Membrane Molecule Reorientation in an Electric Field Recorded by Attenuated Total Reflection Fourier-Transform Infrared Spectroscopy

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ABSTRACT Electric fields play an important role in the physiological function of macromolecules. Much is known about the role that electric fields play in biological systems, but membrane molecule structure and orientation induced by electric fields remain essentially unknown. In this paper, we present a polarized attenuated total reflection (ATR) experiment we designed to study the effect of electric fields on membrane molecule structure and orientation by Fourier-transform infrared (FTIR) spectroscopy. Two germanium crystals used as the internal reflection element for ATR-FTIR experiments were coated with a thin layer of polystyrene as insulator and used as electrodes to apply an electric field on an oriented stack of membranes made of dioleylphosphatidylcholine (DOPC) and melittin. This experimental set up allowed us for the first time to show fully reversible orientational changes in the lipid headgroups specifically induced by the electric potential difference.

INTRODUCTION

Cells are constantly exposed to or surrounded by electric fields that have magnitudes of 100–500 kV cm^{-1} (Tsong and Astumian, 1988) and that are believed to play a major role in biological processes. Proteins and lipids of cell membranes are thus subjected to these electric fields, which, in many instances, regulate their activity (Atwater et al., 1978, 1979; Lechleiter et al., 1991). However, there has been little work done to directly study the effect of electric fields on biomembrane structural changes at a molecular level. This aspect is essential to understand how field-dependent membrane-embedded proteins work during the course of their catalytic cycle.

Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy is a very powerful tool to gain information on the structure of biological molecules (Arrondo et al., 1994; Goormaghtigh et al., 1994a,b, 1999). An advantage of ATR-FTIR to study the structure of biomembranes is that the membrane can be deposited on the surface of the internal reflection element (IRE) as a thin film of highly oriented membranes by evaporation of the water. Using parallel and perpendicular polarized incident light, it is thus possible to detect changes in the orientation of a number of chemical bonds belonging to lipids and proteins (Goormaghtigh et al., 1999). Changes in the secondary structure of proteins (Goormaghtigh et al., 1994a,b) can be evaluated from the amide I band shape.

To analyze the effect of electric fields on biological molecules, several problems arise. The main experimental problem is to apply a transmembrane electric field of the same magnitude as the one existing in physiological conditions. The transmembrane field is typically in the range of −10 to −250 mV over a membrane thickness of 5–7 nm (Tsong and Astumian, 1988). In the case of a typical ATR-FTIR spectroscopy experiment, a stack of ~500 oriented lipid bilayers is built on the IRE. As a consequence, the thickness of the analyzed sample is in the range of 2.5–3.5 μm. It is therefore necessary to apply a potential up to 200 V to mimic physiological conditions.

Another required condition for this experiment is the absence of current flowing through the biomembranes. Indeed, electrical currents could lead to a potential drop and to heating and catalysis of undesirable electrochemical reactions.

In the present work, we used a melittin/dioleylphosphatidylcholine (DOPC) system to study the effect of a transmembrane potential on lipid and protein structure and orientation. DOPC is a common model for unsaturated phosphatidylcholines and melittin is one of the best-studied membrane-interactive peptides (Dempsey, 1990). Melittin is an α-helix peptide that bears an intrinsic dipole moment. In addition to the helix dipole, it has a cluster of four basic amino acids near its carboxyl end, which should make it sensitive to an external electric field. Furthermore, melittin can adopt different orientations in synthetic bilayers depending on their composition (Frey and Tamm, 1991; de Jongh et al., 1994). Lastly, melittin can be organized as tetramers that have voltage-gated channel properties with a specificity for anions over cations (Tosteson and Tosteson, 1981; Hanke et al., 1983; Tosteson et al., 1990).

We present here for the first time an experimental apparatus to analyze the effect of electric fields on biological molecule structure by polarized ATR-FTIR spectroscopy. The ATR-FTIR cell we designed allowed the observation of orientation changes occurring in lipid headgroups, but not in melittin, when an electric field was applied. This phenomenon increases with the strength of the electric field and is fully reversible. The method developed here could be extended to the study of membrane-embedded protein conformational changes.

