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Ca²⁺ refilling of the endoplasmic reticulum is largely preserved albeit reduced Ca²⁺ entry in endothelial cells

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Abstract

In this study the relationship between the efficiency of endoplasmic reticulum (ER) Ca²⁺ refilling and the extent of Ca²⁺ entry was investigated in endothelial cells. ER and mitochondrial Ca²⁺ concentration were measured using genetically encoded Ca²⁺ sensors, while the amount of entering Ca²⁺ was controlled by varying either the extracellular Ca²⁺ or the electrical driving force for Ca²⁺ by changing the plasma membrane potential. In the absence of an agonist, ER Ca²⁺ replenishment was fully accomplished even if the Ca²⁺ concentration applied was reduced from 2 to 0.5 mM. A similar strong efficiency of ER Ca²⁺ refilling was obtained under condition of plasma membrane depolarization. However, in the presence of histamine, ER Ca²⁺ refilling depended on mitochondrial Ca²⁺ transport and was more susceptible to membrane depolarization. Store-operated Ca²⁺ entry (SOCE), was strongly reduced under low Ca²⁺ and depolarizing conditions but increased if ER Ca²⁺ uptake was blocked or if ER Ca²⁺ was released continuously by IP₃. A correlation of the kinetics of ER Ca²⁺ refilling with cytosolic Ca²⁺ signals revealed that termination of SOCE is a rapid event that is not delayed compared to ER refilling. Our data indicate that ER refilling occurs in priority to, and independently from the cytosolic Ca²⁺ elevation upon Ca²⁺ entry and that this important process is widely achieved even under conditions of diminished Ca²⁺ entry.

Keywords

Cameleon; Endoplasmic reticulum Ca²⁺ refilling; Membrane depolarization; Mitochondrial Ca²⁺; Pericam; Store-operated Ca²⁺ entry

1. Introduction

Often, agonist-induced stimulation of non-excitable cells leads initially to the production of inositol 1,4,5-trisphosphate (IP₃) that triggers the release of Ca²⁺ from the endoplasmic reticulum (ER) [1]. Subsequently to the Ca²⁺ depletion of the ER, a Ca²⁺ influx across the plasma membrane through non-voltage gated ion channels is activated (for review see [2]). This phenomenon is known as capacitative Ca²⁺ entry or store-operated Ca²⁺ entry (SOCE; [3,4]) and was shown to be crucial for a variety of cellular processes such as exocytosis, enzyme activity, contraction and gene expression, in addition of being essential for ER Ca²⁺ refilling (for review see [2]). Although several putative mechanisms has been proposed to account for the store-operated opening of plasma membrane Ca²⁺ channels, such like a diffusible factor or a direct coupling between the ER and the SOCE channels, the molecular mechanism(s) is (are) still controversial. Likewise, the molecular identity of the SOCE channels is still not known, but so far the most likely candidates belong to the large family of TRP channels [5-7].

In non-excitable cells such like endothelial cells, cytoplasmic Ca²⁺ elevation is accompanied with membrane hyperpolarization that is due to the opening of Ca²⁺-activated K⁺ channels (e.g. BK_{Ca} channels; [8]), which, in turn, enhances the driving force for Ca²⁺ and, thus, increases the extent of SOCE.

In our previous work, we found that Ca²⁺ entering endothelial cell transits through two different pathways to refill the ER depending whether or not the IP₃ level was elevated [9]. Out from this work it appeared that the cytosolic Ca²⁺ elevation (measurable with i.e. fura-2) is not an appropriate indicator of the ER Ca²⁺ refilling extend. Consequently, the relationship between the quantity of Ca²⁺ entry and the kinetics of ER Ca²⁺ refilling, a process also crucial for SOCE inactivation, is unclear and needs further assessment to elucidate how much Ca²⁺ actually has to enter the cell to achieve complete ER Ca²⁺ replenishment.

In order to determine the efficiency of ER Ca²⁺ refilling in correlation to the extent of Ca²⁺ influx, the amount of Ca²⁺ entering the cells through SOCE was manipulated by varying either the Ca²⁺ concentration of the extracellular medium or the electrical driving force for Ca²⁺ by changing the plasma membrane potential. In addition, the intraluminal Ca²⁺ concentration of the ER was directly measured in single cells using genetically encoded, ER targeted Ca²⁺ sensors. Our results indicate that ER Ca²⁺ refilling in the absence of an agonist is widely achieved even under conditions of strongly diminished Ca²⁺ entry and the lack of a pronounced cytosolic Ca²⁺ elevation due to a constant strong SERCA activity. On the other hand, in the continuous presence of an IP₃ generating agonist ER Ca²⁺ refilling becomes dependent on *trans*-mitochondrial Ca²⁺ fluxes [9] and thus more susceptible to (reduced) Ca²⁺ entry. This adaptability of the ER Ca²⁺ refilling machinery may be of vitally importance to ensure repeatability of ER Ca²⁺ release and to adjust Ca²⁺ dependent functions such as protein folding within the ER [10] according to the extent of entering Ca²⁺ [11].

2. Materials and methods

2.1. Materials

Cell culture chemicals were obtained from Invitrogen (Vienna, Austria). Fetal bovine serum was from PPA Laboratories (Linz, Austria). Dulbecco's modified eagle's medium (DMEM), BHQ, EGTA, KB-R 7943 and Histamine were obtained from Sigma Chemicals (St. Louis, MO, USA). Fura-2/AM was ordered from Molecular Probes Europe (Leiden, The Netherlands). All other chemicals were obtained from Roth (Karlsruhe, Germany).

2.2. Cell culture

All experiments were performed with the human umbilical vein endothelial cell derived cell line, EA.hy926 passage 45. Endothelial cells were grown in DMEM containing 10% fetal bovine serum and 1% HAT (5 mM hypoxanthin, 20 μ M aminopterin and 0.8 mM thymidine) and were maintained in an incubator at 37 °C in 5% CO₂ atmosphere. Cells were plated on 30 mm glass cover slips for experiments and transfection.

