Biochemical and Biophysical Research Communications 414 (2011) 246–251

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Vesicularization of the endoplasmic reticulum is a fast response to plasma membrane injury

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ARTICLE INFO

Article history:
Received 24 August 2011
Available online 17 September 2011

Keywords:
Endoplasmic reticulum
Structure
Tubule
Cell membrane
Permeabilisation
Ca^{2+}

ABSTRACT

The endoplasmic reticulum of most cell types mainly consists of an extensive network of narrow sheets and tubules. It is well known that an excessive increase of the cytosolic Ca^{2+} concentration induces a slow but extensive swelling of the endoplasmic reticulum into a vesicular morphology. We observed that a similar extensive transition to a vesicular morphology may also occur independently of a change of cytosolic Ca^{2+} and that the change may occur at a time scale of seconds. Exposure of various types of cultured cells to saponin selectively permeabilized the plasma membrane and resulted in a rapid swelling of the endoplasmic reticulum even before a loss of permeability barrier was detectable with a low-molecular mass dye. The structural alteration was reversible provided the exposure to saponin was not too long. Mechanical damage of the plasma membrane resulted in a large-scale transition of the endoplasmic reticulum from a tubular to a vesicular morphology within seconds, also in Ca^{2+}-depleted cells. The rapid onset of the phenomenon suggests that it could perform a physiological function. Various mechanisms are discussed whereby endoplasmic reticulum vesicularization could assist in protection against cytosolic Ca^{2+} overload in cellular stress situations like plasma membrane injury.

1. Introduction

The endoplasmic reticulum (ER) derives its name from its microscopic structure, as it forms a highly interconnected network of narrow membrane sheets and tubules. It is spread throughout the cell with in general a higher membrane density around the nucleus than in the cell periphery. Visualization of the ER in live-cell microscopy shows that tubules move, branch, fuse and form de novo [1]. This dynamic stability of the highly fluid ER membranes [2] is not surprising in view of its function in the initial steps of the secretory pathway and in various signaling events involving interactions with other cellular subcompartments [3,4]. For example, the ER is the major intracellular Ca^{2+} store. It is well known that the ER takes part in the generation of spatially and temporally complex cytosolic Ca^{2+} signals by preferentially releasing Ca^{2+} at specific sites and in response to specific messengers [5,6]. The normal ER morphology guarantees a very high surface to volume ratio, facilitating exchange of compounds with the cytosol, and increasing the chances of close apposition to other intracellular organelles in the crowded intracellular context. Various ER regions are located at strategic positions allowing mutual control between the ER membranes and other organelles [4,7,8]. The largest of these regions is the nuclear membrane, which is continuous with the ER. Peripheral ER microdomains form close contacts with the plasma membrane or its subdomains, allowing preferential interactions with specific ion channels, transporters and receptors. Close apposition of ER membranes to mitochondria may allow movement of Ca^{2+} between both organelles via a pathway that is shielded from the cytosol [3,9]. Therefore structural alterations of the ER may have profound effects on its function.

The most common structural alteration of the ER is a transition to a predominantly vesicular morphology. Probably the best known condition causing ER vesicularization is an excessive elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) by application of the ionophore ionomycin [10,11], by application of the ER Ca^{2+} pump inhibitor thapsigargin ([11]), or by overexpression of Ca^{2+} channels [12]. The observation that ER vesicularization may occur during the harsh treatment of cells necessary for their isolation [13], or also following 5 min of inhibition of glycolysis and mitochondrial respiration [14] may similarly be explained by an excessive increase of $[Ca^{2+}]_c$. Also more physiological increases of $[Ca^{2+}]_c$ have been reported to induce a transition of the ER from tubular structures to small vesicles [10,15]. In addition, there exist slower processes causing changes of ER morphology over hours or days, for example ER swelling associated with various forms of cell death [16] or with the overexpression of some specific proteins in cultured cells such as the chloride-iodide transporter pendrin [17] or the vanilloid receptor 1 [12].

