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Electric Field Pulses Can Induce Apoptosis

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Abstract. Injection of electric field pulses of high intensity (kV/cm) and short duration (microsecond range) into a cell suspension results in a temporary increase of the membrane permeability due to a reversible electric breakdown of the cell membrane. Here we demonstrate that application of supercritical field pulses between 4.5 and 8.1 kV/cm strength and 40 μsec duration induce typical features of apoptosis in Jurkat T-lymphoblasts and in HL-60 cells including DNA fragmentation and cleavage of the poly(ADP ribose) polymerase. Apoptosis induction did not depend on the presence of any particular electrolyte in the extracellular medium. However, no apoptosis was observed in solutions without a minimum amount of salt. Apoptotic DNA fragmentation was prevented by the caspase inhibitor zVAD.

Key words: Electropermeabilization — Electroporation — Apoptosis — Jurkat T-lymphoblast — HL-60 — Caspase

Introduction

Field pulse techniques are widely used for the manipulation of the genome and the cytoplasm of suspended cells as well as for fusion of cells after dielectrophoretic alignment [31]. Under optimum conditions high transfection and high hybridization rates can be obtained in a very reproducible manner. Exposure of suspended cells to an exponentially decaying external electric field pulse of high intensity (kV/cm) and short duration (up to about 100 μsec) leads to a reversible electric breakdown of the membrane [32]. The breakdown of the membrane is associated with a temporary increase of the membrane permeability which allows the incorporation of normally membrane-impermeable low- and high-molecular weight xenomolecules into the cytoplasm. Field-treated cells recover their original plasma membrane impermeability within minutes to hours depending on the temperature.

Reversible electric breakdown of the cell membrane occurs at membrane sites in field direction when the membrane voltage exceeds 1 V at room temperature or approximately 0.5 V at 37°C. The area of membrane permeabilization depends — among other things — on the field strength and, because of the temperature-dependence of the resealing process, on the temperature at which the field pulse is applied (reviewed in [31]). Substantial supercritical field strengths lead to cell death [9, 15, 17]. In addition, the reversibility of the high-permeabilization state of the membrane of the pulsed cells critically depends on the ionic composition of the pulse medium. This is due to the loss of the selective osmotic barrier of electropermeabilized cells. External relatively high concentrations of potassium ions are necessary to avoid the collapse of the ion gradients after permeabilization [31], but they can be toxic to the outer surface of the membrane. Thus, the field and medium conditions before and during breakdown, as well as the temperature-dependent resealing process dictate not only the rate and the amount of uptake of xenomolecules, but also the rate of viable cells once the resealing process is completed. Further optimization of the field pulse techniques can be envisaged if the processes are known which lead to cell death due to the electropermeabilization of the cell membrane, particularly at supercritical field strengths.

Cellular death may occur by either necrosis or apoptosis. Whereas necrosis is regarded as a nonphysiologic type of cell destruction, apoptotic cell death occurs both in physiological situations and upon contact with various noxious agents. Apoptosis is a process of cell death characterized by morphological and biochemical alterations of the cell including membrane blebbing, chro-

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matin condensation, DNA fragmentation [27] and the degradation of intracellular proteins such as poly(ADP-ribose) polymerase (PARP), lamin and others [3, 4, 13] and involves the concerted action of many enzymatic steps (reviewed in: [24]). Apoptosis can be induced in a variety of different ways, e.g., by cross-linking of the CD95 molecule [25, 30], ionizing radiation [20, 22, 28], glucocorticoids [2], cytotoxic T cell activity [7, 16, 18], long duration current [12], serum removal [21] and other factors.

In this communication, we demonstrate that application of an electric field pulse of supercritical field strength at 33–37°C results in cell death by apoptosis. The process depends on the field strength and the medium composition. Apoptotic DNA fragmentation and PARP cleavage can be prevented by addition of the caspase inhibitor zVAD to the medium.

