^{31}P NMR Analysis of Membrane Phospholipid Organization in Viable, Reversibly Electropermeabilized Chinese Hamster Ovary Cells†

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ABSTRACT: Chinese hamster ovary (CHO) cells were reversibly permeabilized by submitting them to short, high-intensity, square wave pulses (1.8 kV/cm, 100 μs). The cells remained in a permeable state without loss of viability for several hours at 4 °C. A new anisotropic peak with respect to control cells was observed on ^{31}P NMR spectroscopic analysis of the phospholipid components. This peak is only present when the cells are permeable, and normal anisotropy is recovered after resealing. Taking into account the fusogenicity of electropermeabilized cells, comparative studies were performed on 5% poly(ethylene glycol) treated cells. The ^{31}P NMR spectra of the phospholipids displayed the same anisotropic peak as in the case of the electropermeabilized cells. In the two cases, this anisotropic peak was located downfield from the main peak associated to the phospholipids when organized in bilayers. The localization of this anisotropic peak is very different from the one of a hexagonal phase. We proposed a reorganization of the polar head group region leading to a weakening of the hydration layer to account for these observations. This was also thought to explain the electric field induced fusogenicity of these cells.

The transfer of exogenous material into cells remains a puzzling problem in cell biology and is often a limiting step in many biotechnological processes. In the last few years, a new technique has been proposed for triggering this transfer, "electropermeabilization" (Zimmermann, 1982; Neumann, 1984; Berg, 1985). The membranes of cells (eukaryotes as well as prokaryotes) become permeable to large molecules if the cell suspension is submitted to a short-duration, high-intensity electric field. Plasmids can also be introduced inside the cytoplasm by this technique (Zerbib et al., 1985). This "permeabilization" is only transient and disappears slowly after application of the pulses. This reversibility is a major advantage since the viability of the cells is not affected by the treatment. Furthermore, adjacent cell membranes tend to fuse if the cells are in close contact (Blangero & Teissie, 1983), and high yields of viable hybrid cells can be obtained by this technique (Finaz et al., 1984).

The molecular processes involved in electropermeabilization are still not well understood. The external electric field alters the membrane potential (Bernhardt & Pauly, 1973), and above a given threshold it triggers a change in membrane structure. By considering a biological membrane to be an homogeneous solid (Crowley, 1973) or a viscoelastic fluid (Dimitrov, 1984), the restructuration has been explained by membrane breakdown induced by electrocompression, although no explanation for the reversibility of the process has been advanced. A progressive induction of structural defects in the lipoprotein matrix may be more realistic, and when a critical size of defect is reached, a permeant state is attained (Abidor et al., 1979). These defects would effectively represent a series of transmembranous aqueous pores (Weaver et al., 1984) which alter the dielectric properties of the membrane (Kinosita & Tsong, 1979). In erythrocyte membranes (Kinosita & Tsong, 1979), these electric field induced defects were shown to be long-lived, although this was not observed in pure phospholipid vesicles (Teissie & Tsong, 1981). This "permeabilization" was thought to be associated with the rotation of "building blocks" (Sugar & Neumann, 1984). Phospholipids appeared as a specific target for restructuration in agreement with our experimental observation that unilamellar phospholipid vesicles can be transiently "electropermeated" (Teissie & Tsong, 1981). It should be emphasized that all these explanations are based on thermodynamic considerations and are essentially theoretical. No direct experimental observation of the events affecting the membrane at the molecular level during reversible permeabilization has been reported. Furthermore, it has been shown recently that electropermeabilized erythrocyte ghosts (Sowers, 1986) and CHO[1] cells (Teissie & Rols, 1986) are fusogenic, although the mechanism is unclear.

Techniques such as freeze-fracture electron microscopy (Verkleij, 1984) or small-angle X-ray diffraction (Marsh & Seddon, 1982) can provide information on the structural arrangement of lipids, but they only provide a static view of the membrane after harsh treatments. In a recent study by quick-freezing electron microscopy, the precise ultrastructural events affecting Pronase-treated erythrocytes during electrofusion have been observed (Stenger & Hui, 1986). But as pointed out by the authors, the observed morphological alterations were probably not those responsible for the long-lasting permeation to solutes.

A dynamic picture can be provided by fluorescence and ESR spectroscopy but only by use of an external probe. This technique is prone to artifacts from structural alterations induced by the exogenous indicator.