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doi:10.1016/j.bbrc.2011.09.065

We here document that in cultured cells ER vesicularisation is a rapid response to membrane permeabilization either by saponin treatment or by mechanical injury and show that this response occurs independently of changes of cytosolic Ca^{2+}. The possibility is discussed that this structural change could help to counteract cytosolic Ca^{2+} overload and thereby promote cell survival.

2. Materials and methods

2.1. Cell culture and transfection

Cells of the HaCaT human keratinocyte cell line [18], HeLa cells and COS-1 cells were seeded into chamber slides (Nunc Intermed, Roskilde Denmark) at a density of 3×10^4/well and grown in a humidified incubator at 37 °C and 5% CO_2. The growth medium consisted of DMEM, supplemented with 10% fetal bovine serum, 3.5 mM L-glutamine, 88 U/ml penicillin and 88 μg/ml streptomycin.

After 1 day the cells were transfected with the cameleons YC3.3ER or GT-YC3.3 (gifts from R.Y. Tsien, University of California, San Diego) for imaging the endoplasmic reticulum and FRET measurements [19]. Transfection of the cameleons in the expression vector pcDNA3 was carried out using the transfection reagent GeneJuice (Novagen, WI, USA). The experiments were performed between 2 and 3 days after transfection.

2.2. Solutions and chemicals

Krebs solution contained (mM): 135 NaCl, 6 KCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 12 glucose and 12 Na/Hepes (pH 7.3). High K^+ solution was obtained by replacing NaCl with KCl and Ca^{2+}-free conditions by replacing $CaCl_2$ by 2 mM EGTA. Saponin-was dissolved at 1 mg/100 ml in a solution containing (mM): 120 KCl, 30 imidazole, 2 EGTA, 2 $MgCl_2$, 1 ATP (solution S).

Saponin, ionomycin and ATP were purchased from Sigma (St. Louis, MO, USA). Fura-2 AM, TO-PRO3 and ER-Tracker Blue-White DPX were obtained from Molecular Probes (Invitrogen, Eugene, OR, USA).

2.3. Microscopy

Transfected live cells were imaged on an Olympus IX-81 inverted microscope operated by the Cell^R system. The whole setup, including the microscope, the solutions and the perfusion system were enclosed in a temperature-controlled Lucite chamber maintained at 37 °C. Fluorescence was recorded by a FView cooled CCD camera (Olympus), using a 40× UApo (NA 1.4) oil-immersion objective. For standard fluorescence observations we used a triple emission filter (460, 530 and 630 nm) and a 492/18 (for cameleons) or 572/23 nm (for TO-PRO3) excitation filter.

Mechanical stabbing of a single cell was done by briefly touching the cell with a 30G × 1/2" needle (Becton Dickinson, NJ) mounted on a micromanipulator.

FRET signals from the cameleon probe were determined by emission ratio measurements using a 436/15 excitation filter and simultaneous detection of emission at two wavelengths via a Dual-View™ imager (Optical Insights, Santa Fe, NM), via a 505 nm dichroic mirror and 480/30 and 535/40 emission filters. In selected regions of the image, the fluorescence ratio of both channels after background subtraction was then calculated using the Cell^R software.

For intracellular Ca^{2+} measurements, cells were loaded in Krebs containing 1 μM Fura-2 AM at room temperature for 30 min. After washing the excess of dye, the fluorescence ratio was recorded at 510 nm using 340 and 380 nm excitation filters. TO-PRO3, a red-fluorescent cell impermeant nucleic acid stain was used to record the loss of the permeability barrier of the plasma membrane. To visualize the ER in live non-transfected cells, ER-Tracker Blue–White DPX was applied at 1 μM for 30 min at 37 °C.

3. Results

3.1. Saponin permeabilization causes ER restructuring

Live cells expressing the ER-targeted cameleon YC3.3ER clearly reveal the structure of the ER, including a fine meshwork of narrow tubules that continuously moves and restructures at high resolution but whose general topology remains stable for several hours during standard observation conditions (superfusion with normal Krebs solution at 37 °C), as described previously [1].

