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Cancer Res 2012;72:1336-1341. Published OnlineFirst January 26, 2012.

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Priority Report

Direct Therapeutic Applications of Calcium Electroporation to Effectively Induce Tumor Necrosis

Stine Krog Frandsen¹, Hanne Gissel², Pernille Hojman³, Trine Tramm⁴, Jens Eriksen^{1,5}, and Julie Gehl¹

Abstract

Electroporation of cells with short, high-voltage pulses causes a transient permeabilization of cell membranes that permits passage of otherwise nonpermeating ions and molecules. In this study, we illustrate how electroporation with isotonic calcium can achieve highly effective cancer cell kill *in vivo*. Calcium electroporation elicited dramatic antitumor responses in which 89% of treated tumors were eliminated. Histologic analyses indicated complete tumor necrosis. Mechanistically, calcium electroporation caused acute ATP depletion likely due to a combination of increased cellular use of ATP, decreased production of ATP due to effects on the mitochondria, as well as loss of ATP through the permeabilized cell membrane. Taken together, our findings offer a preclinical proof of concept for the use of electroporation to load cancer cells with calcium as an efficient anticancer treatment. Electroporation equipment is already used clinically to enhance the delivery of chemotherapy to superficial tumors, with trials on internal tumors in progress, enabling the introduction of calcium electroporation to clinical use. Moreover, the safety profile, availability, and low cost of calcium facilitate access to this technology for many cancer patients in developed and developing countries. *Cancer Res*; 72(6); 1336–41. ©2012 AACR.

Introduction

Calcium is a ubiquitous second messenger involved in many cellular processes, including regulation of transcription, metabolism, proliferation, muscle contraction, and cell death (apoptosis and necrosis; refs. 1–3). Due to the many effects of calcium, the intracellular calcium concentration is tightly regulated, and the effects of calcium are dependent on time, place, amplitude, frequency, and duration of the calcium signal (1, 2, 4–6). Cellular uptake of calcium can be facilitated by electroporation in which cells are exposed to an electric field exceeding the dielectric strength of the cell membrane, resulting in generation of reversible permeabilization structures in the membrane (7). In eukaryotic cells, the concentration of free intracellular calcium is very low (10^{-7} mol/L), in striking contrast to the concentration of free calcium in plasma (10^{-3} mol/L; ref. 5). Thus, even a small increase in the permeability of the membrane may increase the concentration of free intracellular calcium drastically. Increase in intracellular calcium concentration due to electroporation has previously been shown (8, 9), but its use in cancer treatment was not investigated.

Here, we document that calcium electroporation can be highly efficient in eradicating tumors *in vivo*, and we

suggest that the mechanistic explanation is acute energy depletion.

Materials and Methods

In vitro electroporation

Three cell lines were used for *in vitro* experiments, DC-3F, a transformed Chinese hamster lung fibroblast cell line; K-562, a human leukemia cell line; and Lewis Lung Carcinoma, a murine lung carcinoma cell line (all tested negative for mycoplasma). Cells were grown in RPMI-1640 culture medium (Gibco, Invitrogen), 10% fetal calf serum (Gibco, Invitrogen), penicillin, and streptomycin at 37°C and 5% CO₂. After harvesting, cells were washed and diluted in HEPES buffer [10 mmol/L HEPES (Lonza), 250 mmol/L sucrose, and 1 mmol/L MgCl₂ in sterile water]. Cell suspension (270 μL; 6.1×10^6 cells/mL) with 30 μL CaCl₂ or HEPES buffer (controls) were electroporated in 4 mm cuvettes with aluminium electrodes (Molecular BioProducts, Inc.). Cooled cells (8°C) were exposed to 8 pulses of 99 μs with respectively 1.2 kV/cm (DC-3F and K-562) and 1.4 kV/cm (Lewis Lung Carcinoma) using a BTX T820 square wave electroporator. Electroporation parameters were optimized for high permeabilization and cell survival. After 20 minutes at 37°C and 5% CO₂, cells were diluted in culture medium (as above) and seeded in 96-well plates (3.1×10^4 cells per 100 μL). After 1 and 2 days, respectively, MTT assay was conducted with Multiskan-Ascent ELISA reader (Thermo LabSystems).

