CrossMark

Control by Low Levels of Calcium of Mammalian Cell Membrane Electropermeabilization

Florin Ciobanu[1,2] · Muriel Golzio[1] · Eugenia Kovacs[2] · Justin Teissié[1]

Abstract Electric pulses, when applied to a cell suspension, induce a reversible permeabilization of the plasma membrane. This permeabilized state is a long-lived process (minutes). The biophysical molecular mechanisms supporting the membrane reorganization associated to its permeabilization remain poorly understood. Modeling the transmembrane structures as toroidal lipidic pores cannot explain why they are long-lived and why their resealing is under the control of the ATP level. Our results describe the effect of the level of free Calcium ions. Permeabilization induces a Ca^{2+} burst as previously shown by imaging of cells loaded with Fluo-3. But this sharp increase is reversible even when Calcium is present at a millimolar concentration. Viability is preserved to a larger extent when submillimolar concentrations are used. The effect of calcium ions is occurring during the resealing step not during the creation of permeabilization as the same effect is observed if Ca^{2+} is added in the few seconds following the pulses. The resealing time is faster when Ca^{2+} is present in a dose-dependent manner. Mg^{2+} is observed to play a competitive role. These observations suggest that Ca^{2+} is acting not on the external leaflet of the plasma membrane but due to its increased concentration in the cytoplasm.

Ciobanu Florin and Golzio Muriel contributed equally to the work.

✉ Justin Teissié
justin.teissie@ipbs.fr

1 Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse, CNRS, UPS, Toulouse, France

2 University Carol Davila, Bucarest, Romania

Exocytosis will be enhanced by this Ca^{2+} burst (but hindered by Mg^{2+}) and occurs in the electropermeabilized part of the cell surface. This description is supported by previous theoretical and experimental results. The associated fusion of vesicles will be the support of resealing.

Introduction

Electric pulses, when applied to a cell suspension, induce a reversible permeabilization of the plasma membrane (electropermeabilization, electroporation). This permeabilized state is a long-lived process (minutes) but cells can recover the electrically mediated stress. The biophysical molecular mechanisms supporting the membrane reorganization associated to its permeabilization remain poorly understood (Teissie et al. 2005; Sersa et al. 2015; Sweeney et al. 2016; Wegner et al. 2015). There is now a general agreement that the generation of field-dependent toroidal lipidic pores cannot support the complexity of the observations on cells. More basic research is needed. Several steps are present along electropermeabilization with two main classes: those present during the field pulse delivery (trigger, expansion) and those that follow and are involved in the stabilization, the resealing and the final repair of the pulse-induced membrane defects.

Electropermeabilization not only affects the organization of the plasma membrane by inducing conductive pathways but also permits transmembrane transport. A dramatic alteration of the cytoplasmic concentration of metabolites would result. This was reported more than 30 years ago as an elegant method to "gain access to the

cytoplasm" (Knight and Scrutton 1986; Knight and Baker 1982).

Ca^{2+} was a focus of a large interest of investigators on the cellular aspects of electropermeabilization due to its role as a second messenger and its interfacial properties when interacting with charged species (Craviso et al. 2012; Semenov et al. 2013a, b; Pakhomova et al. 2014). One of the more dramatic structural effects of Ca^{2+} uptake on the cell organization was the induction of massive cytoplasmic vesicle fusion (Niessen et al. 1994; Huynh et al. 2004). The most reported of Ca^{2+} consequences in electropermeabilization is the induction of cell death (Rols et al. 1990; Escande-Géraud et al. 1988; Golzio et al. 2003). This appears to result from the high uptake of Ca^{2+} in the cytoplasm that triggers a dramatic Calcium-induced Calcium release. The resulting cytosolic free Calcium concentration cannot be recovered by the Ca^{2+} plasma membrane pumps (Pakhomova et al. 2014). Moreover, other effects associated to electric pulse-induced cell death are controlled by the level of Ca^{2+}. Defense mechanism of electropermeabilized involved the generation of reactive species. These responses are affected by the Ca^{2+} content of the pulsing buffer (Gabriel and Teissié 1994).

Preclinical applications are under development to take advantage of the induced cell death by using Ca^{2+} as a drug with a localized effect on the electropermeabilized tissue (Frandsen et al. 2012) or in controlling gene expression by a transient destruction of the transfected tissue (Hojman et al. 2012). These translational developments are supported by systematic investigations of the effects of the pulsing parameters, of the Ca^{2+} dose, and of the cells on this Ca^{2+} electrically mediated cell death (Golzio et al. 2003; Frandsen et al. 2014; Hansen et al. 2015; Zielichowska et al. 2016; Dermol et al. 2016).

Biophysical methods reported the complexities of the transport pathways associated to Ca^{2+} transfer by direct imaging approaches (Tekle et al. 1994; Gabriel and Teissié 1999). The conclusions were that Ca^{2+} was electrophoretically driven during the pulse but that a massive uptake was present during the post-pulse step by a concentration-driven diffusion (the resting cytoplasmic Ca^{2+} concentration being about 200 nM is lower than the pulsing buffer level). This conclusion of Ca^{2+} transport after the pulse when membrane defects (supporting the transport) are resealing highlights the critical role of the repair process in the control of the Ca^{2+} effects on the cell survival (Son et al. 2016).

