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Hemolysis of human erythrocytes by a transient electric field

(Joule-heating/membrane/pores/ionic permeability)

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ABSTRACT **Exposure of human erythrocytes, under isotonic conditions, to a high voltage pulse of a few kV/cm leads to total hemolysis of the red cells. Experiments described herein demonstrate that the hemolysis is due to the effect of the electric field. Neither the effect of current nor the extent of the rapid Joule-heating to the suspending medium shows a direct correlation with the observed hemolysis. Voltage pulsation of the erythrocyte suspension can induce a transmembrane potential across the cell membrane and, at a critical point, it either opens up or creates pores in the red cells. In isotonic saline the pores are small. They allow passage of potassium and sodium ions but not sucrose and hemoglobin molecules. The pores are larger in low ionic conditions and permit permeation of sucrose molecules, but under no circumstances can hemoglobin leak out as the direct result of the voltage pulse. Kinetic measurements indicate that the hemolysis of the red cells follows a stepwise mechanism: leakage of ions leads to an osmotic imbalance which in turn causes a colloidal hemolysis of the red cells. Other effects of the voltage pulsation are also discussed.**

In the past few years, numerous studies bearing on the effects of high voltage pulsation and rapid Joule-heating on suspensions of cells and phospholipid bilayers have appeared in the literature (1–10). These studies have focused on two different aspects of the voltage pulsation: one deals with the lysis of, and the release of molecules from, cells treated with high electric fields (1–5), and the other deals with the relaxation phenomena of cell suspensions accompanying rapid Joule-heating (6–10). Both types of experiment foster the idea that the molecular release and the relaxation phenomena observed may be related to basic processes of membrane and nerve functions. Because the voltage pulsation in these studies introduces electric field and temperature jump at the same time, and because each of them can perturb the system in several different ways, the interpretation of the experimental data has been ambiguous. Possible perturbations of a cell suspension by a voltage pulse have been discussed elsewhere (4, 9, 11), and are summarized in a more comprehensive way in Table 1.

Among these studies, we have reported that a rapid temperature jump of an isotonic suspension of erythrocytes leads to hemolysis of red cells (4, 9). It was observed that glucose permeation occurred prior to hemolysis of the red cells. Because the magnitude of the temperature jump was small (<2°) and none of these phenomena were seen in a slow heating experiment, it was concluded that either the thermal osmosis effect or the electric field effect contributed to the lysis of the red cells (4). Distinction of the two effects was not possible.

In this communication, we report a controlled experiment in which the hemolysis of the red cells treated with a high voltage pulse is shown to be due to the electric field. Kinetic measurements indicate that the hemolysis occurs because of the osmotic imbalance generated by the leakage of ions and small molecules.

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MATERIALS AND METHODS

Human blood was obtained from healthy young adults by venipuncture in the presence of heparin. Erythrocytes were washed three times with a solution containing 150 mM NaCl and 7 mM phosphate buffer, pH 7.0. After washing, the cells were resuspended in mixtures consisting of various ratios of the above NaCl solution and a 272 mM sucrose solution (both solutions have approximately the same osmolarity, 300 milliosmolals (mOsm), which is supposed to be isotonic). The suspensions were kept at 0–4° until just prior to the pulsation. All experiments were done within the day of preparation.

The device for high voltage pulsation is shown in Fig. 1. A single electric pulse was applied to each sample after equilibration at room temperature (25 ± 2°). The intensity of the applied electric field E and the current density i were calculated from the known dimensions of the pulsation cell and the measured voltage and current. The magnitude of the temperature jump ΔT was obtained by the relation $\Delta T = i^2 r \Delta t/\rho C$, with ρ and C taken as unity and $r = E/i$ (refer to Table 1 for symbols).

The extent of hemolysis was determined from the absorption at 410 nm of hemoglobin in the supernatant. In kinetic studies, hemolysis was followed by turbidity at 700 nm of the whole suspension or colorimetry by eye of the sample spun down in a microhematocrit tube.

Sodium and potassium were determined by flame photometry after solubilization in Li_2SO_4. Radioassay of [^{14}C]sucrose was made on samples bleached with hydrogen peroxide/perchloric acid and dissolved in Hydromix (Yorktown Research). A detailed account of the molecular permeation experiments as well as other kinetic measurements will be given elsewhere (K. Kinosita, Jr. and T. Y. Tsong, unpublished data).