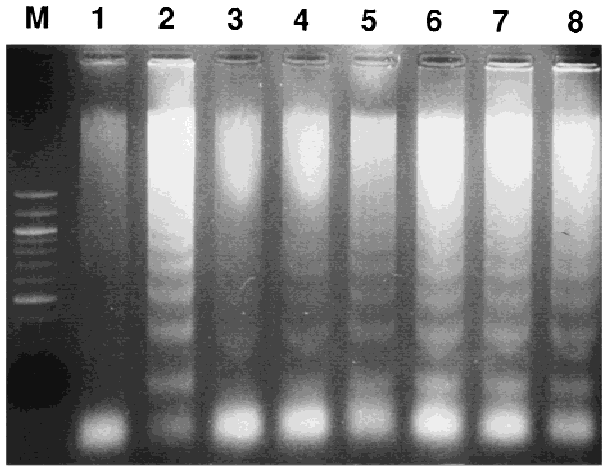

Fig. 1. Electric field pulses induce apoptosis in Jurkat cells. Jurkat T cells were treated in R-10 medium with electric field pulses of increasing strength or with an apoptosis-inducing concentration of Triton X-100 and LMW DNA prepared as described in Materials and Methods. Lanes: M: 100 bp ladder DNA marker; 1: untreated cells, 2: Triton-treated cells; 3: 3.6 kV/cm; 4: 4.5 kV/cm; 5: 5.4 kV/cm; 6: 6.3 kV/cm; 7: 7.2 kV/cm; 8: 8.1 kV/cm.

Materials and Methods

Cells

The human T-lymphoblast cell line Jurkat E6-1 [26] and the human promyeloblastic cells HL-60 were obtained from the American Tissue Culture Collection. The cells were cultured in RPMI-1640 culture medium supplemented with antibiotics, 10 mM HEPES buffer and 10% (R-10) or 15% (R-15) FCS. The radius of the cells was measured electronically using the Casy-1 instrument (Schärfe, Reutlingen, Germany) based on the Coulter Counter principle. The average radii of the Jurkat and the HL-60 cells were 9.27 ± 0.04 μm and 6.38 ± 0.03 μm, respectively.

Electropermeabilization of Cells

For electropermeabilization (also termed electroporation, electroinjection), cells were centrifuged for 5 min at 300 × *g*, washed and resuspended in pulse medium at a concentration of 1.5–2 × 10^6 cells/ml. The media used were either FCS-supplemented RPMI-1640 culture medium or phosphate-buffered (K_2HPO_4 0.85 mM, KH_2PO_4 0.3 mM, pH 7.4) aqueous solutions. The buffered solutions contained either no additional salt, NaCl, KCl, sodium acetate, potassium acetate, LiCl, or $MgSO_4$ at various concentrations. The osmolality of the solutions was determined using a cryoscope (Osmomat 030, Gonotec, Berlin, Germany) and adjusted to 280–300 mOsmol with myo-inositol (Boehringer Mannheim, Mannheim, Germany). The cell suspensions were transferred into a prewarmed discharge chamber which consists of two flat, circular, stainless-steel electrodes (5.5 mm apart; volume 1.2 ml). Cells were subjected to a single, exponentially decaying pulse of up to 8.1 kV/cm strength and a decay time constant of 40 μsec by using a high-voltage pulser (Biojet MI, B. Braun, Melsungen, Germany). Pulsing was performed at 33–37°C. The cells were transferred back into R-10 or R-15 culture medium and incubated for 4 hr at 37°C. Cells were then processed for analysis of DNA fragmentation or PARP cleavage. As a positive control for apoptosis, cells were treated with the anti-CD95 mAb CH-11 (800 ng/ml, Coulter Immunotech, Hamburg, Germany) or with 0.0075% Triton X-100 which readily induces apoptosis in a variety of different cells ([1], Strupp and Jassoy, *manuscript in preparation*). For inhibition experiments, the synthetic caspase inhibitor carbobenzoxy-Val-Ala-Asp-fluoromethylketone (zVAD; Enzyme System Products, Livermore, CA) was used at 50 mM and added to Jurkat cells immediately after the pulse.

