Electroporation of the photosynthetic membrane

A study by intrinsic and external optical probes

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ABSTRACT The study examines the relationship between electric field–induced conductivity and permeability changes in a biological membrane (electroporation) and the amplitude-duration parameters of the externally applied electric field. These reversible changes were characterized in giant photosynthetic membrane vesicles by means of the calibrated response of an intrinsic voltage-sensitive optical probe (electrophotoluminescence) and by the uptake studies of dextran-FITC fluorescent probes of different molecular weights. We quantitatively monitored electric field–induced conductivity changes by translating the electrophotoluminescence changes into conductivity changes. This was carried out by measuring the attenuation of the electrophotoluminescent signal after the addition of known amounts of gramicidin. The results demonstrate that electroporation involves the reversible formation of discrete holes in the membrane having radii <5.8 nm. The total area of the electric field–induced holes was 0.075% of the total surface of the vesicle. The formation of the electropores was affected differently by the electric field strength than by its duration. Increase in electric field strength caused increase in the total area of the vesicle that undergoes electroporation. Increase in the duration of the electric field increases the area of single electropores. Each of the two electric parameters can be rate limiting for the dynamics of electropore formation. These results are in accordance with the model of electroporation based on electric field–induced expansion of transient aqueous holes.

INTRODUCTION

Short exposure of cells or membrane vesicles to a high external electric field leads to a reversible transient increase in the permeability of the membrane to ions and molecules. This process has been defined by equivalent terms such as reversible electric breakdown (REB) or electroporation (for general review on electroporation see Zimmerman, 1982; Tsong, 1983; Neumann, 1984). Reversible electric breakdown has been studied in two types of membranous systems: planar black lipid bilayers and vesicular membranes. One of the basic differences in the electroporation process induced in these two systems arises from the fact that whereas an identical transmembranal potential difference is induced across the planar lipid bilayer, a continuous spectrum of transmembranal potential values is induced in a vesicular membrane exposed to a homogeneous external electric field. This nonhomogeneous distribution results from the angular dependence of the induced electric field in the membrane having a maximal amplitude in the pole areas along the externally applied field and vanishing towards the equators (Farkas et al., 1984; Gross et al., 1986; Ehrenberg et al., 1987; Kinosita et al., 1988). This situation complicates the detailed characterization of the electroporation process (e.g., electropore diameters distribution in relation to the amplitude-duration characteristics of the external electric field and the life times of the electropores). The knowledge of the dependence of electric field–induced permeability changes on the amplitude-duration characteristics of the electric field is one of central issues in the efficient electric-induced gene transfer into mammalian cells (e.g., Neumann et al., 1982; Collins, 1988) and plant cells (e.g., Lurquin and Paszty, 1988).

In the present study we examined the relationship between the electric field–induced conductivity and permeability changes and the amplitude-duration parameters of the electric field in giant unilamellar vesicles prepared from thylakoid membranes of chloroplasts. This study has been carried out by means of the calibrated response of an intrinsic voltage sensitive optical probe (electrophotoluminescence—EPL) in addition to the uptake studies of fluorescent probes of different molecular weights.

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EXPERIMENTAL

Materials

Broken chloroplasts were prepared from spinach, pea, or tobacco according to Avron (1960). The broken chloroplasts were stored at −180°C to preserve their photosynthetic activity for a long period (Farkas and Malkin, 1979). In every set of experiments the concentrated thylakoids were thawed at room temperature and then incubated at 50°C for 3 min (heat inactivation) to deplete the thylakoids from most of their photosystem II related electrophotoluminescence activity, which is less sensitive to the electric field (Symons et al., 1984), while preserving the photosystem I–related activity (Symons et al., 1985). After heat inactivation the thylakoids were resuspended under hypotonic conditions. The concentration of the stored stock of broken thylakoids was 6–10 mg/ml, and they were diluted 1,000-fold in 5 mM MES-NaOH buffer pH 5.5 ($\sim 4 \times 10^6$ vesicles/ml). After 15 min at room temperature they were kept on ice. This swelling process yields spherical vesicles known as swollen thylakoids. Because these vesicles are formed even in extreme hypotonic medium (e.g., distilled water) we have to assume the abolishment of the initial osmotic difference during and/or after the swelling process. These vesicles, composed of a single membrane with occasional patches on it, have a size distribution of radii of 1–10 μm, with an average radius of 4 μm. The analysis of size distribution was done by microscopic visualization. Gramicidin D (Dubos, Sigma Chemical Co., St. Louis, MO) was used for calibration at concentrations of 10^{-8} M.

Electrophotoluminescence studies

The experimental set up for electrophotoluminescence measurements was described elsewhere (Farkas et al., 1984). The experiment was initiated by a preillumination, for 120 ms with a light projector. The light was filtered by a 4-96 glass filter (Corning Glass Works, Corning, NY) limiting the exciting wavelength to ~400–600 nm. After a dark time of 230 ms an external electric field pulse was applied. The electric field–induced luminescence was filtered by a model RG 665 cut-off filter (Schott Glass Technologies Inc., Duryea, PA) and was monitored on a fast oscilloscope (model 2430A, Tektronix, Inc., Beaverton, OR) interfaced to a compatible IBM PC computer. In all cases the amplitude of a particular EPL signal was taken at its two pulses which varied between 100 μs to 70 ms. The applied pulse shape, intensity, and kinetics were monitored by use of an inductive current probe. All experiments were carried out at a temperature of 5°C maintained by a thermostated bath Techne RB-12.