RESULTS AND DISCUSSION

Hemolysis is due to the field-dependent perturbation of the cell membrane

As shown in Table 1, possible effects of an electric pulsation can be classified into those due primarily to the externally applied electric field (row 1) and those resulting from the gross heating of the medium (row 2). Effects in column A can be observed in ordinary solutions as well as in cell suspensions. In the latter, however, the presence of membranes introduces several distinct possibilities, which are listed in column B. Thus, in 1B, the cell membrane as an electric insulator allows the total polarization of the cell interior, which results in a large transmembrane potential as can be shown by solving the Laplace equation (1, 4, 5). In a spherical cell with a 3-μm radius, for example, the electric field so induced across the membrane would be approximately 500 times as large as the applied field (see the equation in Table 1). Therefore, any field-dependent effects are highly intensified within or on the surface of the membrane.

Table 1. Perturbations of a cell suspension by a high voltage pulse

	A. Effects common to all systems	B. Effects specific to cell suspensions
1. Effects due to electric field	Electrophoresis Field-induced orientation Field-induced dissociation*	Field-induced transmembrane potential $\Delta V_{max} = 1.5aE$† Amplification of common effects within cell membrane Electrocompression of membrane‡ Large transmembrane current
2. Effects due to temperature jump of medium $\Delta T = i^2 r \Delta t / \rho C$*	Shift of chemical equilibrium $\Delta K/K = (\Delta H/RT^2)\Delta T$* Solvent expansion (shock waves) $\Delta P = (\alpha/\kappa)\Delta T$*	Temperature gradient across membrane† Colligative effect $\Delta\pi = cR\Delta T$† Thermal osmosis effect $\Delta P = -(Q/\bar{v}T)\Delta T$†

(1A) In ordinary solutions the electric field induces electrophoresis or orientation of solute (and solvent) molecules, dissociation of ion pairs, etc. In cell suspensions, the electromechanical force which acts on the whole cell may move, reorient, or deform the cells. (2A) Passage of current raises the temperature of the system by the Joule-heating effect. The rapid temperature jump induces chemical reactions toward a new equilibrium. A jump faster than the thermal expansion of the system can create a large pressure, which dissipates as shock waves. (1B) When the system contains membranes in closed vesicular form, the electric field is sustained primarily by the membrane, generating a large transmembrane potential. Thus, the common effects in 1A are much intensified in the vicinity of the membrane. The transmembrane potential also exerts such force as to thin the membrane (electrocompression). Passage of ions through the membrane may be promoted to a greater extent by the potential. (2B) The rapid temperature jump of the medium creates a temperature gradient across the membrane that may be sustained for a certain period of time. This results in an osmotic pressure difference between the inside and the outside of the cell due to the colligative effect, inducing a flow of water out of the cell. On the other hand, the flow of heat across the membrane accompanies the flow of water through the thermal osmosis effect. This effect might be larger than the colligative effect, and acts in the opposite direction. a, radius of the cell; C, specific heat of the suspension; c, concentration of the solutes; E, intensity of the applied electric field; ΔH, enthalpy of the reaction; i, current density; K, equilibrium constant; ΔP, pressure increment or pressure difference across the membrane; Q, heat of transfer of solvent molecules across the membrane; R, gas constant; r, specific resistivity of the suspension; T, temperature; ΔT, temperature increment or temperature difference across the membrane; Δt, duration of the pulse; ΔV_{max}, maximal transmembrane potential for a spherical cell; $\bar{v}$, partial molar volume of the solvent; α, expansion coefficient of the suspension; κ, compressibility of the suspension; ρ, density of the suspension; $\Delta\pi$, osmotic pressure difference.

* See ref. 12.

† See refs. 1, 4, 5, and 9 and those therein.

‡ See ref. 13.

In 2B, on the other hand, the membrane as a poor heat conductor can sustain the temperature gradient across the membrane generated by the Joule-heating. The resultant flow of water (see Table 1 and refs. 4 and 9) across the membrane might create such a pressure difference as to disrupt the cell.