Determination of Cell Membrane Breakdown by Fluorescence Spectroscopy

Cells (10^6) were washed, resuspended in 1.2 ml pulse medium and propidium iodide (Sigma, Deissenhofen, Germany) was added at a final concentration of 25 μg/ml. Cells were transferred to a pulse cuvette that contains two stainless steel electrodes at a distance of 10 mm. The cuvette was placed into a fluorescence spectrometer (Luminescence Spectrometer LS 50, Perkin Elmer-Applied Biosystems, Weiterstadt, Germany) and pulses of defined field strengths applied to the cells. Fluorescence was measured at 610 nm in time-drive mode for up to 10 min. The excitation wave length was 560 nm.

Determination of Intracellular DNA Fragmentation

The low molecular weight (LMW) DNA was prepared as described previously [11]. Briefly, cells were lysed in a 20 mM Tris/EDTA solution containing 0.5% Triton X-100. The high-molecular weight DNA was removed by high speed centrifugation and the proteins by phenol/chloroform extraction. After precipitation in isopropanol over night, the LMW DNA was resolved in a 10 mM Tris/EDTA buffer containing 0.5% SDS. The DNA was analyzed by agarose gel electrophoresis using 1.8% gels and a 100 base pair ladder molecular weight marker. Nucleic acids of a size of below 100 DNA base pairs were regularly obtained upon preparation of LMW DNA from both intact and apoptotic cells. The intensity of this nucleic acid band corresponded to the amount of processed cells. It was reduced when cells were lysed before preparation of the LMW DNA.

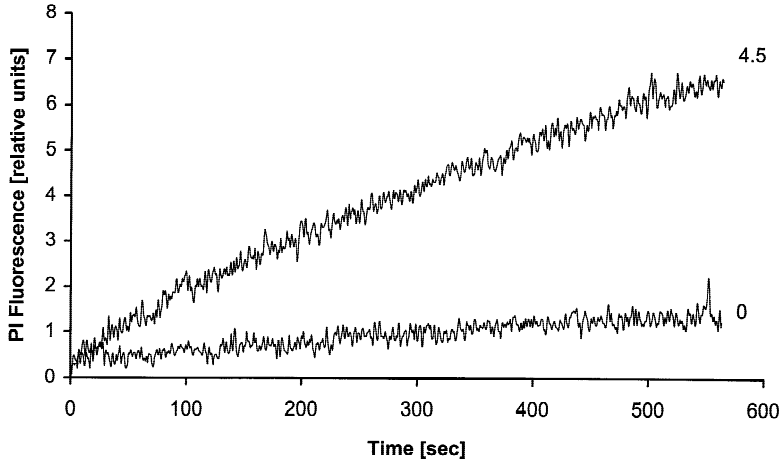

Fig. 2. Membrane permeabilization by a field pulse in low conductivity medium. Jurkat cells in potassium phosphate-buffered iso-osmolar low conductivity medium containing inositol but no additional salt were treated with an electric field pulse of 4.5 kV/cm (4.5). As control, cells were not treated with a field pulse (0). Propidium iodide uptake was determined by fluorescence spectroscopy.

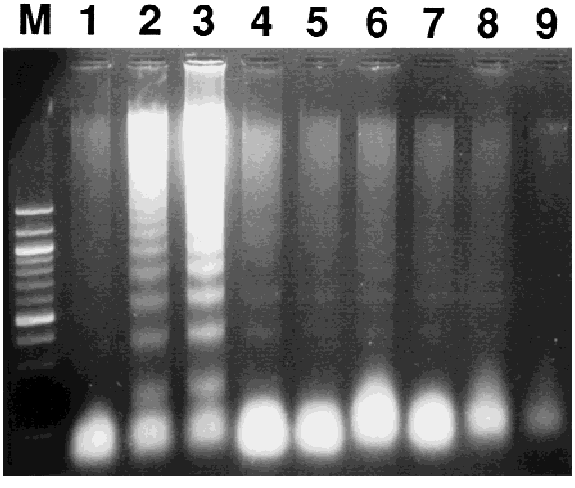

Fig. 3. Apoptosis is not induced in low conductivity medium. Jurkat cells were treated with field pulses of increasing strength in an isotonic potassium phosphate-buffered low conductivity medium. As controls, cells were treated with 6.3 kV/cm or Triton X-100 in R-10 medium. Lanes: M: 100 bp ladder DNA marker, 1: untreated cells; 2: cells treated with a field pulse in R-10 medium; 3: cells treated with Triton; 4: 0.9 kV/cm; 5: 1.8 kV/cm; 6: 2.7 kV/cm; 7: 3.6 kV/cm; 8: 4.5 kV/cm; 9: 5.4 kV/cm.