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MATERIALS AND METHODS

Materials

Melittin, DOPC, and dimyristoylphosphatidylcholine (DMPC) were purchased from Sigma (St. Louis, MO) and used without any further purification.

Preparation of the IRE

The experimental set up is described in Fig. 1 *A*. To apply an electric field on a sample in a way compatible with ATR-FTIR spectroscopy measurements, we took advantage of the electrical properties of the germanium crystal, the IRE used for the measurements. Germanium in its form available for ATR-FTIR has an electrical conductivity of 0.022 S cm^{-1} and was therefore used as an electrode. The 50 × 20 × 2 mm IRE with a 45° incidence angle yielding 25 internal reflection (Fig. 1 *A*1) was obtained from S.A.F.I.R. (Puurs, Belgium). The 20 × 12 × 2 mm counter electrode (Fig. 1 *A*3) was cut from an IRE similar to Fig. 1 *A*1. The IRE were first washed with a basic detergent, then with methanol, and finally with chloroform. Before use, they were placed for 3 min in a plasma cleaner (Harrick, Scientific Corporation, Ossining, NY, 100W PDC-23G), an important step for making the surface clean and hydrophilic. Polystyrene (Fina, Brussels, Belgium, crystal N1280) dissolved in chloroform (0.420 mg/ml) was used to coat the two germanium crystals to obtain a film of 13 μg of polystyrene/cm^2. The germanium crystals were covered with the polystyrene solution and placed in a covered flask to allow a slow evaporation of chloroform. This was found to be necessary to obtain a homogeneous layer of polystyrene with a thickness in the range of 165 nm and to maintain a dust-free environment (Fig. 1 *A*2). Wires were attached to germanium using a conducting glue (Nickel print, GC Electronics, Rockford IL, item 22–207) or simply squeezed between the holder and the IRE.

Preparation of liposomes

DOPC (100 μg) was first dissolved in chloroform, dried under N_2 to form a thin film, and resuspended in H_2O at a concentration of 20 mg/ml. The suspension was freeze-thawed fivefold. Then, 10 μg of melittin was mixed with the liposomes and incubated 10 min at room temperature. In these conditions, all the melittin is associated with the vesicles.

Multilayer preparation

An oriented stack of membrane multilayers was obtained by slowly evaporating the water from a DOPC/melittin suspension on a area of 15 × 3 mm^2 on the IRE with extreme care not to scratch the polystyrene layer. The resulting mean thickness of the sample is 2.4 μm (Fig. 1 *A*4). The counter-electrode was then deposited on the sample film. Finally, this assembly cell was sealed in a vertical holder electrically isolated with tape, and the system was tested for short circuits by applying a voltage of 100 V. Neither at this point nor later in the course of the experiment was any current flowing through the cell (<5 nA). The sample was then left for at least 2 h under a constant purge in the spectrophotometer.

IR spectroscopy

ATR-FTIR spectra were recorded on a Bruker Equinox-55 IR spectrophotometer equipped with a liquid-nitrogen-cooled mercury-cadmium-telluride detector, a reflectance accessory, and a polarizer mount assembly under computer control. Backgrounds of the IRE coated with polystyrene were first collected for each polarization. All the spectra were collected versus these backgrounds. Thus, the polystyrene contribution was removed in the final spectra. A total of 256 scans were averaged for each spectrum to improve the signal/noise ratio. Spectra were recorded at a nominal resolution of 4 cm^{-1}. The spectrophotometer was continuously purged with air dried on a FTIR purge gas generator 75–62 Balston (Maidstone, UK) at a flow rate of 8 L/min. Spectra were transferred on a PC computer with the OPUS 2.1 software provided by Bruker.