Experimental discrimination between the field-dependent effects and the gross heating effects, the latter being dependent on the applied current, is straightforward because the field strength and the current density can be varied independently by changing the ionic strength of the medium. Because any of these effects may well depend on time, the two alternatives should be compared under the same pulse duration. Fig. 2 shows an example of such comparison, where the hemolysis of human erythrocytes was examined under various combinations of field and current intensities. In Fig. 2A the extent of hemolysis is plotted against the temperature increment ΔT. Obviously the data do not conform to the idea that the hemolysis is due to the temperature jump of the medium: in isotonic NaCl, ΔT of 2° is required for 50% hemolysis, while ΔT as small as 0.07° can cause hemolysis at the lowest ionic strength studied. When the same set of data is plotted against the field intensity, all curves coincide as shown in Fig. 2*B*. Clearly, the hemolysis is the result of a field-dependent process. The data presented here also eliminate the possible hemolyzing effect of electrolytic products which may accumulate at the electrodes in proportion to the total current. As a control, an erythrocyte sample was suspended in an isotonic saline pretreated with ten 20-microsecond (μs) pulses at 5 kV/cm. No lysis of the cells was observed.

At field intensities around a few kV/cm, the effects listed in Table 1 (1A) can hardly induce hemolysis: e.g., the electrophoretic velocity of a whole erythrocyte would be as small as a few mm/s; field-induced dissociation is significant only at fields much greater than 10 kV/cm. Therefore, a sole possibility seems to be the effects of the field-induced transmembrane potential.

For human erythrocytes, in fact, an applied electric field of 2.5 kV/cm generates a transmembrane potential of about 1 V (see Table 1). Synthetic phospholipid bilayers are known to break down at a transmembrane potential of a few hundred mV (14). A breakdown of *Valonia utricularis* at 0.85 V has been reported by Coster and Zimmerman (15). It is concluded that the hemolysis of erythrocytes is the result of the field-induced transmembrane potential. As suggested in Table 1 (1B), the transmembrane potential will perturb the cell membrane either through the interaction with charged molecules, dielectric forces, or the local heating due to highly promoted membrane currents.

Mechanism of the field-dependent hemolysis

How does a transient transmembrane potential (20 μs for the data given in this report) induce the hemolysis of red cells? Does it indiscriminately break down the cell membrane so that the cytoplasmic contents leak out at the moment of the voltage pulsation? Previous studies have revealed very little in this aspect and the question remains to be answered.

Under certain experimental conditions, the rate of the hemolysis is very slow. High ionic strength in pulsation medium, for example, markedly reduces the rate of hemolysis. When erythrocytes were treated with a 20 μs pulse at 3.7 kV/cm and then transferred into an isotonic NaCl solution, 50% hemolysis was attained, respectively, at 0.2 min, 0.6 min, 13 min, and 4 hr for the pulsation media containing 3, 10, 30, and 100% isotonic NaCl. Thus, the data in Fig. 2 were taken at 15 hr after the pulsation, in order to assure the completion of hemolysis reaction.