There is a need to obtain more information on the control of electropermeabilized membrane resealing by Ca^{2+}. Membrane resealing can be observed from the kinetics of a reporter dye uptake (Pucihar et al. 2008), by the post-pulse membrane conductance change (Hibino et al. 1993; Hai and Spira 2012) or by a delayed addition of the dye (Teissie and Rols 1988). Electropermeabilization resealing appears as an event of 3 first-order processes but as the first two are very fast (less than 1 s) (Pucihar et al. 2008), in most studies only the slow one (of the order of minutes) is described. It will be the focus of the present study where the post-pulse addition of the dye was used.

Taking into account the known toxic effects of Ca^{2+} concentration higher than 1 mM in the pulsing buffer, our analysis would be on the use of lower concentrations.

Materials and Methods

Cells

Cells were checked to be mycoplasma free. Chinese hamster ovary (CHO) cells (Wild-Type Toronto WTT clone) were grown in suspension in Eagle's minimal essential medium (MEM 0111, Eurobio, Les Ulis, France) supplemented with 10% fetal bovine serum (GIBCO/Life Technologies, Grand Island, NY), L-glutamine (0.58 g/L, GIBCO/Life Technologies), 2.95 g/L tryptose-phosphate (Sigma-Aldrich, St. Louis, MO), BME vitamins (Sigma-Aldrich), 3.5 g/L glucose (Sigma-Aldrich), and the antibiotics penicillin (100 U/mL) and streptomycin (100 μg/mL, GIBCO/Life Technologies) in a hermetic closed spinner (Corning Inc, Corning, NY, USA) at 37 °C with soft stirring. Every day, the cell culture was diluted twofold to stay at 0.5×10^6/mL. Growing cells in the suspension avoided a trypsinization step before the delivery of electric pulses. The extracellular matrix was preserved (Gabriel and Teissié 1995).

Chemicals

Propidium iodide (PI: molecular weight 668.4), HEPES, sucrose, $MgCl_2$, and $CaCl_2$ were purchased from Sigma Chemical Co. (St. Louis, MO). Fluo-3 AM was obtained from Molecular Probes (Eugene, OR). Standard isoosmotic pulsing buffer (PB) was 250 mM sucrose and 10 mM HEPES buffer (pH 7.4). HEPES buffer was selected to avoid Ca^{2+} chelation present with a phosphate buffer. Conductivity and osmotic pressure of PB were 1.5 mS/cm and 310 mOs/kg, respectively. Different concentrations of $CaCl_2$ were added giving a slight increase in conductivity.

Calcium Assay

The cell-permeant Fluo-3 AM (2.2 M) was loaded (in the presence of 0.2% w/v F-127 pluronic acid) by incubating the cells for 15–20 min in a culture medium at room temperature and under gentle agitation. Excess external dye was washed off after incubation, and the cells were

suspended in 1 mM $CaCl_2$-containing PB. When external Ca^{2+} ions enter the electropermeabilized cell, they immediately bind to pre-loaded Fluo-3 indicators inside the cells and cause enhancement of fluorescence.

Cell Electropermeabilization

Cells were harvested by centrifugation for 10 min at $100 \times g$ (700 rpm, C500 centrifuge, Jouan, St. Herblain, France), washed with PB, and resuspended in PB containing the PI (0.1 mM; 10^6 cells/mL).

Propidium is classically used to quantify long-lived cell electropermeabilization. When the dye enters the electropermeabilized cell, it binds to intracellular targets (DNA and/or RNA) with an increase in quantum yield and its fluorescence strongly increases (Gabriel and Teissié [1997]).

The PS10 CNRS electropulser (Jouan) was used to supply square-wave electric pulses. Pulse parameters (voltage and duration) were monitored online through a 15-MHz oscilloscope (Enertec, St. Etienne, France).

CHO cells were collected from the spinner. The cells were suspended in pulsation buffer (PB) at a concentration of 5×10^6 cells/mL. 100 μL of the cell solution was dropped between two stainless steel, flat, parallel electrodes (0.4 cm gap) in contact with the bottom of a culture dish (Nunc, Denmark). This configuration built an open pulsing chamber, where it was easy to add PI or $CaCl_2$ after pulsing the suspension (Fig. S2).

Eight pulses of 1.4 kV/cm lasting 0.1 ms were delivered at 1 Hz frequency. They were previously shown to induce a high level of membrane permeabilization (70–80%) while preserving the cell viability to a large extent (80–85%) in a Ca^{2+}-free buffer (Fig. S1).

Membrane resealing was monitored by a delayed addition of PI giving a final concentration of 0.1 mM.

Fluorescence Imaging

For microscopic observations, the microscope glass coverslip with a drop of cells was placed on the stage of an inverted digitized fluorescence video microscope (Leica DMIRB, Wetzlar, Germany). The cells were observed with a Leica $100\times$, 1.30 oil immersion objective. The wavelengths were selected using the Leica N2.1 filter block for PI-labeled cells. Images were recorded with a Photometrics cooled CCD camera (Princeton instrument, Inc., Trenton, NJ, USA) and a computer-controlled excitation light source (Leica, EL 6000, Wetzlar, Germany). The shutter limited photobleaching. Digitized images were processed using MetaMorph Acquisition software (Version 7.04r4 © 1992–2006 Molecular Devices, Downingtown, PA, USA) run on a DELL computer under Microsoft Windows XP. The cells were considered as permeabilized when their cytoplasmic PI emission was larger than twice the background level and called PI positive.