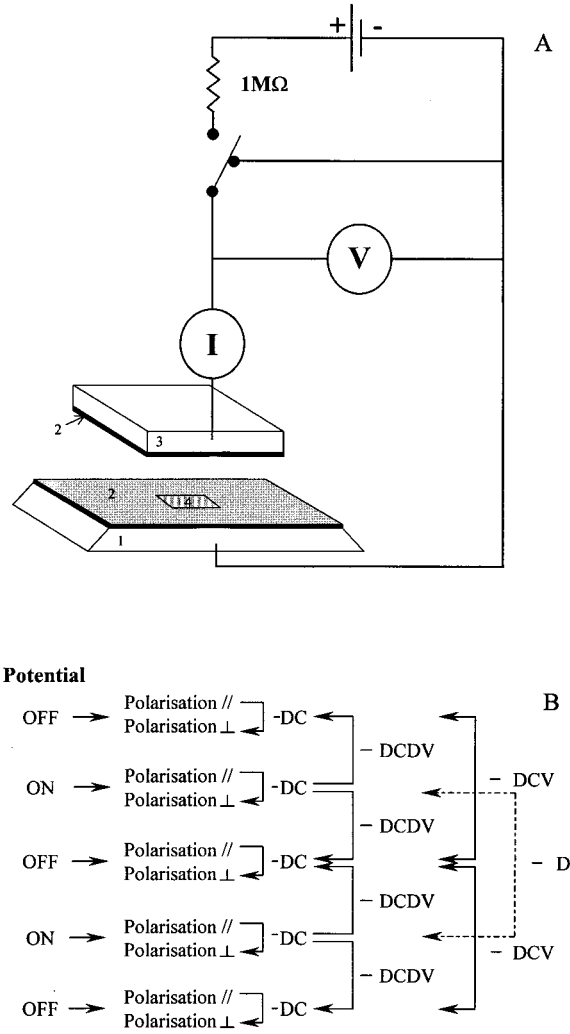

FIGURE 1 (*A*) The experimental assembly. The (20 × 48 × 2 mm) germanium internal reflection element (IRE) (1) was coated with a 165-nm-thick polystyrene layer (2). The oriented multilayers were coated on a small area (15 × 3 mm) (4) of the IRE and the counter electrode, a (20 × 12 × 2 mm) germanium crystal also coated with polystyrene (3), was deposited delicately above. When the switch was in the off position, the two electrodes (1) and (3) were connected to ensure that no potential remained between them. (*B*) Protocol used for data collection and analysis. Spectra were collected with parallel and perpendicular polarized light in the absence and the presence of an electric field. From these spectra, dichroic spectra (DC) were obtained by subtracting spectra collected with perpendicular polarized light from spectra collected with parallel polarized light by canceling the area of the lipid C═O band (1770–1710 cm^{-1}) (Bechinger et al., 1999). DCDV spectra were obtained by subtracting the DC spectra from data collected without potential from the DC spectra recorded with the potential applied. DCV spectra were obtained by subtracting dichroic spectra collected either with or without a potential applied at different time during the course of the experiment.

For each potential investigated, the sequence of the spectra recorded is shown on Fig. 1 *B*. A macro program written for the Opus software from

Bruker handled the recording of the spectra, the selection of the corresponding backgrounds, and the polarizer changes. A standard 8-bit digital analog card was used through the macro program to turn on and off the potential. Because of the high voltages used here, a relay was installed between the digital analog card and the power supply (Bio-Rad Laboratories, Hercules, CA, model 3000 Xi). When the switch was in the off position, the two electrodes (IRE) were connected to ensure that no potential remained between them (Fig. 1 *A*).

Spectra were collected after a waiting time of at least 1 min after switching on or off the potential for either the parallel or the perpendicular polarized light. This cycle (polarized spectra collected without applied potential plus polarized spectra collected with an applied potential) was repeated at least three times, and at last, a set of spectra was collected in the absence of potential. This entire process was repeated using higher and higher potentials until a short circuit occurred. Breakdown of the insulator was always sudden and irreversible.

