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Control of Electric Field Induced Cell Membrane Permeabilization by Membrane Order

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ABSTRACT: Cells can be made temporarily permeable if pulsed by high-intensity short-duration electric fields. The molecular mechanisms underlying this electropermeabilization are still unknown. The kinetic events may be described by four successive steps: induction, expansion, stabilization, and resealing. On one hand, cell electropermeabilization is detected only under more stringent conditions when cells have been treated by ethanol. On the other hand, lysolecithin is observed to facilitate cell electropermeabilization. More precisely, these molecules that modify membrane order, when used in concentrations compatible with cell viability, are shown to affect only the expansion and resealing steps. Electropermeabilization is inducing a transition in the membrane organization. Membrane order is modulating the energy barrier needed to evoke this membrane transition which occurs when cells are submitted to a field larger than a characteristic threshold (expansion step). Less order would increase the magnitude of this energy barrier; more order would decrease it.

The cytoplasmic content of cells is protected from exogenous molecules by the selective permeability of the plasma membrane. This barrier function can be temporarily removed by applying short high-intensity electric pulses to the cell suspension (Neumann & Rosenheck, 1972). Such "electropermeabilization" is very easy to handle and is now routinely used in cell biotechnology to introduce plasmids into the host cell genome (electrotransformation) for genetic manipulation (Neuman et al., 1982; Potter, 1988). This approach is valid for almost any cell system [mammalian cells, plant, yeast, and bacterial protoplasts (Neumann et al., 1982; Shillito et al., 1985; Shivarova et al., 1983)] and was recently shown to be operative with walled systems (Taketo, 1988). Another property of electropermeabilization is the creation of a fusogenic state in the cell membrane. It has been known since 1980 that pulsation of cells held in close contact would cause them

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to fuse (Neumann et al., 1980; Zimmermann, 1982; Teissié et al., 1982), but more recently, it was shown that fusion occurs even if the contact is established after the pulse (Sowers, 1986; Teissié & Rols, 1986).

The field of applied biotechnology is exploiting these properties of cell electropulsation (Teissié, 1988), but very little is known about the molecular aspects of the processes. The physical trigger is linked to potential modulation of a membrane by the external field (Bernardt & Pauly, 1973; Loew et al., 1986; Kinosita et al., 1988). When intensity reaches a certain threshold, dramatic restructuration of the lipid matrix occurs, giving a high degree of conductivity to the membrane (Kinosita et al., 1988; Abidor et al., 1979; Weaver et al., 1984). This was believed to be due to a "breakdown" related to electrocompression (Crowley, 1973) or to structural defects causing hydrophilic pores (Chernomordik et al., 1987). These theoretical models were developed from experimental data about lipid bilayers and took the fact that large pure phospholipid unilamellar vesicles can be electropermeabilized into account (Teissié & Tsong, 1981). Very few results deal with direct observation of what affects the cell. Electron microscopy has shown that very short lived lipidic particles (less than 1 s, i.e., not correlated with the permeability created) may be present after the pulsation of Pronase-treated erythrocytes (Stenger & Hui, 1986) and that an eruption of villi may be observed on the surface of electropermeabilized cells (Escande-Geraud et al., 1988). Their lifespan is directly related to the period of permeability. The ^{31}P nuclear magnetic resonance (NMR)[1] studies of the organization of plasma membrane phospholipids demonstrated a reversible transient change in the orientation of the polar head groups during the permeable state (Lopez et al., 1988). The fatty acid chain region was not apparently affected, and the classical bilayer organization was maintained. This change in the polar head group array provides physical support to the fusogenicity of electropermeabilized cells. Cell aggregation depends on the interactive energy of the approaching membranes, but a very strong repulsive force (the hydration force) hinders close contact (Parsegian & Rau, 1984). This repellent force comes from the organization of the interfacial water molecules whose dipoles are oriented by the local field generated by the polar head groups (Gruen & Marcelja, 1983). The membrane-induced regular organization of this array creates a well-organized lattice of water molecules which generates this repulsive force when cells are in closed approach. The ^{31}P NMR results showed that the polar head array is in a new configuration in the permeabilized cells, and, consequently, the lattice of water molecules may no longer be present or may be modified, making the interface more hydrophobic, as shown in studies on electropermeabilized plant protoplasts (Hahn-Hagerdal et al., 1986). The hydration forces will not be strong enough, if still present, to hinder close contact and fusion of cells.