Difference between electroporated and nonelectroporated cells in identical buffer was assessed using 2-way ANOVA with post least-squares-means test with Bonferroni correction.

ATP assay

DC-3F cells were electroporated as described above, with 1 mmol/L calcium. Cells electroporated with HEPES buffer,

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doi: 10.1158/0008-5472.CAN-11-3782

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nonelectroporated cells with 1 mmol/L calcium, and untreated cells were used as controls. Cell death induced by irreversible electroporation (8 pulses of 99 μ s and 6.6 kV/cm) was used as negative control. Cells seeded in 96-well plates as above were lysed with Cell-Based Assay Lysis Buffer (Cayman Chemical), and ATP content was determined after 1, 4, and 8 hours incubation by adding 100 μ L rL/L Reagent (ENLITEN ATP Assay; Promega) and measuring light emission using a luminometer (LUMIstar; Ramcon).

Difference in ATP level after different treatments was assessed by 2-way ANOVA with post least-squares-means test with Bonferroni correction.

Tumor volume and tumor fluorescence intensity

In vivo experiments were conducted in accordance with European Convention for the Protection of Vertebrate Animals used for Experimentation and with approval from the Danish Animal Experiments Inspectorate.

For *in vivo* experiments, the H69 human small cell lung cancer cell line stably transfected with enhanced green fluorescent protein (EGFP) regulated by the cytomegalovirus promoter (10) was kindly provided by the Department of Radiation Biology, Copenhagen University Hospital. Cells were tested by rapid MAP27 panel (Taconic) without signs of infection. Cells were grown *in vitro* as described above. A total of 1.5×10^6 cells per 100 μ L PBS were injected s.c. in both flanks of 9- to 11-week-old NMRI-*Foxn1^{nu}* mice (Harlan). Tumor pieces were transplanted to the right flank of recipient mice. Hypnorm-dormicum (VetaPharma and Roche) was used for anesthesia complemented with rimadyl (Pfizer Aps) and lidocaine in the incision. Mice were randomized at an average tumor diameter of 6.2 mm (range, 5.5–6.9 mm) and treated with (i) injection of isotonic calcium chloride solution (168 mmol/L CaCl_2) and electroporation (8 pulses of 100 μ s at 1.0 kV/cm and 1 Hz; refs. 7, 11) using a 6-mm plate electrode and a square wave electroporator, from Cliniporator, IGEA; (ii) calcium-free physiologic saline injection and electroporation with the same parameters as above; (iii) isotonic calcium chloride injection; or (iv) calcium-free physiologic saline injection. Atomic absorption spectrophotometry (Solaar AAS; Thermo) confirmed absence of calcium in physiologic saline. Tumor volume was calculated as $ab^2\pi/6$ (a , largest diameter; b , largest diameter perpendicular to a). Initially, tumors were injected with a volume equivalent to the tumor volume but as the Ca^{2+} -EP group showed skin necrosis, the injected volume was changed to half the tumor volume for all groups. Solutions were injected through the side of the firm tumor, and the needle was moved around inside the tumor to secure injection all over the tumor. Tumor size measurements (Vernier calliper) and bioimaging using the Optix MX-2 optical molecular image system (ART) with a scan resolution of 1.5 mm were carried out before treatment and 3 times a week after treatment. Background fluorescence (opposite flank) was subtracted from fluorescence intensity of tumors, and fluorescence intensity below 100 normalized counts (NC) was filtered away with Optiview version 2.02 (ART).

Differences in tumor volume and fluorescence intensity in the 4 treatment groups were evaluated as repeated measure-

ments, validated, and analyzed with an exponential decrease model with Bonferroni correction using SAS software (version 9.1). "Group," "days," and "mouse" were considered factors, and baseline levels of tumor volume or fluorescence intensity were used as covariant. Fluorescence intensity values were log transformed before analysis.

Histology

At an average diameter of 6.1 mm (range, 5.8–6.6 mm) tumors were treated with CaCl_2 injection and electroporation as described above. Tumors were removed 2 hours, at days 1, 2, and 6, respectively, after treatment, fixated in formalin (10% neutrally buffered), and paraffin embedded. Tissue sections (3 μ m) were hematoxylin and eosin (H&E) stained, and a fraction of necrosis was estimated by stereologic point counting using a light microscope, and evaluated by a pathologist who was blinded with respect to treatment status.