RESULTS

First attempts to apply an electric field through a stack of lipids bilayers sandwiched between two germanium electrodes failed because a net current flow was observed for the lowest applied voltage. In a first step, we searched through various substances able to electrically insulate the electrodes. To prevent a major potential drop in the insulator, these molecules either should have a high enough dielectric constant or should form thin films. We found polystyrene to be the most suitable compound for this purpose. A 165-nm-thick polystyrene film was formed on the germanium surface as described in Materials and Methods. The IR spectrum of polystyrene is reported in Fig. 2 *A*. Although it is well described that membranes orient themselves parallel to the germanium surface (Goormaghtigh et al., 1999), it was necessary to check their orientation when spread on a polystyrene layer. This was checked by recording IR spectra of melittin embedded in DOPC or DMPC with parallel and perpendicular polarized lights. Melittin is known to adopt different orientations in lipid bilayers depending on the lipids composition and hydration state (Frey and Tamm, 1991; de Jongh et al., 1994). In the present paper, the film was kept in the dry state. Yet we showed previously (de Jongh et al., 1995) that a significant number of water molecules remain bound to the proteins and that this number of bound water molecules is sufficient to maintain the structure and dynamics of the membrane molecules. This matter has been discussed at length in a recent review on ATR (Goormaghtigh et al., 1999). In DOPC, the melittin amide I peak ($\sim$1656 cm^{-1}) has a weak perpendicular polarization, whereas in DMPC, the melittin amide I peak has a marked parallel dichroism indicating a transmembrane orientation of the helix (de Jongh et al., 1994). This was verified here in the presence of the polystyrene layer.

The unpolarized IR spectrum of melittin in DOPC is reported on Fig. 2 *B* between 3200 and 800 cm^{-1} with some assignments (Fringeli and Günthard, 1981). The dichroic (DC) spectrum (Fig. 3 *C*) was obtained by subtracting the spectrum recorded with perpendicular polarized light (Fig. 3 *A*) from the spectrum recorded with parallel polarized light (Fig. 3 *B*) with a subtraction coefficient computed so that the area of the lipid C═O peak (1770–1710 cm^{-1}) in the difference spectrum was zero. This procedure has been discussed at length before (Bechinger et al., 1999; Goormaghtigh et al., 1999). A negative deviation indicates a dipole orientation perpendicular to the IRE normal and a positive one indicates a dipole parallel to the IRE normal. In the case of melittin in DOPC, helix orientation can be estimated using amide I peak dichroism (Marsh et al., 2000). No clear deviation is shown in this region (Fig. 3 *C*, dotted line) in agreement with previous data (de Jongh et al., 1994). The same experiment was carried out using DMPC instead of DOPC. In the latter case, a strong parallel polarization of amide I (data not shown) was observed as previously described (de Jongh et al., 1994). Therefore, because polystyrene was shown to be a good insulator and allowed the parallel orientation of the stack of membranes with respect to the IRE surface, we used it for all the following experiments.

Changes in orientation were investigated as described on Fig. 1 *B*. DC spectra were generated as explained above (Fig. 3 *C*). Then, DC spectra collected in the absence of applied voltage were subtracted from high-voltage DC spectra to produce DC voltage difference (DCDV) spectra (Fig. 3 *D*, solid line). The subtraction coefficient was first established for the sample in the absence of potential and used for all the experiments carried out on the same sample in the presence of a field. To analyze whether the differences observed in the DCDV spectra were due to the electric field and not to an irreversible change occurring in the sample, we also subtracted DC spectra collected at different times during the course of the experiment at identical potentials to produce the DCV spectra (Fig. 3 *D*, dotted line). The orientation changes were strictly limited to the polar headgroups of the lipids. In particular, no change was apparent in the lipid C-H stretching vibrations in the 3000–2800-cm^{-1} region of the spectrum (not shown).

DISCUSSION

The electrical breakdown of cell and of pure lipid membranes (e.g., Teissie and Tsong, 1981; Chernomordik et al., 1987) indicates the sensitivity of the lipid assembly to the presence of an electric field. The electric field creates a compressive stress in the membrane that can lead to changes in the dimensions of membrane structure (Coster, 1975). The voltage-induced thickness change (compression) of planar black lipid membranes on DOPC has been carefully investigated by Bamberg and Benz (1976) through capacity changes. However, the presence of *n*-decane in these experiments may have affected the results. More insight into molecular consequences of the electric field has been brought by Sugar (1979, 1981) by modeling. Although compression of the membrane is present, its amplitude remains small (Sugar, 1981), but the relative area occupied