Plasma membranes are typically enriched in cholesterol. Permeabilization of cells with cholesterol-complexing compounds like saponin or digitonin is a frequently used procedure to gain direct access to intracellular organelles by selective permeabilization of the plasma membrane while preserving the permeability barrier of the endoplasmic reticulum and mitochondria (see [20,21] for review). Cameleon-transfected HaCat cells were pretreated with Ca^{2+}-free Krebs solution and then exposed to Ca^{2+}-free, ATP-containing high-K^+ solution (S solution) supplemented with a low concentration of saponin to selectively permeabilize the plasma membrane. It is confirmed in Fig. 1 that this procedure conserves the functional integrity of the ER membrane barrier. As the YC3.3ER indicator [22] has a relatively high affinity for Ca^{2+} (K_d < 10 μM), the experiment demonstrates the complete Ca^{2+} depletion of the ER following the exposure to saponin. An increase

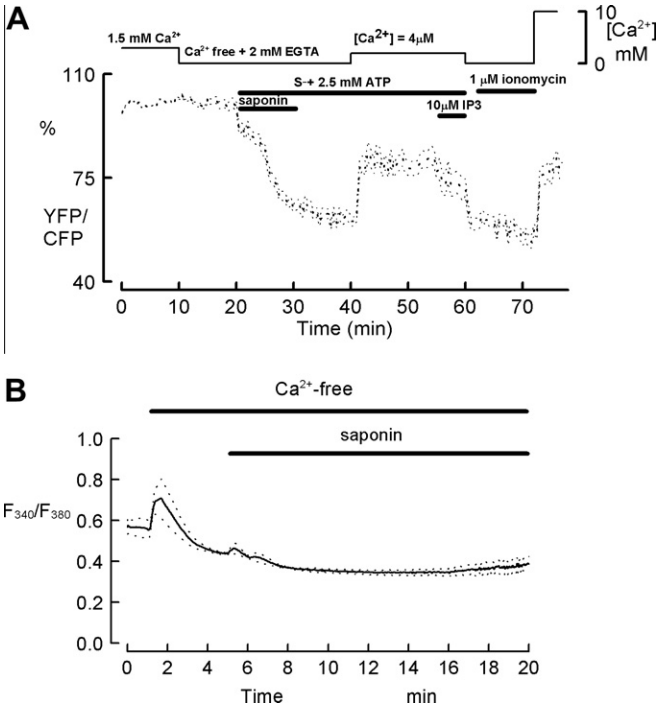

Fig. 1. Changes of the free Ca^{2+} concentration during saponin treatment. (A) FRET measurements of the ER-targeted YC3.3ER cameleon show that the permeability barrier of the ER and the function of its Ca^{2+} pumps are maintained despite the ER vesiculation. Cells were treated for 10 min with 10 μg/ml saponin in S solution with ATP. After addition of 4 μM free Ca^{2+} to the empty stores, the ER can take up Ca^{2+} again due to the SERCA pump activity. Addition of 10 μM IP_3 induced a partial Ca^{2+} release. At the end, minimum and maximum emission ratios were determined by application of ionomycin in EGTA or in 10 mM Ca^{2+}. The curve represents the mean of 6 cells. (B) Changes of $[Ca^{2+}]_c$ measured with Fura-2 in the same conditions as in (A). Changing to Ca^{2+}-free solution induced a rise of $[Ca^{2+}]_c$. Further addition of saponin had a negligible effect.

of the Ca^{2+} concentration to 4 μM in these permeabilized cells caused a rapid refilling of the ER due to SERCA Ca^{2+} pump activity and the addition of IP_3 induced a release of Ca^{2+}, confirming the functional integrity of the ER permeability barrier and its Ca^{2+} pumps and channels, as reported previously using tracer $^{45}Ca^{2+}$ fluxes [23]. Measurements of cytosolic Ca^{2+} in parallel experiments showed that $[Ca^{2+}]_c$ transiently increased on superfusion of cells with Ca^{2+}-free solution (Fig. 1B). This effect may be caused by the opening of connexin hemichannels, which are opened when the extracellular Ca^{2+} concentration decreases below 1.5 mM [24]. The subsequent addition of saponin to the Ca^{2+}-free solution had little effect on $[Ca^{2+}]_c$.