^{31}P NMR spectroscopy is a powerful tool for the study of cellular phosphorus-containing molecules. Metabolites, such as ATP or ADP or phospholipids in very small membrane

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[1] Abbreviations: CHO, chinese hamster ovary; NMR, nuclear magnetic resonance; PEG, poly(ethylene glycol) (M_r 1450); ESR, electron spin resonance; FID, field induction decay; SUV, small unilamellar vesicules; DANTE, delays alternating with nutations for tailored excitation; LIP, lipidic intramembranous particles; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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vesicles, tumble very fast in the cytoplasm and as such give a narrow resonance peak. Phospholipids present in membranes of larger systems such as the plasma membrane give broad resonance signals because they experience movements which are slow on the NMR time scale. The two responses (sharp peaks and broad signals) are present superimposed in the NMR spectra of living cells (Sabelnikov et al., 1985; Thoma et al., 1985; De Kruijff et al., 1980). But it is possible to manipulate the free induction decay and selectively reduce the contribution of broad resonance by use of low-power selective irradiation prior to the application of the nonselective hard pulse which triggers the field induced decay (FID) (De Kruijff et al., 1980). When the ensemble is irradiated at a given frequency, only those phospholipids which have an orientation with respect to the field such that their chemical shift coincides with this frequency are saturated. But due to their movement by lateral diffusion, the orientation of phospholipids will change with time. If the duration of the saturating pulse is chosen long enough when compared to the reorientation correlation time of the phospholipids, then all the lipid phosphorus nuclei will experience the irradiation and will be saturated out. This is not the case with fast-tumbling phosphorus nuclei, where the reorientation is fast on the NMR time scale. Their narrow peaks will remain unaffected if the selectivity of the saturating pulse train is good. Therefore by saturation of the contribution of the slow-moving components, the subtraction of the FID obtained in the presence of the saturating irradiation from the one without it should give only the broad resonance which is associated to the membrane phospholipids.

The theoretical work of Thayer and Kohler (1981) established a link between the conformation of the phospholipid molecules and the observed NMR spectrum. A nonbilayer phospholipid organization (hexagonal phase H_{II} for example) or a change in the orientation of the polar groups (out of equilibrium configuration) of the phospholipids in a bilayer would produce a nonclassical anisotropic NMR spectrum. A change in the broad anisotropic NMR signal would directly reflect a change in the organization of the phospholipid membrane. ^{31}P NMR therefore appears to be suited to an investigation of phospholipid configuration alterations in electropermeabilized cell membranes.

In the present study, ^{31}P NMR spectroscopy was used to study alterations in membranes of CHO cells, electropermeabilized under mild conditions. The conditions were chosen such that cell viability was unaffected over the long periods required for the NMR studies.

Materials and Methods

Materials

Chinese hamster ovary cells (CHO cells) were grown in suspension at 37 °C under gentle agitation (100 rpm) in Eagle's minimum essential medium (MEM 0111, Eurobio, France) supplemented with 8% newborn calf serum (Boehringer), penicillin (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (1.16 mg/mL). Poly(ethylene glycol) (PEG 1450) was obtained from Sigma (P 5402) and used without purification. All chemicals were of analytical grade. Egg lecithin was purified as described by Kates (1972). NMR tubes were obtained from Wilmade Inc. (Buena, NJ).

Sample Preparation

Sample Preparation for Permeability and Viability Tests. Cells in monolayer were obtained by plating them on Petri dishes (35 mm in diameter, Nunc, Denmark) and were kept at 37 °C in a 5% CO_2 atmosphere. The cell density was 200 cells/mm^2, and the volume of the culture medium was 2 mL. Cells in suspension were centrifuged for 5 min at 350*g* (1000 rpm, Jouan C 500 centrifuge, France) at room temperature and resuspended in a pulsation medium (250 mM sucrose, 10 mM Tris-HCl, pH 7.6) (Blangero & Teissie, 1983). Small unilamellar vesicules (SUV) were prepared by sonification and centrifugation (De Kruijff et al., 1980).

Sample Preparation for NMR. A 400-mL aliquot of a suspension at 8.5×10^5 cells/mL was centrifuged at 350*g* for 10 min at 4 °C, resuspended in 500 μL of a 10 mM Tris-HCl, 250 mM sucrose, and 20% D_2O, pH 7.7, buffer in a 10-mm NMR tube, and kept on ice. PEG (5% w/v) was added to the medium when required. Electropulsation was carried out on this very dense cell suspension (1.8 kV/cm, 100 μs, 5 times, delay 1 s). These sample preparations were directly utilizable for ^{31}P NMR spectroscopy.

Methods

Viability was measured by observing the growth of the plated cells over 48 h under an inverted phase-contrast light microscope.

Penetration of Trypan blue (4 mg/mL in the pulsing medium) was used to monitor permeabilization. One milliliter of the dye solution was added to the dish, and after 5 min the cells were washed with pulsing buffer. Microscopic examination could easily distinguish the permeated cells by the blue staining of the cellular components.