Uptake studies

The preparation of the swollen thylakoids for uptake studies was basically the same as in the electrophotoluminescence ones with one modification where a dilution of 100 of the stored concentrated thylakoids was used instead of 1,000-fold dilution. The experiments were initiated after the addition of the fluorescent dye probe. In the uptake measurements the following probes were used: 5(6)-carboxyfluorescein (CF), mol wt 376 D, from Eastman; dextran-FITC molecules of 4,000, 10,000, 20,000, 40,000, and 70,000 D from Sigma Chemical Co. All reagents used were of analytical grade. The control suspension was incubated under the same conditions as the experimental one but without exposure to the external electric field. For each treatment a single aliquot of 0.5 ml ($\sim 4 \times 10^7$ vesicles/ml) was used. The experimental suspension was exposed to 100 square pulses at intervals of 5 s, delivered by the high-voltage pulse generator. After the exposure, both the treated and the control samples were centrifuged at 6,100 *g* (202 MK centrifuge, Sigma Chemical Co.) for 4 min, and were washed twice with 1 mM Tris-HCl, pH 8.0 (pre-filtered by a 0.22-μm filter). The uptake of the different optical probes into single vesicles was measured on a flow cytometric device in the fluorescence mode. The flow cytometric device used was a FACS IV with consort 40 data analysis system (Becton Dickinson Corp.). Samples were excited by a 488-nm light of a 200-mW argon laser. Forward scattered light was filtered through a 1-OD neutral density filter. Fluorescence was measured at 530 nm on a logarithmic scale.

RESULTS

Electric field–induced conductance-permeability changes in the thylakoid membrane were studied by two independent approaches. The first approach aims at measuring electric field–induced conductance changes in a membrane by the use of a voltage-sensitive optical probe. This methodology relies on one side on the relationship between the local electric field induced in the membrane and the concomitant changes in the spectroscopic properties of the voltage-sensitive optical probe and on the other side on the dependence of the strength of the induced electric field on membrane conductivity. Thus, upon exposure to the same external electric field, a lower electric field will be induced in a membrane of a high conductance than in a membrane of a lower one. In this case a smaller spectroscopic amplitude change of the voltage-sensitive probe is expected to take place during the electroporation process than before its occurrence. This principle was applied in previous studies of ionophore-mediated ion transfer (Farkas et al., 1982) and in studies on electroporation of the photosynthetic membrane (Korenstein et al., 1984). More recently, this principle was demonstrated in spatial studies of the electroporation process by utilizing fast image analysis techniques (Kinosita et al., 1988). The second approach applied in the present study is based on electric field–induced uptake of fluorescent probes of different molecular weights. This method allows not only the detection of membrane permeability changes but also the estimation of the sizes of these permeability pathways through the membrane (Sowers and Lieber, 1986; Weaver et al., 1988).

Study by electrophotoluminescence

Electrophotoluminescence (EPL) is explained by an enhancement of delayed luminescence of photosystem I due to an increase of induced charge recombination rate in one hemisphere of a swollen thylakoid by the electric field after preillumination (Farkas et al., 1982). Thus, during the application of a bipolar pulse the electric field acts alternatingly on the existing (light-induced) luminescence precursors in the two hemispheres. If charge recombination is the only process induced by the electric

field one would expect to obtain two equal EPL signals (provided the spacing between the pulses is much shorter than both the dark time elapsed from preillumination and the rotational diffusional time of the spherical thylakoid membrane). Reversible electric breakdown of the photosynthetic membrane was carried out by exposing a suspension of swollen thylakoids to two consecutive high electric fields of identical strength and duration, but of opposite polarity. As a result of the exposure we observed two EPL signals, where the second signal was attenuated in comparison to the first one (Fig. 1). A third single electric field pulse of the same characteristics gave an EPL signal of an equal amplitude to the first one, demonstrating the reversibility of the process. We attribute the attenuation in the second EPL signal to a transient change in membrane conductance due to a reversible electric breakdown which takes place during and after the first pulse. To translate the relative attenuation of the EPL signal into a conductance change we used the same strategy whose principles enabled us to measure ionomycin-mediated conductivity changes (Rosemberg and Korenstein, 1990). The EPL signal is an explicit function of the electric field induced in the membrane rather than of membrane conductance itself. The induced local electric field in the membrane has been calculated for spherical vesicles by solving Laplace's equation with the appropriate boundary conditions (Farkas et al., 1984; Ehrenberg et al., 1987). For the case where the specific conductivities of the inner and outer media (σ_i and σ_o, respectively) are equal and where the vesicle's radius (R) is much bigger than membrane thickness (d) one obtains an expression for the time-independent induced electric field (E_m):

$$E_m = \frac{3(R/d)\cos\theta\, E_{ex}}{2 + 3(R/d)\cdot(\sigma_m/\sigma_o)}, \tag{1}$$

where E_{ex} is the externally applied electric field intensity and θ is the angle between the applied electric field direction and the radius vector of the vesicle to a certain point on the membrane where the local electric field is induced. By introducing

$$K = \frac{3(R/d)\cos\theta}{2 + 3(R/d)(\sigma_m/\sigma_o)}, \tag{2}$$

we obtain

$$E_m = K \cdot E_{ex}. \tag{3}$$

To calibrate the EPL signal against the E_m, we measured the dependence of the EPL on the externally applied electric field (E_{ex}). As can be seen from Fig. 2, the EPL signal, as a whole, is not a linear function of the E_{ex}, but, it can be taken as such in the range 1,000–2,000 V/cm (correlation coefficient >0.95). Because the EPL is a function of E_m, it depends on the product $E_{ex} \cdot K$. Thus, an attenuation in the EPL signal reflects parallel attenuation of this product. The attenuation of the product can be measured from the dependence of the EPL on E_{ex}, in the linear range, which is used as a calibration curve. For this purpose we define a new term, the effective electric field, E_{ef}, given by the relationship $E_{ef} \cdot K_1 = E_{ex} \cdot K_2$, or $E_{ef} = E_{ex} \cdot K_2/K_1$, where K_1 is the K value before electric breakdown and K_2 is the K value during the electric breakdown. E_{ef}, which is obtained directly from the calibration curve, relates the attenuation of the EPL signal to a certain ratio between σ_{m2} and σ_{m1} (the specific membrane conductivity during and before the REB,