However, following the addition of saponin the ER transformed after a few minutes from a fine network into a large number of small vesicles spread-out over the entire cytoplasm, which thereupon combined to form giant vesicles (Figs. 2B and 3B). This vesicularization was maximal after about 8–15 min. Parallel experiments on non-transfected cells using a live-cell ER tracker that labels the membranes of the ER instead of the lumen showed the same effect, except for the fact that, as expected for a vesicularization process, the round structures did not appear as filled circles but as fluorescent membranes surrounding a dark space (data not shown). In view of the rapid onset of the structural change, we investigated how it correlates with the saponin-induced plasma membrane permeabilization by using the fluorescent nuclear dye TO-PRO3, which is excluded from intact cells. Surprisingly, the restructuring preceded by several minutes the staining of the nucleus (Fig. 2B). This observation indicates that the ER structure may be very sensitive to slight perturbations of the surface membrane integrity. Omission of saponin from solution S did not affect the ER morphology (data not shown).

Remarkably, when cells were subjected to a transfection protocol, those cells that successfully took up the expression vector and expressed the cDNA insert were slower to take up the TO-PRO3 reporter than non-transfected cells (Fig. 2B). TO-PRO3 permeated most non-transfected cells several minutes before the dye appeared in transfected cells. This difference was not related to the cell type or to the specific cDNA used for transfection, since it was also observed in COS-1 and HeLa cells, and after transfection with a Golgi-targeted cameleon or with EGFP expressed in the cytosol (data not shown). We did not further investigate this phenomenon.

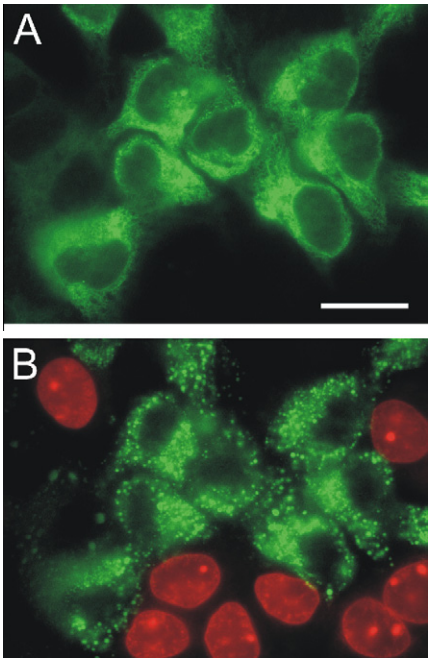

Fig. 2. Fluorescence images of live HACAT cells expressing the ER-targeted YC3.3ER cameleon. (A) In normal Krebs solution. (B) 8 min after superfusion with S solution containing 10 μg/ml saponin and 1 mM ATP, showing the ER vesiculation (green). At this stage the transfected cells are not yet permeabilized, as demonstrated by the exclusion of TO-PRO3 (red). In contrast, most non-transfected cells are permeabilized. Scale bar = 20 μm.