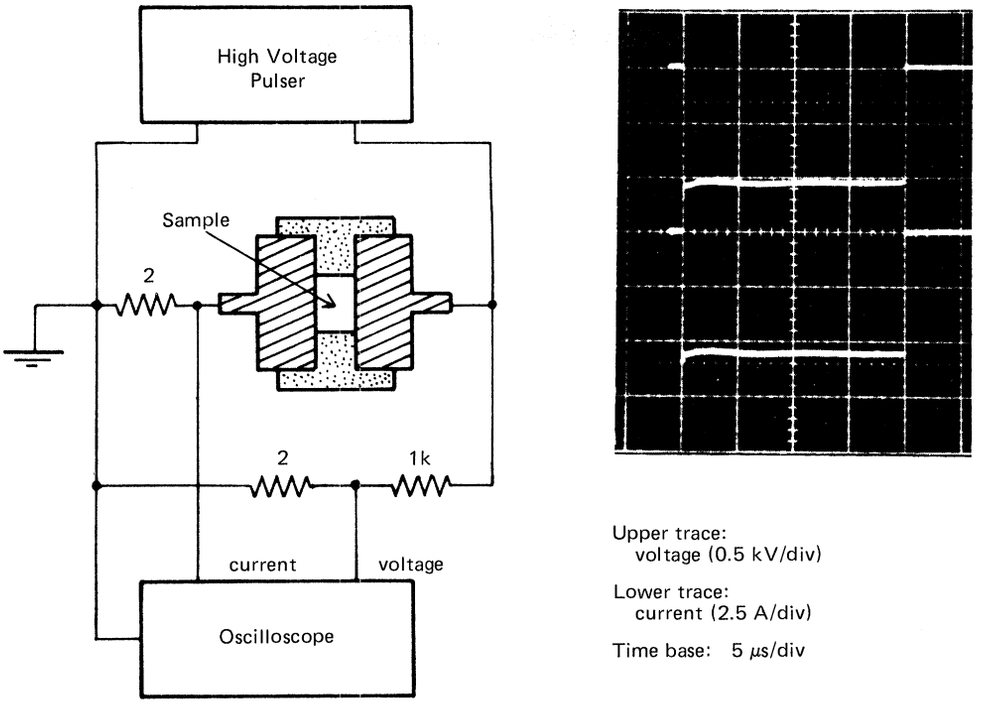

FIG. 1. A schematic diagram of the high voltage pulsation device (*Left*) and its waveforms (*Right*). A sample suspension is placed in a cylindrical cavity enclosed by a pair of stainless steel electrodes and a Plexiglas cell; several cells of different dimensions provide different combinations of the width and cross section of the cavity, ranging from 2 to 10 mm for the width and from 50 to 200 mm^2 for the cross section. The electrodes are connected to a Cober model 605P high-voltage pulser, which delivers a rectangular pulse of voltage up to 2.2 kV and duration of 50 ns to 10 ms. Upon every pulsation, both the voltage and current waveforms are recorded by a dual trace storage oscilloscope, as shown *Right*. Upper trace (*Right*), voltage (0.5 kV/division); lower trace, current (2.5 A/division); time base, 5 μs/division.

On the other hand, the pellet obtained by centrifugation in the course of hemolysis always consisted of two distinct layers, unlysed cells that retained the whole hemoglobin and the fully lysed "ghosts." This indicates that, under all conditions examined, release of hemoglobin from individual cells takes only a short time, less than some 10 s as confirmed under a microscope. Thus, the electric field lyses the cells only after a certain latency period that can be as long as hours, depending on the ionic strength. Obviously the membrane potential does not directly rupture the membrane.

The long latency period for the pulsation in isotonic NaCl enables us to observe several reactions preceding cell lysis. In Fig. 3A, curve D gives a time course of hemolysis by a 3.7 kV/cm, 20-μs pulse, and shows that more than 97% of the red cells still retained hemoglobin even after 20 min. On the other hand, the sodium content of the treated cells increased from that of the untreated cells, 10 ± 3 milliequivalents/liter of packed cells (about 6 in the ordinate scale of Fig. 3A, and remained constant over the 120-min period), to 104 ± 5 milliequivalents/liter of packed cells within a few min, as shown in

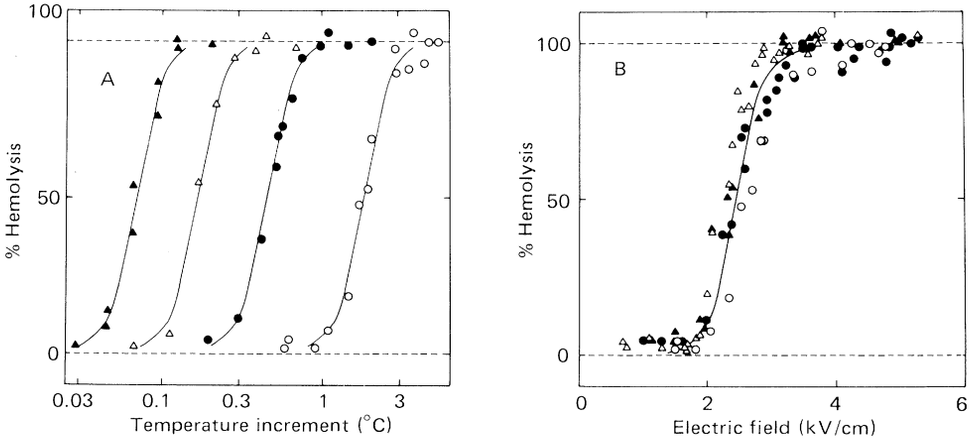

FIG. 2. Hemolysis at different ionic strengths plotted against (*A*) the magnitude of temperature jump and (*B*) the intensity of the applied electric field. Washed human erythrocytes were suspended in 100 volumes of isotonic NaCl/sucrose solutions, the relative NaCl content being: ○, 100%; ●, 30%; △, 10%; ▲, 3%. Aliquots of the suspensions were subjected to a single electric pulse of various intensities in the device shown in Fig. 1. After the pulsation the samples were rapidly diluted into 30 volumes of isotonic NaCl to provide an identical environment, in which hemolysis took place (essentially the same result was obtained with samples left in the original media). After 15 hr, the erythrocytes were spun down at 10,000 × *g* for 10 min, and the extent of hemolysis was determined from the absorption of hemoglobin in the supernatant. The value for 100% hemolysis was obtained by hypotonic hemolysis. Temperature was 25 ± 2°, and the pulse duration was 20 μs.