2.3. Transfection

Cells of approximately 70–80% confluence were transiently transfected with 1.5–2.5 μ g cDNA of the pcDNA3 vector encoding for either vYC4er, apoK1er, D1-ER or RP-mt using TransFast™ Transfection Reagent (Promega, Mannheim, Germany). Approximately 36 h after transfection cells were used for experiments.

2.4. Solutions

The loading buffer solution (LB) contained (in mM): 135 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 hepes acid, 2.6 NaHCO₃, 0.44 KH₂PO₄, 10 glucose with 0.1% vitamins and 0.2% essential amino acids, 1% penicillin/streptomycin, 1% fungizone, pH adjusted to 7.4 with NaOH. The experimental buffer (EB) for control experiments was composed of (in mM): 138 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose and 10 hepes acid, pH adjusted to 7.4 with NaOH. For the low Na⁺ experiments NaCl in the EB was reduced to 13 and 125 mM of cholin chloride (C₅H₁₄NO·Cl) was added to obtain an isotonic solution. The high K⁺ EB was composed of (in mM): 13 NaCl, 130 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose and 10 hepes acid, pH adjusted to 7.4 with KOH. Ca²⁺ free EB contained 1 mM EGTA instead of CaCl₂. The solutions containing less than 2 mM Ca²⁺ were prepared from nominal Ca²⁺ free EB without EGTA.

2.5. Cytosolic Ca²⁺ measurement ([Ca²⁺]_{cyto})

Changes in [Ca²⁺]_{cyto} were monitored using the fura-2 technique as described previously [12,13]. Briefly, cells were loaded with 2 μ M fura-2 AM for 45 min in LB at room temperature in the dark. Before experiments cells were washed twice with LB, and equilibrated for further 15–20 min in the same buffer. Subsequently cells were illuminated alternatively at 340 and 380 nm (either by filters: 340HTI15 and 380HTI15; Omega Optical Brattleboro, VT, USA or utilizing a monochromator: DeltaRam, Photon Technology Inc., Monmouth Junction, NJ, USA) and emitted light was collected at 510 nm using the 510WB40 emission filter from Omega Optical.

2.6. ER Ca^{2+} measurement ($[\text{Ca}^{2+}]_{\text{ER}}$)

For measuring the free Ca^{2+} concentration within the lumen of the ER ($[\text{Ca}^{2+}]_{\text{ER}}$) Ca^{2+} dependent fluorescence energy transfer (FRET) of the ER targeted cameleon constructs YC4er [14], apoK1er [15] or D1-ER [16] was monitored. Transfected cells were excited at 440 ± 15 (440AF21, Omega Optical) and emission was monitored simultaneously at 535 and 480 nm with one given camera using an optical beam splitter (535 and 480, Dual-View MicroImager™, Optical Insights, Visitron Systems, Puchheim, Germany).

2.7. Ca^{2+} measurements of free mitochondrial Ca^{2+} ($[\text{Ca}^{2+}]_{\text{mito}}$)

Cells stably expressing ratiometric pericam (RP-mt) were used to monitor $[\text{Ca}^{2+}]_{\text{mito}}$. RP-mt was excited at 433 nm (433DF15, Omega Optical) and emission was collected at 535 nm (535AF26, Omega Optical). $[\text{Ca}^{2+}]_{\text{mito}}$ was expressed as $1 - F_{430}/F_0$ as previously shown [9,17].

2.8. Imaging system, data acquisition and analysis

All experiments were performed with adherent cells on glass cover slips in the dark at room temperature on an inverted microscope (Eclipse 300 TE, Nikon, Vienna) as described previously [12]. The microscope was equipped with a 40×1.3 N.A. oil-immersion objective (Plan Fluor, Nikon, Vienna), a computer controlled z-stage (Ludl Electronic Products, Hawthorne, NY, USA) and an epifluorescence system (150 W XBO; Optiquip, Highland Mills, NY, USA). Excitation filters were changed with a Ludl filter-wheel device. Fluorescence images were collected using a cooled charge-coupled device camera (-30°C ; Quantix KAF 1400G2, Roper Scientific, Acton, MA, USA) that allowed image resolution of $0.171 \mu\text{m pixel}^{-1}$. Alternatively, a monochromator-based imaging system (Visitron Systems) was used as previously described [18]. All devices were controlled by Metafluor 4.0 software (Molecular Devices, Visitron Systems). Fluorescence dye bleaching was corrected by calculating the bleaching function R_0 or F_0 for each individual cell using a one phase exponential decay equation for curve fitting according to the individual decay of Ratio F_{340}/F_{380} for fura-2, F_{535}/F_{480} for the ER Ca^{2+} sensors or $1 - F_{430}$ for RP-mt that were collected at the beginning and end of each experiment. Accordingly, $[\text{Ca}^{2+}]_{\text{cyto}}$ is expressed as Ratio $(F_{340}/F_{380})/R_0$, $[\text{Ca}^{2+}]_{\text{ER}}$ is shown as Ratio $(F_{535}/F_{480})/R_0$ and $[\text{Ca}^{2+}]_{\text{mito}}$ is expressed as $1 - F_{430}/F_0$.

2.9. Statistics

Analysis of variance (ANOVA) was performed and statistical significance was evaluated using Scheffé's *post hoc* *F*-test. Level of significance was defined as $P < 0.05$.