Electropulsation was performed on a CNRS cell electropulsator. High-voltage pulses generated by the pulse generator were applied to two parallel thin stainless steel electrodes (12 mm long, 2.5 mm apart). The electrodes were sealed into wells (Limbro) which contained 250 μL of the cell suspension. The chamber was placed on an ice bath. The pulse generator and chamber were similar to those described elsewhere (Zerbib et al., 1985). Five consecutive pulses of 100 μs were applied at a constant intensity of 1.8 kV/cm. Cells were maintained on ice. For the viability test, 1 mL of a cell suspension in the pulsing buffer at 4×10^6 cells/mL was treated by pooling four consecutive samples. At intervals, an aliquot of 50 μl was put into the culture in a Petri dish with 2 mL of culture medium and incubated at 37 °C in a 5% CO_2/95% air atmosphere. Control and PEG-treated cells were incubated under identical conditions.

Plated cells were used for monitoring the permeability as described below (Blangero & Teissie, 1983; Zerbib et al., 1985). Just before application of the pulse, the culture medium was replaced by 1 mL of the pulsing buffer. Five short-duration pulses (100 μs) at constant intensity (1 kV/cm) were applied to the two electrodes (10 mm long, 10 mm apart). They were dipped in the buffer and seated on the bottom of the culture dish which was placed on ice. After application of pulses, the Petri dishes were incubated at 4 °C. The cells were tested for permeability to Trypan blue at various incubation times.

NMR Measurements. ^{31}P NMR measurements were performed on a BRUKER WM 250 spectrometer equipped with an RTC pulse programmer at 101 MHz with a multinucleus 10-mm probe using 40-μs (90°) pulses, an interpulse delay of 2.2 s, and a sweep width of 10 kHz. Bilevel, high-power broad-band ^1H decoupling (0.3 W between pulses and 4 W during data acquisition) was employed to remove ^1H–^{31}P dipolar coupling.

Each spectrum represented an accumulation of 3840 transients (600 in the case of SUV). Before Fourier transformation, an exponential multiplication corresponding to 30-Hz line broadening was applied to the free induction decay in order

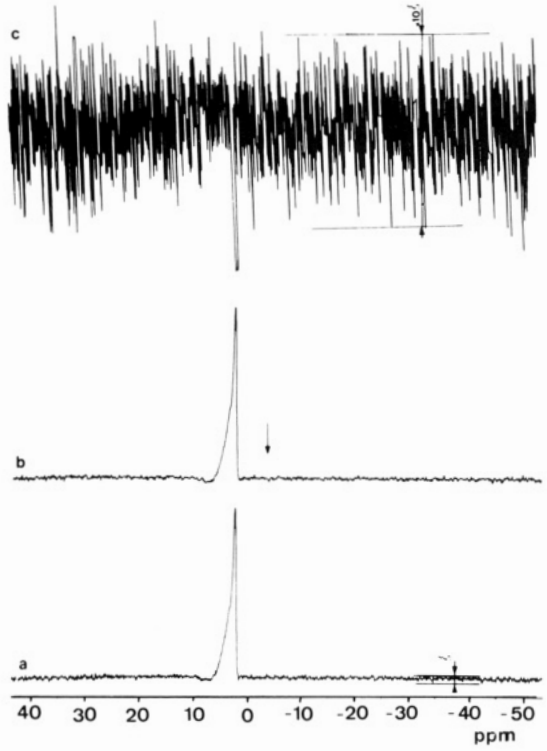

FIGURE 1: ^{31}P NMR spectra of egg lecithin SUV. Experiments were run at 25 °C. (a) Normal spectrum. (b) Spectrum with low power selective presaturation. (The spectrometer settings were the same as those used with CHO cells.) (c) Difference spectra with a 10×-enhanced scale.

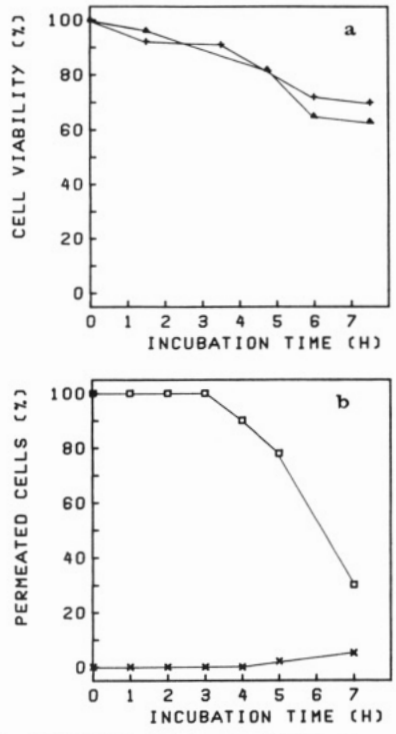

FIGURE 2: Postfield CHO cell behavior. CHO cells were pulsed and incubated at 4 °C. Experimental conditions are detailed under Materials and Methods. (a) Viability of the cells. This was checked from the growth over 48 h after the indicated incubation time [(+) control cells; (Δ) pulsed cells]. (b) Time course of membrane resealing. Trypan blue test was used to indicate the permeated state of the membrane [(□) control cells; (×) pulsed cells].

to increase the signal to noise ratio.

