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Reversible and irreversible modification of erythrocyte membrane permeability by electric field

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Electric fields of a few kV/cm and of duration in microseconds are known to implant pores of limited size in cell membranes. We report here a study of kinetics of pore formation and reversibility of pores. Loading of biologically active molecules was also attempted. For human erythrocytes in an isotonic saline, pores allowed passive Rb^+ entry formed within 0.5 μs when a 4 kV/cm electric pulse was used. Pores that admitted oligosaccharides were introduced with an electric pulse of a longer duration in an isosmotic mixture of NaCl and sucrose. These pores were irreversible under most circumstances, but they could be resealed in an osmotically balanced medium. A complete resealing of pores that admitted Rb^+ took approximately 40 min at 37°C. Resealing of pores that admitted sucrose took much longer, 20 h, under similar conditions. In other cell types, resealing step may be omitted due to stronger membrane structures. Experimental protocols for loading small molecules into cells without losing cytoplasmic macromolecules are discussed.

Introduction

Cell membranes are inherently electrical, and because of their compositions and geometry cells are especially susceptible to an electric field [1–4]. Recent work has shown that an electric field can open or close membrane ionic channels [2–5], activate transport and synthesis ATPases [6–10], induce cell fusions [3,11,12], stimulate cell proliferations [3] and modify membrane permeability [13–15]. This latter effect of an electric field is fundamental for understanding membrane structures and organizations. It also allows loading of molecules into cells with a minimum perturbation of the normal function of cells. The method has already found applications in molecular and cell biology researches, e.g., to transfer gene into cells [16] or to inject Ca^{2+} into sea urchin eggs [17]. In addition, pore formation is believed to be an early and a critical event for the electric induced cell fusion [3,11,12]. Previously, we used cell swelling as a criterion of molecular permeation into electrically perforated erythrocytes [13]. Complete restoration of the permeation barrier of the electrically treated cells was demonstrated only in few cases [14,15]. In view of the recent interest in electric cell fusion and electric loading of molecules it is essential that effects of electric field on cell membrane permeability be examined in a greater detail. Unless fused cells or drug-loaded cells fully recover from the electric damage, minor as it may seem, leakage of cations will continue and the survivability of cells can not be assured. Here, we have monitored membrane permeability change using Rb^+ and carbohydrate tracers during and after the electric field treatment. It is

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

shown that electrically introduced pores are stable at 4°C. However, the size of these pores reduces rapidly and under an appropriate condition a complete resealing can be achieved for small pores.

Materials and Methods

Chemicals

$^{86}Rb^+$, $^{22}Na^+$, ^{14}C-labeled sucrose and other carbohydrates were purchased from Amersham. Other chemicals and reagents were of highest purity commercially available.

Erythrocyte samples

Red cells from freshly drawn human blood were washed three times with 5 volumes of 150 mM NaCl in 20 mM Hepes (pH 7.2). The cells were then washed with a medium of the same composition as that used in voltage pulse experiments, except that radioactive tracer was omitted. The cells were kept in the same medium on ice at 50% hematocrit before use. In cases where preloading of $^{86}Rb^+$ was done, red cells were suspended in 140 mM NaCl/10 mM RbCl and $^{86}Rb^+$/50 μg per ml chloramphenicol in 20 mM Hepes (pH 7.2) at 10% hematocrit overnight at 4°C. Cells were then washed 4–5-times with a nonradioactive medium of proper composition, and stored on ice before experiments.

Electric modification of membrane permeability

The electric-pulse instruments used for voltage perforation of erythrocyte membrane has been described [13]. Briefly, erythrocytes in an isosmotic suspension was placed in a cylindrical chamber made of plexiglas. On the two ends of the chamber were two black-coated platinum sheets which served as electrodes. Temperature of the sample was controlled with circulating water through two hollow brass blocks in tight contact with the two electrodes and was monitored when necessary with a microthermistor. The electrodes were connected to a Cober 605P voltage generator which can deliver up to 2.2 kV. The distance between the two electrodes can be adjusted to achieve an electric field strength of greater than 10 kV/cm. The rise-time of electric pulses was found to be less than 0.1 μs. The waveform of electric pulses was monitored simultaneously for the field strength and the current, from the latter temperature jump of the suspension can be calculated [13]. Previous work has shown conclusively that all of the phenomena we are discussing here result from effects of the electric field and are unrelated to Joule heating [4,13].