The organization of the membrane should be a leading factor in the processes creating electropermeabilization. It has been shown that lipids are involved (Teissié & Tsong, 1981), but in one case, an intrinsic membrane protein was affected (Teissié & Tsong, 1980). The proposal that electropermeabilization is linked to percolation suggests that lipid–protein interactions play a regulatory role (Sugar et al., 1987). As a consequence and for greater clarity, we call this labile organization temporarily permeable structures (TPS) (Teissié & Rols, 1986). In this report, the effect of molecules affecting membrane order on cell electropermeabilization was investigated. Such molecules were shown to affect the electrofusion of Chinese hamster ovary (CHO) cells already in contact during pulsation (Orgambide et al., 1986), and similar results were obtained on plant protoplasts (Nea et al., 1987; Christov & Vaklinova, 1987). The results on mammalian cells were tentatively explained by some alteration in the extent of electropermeabilization. Such a model is challenging, and the present study sets out to check it through experiments and obtain further details on the causes of electropermeabilization. Plated CHO cells have been chosen because considerable information about the electropermeabilization of that strain is available (Blangero & Teissié, 1983, 1985; Teissié & Blangero, 1984; Orgambide et al., 1986; Lopez et al., 1988; Escande-Geraud et al., 1988; Teissié & Rols, 1986, 1988a,b; Rols & Teissié, 1989) and because of the experimental advantages of working with plated cells.

Materials and Methods

Chemicals. Salts were analytical grade (Prolabo, France). Alcohol was puriss (Fluka, Switzerland) or was distilled in the laboratory (Prolabo, France). Lysolecithin was purchased from Sigma.

Ultrapure water from a Millipore Milli Q unit was used to prepare buffers.

Cells. Chinese hamster ovary (CHO) cells have been adopted by a large number of somatic cell genetic laboratories [see Gottesmann (1985) for a review]. The WTT clone, which was kindly given to us by Prof. Zalta (of this institute), was selected for the present study. Cells were grown on culture dishes (Nunc, Denmark) in Eagle's minimum medium (MEM 0111, Eurobio, France) supplemented with 6% newborn calf serum (Boehringer, Federal Republic of Germany), antibiotics, and glutamine, at 37 °C, in an air/CO_2 incubator (Jouan, France). The generation time was 16–18 h.

Electropermeabilization. The "electropermeabilization" method used has previously been described (Teissié & Rols, 1988b). In this approach, the electric pulse is applied directly to cells growing in monolayers on culture dishes (Nunc, Denmark). The field is homogeneous, being generated by a high-voltage pulse of electronically selected duration applied to two flat parallel stainless-steel electrodes (distance 5 mm, length 20 mm) which are in contact with the bottom of the dish (CNRS cell electropulsator, marketed by Jouan, France). Repetitive square wave pulses (100-μs duration) of 1-Hz frequency are applied at a selected intensity. The pulsing medium (2 mL) is 10 mM phosphate buffer (pH 7.5), 250 mM sucrose, and 1 mM $MgCl_2$. The cells are in contact with the pulsing medium only during the pulsation, i.e., about 2 min.

Prepulse incubation was carried out as follows. The cells at a density of 800 cells/mm^2 were incubated for 30 min (just before the electric treatment) at 37 °C in a culture medium containing the membrane-active molecules at the indicated concentrations. Incubations were always carried out in an air/CO_2 95/5 atmosphere. The cells were then washed out and bathed with the pulsing buffer. Additive was not present in the pulsing medium, and the effect was introduced during the prepulse incubation. Control experiments were always run with no treatment during the prepulse incubation period.

Electropermeabilization of CHO cells was quantified by two different methods: (i) penetration of Ca^{2+} into cells, leading to lysis; (ii) penetration of Trypan blue (M_r 960), staining the nuclei blue (Teissié & Rols, 1988b).

In case i, cells were pulsed in a pulsing buffer containing 3 mM $CaCl_2$ and Trypan blue (0.4% w/v). They were incu-

[1] Abbreviations: CHO, Chinese hamster ovary; NMR, nuclear magnetic resonance; TPS, temporarily permeable structure(s); DPH, 1,6-diphenyl-1,3,5-hexatriene.

bated for 5 min after the pulsation, and the number of blue-stained cells was counted in the population. In that case, Trypan blue was used to monitor cell lysis. Sieving of TPS was just big enough to allow Ca^{2+} to penetrate.