Difference in fraction of necrosis was assessed by 1-way ANOVA with post least-squares-means test with Bonferroni correction.

Results and Discussion

To test the effect of calcium overloading *in vitro*, we electroporated 3 cell lines from different species and of different tissue origin in buffers with increasing calcium concentrations (Fig. 1). There was a dose-dependent, dramatic decrease in viability for all electroporated cell lines with half-maximal effective concentration (EC_{50}) whereas EC_{50} being 0.57 mmol/L Ca^{2+} (range, 0.35–0.79 mmol/L) was not reached without electroporation. As expected, due to differences in, for example, cell size and homogeneity, there was a differential effect of the electroporation procedure alone on the different cell lines (12), as electroporation alone reduced viability by 0% (DC-3F), 6% (Lewis Lung Carcinoma), and 23% (K-562), respectively. Consequently, values are listed as a percentage of electroporated versus nonelectroporated controls, respectively. The decrease in viability after calcium electroporation was similar to the effect induced by electroporation with the chemotherapeutic agent bleomycin in concentrations from 0.1 μ mol/L (data not shown).

After having shown a robust anticancer effect *in vitro*, the effect of calcium electroporation *in vivo* was tested. Fluorescent tumors (10) were treated with isotonic calcium chloride injection and electroporated (Ca^{2+} -EP, or in the case of controls, injected with calcium-free physiologic saline and electroporated (NaCl-EP), only injected with isotonic calcium chloride (Ca^{2+}) or calcium-free physiologic saline (NaCl; Fig. 2A). Strikingly, Ca^{2+} -EP treatment eliminated 89% (8 of 9) of the treated tumors. Ulceration occurred in all Ca^{2+} -EP-treated tumors, with healing at an average of 18 days (range, 9–24 days). Tumor volume was measured including the ulceration, giving the impression that tumor volume was increasing just after treatment; however, fluorescence intensity showed acute decrease. The volume of tumors treated with Ca^{2+} -EP was significantly different from controls treated with Ca^{2+} ($P < 0.0001$) and NaCl-EP ($P < 0.01$). Tumor volume of all nonelectroporated tumors increased with a doubling