For the uptake of carbohydrates, red blood cells were treated with one electric pulse of up to 4 kV/cm and of duration up to 100 μs, at 25°C. The sample was transferred to a cooled tube and kept in ice. At a designated time, an aliquot of sample was drawn and diluted into a loading medium containing a ^{14}C-labeled carbohydrate and the isosmotic concentration of the carbohydrate. At intervals, an aliquot was centrifuged in a microhematocrit tube and radioactivity in the cells and supernatant determined. The hematocrit of loading sample was almost constant at 18% throughout the assay period, indicating that swelling did not occur. Slight variation in solvent condition is included in figure legends. Na^+ and Rb^+ uptake experiments followed similar procedure.

Resealing of pores

Red cells treated with an electric pulse of appropriate strength and duration were transferred into a resealing medium and incubated at 37°C. The resealing medium was composed of 2 mM adenine/3 mM inosine/10 mM glucose/1 mg per ml bovine serum albumin/15 mM sucrose/94 mM NaCl/5 mM RbCl/25 mM stachyose/20 mM Hepes at pH 7.2. Variation from this composition will be mentioned in the figure legends. At an indicated time, an aliquot was transferred into a solution consisting of 140 mM NaCl/10 mM RbCl and $^{86}Rb^+$ tracer/20 mM Hepes (pH 7.2) and incubated at 25°C. Uptake of tracer was determined for washed cells as a function of time and compared with control samples. Complete resealing is defined as a complete restoration of the membrane permeation barrier against Rb^+ penetration.

Loading and retention of molecules

Loading and resealing of red cells followed the procedures described above. [^{14}C]penicillin G, [^3H]dexamethasone, [^3H]cystosine-*b*-D-arabinoside and [^3H]actinomycin were each loaded into mouse erythrocytes (from AKR/J female) for in vivo

retention experiment. Drug-loaded and resealed mouse red cells were suspended at 35% hematocrit in a medium containing 150 mM NaCl/8 mM KCl/2 mM Mg_2SO_4/2 mM $CaCl_2$/10 mM D-glucose/1 mM adenosine/1 mM inosine/0.1 mg per ml chloramphenicol/6 mM phosphate buffer at pH 7.5. 150–300 μl of the suspension was injected into a tail vein of a mouse. In control mice, free drug in the same medium was injected. At intervals after injection, approx. 40 μl of blood was withdrawn, and activities in total red cells and in total plasma in the mouse were estimated as described in Ref. 15.

Results

Electric modification of membrane permeability

Application of an electric pulse of 0.5–10 μs at different field strength caused a rapid influx of $^{86}Rb^+$ or $^{22}Na^+$ into the red cells. Data in Fig. 1 give $^{86}Rb^+$ uptake 10 min after the voltage pulse experiment. Electric pulses shorter than 0.5 μs were found to have little effect on the membrane permeability when the field strength was limited to below 4 kV/cm. The extent of ion influx depended both on pulse duration and field strength. As shown in Fig. 2, for pulse durations up to 10 μs, a minimum voltage of 2.1 kV/cm was required for introducing pores in the erythrocyte membranes. Electric field of less than 2.1 kV/cm was ineffective for pore induction. Note that data in Figs. 1 and 2 indicate the relative influx of ions. Voltage-induced pores were stable at temperatures below 4°C, and the intra- and extracellular ionic compositions approach a Donnan equilibrium within 10 min under an osmotically balanced condition [13,18].