In case ii, cells were pulsed in a pulsing buffer to which Trypan blue (0.4% w/v) was added. They were incubated for 5 min at room temperature after the pulsation, and the percentage of blue-stained cells was determined. In that case, sieving of TPS was big enough to allow Trypan blue to pass.

Reversibility of the electropermeabilization was assayed by the Trypan blue test. The cells were pulsed in the "pulsing" buffer which was replaced by the Trypan blue containing buffer after the indicated time. Cells were incubated for 5 min thereafter. Staining of cells was indicative that TPS with a sieving large enough for the penetration of the dye were still present.

The samples were observed under an inverted microscope (Leitz, Federal Republic of Germany) with video monitoring (JVC, Japan). Control (unpulsed) cells were present on the same dish as the pulsed ones but outside the interelectrode space. No treatment was needed for the cell observation except for washing out of the Trypan blue solution, because the dish was moved directly onto the microscope stage. A total of 2 × 500 cells (pulsed and control) were routinely observed per experimental condition in order to obtain statistically significant values. Due to some drifts in the results with the aging of the culture, comparative experiments were always run on the same day. Cell viability after pulsing was obtained by observing the growth 24 h after the pulses, which were operated in sterile conditions under a laminar flowhood to avoid contamination.

Fluorescence polarization experiments were performed by using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a fluorescent probe for the hydrocarbon region of biological membranes (Shinitzki & Inbar, 1974). Cells in suspension, in some cases preincubated with ethanol or lysolecithin, were washed by centrifugation and resuspended in pulsing buffer in the presence of 1 μM DPH. After an appropriate incubation delay, determinated to be 10 min at 21 °C, cells were washed and resuspended at a concentration of 10^6 cells/mL in pulsing buffer. Microscopic examination revealed no damage to the cells and a uniform fluorescence strictly localized at the plasma membrane level. Polarization of fluorescence was measured at 21 °C with a Jobin Yvon spectrofluorometer equipped with polarizers. Excitation was at 360 nm and emission at 430 nm. Correction of measurements was performed as described by Harris and Bashford (1987).

Results

Morphological Alterations of Plated CHO Cells following Membrane-Active Molecule Treatment. In a previous study, we showed how the CHO strain used in this study was not morphologically affected by 400 mM ethanol treatment (Orgambide et al., 1986). If a high ethanol concentration was used (1 M), cells were no longer plated at the end of the incubation period (30 min, 37 °C), but if they were then brought back to a fresh culture medium, they were observed to be plated one again following overnight incubation. There was no immediate propanol and butanol effect (0.4 M) after the 30-min incubation period, but a majority of cells were observed to be spherical 24 h after the alcohol treatment. Cells were not able to withstand 0.05 M benzyl alcohol or octanol treatment.

Lysolecithin is known to be lytic for red blood cells at a 0.1–1 mg/mL concentration (Chernomordik et al., 1987). We therefore ran the assay at a lower concentration (0.02 mg/mL)

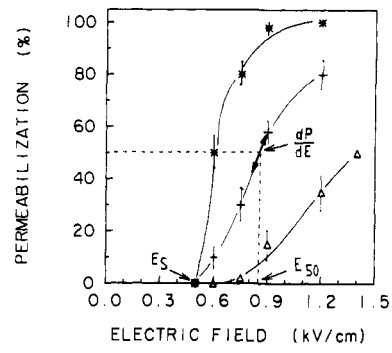

FIGURE 1: Extent of permeabilization as a function of field intensity. Plated CHO cells were pulsed 10 times (pulse duration 100 μs, time interval 1 s) in a low ionic content pulsing buffer (10 mM phosphate, 1 mM $MgCl_2$, and 250 mM sucrose). Permeabilization was detected by the penetration of Trypan blue and is expressed as the percentage of the cell population which is blue stained. Cells were control samples (+) or pretreated either by 400 mM ethanol (30 min at 37 °C) (Δ) or by 20 μg/mL lysolecithin (5 min at 21 °C) (*). Threshold field intensity (E_s) is the smallest field intensity needed to detect pulse-created permeabilization, the half-effect intensity (E_{50}) is the field strength which induces the permeabilization of 50% of the pulsed population, and dP/dE is the relative change in permeabilization versus field intensity (for field intensity equal to E_{50}).

and did not observe any effect.