3. Results

3.1. Modulation of store-operated Ca^{2+} entry by membrane depolarization or decreased extracellular Ca^{2+} concentration

We first tested the impact of a complete membrane depolarization on Ca^{2+} entry, by increasing the extracellular K^+ concentration from 5 to 130 mM. To activate store-operated Ca^{2+} influx, ER was emptied by cell stimulation with 100 μM of the IP_3 generating agonist

histamine in the absence of extracellular Ca^{2+} , followed by Ca^{2+} re-addition. The amplitude of the Ca^{2+} elevation was used as a measure of SOCE. When the cells were depolarized, cytosolic Ca^{2+} elevation was reduced by approx. 70% (Fig. 1A), indicating that membrane depolarization and, thus, the disruption of the electrical driving force for Ca^{2+} entry, impaired the Ca^{2+} entry into non-excitable cells. Since the high K^+ solution was kept isotonic and, thus, Na^+ was replaced by K^+ , the Na^+ concentration in this buffer was 13 mM. In order to assess whether the reduction of extracellular Na^+ might contribute to the results described above, the extracellular Na^+ concentration was reduced to 13 mM in normal K^+ solution (i.e. 5 mM K^+). Low extracellular Na^+ and physiological K^+ conditions did not affect the cytosolic Ca^{2+} elevation upon Ca^{2+} re-addition to pre-stimulated cells ($n = 35$; data not shown). In line with these results, inhibition of the reversed mode of the plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX_{pm}) by 10 μM KB-R 7943 [19] did not mimic the effect of high K^+ ($n = 43$; data not shown).

In order to imitate the consequences of membrane depolarization on Ca^{2+} entry, different extracellular Ca^{2+} concentrations were applied to pre-stimulated cell in normal K^+ medium (Fig. 1B, left panel). The cytosolic Ca^{2+} elevation in response to Ca^{2+} re-addition showed a clear concentration dependency with an EC_{50} of 0.71 mM (0.60–0.83 mM) (Fig. 1B, right panel), indicating that manipulating the extracellular Ca^{2+} concentration is a proper tool to control the amount of Ca^{2+} that enters the cell upon ER depletion. If the impact of membrane depolarization and that of the reduction of extracellular Ca^{2+} were compared in terms of the attenuation of cytosolic Ca^{2+} elevation, depolarization mimicked a condition that equals the addition of approx. 0.5 mM extracellular Ca^{2+} (Fig. 1C).

3.2. ER Ca^{2+} refilling is largely assured even under conditions of reduced Ca^{2+} influx

From the data presented above, it needs to be assessed whether the reduction in the cytosolic Ca^{2+} elevation had an impact on the extent of ER refilling during the Ca^{2+} re-addition phase. A simple protocol to assess the efficiency of ER refilling was to re-stimulate the cells with histamine in Ca^{2+} free medium after the Ca^{2+} re-addition. Surprisingly, the second histamine-induced Ca^{2+} elevation was not impaired even under depolarizing conditions (Fig. 2A). In order to elucidate ER Ca^{2+} dynamics upon Ca^{2+} re-addition directly, cells were transiently transfected with vYC4er. As shown in Fig. 2B, neither the amplitude nor the kinetics of ER Ca^{2+} refilling were altered in depolarizing conditions.

Similar experiments were performed by lowering the Ca^{2+} concentration added after histamine stimulation. The EC_{50} value for extracellular Ca^{2+} to achieve Ca^{2+} refilling was 0.19 (0.17–0.23) mM (Fig. 3A and C). Notably, even if the Ca^{2+} concentration was reduced from 2 to 0.5 mM, ER Ca^{2+} refilling was fully accomplished (Fig. 3A) whereas its kinetics was more sensitive to a reduction of extracellular Ca^{2+} (Fig. 3B). The analysis of the kinetics of ER Ca^{2+} refilling (Fig. 3B, upper panel) and calculating the rate constants of ER Ca^{2+} refilling at various concentrations of extracellular Ca^{2+} (Fig. 3B, lower panel) revealed an EC_{50} of 0.52 (0.43–0.67) mM on the maximal rate (Fig. 3C). At extracellular Ca^{2+} concentrations lower than 0.5 mM, ER Ca^{2+} refilling was incomplete within 3 min and could be accelerated and fully restored by increasing the extracellular Ca^{2+} concentration to 2 mM (Fig. 3A). Thus, even under conditions of reduced Ca^{2+} entry, the extent of ER Ca^{2+}

refilling is largely preserved despite and reduced rate of the refilling process. These data indicate that ER Ca^{2+} refilling occurs in priority to, and independently from the cytosolic Ca^{2+} elevation.

In order to strengthen this hypothesis, cells were stimulated a second time by histamine in the absence of extracellular Ca^{2+} after an intermediate Ca^{2+} refilling period in which different extracellular Ca^{2+} concentrations were added (Fig. 3D). Under these conditions, the amount of cytosolic Ca^{2+} elevation upon the second histamine stimulation reflects the efficiency of ER Ca^{2+} loading during the intermediate Ca^{2+} refilling period. Consistently with our data on ER Ca^{2+} content (Fig. 3A), the EC_{50} for extracellular Ca^{2+} for the second histamine-induced cytosolic Ca^{2+} elevation was 0.14 (0.10–0.19) mM (Fig. 3E).

3.3. Entering Ca^{2+} is redirected towards the cytosol upon SERCA inhibition

From the data presented above, it is tempting to speculate that ER Ca^{2+} refilling is a procedure that operates in priority compared to an elevation of cytosolic Ca^{2+} . In order to investigate this point, we performed similar experiments as previously described but in the presence of BHQ, a potent SERCA inhibitor, which prevents ER refilling. It should be noticed that the degree of ER Ca^{2+} depletion in the absence of extracellular Ca^{2+} did not differ whether histamine, BHQ or a combination of both was used [9]. Under depolarizing conditions, BHQ largely preserves cytosolic Ca^{2+} elevation upon Ca^{2+} re-addition to pre-stimulated cells (Fig. 4A). In line with these findings, in the presence of BHQ, the sensitivity of cytosolic Ca^{2+} elevation to extracellular Ca^{2+} was significantly increased (Fig. 4B, EC_{50} = 0.22 (0.18–0.28) mM) compared with that in the absence of the SERCA inhibitor (Fig. 1B). These data indicate that if ER Ca^{2+} refilling is prevented, entering Ca^{2+} , which normally is taking up by the ER for its refilling, is directed towards the cytosol.