Analysis of Poly(ADP-Ribose) Polymerase Cleavage

Field pulse-treated Jurkat cells (1.5–2 × 10^6 cells) were lysed in 30 μl 20 mM Tris/EDTA solution containing 0.5% Triton X-100. The protein content of the samples was determined by the Bradford method and equal amounts of protein from each sample were separated by SDS polyacrylamide gel electrophoresis [19]. The proteins were then analyzed by Western blotting, using the anti-PARP mAb C2-10 (diluted 1:2000, Pharmigen, San Diego, CA), which recognizes both the complete 116 kD PARP molecule and the 85 kD cleavage fragment. A peroxidase-conjugated rabbit anti-mouse antiserum (diluted 1:500, Dako, Hamburg, Germany) was used as a secondary antibody. To visualize the PARP protein, a freshly prepared mixture of 0.02% 3-amino-9-ethyl-carbazole in 5% dimethylformamide and 0.015% H_2O_2 in 50 mM sodium acetate (pH 5.0) was used as substrate.

Results

Electric Field Pulses Induce DNA Fragmentation

Jurkat cells suspended in R-10 culture medium were subjected to single exponentially decaying 40 μsec field pulses of increasing strength from 3.6 to 8.1 kV/cm at 33–37°C. These field strengths were above the breakdown voltage as determined by propidium iodide uptake and incorporation into the cellular DNA by fluorescence spectroscopy (*data not shown*). Treatment of the cells with field pulses above 4.5 kV/cm readily induced DNA fragmentation. The degree of fragmentation observed increased up to a maximum at a field strength of 6.3 kV/cm (Fig. 1). Similarly, electrical field pulses induced DNA fragmentation in HL-60 cells in a voltage-dependent fashion. Maximum DNA fragmentation in these cells was observed at a field strength of 6.9 kV/cm (Fig. 5*C* and *data not shown*).

Field-induced Apoptosis is Determined by the Ionic Composition of the Pulse Medium

To identify environmental factors that are required for the execution of apoptosis by an electric field pulse, Jurkat cells were pulsed in aqueous solutions of different electrolyte compositions. In the first set of experiments, we used a potassium phosphate-buffered iso-osmolar, low conductivity medium containing inositol but no additional salts. Propidium iodide uptake experiments were performed in order to substantiate that the membrane was rendered permeable by the electric field pulse. In these experiments, it was observed that in the low conductivity medium the field strength critical for the Jurkat cell membranes was reached at approximately 3 kV/cm. An example of membrane permeabilization and propidium iodide uptake at 4.5 kV/cm is shown in Fig. 2.