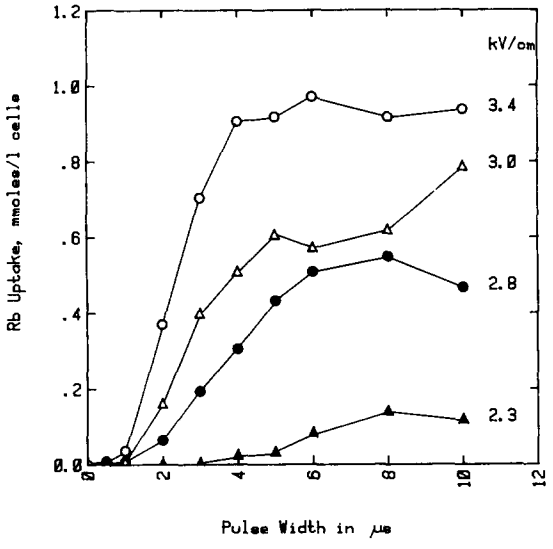

Fig. 1. Electric modification of erythrocyte membrane permeability to Rb^+. Red blood cells were suspended at 6–9% hematocrit in a solution containing 140 mM NaCl/10 mM RbCl/ $^{86}Rb^+$ tracer at 6 cpm/pmol at 20 mM Hepes (pH 7.2). 10 min after the suspension was treated with a single electric pulse of indicated field strength and pulse duration at 27°C, cells were washed three times with a medium of the same composition, except that the radioactive tracer was omitted. $^{86}Rb^+$ content of the washed cells was determined. Cells without exposing to an electric field were incubated and washed through the same procedure for control. Rb^+ uptake in the control samples were found to be insignificant. The net uptake of electrically treated cells is expressed as mmoles/l cells. Results represent three independent runs each in duplicate. Electric field strengths, in kV/cm, for different curves are given in the figure. Pores opened by the electric pulse were relatively stable and a slight leakage was observed in the washing. The values shown here are not absolute permeabilities of these membranes, but are relative permeabilities.

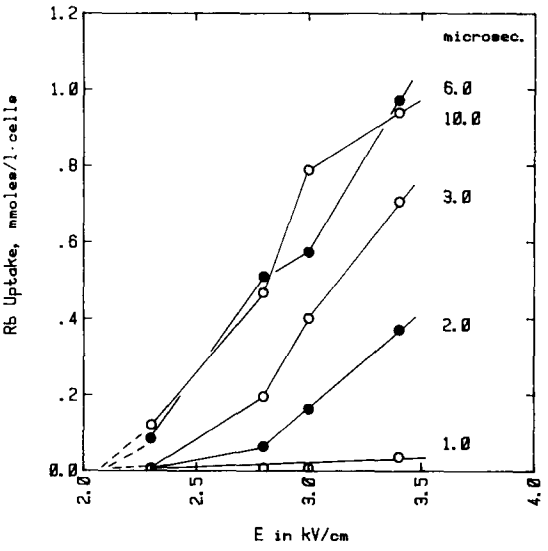

Fig. 2. Threshold voltage of pore formation. Rb^+ inward movement is plotted against the applied field strength for pulse durations greater than 1 μs. Note that the abscissa starts at 2 kV/cm, and a threshold field strength for the electric perforation of erythrocyte membranes is seen around 2.1 kV/cm. Each curve represents results of a fixed pulse width, indicated in the figure, and a varied field strength.