A more quantitative analysis was performed by flow cytometry (Becton–Dickinson FACScan; Becton–Dickinson Biosciences, Franklin Lakes, NJ, USA). Both the percentage of fluorescent cells (i.e., the percentage of permeabilized cells, PI-positive cells) and the level of fluorescence associated with this permeabilization (i.e., the efficiency of permeabilization) are obtained on a large population (5×10^3 cells). The cells were resuspended in 1 mL of buffer and analysis by flow cytometry was gated with the scatters (forward scatter and side scatter) to exclude debris. Excitation was obtained with an argon laser (488 nm) and the fluorescence of intracellular propidium iodide was collected in FL-2 channel (bandpass 585 ± 42 nm). Acquisition was obtained in list mode and analyzed with CellQuest software (Becton–Dickinson).

The critical fluorescence threshold for selecting PI-positive cells (gating) was set manually. Information such as the percentage of permeabilized cells (gated as PI positive) and the mean fluorescence of the PI-positive cells was collected.

In all the experiments, control cells were treated exactly in the same way apart from being exposed to electric pulses.

Data analysis

Experiments were performed on different days and as such on different cultures. The level of permeabilization (mean fluorescence) was obtained with the flow cytometer on the subpopulation, which was larger than the manually set critical threshold. A total number of 5000 cells were observed. As fluctuations were present due to the day-to-day cell culture conditions, the relative values were obtained normalizing the data to the samples with no Ca++ added. Data were expressed as mean ± standard deviation (SD) values shown as error bars on the graphs. All experiments were repeated on 4 different days (more than 5000 cells were observed each time) giving a standard deviation of less than 10%. Ca++-treated samples (0.1–0.5 mM Ca++) were compared to control (no Ca++) using single-factor ANOVA test implemented in Excel Analysis ToolPak (Microsoft Inc., Redmond, WA)). This software checked when the probability P was 0.05 or less. The significant comparisons are shown by a * on the graphs.

Results

Calcium inflow resulting from cell electropermeabilization was observed using Fluo-3-loaded cells as described in Golzio et al. ([2003]). A large burst in fluorescence was

observed under the fluorescence microscope that disappeared in the minutes following the pulse delivery. The cell viability was affected and all permeabilized cells were killed when the Ca^{2+} concentration was 5 mM. Confirmative results of the previous study were obtained in the present study, i.e., the calcium burst was transient when the external Ca2 + concentration was in the submillimolar range (data not shown).

Calcium Effects on Electropermeabilization

Cells were treated in a PI-containing buffer. A background level of PI-positive cells (10% or higher) was present in the unpulsed control cells as it was the case in many previous reports where similar external concentrations of PI were used. Pulse delivery induced a sharp increase in the percentage of PI-positive cells up to 80%. This was observed under the microscope but a systematic quantitative analysis was performed by flow cytometry. This percentage was affected when Ca^{2+} was present with concentrations increasing up to 0.5 mM, dropping to 60%. The PI fluorescence mean emission was therefore evaluated on 5×10^3 cells for each experiment. The PI emission in each single cell was observed to slightly decrease in a statistically non-significant way when the Ca^{2+} concentration was equal to or higher than 0.2 mM (Fig. 1).

In a previous study using larger Ca^{2+} concentrations (up to 5 mM), it was reported a dose-dependent increase in the PI fluorescence emission (Golzio et al. 2003) due to the increase in toxicity. PI emission in dead cells is much higher than that in reversibly permeabilized cells.

When Ca^{2+} was added a few seconds after the train of pulses were delivered in a PI-containing buffer, the

percentage of PI-positive cells was observed to only slightly decrease in a statistically non-significant way with an increase in the Ca^{2+} concentration (Fig. 2a). A 10% decrease was observed when the Ca^{2+} final concentration after the pulses was 0.5 mM. PI mean fluorescence of PI-positive cells was therefore evaluated on 5×10^3 cells.

The PI fluorescence emission in each single cell was observed to decrease significantly when the Ca2 + concentration was higher than 0.1 mM (Fig. 2b).

