding kinetics [61], we hypothesized that also murine mesothelial cells might express this protein and that its presence might affect the Ca\(^{2+}\) oscillations. However, calretinin protein expression levels in mouse primary mesothelial cells were found to be below the detection limit of our Western blot analysis [62], i.e. lower than approximately 100 nM, thus unlikely to affect the oscillations. In support the oscillation patterns (frequency, amplitude, duration) in primary mesothelial cells derived either from calretinin knockout (CR/−/−) or from wildtype mice were indistinguishable (data not shown). Nevertheless, the effect of a synthetic fast buffer, BAPTA (Kd = 0.2 μM; koff = 500 μM–1 s–1), was tested; BAPTA significantly reduced the amplitude of Ca\(^{2+}\) oscillations, but broadened (increased half-width) the Ca\(^{2+}\) transients (Fig. 2G). A similar effect had been modeled for repetitive (20 Hz) Ca\(^{2+}\) transients in dendrites of neurons with or without BAPTA (see Fig. 3 in [61]).

The Ca\(^{2+}\) influx is mediated by PMCA and NCXs. The NCXs have low Ca\(^{2+}\) affinities, but high capacity for Ca\(^{2+}\) transport, whereas the PMCAs have a high Ca\(^{2+}\) affinity, but a low transport capacity [63]. Despite the low Ca\(^{2+}\) affinity of NCXs, they play an important role in the Ca\(^{2+}\) efflux in primary mesothelial cells. NCXs use the energy that is stored in the electrochemical gradient of Na\(^+\) by allowing Na\(^+\) to flow down its gradient across the plasma membrane in exchange for the counter transport of Ca\(^{2+}\) ions. Thus, the removal of Na\(^+\) ions from the extracellular space was assumed to impair the function of NCXs and to reduce the Ca\(^{2+}\) efflux. Consequently, replacing the extracellular Na\(^+\) ions with N-methyl-D-glucamine strongly inhibited Ca\(^{2+}\) oscillations (Fig. 2H).

3.2. Modeling the effect of serum re-administration on cytoplasmic and luminal [Ca\(^{2+}\)] in primary mesothelial cells

In mesothelial cells, serum re-administration induced both, Ca\(^{2+}\) influx through the plasma membrane and Ca\(^{2+}\) release from the ER due to InsP3 production. Therefore, both independent variables ([InsP3] and [InsP3]) changed after serum re-administration. We selected two recordings showing oscillations, one with a small amplitude and one with a big amplitude. Fitting our model to these recordings revealed that these oscillations are essentially based on different [InsP3], i.e. \(\text{InsP3,MAX}\) is either 0.24 μM (Fig. 3A) or 1.8 μM (Fig. 3B and Supplementary Excel document). Nevertheless, we can’t exclude that differences in the Ca\(^{2+}\) efflux activities between these two cells might also contribute to the differences in amplitudes.

The model also predicted that serum-induced stimulation of prMC causes a partial depletion of c\(_{\text{ER}}\) and a sawtooth wave starting at c\(_{\text{ER}}\) lower than basal c\(_{\text{ER}}\) (Fig. 3A, B; lower traces). This is exactly in line with the experimental findings (Fig. 2C).

3.3. Pharmacological identification of Ca\(^{2+}\) influx components and modeling the effect of the magnitude of Ca\(^{2+}\) influx (JIN) on the oscillation frequency

Ionomycin is a Ca\(^{2+}\) ionophore forming pores in cell membranes. At low concentrations (≤500 nM) ionomycin evokes a transient Ca\(^{2+}\) signal that mostly reflects the depletion of ER stores and not the Ca\(^{2+}\) influx across the plasma membrane, since after ionomycin treatment the magnitude of the Ca\(^{2+}\) transients were essentially unchanged
when the experiment was carried out at very low (~1 μM) external Ca2+ (Fig. 4A, red and green traces). The surface area of ER compartments is approximately 25–50 times larger (based on the data presented by Schmucker et al. [64]) than the surface of the plasma membrane; therefore the ionomycin-evoked Ca2+ leakage from the ER is more pronounced compared to the leakage through the plasma membrane. SERCA activity can’t compensate for the elevated leakage from the ER and consequently the ER remained in a depleted state (Fig. 4A, blue trace). However, cys was recovered to near resting values, because the plasmalemmal extrusion system is able to cope with the increased Ca2+ influx. Although the ER was in a depleted state, the re-administration of serum after ionomycin treatment (500 nM) evoked an additional Ca2+ transient, indicative of the activation of ER load-independent serum-activated plasmalemmal Ca2+ channels (Fig. 4A, red trace). To test whether store-operated (SOC) i.e. ER load-dependent Ca2+ entry was also occurring in prMC, we employed THAPS, which causes passive Ca2+ entry of ER stores that is expected to activate SOC-mediated Ca2+ entry. Cells were initially incubated in a Ca2+-free solution; THAPS (5 μM) induced a slow increase in cys marking the depletion of the ER stores. Increasing [Ca2+]o to 1 mM resulted in a second increase of cys most probably mediated via SOC entry (Fig. 4B red trace). In the absence of THAPS, an increase in [Ca2+]o to 1 mM caused a minute, but prolonged increase in cys (Fig. 4B black trace).