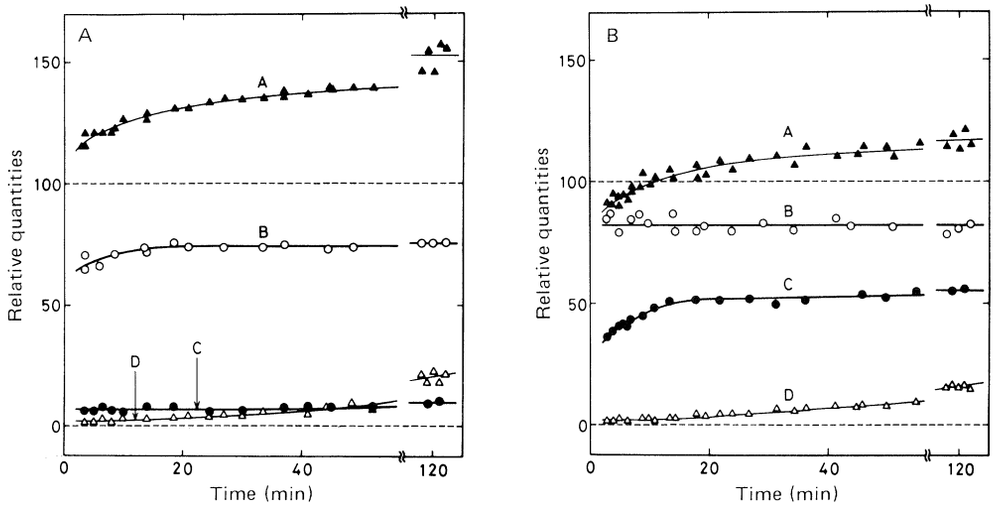

FIG. 3. (*A*) Permeation of ions and sucrose molecules and the swelling of the erythrocytes prior to the field induced hemolysis. Figure (*A*) gives data in isotonic NaCl, and figure (*B*) gives data in isotonic NaCl/sucrose (30%/70% mixture). Curve A, the volume of the unlysed cell relative to the untreated; B, sodium ion penetration; C, sucrose penetration; D, the extent of hemolysis. Sodium and sucrose penetrations are defined as 100 × (the amount of sodium or sucrose per liter of packed unlysed cells)/(the amount per liter in external medium). Washed erythrocytes were suspended in five volumes of either an isotonic NaCl or a NaCl/sucrose mixture (in sucrose penetration experiments, a trace of [^{14}C]sucrose was added to the medium). Aliquots were subjected to a single electric pulse of intensity 3.7 kV/cm and duration 20 μs. At various intervals after the pulsation, the samples were spun down in microhematocrit tubes for 10 min at the maximum speed of an International clinical centrifuge. The extent of hemolysis was estimated by colorimetry of the supernatant; the erythrocyte volume was read as the height of the pellet and corrected for hemolysis. Measured portions of the pellet as well as the supernatant were cut out and assayed for sodium (National Instrument Laboratories flame photometer) or [^{14}C]sucrose (Beckman LS-230 liquid scintillation counter). Abscissas of the figures refer to the time between the pulsation and the beginning of the centrifuge plus 2 min, the time required for approximate packing. Curve D eventually reached 100. Other curves were not followed after 120 min because of increasing errors due to the hemolysis. Temperature 25°.

curve B. The latter value indicates a near equilibration of sodium ions across the cell membrane, when allowance is made for the existence of hemoglobin in the cells at about 30% wt/wt (sodium concentration in the medium dropped from 160 to 140 milliequivalents/liter as the ions moved into the cells). As the penetration of sodium increased, the potassium content of the cells decreased from 95 ± 5 to 15 ± 3 milliequivalents/liter packed cells within a few min. Since both the active and passive transports of these ions in intact erythrocytes are as slow as a few milliequivalents/liter of packed cells per hr (16), the above observation indicates a roughly 1000-fold increase in the cation permeability upon the voltage pulsation.