3.4. In case of reduced Ca^{2+} influx, cytoplasmic Ca^{2+} elevation is preserved due to *trans*-ER Ca^{2+} flux

The data presented above reveal that in high K^{+} medium, a rather large difference in the cytosolic Ca^{2+} elevation occurred, depending whether or not the SERCA are functional. Here, we investigated the effect of a prolonged exposure of elevated IP_3 levels (i.e. continuous histamine application) on the cytosolic Ca^{2+} rise. In the continuous presence of histamine, the cytosolic Ca^{2+} elevation was much less reduced compared to conditions where histamine was washed-out prior the Ca^{2+} re-addition (Fig. 5A). The combination of BHQ and histamine under depolarizing conditions failed to further increase the cytosolic Ca^{2+} signal upon Ca^{2+} re-addition ($n = 63$; data not shown), indicating that the amount of Ca^{2+} that was pumped into the ER could be entirely mobilized by histamine. The findings that the reduction of cytosolic Ca^{2+} elevation upon Ca^{2+} re-addition to (pre-)stimulated cells was less when histamine was present, suggested that Ca^{2+} that refills the stores was released back to the cytosol due to the continuous presence of IP_3 . To challenge the hypothesis of such *trans*-ER Ca^{2+} flux, we stimulated the cells with histamine in the presence of extracellular Ca^{2+} , in low (i.e. physiological) and high K^{+} medium, and measured the cytosolic and the ER Ca^{2+} concentrations (Fig. 5B). In line with the concept of *trans*-ER Ca^{2+} flux, plasma membrane depolarization only weakly affected histamine-induced elevation in $[\text{Ca}^{2+}]_{\text{cyto}}$ (Fig. 5B, left panel), while the depletion of the ER was much more

pronounced in high K^+ medium (Fig. 5B, right panel). These data suggest that in the presence of IP_3 , ER becomes more depleted and does not get refilled if Ca^{2+} influx during cell stimulation is reduced. Moreover, it is tempting to speculate that the continuous ER depletion in the presence of an agonist, subsequently results in an long-lasting SOCE activation that, in turn, elevates cytosolic Ca^{2+} concentration.

3.5. In the presence of IP_3 , ER Ca^{2+} refilling depends on trans-mitochondrial Ca^{2+} flux that is more susceptible to a reduction of Ca^{2+} entry

In our previous work, we described that depending on the presence of an agonist (IP_3) the routes of Ca^{2+} to achieve ER Ca^{2+} refilling are different. While after washout of the agonist a direct Ca^{2+} cross talk at the junction of superficial domains of ER with the plasma membrane accounts for ER Ca^{2+} replenishment, in the presence of the agonist ER Ca^{2+} refilling vitally depends on mitochondrial Ca^{2+} supply, which is fueled by Ca^{2+} entry [9]. Our data presented in Figs. 2 and 3 demonstrate that ER replenishment via junctional Ca^{2+} transfer between SOC channels and superficial ER domains is slower but largely preserved even at conditions of reduced Ca^{2+} entry. In contrast, under conditions in which mitochondrial Ca^{2+} supply is responsible for ER Ca^{2+} refilling (i.e. in the presence of an agonist), the ER became depleted over time in conditions of reduced Ca^{2+} entry (Fig. 5B, right panel), thus, indicating that this path of ER Ca^{2+} refilling is more sensitive to attenuated Ca^{2+} entry. This assumption was further confirmed by Ca^{2+} re-addition experiments in high K^+ that revealed diminished mitochondrial Ca^{2+} elevation (Fig. 6A) and insufficient ER Ca^{2+} replenishment (Fig. 6B) in the presence of histamine. Remarkably, the fast switch in the Ca^{2+} route from the lower Ca^{2+} -sensitive mitochondrial Ca^{2+} transit to the high Ca^{2+} -sensitive junctional ion transfer between the ER and the plasma membrane became visible upon histamine washout (Fig. 6B). These two different Ca^{2+} paths directed towards the ER, their sensitivity to Ca^{2+} entry and their efficiency to accomplish ER refilling are schematically illustrated in Fig. 6C.

3.6. Termination of SOCE after agonist washout

The experimental conditions of reduced Ca^{2+} entry, in which the Ca^{2+} refilling of the ER remained efficient while cytosolic Ca^{2+} did only marginally increase, represent a suitable mode to challenge the concept that SOCE terminates exclusively by ER Ca^{2+} refilling but not by cytosolic processes that depend on global Ca^{2+} elevation. The correlation between ER Ca^{2+} content and SOCE activity was elucidated in experiments in which ER Ca^{2+} refilling was prevented by BHQ (Fig. 7A). Under these conditions, the addition of extracellular Ca^{2+} in high K^+ medium did not achieve termination of SOCE and, consequently, the switch to low K^+ buffer yielded strong enhancement of cytosolic Ca^{2+} elevation. This additional Ca^{2+} elevation may reflect the increased Ca^{2+} entry due to the restoration of the membrane potential. In contrast, in cells with emptied ER and after agonist washout the addition of Ca^{2+} even under high K^+ conditions was sufficient to terminate SOCE (by ER Ca^{2+} refilling) as indicated by no further cytosolic Ca^{2+} elevation upon the switch to low K^+ medium (Fig. 7B).