Sample temperature was regulated to 4 °C (±0.1 deg) by a stream of thermostated nitrogen.

Selective saturation of ^{31}P resonances was achieved with a DANTE pulse sequence (Morris & Freeman, 1978; De Kruijff et al., 1980). The parameters used for the DANTE saturation sequence were chosen to ensure Morris and Freeman conditions. The duration of the saturation was empirically selected to be long relative to molecular reorientation (via tumbling or lateral diffusion) but short with respect to T_1 of the saturated resonance.

Practically, the pulse sequence was [D0–(P1–D1)m–D2–PW–acquisition]Ns, where D0 is the relaxation delay (2.2 s), P1 is the saturation pulse (1 μs), D1 is the delay between two saturation pulses (100 μs), m is the number of saturation pulses (3000), $D2$ = 5 μs, PW is the 90° radio frequency pulse (40 μs), and Ns is the number of pulses for one spectrum. The arrow in all figures indicates the carrier frequency at which the saturation was applied.

In order to eliminate possible effects of sample degradation with time, spectra a and b were obtained by an interlaced method; blocks of 16 scans were alternately accumulated in the normal way or together with the saturation pulse; 240 blocks were collected and then summed. (Ns = 3840 = 16 × 240).

Two successive accumulations of 7680 scans were performed, the first over the 6 h following the pulses and the second from the 6th to the 12th hours.

Spectral subtractions were performed with the standard Brucker software.

Chemical shifts in parts per million are relative to external H_3PO_4 at 85% w/v.

As can be seen on the figures, a high selectivity in the rejection of the broad anisotropic signals was obtained with our sequence, and a complete elimination of the non-phospholipid phosphate contribution was present after subtraction. As a control, we ran similar experiments on phospholipid small unilamellar vesicles, and a flat line was obtained after subtraction (Figure 1). This improvement in the quality of the rejection when compared to the results by De Kruijff et al. (1980) was linked to the use of a WM 250 spectrometer. For comparison, this was not possible when we tried to repeat the experiments with a WB 300 spectrometer.

For computer simulations, a computer program, coded by us, was run on an AT-Normerel microcomputer linked to a Hewlett-Packard plotter.

RESULTS

Permeabilization of CHO Cells. CHO cells in suspension are transiently permeabilized when the suspension is submitted to five consecutive pulses with an intensity of 1.6 kV/cm and a duration of 100 μs. The field pulse shape was a square wave and was monitored on an oscilloscope incorporated in the cell pulsator. Permeabilization was monitored by penetration of Trypan blue. If kept at a low temperature, the cell membrane remains permeable (Figure 2a) for about 6 h. The viability of the cells was unaffected by the electric pulse treatment (Figure 2b). This apparent stability of the permeabilized state was a considerable advantage for the NMR studies since it was possible to average the sweeps over 6 h in order to improve the signal to noise ratio. CHO cell fusion was not observed under these experimental conditions.

^{31}P NMR Analysis of CHO Cells. As described under Methods, ^{31}P NMR spectra were obtained with a dense CHO cell suspension. The results are shown in Figure 3. All phosphorus-containing molecules contribute to the direct spectra. Phosphorylated metabolites gave sharp isotropic signals of different magnitudes in the first and second acquisition sequences (cf. spectra b and c of Figure 3). The peaks