Polarization of fluorescence (p) of DPH in CHO cells was found to be equal to 0.29 (±0.01) for control cells, 0.27 (±0.01) for cells pretreated with ethanol, and 0.31 (±0.01) for cells preincubated with lysolecithin and was stable for minutes after the incubated cells were washed. That shows a long-term alteration of the membrane order and its associated parameter fluidity which is increased in the case of ethanol-treated cells and decreased with lysolecithin.

Alteration of CHO Electropermeabilization by a Prepulse Ethanol Treatment. It was previously shown (Escande-Geraud et al., 1988) that the percentage of electropermeabilized cells was controlled by the field intensity, pulse duration, and pulse number. No permeabilization was observed whatever the pulse duration or number when the field strength was under the characteristic threshold, E_0. Increasing the field above the E_0 value increased the percentage of permeabilized cells. This process is associated with a permeabilization vs field intensity plot with a sigmoidal shape and can be described with several parameters such as the field intensity (E_s) required to obtain permeabilization ("apparent threshold value"), the field intensity giving 50% of permeabilized cells (E_{50}), or the derivative of the change in permeabilization versus field strength (dP/dE) at E_{50}. Treating the cells with 0.4 M ethanol strongly affects their electropermeabilization (Figure 1). For 10 pulses of 100-μs duration, the threshold E_s is slightly affected, and the permeabilization curve is shifted toward higher field strengths with an associated increase in E_{50} and a decrease in dP/dE.

This effect was controlled by the ethanol concentration but can be detected even at 20 mM ethanol treatment.

The extent of permeabilization is controlled by the pulse duration for a given number and field strength. As shown in Figure 2, longer pulses were needed to induce the same level of permeabilization as in the control cells when a 0.4 M ethanol treatment was operated.

At a given pulse duration and field intensity, the extent of permeabilization was increased by applying more and more pulses to the culture. Again, more drastic conditions are needed to get the same permeabilization level of the cell population after a 0.4 M ethanol treatment (Figure 3). The characteristic permeabilization threshold E_0 ("the real

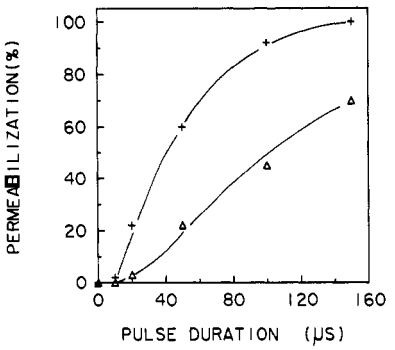

FIGURE 2: Effect of pulse duration on the extent of permeabilization. Cells were pulsed 10 times (field intensity 1.2 kV/cm, 1-s time interval). Control cells are (+), and cells incubated in ethanol prior to pulsing (400 mM, 30 min, 37 °C) are (Δ).

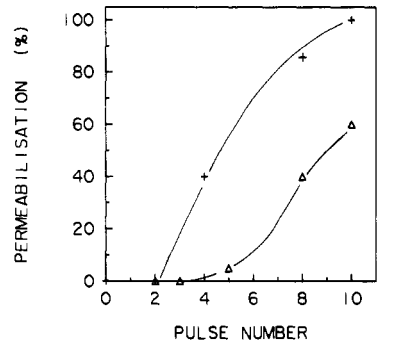

FIGURE 3: Effect of number of pulses on the extent of permeabilization. Cells were pulsed as indicated (other settings are 1.2 kV/cm, 100 μs, 1-s time interval). Control cells are (+), and cells incubated in ethanol prior to pulsing (400 mM, 30 min, 37 °C) are (Δ).