The absence of extracellular Ca2+ ([Ca2+]o ≈ 0 mM), ionomycin (50 nM) evoked a rise in cys from basal levels to 350–400 nM (data not shown) together with an 80–90% depletion of luminal Ca2+ in the ER [65]. Using Eq. (6) we calculated a γ of around 800–1300. Knowing that this equation somewhat overestimated γ, in our model we used a lower value, of γ = 450.

In the absence of extracellular Ca2+ (low Ca2+ solutions supplemented with 50 mM EGTA), prMC didn’t show any Ca2+ response to 10% FCS administration in most cases. Only few cells displayed an initial small rise in cys, without signs of Ca2+ oscillations (data not shown). In experiments, where the serum was added to Ca2+-containing solutions [Ca2+]o ≈ 1 mM and subsequently 10 mM EGTA was added to lower the Ca2+ to 10 μM, the oscillations halted after one final Ca2+ spike (Fig. 4C). When EGTA (0.2 mM final) was added lowering [Ca2+]o to ≈0.8 mM, a decrease in the oscillation frequency was observed (Fig. 4D). Contrarily, when [Ca2+]o was raised from 1 mM to 2 mM, the oscillation frequency was increased (Fig. 4E). ML-9, an inhibitor of TRPC channels [66] that also inhibits myosin light chain kinase [67] and furthermore inhibits store-operated Ca2+ entry by inhibiting STIM1 recruitment [68] had a particular effect on the oscillations. ML-9 increased basal cys, which resulted either in the stopping of the oscillations or in oscillations with a lower frequency, but with increased Ca2+ amplitudes and moreover, elevated basal cys levels (Fig. 4F). Our results indicate that the main effect of ML-9, an inhibitor of limited specificity, was not the inhibition of the Ca2+ influx, but rather reducing the Ca2+ efflux rate, based on the slightly elevated basal cys after ML-9 administration (Fig. 4F), SKF 96365 (100 μM), a compound that blocks both SOCs and DAG-activated TRPC channels [66,69] reducing the Ca2+ influx across the plasma membrane [70], either completely blocked (Fig. 4G, black trace) or strongly decreased the oscillation frequency. In conditions, when oscillations were not blocked, the amplitude of the Ca2+ signals was not affected (Fig. 4G, red trace). Interestingly, immediately after SKF 96365 administration, the drug evoked a brief Ca2+ transient in each cell, which either halted oscillations or resulted in frequency-reduced Ca2+ oscillations. Our model recapitulated the effect of SKF 96365 on Ca2+ oscillation and revealed that the oscillation frequency depended on the magnitude of Ca2+ influx. Decreasing the Ca2+ influx through plasma membrane Ca2+ channels decreased the frequency of Ca2+ oscillations (Fig. 4H, red trace). Completely switching off the influx (Jin = 0) resulted in a stop of the oscillations (Fig. 4H, black trace).

3.4. Effect of blocking SERCA pumps on the oscillation frequency

While thapsigargin administered at high concentration (≥2 μM) completely blocked the Ca2+ oscillations, at lower concentrations, it modified the oscillation frequency in both directions, i.e. increasing it in some cells and lowering it in others (Fig. 5A). Both situations could be modeled by changing the parameter 1/r2 from 22 nM/s to 26.5 nM/s (Fig. 5B, C). The direction of the change caused by partial blocking of SERCA pumps was dependent on [InsP3]. At lower concentration ([InsP3] = 0.18 μM), the oscillation frequency was increased (Fig. 5B), while at higher concentrations ([InsP3] = 5 μM) the oscillation frequency was decreased (Fig. 5C). Of note, the partial block of SERCA pumps resulted in an increase of basal cys, both in the model and in the experimental measurements.