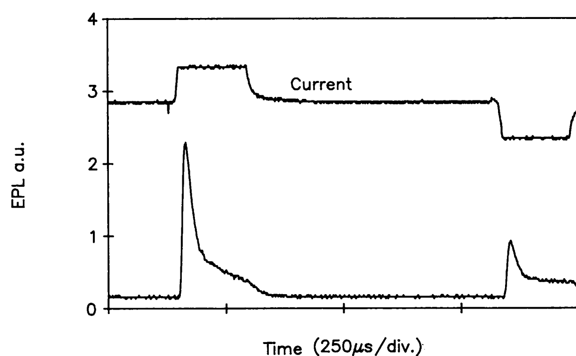


FIGURE 1 Attenuation of the EPL signal upon exposure of a suspension of swollen thylakoid vesicles to two consecutive electric fields of the same strength and duration but of opposite polarity. (*a*) *Upper curve.* The current produced in the medium, by the application of a bipolar electric field (recorded by a current probe). (*b*) *Lower curve.* EPL signal (E_{ex} = 1,100 V/cm; pulse duration = 150 μs); the thylakoid vesicles (~5 μg/ml chlorophyll) were suspended in 5 mM MES-buffer pH 5.5 at 5 ± 1°C.

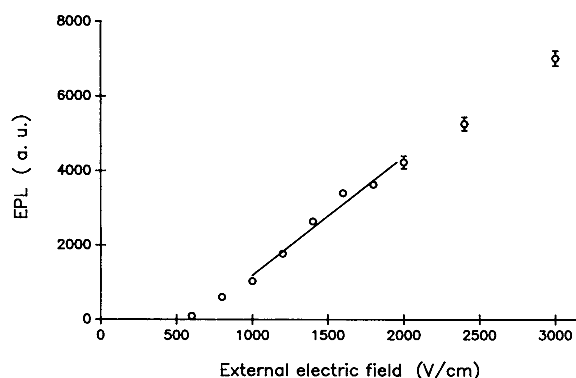


FIGURE 2 EPL signal (produced by the first pulse) as a function of the external applied electrical field. Conditions same as in Fig. 1. (The absence of error bars implies that the open circle is bigger than the standard deviation).

respectively). To calculate the values of σ_{m1} and σ_{m2}, an additional equation involving σ_{m1} and σ_{m2} is needed. To accomplish this, we carried out an additional experiment in which we induced a known change in membrane conductance, by the addition of a known amount of gramicidin, whose ion single-channel conductance is known (Neher et al., 1978). The effects of gramicidin on EPL were previously studied (Farkas et al., 1982; Farkas et al., 1984). This experiment, based on a titration with gramicidin, yields an additional equation from which σ_{m1} and σ_{m2} can be calculated. A value of 171 ± 56 nS/cm ($n = 10$) could be calculated for the specific conductance at the maximum of the first EPL signal (σ_{m1}). We used this average value of σ_{m1} for further calculation of the change in membrane conductance after the exposure to the electric field. Based on the above approach we studied the dependence of the electric field–induced change in membrane conductance, on the electric field strength and duration. The dependence of membrane conductance changes during electroporation on electric field strength is shown in Fig. 3. We obtain a linear relationship between membrane conductance increase and the strength of the applied electric field (E_{ex}). (Correlation coefficients of >0.99 and >0.92 for durations of 40 and 150 μs, respectively). This linear relationship seems to be independent of pulse duration (Fig. 3). Below a certain threshold value of the electric field strength (700 V/cm) no significant change in membrane conductance could be observed. However, the change in membrane conductance as a function of the exposure time to the electric field, shows a nonlinear dependence (Fig. 4). This nonlinearity is independent of the strength of the applied electric field. For long pulse durations, the effect of an incremental increase in pulse duration was less than a similar increment for short durations. At very short pulse durations (<20 μs), no significant change in membrane conductance could be measured.

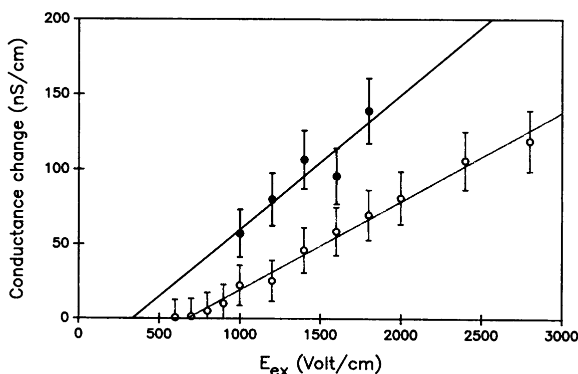


FIGURE 3 Relative change in membrane conductance as a function of the electric field strength. The change in membrane conductance was calculated from the attenuation in the EPL signal by the procedure described in the text. (○) 40-μs pulse (interpulse interval of 150 μs); (●) 200-μs pulse (interpulse interval of 600 μs). (The absence of error bars implies that the open circle is bigger than the standard deviation).

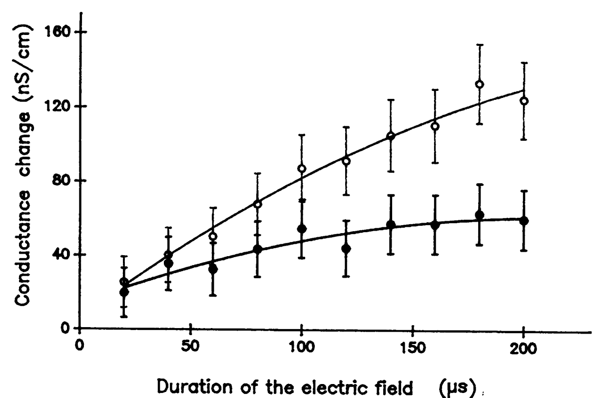


FIGURE 4 Relative change in membrane conductance as a function of the electric field duration. The change in membrane conductance was calculated from the attenuation in the EPL signal by the procedure described in the text. (○) Electric field strength of 1,800 V/cm; (●) Electric field strength of 1,000 V/cm. Other conditions same as in Fig. 1.