Toxicity of Low Concentrations of Ca^{2+} During Electropermeabilization

The observation of cell fluorescence with an addition of PI 30 min after the pulse delivery in a PI-free buffer, a
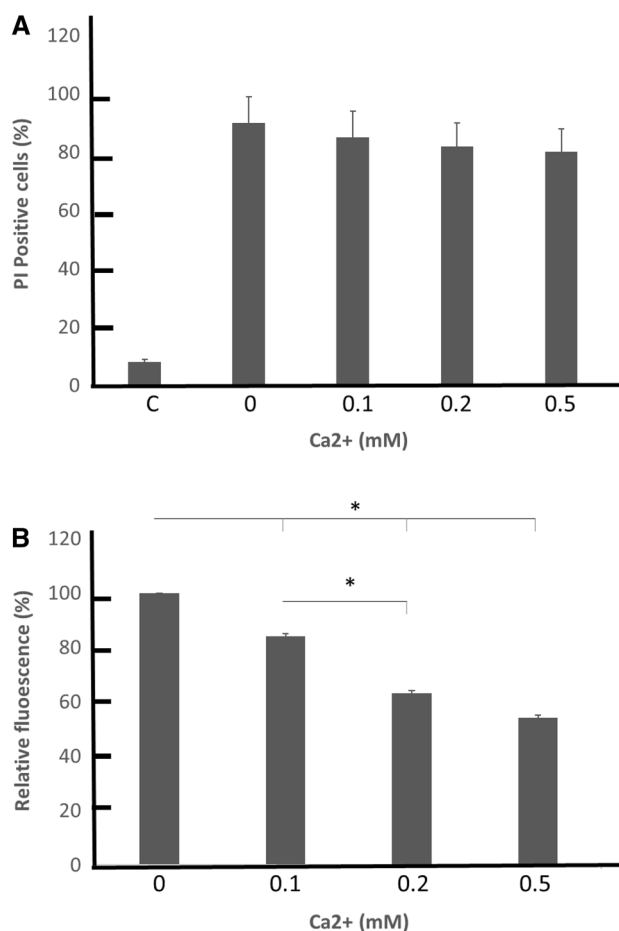
Fig. 2 Electropermeabilization with a post pulse addition of Ca^{2+}. Cells were pulsed (1.4 kV/cm, 0.1 ms, 8 times, 1 Hz) in PB (0.1 mM PI). Different levels of Ca^{2+} were added post pulse (final concentrations on the axis). **a** Percentage of PI positive cells. (c control with no EP). **b** The relative value of mean fluorescence emission of PI positive cells were obtained normalizing data to the samples with no Ca++ added using the emission with no Ca^{2+} as 100%. The statistical significance on the top line is indicative of statistically significant difference between control and all the other samples ($n = 4$)
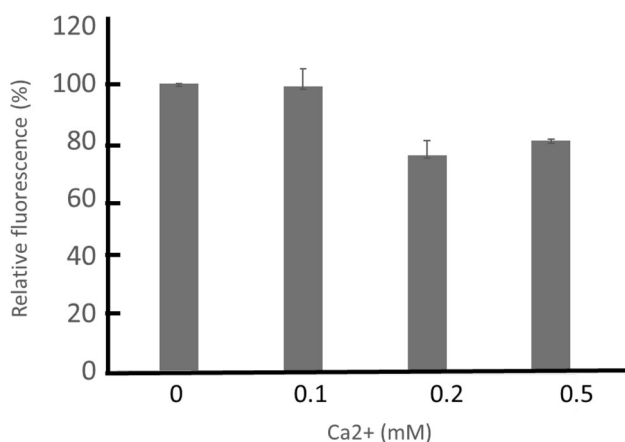

Fig. 1 Effect of Calcium concentration on the intensity of the fluorescence emission of PI positive cells. Cells were pulsed (1.4 kV/cm, 0.1 ms, 8 times, 1 Hz) in a buffer containing PI (0.1 mM) and different levels of Ca^{2+}. The relative mean fluorescence emission of PI positive cells is reported using the emission with no Ca^{2+} as 100% ($n = 4$)

condition where resealing is assumed to be completed, showed that the percentage of cells that were not able to recover was only slightly affected when the Ca^{2+} concentration present during the pulse delivery was less than 0.2 mM (Fig. 3).

When the Ca^{2+} level was 0.5 mM viability decreased more than twofold (from 13 to 30% PI-positive cells), however the decrease was not statistically significant ($P = 0.07$), as compared with the results in a calcium-free buffer, which is in line with a 2003 study (Golzio et al. 2003).

Effect of Calcium Concentration on Membrane Repair

In our previous study of the effect of Ca^{2+} on membrane electropermeabilization, high (>1 mM) Ca^{2+} concentrations were investigated (Golzio et al. 2003). A key problem was that such Ca^{2+} amounts were inducing a high loss of viability in the permeabilized cells. There was a competition between resealing and loss of viability in the kinetics of the uptake of PI. Nevertheless, the early steps of resealing appeared faster in a 1 mM Ca^{2+} buffer than in a Ca^{2+}-free buffer. Indeed, we observed that some electropermeabilized cells could survive when pulses were delivered in a 1 mM Ca^{2+} buffer. Considering our results on the low toxicity associated with pulse delivery in a buffer containing 0.5 mM or less Ca^{2+}, we investigated further the effect of those low concentrations on the kinetics of resealing.
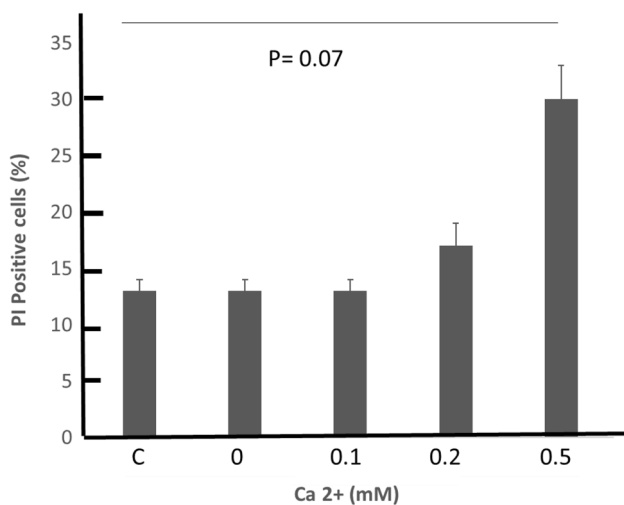