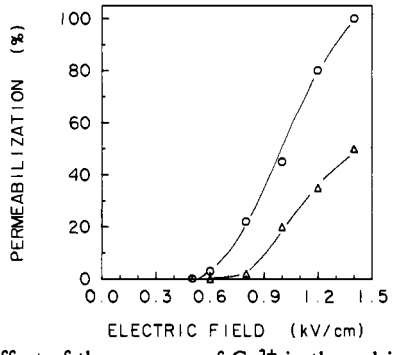

FIGURE 4: Effect of the presence of Ca^{2+} in the pulsing buffer on electropermeabilization of the cells. Cells were incubated with ethanol prior to pulsing. Permeabilization was created with the indicated field intensity (10 times, 100 μs, 1-s time interval). The pulsing buffer contained $CaCl_2$ (3 mM) (O) or no $CaCl_2$ (Δ).

threshold value") was unaffected by the prepulse ethanol treatment: for longer pulse duration and number, E_s is equal to the limit value E_0 of 0.5 kV/cm.

As the size of molecules able to cross the plasma membrane of an electropermeabilized cell is controlled by the pulse parameters (intensity, duration, number) (Kinosita & Tsong, 1978; Schwister & Deuticke, 1985; Escande-Geraud et al., 1988), we checked whether the reduction of electropermeabilization described here linked to ethanol incubation was due to cancelling out of the process or to a reduction of sieving. Ca^{2+} penetration which is caused by electropermeabilization was therefore used. As shown in Figure 4, permeabilization was more easily obtained when Ca^{2+} was present in the pulsing buffer. It should be noticed that the threshold field E_0 was not affected and was the same as in control experiments.

Resealing of the Temporarily Permeable Structures Associated with Electropermeabilization. One of the major

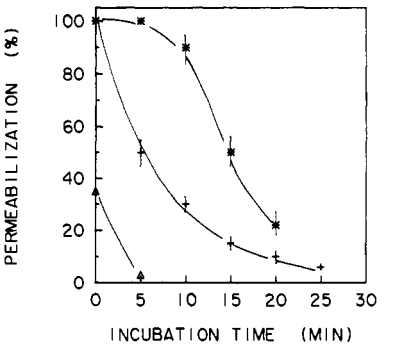

FIGURE 5: Resealing of the temporarily permeable structures. Change in permeabilization during incubation following pulsation was observed. Cells were pulsed (1.2 kV/cm, 100 μs, 1-s time interval, 10 times) and then incubated at 21 °C. Prior to pulsing, the cells were treated as follows: control (+); ethanol (400 mM, 30 min, 37 °C) (Δ); lysolecithin (20 μg/mL, 5 min, 21 °C) (*).

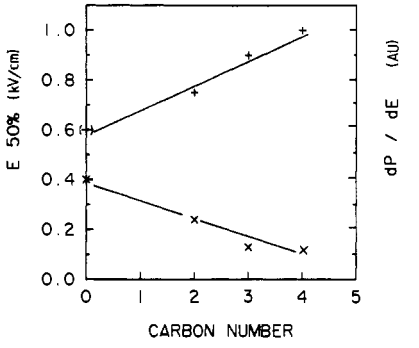

FIGURE 6: Dependence of the permeabilization parameters on the carbon chain length of the alcohols. Cells were pulsed (10 times, 100 μs, 1-s time interval) after a 30-min incubation at 37 °C in the presence of alcohol at a 0.2 M concentration. E_{50} (+) and dP/dE (×) were obtained from the change of permeabilization as a function of the field intensity as shown in Figure 1. Carbon 2 was relative to ethanol, 3 to propanol, and 4 to butanol; 0 means no treatment.

advantages of electropermeabilization for applied purposes is its reversibility. Access to the cytoplasmic content is provided under conditions which do not affect the cell viability. As shown in Figure 5, 0.4 M ethanol incubation does not inhibit the reversibility of electropermeabilization. Nevertheless, these experimental results suggest that, apparently, the loss of the temporarily permeable structures is speeded up when cells are treated with ethanol.

Effects of Longer Chain Alcohols. Taking into account the above reported effects of alcohol treatment on CHO morphology, a comparison of the potency of various alcohols was made.

Effects similar to those associated with ethanol treatment were observed: same E_0, increase in E_{50}, decrease in dP/dE (Figure 6).

The shift in the values of these parameters was found to depend on the chain length of the alcohol. Alcohols with longer chain lengths caused a stronger inhibition of the extent of electropermeabilization. A strong increase of the effect is noticeable between C2 (ethanol) and C3 (propanol) alcohols.