Studies by uptake

Electric field–induced permeability changes of swollen thylakoids were examined by measuring the uptake of a series of fluorescent dyes (5[6]-carboxyfluorescein or dextran-FITC molecules) into these vesicles. In these experiments the vesicles were exposed to a number of pulsed electric fields, possessing the same characteristics as used in the EPL studies. A low repetition rate (1 pulse/5 s) was used to allow the vesicles to reseal between successive pulses (we could estimate from the EPL studies that resealing process was terminated within 1 s) and to avoid other nonspecific effects (e.g., temperature rise). A control suspension was incubated under the same conditions but without the exposure to the electric field. The level of fluorescence in single vesicles was measured in a flow cytometry apparatus. As a result of the exposure to the electric field we observed significant uptake of the dye into the swollen thylakoids. This is evident from the higher level of fluorescence observed in the exposed suspension (Figs. 5 and 6). We studied the dependence of uptake efficiency on electric field duration (Fig. 7) and strength (Fig. 8). The results obtained had basically similar trends in both cases. We observed an increase in the uptake of the dye when we increased either the strength (Fig. 8) or the duration (Fig. 7) of the external electric field. To further characterize the dimensions of the structural permeability changes in thylakoid membranes during electroporation, we studied the influence of

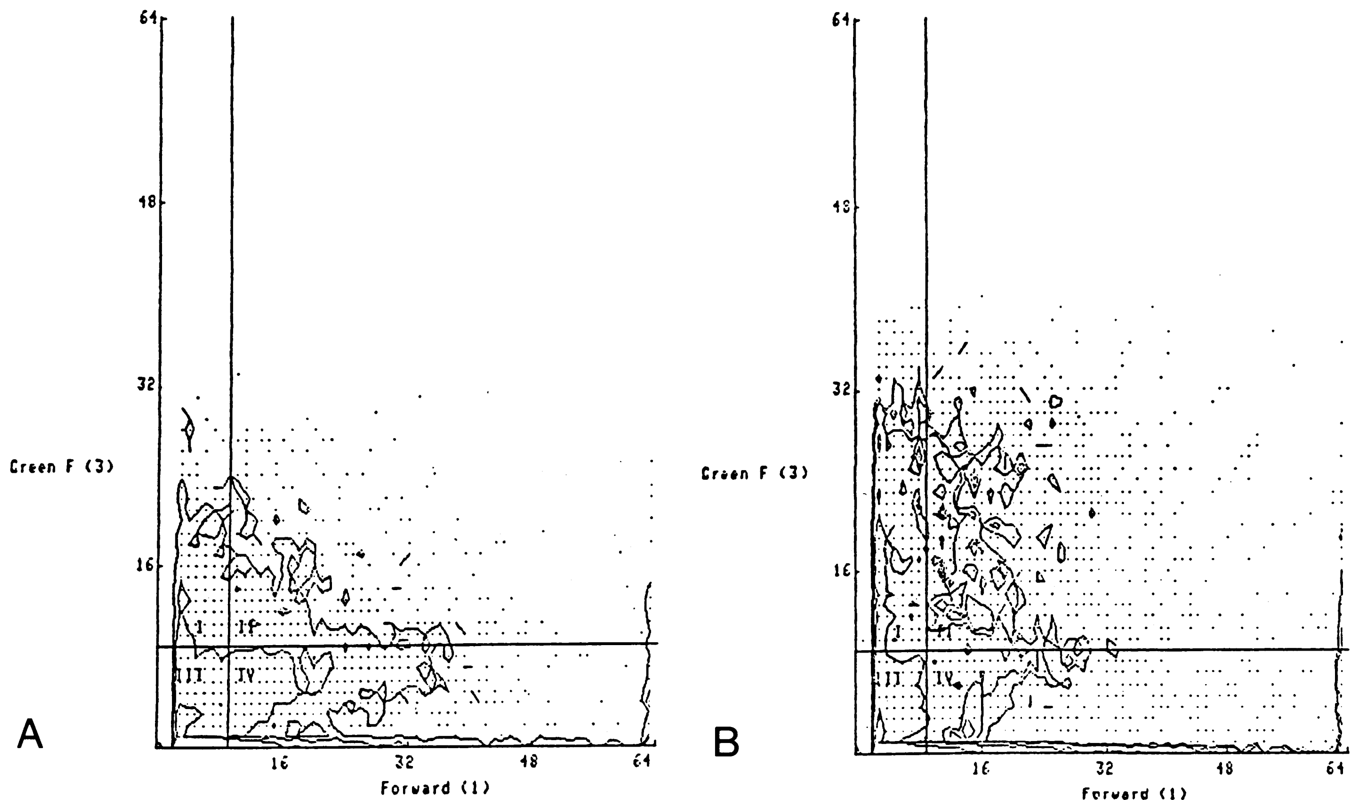
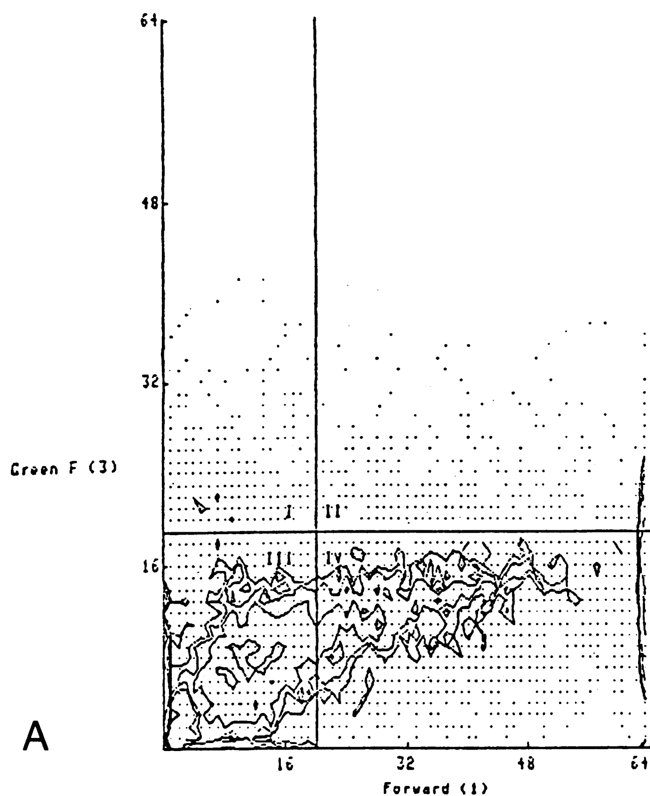
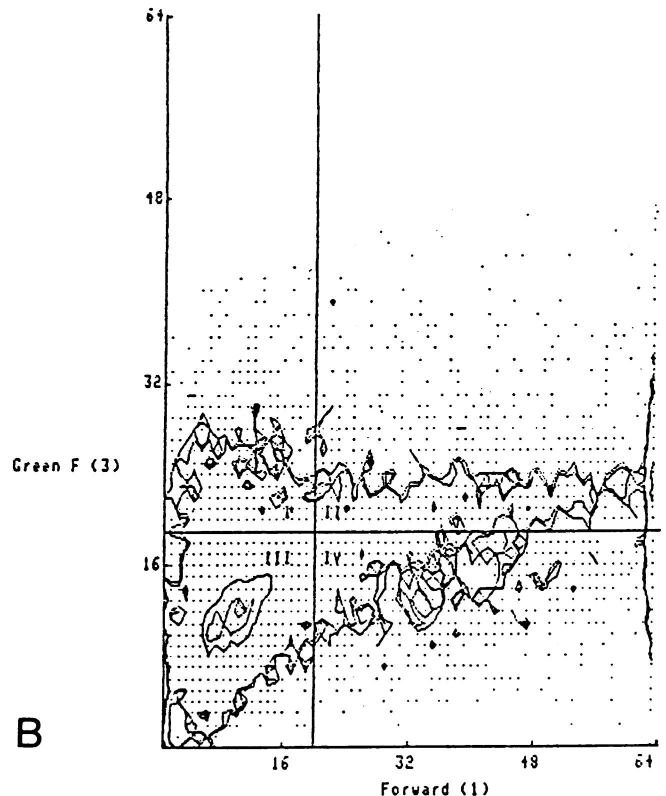