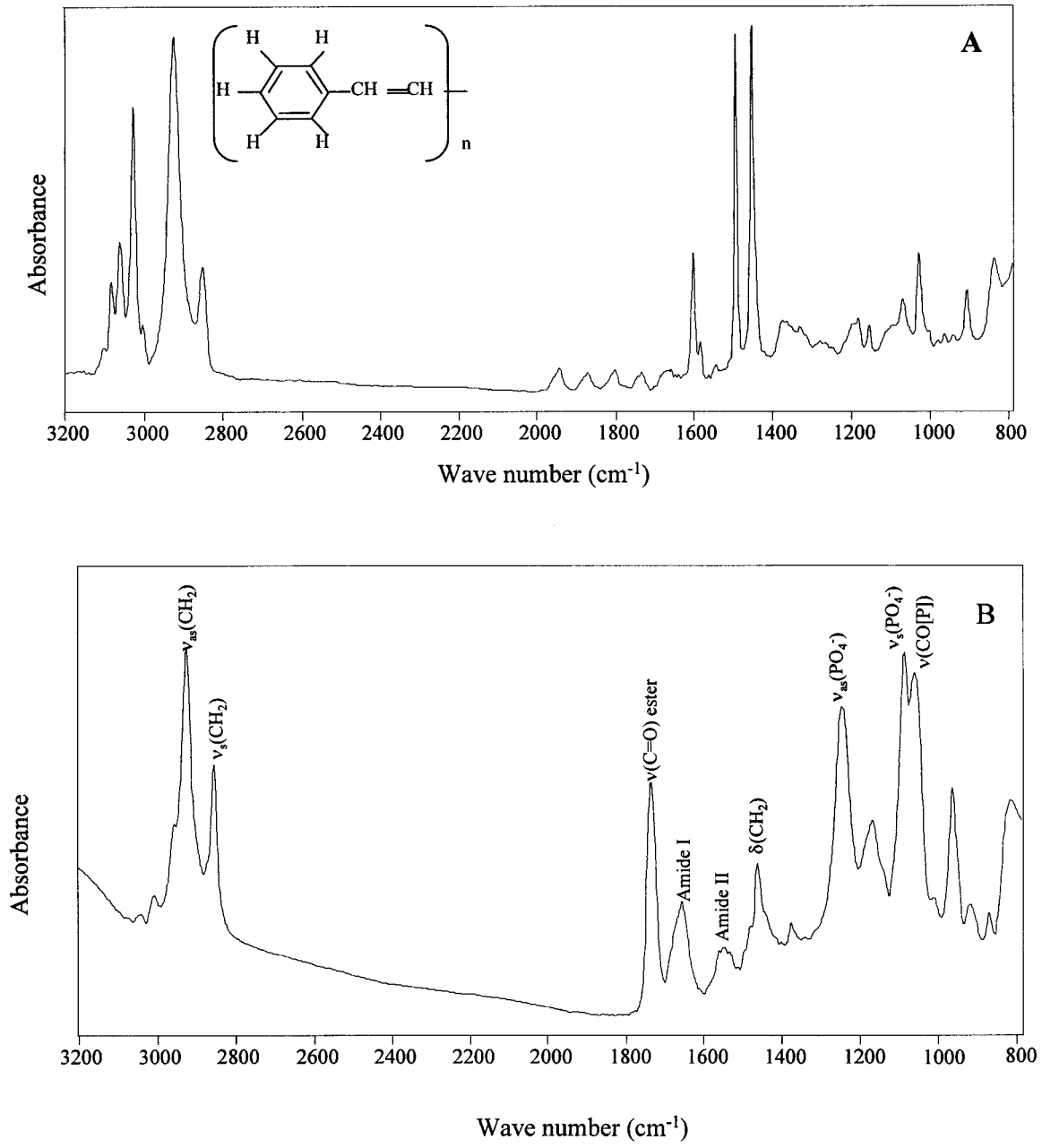

FIGURE 2 IR spectra of polystyrene (*A*) and of melittin:DOPC (*B*). This spectrum was collected versus a background spectrum corresponding to the IRE coated with polystyrene.

per molecule increases. From the details of the analysis, it can be stated that lateral pressure and external voltage act on the membrane conformation in opposite direction (Sugar, 1979). High voltages result in membrane breakdown as experimentally observed. How the different regions of the lipid molecule are influenced by the electric field remains to be determined experimentally.