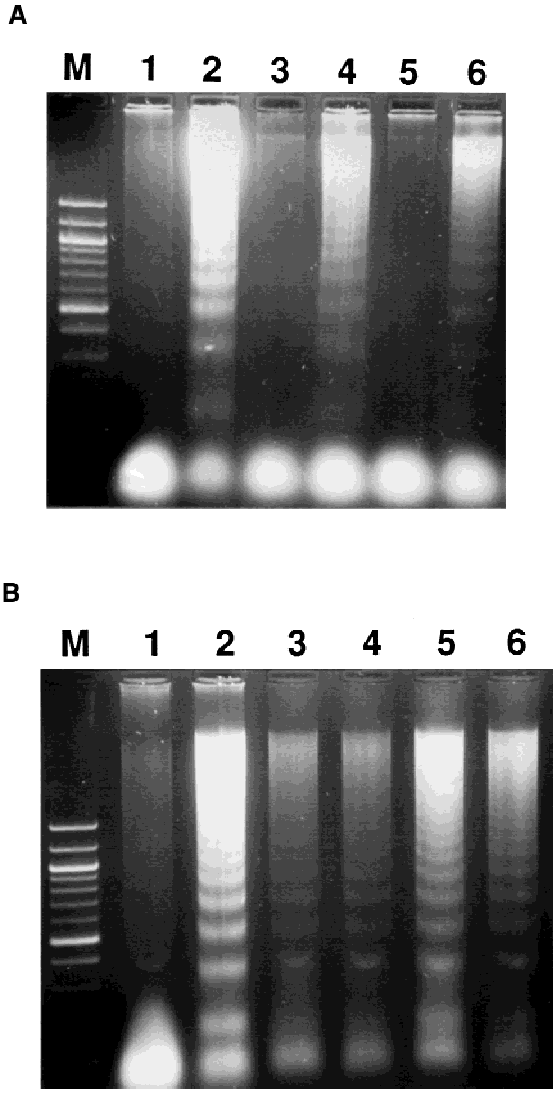

Fig. 4. Induction of apoptosis by a field pulse depends upon the ionic milieu. Jurkat cells were treated with a field pulse in potassium phosphate-buffered solutions containing different concentrations of NaCl, potassium acetate (KAc) or $MgSO_4$. Untreated cells and cells pulsed with 6.3 kV/cm in R-10 medium were used as controls. (*A*) Lanes: M: 100 bp ladder DNA marker; 1: untreated cells; 2: cells treated with a field pulse in R-10 medium; 3: 40 mM NaCl, 4.5 kV/cm; 4: 140 mM NaCl, 6.3 kV/cm; 5: 40 mM KAc, 4.5 kV/cm; 6: 140 mM KAc, 6.3 kV/cm. (*B*) Lanes: M: 100 bp ladder DNA marker, 1: untreated cells; 2: cells treated with a field pulse in R-10 medium; 3: 5 mM $MgSO_4$, 2.2 kV/cm; 4: 5 mM $MgSO_4$, 2.8 kV/cm; 5: 15 mM $MgSO_4$, 3.6 kV/cm; 6: 15 mM $MgSO_4$, 4.5 kV/cm.

However, no DNA fragmentation was observed in this medium at field strengths of up to 5.4 kV/cm. Increasing destruction of the cells at higher voltages is additionally mirrored by the reduced yield of RNA upon processing of the cells treated with field strengths above 3.6 kV/cm. At 5.4 kV/cm, cells were almost completely destroyed and few nucleic acids could be recovered from the remaining cell pellet (Fig. 3).

In the following experiments, part of the inositol in the basic medium was replaced by iso-osmolar amounts of monovalent salts such as NaCl or potassium acetate. Apoptosis was observed in these electrolyte solutions at high salt concentrations but not in solutions containing 40 mM of the salts (Fig. 4). Similar results were obtained with KCl, LiCl, or sodium acetate solutions (*data not shown*) indicating that induction of apoptosis was independent of any particular electrolyte. In contrast to that with monovalent electrolytes, DNA fragmentation in solutions containing $MgSO_4$ was detected at salt concentrations as low as 5 mM, and maximum fragmentation was observed at 15–25 mM (Fig. 4). Additional experiments were performed to specifically address the role of calcium. DNA fragmentation was not inhibited in the presence of 1.5–8 mM EGTA (*data not shown*).

Electric Field Pulses Activate Caspases

To examine whether other features characteristic of apoptosis were induced by treatment with electric field pulses, we examined the degradation of the PARP molecule. This enzyme is a substrate of several caspases which mediate and execute the apoptotic pathway [24]. Treatment with an electric field pulse resulted in cleavage of the PARP protein. Moreover, inhibition of caspases by the synthetic peptide zVAD blocked the cleavage of the PARP molecule and prevented DNA fragmentation induced by the electric field pulse in both Jurkat and HL-60 cells (Fig. 5).