FIGURE 5 Population distribution of fluorescence intensity in swollen thylakoid vesicles after incubation with 0.05 mM dextran-FITC 10,000 D fluorescent dye and exposure to 100 repetitive electric field pulses at intervals of 5 s. The contours plots of green fluorescence vs. forward scatter with identical number of events were chosen arbitrarily to reflect the main features of population distribution. Thylakoid vesicles (50 μg/ml chlorophyll) were suspended in 1 mM Tris-HCl pH 8.0 at 25°C. (*A*) Control, where the suspension was not exposed to the external field. The thylakoid vesicles were incubated with the dye for 10 min. ***Quadrant I,*** low forward scattering particles of high fluorescence intensity (462 events = 4.5%). ***Quadrant II,*** high forward scattering particles of high fluorescence intensity (551 events = 5.4%). ***Quadrant III,*** low forward scattering particles of low fluorescence intensity (probably this quadrant represents mostly very small and broken vesicles) (6,288 events = 61.4%). ***Quadrant IV,*** high forward scattering particles of low fluorescence intensity (2,939 events = 28.7%). (*B*) The suspension was exposed to electric field strength of 1,600 V/cm and duration of 200 μs. The exposure to the electric field was repeated 100 times at intervals of 5 s. ***Quadrant I,*** low forward scattering particles of high fluorescence intensity (1,576 events = 15.4%). ***Quadrant II,*** high forward scattering particles of high fluorescence intensity (1,492 events = 14.6%). ***Quadrant III,*** low forward scattering particles of low fluorescence intensity (probably this quadrant represents mostly very small, and broken vesicles) (4,774 events = 46.6%). ***Quadrant IV,*** high forward scattering particles of low fluorescence intensity (2,398 events = 23.4%).

the macromolecular size of the fluorescent probe on the electric field–induced uptake. For this purpose, we added to suspensions of swollen thylakoids 0.1 mM dextran-FITC of different molecular weights, and exposed them to repetitive pulses of electric field. All the suspensions that were exposed to the electric field (1,600 V/cm for 200 μs) showed a higher level of fluorescence as compared to the control suspensions, except for the suspension that contained a dextran-FITC of 70,000 D (Fig. 9). In this case the fluorescence level of the exposed suspension was almost the same as in the control suspension. We examined the relation between the pulse duration and the size of the molecule that could undergo an uptake process. For this purpose we exposed different suspensions of swollen thylakoids to electric fields of different durations, where the suspension contained either 5(6)-carboxyfluorescein or dextran-FITC of 70,000 D. The results are given in Fig. 10. At a duration of 40 μs no significant uptake of dextran-FITC could be detected. However, when the duration of the pulse was increased to 500 μs the suspended vesicles showed higher level of uptake. When 5(6)-carboxyfluorescein was added instead of dextran-FITC 70,000 D an increased uptake of the dye was induced by the exposure even with electric fields of 40 μs duration. To examine whether the size of electropores depends on electric field strength, we exposed suspension of thylakoid vesicles to 5(6)-carboxyfluorescein and to dextran-FITC 40,000 D (Fig. 10 *b*). For exposures of 100 μs at 700 V/cm an uptake of 5(6)-carboxyfluorescein was observed, whereas no significant uptake of dextran-FITC