In order to visualize that SOCE is terminated even under high K^+ conditions where no strong cytosolic Ca^{2+} elevation takes place, cells were stimulated with histamine in Ca^{2+} -

free medium for 4.5 min followed by Ca^{2+} re-addition in high K^+ medium that was replaced after 15, 20, 30 or 45 s by low K^+ containing solution (inset of Fig. 7C). As shown in Fig. 7C, the increase in cytosolic Ca^{2+} level gradually reduced with increased delay of the switch to low K^+ medium, thus, indicating that within approx. 45 s in Ca^{2+} -containing high K^+ solution SOCE activity is terminated. Since the kinetics of SOCE inactivation in high K^+ medium was faster than the ER Ca^{2+} refilling dynamics under this condition which takes 2.2 ± 0.17 min for full Ca^{2+} refilling (see Fig. 2B and 3A), we conclude that SOCE termination does not need complete ER Ca^{2+} refilling and occurs once the ER is approx. 70% refilled.

4. Discussion

In this study we investigated the efficiency of ER Ca^{2+} refilling during Ca^{2+} re-addition protocol. We showed that the refilling of the ER is a very potent process that occurred without apparent alteration even under condition of reduced Ca^{2+} entry. In the presence of 0.5 mM extracellular Ca^{2+} concentration, or under depolarizing conditions, the extent of ER Ca^{2+} refilling was essentially unaltered, despite the rate of ER Ca^{2+} refilling and cytosolic Ca^{2+} elevation were significantly reduced. Another important finding was that the termination of SOCE was fast and followed the time course of ER Ca^{2+} replenishment while cytosolic Ca^{2+} elevation appeared not to be involved in the shutdown of the SOCE.

While the molecular mechanisms and putative messengers for an activation of SOCE are frequently investigated (for review see [2]), the kinetics of SOCE termination and the correlation between the amount of cytosolic Ca^{2+} elevation and the efficiency of ER refilling, and, thus, termination of SOCE is not clear. Here we investigated this particular point on an endothelial cell line derived from human umbilical vein. In a previous paper, we showed that the amount of cytosolic Ca^{2+} elevation measured and the extent of ER refilling do not strictly correlate [9]. In this study, we used two simple ways to reduce Ca^{2+} entry. Either the plasma membrane was depolarized by increasing the extracellular K^+ concentration, or the extracellular Ca^{2+} concentration and, thus, the source for Ca^{2+} to enter the cell, was reduced. To visualize Ca^{2+} entry, the classical Ca^{2+} re-addition protocol [4,20] was examined that allows isolation of SOCE from other possible route of Ca^{2+} entry.

Membrane depolarization reduced the cytosolic Ca^{2+} elevation upon Ca^{2+} re-addition to pre-stimulated cells by approx. 70%. This inhibition was found to be in a similar extent than that obtained if the Ca^{2+} content of the extracellular medium added was reduced from 2 to 0.5 mM.

In contrast to the strongly diminished cytosolic Ca^{2+} elevation upon Ca^{2+} re-addition to pre-stimulated cells under depolarizing/low Ca^{2+} conditions, the competence of ER Ca^{2+} refilling, measured with genetically encoded fluorescent probes (i.e. vYc4er [14], apoK1er [15] or D1_{ER} [16]) was not altered in either of these conditions. However, whilst the final equilibrium Ca^{2+} level in the ER was less sensitive to a reduction of Ca^{2+} entering, the rate of refilling was highly sensitive to reduced Ca^{2+} entry. These data point to a very high efficiency of ER Ca^{2+} refilling even in conditions of reduced Ca^{2+} entry and suggest that the reduced rate of ER Ca^{2+} loading is compensated by a prolongation of the Ca^{2+} sequestration period. This shape of ER Ca^{2+} changes has been described to be most probably due to a

steep dependency of SERCA on the Ca^{2+} concentration, which is achieved in the ER lumen during Ca^{2+} re-addition [21,22].

The discrepancy between the sensitivity of cytosolic Ca^{2+} elevation and the robustness of ER Ca^{2+} refilling to depolarizing/low Ca^{2+} conditions, might be due to the activity of the SERCA pumps that master ER Ca^{2+} refilling despite limited Ca^{2+} entry, or to a store refilling from the extracellular space directly to the ER lumen [23]. Such direct ER Ca^{2+} refilling was initially postulated when the capacitative Ca^{2+} entry model was proposed [3,24]. Recently, data were presented that indicate that the ER can get refilled even in the presence of SERCA inhibitors like thapsigargin or BHQ, and that the cytosolic Ca^{2+} elevation that took place during SOCE, was due to a “secondary” release of Ca^{2+} from the ER [25]. While the concept that Ca^{2+} entering the cell “tunnels” directly into the ER, and, thus, by-passes the cytosol is attractive, several other reports could not elaborate supportive data (for review see [2]). In line with the latter reports, a direct Ca^{2+} tunneling into the ER does not appear to take place in the cell used in this study, as in the presence of SERCA inhibitors, no refilling of the ER occurred [26]. Additionally, our findings that in the presence of BHQ, a larger cytosolic Ca^{2+} elevation was found, stand against a direct refilling procedure.

From our data that under conditions with limited Ca^{2+} entry ER Ca^{2+} refilling, but not cytoplasmic Ca^{2+} elevation remained unchanged, we can conclude that in the absence of BHQ or histamine, an important part of Ca^{2+} entering the cell directly refills the ER via SERCA pumps, while only the excessive Ca^{2+} accounts for the cytosolic Ca^{2+} elevation that is measurable with fura-2. However, our findings that in the presence of BHQ cytoplasmic Ca^{2+} elevation under depolarized/low Ca^{2+} conditions was largely preserved indicate that if the ER cannot get refilled due to an inhibition of the SERCA pumps entering Ca^{2+} solely elevates the Ca^{2+} concentration in the cytosolic compartment.