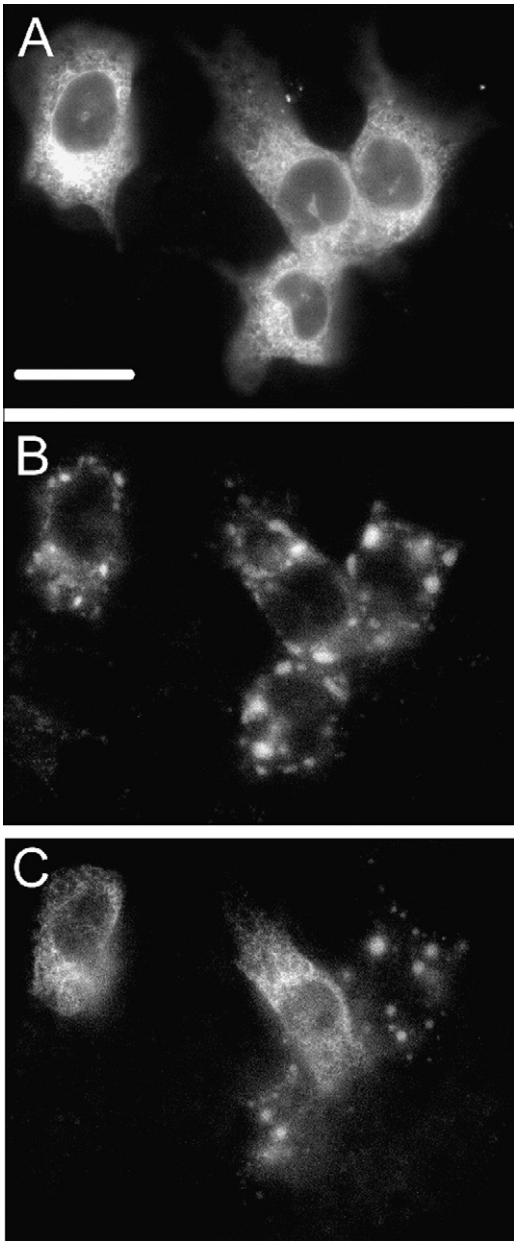

Fig. 3. Reversibility of ER restructuring induced by saponin. (A) Cameleon-transfected HaCaT cells in normal Krebs solution. (B) The same cells showing large ER vesicles after exposure for 15 min to 10 μg/ml saponin in S solution. (C) Recovery for 75 min in cell culture medium. Two of the cells reformed a fine network ER, whereas two others failed to recover. Scale bar = 20 μm.

The saponin-induced ER restructuring is slowly reversible provided the time of exposure to the detergent is not too long. Fig. 3 shows four cells exposed for 15 min to saponin. Two of these cells did recover a normal network ER after 75 min of saponin washout. In contrast to the dramatic restructuring of the ER, saponin had little if any effect on the morphology of the Golgi apparatus as observed with a Golgi-targeted cameleon (data not shown).

3.2. Effect of mechanically-induced membrane injury on ER structure

Since the experiments with saponin indicate that the perturbation of plasma membrane integrity may be a trigger for ER vesiculation, we studied the effect of a mechanically-induced membrane trauma. HeLa cells were mechanically stimulated with a steel needle mounted on a micromanipulator. Since we wanted to exclude the possibility that any ER rearrangement caused by the mechanical disturbance would be mediated by a strong rise of $[Ca^{2+}]_c$ [11] and also to mimic the conditions of the saponin-induced vesiculation, the experiments were conducted after pre-incubation in Ca^{2+}-free solution for at least 20 min. A typical experiment is shown in Fig. 4 and movie1 in the Supplementary Material. ER vesicularization started within seconds in the region near the zone touched by the needle. After 9 s, ER restructuring had occurred all over the cell. The plasma membrane of the cell shown in Fig. 4 was probably not excessively damaged because there was no observable loss of cell content and the cell maintained its shape. The ER did not return to a tubular structure within the observation period of 45 min, except for one cell out of 15 (not shown).

Mild deformation of the cell shape by gentle touching of the cell or by a sudden stream of solution form a 0.3 mm needle positioned a few microns away from the cell did not alter the network architecture of the ER (data not shown).