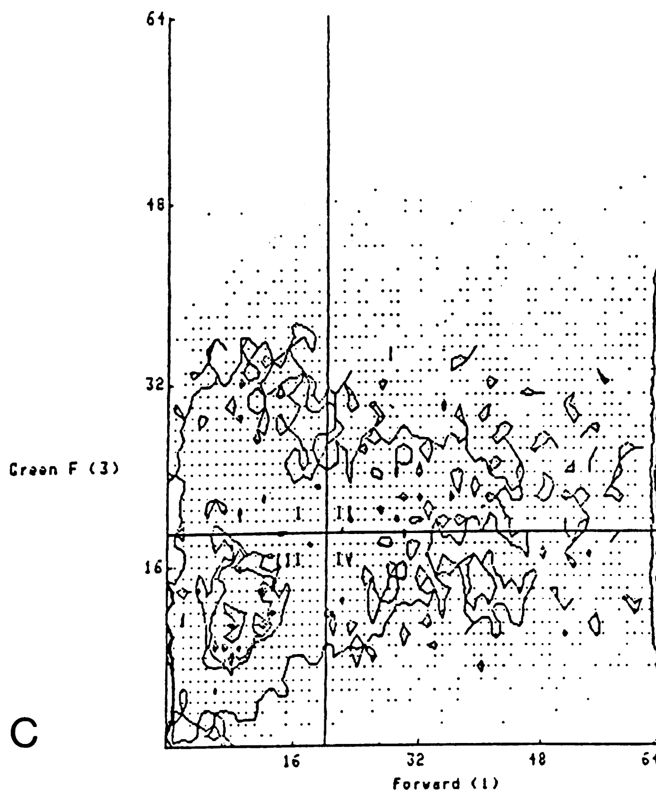


FIGURE 6 Population distribution of fluorescence intensity in swollen thylakoid vesicles after incubation with 0.1 mM 5(6)-carboxyfluorescein. The contours plots of green fluorescence vs. forward scatter with identical number of events chosen arbitrarily to reflect the main features of population distribution. (*A*) Control, where the suspension was not exposed to the external electric field. The thylakoid vesicles were incubated with the dye for 10 min. ***Quadrant I,*** low forward scattering particles of high fluorescence intensity (377 events = 3.7%). ***Quadrant II,*** high forward scattering particles of high fluorescence intensity (469 events = 4.6%). ***Quadrant III,*** low forward scattering particles of low fluorescence intensity (probably this quadrant represents mostly very small and broken vesicles) (5,352 events = 52.3%). ***Quadrant IV,*** high forward scattering particles of low fluorescence intensity (4,042 events = 39.5%). (*B*) The suspension was exposed to electric field strength of 1,500 V/cm for of 150 μs. The exposure to the electric field was repeated 100 times at intervals of 5 s. ***Quadrant I,*** low forward scattering of high fluorescence intensity (985 events = 9.6%). ***Quadrant II,*** high forward scattering of high fluorescence intensity (1,578 events = 15.4%). ***Quadrant III,*** low forward scattering particles of low fluorescence intensity (probably this quadrant represents mostly very small and broken vesicles) (6,097 events = 59.5%). ***Quadrant IV,*** high forward scattering particles of low fluorescence intensity (1,580 events = 15.4%). (*C*) The suspension was exposed to electric field strength of 1,500 V/cm and duration of 500 μs. The exposure to the electric field was repeated 100 times at intervals of 5 s. ***Quadrant I,*** low forward scattering particles of high fluorescence intensity (1,800 events = 17.6%). ***Quadrant II,*** high forward scattering particles of high fluorescence intensity (2,058 events = 20.1%). ***Quadrant III,*** low forward scattering particles of low fluorescence intensity (probably this quadrant represents mostly very small and broken vesicles) (4,954 events = 48.4%). ***Quadrant IV,*** high forward scattering particles of low fluorescence intensity (1,428 events = 13.9%).

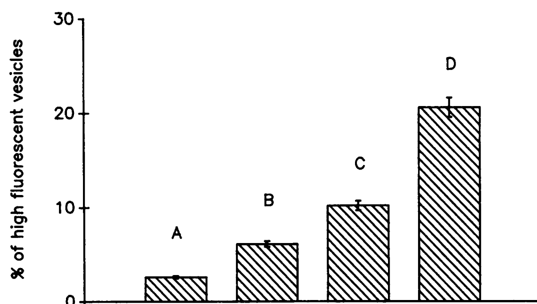


FIGURE 7 Change in the population distribution of fluorescence intensity due to electric field–induced uptake of 5(6)-carboxyfluorescein into thylakoid vesicles as a function of electric field duration. The externally applied electric field was 1,600 V/cm. The analysis was carried out from the histogram of fluorescence intensity vs. the number of events (independent of the forward scattering). The analysis was made by choosing a threshold fluorescence intensity above which we determined the percentage of fluorescent vesicles out of the total population. Other conditions as in Fig. 5. (*A*) Control, without exposure to the electric field. (*B*) Exposed to pulse of 100 μs. (*C*) Exposed to pulse of 200 μs. (*D*) Exposed to pulse of 500 μs.

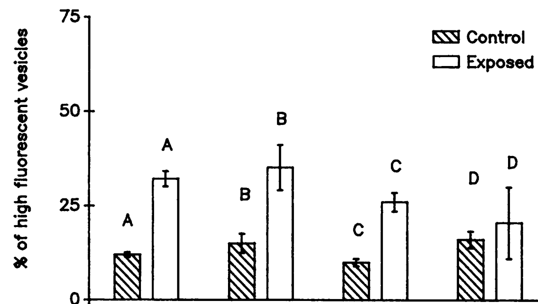


FIGURE 9 Change in the population distribution of fluorescence intensity due to electric field–induced uptake of dextran-FITC molecules having different molecular weights. The sample was exposed to external electric field amplitude of 1,600 V/cm and 200 μs duration. The analysis was carried out from the histogram of fluorescence intensity vs. the number of events (independent of the forward scattering). The analysis was made by choosing a threshold fluorescence intensity above which we determined the percentage of fluorescent vesicles out of the total population. (*A*) Dextran-FITC 1,000 D; (*B*) dextran-FITC 20,000 D; (*C*) dextran-FITC 40,000 D; (*D*) dextran-FITC 70,000 D. The uptake studies with dextran-FITC molecules of different molecular weights were carried at concentrations of 0.05 mM. However, the different dextran had different fluorescence yields. Thus, only the relative changes between "exposed" and "control" should be compared. Other conditions similar to those in Fig. 5.

could be detected. However, when increasing the field strength to 1,600 V/cm a significant increase in the uptake of dextran-FITC of 40,000 D could be observed.