On the other hand, our findings that in the presence of histamine, cytosolic Ca^{2+} elevation upon Ca^{2+} re-addition under depolarizing conditions by far exceed that obtained in the absence of histamine suggest that in case of the presence of an agonist during the re-addition phase, Ca^{2+} entry refills the ER but is, subsequently, released to the cytosol, due to the continuous presence of IP_3 . This two-phases phenomenon was elegantly described by van Breemen et al. in vascular smooth muscle cells [24,27]. By measuring the contraction developed during Ca^{2+} entry this group showed that if the ER was filled, depolarization-induced Ca^{2+} entry leads to immediate muscle contraction as a measure of cytosolic Ca^{2+} elevation, whereas if the ER was previously emptied, the contraction was delayed due to the initial ER Ca^{2+} refilling [27].

Interestingly, when endothelial cells were stimulated with histamine in the presence of extracellular Ca^{2+} , the effect of membrane depolarization on the plateau phase was rather weak, and even several minutes after stimulation in high K^+ buffer the cytosolic Ca^{2+} concentration remained elevated. This modest effect of high K^+ medium on the cytosolic Ca^{2+} plateau due to histamine stimulation was surprising, as changing membrane potential strongly attenuates the driving force for Ca^{2+} entry [28]. The importance of the membrane hyperpolarization for Ca^{2+} -dependent endothelial cell function such like the formation of

NO[•] has been early described [29]. As NO[•] production by endothelial nitric oxide synthase mainly depends on the influx of Ca²⁺, the augmentation of Ca²⁺ entry by membrane depolarization consequently results in a diminution in the NO[•] production. More recently, Kamouchi et al. [30] measured simultaneously the membrane potential and the intracellular Ca²⁺ concentration in endothelial cells and directly demonstrated the strong interdependence between both parameters. In particular, the interrelation between membrane potential and Ca²⁺ entry was illustrated by experiments in which increasing the extracellular K⁺ concentration markedly decreased the Ca²⁺ plateau due to agonist stimulation. In variation to this report, we did not observed such a high impact of membrane depolarization on the Ca²⁺ plateau level. Since, under high K⁺ conditions, the depletion of the ER was much more pronounced compared with that obtained using normal K⁺-containing medium, it appeared that the cytosolic Ca²⁺ elevation remained at a high level, but at the “expense” of more pronounced store depletion.

The discrepancy between our results presented herein and previously published data [30], may reflect a different contribution of Ca²⁺ entry to cytosolic Ca²⁺ elevation under the given experimental protocols. Under conditions in which the Ca²⁺ cycle between the ER, the mitochondria and the cytosol takes place [9,26], the cell does not loose much Ca²⁺ by plasma membrane Ca²⁺ extrusion mechanisms and, thus, only limited Ca²⁺ entry would be sufficient to fuel the longtermed elevation in cytosolic Ca²⁺ under agonist stimulation. Evidence for the existence of such cellular Ca²⁺ circuit are provided in our findings that the ER is efficiently refilled even in the presence of an agonist despite increased cytosolic Ca²⁺ levels (Fig. 4B). Additionally, inhibition of SERCA unmasks the cellular Ca²⁺ circuit as it resulted in a more transient cytosolic Ca²⁺ elevation upon histamine in the absence of extracellular Ca²⁺ (Fig. 3A), thus, indicating that once Ca²⁺ cannot cycle back into the ER it is lost to the outer space. On the other hand, under conditions in which the cellular Ca²⁺ circuit is impaired by either divergent cell culture conditions, which may alter the expression level of Ca²⁺ shuttling proteins, or by the experimental conditions or the organelle's architecture, the contribution of Ca²⁺ entry to long-termed cytosolic Ca²⁺ elevation upon stimulation with an agonist gains importance. It is tempting to speculate that under these conditions, the cytosolic plateau phase upon stimulation with an agonist is more sensitive to a diminution of Ca²⁺ entry by membrane depolarization.

Nevertheless, depending on the presence of the agonist/IP₃, there are obviously two different routes by which external Ca²⁺ is directed into the ER [9]. Namely, after washout of the agonist ER Ca²⁺ refilling is achieved by a direct Ca²⁺ cross-talk at the junction of superficial domains of the ER with the plasma membrane. Our data presented herein demonstrate that this route for ER replenishment, which builds on a junctional Ca²⁺ transfer between SOC channels and superficial SERCA, is not affected by membrane depolarization. On the other hand, in the presence of the agonist/IP₃, mitochondrial Ca²⁺ supply is pivotal for ER Ca²⁺ refilling. Since under these conditions the ER Ca²⁺ content as well as the mitochondrial Ca²⁺ elevation were found to be highly susceptible to reduced Ca²⁺ entry by membrane depolarization, we speculate that this path of ER Ca²⁺ recovery is more susceptible to attenuated Ca²⁺ entry most likely due to the low Ca²⁺ sensitivity of mitochondrial Ca²⁺ uniporter [31].

Recently we reported that protein folding within the ER is accomplished even during cell stimulation with an IP₃ generating agonist by mitochondria dependent ER Ca²⁺ replenishment [10]. As this process seems to be highly dependent on the extracellular Ca²⁺ concentration, pathological conditions of severe ionized hypocalcaemia induced by e.g. colloid induced hemodilution of traumatized patients [32] might lead to complications due to insufficient ER Ca²⁺ refilling.

Besides the contribution of Ca²⁺ entry to ER refilling and cytosolic Ca²⁺ elevation, our experiments in which the external solution was switched from high to physiological K⁺ concentration at given time points, allowed estimation of SOCE termination. It is believed that SOCE is switched off once the ER gets refilled but the actual kinetics of this process is unclear. The inactivation of Ca²⁺-release-activated Ca²⁺ channels (CRAC) has been found to be a rather slow process that is initiated either by ER Ca²⁺ refilling [33] or by a slow feedback by elevated cytosolic Ca²⁺ [34]. Although it is not known whether the SOCE results from CRAC activity in our cell model, we could observe that its inactivation correlates with the ER Ca²⁺ refilling. Indeed, in high K⁺ as well as in physiological K⁺ medium, the ER is completely refilled within 2 min, while already after 1 min no more Ca²⁺ entry could be observed, thus, indicating that the termination of SOCE is a rapid event which is not delayed compared to ER refilling. These data are in line with previous reports that described that in endothelial cells only parts of the ER accounts for activation of SOCE [35]. In addition, such fast termination of SOCE supports the concept of biological labile second messenger for SOCE activation [36-38] which could explain SOCE shutdown without the generation of an inhibitory messenger.