Effect of Lysolecithin Treatment on CHO Electropermeabilization. Lysolecithin was previously described as a potent agent capable of modifying voltage-induced permeabilization of black lipid membranes (Chernomordik et al., 1987) and the electrofusion of plant protoplasts (Nea et al., 1987). Parallel experiments to those on the effect of alcohols were conducted.

The threshold field intensity required for the induction of electropermeabilization, E_0, was not affected by the lysolecithin treatment, but the E_{50} was decreased and the dP/dE was increased. As a general conclusion, electropermeabilization

of all cells is obtained under milder conditions (weaker field intensity) when CHO cells have been preincubated with lysolecithin (Figure 1).

The resealing process was also affected by the preincubation step (Figure 5). The temporarily permeable structures were apparently stabilized, and the lifetime of the permeable state was longer when the cells were treated with lysolecithin before pulsing.

Discussion

The creation of the permeable state in a membrane by the electropermeabilization process can be described by the creation of temporarily permeable structures (TPS) in which the molecules involved are shifted from the normal impermeable state "I" to a new permeable one "P".

Little information is available on this new "P" organization. Electron microscopy suggested it may be associated transiently to lipidic particles (Stenger & Hui, 1986) but more probably to an induction of villi (Escande-Geraud et al., 1988). ^{31}P NMR studies showed that the organization of the plasma membrane phospholipid was altered in the permeable state (Lopez et al., 1988).

Electropermeabilization obeys

$$I \underset{K^-}{\overset{K^+}{\rightleftharpoons}} P \qquad (1)$$

K^+ is controlled by the electric field intensity with the well-known fact that $K^+ = 0$ if $E < E_0$, which simply translates the observation that the field must be greater than the threshold to trigger permeabilization.

The dependence of K^+ upon field intensity is not known, but conductance studies either on black lipid membranes (Chernomordik et al., 1983), on fibroblasts (Glaser et al., 1988), or on erythrocytes (Kinosita & Tsong, 1979) suggest that it is a nonlinear relationship.

As resealing is spontaneous, i.e., present without the application of the field, we can conclude that K^- is not a function of field intensity. When the field is applied (i.e., during the pulse), both processes are present and are competing. Our observations of the effect of ethanol on one hand and of lysolecithin on the other show that the total effect is to shift the equilibrium (eq 1) to the "I" side when ethanol is present and to the "P" side when lysolecithin is added. This observation can be explained by a change in K^+ (decrease with ethanol, increase with lysolecithin), in K^-, or a combination of both.

The resealing process is controlled only by K^-, but conclusions on changes in K^- drawn from experimental results may be misleading if one neglects that resealing is dependent on the extent of permeabilization. When cells are electropermeabilized, resealing is observed to be faster if the field intensity is smaller, i.e., if the extent of permeabilization and the associated sieving of the TPS are smaller. This is clearly shown in Figure 7 and was previously shown with red blood cells (Mishra & Sinh, 1986). When using the results in Figure 5, one should correct the observation by comparing the results of treated cells with those of a control sample in which the same permeabilization has been triggered by the pulse. This correction was previously described (Rols & Teissié, 1989) and is very important for accurate description of the resealing processes. In the case of ethanol-treated cells, the conditions in the resealing experiment (10 times a 100-μs pulse with an intensity of 1.2 kV/cm) give a permeabilization of 35% (Figure 1), which is obtained in a nontreated sample by much milder conditions [10 times a 100-μs pulse with an intensity of only 0.8 kV/cm (Figure 1)]. From Figure 7, it can be concluded that resealing for nontreated cells which were electropermeabilized by fields with intensities of 0.8 kV/cm is very fast. Resealing is almost complete in less than 1 min. With ethanol-treated cells which were electropermeabilized to the same extent (i.e., using an intensity of 1.2 kV/cm), resealing is much slower for only 50% of the permeabilized cells are resealed after 3 min (Figure 5). This means that K^- is smaller in ethanol-treated cells than in the control cells. A direct implication of this observation is then that K^+ is decreased too. Prepulse ethanol incubation creates a state in the cell membrane which makes electropermeabilization less potent. Using the same approach with lysolecithin-treated cells shows that such a treatment induces a membrane organization much more sensitive to electropermeabilization.