DISCUSSION

The calculated membrane conductance at the maximum of the first EPL signal yielded a value of 171 ± 56 nS/cm.

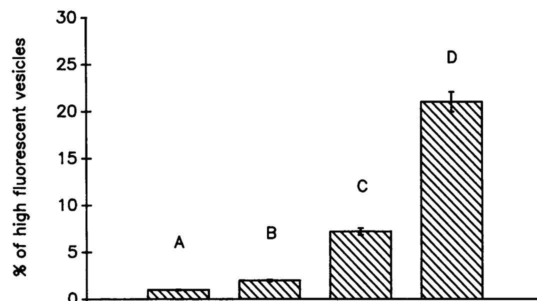


FIGURE 8 Change in the population distribution of fluorescence intensity due to electric field–induced uptake of 5(6)-carboxyfluorescein into thylakoid vesicles as a function of electric field strength. The analysis was carried out from the histogram of fluorescence intensity vs. the number of events (independent of the forward scattering). The analysis was made by choosing a threshold fluorescence intensity above which we determined the percentage of fluorescent vesicles out of the total population. Other conditions as in Fig. 5. (*A*) Control, without exposure to the electric field. (*B*) Exposed to electric field of 700 V/cm. (*C*) Exposed to electric field of 1,600 V/cm. (*D*) Exposed to electric field of 2,000 V/cm.

(The standard deviation around the average of the value results from the high variation among the different experiments. However, the variation of the delta σ_m is much smaller [17.7 ± 0.5 nS/cm].) This value is higher than the value of 2.5 nS/cm, obtained from electric field–induced rotation of thylakoid vesicles (Arnold et al., 1985), and from values in the range of 0.5–5 nS/cm obtained from direct electrophysiological measurements (Campo and Tedeschi, 1985). A possible explanation for these differences may arise from the fact that while both previous studies were performed under relatively low electric fields, the present study was carried out under high electric field strength. Thus, when the EPL signal reaches its maximum after ~20 μs (see Fig. 1), an initial process of electroporation may have already taken place causing an increase in membrane conductivity. However, this conductivity change is still smaller than the one which occurs at times >20 μs. This suggestion is supported by the dependence of electroporation on pulse duration. At pulse duration of 20 μs, only a marginal conductivity change could be observed even at the highest applied electric field (data not shown).

The observed linear relationship between the induced conductivity change and the strength of the applied electric field, both for short and long time exposures (Fig. 3) may be rationalized in terms of the linear increase in the vesicle's area that undergoes permeabiliza-

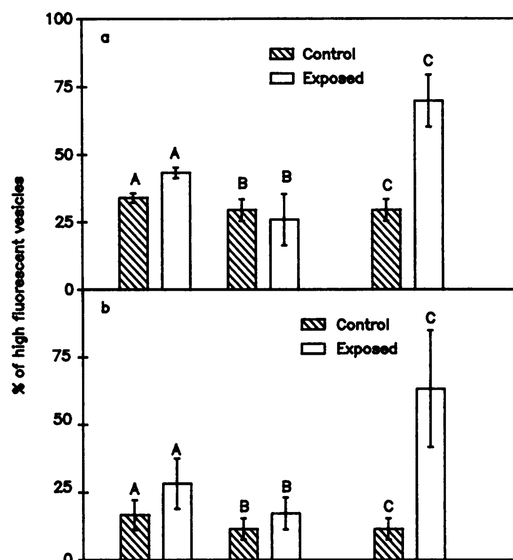


FIGURE 10 (*a*) Change in the population fluorescence intensity due to electric field–induced uptake of dextran-FITC 70,000 D or 5(6)-carboxyfluorescein as function of electric field duration (E_{ex}, 1,600 V/cm). The analysis was carried out from the histogram of fluorescence intensity vs. the number of events independent of the forward scattering. The analysis was made by choosing a threshold fluorescence intensity above which we determined the percentage of fluorescent vesicles out of the total population. (*A*) Duration of the electric field: 40 μs, fluorescent dye: 5(6)-carboxyfluorescein. (*B*) Duration of the electric field: 40 μs, fluorescent dye: 70,000 D dextran-FITC. (*C*) Duration of the electric field: 500 μs, fluorescent dye: 70,000 D dextran-FITC. (*b*) Change in population distribution of fluorescence intensity due to electric field–induced uptake of dextran-FITC 40,000 D or 5(6)-carboxyfluorescein as a function of electric field strength. The analysis was carried out from the histogram of fluorescence intensity vs. the number of events (independent of the forward scattering). The analysis was made by choosing a threshold fluorescence intensity above which we determined the percentage of fluorescent vesicles out of the total population. (*A*) Fluorescent dye: 5(6)carboxyfluorescein; electric field 700 V/cm; pulse 100 μs. (*B*) Fluorescent dye: dextran-FITC 40,000 D; electric field 700 V/cm; pulse 100 μs. (*C*) Fluorescent dye: dextran-FITC 40,000; electric field 1,600 V/cm; pulse 100 μs.