4. Discussion

We have shown that saponin skinning or mechanical injury of various cultured cell types rapidly results in ER vesicularization and that this restructuring is independent of changes of intracellular Ca^{2+} concentrations. This process is especially fast in case of mechanically-induced trauma, which results in dramatic restructuring on a time scale of subseconds. The vesicularization elicited by saponin did not depend on the presence of the cameleon because it was also seen in non-transfected cells in which the ER was visualized with a live-cell ER tracker dye. Neither did the expression of the ER-targeted cameleon protein on its own induce ER vesicularization in the majority of the cells, although cells with vesicular ER were seen along with normal cells. Since ER vesicularization precedes various forms of cell death [16], these cells could be dying cells and were therefore not further analyzed. The restructuring we observed is thus independent of protein overexpression, as has been observed for example in cells transfected with cDNA encoding pendrin [17] or vanilloid receptor 1 [12].

We could clearly show that the trigger mechanism responsible for structural changes of the ER in our experimental conditions is Ca^{2+}-independent. These results thus demonstrate that besides the Ca^{2+}-induced ER vesiculation previously reported [10,11, 13,15], there also exists a Ca^{2+}-independent but as yet unidentified trigger mechanism. A rise of $[Ca^{2+}]_c$ was prevented by eliminating both Ca^{2+} influx and Ca^{2+} release from the ER, respectively by incubation in Ca^{2+}-free EGTA-containing solution and by depletion of ER lumenal Ca^{2+}, which by itself had no effect on ER structure. The Ca^{2+}-free solution did not increase the sensitivity of the ER vesiculation to plasma membrane vesiculation since it was likewise observed in normal Krebs solution (data not shown). The structural alteration of the ER induced by plasma membrane injury in conditions of very low and stable free Ca^{2+} concentrations has to our knowledge not been reported, except for saponin-induced vesicularization observed by electron microscopy in liver cells in suspension (but strangely this was not observed in liver cells attached to coverslips) [25].

Tubule formation from vesicular membranes requires energy [26] and the presence of curvature-stabilizing proteins: the reticulons and DP1/Yop1p [27,28] and rab5 [29]. Branching of the tubules depends on the GTPase atlastin [30]. The present experiments show, however, that the maintenance of the tubular network can be rapidly disturbed. We can only speculate whether ER vesicularization plays a specific physiological role. If so, it could contribute to cell survival, together with other documented repair systems, like recruitment of annexins [31], Ca^{2+}-regulated endocytosis [32] and exocytosis of lysosomes [33]. An excessive elevation of $[Ca^{2+}]_c$. obviously occurs *in vivo* following membrane permeabilization. One necessary consequence of ER vesiculation, independent of whether or not the continuity of the ER lumen is broken,