The advantage of ATR-FTIR is that this technique is able to monitor the reorientation of a number of chemical bonds upon application of a transmembrane potential. As shown on Fig. 3, *C* and *D*, the potential induced changes visible on the DC spectra. They appear even more clearly on the difference between DC spectra recorded at different potentials (DCDV spectra). These changes were specifically due to the potential applied. Indeed, on the DCV spectra, no significant changes could be observed. Interestingly, the changes occurred only in the vibrations of the polar headgroup of DOPC: the phosphate and choline groups. The changes at 1252 cm^{-1} and 1086 cm^{-1} correspond to a modification in the orientation of the antisymmetric and

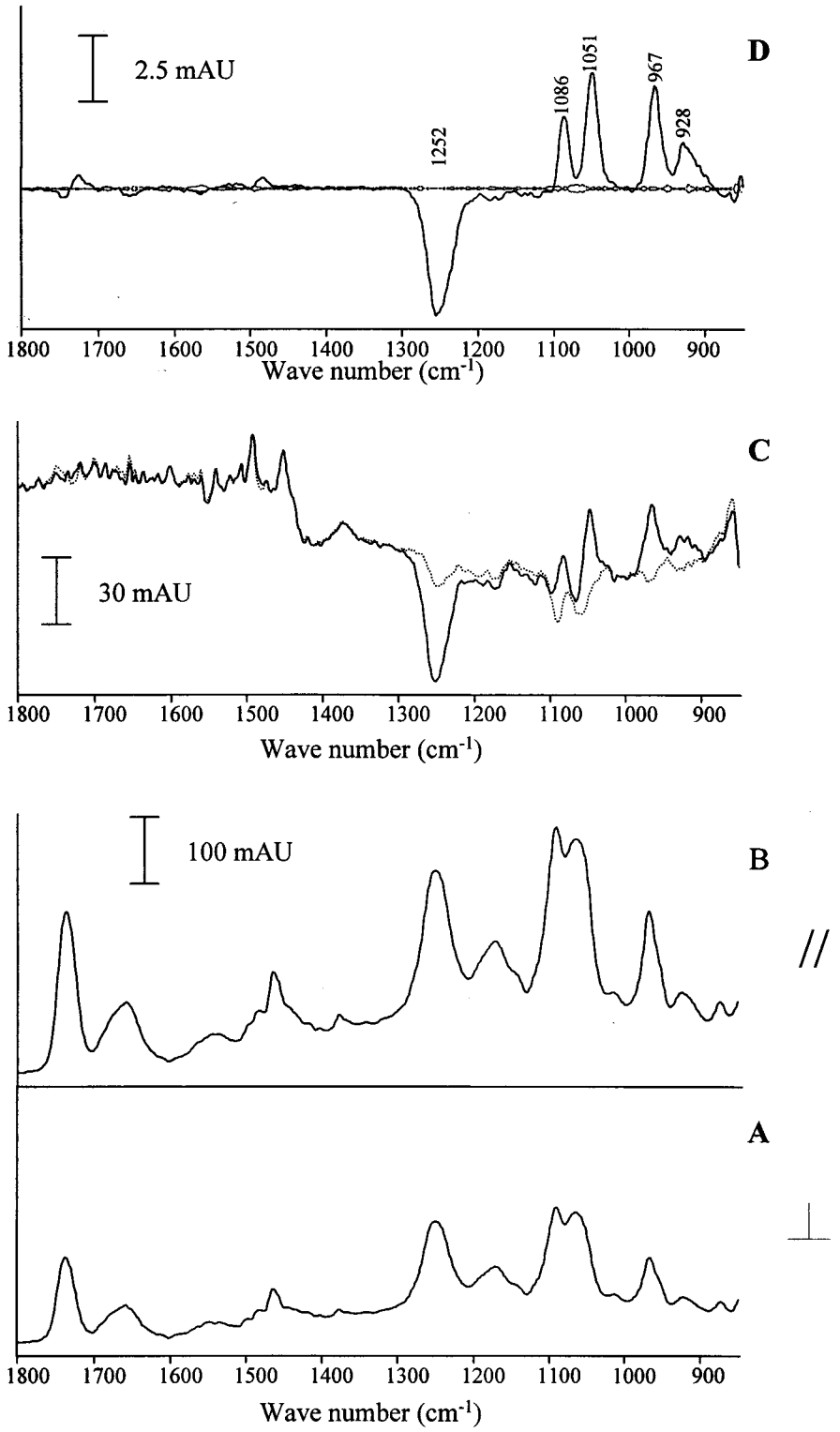

FIGURE 3 IR spectra of melittin:DOPC collected in the absence and in the presence of a potential of 200 V. (*A*) Absorbance spectrum obtained with perpendicular polarized light in the presence of the potential. (*B*) Absorbance spectrum collected with parallel polarized light in the presence of the potential. (*C*) Dichroic spectra (DC) obtained as described in Fig. 1 *B*. - - -, DC spectrum obtained from spectra collected in the absence of potential. ——, DC spectrum obtained from spectra collected in the presence of a potential of 200 V. (*D*) DCDV and DCV spectra obtained as described in Fig. 1 *B*. The spectrum shown by a dark line is the average of 8 DCDV spectra from the experiment carried out with a potential of 200 V. The spectra shown by gray lines are the average of 10 DCV spectra collected either at 0 V or at 200 V. The optical density amplitude of spectra in *C* and *D* has been increased 7.6 times with respect to spectra in *A* and *B*.

symmetric PO_2^- double-bond stretching, respectively. In the absence of potential, the antisymmetric PO_2^- double-bond stretching has no marked dichroism whereas the corresponding symmetric PO_2^- double-bond stretching at 1086 cm^{-1} shows a negative dichroism. When an electric field was applied, the antisymmetric PO_2^- double-bond stretching (1252 cm^{-1}) adopted a defined perpendicular polarization (negative dichroism on Fig. 3, *C* and *D*). On the contrary,