Discussion

In this study, we demonstrate that electric field pulses of 40 μsec and a field strength above 4.5 kV/cm induce cell death by apoptosis in Jurkat T-lymphoblasts and in HL-60 cells in normal culture medium such as RPMI-1640. Electroinjection of xenomolecules into living cells is usually performed at 4°C [31]. The reason is that the duration of the high-permeability state of the membrane after application of the field pulse decreases with increasing temperature because of a more rapid resealing of the membrane. On the other hand, the release of intracellular solutes is more pronounced at low than at high temperatures, a process that affects cell viability. We chose to study the effect of field pulses at temperatures above 30°C to minimize effects that would complicate the interpretation of the apoptosis induction. In addition, due to the facilitated resealing at this temperature, field strengths higher than usual could be applied without overt cytolysis [5]. Finally, many myeloma and hybridoma cell lines do not tolerate low temperatures for a long

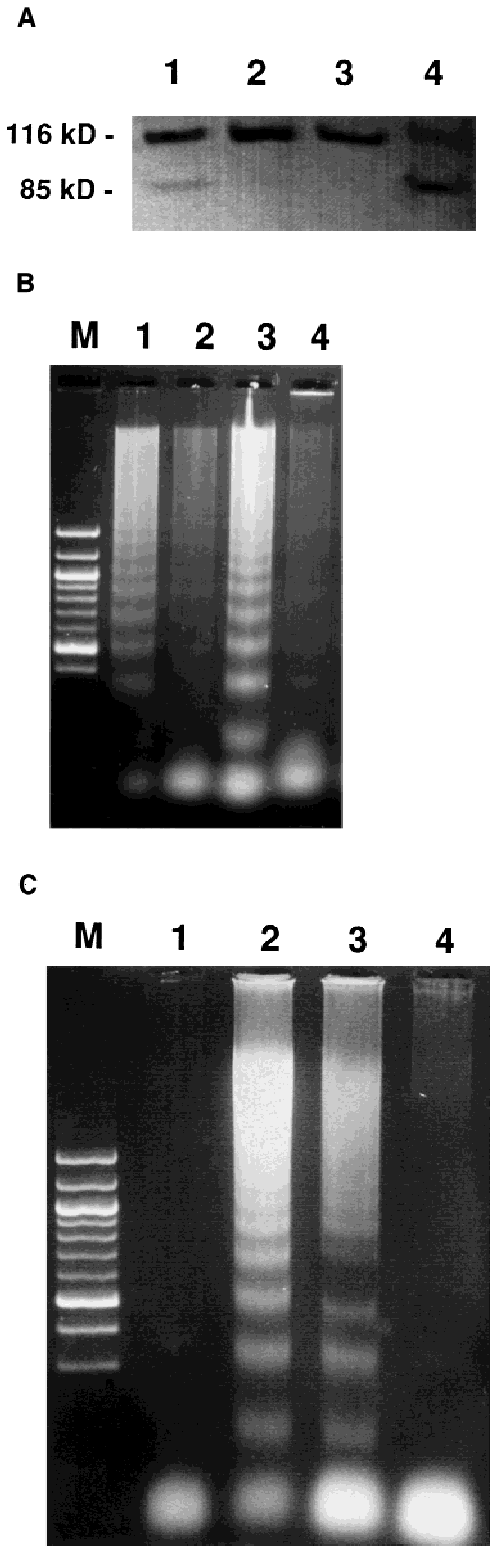

Fig. 5. Inhibition of caspases prevents field pulse-induced PARP cleavage and DNA fragmentation. (*A*) Jurkat cells were treated with an electric field pulse of 6.3 kV/cm or with an apoptosis-inducing concentration of Triton X-100 and cleavage of the PARP molecule analyzed by Western blotting. Field pulse-treated cells were analyzed in the absence or presence of the caspase inhibitor zVAD (50 mM). Lanes: 1: field pulse-treated cells; 2: field pulse-treated cells and zVAD; 3: untreated cells; 4: Triton-treated cells. (*B*) Jurkat cells were treated in R-10 medium with an electric field pulse of 6.3 kV/cm in the absence or presence of zVAD or with the apoptosis-inducing anti-CD95 mAb CH-11. Lanes: M: 100 bp ladder DNA marker; 1: anti-CD95 mAb-treated cells; 2: untreated cells; 3: field pulse-treated cells; 4: field pulse-treated cells with zVAD. (*C*) HL-60 cells were treated in R-15 medium with an electric field pulse of 6.9 kV/cm in the absence or presence of zVAD or with an apoptosis-inducing concentration of Triton X-100. Lanes: M: 100 bp ladder DNA marker; 1: untreated cells; 2: Triton-treated cells; 3: field pulse-treated cells; 4: field pulse-treated cells with zVAD.

time period, even without pulsing [31] and electroinjection at higher temperatures would be an advantage.