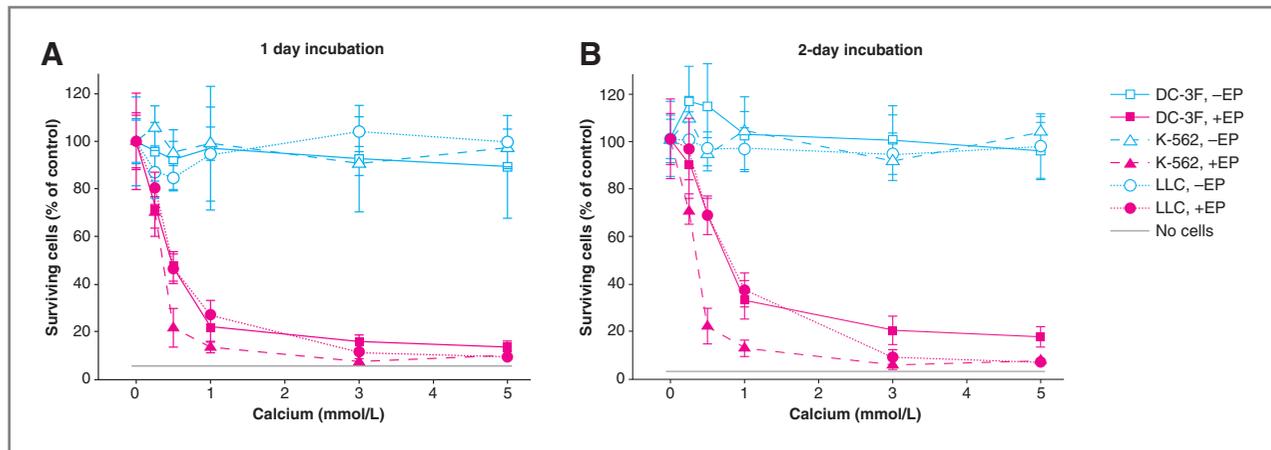


Figure 1. Calcium overloading induces cell death *in vitro*. Cell viability in 3 cell lines, DC-3F, a transformed Chinese hamster lung fibroblast cell line (■); K-562, a human leukemia cell line (▲); and Lewis Lung Carcinoma (LLC), a murine lung carcinoma cell line (●) after treatment with increasing calcium concentrations either electroporated (red) or not (blue). MTT viability assay was conducted 1 (A) and 2 days (B), respectively, after treatment. Results are depicted as percentages of controls (electroporated or nonelectroporated cells in 0 mmol/L calcium; means \pm SD, $n \geq 6$). The viability decreased significantly ($P < 0.01$) starting from 0.5 mmol/L for all cell lines treated. EP, electroporation.

time of 3.9 days (NaCl) and 6.3 days (Ca^{2+}), respectively. Tumors treated with NaCl-EP decreased in size in the first day after treatment, but tumors started increasing in size from

around day 7, except for 2 tumors that were eliminated, indicating that electroporation alone could modulate tumor growth.

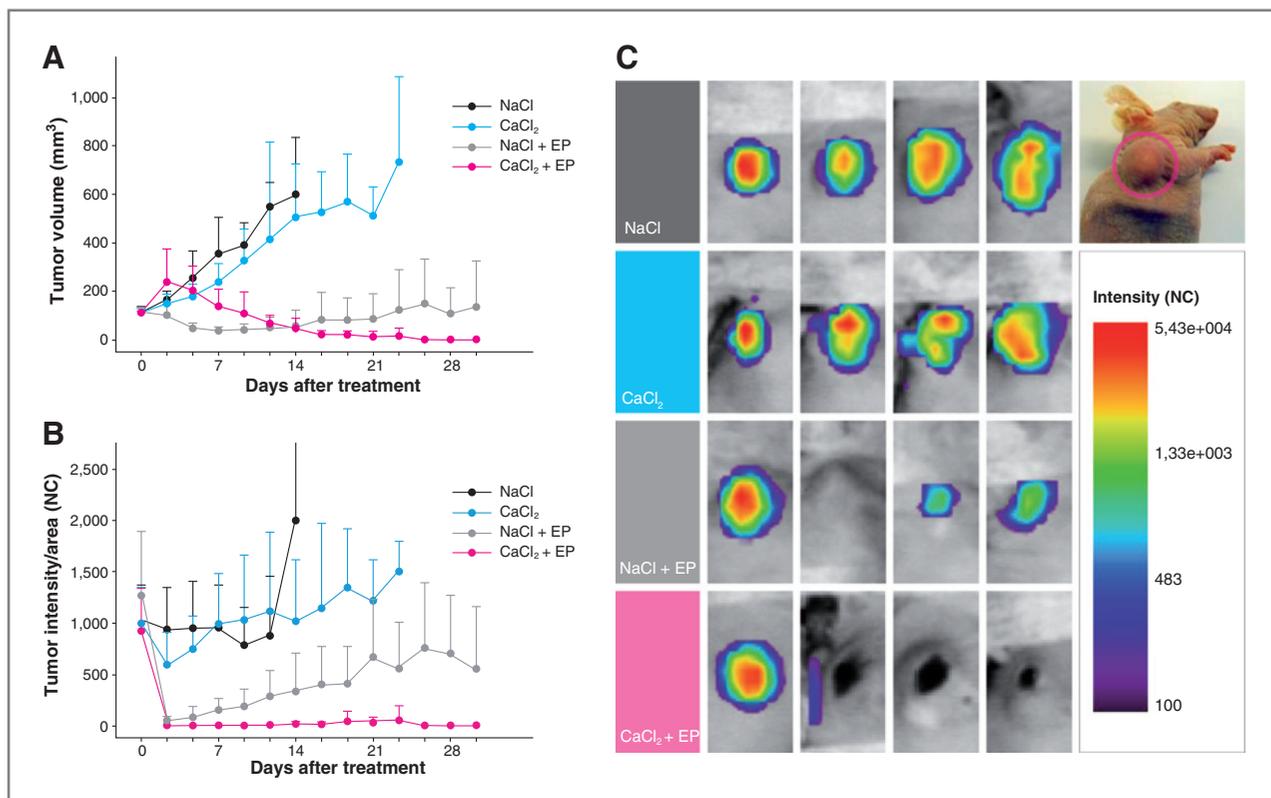
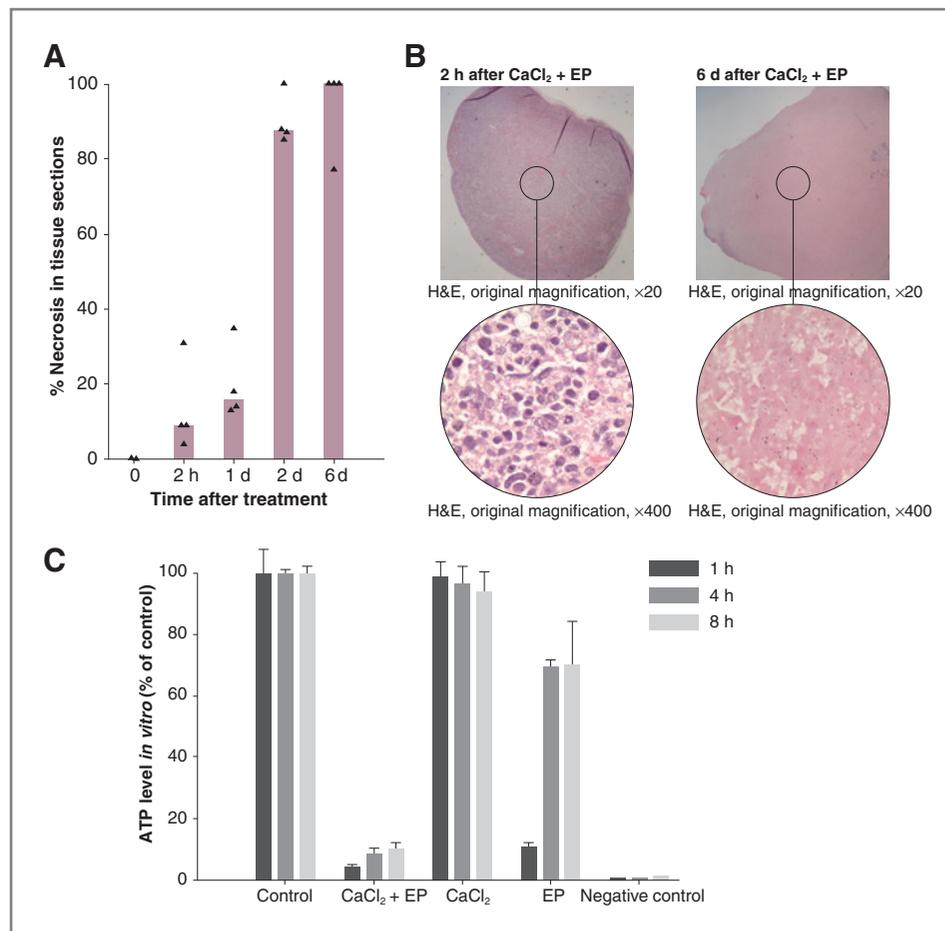