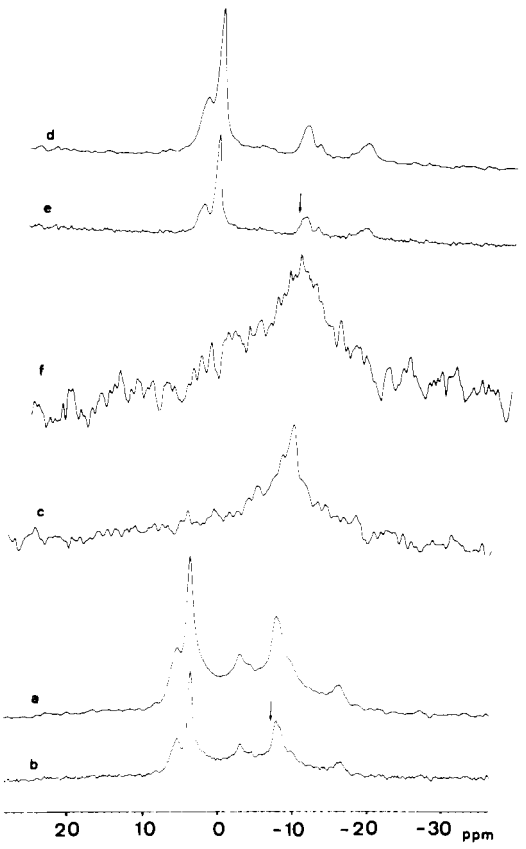

FIGURE 3: ^{31}P NMR spectra of living CHO cells in the absence of electric field (reference spectra). First acquisition period (0–6 h): (a) normal spectrum; (b) spectrum with low power selective presaturation; (d) difference spectrum. Second acquisition period (6–12 h): (d) normal spectrum; (e) spectrum with low power selective presaturation; (f) difference spectrum.

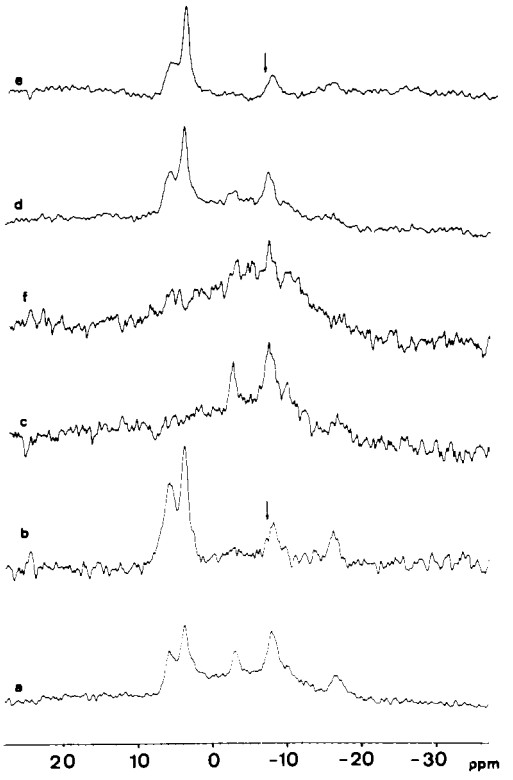

FIGURE 4: ^{31}P NMR spectra of living CHO cells after electropermeabilization. First acquisition period (0–6 h): (a) normal spectrum; (b) spectrum with low power selective presaturation; (c) difference spectrum. Second acquisition period (6–12 h): (d) normal spectrum; (e) spectrum with low power selective presaturation; (f) difference spectrum.

associated with ATP and phosphoesters disappear, and the one associated with inorganic phosphorus increases. In contrast to these sharp signals, a broad anisotropic signal of constant amplitude is associated with phospholipids. As this signal is difficult to observe on the direct spectrum, it was extracted by a saturation-transfer technique (Morris & Freeman, 1978) (see Methods).

The spectrum associated with the phospholipids (Figure 3c,f) corresponds to an axially symmetrical, partially averaged motion on the NMR time scale consistent with a lamellar configuration of the phospholipid molecules (Seelig, 1978). This "signature" was reproducible on the same sample over two consecutive accumulations (cf. spectra c and f of Figure 3). Nonviable cells, which are permanently permeable to Trypan blue, gave the same phospholipid signature as the control sample described above (data not shown). The apparent residual chemical shift is close to 40 ppm in agreement with previous results of natural membranes (Ellena et al., 1986; Thoma et al., 1985). The sharp upfield peak is localized as reported in intact tissues (Thoma et al., 1985). Our observation that the chemical shift anisotropy was nevertheless smaller than in the case of model systems was in agreement with what was observed in microsomal membranes (Van Duijn et al., 1986).

^{31}P NMR Analysis of Electropermeabilized CHO Cells. The sharp peaks associated with small metabolites are present in the spectra of the electrically pulsed cells and look very similar to those of the control samples (Figure 4b,e). However, the broad anisotropic contribution observed with the saturation-transfer technique is quite different (Figure 4c). A new and distinctive anisotropic signal with a maximum around −3.4 ppm is observed in addition to a spectra similar to the one of the unpulsed samples. However, it was only detectable during the first acquisition sequence, which was when the CHO cells were both permeable and viable (Figure 2a,b). During the second acquisition sequence, the NMR signal was identical with that of the control sample (compare Figure 3c,f to Figure 4f). The phospholipid spectral signature of an extended lamellar configuration was likewise observed. In summary, the new anisotropic signal observed just after the pulses (Figure 4c) is transient, and the classical anisotropic signature is observed again 6 h after the pulses. This feature was highly reproducible along repeated experiments with the same localization at −3.4 ppm and with the same signal to noise ratio close to 3 (the signal being taken above the broad shoulder).