We previously reported that cell membranes are more sensitive to an electric field pulse in solutions of low conductivity [6]. To analyze membrane destruction under low conductivity conditions, field pulses were applied to cells in phosphate-buffered iso-osmolar inositol solutions containing no additional electrolytes. No apoptosis was observed in these pulse media although cells were effectively rendered permeable and finally destroyed by the field pulse. The fact that apoptosis was not observed in the low conductivity medium was not due to the inability to induce DNA fragmentation in this solution because control experiments demonstrated that an anti-CD95 mAb readily induced DNA fragmentation in this medium (*data not shown*). This observation indicates that induction of apoptosis by an electric field pulse is not merely a consequence of the permeabilization of the cell membrane but requires additional factors.

To examine the role of extracellular electrolytes for apoptosis induction in the electric field, we added various salts to the low conductivity medium. Since larger areas of the membrane are rendered permeable in media of low conductivity [23], lower field strengths were applied to cells in less concentrated salt solutions. We observed that the addition of any of a wide variety of salts to the pulse medium was sufficient to allow the induction of DNA fragmentation. However, a prerequisite for the occurrence of apoptosis in the electric field was that the concentration of monovalent salts in the pulse medium exceeded approximately 60 mM. In the case of the divalent salt $MgSO_4$, apoptosis occurred at much lower concentrations. This suggests that the ionic strength is critical for the induction of apoptosis by the field pulse.

The concentration and nature of the pulse medium ingredients are crucial for electroporation and electrofusion because of the loss of the selective osmotic barrier in permeabilized cells. For instance, the potassium concentration in the external pulse medium is an important factor for the survival of the pulsed cells [31]. An external relatively high concentration of potassium ions is necessary to avoid the collapse of the ion gradient after

permeabilization, but it can be toxic to the outer surface of the membrane. We recently recommended a medium for electropermeabilization that contains 30 mM KCl [31]. No apoptosis was observed when field pulses were applied in this solution at 33–37°C (*data not shown*). Our observations suggest that this is due to the low concentration of electrolytes in the medium proposed. The results obtained in this study indicate that media that contain more than 60 mM KCl or NaCl such as those that were used previously by others [10, 14, 29] may cause increased damage to the cells through apoptosis.

We observed that field pulse-induced signs of apoptotic cell death including DNA fragmentation and cleavage of the PARP molecule were prevented by the caspase inhibitor zVAD. This demonstrates that caspases are involved in field-induced apoptosis, a feature characteristic of other types of apoptosis [24]. It remains to be determined whether inhibitors of caspases improve cell viability in this situation.

Hypo-osmolality increases the transfection and hybridization rates considerably and allows the use of inexpensive field instruments which deliver microsecond pulses of only up to about 1.29 kV [8]. However, cells do not tolerate hypo-osmolarity for a long period of time. To improve the conditions for electropermeabilization, it will be important to extend studies on induction of cell death to strongly hypo-osmolar media. In this regard, preliminary experiments with the Multiporator (Eppendorf, Hamburg, Germany) indicate that such media are beneficial, but apoptosis can occur in certain cells when cells are exposed to this environment at 37°C for longer than approximately 15 minutes (*unpublished data*).

In conclusion, electric field pulses applied at 33–37°C induce apoptotic cell death in the presence of a minimum concentration of electrolytes but not in low conductivity and low salt media. No particular electrolyte in the extracellular medium is required for apoptosis induction. Membrane permeabilization alone is insufficient to activate the apoptotic process. To examine whether this observation is relevant to the currently used electropermeabilization protocols, additional experiments have to be performed at lower temperatures. If apoptosis similarly occurs when cells are pulsed at low temperature, the application of agents that prevent apoptosis may enhance the efficiency of electropermeabilization.

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