3.5. Applying the model to experiments done by others

We simulated the effect of a transient brief [InsP3] increase (“InsP3 step”) on baseline spiking oscillations as previously measured in HEK293 cells (see Fig. 2B in [71]). [InsP3] was held constant except for a 30 s-theoretical InsP3 step characterized by an immediate on/off step. During the baseline spiking oscillations, this instantaneous increase caused an immediate spike and a delay selectively of the next Ca2+ transient with no changes in the oscillation frequency of the following spikes (Fig. 6A). When the InsP3 step was applied during high-frequency oscillations, this caused an increase in oscillation frequency (see Fig. 2A in [71]). In our model, at high Ca2+ influx rates (Jin > 12 nM/s), high [InsP3] (>5 μM) and a raise of [InsP3] to 50 μM, this caused a transient elevation of the Ca2+ spike amplitude and increased the oscillation frequency during the period of elevated [InsP3] (Fig. 6B). Previous studies have reported on a critical cys for spike development (see Fig. 4A in [72]). Our model predicts that the Ca2+ spike generation is Ca2+-influx-independent within a certain range of cys values and for this, basal cys was set to 90 nM. If the Ca2+ influx was switched off at cys = 95 nM (Jin = 0), then no Ca2+ spike developed (Fig. 6C, part a), however if the Ca2+ influx was blocked at cys = 100 nM (parts b,c), the amount of intracellular Ca2+ was sufficient to cause ER-mediated Ca2+ release representing the major component of the Ca2+ spike (Fig. 6C). Furthermore, during Ca2+ oscillations, a slight delay between the time points of maximum of cys and the minimum of cys was observed (see Fig. 4C in [27]). Our model also showed this phenomenon.

Fig. 2. Ca2+ oscillations in mesothelial cells after serum re-administration. A. Single-cell fluorescent recordings from time-lapse videos show Ca2+ oscillations after serum re-administration. Each color track represents a single cell. B. For the calculations of the frequency (black trace) and amplitude (red trace) after serum re-administration, 100 GCaMP3-expressing mesothelial cells were evaluated. For determining the slopes, the time period from 2 min after serum addition until 13 min was considered. While the amplitude slope is not significantly different from zero (p > 0.05), the frequency decreases with time after serum addition (p = 0.0001). C. Two representative recordings from a DfER-expressing cell show a rapid decrease in cys resulting from serum re-administration followed by a sawtooth wave in cys. D. A single-cell fluorescent recording from time-lapse videos shows Ca2+ oscillations after serum re-administration. Frequency and amplitude of the oscillation remained unchanged after administration of ryanodine (50 μM). E. A representative recording from a GCaMP3-expressing cell shows an oscillation arrest after administration of U-73122, a phospholipase C inhibitor. F. A representative recording from a GCaMP3-expressing mesothelial cell shows an increase in oscillation frequency after elevating the serum concentration from 1% (left) to 10% (right). F. A representative single-cell fluorescent recording shows Ca2+ oscillations after serum re-administration. Administration of BAPTA-AM (15 μM) to the medium results in oscillations with lower amplitudes, but increased half-width of Ca2+ transients. H. In the absence of extracellular Na+ ions ([Na+]o = 1 mM), in most cases only the initial Ca2+ spike is observed (red trace). In few cells, additional Ca2+ transients are detected (black trace). Two representative single-cell fluorescent recordings were selected.
The five phases of one oscillatory cycle are depicted in Fig. 6D and more details are provided in Table 2.

4. Discussion

Oscillatory changes in c_{cyt} occur in excitable, as well as in non-excitatory cells, including hepatocytes [73], embryonic intestinal epithelial cells [74] and pancreatic acinar cells [75]. The reason for not having observed Ca^{2+} oscillations in mesothelial cells before might be manifold: measurement of global Ca^{2+} signals (cuvette recordings) [3–6] vs. recordings in individual cells, the use of non-optimized Ca^{2+} indicator dyes with respect to K_{D}, k_{on} or high indicator concentrations, which were shown to modify Ca^{2+} oscillations [76,77]. Based on the large diversity of Ca^{2+} oscillation patterns in various cell types and moreover even within an apparently homogeneous cell population [76], many different models were developed to describe these oscillations [78]. Despite the large number of studies reporting on the underlying processes leading to Ca^{2+} oscillations, no broad general consensus has been reached yet; however the various oscillation models can be classified into subgroups [79]. Our model is a channel-based, deterministic, open-cell model. With the modeling, we aimed to demonstrate how the measured dependent variables (c_{cyt} and c_{ER}) vary as the result of changes of the independent variables J_{IN} and [InsP_{3}].