^{31}P NMR Analysis of PEG-Treated CHO Cells. PEG is known to increase the fusogenicity of cell membranes as does electric pulsation. The alterations of membrane properties induced by electric pulse treatment were therefore compared to those induced by PEG. CHO cells in suspension were treated with PEG 1450 (5% w/v) for 2 h at 4 °C. Viability was not affected by treatment with low PEG concentrations as shown by both the Trypan blue exclusion test (short-term control) and the growth rate over the 48 h following treatment with PEG (long-term control). During the contact between CHO cells and PEG 1450 (5% w/v), no incorporation of Trypan blue was observed, showing that no permeability was induced by this treatment. No fusion was observed even when the PEG concentration was increased to 10%.

The sharp peaks associated with the small metabolites and the broad anisotropic signal due to the phospholipids (Figure 5) were again observed on the ^{31}P NMR spectra. The isotropic signals were very similar to those observed on the control samples (Figure 5b,e). On the other hand, the phospholipid

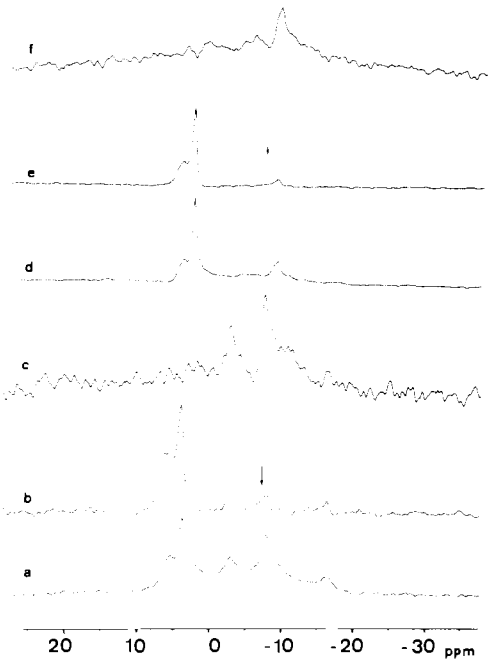

FIGURE 5: ^{31}P NMR spectra of living CHO cells in the absence of electric field but with 5% PEG in the medium. First acquisition period (0–6 h): (a) normal spectrum; (b) spectrum with low power selective presaturation; (c) difference spectrum. Second acquisition period (6–12 h): (d) normal spectrum; (e) spectrum with low power selective presaturation; (f) difference spectrum.

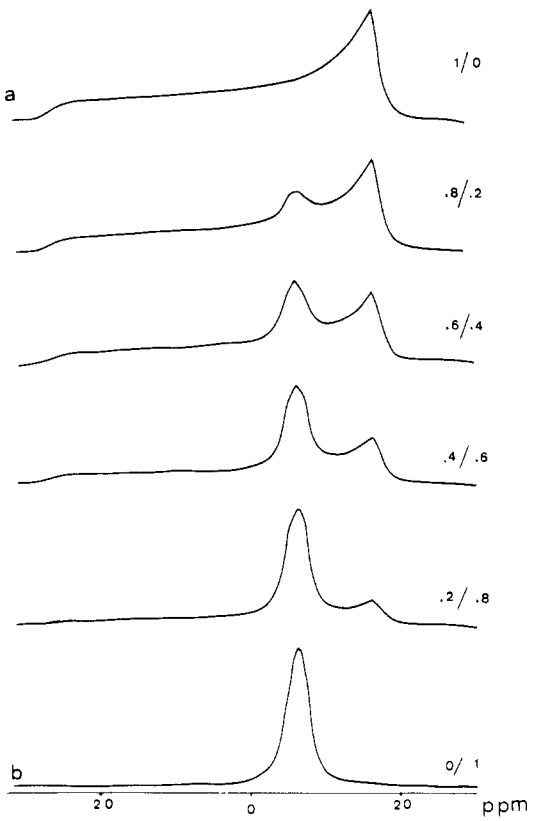

FIGURE 6: Simulation of ^{31}P NMR spectra. The assumption was that two phospholipid polar head conformations were present in the cell. The results described for phosphatidylethanolamine were used for computation (Thayer & Kohler, 1981). The resulting line shapes for different ratios between phospholipids with classical conformation (a) and those with the altered local conformation of the head groups (b) are shown.

signature obtained from the saturation procedure was significantly different from that of the control samples (Figure 5c). As for the electropermeabilized CHO cells, a significant change in the value of the effective chemical shift anisotropy was seen, and a new anisotropic peak was present at −3 ppm in addition to a spectrum similar to what is classically observed with a phospholipid bilayer.