Fig. 3 Cell mortality resulting from electropermeabilization as a function of a low Ca^{2+} level. Cells were pulsed (1.4 kV/cm, 0.1 ms, 8 times, 1 Hz) in a PB buffer containing different levels of Ca^{2+}. Lysis (mortality) was evaluated by the percentage of cells that remained PI positive with a 30 min delayed addition of PI (0.1 mM). C no pulse delivered ($n = 4$)

Resealing, i.e., repair of membrane injuries after pulse delivery, was followed by counting the percentage of cells that remained PI positive at a given time t after being pulsed by a field with a strength E, $P (E,t)$. Only the slow step was analyzed for technical reasons as mentioned in the Introduction.

It is observed that it is described by

$$P(E,t) = L(E) + [(P(E,0) - L(E)) \exp(-Kt)],$$

where $L(E)$ is the percentage of cells in the pulsed population that cannot survive (lysed cells) (Rols and Teissié 1990) (Fig. 3).

Under low Ca^{2+} concentration conditions, we just observe that $P(E,0)$ is not affected to a large extent by the concentration of Ca^{2+} present in the pulsing buffer.

The kinetics of resealing is a first-order process that can be quantified by the ratio:

$$(P(E,t) - L(E))/(P(E,0) - L(E))$$

i.e., $\exp(-Kt)$ at a given time t.

A decrease in this ratio is indicative of faster resealing (a higher value of K, i.e., a shorter time constant in resealing, the reciprocal of K).

The 5 min characteristic time of the early events was selected from our previous studies on CHO resealing (Rols and Teissié 1990). PI was added 5 min after the pulse delivery in a PI-free buffer containing different levels of Ca^{2+} to observe the resealing (P(5)).

A dramatic significant effect of the increase in the low concentration of Ca^{2+} on the resealing was observed (Fig. 4). Resealing is very fast in the 0.5 mM buffer where the loss of viability remains low and was almost completed within 5 min (Fig. 3). Therefore, the PI uploading process is short-lived and the number of PI molecules that reach the cytoplasm is less than that in a Ca^{2+}-free pulsing buffer. This is supported by our observation that the level of permeabilization ($P(E)$) and the mean fluorescence emission of PI-positive cells were affected to some extent when Ca^{2+} was present at 0.5 mM (Figs. 1, 2).

Mg^{2+} is a Competitor of the Positive Effect of Ca^{2+} on Resealing

To investigate if the effect of Ca^{2+} on resealing was due its double charge, Mg^{2+} (1 mM) was added to PB. It should be reminded that Mg^{2+} at that low concentration is always present in the pulsing buffer where the pH is controlled by a phosphate buffer (a strong chelator of Ca^{2+}). Under our pulsing conditions (1.4 kV/cm, 0.1 ms, 8 times, 1 Hz), pulsing CHO cells in that buffer did not affect the cell viability (Golzio et al. 2003). It was previously shown that that higher Mg^{2+} led to higher electropermeabilization for PI and higher viability (Haberl et al. 2010). Our
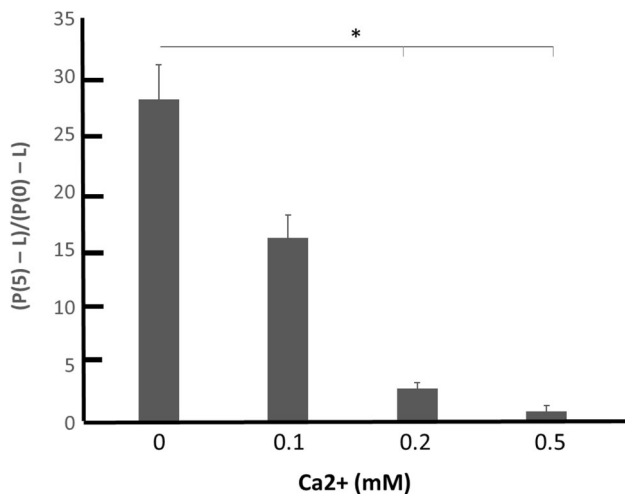
Fig. 4 Effect of Calcium concentration on resealing of electropermeabilized cells. Cells were pulsed (1.4 kV/cm, 0.1 ms, 8 times, 1 Hz) in PB containing different levels of Ca^{2+}. Resealing was quantified by the ratio of permeabilized cells detected at 5 min post pulse (P(5)) to the initial level (P0)) (with a correction of the background) (in percent). In the two cases, the final concentration was PI 0.1 mM ($n = 4$)