tion upon the increase of electric field strength. However, a nonlinear increase in membrane conductivity is observed when maintaining the electric field strength constant while changing the exposure duration (Fig. 4). In this case we would expect the vesicle's area that undergoes electroporation to be constant, whereas the areas defined by single electropores would increase with the length of the exposure time. This time-dependent process seems to be a nonlinear one. It is evident from Fig. 4 that for short exposure times (e.g., 20 μs), the induced conductivity change is only weakly dependent on the electric field strength. The capacitive charging time of a vesicular membrane can be estimated according to Cole (1972):

$$t = RC_m(1/\sigma_i + 2/\sigma_o),$$

where t is the charging time, R is the radius of the vesicle, C_m is the specific capacitance of the membrane, and σ_i, σ_o are the inner and outside specific conductivities of the medium. For values of $R = 4$ μm, $\sigma_m \ll \sigma_i = \sigma_o = 200$ μS/cm, and $C_m = 1$ μF/cm^2, we obtain a charging time of ≈ 3 μs. The use of the above equation is valid even if an initial electroporation has already started since σ_{ml} (171 ± 56 nS/cm) is still three orders of magnitude smaller than σ_o ($\approx$200 μS). This apparent difference between the charging time and the time when we begin to detect the induced conductivity change in the membrane reflects the nonlinear dependence of the induced local electric field on the σ_m/σ_o ratio (see Eq. 1 and Farkas et al., 1982). Thus, for electric field–induced changes in σ_m, where σ_m/σ_o is $<10^{-3}$, only a small attenuation in E_m and consequently in EPL is to be expected. This theoretically limits the possibility to study the initial development of the electropore when employing voltage-sensitive optical probe. Taking it into account, our observations may suggest that at short exposures the electropores do not expand sufficiently to contribute to a conductivity change, whereas under conditions of long exposure times the electropores have enough time to grow in size so as to lead to a more significant conductivity change.The overall permeability increase as a function of pulse duration, as monitored by the uptake studies, agrees with (Figs. 6 and 7) to the EPL-based studies (Fig. 4). A similar situation is reflected in the increase of membrane permeability as a function of electric field strength (Fig. 3 and Fig. 8). The uptake studies of the different fluorescent dextran molecules aimed not only to examine the electric-induced permeability changes but also to estimate the average cross-section areas of the individual electropores. Fig. 5 demonstrates the uptake of dextran-FITC 10,000 D when exposed to external electric field of 1,600 V/cm for 200 μs. The observed change in the population distribution fluorescent intensity reflects the formation of electropores possessing a radius >2.4 nm. The correspondence between the molecular weights of the different dextran and their molecular diameter was taken from Laurent and Granath (1967) and Sowers and Lieber (1986). Thus, exposures to an electric field of 1,600 V/cm and for 200 μs induces largest electropores of an average radius (R) of $4.8 < R < 5.8$ nm. Extending the exposure time leads to an increase in the average area of the electropore. This is evident from Fig. 10 *a*, where no uptake of dextran-FITC 70,000 D molecules is observed for exposure of 40 μs (Fig. 10 *a*, *B*), whereas a significant uptake is observed when the duration is extended to 500 μs (Fig. 10 *a*, *C*). An early attempt to estimate electropore size in erythrocytes was carried out by Kinosita and Tsong (1977). To test the contribution of electric field strength to the expansion in the areas of electropores, we studied the uptake of 40,000 D dextran-FITC in suspensions exposed

to 700 V/cm and 1,600 V/cm (100 μs duration). An increased uptake of the fluorescent probe is observed at the higher electric field, whereas no significant uptake was observed at the lower one (Fig. 10 *b*). This experiment suggests that electric field strength contributes to the expansion of the average area of the electropore, at least for the relatively long exposure times.

From the combined knowledge of the induced conductivity change (EPL studies) and average areas of the electropores (uptake studies), carried under identical electric field exposure characteristics, we can estimate the induced number of electropores. Thus, we measured a conductivity change of 111 nS/cm when exposing the membrane vesicles to electric field strength of 1,600 V/cm for 200 μs. From Ohm's law we calculate an average electric field–induced conductance change of $\approx$450 nS per single vesicle (where the number of vesicles was 2.8×10^6 and the area of a vesicle having an average radius of 4 μm was 2.03×10^{-6} cm^2). The conductance of a single aqueous pore ($\cap$) in a bilayer can be estimated again from Ohm's law (e.g., Benz, 1984): $\cap = \sigma_0 \cdot R^2/d$, where σ_0 is the specific conductivity of the medium ($\sigma_0 = \sigma_i$), R is the pore's radius, and d is membrane thickness. Because under the above conditions of electroporation we obtain that the largest electropores have a radius of $4.8 < R < 5.8$ nm, the calculated single pore conductance is $215 < \cap < 314$ pS. Therefore, the minimal average number of electropores (n) in a single vesicle is $2{,}100 > n > 1{,}340$. This number of electropores is of the same order of magnitude as reported by Sowers and Lieber (1986) or Kinosita and Tsong (1977) for erythrocytes. Furthermore, the total area of electroporation is only 0.075% of the total surface of a swollen thylakoid. This value of the total pore area fraction per vesicle is similar to the value of 0.06% (exponential pulse of 7,000 V/cm and pulse duration of 1.2 ms) obtained for erythrocytes (Sowers and Lieber, 1986) and to 0.01–0.1% (rectangular pulse of 400 V/cm and pulse duration of 25 μs) obtained for eggs of sea urchins (Kinosita et al., 1988). Thus it seems that for some aspects of electroporation the thylakoid swollen vesicle can serve as a good model for the natural cell membrane. However, it should be pointed out that thylakoid vesicles differ from cells in two main aspects: the composition of membrane lipids and the absence of any cytoskeletal elements.

In summary, the results obtained in this study demonstrate that the process of electroporation involves the reversible formation of discrete holes in the membrane. The formation of electropores is a dynamic process which is modulated by both the electric field strength and its duration. However, the influence of each of the two parameters of the electric field on the electroporation process is different. Electric field strength affects the total area of the vesicle that undergoes electroporation. When applied in combination with relatively long pulse durations it also increases the area of the single electropores themselves. Each of these two parameters can be rate limiting in the dynamics of the electropore formation. These conclusions support the theoretical model of the transient aqueous pores (Abidor et al., 1979; Powell et al., 1986; Glaser et al., 1988).

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