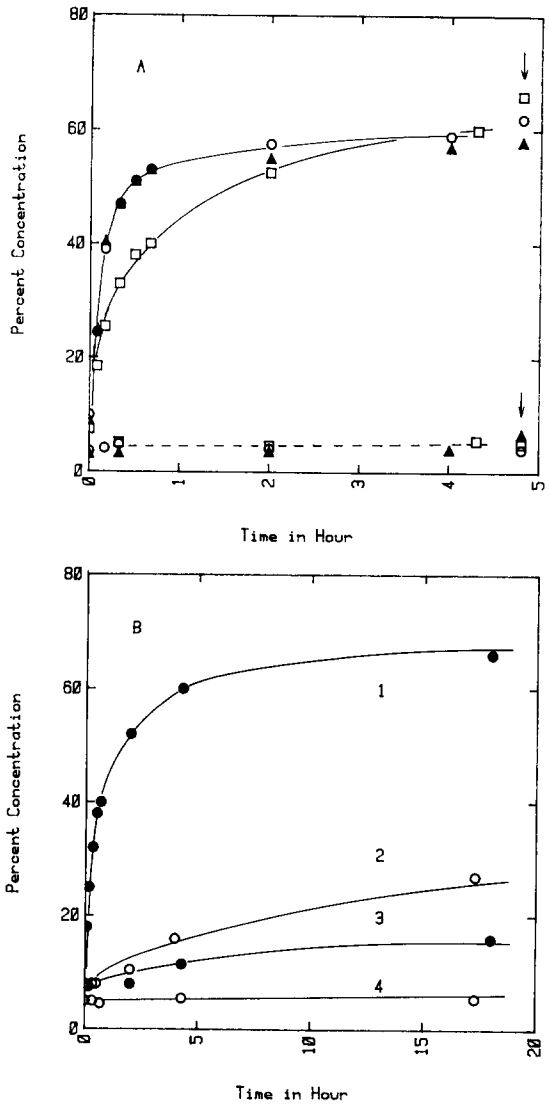

Fig. 3. Electric induction of larger pores. (A) Red cells were suspended in a medium containing 131 mM NaCl/34 mM stachyose/6 mM sodium phosphate were exposed to a 80 μs electric pulse at 3.0 kV/cm, at 25°C. The cells were then cooled down to ice temperature, and ^{14}C-labeled isotonic suspension of a carbohydrate was added to 8 mM concentration. At intervals, an aliquot was centrifuged in a microhematocrit tube, and radioactivity in the cells and supernatant were determined. The ordinate, percent concentration denotes the ratio of radioactivity in equal volume of the packed cells and the supernatant, expressed in %. The dashed curve is for control samples, i.e., red cells without an electric pulse treatment. Data pointed by arrows are obtained after 18 h incubation in radioactive medium. Symbols used are data for sucrose (□), mannitol (○) and xylitol (▲). (B) A similar experiment that followed sucrose permeation into red cells. Curve 1, red cells treated with a 80 μs pulse of 3.0 kV/cm; curve 2, red cells treated with a 10 μs pulse of 3.0 kV/cm; curve 3, red cells treated with a 80 μs pulse of 3.0 kV/cm followed by an incubation at 37°C for 30 min before the sucrose penetration experiment was done; curve 4, a control experiment. Comparison of curves 1 and 3 indicates that pores shrank quickly at 37°C. Note that sucrose penetration experiment was done at ice temperature.

Loading of carbohydrates

Reduced ionic strength of an isosmotic cell suspension increases the effectiveness of electric perforation [13], and pores large enough to admit tetrasaccharides can be generated [14]. Time-course of carbohydrate loading after treatment with a 80 μs pulse at 3 kV/cm is given in Fig. 3A. Red cell membranes treated under these conditions were impermeable to stachyose, a tetrasaccharide, and the suspension contained 34 mM of stachyose to compensate for the colloid osmotic pressure of cytoplasmic hemoglobin. Osmotically balanced medium prevented water influx [13,18], and cell volume remained unchanged after electric pulse treatment. Data show that xylitol, mannitol and disaccharide, sucrose continued to enter the red cells after more than 10 h of incubation at 4°C. This means these pore were stable at low temperatures. The size of pores shrank rather rapidly at a higher temperature. Data in Fig. 3B indicate that an incubation of the voltage-treated sample at 37°C for 30 min restored to a large extent the permeation barrier of the membranes to sucrose. As will be shown below, restoration of the permeation barrier to Rb^+ took much longer.

Resealing of electrically perforated membranes

Complete resealing of pores were examined by the return of membrane permeation barrier to Rb^+, an ion most sensitive to membrane damage. Since in the red cell membrane, an inward pumping mechanism of Rb^+ exists, i.e., the (Na, K) ATPase, the experiment was done to compare relative rate of Rb^+ entry for samples treated and untreated with the electric pulse. Pores induced with a 4-kV/cm 20-μs pulse were small enough to prevent permeation of sucrose [13,14]. Incubation of the sample at 37°C in an osmotically balanced medium quickly restored the membrane permeation barrier to Rb^+. No hemolysis was observed throughout the entire course of the experiment.

The ratio of Rb^+ entry by the pulsed sample and the untreated sample as a function of resealing time at 37°C is given in Fig. 4 (data in open circles). The time constant for the resealing was 15 min, and a complete resealing took approx. 40 min.

Resealing of a sample treated under a more drastic condition took much longer. Pores opened with a 3.7-kV/cm 20-μs pulse were large enough to admit sucrose [14]. 10 min after the electric pulsation, the sample was found to retain 3 mM of sucrose, and no slow leakage of the entrapped sucrose was observed many hours after the voltage experiment. On the other hand, these cells remained permeable to Rb^+ even after incubation in a proper resealing medium for more than 20 h. The resealing of these pores took much longer (data in closed circles of Fig. 4). Previously, we found that a similarly treated mouse erythrocytes survived in the mouse circulation with a normal lifetime and retained the sucrose tracer [15]. For erythrocytes, pores larger than that discussed here have been found to be difficult to reseal without losing part of the hemoglobin content.