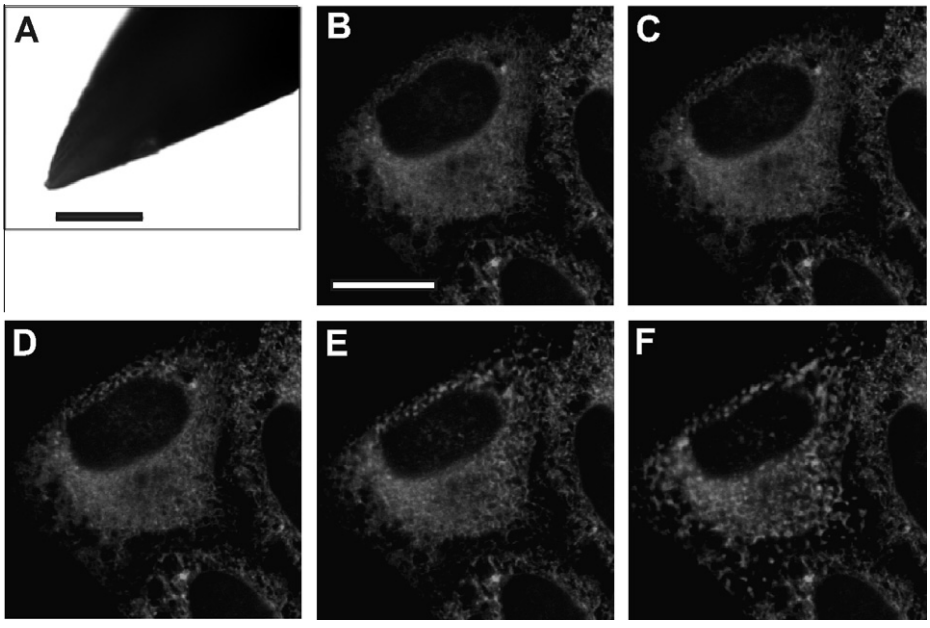

Fig. 4. HeLa cell in Ca^{2+}-free solution mechanically stubbed with a needle. (A) Needle tip used for mechanical stabbing. Scale bar = 50 μm. (B) 0.3 s before weak stimulus. Scale bar = 0 μm. (C) 0.6 s after weak stimulus. (D) Stronger stimulus 2.4 s after the first stimulus. (E) 3.6 s after 2nd stimulus. (F) 9 s after 2nd stimulus. See supplementary movie for complete sequence.

is an increase of the lumenal volume for the same membrane area, which therefore results in a decrease of the lumenal Ca^{2+} concentration even in the absence of any Ca^{2+} efflux. For example, when the membrane of tubules with a typical inner diameter of 60 nm [28] and a total length of 5 μm assume a round shape, the resulting single vesicle has a diameter of about 0.7 μm and a volume that is ~6-fold larger than that of the tubule (Supplementary Fig. S1A). The relative decrease of the lumenal Ca^{2+} concentration will be less than the inverse of the relative increase in volume because of buffering by lumenal Ca^{2+}-binding proteins. The new concentration depends on the initial value, on the concentration of the lumenal buffers and on their affinity for Ca^{2+}. This dependence was calculated as given in the legend of Supplementary Fig. S1C, assuming a low-affinity Ca^{2+} buffer with a single class of binding sites. For reasonable parameter values, for example an initial free Ca^{2+} concentration of 0.5 mM, a buffer with a K_d of 0.5 mM and a total Ca^{2+}-binding site concentration of 5 mM, free Ca^{2+} in the example given above would in theory decrease from 0.5 to 0.23 mM. A decrease of the lumenal Ca^{2+} concentration results in a lower Ca^{2+} efflux because of the smaller driving force and because the open probability of the Ca^{2+}-release channels, the inositol 1,4,5-trisphosphate receptors and the ryanodine receptors decreases (for review see [34,35]. Additionally, the lower ER luminal Ca^{2+} will exert less inhibition on the SERCA Ca^{2+} pump and thus will stimulate the rate of Ca^{2+} uptake. Vesicularization may thus increase the Ca^{2+}-storage capacity of the ER, increase its total Ca^{2+} load and thus counteract a rise of $[Ca^{2+}]_c$ to harmful levels. Conversely however, the decrease of lumenal Ca^{2+} could also lead to enhanced Ca^{2+} influx into the cell via capacitative Ca^{2+} entry channels activated by the movement of the ER lumenal Ca^{2+} sensors STIM1 and STIM2 to sites in close apposition to the surface membrane [36–38]. However, this system may be switched off as well by ER vesicularization because this gross structural change would likely result in disruption of the close contacts between the ER and the plasma membrane. ER-mitochondria junctions may be broken as well. These contact sites play an important role in the generation of pro-apoptotic signals by facilitating the transfer of Ca^{2+} from the ER to mitochondria, a cause of the release of apoptosis-inducing cytochrome *c* [39]. The effects of ER vesicularization could thus also include the disruption of ER-mitochondria contact sites, which in combination with the expected decrease of the ER lumenal Ca^{2+} concentration and the decrease of capacitative Ca^{2+} entry would affect the fate of the cell.

Acknowledgments

We thank Catheleyne D'hondt for assistance in the initial experiments on mechanical stimulation. This research was financed by the Research Foundation-Flanders (FWO-Vlaanderen) G.0646.08 and by the Interuniversity Attraction Poles Program P6/28 of the Belgian State, Federal Office for Scientific Technical and Cultural Affairs.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.09.065.

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