the electric field induced a parallel polarization of the symmetric PO_2^- double-bond stretching (1086 cm^{-1}) (positive dichroism on Fig. 3, *C* and *D*). It is important to realize that the dipoles associated with these bands are respectively parallel and perpendicular to the O-P-O bisector (Fringeli and Günthard, 1981). Reorientation of the phosphate group is therefore expected to have opposite effects on the dichroism of these two bands. Finally, we observed a change at 1051 cm^{-1}. This modification corresponds to a change in the orientation of the transition dipole of the C-O stretching, which also involves the P-O single-bond stretching of the phosphate group (Fringeli and Günthard, 1981). Application of an electric potential difference led to a parallel dichroism of this vibration. We can conclude from these observations that the C-O-P-O-C axis rotates from an orientation parallel to the membrane to an orientation more perpendicular to the membrane plane.

The DCDV peaks at 967 and 928 cm^{-1} correspond to changes in the orientation of the choline part of the polar headgroup. Assuming local C_3 symmetry in the choline part, these two peaks can be assigned to the asymmetric and to the symmetric stretching vibration of the N-$(CH_3)_3$ moiety, respectively (Fringeli and Günthard, 1981). These transition dipole moments appear to be respectively parallel and poorly oriented with respect to the plane of the membrane in the absence of potential. The application of a potential led to a change in the orientation of the transition dipole moment of the N-$(CH_3)_3$ moiety in such a way that these two vibrations adopted a more parallel polarization (positive dichroism) with respect to the IRE surface (Fig. 3, *C* and *D*). The increased dichroism of ν_s(N-CH_3) is consistent with a rotation of the polar headgroup away from the membrane plane. It must be noted here that the dichroism of the 967-cm^{-1} band would be expected to move in the opposite direction, at odds with the observation reported in Fig. 3. This apparent discrepancy might originate from overlaps of ν_{as}(CN), ν_s(CNC) and δ_{as}(N-CH_3) in this spectral region (Fringeli and Günthard, 1981).