As InsP_{3}Rs are biphasically regulated by c_{cyt} [80], the InsP_{3}R-based models usually assume that InsP_{3}Rs are quickly activated, but slowly inactivated by Ca^{2+} [81]. Consequently the period length of Ca^{2+} oscillations depends on the time required for InsP_{3}R to recover from Ca^{2+}-induced inhibition. Thus, these models mostly fail to explain why the frequency of Ca^{2+} oscillations even in a single cell can vary in a very wide temporal range [79]. Other theories based on the function of putative InsP_{3}R inhibitors [17,82] are faced with the same problem. If such an inhibitor inactivates the receptor at high c_{cyt}, then the next activation at low c_{cyt} will be governed by the K_{D} of the putative inhibitor. For instance, calmodulin was reported to inhibit InsP_{3}R at elevated c_{cyt} [82], however this doesn’t play a direct role in the biphasic modulation of InsP_{3}R by c_{cyt} [83].

We solved this problem by taking into account the regulatory effect of c_{ER} on InsP_{3}R. High c_{ER} increases the open probability rate of InsP_{3}R; low c_{ER} decreases the open probability [84–86]. The “store loading Ca^{2+} oscillatory” theory [71,46] takes into account the InsP_{3}R regulation by c_{ER}. After a Ca^{2+} spike, c_{ER} is decreased and has to recover before InsP_{3}Rs are resensitized to deliver the next Ca^{2+} pulse. Thus, the interspike interval is essentially determined by the velocity of ER load- ing. Although this theory allows for a constant c_{cyt} and an increasing c_{ER} during interspike periods [4], the mathematical model based on this theory does not show this phenomenon [87]. Because our experimental data confirmed that in mesothelial cells a sawtooth wave in c_{ER} is paralleled by a baseline spiking oscillation in c_{cyt}, we integrated the “store loading Ca^{2+} oscillatory” concept into our model. The saw- tooth wave in c_{ER} was also found in BHK-21 fibroblast cells after bradykinin stimulation [88].

In our simulations a Ca^{2+} spike starts with a small elevation in c_{cyt}, and with a little delay c_{ER} reaches its maximum before it starts to decrease. In this case, the slow rise of c_{cyt} precedes the decrease of c_{ER} (Fig. 6D), as was also proven experimentally [89]. The foot period of the spike (Fig. 6D) lasts until a critical level c_{cyt, crit} is reached. At c_{cyt} lower than the c_{cyt, crit} value, the spike development is based on Ca^{2+} influx, but the further development of the spike is influx-independent, i.e. the activation of InsP_{3}R is mediated exclusively by the increased c_{cyt}. This self-sustaining phenomenon is called “Ca^{2+}-induced Ca^{2+} release”. 