In summary, our data reported herein point to the high efficiency and flexibility of the ER Ca²⁺ uptake machinery in non-excitabile cells. Notably, in the absence of an agonist/IP₃ ER Ca²⁺ refilling was found to be accomplished even under conditions of strongly diminished Ca²⁺ entry and the lack of a pronounced cytosolic Ca²⁺ elevation. However, in the presence of an agonist/IP₃, ER replenishment is more susceptible for reduced Ca²⁺ entry which is likely due to the limited mitochondrial Ca²⁺ supply that relays entering Ca²⁺ towards the ER under these conditions. Moreover, the termination of SOCE strictly correlated with ER Ca²⁺ refilling and occurred rather independently from cytosolic Ca²⁺ elevation. This data indicate that ER Ca²⁺ refilling represents a privileged process that does not necessarily correlate with the cytosolic Ca²⁺ dynamics.

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Abbreviations

BHQ	2,5-di- <i>tert</i> -butylhydroquinone
[Ca²⁺]_{cyto}	free cytosolic Ca ²⁺ concentration

[Ca²⁺]_{er}	free intraluminal ER Ca ²⁺ concentration
[Ca²⁺]_{mito}	free mitochondrial Ca ²⁺ concentration
SOCE	store-operated Ca ²⁺ entry
ER	endoplasmic reticulum
IP₃	inositol 1,4,5-triphosphate
NCX_{pm}	plasma membrane Na ⁺ /Ca ²⁺ exchanger
RP-mt	mitochondrial targeted ratiometric pericam
PMCA	plasma membrane-Ca ²⁺ ATPase(s)
SERCA	sarcoplasmic/endoplasmic reticulum Ca ²⁺ pumps
vYC4er	ER-targeted venus cameleon 4

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Fig. 1.

Modulation of store-operated Ca^{2+} entry (SOCE) by membrane depolarization or decreased extracellular Ca^{2+} concentration. Cytosolic Ca^{2+} signals were recorded using fura-2. In order to deplete the ER, cells were stimulated with 100 μM histamine in the absence of extracellular Ca^{2+} . As a measure of SOCE, cytosolic Ca^{2+} elevation upon Ca^{2+} re-addition after agonist washout was obtained. (A) To induce plasma membrane depolarization, extracellular K^+ concentration was increased from 5 to 130 mM 2 min prior Ca^{2+} re-addition (filled symbols, dotted line, $n = 47$). For comparison, experiments in physiological medium

(i.e. 5 mM K^+) are shown (open symbols, continuous line, $n = 43$). $*P < 0.05$ vs. control. (B) Left panel: as indicated, different Ca^{2+} concentrations ranging from nominal Ca^{2+} -free to 2 mM Ca^{2+} were added ($n = 32-91$). Right panel: concentration–response curve of the cytosolic Ca^{2+} elevation achieved by the various extracellular Ca^{2+} buffers. (C) Left panel: a comparison of the effect of membrane depolarization with that of various extracellular Ca^{2+} concentration (values are expressed in percentage while the maximal effect under control conditions was set to 100%). Right panel: schematic illustration of the three experimental conditions: maximal $[Ca^{2+}]_{cyto}$ elevation was obtained in normal K^+ -containing buffer and addition of 2 mM Ca^{2+} (a, control), reduced extracellular Ca^{2+} in normal K^+ -containing buffer (b, low $[Ca^{2+}]_e$) and depolarizing conditions in normal Ca^{2+} -containing solution but high extracellular K^+ (c, 130 mM K^+).

Fig. 2.

ER Ca^{2+} refilling is largely preserved under conditions of membrane depolarization. (A) Following the experimental protocol shown in Fig. 1A in which the ER of pre-stimulated cells was refilled by the addition of 2 mM Ca^{2+} in physiological extracellular K^+ (i.e. 5 mM K^+ , open symbols, continuous line, $n = 43$) or under high extracellular K^+ conditions (i.e. 130 mM K^+ , filled symbols, dotted line, $n = 47$) (grey in the present figure), ER Ca^{2+} content was estimated by a further stimulation with 100 μM histamine in the absence of extracellular Ca^{2+} . * $P < 0.05$ vs. cytosolic Ca^{2+} elevation upon Ca^{2+} re-addition (grey line). (B) In EA.hy 926 cells that were transiently transfected with vYC4er, ER Ca^{2+} content was monitored upon stimulation with 100 μM histamine followed by Ca^{2+} refilling after agonist washout under control conditions (open symbols, continuous line, $n = 12$) and in high K^+ -containing buffer (filled symbols, dotted line, $n = 15$).

Fig. 3.

ER Ca^{2+} refilling is largely assured even under conditions of reduced Ca^{2+} influx. (A) The impact of the concentration of extracellular Ca^{2+} on ER Ca^{2+} refilling was assessed in cells transiently transfected with YC4er ($n = 6-15$). (B) Calculation of the rate of ER Ca^{2+} refilling from the external Ca^{2+} concentration. The kinetics of ER Ca^{2+} loading under conditions with different external Ca^{2+} concentrations were fitted according to a Boltzmann sigmoid curve (upper panel) and used to calculate the derivation $d(\text{Ratio})/dt$. (C) Concentration-response curves of the rate (bold line, $n = 6-15$) and extent of ER Ca^{2+} refilling (bold dotted line, $n = 6-15$) and $[\text{Ca}^{2+}]_{\text{cyto}}$ elevation (grey line and symbols; Fig.