All of these effects were specifically observed in the presence of an electric field and fully reversible. The amplitude of the peaks on the DCDV spectra increased as the strength of the electric field applied increased as shown on Fig. 4. A correlation between the strength of the potential applied and the amplitude of the peaks clearly appears from Fig. 4. Plotting the amplitude of the dichroic peaks visible on Fig. 4 versus the potential difference (not shown) displays an almost linear relationship, indicating that the maximal effect was not reached. Therefore, we could expect a bigger effect with a higher potential applied. All the results together suggest that consecutive to the application of the potential, the polar headgroups change their orientation in such a way that the phosphate group and the N-$(CH_3)_3$ moiety will adopt a more perpendicular orientation with respect to the membrane surface and thus move away from the hydrocarbon chains. No change was observed for melittin. This could come either from the inability of this peptide to change its orientation in the presence of an electric field or to a too weak electric field. Interestingly, the results obtained on DMPC were identical to the results obtained on DOPC for both the lack of melittin reorientation and with respect to the PC headgroup reorientation. Yet the starting orientation of the melittin helix is now perpendicular to the bilayer plane.

As indicated on Fig. 4, the amplitude of the absorbance changes for the largest potential applied in the range of a few mOD units whereas the absorbance of the original bands reached ~300 mOD units. Although difference spectroscopy is very sensitive, changes of such a small amplitude result in nonsignificant changes of the dichroic ratio defined as $A^{//}/A^{\perp}$. In turn it is not possible to obtain a reasonable estimate of the orientation change in degrees.

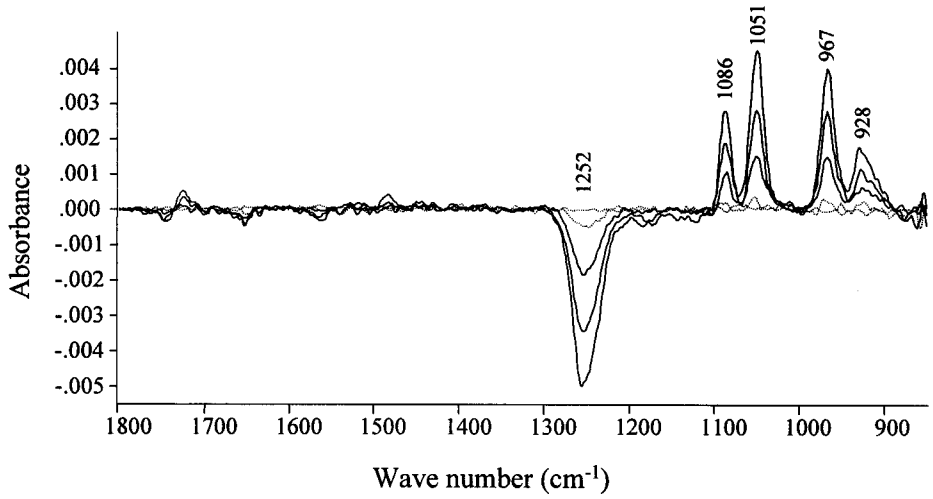

FIGURE 4 Amplitude of the effect is a function of the potential applied. Each spectrum is the average of eight DCDV spectra collected in different conditions of potential: black line, 200 V; dark gray, 150 V; light gray, 100 V; dashed dark, 50 V; and dashed gray, 0 V.

The very weak dichroic ratio change also explains the linearity between the field and the angular orientations.

Data present in the literature are scarce. Bechinger and Seelig (1991) investigated by ^{2}H and ^{31}P NMR the effects of the lipophilic dipolar molecules of phloretin and analogs on the structure of POPC. Incorporation of phloretin induced only a small disordering of the hydrocarbon chains but induced an orientation change of the phosphocholine headgroup. Although the (−)P-N(+) dipole is parallel to the bilayer for pure POPC, addition of phloretin induced a rotation of this dipole toward the membrane normal (Bechinger and Seelig, 1991). This experiment indicates that the polar headgroup of the phosphatidylcholine is the first structural element to be affected by the external dipole. These results are in line with ESR and ^{31}P NMR studies on oriented DMPC multilayers, which concluded that the polar headgroup is greatly affected by the field but without significant effects on the structure and dynamics of the hydrocarbon chains (Stulen, 1981). These results are in good agreement with the ATR-FTIR data presented here.

In conclusion, the changes observed in the polar headgroups of DOPC and DMPC (data not shown) let us believe that the experimental assembly we designed here could be applied for the study of the conformational changes of voltage-regulated membrane-embedded proteins.

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