experiments were focused on the effect of Mg^{2+} (1 mM) when low concentrations of Ca^{2+} were present. The resealing kinetics were strongly affected and slow resealing was observed with no added Calcium (Fig. 5). This is in agreement with the previous observations using a phosphate buffer (Rols and Teissié 1990). No calcium-enhancing effect was observed with the addition of 0.1 mM Calcium. It was detected at higher concentrations but to a
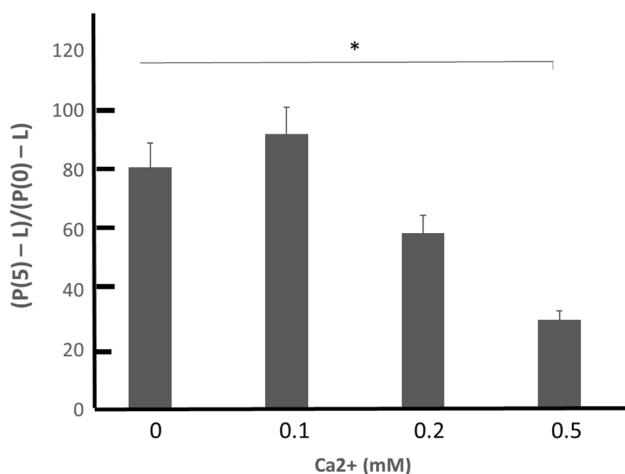

Fig. 5 Effect of 1 mM $MgCl_2$ on the resealing at different Ca^{2+} concentrations. Cells were pulsed (1.4 kV/cm, 0.1 ms, 8 times, 1 Hz) in PB (PI 0.1 mM) containing $MgCl_2$ (1 mM) with different levels of Ca^{2+}. Resealing was quantified by the ratio of permeabilized cells detected at 5 min post pulse PI addition (P(5)) to the initial level (P(0)) (with a correction of the background) (in percent). In the two cases, the final concentration was PI 0.1 mM ($n = 4$)

lower extent to what was observed in the Mg^{2+}-free buffer (Fig. 4).

Mg^{2+} and Ca^{2+} appear to have competitive effects on the resealing of electropermeabilized cells.

Discussion

Low concentrations (less than 0.5 mM) of Ca^{2+} affect the events linked to cell electropermeabilization performed with the classical approach (pulse duration of 0.1 ms as for electrochemotherapy). The viability of pulsed cells remains preserved. In our previous study at millimolar concentrations of Ca^{2+}, a more dramatic effect was observed as 80% of the electropermeabilized cells were dead after being pulsed in a 5 mM Ca^{2+} buffer (Golzio et al. 2003). A similar high toxicity was recently reported at 5 mM Ca^{2+} on other cell systems when using 1.2–1.6 kV/cm field strengths with the same pulse duration and number as used in the present study (Frandsen et al. 2014). The main effects of the submillimolar Ca^{2+} are not on the processes present when the field is present, i.e., trigger and expansion of permeabilization (Teissie et al. 2005), but affect the resealing process. Cells remain in the electropermeabilized state for a shorter time when Ca^{2+} is present in this low concentration range. Calcium-induced toxicity is present only above a significant concentration (0.2 mM). Most of the associated uptake of exogenous molecules (PI in the present study) is reduced. The effect of Calcium is prevented if Mg^{2+} is present (as it is the case in most investigations). A previous report using electrical recording of the resealing observed that it proceeded as an exponential process interrupted by abrupt recovery steps. These abrupt events were considered as a consequence of the "membrane patch model" of membrane repair in which patches of intracellular membrane fused with the plasma membrane at the site of injury (Hai and Spira 2012).

This action of Ca^{2+} on the resealing processes is in line with the reports describing the reversibility of electropermeabilization not as an intramembrane mechanical recovery but as membrane repair, where exocytosis plays a key role (McNeil and Steinhardt 2003; Cooper and McNeil 2015; Terasaki et al. 1997). Exocytosis was thought to promote resealing by 'patching' the plasma membrane lesion or by facilitating bilayer restoration through reduction in membrane tension (Idone et al. 2008a, b; Huynh et al. 2004). Resealing is associated to the fusion of cytosolic vesicles in the part of the cell membrane affected by the permeabilization process after the pulse delivery. Membrane repair is associated to a rapid Ca^{2+}-dependent endocytic pathway (Idone et al. 2008a, b.). This physiological fusion associated to exocytosis involves the action of Ca^{2+} ions but is hindered by Mg^{2+} ions (Reddy et al.

2001; Idone et al. 2008a, b). This competition between Ca^{2+} and Mg^{2+} is observed in our study. Spontaneous fusion of small lipid vesicles (LUV, large unilamellar vesicles) to electropermeabilized cells was obtained and was observed to be controlled by Ca^{2+} ions (Ramos et al. 2002).

This involvement of Ca^{2+} in electropermeabilization resealing was mentioned in nanosecond pulsed electric field effect and on more complex biological systems (Pakhomova et al. 2014; Swezey and Epel 1989; Boonen et al. 1992, 1993).