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Fig. 3. Modeling of serum-induced Ca^{2+} oscillations. A. A mesothelial cell recording (red trace) showing low [InsP_{3}] based oscillations was selected for the fitting. The parameters for J_{IN} and [InsP_{3}] are: \( \Delta J_{IN} = 70 \mu M/s, \Delta \text{InsP}_{3} = 5 \mu M, r_{k, \Delta J_{IN}} = 0.35 \mu M/s, K_{D, \Delta J_{IN}} = 0.005, K_{D, \Delta \text{InsP}_{3}} = 10^{-8}, k_{p, \Delta \text{InsP}_{3}} = 0.24 \mu M, K_{p, \Delta J_{IN}} = 0.01 \). For the modeling, the initial c_{cyt} was set at 257 \mu M, resulting in a slightly lower resting c_{cyt} than in the standard simulations, where c_{cyt} was set at 260 \mu M. The simulations for c_{cyt} (black solid line) and for c_{ER} (black dashed line) result in a pattern closely resembling the one of c_{cyt} obtained from the experimental recording. B. A recording showing high [InsP_{3}] based oscillations (red trace) was selected for the model fitting. Black lines (solid and dashed) represent the modeled c_{cyt} and c_{ER}, respectively. The small inset represents the changes in independent parameters for the Ca^{2+} influx and [InsP_{3}]: \( \Delta J_{IN} = 60 \mu M/s, \Delta \text{InsP}_{3} = 72 \mu M, r_{k, \Delta J_{IN}} = 1.05 \mu M/s, K_{D, \Delta J_{IN}} = 0.01, K_{D, \Delta \text{InsP}_{3}} = 2, k_{p, \Delta \text{InsP}_{3}} = 1.8 \mu M, K_{p, \Delta J_{IN}} = 0.1 \). Note the close match between the modeled and measured traces.
As reported before [72], Ca$^{2+}$ entry is necessary for sustained oscillatory activity, but its contribution and thus importance changes during the rising phase of a Ca$^{2+}$ spike: the slow rise in $c_{\text{cyt}}$ at the beginning requires Ca$^{2+}$ entry, but the rapid upstroke phase doesn’t. Our model also highlights that the maximum of $c_{\text{cyt}}$ precedes the minimum of $c_{\text{ER}}$, similar to what was found by Shmigol et al. [91]. Dagnino-Acosta
and Guerrero-Hernández [92], Palmer et al. [27] and Ishii et al. [89], all of whom reported a slight delay between the maximum of ccyt and the minimum of cER. The changes of the different parameters during the five phases of an oscillation are summarized in Fig. 6D and Table 2.