1C) upon Ca^{2+} re-addition. (D) After addition of different extracellular Ca^{2+} concentration (nominal Ca^{2+} free to 2 mM) to pre-stimulated cells (grey lines represent this protocol already described under Fig. 1B), cells were stimulated a second time with 100 μM histamine in the absence of extracellular Ca^{2+} ($n = 32\text{--}91$). (E) Concentration–response curves of the second histamine-induced cytosolic Ca^{2+} elevation (bold continuous line) compared with recurrences of the ER Ca^{2+} refilling (grey dotted line) and $[\text{Ca}^{2+}]_{\text{cyto}}$ elevation upon Ca^{2+} re-addition (grey continuous line). * $P < 0.05$ vs. cytosolic Ca^{2+} elevation upon Ca^{2+} re-addition (grey line).

Fig. 4.

Entering Ca^{2+} is directed towards the cytosol upon SERCA inhibition. (A) The impact of SERCA inhibition with $15 \mu\text{M}$ BHQ on intracellular Ca^{2+} mobilization to $100 \mu\text{M}$ histamine followed by an addition of 2 mM extracellular Ca^{2+} in high K^+ -containing buffer. $[\text{Ca}^{2+}]_{\text{cyto}}$ signals were assessed in fura-2 loaded cells. Traces represent average curves of 15 measurement in the absence of BHQ (filled symbols, continuous line) and 63 recordings in the presence of BHQ (open symbols, dotted line). $*P < 0.05$ vs. in the absence of BHQ. (B) Left panel: in low K^+ medium (i.e. 5 mM), different extracellular Ca^{2+} concentration ranging from nominal free Ca^{2+} to 2 mM were added to pre-stimulated cells in the continuous presence of $15 \mu\text{M}$ BHQ ($n = 29-45$). Right panel: the peak magnitudes reached upon the addition of given extracellular Ca^{2+} concentration in the presence of BHQ were normalized to the maximal amplitude and used for calculation of a concentration-response curve. For comparison replications from Fig. 1D and C are presented as grey curves. $*P < 0.05$ vs. cytosolic Ca^{2+} elevation upon Ca^{2+} re-addition in the absence of BHQ (grey line).

Fig. 5.

A *trans*-ER Ca^{2+} flux maintains increased $[Ca^{2+}]_{cyto}$ levels under depolarizing conditions. (A) 2 mM extracellular Ca^{2+} were added to pre-stimulated cells (100 μ M histamine) in the continuous presence of the agonist under conditions of physiological extracellular K^+ (i.e. 5 mM K^+ , open circles, continuous line, $n = 45$) or in the presence of high extracellular K^+ (i.e. 130 mM K^+ , closed circles, dotted line, $n = 59$), while $[Ca^{2+}]_{cyto}$ signals were recorded using fura-2. Alternatively histamine was removed before 2 mM Ca^{2+} was readded in the presence of 130 mM extracellular K^+ (filled squares, dashed line, $n = 15$). $*P < 0.05$ vs. control. $\#P < 0.05$ vs. in the absence of histamine. (B) Left panel: as indicated, cells were stimulated with 100 μ M histamine in the presence of 2 mM extracellular Ca^{2+} under conditions of low extracellular K^+ (i.e. 5 mM K^+ , open symbols, continuous line, $n = 43$) or in high extracellular K^+ buffer (i.e. 130 mM K^+ , filled symbols, dotted line, $n = 41$). $*P <$

0.05 vs. control. Right panel: cells transiently transfected with D1_{ER} were used to investigate changes of the ER Ca²⁺ content upon stimulation with 100 μM histamine in low extracellular K⁺ buffer (i.e. 5 mM K⁺, open symbols, continuous line, *n* = 13) and high extracellular K⁺ medium (i.e. 130 mM K⁺, filled symbols, dotted line, *n* = 17). **P* < 0.05 vs. control.

Fig. 6.

Two routes for ER Ca^{2+} refilling. (A) Cells expressing RP-mt were used to measure mitochondrial Ca^{2+} sequestration upon Ca^{2+} re-addition in the presence of 100 μM histamine under conditions of physiological (i.e. 5 mM; $n = 8$) and high (i.e. 130 mM; $n = 22$) external K^+ . (B) Cells were transiently transfected with D1_{ER} and ER replenishment was monitored upon Ca^{2+} re-addition in the presence of 100 μM histamine under conditions of physiological ($n = 6$) and high ($n = 5$) external K^+ . (C) Schematic illustration of the two different Ca^{2+} paths that achieve ER Ca^{2+} refilling upon Ca^{2+} re-addition after agonist

washout in physiological K^+ (I) or high K^+ buffer (II), and in the presence of histamine in physiological K^+ (III) or high K^+ buffer (IV). Accordingly, the respective conditions are indicated in (A) and (B) and Fig. 2B.

Fig. 7.

Termination of SOCE. (A) ER Ca^{2+} stores were emptied by application of $15\text{ }\mu\text{M}$ BHQ, and 2 mM Ca^{2+} was then readded in high K^+ buffer (130 mM K^+). After 4 min, the medium was switched to physiological K^+ concentration (5 mM K^+) ($n = 14$). (B) ER Ca^{2+} stores were emptied by the application of $100\text{ }\mu\text{M}$ histamine and after the agonist washout, 2 mM Ca^{2+} were readded in high K^+ buffer (130 mM K^+). After 3 min, the medium was switched to physiological K^+ concentration (5 mM K^+) ($n = 10$). (C) The inset represents the protocol used to evaluate the termination of SOCE. After ER depletion, 2 mM Ca^{2+} were readded in

high K^+ buffer (130 mM K^+), and after different time points (15 and 45 s are represented), the medium was switched to normal K^+ concentration (5 mM K^+). Columns represents the statistic evaluation of the impact of reducing the K^+ concentration to normal level (5 mM K^+) at different time points (15, 20, 30 and 45 s) after Ca^{2+} re-addition ($n = 16-48$).