Once the cell membrane becomes permeable to ions, the excess osmotic pressure due to the presence of hemoglobin inside causes the swelling of the erythrocytes. This is shown in Fig. 3*A* (curve A) which represents the volume of the pulse-treated cells relative to the untreated ones. When the volume reaches a limit, the membrane is punctured because of the pressure difference, and the release of hemoglobin follows. This type of hemolysis reaction has been known as colloid osmotic hemolysis (16, 17). The following observations support this view: (*i*) Hemolysis was always preceded by the leakage of sodium and potassium ions, and the leakage always accompanied hemolysis. Under the experimental conditions of Fig. 2, no leakage was detected with pulses of field intensity less than 1.5 kV/cm, even after 12 hr; complete prelytic exchange of sodium and potassium was observed with pulses greater than 3.0 kV/cm. (*ii*) After a pulsation in isotonic NaCl, addition of 30 mM of sucrose immediately stopped the swelling of the erythrocytes; further addition even induced shrinkage. Since sucrose hardly penetrates the cell membrane under this condition, as seen in curve C of Fig. 3*A*, it can counterbalance the osmotic pressure of hemoglobin. (*iii*) Addition of large molecules such as bovine-serum albumin (30% wt/wt) or stachyose (tetrasaccharide, 30 mM) totally prevented the hemolysis at least over a period of 48 hr; sucrose was slightly less effective.

The drastic change in ionic permeability suggests that the transmembrane potential either creates or opens up, in the erythrocyte membrane, pores of limited size which allow the passage of small ions but block large molecules such as stachyose, albumin, or hemoglobin. Sucrose appears to approximate the critical size. In contrast, larger pores which admit [^{14}C]sucrose, but not hemoglobin, were obtained by pulsation in 30% NaCl–70% sucrose isotonic mixture (curve C of Fig. 3*B*). Because the permeation of sucrose is slower than that of sodium or potassium ions, as is seen in the figure, the erythrocytes first shrank due to the loss of ions and then swelled again as the sucrose entered the cells (curve A). The half-time of hemolysis was 6 hr in this case. The hemolysis reaction can be accelerated by transferring the treated cells into an isotonic NaCl solution: as mentioned before, 50% of the cells hemolyze after 13 min. However, if this solution contains bovine-serum albumin at 30% wt/wt, hemolysis is completely prevented (stachyose only retards hemolysis).

Transmembrane potential undoubtedly plays important roles in living organisms. It opens up the so-called sodium and potassium channels in nerve membrane (18); it triggers the contraction of muscle by inducing the release of calcium from sarcoplasmic reticulum (19). Here we have generated transient transmembrane potentials across erythrocyte membranes simply by applying electric pulses to cell suspensions. The experimental results suggest that a transmembrane potential in the order of 1 V creates or opens up pores in the membranes. Preliminary measurements on permeation of 10 different carbohydrate molecules have confirmed this view: permeability of pulse-treated cells to these molecules decreased in increasing order of the molecular size to a virtual impermeability at a critical size; an exception was D-glucose which is carried by a specific transport system (16); the critical size could be controlled by changing the ionic strength of the solution, the intensity, or the duration of the applied electric pulse. Although the physiological implications of these pores are not clear, the

system could serve as a useful model for studying molecular permeations mediated by membrane-potentials in cellular systems.

The detailed process of the pore formation is still under investigation. However, the experiments described here clarify one important aspect. The ultimate extent of hemolysis is a function only of the applied field intensity (Fig. 2*B*). In other words, whether the pores are opened at all or not is determined solely by the magnitude of the transmembrane potential. In contrast, the size of these pores formed under the same field intensity can be quite different depending on the ionic strength (Figs. 3*A* and *B*). These observations suggest that at least two steps are involved in the formation of the pores: the initiation, and the subsequent growth of the pore size. The initiation of pores requires a transmembrane potential greater than a threshold (approximately 1 V), whereas the latter process is governed by various factors such as the ionic strength, the potential, or the pulse duration. Ionic strength is known to affect the cation permeability of intact erythrocytes (20); a common mechanism might operate also in the process of the pore growth.

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