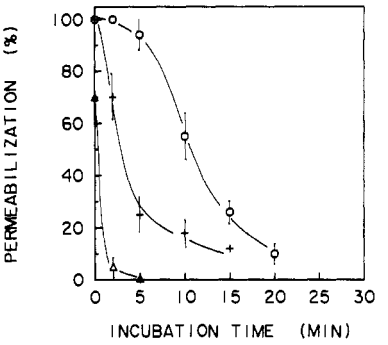

FIGURE 7: Resealing of the temporarily permeable structures as a function of the electric field intensity. The change in electropermeabilization was observed during the postpulse incubation at 21 °C. Cells were pulsed 10 times (pulse duration 100 μs, 1-s time interval) with the following intensity: 0.9 kV/cm (Δ); 1.5 kV/cm (+); 1.8 kV/cm (O).

Electropermeabilization is known to be a two-step process: (1) creation of critical structural defects; (2) expansion of the defect with the creation of a temporarily permeable structure (Kinosita & Tsong, 1979; Chernomordik et al., 1983; Mishra & Sinh, 1986; Glaser et al., 1988; Rols & Teissié, 1989).

Step 1 is present as soon as the field intensity is larger than the threshold E_0 and is a very fast process (always with a shorter duration than 1 μs, which is the time resolution of the observation procedure).

Step 2 is controlled by the field intensity (which must be greater than E_0), the pulse duration, and the number of pulses.

Our experimental observation that E_0 is affected neither by alcohols nor by lysolecithin suggests that step 1 is not altered by these chemicals and that step 2 is the specific target of their action. Lysolecithin was shown to facilitate electrofusion, and ethanol to inhibit it (Orgambide et al., 1986; Nea et al., 1987; Christov & Vaklinova, 1987). This could be explained by the fact that as fusion is a direct consequence of permeabilization, chemical treatment affecting membrane permeabilization should also affect fusion, as we suggested previously (Orgambide et al., 1986).

Ethanol and other alcohols affect the order of the membrane organization and its associated parameters such as fluidity (Zavoico et al., 1985) as confirmed by our experiments with DPH. Ethanol is known to alter the packing of phospholipids close to the ester linkage by inducing expansion of the lipid matrix (Zavoico et al., 1985; Jain et al., 1978). An associated decrease in the order is therefore present throughout the fatty acid chains (Jain et al., 1973; Gruen, 1980), but a new orientation of the polar head groups may be present (Jain et al., 1978). The disordering effect of ethanol has a dramatic influence on the properties of biological membranes by affecting the lipid–protein interactions (Grisham & Barnett, 1972, 1973) and the water permeability of the lipid bilayer (Jain et al., 1973).

Electropermeabilization is inducing a transition in the membrane organization. Such a structural transition is associated with an energy barrier which must be overcome to bring membranes from the normal (impermeant) state to the permeabilized one. Chemical treatment by membrane order affecting agents alters the behavior of the membrane against the permeabilizing field. The rate of the expansion step is controlled by this transition energy barrier according to the Arrhenius law. In our experiments, this rate is directly related to the total pulse duration under a given field strength. Less order makes the permeabilization more difficult and requires more stringent pulsing conditions to obtain the same expansion as with control sample: more energy must be provided (longer pulse duration). This conclusion means that the energy barrier which must be overcome is larger in the treated cell. The opposite fact is observed when more order is present. The rate of the expansion step of the permeabilization process is then strictly dependent on membrane order; it decreases with ethanol and increases after lysolecithin treatment.

The resealing process kinetics are also controlled by the energy barrier needed for the creation of the permeant state (induction of TPS). Ethanol, which increases the associated free energy of TPS creation, would decrease the kinetic constant of the resealing step, which is what we observed, and lysolecithin should have the reverse effect, and it did.

As far as molecular structure is concerned, it is suggested that TPS occurs by percolation (Sugar et al., 1987). Alteration of the membrane order will affect increase of defects. Our observation that lysolecithin and ethanol play opposite roles in electropermeabilization therefore lends experimental support to the biophysical contention of percolation.

The mechanisms involved in the electropermeabilization of cells still remain obscure, but the results presented here may help improve the electric field technique for cell permeabilization and transformation.