Figure 2. Calcium overloading induces cell death *in vivo*. H69 (EGFP-transfected human small cell lung cancer cell line) tumors induced on nude mice were treated with calcium chloride and electroporation (red), calcium-free physiologic saline and electroporation (gray), calcium chloride alone (blue), or calcium-free physiologic saline alone (black). Tumor size (A) and fluorescence intensity in bioluminescence (B) were measured before treatment and 3 times a week after treatment, (means \pm SD, $n = 3-9$). C, representative images of fluorescence intensity in the tumors; placement of the mouse in the scanner and location of the tumor are shown in the top right corner, and intensity bar is shown as a logarithmic scale. EP, electroporation.

Figure 3. Calcium overloading induces tumor necrosis and ATP depletion. **A**, the fraction of necrosis in tumors after calcium electroporation determined by stereologic point counting, [median, individual data points (\blacktriangle), $n = 4$ for treated tumors and $n = 2$ for untreated tumors] is shown. **B**, light microscope images of H&E sections of tumors 2 hours or 6 days, respectively after calcium electroporation. **C**, ATP level in DC-3F cells 1 (dark gray), 4 (gray), and 8 hours (light gray) after treatment with calcium electroporation, calcium alone, or electroporation alone, in control (untreated cells), and negative control (dead cells). ATP level in percentage of control (means \pm SD, $n = 6$). Calcium electroporation resulted in an immediate and severe drop in ATP level, which stayed low, at 10.3% ($P < 0.0001$) of control levels up to 8 hours after treatment. EP, electroporation.