Loading of molecules

The cells loaded with radioactive sucrose were judged to be indistinguishable from the intact red cells as far as cell shape, cell volume, and ability for active transport of Na^+ and K^+ and facilitated transport of glucose are concerned [14,15]. Thus, we have tested the use of erythrocytes as drug carriers. Four drugs of which radioactive derivatives were commercially available were selected for test. They were [^{3}H]cytosine-β-D-arabinoside, [^{14}C]penicillin G, [^{3}H]actinomycin D and [^{3}H]dexamethasone. In all cases loading and resealing were successful. However, these drugs were found to be membrane permeant at 37°C, and they leaked out from resealed red cells in several hours. That these chemicals are membrane-permeant was verified by an incubation of drugs with intact erythrocytes. It was found that they permeated into red cells at 37°C in a few hours without voltage treatment. Similar observation was made by Kaibara and Tsong (Kaibara, M. and Tsong, T.Y., unpublished data). They loaded millimolar concentrations of antisickling reagents, phenylalanine and alkylurea, into SS-erythrocytes. At 4°C, these drugs were retained for 1–2 days in the erythrocytes. When the drugs loaded SS-erythrocytes were depleted of oxygen, by diluting into an oxygen-free solution, they re-

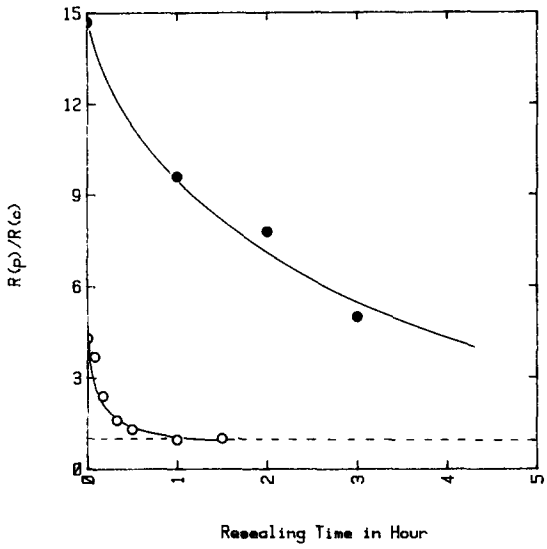

Fig. 4. Complete restoration of electrically perforated membranes. Resealing of small pores (open circles): red cells at a hematocrit of 5% were treated with a single electric pulse of 4 kV/cm and of duration 2.0 μs, in a medium containing 140 mM NaCl/10 mM RbCl/20 mM Hepes (pH 7.2), at 27°C. Afterward, cells were incubated in the same medium at 37°C for the time indicated for each sample. $^{86}Rb^+$ was then added to the suspension, and the uptake of Rb^+ tracer was determined 15 min after. The ratio of Rb^+ uptake in the pulsed samples, R(p), and the control sample, R(c), is plotted against resealing time. A complete resealing against Rb^+ permeation, i.e., $R(p)/R(c) = 1.00$, was achieved in 60 min; the time constant of resealing was 15 min. Resealing of large pores (closed circles): red blood cells at 10% hematocrit were treated with a single electric pulse of 3.7 kV/cm for 20 μs in a medium containing 15 mM sucrose/[^{14}C]sucrose/139 mM NaCl/20 mM Hepes (pH 7.2) at 4°C. After washing with a medium of the same composition except sucrose tracer, the cells were transferred into a resealing medium, at 2–3% hematocrit, at 37°C. The resealing medium contained 2 mM adenine/3 mM inosine/10 mM glucose/1 mg per ml bovine serum albumin/15 mM sucrose/94 mM NaCl/5 mM RbCl/25 mM stachyose/10 mM Hepes (pH 7.2). At indicated time, aliquots were drawn and transferred into a medium containing 140 mM NaCl/10 mM RbCl/$^{86}Rb^+$/20 mM Hepes at pH 7.2 for Rb^+ permeation measurement. $^{86}Rb^+$ incorporation was determined in washed cells 20 min after. The red cells were found to entrap 3 mM sucrose 10 min after the electric pulse experiment, and no leakage of sucrose tracer was observed in the $^{86}Rb^+$ incorporation experiment. The ratio of Rb^+ entry is then plotted. A complete resealing took more than 20 h in this case.

sisted sickling at 25°C. Controlled samples sickled within a minute. However, at 37°C, the antisickling effect disappeared in a few hours because of drug leakage. Note that experiment with sucrose shows that membrane impermeant molecules can be retained in properly resealed red cells for along as the normal life span of these red cells [15].

Loading of ions or other molecules into cells other than erythrocytes has also been attempted in our laboratory. Rossignol et al. have loaded Ca^{2+} into sea urchin eggs to induce formation of partial fertilization envelopes [17]. Knox et al. have loaded discoideum with ATP and followed the metabolic pathway of this compound (unpublished results). The ability of the egg and the discoideum to develop was not impaired by the electric treatment. A resealing step was not required in these systems.