Conclusions

The final conclusions are that low Ca^{2+} concentrations are involved in electropermeabilization in conditions that are not toxic. The same effect appears to be present at higher concentrations (see the observations at 0.5 mM), but it is masked by the toxicity linked to the high cytoplasmic Ca^{2+} concentration. Ca^{2+} influx through plasma membrane electro-defects triggers a rapid repair response that is essential for cell survival. This effect of Ca^{2+} supports the description of resealing as a membrane repair of damaged cells, i.e., supported by the metabolic activity of cells (Rols et al. 1998).

References

Boonen GJ, van Steveninck J, Dubbelman TM, van den Broek PJ, Elferink JG (1992) Exocytosis in electropermeabilized neutrophils. Responsiveness to calcium and guanosine 5′-[gamma-thio]triphosphate. Biochem J 287:695–700

Boonen GJ, VanSteveninck J, Elferink JG (1993) Strontium and barium induce exocytosis in electropermeabilized neutrophils. Biochim Biophys Acta 1175(2):155–160

Cooper ST, McNeil PL (2015) Membrane repair: mechanisms and pathophysiology. Physiol Rev 95(4):1205–1240. doi:10.1152/physrev.00037.2014

Craviso GL, Choe S, Chatterjee I, Vernier PT (2012) Modulation of intracellular Ca^{2+} levels in chromaffin cells by nanoelectropulses. Bioelectrochemistry 87:244–252. doi:10.1016/j.bioelechem.2011.11.016

Dermol J, Pakhomova ON, Pakhomov A, Miklavčič D (2016) Cell electrosensitization exists only in certain electroporation buffers. PLoS ONE 11(7):e0159434. doi:10.1371/journal.pone.0159434

Escande-Géraud ML, Rols MP, Dupont MA, Gas N, Teissié J (1988) Reversible plasma membrane ultrastructural changes correlated with electropermeabilization in Chinese hamster ovary cells. Biochim Biophys Acta 939(2):247–259

Frandsen SK, Gissel H, Hojman P, Tramm T, Eriksen J, Gehl J (2012) Direct therapeutic applications of calcium electroporation to effectively induce tumor necrosis. Cancer Res 72(6):1336–1341. doi:10.1158/0008-5472.CAN-11-3782

Frandsen SK, Gissel H, Hojman P, Eriksen J, Gehl J (2014) Calcium electroporation in three cell lines: a comparison of bleomycin and calcium, calcium compounds, and pulsing conditions. Biochem Biophys Acta 1840:1204–1208

Gabriel B, Teissié J (1994) Generation of reactive-oxygen species induced by electropermeabilization of Chinese hamster ovary cells and their consequence on cell viability. Eur J Biochem 223(1):25–33

Gabriel B, Teissié J (1995) Control by electrical parameters of short- and long-term cell death resulting from electropermeabilization of Chinese hamster ovary cells. Biochim Biophys Acta 1266(2):171–178

Gabriel B, Teissié J (1997) Direct observation in the millisecond time range of fluorescent molecule asymmetrical interaction with the electropermeabilized cell membrane. Biophys J 73(5):2630–2637

Gabriel B, Teissié J (1999) Time courses of mammalian cell electropermeabilization observed by millisecond imaging of membrane property changes during the pulse. Biophys J 76(4):2158–2165

Golzio M, Gabriel B, Boissier F, Deuwille J, Rols MP, Teissié J (2003) Calcium and electropermeabilized cells. J Soc Biol 197(3):301–310

Haberl S, Miklavcic D, Pavlin M (2010) Effect of Mg ions on efficiency of gene electrotransfer and on cell electropermeabilization. Bioelectrochemistry 79(2):265–271. doi:10.1016/j.bioelechem.2010.04.001

Hai A, Spira ME (2012) On-chip electroporation, membrane repair dynamics and transient in-cell recordings by arrays of gold mushroom-shaped microelectrodes. Lab Chip 12(16):2865–2873. doi:10.1039/c2lc40091j

Hansen EL, Sozer EB, Romeo S, Frandsen SK, Vernier PT, Gehl J (2015) Dose-dependent ATP depletion and cancer cell death following calcium electroporation, relative effect of calcium concentration and electric field strength. PLoS ONE 10(4):e0122973. doi:10.1371/journal.pone.0122973

Hibino M, Itoh H, Kinosita K Jr (1993) Time courses of cell electroporation as revealed by submicrosecond imaging of transmembrane potential. Biophys J 64(6):1789–1800

Hojman P, Brolin C, Gissel H (2012) Calcium influx determines the muscular response to electrotransfer. Am J Physiol Regul Integr Comp Physiol 302(4):R446–R453. doi:10.1152/ajpregu.00383.2011

Huynh C, Roth D, Ward DM, Kaplan J, Andrews NW (2004) Defective lysosomal exocytosis and plasma membrane repair in Chediak-Higashi/beige cells. Proc Natl Acad Sci USA 101(48):16795–16800

Idone V, Tam C, Andrews NW (2008a) Two-way traffic on the road to plasma membrane repair. Trends Cell Biol 18(11):552–559. doi:10.1016/j.tcb.2008.09.001

Idone V, Tam C, Goss JW, Toomre D, Pypaert M, Andrews NW (2008b) Repair of injured plasma membrane by rapid Ca^{2+}-dependent endocytosis. J Cell Biol 180(5):905–914. doi:10.1083/jcb.200708010