According to our model, the frequency of the baseline spiking oscillations depends on (I) the Ca$^{2+}$ influx; the stronger the Ca$^{2+}$ influx, the higher the frequency. This is in line with previous findings that an increase in extracellular Ca$^{2+}$ during serotonin-induced oscillations in bowly salivary glands accelerates Ca$^{2+}$ oscillations without increasing [InsP$\text{3}$] [93]. It further depends on (II) the amount of Ca$^{2+}$ that is removed from the cell during one Ca$^{2+}$ spike (the larger the Ca$^{2+}$ removal, the slower the oscillation) and (III) the rate of change in [InsP$\text{3}$] (the faster the rate of InsP$\text{3}$ production, the faster the oscillation). Of interest, the mere rise in [InsP$\text{3}$] without Ca$^{2+}$ influx ($J_{\text{IN}} = 0$) leads to few oscillations that stop once the elevated steady-state [InsP$\text{3}$] is reached. At a constant [InsP$\text{3}$] the oscillation frequency is then determined and maintained by IN and EFF processes. Tanimura and Turner [50] demonstrated InsP$\text{3}$-dependent Ca$^{2+}$ oscillations within the lumen of the ER of saponin-permeabilized HSY cells by monitoring cER with the fluorescent Ca$^{2+}$ indicator Mag-fura-2. HSY cells exposed to successively higher concentrations of InsP$\text{3}$ show few oscillations in cER when [InsP$\text{3}$] is increased from 0.3 to 0.6 μM. Then, cER remains constant and moreover, lower than before the increase in [InsP$\text{3}$] and consequently, no oscillations are observed afterwards. However, cER oscillations reappear after an additional increase in [InsP$\text{3}$] to 1 or 3 μM and moreover, the rate of decrease in cER (slope) is higher at higher [InsP$\text{3}$] and this is also paralleled by a higher oscillation frequency (for details, see Fig. 1 in [50]). Of note in intact cells this effect is masked by two additional factors. The higher [InsP$\text{3}$] causes a larger ER depletion, which due to the activation of SOC channels results in a stronger Ca$^{2+}$ influx, and consequently increased oscillation frequency. On the other hand, the increased [InsP$\text{3}$] produces higher peaks in ccyt, i.e. the amount of Ca$^{2+}$ ions that are removed by plasmalemmal pumps is also increased. This in turn, slows down the frequency. In the experiments carried out at lower serum concentration (1%; Fig. 2F) expected to lead to weaker cell activation, the oscillation frequency (after the fast oscillation period of approximately 120 s) was clearly lower and accompanied with a faster recovery of cER from its semi-depleted state. Since the cER depletion level is highly correlated with [InsP$\text{3}$], we assume a time-dependent decrease in the rate of InsP$\text{3}$ production during 1% FCS-induced oscillations. On the other hand, the stronger 10% FCS-induced cell activation leads to higher frequency oscillations, a nearly constant, semi-depleted ER state during the oscillations and presumably to constant [InsP$\text{3}$] levels. Thus, the model reveals that both, a decreased
Model simulations based on experiments previously reported by others. A. In an oscillating airway smooth muscle cell, an instantaneous, flash photolysis-induced increase in \([\text{InsP}_3]\) results in an initial \(\text{Ca}^{2+}\) spike and a delay of only the next peak of the \(\text{Ca}^{2+}\) oscillation without changing the oscillation frequency\cite{71}. Our model simulation demonstrates the effect of a rapid transient \([\text{InsP}_3]\) increase during slow baseline spiking oscillations applied just before the onset of the “regular” spiking. This results in an immediate \(\text{Ca}^{2+}\) spike and a delay of the next \(\text{Ca}^{2+}\) spike, without globally affecting the oscillation frequency. The independent variables are: \(J_{\text{IN}} = 1 \text{nM/s} (t_0 \rightarrow t_{\text{end}})\), changes in \([\text{InsP}_3]\) are shown at the bottom of the x-axis. B. In an oscillating pancreatic acinar cell, the photorelease of \(\text{InsP}_3\) causes a transient increase in oscillation frequency\cite{71}. In our model when the duration of the transient elevation of \([\text{InsP}_3]\) is longer than one oscillatory cycle, a transient elevation of \([\text{InsP}_3]\) from 5 to 50 mM (dashed line) causes a transient increase in the oscillation frequency and in the maximal amplitude \(c_{\text{cyt}}\), evident only during the increased \(\text{InsP}_3\) step. The independent variable is: \(J_{\text{IN}} = 1 \text{2nM/s} (t_0 \rightarrow t_{\text{end}})\). C. The existence of a critical \(c_{\text{cyt}}\) concentration for spike development was proven in sympathetic neurons\cite{72}. Our model shows that during the spike development the initial small rise in \(c_{\text{cyt}}\) depends on extracellular \(\text{Ca}^{2+}\) influx, the following fast rise is caused by ER depletion and is independent of \(\text{Ca}^{2+}\) influx. The independent variables are: \([\text{InsP}_3]\) = 0.3 \(\mu\)M \((t_0 \rightarrow t_{\text{end}})\), the changes in \(J_{\text{IN}}\) are shown by the dashed line. If \(J_{\text{IN}}\) is switched off at \(c_{\text{cyt}} = 95 \text{nM} (a)\), the \(\text{Ca}^{2+}\) spike development is halted. If \(J_{\text{IN}}\) is switched off at \(c_{\text{cyt}} = 100 \text{nM} (b)\) or \(c_{\text{cyt}} = 105 \text{nM} (c)\), a regular full-size \(\text{Ca}^{2+}\) spike develops. D. Details on the kinetics of \(\text{Ca}^{2+}\) spike development (see also Table 2).
Ca\textsuperscript{2+} influx and a decreasing rate of InsP\textsubscript{3} production are likely to be the cause for the decreased oscillation frequency observed at the lower serum concentration.

The partial block of SERCA pumps may affect the oscillations frequencies in both directions. In the same cell culture dish, application of thapsigargin caused an increase in oscillation frequency in some cells, while decreasing it in others (Fig. 5). This phenomenon is also observable in recordings from oscillating pancreatic cells (decreasing frequency in [94], increasing frequency in [36]). Our model revealed that thapsigargin decreases the frequencies at higher spike amplitudes, when oscillations are most probably the result of higher [InsP\textsubscript{3}] (Fig. 5B) and accelerates it at lower amplitudes during low [InsP\textsubscript{3}]-based oscillations (Fig. 5C). Previous experimental data also showed that oscillations at semi-depleted cER states reflecting the increased [InsP\textsubscript{3}] cause the increased amplitudes in c\textsubscript{cyt} [95].