As in the case of electropermeabilized cells, this observation contrasted with the "normal signature", which was observed with the control CHO cells (Figure 3c). In contact with this low PEG concentration, CHO cells retained this new anisotropic signature during the second acquisition sequence (Figure 5f) but with a poorer signal to noise ratio.

DISCUSSION

Electric field induced permeabilization of CHO plasma membranes under viable conditions was shown to be correlated with a modification of the ^{31}P NMR phospholipid spectrum. The new organization of the cell phospholipids, related to a permeabilized state of the cell membrane, is reversible.

From the size of the NMR signal and by comparison with the signal from control samples, we conclude that about 20% (±10%) of the cell phospholipids are in this altered state during the 3 h following the pulse. Comparing this proportion to the percentage of phospholipids in the plasma membrane (about 30% of the cell total) (DeGrella & Simoni, 1982), it would appear that 70% of the phospholipids in the plasma membrane are in an altered configuration. As described in the Appendix, about 70% of the plasma membrane should be permeabilized under our experimental conditions.

The quantitative agreement between this rough calculation and the extent of the transient ^{31}P NMR anisotropic signal suggests that almost all the phospholipids in the permeabilized part of the plasma membrane are in this nonequilibrium structure.

Signatures with inverted anisotropy (vis-à-vis the normal bilayer) are often associated with a tubular H_{II} phase (Killian & De Kruijff, 1985). Although the existence of such polymorphism has been demonstrated in lipid dispersions (Brown et al., 1986), the occurrence of such hexagonal phases in biological membranes over an extended period of time is still speculative even if they can be transiently present as shown by a recent electron microscopy study (Stenger & Hui, 1986). In fact, signatures with inverted anisotropy were observed in the case of *Dictyostelium* but lead the authors to a new interpretation of the phospholipid ^{31}P NMR spectra (Thayer & Kohler, 1981). In our case, the new peak is located at −3 ppm and did not give an inverted anisotropy as observed with hexagonal phases. Theoretical simulations have related the change in ^{31}P NMR anisotropy to a small difference in orientation of a polar head with no concomitant change in the acyl chains (Thayer & Kohler, 1981; Banerjee et al., 1985; Noggle et al., 1982). Spectra associated to the coexistence of two phospholipid polar head conformations can be calculated by the linear combination of the signals described by Thayer for pure phosphatidylethanolamine (Thayer & Kohler, 1981). Different relative proportions between the classical orientation (torsional angle −116.9°) and a slightly distorted one (torsional angle −100.3°) were injected in the computational simulations following the framework described by Thayer (Thayer & Kohler, 1981). Results are shown in Figure 6. A fairly good agreement with our experimental results (Figures 4c and 5c) is obtained when the ratio between the two populations is close to 80/20. This ratio is very similar to what we experimentally observed in the NMR study and computed from the field strength, i.e., the extent of permeabilized plasma membrane.

In our study, a peak with a small change in anisotropy as compared to that of the upfield peak associated with the

classical bilayer organization was observed both with electropermeabilized cells and with 5% PEG treated cells. The mode of action of PEG on cell membranes and model systems is well documented, and at the PEG concentrations used here the following has been shown:

(1) PEG does not affect the dielectric constant of the aqueous buffer (Arnold et al., 1983, 1985).

(2) PEG removes the bound water molecules from lipid head groups (Arnold et al., 1983; Boni et al., 1984a,b); about 5 molecules out of 17 are removed at a PEG concentration of 10% (Arnold et al., 1985).

(3) PEG induces the aggregation of small unilamellar vesicles (SUV) (Tilcok & Fisher, 1982). No major effect has been observed on ^{31}P NMR in model systems: the bilayer configuration is unaffected, and only the rotational mobility of polar head groups is reduced (Tilcock & Fisher, 1982; Boni et al., 1984a,b). These authors have also suggested that PEG induces a change in the relative conformation of the phosphate groups in egg lysolecithin (Boni et al., 1984a).

There is little data on the molecular events affecting cell membranes during PEG-induced fusion. Overall, it seems that low concentrations of PEG have little effect on membrane structure, although slight dehydration and some aggregation may occur. In conclusion, the ^{31}P NMR signal we observed (Figure 5c) probably does not result from hexagonal phases or micellar organization of the phospholipids in the plasma membrane but is rather due to a relative reorientation of the polar head groups and the acyl chains.

This also appeared to be the case for electric field effects on phospholipid bilayers. It has been shown that the conformation of the polar head groups of phospholipids is strongly and reversibly affected by the application of strong electric fields to multilayers, although the hydrocarbon chains remain unaltered (Stulen, 1981).