Knight DE, Baker PF (1982) Calcium-dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric fields. J Membr Biol 68(2):107–140

Knight DE, Scrutton MC (1986) Gaining access to the cytosol: the technique and some applications of electropermeabilization. Biochem J 234(3):497–506

McNeil PL, Steinhardt RA (2003) Plasma membrane disruption: repair, prevention, adaptation. Annu Rev Cell Dev Biol 19:697–731

Niessen HW, Onderwater J, Koerten HK, Ginsel LA, Verhoeven AJ (1994) Immunoelectron microscopy reveals significant granule fusion upon stimulation of electropermeabilized human neutrophils. Cell Signal 6(1):47–58

Pakhomova ON, Gregory B, Semenov I, Pakhomov AG (2014) Calcium-mediated pore expansion and cell death following nanoelectroporation. Biochim Biophys Acta 1838(10):2547–2554. doi:10.1016/j.bbamem.2014.06.015

Pucihar G, Kotnik T, Miklavcic D, Teissié J (2008) Kinetics of transmembrane transport of small molecules into electropermeabilized cells. Biophys J 95(6):2837–2848. doi:10.1529/biophysj.108.135541

Ramos C, Bonato D, Winterhalter M, Stegmann T, Teissié J (2002) Spontaneous lipid vesicle fusion with electropermeabilized cells. FEBS Lett 518(1–3):135–138

Reddy A, Caler EV, Andrews NW (2001) Plasma membrane repair is mediated by Ca(2 +)-regulated exocytosis of lysosomes. Cell 106(2):157–169

Rols MP, Teissié J (1990) Electropermeabilization of mammalian cells. Quantitative analysis of the phenomenon. Biophys J 58(5):1089–1098

Rols MP, Dahhou F, Mishra KP, Teissié J (1990) Control of electric field induced cell membrane permeabilization by membrane order. Biochemistry 29(12):2960–2966

Rols MP, Delteil C, Golzio M, Teissié J (1998) Control by ATP and ADP of voltage-induced mammalian-cell-membrane permeabilization, gene transfer and resulting expression. Eur J Biochem 254(2):382–388

Semenov I, Xiao S, Pakhomova ON, Pakhomov AG (2013a) Recruitment of the intracellular Ca^{2+} by ultrashort electric stimuli: the impact of pulse duration. Cell Calcium 54(3):145–150. doi:10.1016/j.ceca.2013.05.008

Semenov I, Xiao S, Pakhomov AG (2013b) Primary pathways of intracellular Ca(2+) mobilization by nanosecond pulsed electric field. Biochim Biophys Acta 1838(3):981–989. doi:10.1016/j.bbamem.2012.11.032

Sersa G, Teissie J, Cemazar M, Signori E, Kamensek U, Marshall G, Miklavcic D (2015) Electrochemotherapy of tumors as in situ vaccination boosted by immunogene electrotransfer. Cancer Immunol Immunother 64(10):1315–1327. doi:10.1007/s00262-015-1724-2

Son RS, Gowrishankar TR, Smith KC, Weaver JC (2016) Modeling a conventional electroporation pulse train: decreased pore number, cumulative calcium transport and an example of electrosensitization. IEEE Trans Biomed Eng 63(3):571–580. doi:10.1109/TBME.2015.2466234

Sweeney DC, Reberšek M, Dermol J, Rems L, Miklavčič D, Davalos RV (2016) Quantification of cell membrane permeability induced by monopolar and high-frequency bipolar bursts of electrical pulses. Biochim Biophys Acta 1858(11):2689–2698. doi:10.1016/j.bbamem.2016.06.024

Swezey RR, Epel D (1989) Stable, resealable pores formed in sea urchin eggs by electric discharge (electroporation) permit substrate loading for assay of enzymes in vivo. Cell Regul 1(1):65–74

Teissie J, Rols MP (1988) Electropermeabilization and Electrofusion of Cells. In: Latruffe N, Gaudemer Y, Vignais P, Azzi A (eds) Dynamics of membrane proteins and cellular energetics. Springer, New York, pp 251–268

Teissie J, Golzio M, Rols MP (2005) Mechanisms of cell membrane electropermeabilization: a minireview of our present (lack of?) knowledge. Biochim Biophys Acta 1724(3):270–280

Tekle E, Astumian RD, Chock PB (1994) Selective and asymmetric molecular transport across electroporated cell membranes. Proc Natl Acad Sci USA 91(24):11512–11516

Terasaki M, Miyake K, McNeil PL (1997) Large plasma membrane disruptions are rapidly resealed by Ca2 + -dependent vesicle-vesicle fusion events. J Cell Biol 139(1):63–74

Wegner LH, Frey W, Silve A (2015) Electroporation of DC-3F cells is a dual process. Biophys J 108(7):1660–1671. doi:10.1016/j.bpj.2015.01.038

Zielichowska A, Daczewska M, Saczko J, Michel O, Kulbacka J (2016) Applications of calcium electroporation to effective apoptosis induction in fibrosarcoma cells and stimulation of normal muscle cells. Bioelectrochemistry 109:70–78. doi:10.1016/j.bioelechem.2016.01.005