Optical bioimaging was used *in vivo* to consecutively track the amount of tumor tissue expressing EGFP (10). Fluorescence intensity of Ca²⁺-EP-treated tumors decreased drastically after treatment and stayed at background levels for the remainder of the experiment, being significantly different from tumors treated with NaCl-EP ($P < 0.01$) and from control groups treated without electroporation ($P < 0.0001$). As expected, fluorescence intensity of the nonelectroporated tumors increased over time. The fluorescence intensity of tumors treated with NaCl-EP decreased 2 to 3 days after treatment and was significantly different from that of tumors treated with NaCl ($P < 0.05$); thereafter, the fluorescence intensity increased and was not significantly different from that of nonelectroporated tumors (Fig. 2B and C).

Histologic analysis was conducted on tissue sections, and the fraction of necrosis was estimated. The analysis of Ca²⁺-EP-treated tumors showed progressive necrosis, which was highly significant 2 days after treatment ($P < 0.0001$) and complete 6 days after treatment (Fig. 3A and B).

Because Ca²⁺-EP treatment leads to highly efficient cell death independently in different cell lines and also leads to uniform necrosis across tumors within 6 days, a condition fundamental for cell survival must be involved. Previous work showed that ATP decreased significantly in tissue exposed to high-voltage pulses (13, 14). Determination of ATP levels in

cells after treatment showed that Ca²⁺-EP treatment resulted in an immediate and severe drop in ATP level (Fig. 3C). Cells treated with electroporation alone exhibited a similar drop in ATP level but with a marked recovery 4 hours after treatment to levels significantly higher than those of Ca²⁺-EP-treated cells ($P < 0.0001$). Calcium without electroporation did not affect ATP levels. Cells electroporated with calcium-free physiologic saline showed a similar drop and recovery in ATP level as cells treated with electroporation alone (data not shown).

Here, we show that calcium electroporation leads to acute ATP depletion and cell death (*in vitro*) as well as massive tumor necrosis *in vivo*. As shown in Fig. 4, ATP depletion in relation to increased intracellular levels of free calcium may be caused by greatly increased activity of the Ca²⁺-ATPase, leading to high consumption of ATP (2, 5). Furthermore, a high intracellular calcium level may induce opening of permeability transition pores (PTP) in the mitochondrial membrane, resulting in loss of the electrochemical gradient, the driving force for ATP production, and thereby uncoupling mitochondrial formation of new ATP (2, 5, 6). Other cellular effects associated with calcium overload include activation of lipases and proteases, and generation of reactive oxygen species (ROS), which may also contribute to cell death (2, 5, 6). Finally, the electroporation procedure itself may lead to some increased ATP consumption as the influx of sodium (directly or due to sodium