Discussion

Electric induction of pores and resealing

Voltage induction of pores requires an electric field greater than a threshold value. Data in Fig. 2 indicate that the threshold field for human erythrocytes in an isosmotic suspension is around 2.1 kV/cm. 2.1 kV/cm can generate a maximum transmembrane potential of approx. 1.1 V [3,4,13]. Once the membrane is punctured by an electric field, the red cells eventually lyse and release their cytoplasmic content [13,18]. Our study shows that the hemolysis is caused by the colloidal osmotic swelling of the red cells, and can be completely prevented by adding molecules larger than the size of pores to the suspension to counterbalance the osmotic pressure of cytoplasmic macromolecules, in this case, hemoglobin [13,18]. Only in an osmotically balanced medium can the electrically induced pores be resealed. Resealing is extremely slow, e.g., days at 4°C, but is relatively fast at 37°C (Fig. 4). Resealing of large pores are difficult for erythrocytes, but may not be difficult for cells with stronger membrane structures or with cells walls [3]. Linear and circular plasmid DNA containing the herpes simplex thymidine kinase have been loaded into mouse L cells by a similar procedures described here by Neumann and co-workers, and the loaded DNA was expressed [16]. Mechanisms of the voltage induced Pore formation have been worked out to certain details by Kinosita and Tsong [13–15,18,19].

The fact that electrically induced pores are stable at low temperatures means that these pores are not reversible. Resealing of these pores takes special attention. When electric field is employed for cell fusion, similar precautions needs be taken to ensure that fused cells restore their permeation barrier [11,12]. Without such precautions, fused cells usually swell and their cytoplasmic contents leak out. Recently, Lo et al. [12] have used receptor-mediated electrically induced cell fusion to prepare monoclonal antibodies. Survival of hybridomas after fusion, however, was found to depend on a proper annealing procedure.

Several factors influence the pore size [4,13,14]. The first one is the magnitude of transmembrane potential generated by an electric pulse. Most cells require about 1 V of transmembrane potential, or greater, to perforate the membrane when microsecond electric pulses are used [3,4]. The second factor is pulse duration. The longer the pulse duration, the large the pores open. Another factor is the ionic strength of the medium. Lower ionic strength leads to larger pores. High ionic strength caused a severe Joule heating when a long pulse width was used. We have avoided such a condition. Finally, each cell type exhibits a characteristic resistance to the electric perforation [3,4].

The question of what is the limit of molecular size which can be loaded and pores can still be resealed properly cannot be answered in a simple term. For human erythrocytes molecules smaller than trisaccharides may be loaded and properly resealed [13,14]. In plant cells, Zimmermann and co-workers have entrapped chlorophylls into protoplasts [3], although the intactness of protoplast membranes after the loading has not been demonstrated. It is recognized, however, that plant cells or yeast can survive a much drastic treatment by electric pulses [3].

Kinetics of pore formation

In an earlier publication, Kinosita and Tsong have followed time-dependent evolution of a voltage induced membrane conductance, and observed two kinetic processes [19]. The fast relaxation occurred in less than 1 μs was attributed to a pore initiation process and the slower relaxation oc-

curred in 50 μs was attributed to a pore expansion process. Membrane conductance measurements are subjects to different interpretations and cannot be taken as direct proof. These relaxations could simply reflect surface charge redistributions [1,3]. Our present result, however, verifies that with a 4 kV/cm electric pulse, pores that admit Rb^+ or Na^+ formed in less than 1 μs, and electric pulses less than 0.5 μs were ineffective in modifying membrane permeability to these ions (Figs. 1 and 2). Pores large enough to admit sucrose formed only with electric pulses in the 10 μs time range (Fig. 3A), again, consistent with our earlier interpretation that the 50 μs relaxation time measured a pore expansion process. It is not clear whether the 0.5 μs observed here reflects a time constant for generating a membrane potential in erythrocytes. We have estimated such time constant to be around 0.1–0.5 μs [19]. Pores formed in pure lipid bilayers are either irreversible and cause an immediate rupture or planner bilayers or reversible within less than 1 ms [21,22].

The rate of ion and molecular permeation across the electrically perforated membranes depends on two factors: pore size and number of pores. Pore size was measured by relative permeability to molecules of different size [14] and number of pores was estimated by rate of K^+ diffusion under an osmotically balanced condition [19]. We have estimated the number of pores open by an electric pulse to be in the order of a few hundreds per cell [19]. In this regard, it is interesting to note that hypotonic hemolysis induces only a single pore in erythrocyte [23,24].

Acknowledgement

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