In our model, the plasmalemmal Ca\textsuperscript{2+} influx (J\textsubscript{in}) and [InsP\textsubscript{3}] are the independent parameters; changing these parameters in time leads to Ca\textsuperscript{2+} oscillations ranging from fast, nearly sinusoidal to slow baseline spiking oscillations, i.e. the interspike period may vary from <10 s to extended time periods, i.e. to a stop of the oscillations. For faster, overlapping or irregular oscillations it might be necessary to expand our model in space and taking into account that the synchronization between the individual oscillatory units might be lost at higher frequencies. The Ca\textsuperscript{2+} influx (J\textsubscript{in}) was modeled with Michaelis–Menten kinetics and an exponential decay ending on a plateau (peak-plateau shape) (inset in Fig. 3B). Experimentally we found that J\textsubscript{in} contains also a SOC-independent i.e. an ER load-independent component (Fig. 4A). In our simulation, the ER load-independent Ca\textsuperscript{2+} influx results in the brief maximal influx rate, while the ER load-dependent SOC entry is responsible for the plateau phase of J\textsubscript{in} (inset in Fig. 3B).

The change in [InsP\textsubscript{3}] was simulated as a rapid increase quickly leveling off. Kinetics of [InsP\textsubscript{3}] changes measured with a specific indicator protein showed Michaelis–Menten kinetics upon stimulation with endothelin-1 [96] in rat oocytes. Matsu-ura et al. [97] also found that [InsP\textsubscript{3}] gradually accumulated in the cytosol with little or no fluctuations during Ca\textsuperscript{2+} oscillations. Experimentally observed small fluctuations in [InsP\textsubscript{3}] are considered as passive reflections of the Ca\textsuperscript{2+} oscillations (increased PLC activity and InsP\textsubscript{3} production during peak c\textsubscript{cyt} and not essential for the driving of the Ca\textsuperscript{2+} oscillations [54]).

Sneyd et al. [71] reported that pulmonary acinar cells show carbachol-induced Ca\textsuperscript{2+} oscillations. An instantaneous, flash photolysis-induced increase in [InsP\textsubscript{3}] during ongoing oscillations results in a delay before the spike without a change in oscillation frequency. However, a similar increase in [InsP\textsubscript{3}] causes a transient increase in oscillation frequency in airway smooth muscle cells. The authors thus proposed different underlying mechanisms and thus different models for Ca\textsuperscript{2+} oscillations present in these two cell types. Our model can recapitulate both phenomena: an InsP\textsubscript{3} step during baseline spiking oscillations leads to a delay of the next spike (Fig. 6A), while a similar step during fast oscillations increases the oscillation frequency during the step duration (Fig. 6B), i.e. the effects are InsP\textsubscript{3},R-based in both cases.

Although we didn’t incorporate mitochondria in our model, these organelles influence Ca\textsuperscript{2+} oscillations in two ways. First, mitochondria produce ATP, required for SERCA and PMCA functions. Second, the Ca\textsuperscript{2+} concentrations in the matrix of the mitochondria also change and slightly oscillate during cytoplasmic Ca\textsuperscript{2+} oscillations [89]. In regard to our model, the mitochondria act in a similar way as the extracellular space. During the spikes, Ca\textsuperscript{2+} ions move from the cytoplasm into the mitochondrial matrix and during the interspike intervals Ca\textsuperscript{2+} ions are transported from the mitochondrial matrix to the cytoplasm. In some cell types, the matrix volume and their Ca\textsuperscript{2+} storing capacity might be so effective as to maintain the Ca\textsuperscript{2+} oscillations even in absence of extracellular Ca\textsuperscript{2+} [89] or by blocking IN and EFF by lanthanide [98]. In mesothelial cells, however, mitochondrial Ca\textsuperscript{2+} uptake appears to play a marginal role in Ca\textsuperscript{2+} oscillations. Removal of extracellular Ca\textsuperscript{2+} ions by EGTA chelation causes an arrest in Ca\textsuperscript{2+} oscillations and Ru360, a blocker of mitochondrial Ca\textsuperscript{2+} uniporter (MCU), didn’t stop these oscillations. Nevertheless, further experiments need to be performed to decipher the exact role of mitochondria in Ca\textsuperscript{2+} oscillations.

**Conflict of interest statement**

1. We have included in the Acknowledgement all third-party financial support for the work in the submitted manuscript.
2. All financial relationships with any entities that could be viewed as relevant to the general area of the submitted manuscript have been added in the Acknowledgement.
3. This includes all sources of revenue with relevance to the submitted work, who made payments to us, or our institution on your behalf, in the 36 months prior to submission.
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6. There are no other relationships or affiliations that may be perceived by readers to have influenced, or give the appearance of potentially influencing, what you wrote in the submitted work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online.

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