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$P^+Q_A^-$ and $P^+Q_B^-$ Charge Recombinations in *Rhodopseudomonas viridis* Chromatophores and in Reaction Centers Reconstituted in Phosphatidylcholine Liposomes. Existence of Two Conformational States of the Reaction Centers and Effects of pH and *o*-Phenanthroline

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ABSTRACT: The $P^+Q_A^-$ and $P^+Q_B^-$ charge recombination decay kinetics were studied in reaction centers from *Rhodopseudomonas viridis* reconstituted in phosphatidylcholine bilayer vesicles (proteoliposomes) and in chromatophores. P represents the primary electron donor, a dimer of bacteriochlorophyll; Q_A and Q_B are the primary and secondary stable quinone electron acceptors, respectively. In agreement with recent findings for reaction centers isolated in detergent [Sebban, P., & Wraight, C. A. (1989) *Biochim. Biophys. Acta 974*, 54-65] the $P^+Q_A^-$ decay kinetics were biphasic (k_{fast} and k_{slow}). Arrhenius plots of the kinetics were linear, in agreement with the hypothesis of a thermally activated process (probably via P^+I^-; I is the first electron acceptor, a bacteriopheophytin) for the $P^+Q_A^-$ charge recombination. Similar activation free energies (ΔG) for this process were found in chromatophores and in proteoliposomes. Significant pH dependences of k_{fast} and k_{slow} were observed in chromtophores and in proteoliposomes. In the pH range 5.5-11, the pH titration curves of k_{fast} and k_{slow} were interpreted in terms of the existence of three protonable groups, situated between I^- and Q_A^-, which modulate the free energy difference between P^+I^- and $P^+Q_A^-$. In proteoliposomes, a marked effect of *o*-phenanthroline was observed on two of the three p*K*s, shifting one of them by more than 2 pH units. On the basis of recent structural data, we suggest a possible interpretation for this effect, which is much smaller in *Rhodobacter sphaeroides*. The decay kinetics of $P^+Q_B^-$ were also biphasic. Marked pH dependences of the rate constants and of the relative proportions of both phases were also detected for these decays. The major conclusion of this work comes from the biphasicity of the $P^+Q_B^-$ decay kinetics. We had suggested previously that biphasicity of the $P^+Q_A^-$ charge recombination in *Rps. viridis* comes from nonequilibrium between protonation states of the reaction centers due to comparable rates of the protonation events and charge recombination. This hypothesis does not hold since the $P^+Q_B^-$ decays occur on a time scale ($\tau \approx 300$ ms at pH 8) much longer than protonation events. This leads to the conclusion that k_{fast} and k_{slow} (for both $P^+Q_A^-$ and $P^+Q_B^-$) are related to conformational states of the reaction centers, existing before the flash. In addition, the fast and slow decays of $P^+Q_B^-$ are related to those measured for $P^+Q_A^-$, via the calculations of the $Q_A^-Q_B \rightleftarrows Q_AQ_B^-$ apparent equilibrium constants, K_2. Finally, these two "conformations", which could arise from different interactions between Fe^{2+} (situated between Q_A and Q_B) and the quinone acceptors or between I and Q_A, behave as two independent components. They have their own ΔG, pH dependence, pH dependence of K_2, and slightly different absorption change spectra, which can be separated near the isosbestic point at 833 nm. Ionic conditions, pH, and *o*-phenanthroline have notable effects on the relative proportions of the two phases. However, the meaning of these two populations is still unclear, as is their possible importance in the reaction center's function.

The electromagnetic energy absorbed by the antenna of photosynthetic organisms is transferred to the photochemical reaction centers. This energy is then stabilized as a transmembrane charge separation. A main step for the understanding of this process has been accomplished with the crystallization and X-ray structural analysis of the reaction center proteins from the purple bacteria *Rhodopseudomonas viridis* (Deisenhofer et al., 1985; Michel & Deisenhofer, 1988; Michel et al., 1986) and *Rhodobacter sphaeroides* (Allen et al., 1988; Chang et al., 1986; Ducruix & Reiss-Husson, 1987; Komiya et al., 1988; Yeates et al., 1988). The reaction center consists of three polypeptides, L, M, and H, with molecular weights between 30 000 and 35 000. In *Rps. viridis*, a tightly bound cytochrome (40 kDa) containing four *c*-type hemes

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