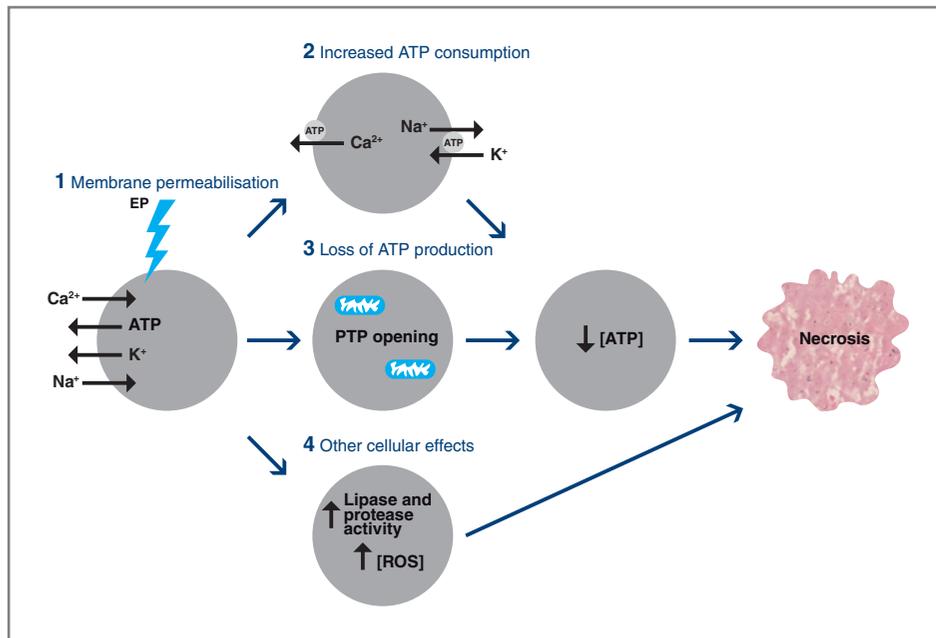


Figure 4. Schematic view of the effect of calcium overloading. Electroporation (EP) generates reversible pores in the cell membrane (A) allowing influx of calcium and sodium, and efflux of potassium and possible also of ATP. Changes in the intracellular ion concentrations lead to high ATP consumption by Ca^{2+} -ATPases (in the plasma and endoplasmic reticulum membranes) and Na^+/K^+ -ATPases (B). Calcium overload may induce PTP opening in the mitochondrial membrane resulting in loss of ATP production due to loss of the electrochemical gradient (C) and activation of lipases and proteases, and generation of ROS (D). This results in severe ATP depletion and necrosis of the cell.

calcium exchange) may increase Na^+/K^+ -ATPase activity (13). A direct loss of ATP through permeabilization structures is also a possible contributor. Together, this may result in cell death (Fig. 4; refs. 2, 5, 6). Severe calcium overload causes cell death, and depending on the cellular ATP level, cells undergo either apoptosis or necrosis. If the majority of mitochondria remain capable of ATP synthesis, the ATP loss may have a transient nature favoring the apoptotic pathway (15–17). On the contrary, if ATP depletion is too severe for apoptosis to occur, the cell will undergo necrosis (15–17). Our results clearly show that calcium overload induced by calcium electroporation results in severe ATP depletion and necrosis (Fig. 3).

Electroporation has been used for local treatment of malignant tumors in combination with chemotherapeutic agents (electrochemotherapy; refs. 11, 18) or plasmids (gene electrotransfer; ref. 7) in clinical trials. The cytotoxic agents bleomycin or cisplatin have been used, either by i.v. or by the intratumoral route, followed by application of electric pulses to the tumor (11, 18). Our suggestion would be to use direct intratumoral calcium injection followed by electroporation according to the standard procedures already published for electrochemotherapy (7). We found that injecting isotonic calcium chloride corresponding to half the tumor volume followed by electroporation was highly efficient. Systemic administration of calcium was not tested in this study, as adsorption to plasma proteins and risk of hypercalcemia were predicted. Because electrochemotherapy has proved efficient in cutaneous tumors, electrodes facilitating the application of electro-

poration for use in colorectal cancer, bone and liver metastases, and brain tumors (19) have been developed, and are now in clinical trials (7). Efforts are also being made to carry out irreversible electroporation of tumors (7) in which addition of calcium could enhance efficacy. Because calcium for injection is commercially available and regularly used at most hospitals and electric pulses are already used clinically, this treatment could easily be implemented. In addition, calcium has an excellent safety profile both for use in patients and for staff, and would not need administration by staff accredited to administer chemotherapy. Finally, cost of cancer treatment is causing global concern (20), and calcium electroporation is both simple and inexpensive and is likely to be of potential benefit in the treatment of local tumors regardless of histology.

Disclosure of Potential Conflicts of Interest

A patent has been submitted (inventors S.K. Frandsen, H. Gissel, P. Hojman, J. Eriksen, and J. Gehl).

Acknowledgments

The authors thank Marianne Fregil, Anne Boye, and Lone Christensen for providing excellent technical assistance.

Grant Support

S.K. Frandsen (scholarship, Danish Cancer Society), P. Hojman (grant, Danish Medical Research Council), H. Gissel (grant, Lundbeck foundation), and J. Gehl (research fellow of the Royal Swedish Academy of Science supported by the Acta Oncologica Foundation).

Received November 16, 2011; revised January 3, 2012; accepted January 19, 2012; published OnlineFirst January 26, 2012.

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