We observed a similar situation in electropermeabilized CHO cells. There is a dramatic alteration of the ^{31}P NMR pattern with the existence of a downfield-shifted transient peak, but the DPH fluorescence anisotropy was found to be the same in control and permeabilized cells (unpublished results). This spectroscopic probe is known to be located in the hydrophobic core, and its fluorescence anisotropy is an indicator of the structural organization of its environment. It is particularly sensitive to phase transitions (Kinosita et al., 1977). A marked consequence of the electropermeabilization of cells is the induction of a long-lived fusogenic state (Sowers, 1986), which has been shown to occur in CHO cells (Teissie & Rols, 1986). Lipidic particles (LIP) were thought to be involved in fusion processes (Verkleij, 1984). They have been transiently observed by quick-freezing electron microscopy of Pronase-treated electropermeabilized erythrocytes (Stenger & Hui, 1986). Their lifetime is very short (0.1–10 s) when compared to the lifetime of the permeabilized state, and they are not thought to be responsible for long-lived electric field induced permeation. From the above considerations, we assumed that LIP were not responsible for our ^{31}P NMR observations.

This associated fusogenicity must be due to a change in the properties of the cell surface. Okhi (1985) has calculated the thermodynamic requirement for membrane fusion. The attractive electric energy between the two membranes must be greater than the energy needed for their dehydration. This dehydration energy is known to fall when the hydrophilic layers become more hydrophobic (Ohki, 1985). This has been shown to be linked to a change in the orientation of the polar heads. This results both from rupture of the hydrogen bonds linking hydration water molecules to the polar head groups and from greater accessibility of the hydrophobic chains to water (Ohki & Ohshima, 1984). This increase in hydrophobicity has been observed in PEG-treated (25% w/v) or electropermeabilized plant protoplasts (Hahn-Hugesdal et al., 1986).

In conclusion, an alteration in the organization of the polar heads may explain the ^{31}P NMR anisotropy and the consequences of permeabilization. It is known that spontaneous fusion cannot occur as long as the hydration forces prevent direct contact between the membranes (Marra & Israelachvili, 1985; Horn, 1984). These hydration forces are thought to result from the sum of the fields of dipoles associated with the regular matrix of polar heads (Marcelja & Radic, 1976). From our observations, it would seem that after electropermeabilization these hydration forces are reduced or eliminated, possibly due to an alteration in the arrangement of the polar heads. A reorientation of the polar heads would affect the hydration forces and account for the observed anisotropy of the ^{31}P NMR signals (Figure 4c). This has been observed to occur after PEG treatment (Maggio et al., 1984), and we showed that electropermeabilization has an analogous effect.

However, alterations in phospholipid organization alone are not sufficient to explain the metastability of the electropermeabilization. Electropermeabilization of phospholipid vesicles (large unilamellar vesicles) is transient (lifetime less than 15 s) (Teissie & Tsong 1981). Membrane proteins or proteins associated with the membrane (cytoskeleton) are probably also involved in the long-term stability (lifetime of more than 6 h).

ACKNOWLEDGMENTS

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APPENDIX

It is recognized that the electric field primarily affects cell membranes. Assuming that a CHO cell in suspension is a sphere, modulation of the plasma membrane potential would obey the relationship (Bernhardt & Pauly, 1973)

$$\Delta\psi(M) = FrE \cos\theta$$

F = proportionality factor; r = radius of the cell; E = intensity of the field; θ = angle between the direction of the field and the radius joining the center to the point M. As the size of the vesicle strongly affects the magnitude of the induced potential, the plasma membrane would be the primary target for electropermeabilization. We have recently confirmed this relationship in a study of different cells and organelles. CHO cells (12 μm in diameter) are permeabilized when submitted to a field of 0.7 kV/cm, *Escheria coli* cells are permeabilized with a field of 2 kV/cm (Teissie, 1986), but 30 kV/cm is required to permeabilize LUV (large unilamellar vesicles, 0.1 μm in diameter) (Teissie & Tsong, 1981). Cytoplasmic vesicles (mitochondria, lysosomes) would be too small to be permeated under our experimental conditions, and the electrical conductivity of the nuclear envelope is so high that its potential is probably not affected by the external field. Under our conditions, only the plasma membrane is likely to be affected.

The extent of the permeabilized area of the plasma membrane is obtained by the value of θ, which is the half-angle of the cone where the potential is above that of the permeation potential (100% being given when θ is equal to $\pi/2$). Since permeabilization is only observed when the field is over 0.7 kV/cm, this means that θ is 0 at this field intensity. We used a field of 1.8 kV/cm in our experiments, and θ was arccos (0.7/1.8). This calculation indicated that about 70% of the

plasma membrane should be permeabilized under our experimental conditions.

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