Proton Lateral Conduction along Lipid Monolayers Is Present Only in the Liquid-Expanded State

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Abstract: Proton lateral conduction along lipid monolayers was observed by fluorescence spectroscopy. Very fast diffusion was detected at the lipid/water interface as compared to the bulk phase. This property was specific to liquid-expanded film organization. When the film was brought to the gel phase, either mechanically for zwitterionic and acidic lipids or by the effect of divalent cations in the case of acidic lipids, no facilitated conduction was present. This was explained by the destruction, associated with the phase transition, of the hydrogen bond network between interfacial water molecules and polar lipid headgroups.

Introduction

Membrane/solution interfaces play a decisive role in the biological functions of cells. Exchange of nutrients, metabolites, and ions occurs at this level. The movement of protons is very important, being involved in membrane transduction energy. Whether this movement is only perpendicular to the plane of the membrane or whether a lateral flow may occur is an open question. A first experimental step in the investigation of this problem was the analysis of such a lateral movement along model lipid membranes.

Lateral proton facilitated movement along lipid monolayers can be observed by fluorescence spectroscopy. This approach allowed us to demonstrate in 1985 that the movement was much faster along the lipid/water interface than in the bulk phase.[1] These results were confirmed in our further studies by using either fluorescence spectroscopy,[2-5] surface pressure,[5,6] or surface potential.[7] This conclusion was supported by the observation in other groups of an enhanced electrical conductance along the interface when a monolayer was present.[8-10] This was in agreement with a much older observation concerning multilayered systems.[11] A fast spectroscopy indirect approach showed a modulation of surface conduction according to the nature of the lipids.[12] It must be pointed out that this conclusion was obtained through the resolution of a complicated set of equations and not by direct observation. All of the direct evidence of facilitated conduction along lipid monolayers gave experimental support to the hypothesis of a "microlocalized" pathway for "chemiosmotic coupling" in energy-transducing membranes as mentioned above.[13] An apparently conflicting observation was recently reported where a decrease in surface electrical conductance was observed upon the compression of a distearoylphosphatidylcholine (DSPC) monolayer.[14] As a different approach was used in this study (conductance) than the one we used in most of our experiments (fluorescence), we decided to try to reproduce their observations and investigate the molecular mechanism that gives such an inhibition. It should be stressed that the fluorescence detection gave very reproducible results as opposed to the conductance approach, in which there were problems.[9,14]

In the present study, we observe that the authors of the conductance experiments missed an essential property of the film in their study, the occurrence of a gel state under compression. In this investigation, we show that facilitated proton lateral conduction is present in the liquid state but is definitively prevented by the transition to the gel state.

Materials and Methods

Chemicals. Phosphatidylethanolamine (PE) from *Escherichia coli*, distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylglycerol (DPPG), and 12-(9-anthroyloxy)stearic acid (12-9-AS) were obtained from Sigma (USA). Synthesis of the pH fluorescent indicator probe, fluorescein phosphatidylethanolaminethiocarbamide (FPE), was described previously.[4] Salts were analytical grade. Ultrapure water free from surfactant was prepared with a Milli-Q system (Millipore, France).

Monolayer Preparation. Buffered saline solutions were prepared with ultrapure water. Lipids were spread from solution in chloroform/methanol (5:1 v/v), and a 5-min period was observed to allow for solvent evaporation. The film surface pressure was monitored by means of a platinum plate (Prolabo, France) connected to a force transducer of our own construction. The sensitivity of the surface pressure determination exceeded 0.2 mN/m. Temperature was 20 °C (±0.5 °C).

Fluorescence Measurements. An interface fluorimeter constructed in the laboratory was used in which the front face fluorescence was monitored.[15,16] The emission from a small illuminated area (about 2 mm in radius) was measured for different compression states of the monolayer. The trough was milled in Plexiglas in order to maintain a low degree of light scattering. Compression was obtained by moving a Teflon barrier automatically in order to change the total surface area of the monolayer. Excitation wavelengths were selected by means of optical fibers. The fluorescence intensity was measured by means of a photomultiplier tube (EMI 9558, England) connected to a data acquisition unit. Fluorescence emission, surface pressure, and film area were simultaneously recorded.

Fluorescence Recovery Measurements. Photobleaching experiments were carried out as follows.[17] The background signal, due to the light scattered by the subphase, was set at zero fluorescence electronically. Then a mixture of probe 12-(9-anthroyloxy)stearic acid and phospholipid (2:98 molar ratio) in a chloroform/methanol mixture (5:1 v/v) was spread at the air/water interface in the dark to avoid photodegradation. After an equilibration period of 5 min to allow for complete solvent evaporation, the film was then compressed to a given pressure and allowed to equilibrate for an additional 5 min. The optical shutter was then opened and the decrease in fluorescence intensity resulting from the dimerization reaction was recorded. After a 30-s bleaching period, the shutter was closed, and recovery was detected by observing the fluorescence signal associated with the irradiation of the monolayer for 1 s at different time lags following the bleaching reactions. The film was then compressed to a new value of the surface pressure, and the bleaching procedure was repeated.

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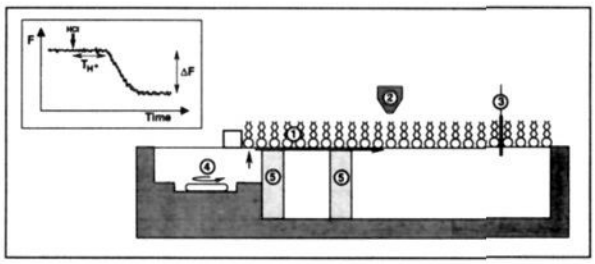

Figure 1. Proton diffusion trough. The monolayer (1) fluorescence and surface pressure are detected in 2 and 3. Proton diffusion is measured by following surface fluorescence change after injection of HCl in the injection compartment (4). The barriers (5) prevent the free diffusion of protons in the bulk phase. The insert shows the kinetics of the fluorescence change following the acid injection.

After photoreaction, the local concentration of monomers in the previously illuminated area was lower than in the nonbleached surface. This concentration gradient then drove the diffusion of fluorescent monomers into the bleached zone. The extent of recovery of fluorescent monomers was a direct function of the lateral diffusion coefficient D of the probe, which was under the control of the order parameter of the lipid matrix.[17] Recovery experiments were analyzed by use of a mathematical approach adapted for uniform disk illumination,[17] after a statistical analysis taking into account the nonlinear relationship between the extent of recovery and the duration of recovery.[18]

Proton Movement Investigation. Proton lateral diffusion experiments were run with the proton "window" jump technique using a trough and an experimental procedure as described in ref 1 and illustrated in Figure 1. Monolayers were obtained by spreading a mixture of phospholipid and FPE (molar ratio 98:2) in a solution of $CHCl_3$/MeOH (5:1 v/v) onto an aqueous subphase (1 mM TES buffer at a well-defined pH). The movement of protons from the injection compartment to the fluorescence observation area was observed by a change in fluorescence emission of the pH-sensitive fluorescent chromophore (FPE at the lipid/water interface). This pH probe was diluted in the host lipid matrix at a low molar ratio (2%) where it is known that its fluorescence is only sensitive to the local concentration of protons.[4] Proton movement was monitored through the dissipation along the monolayer of a localized pH jump in the bulk phase. Proton movement was described by two experimental parameters: T_{H^+}, the time needed for protons to move from the injection compartment to the observation spot, which were 4.2 cm apart (i.e., the velocity) (this was the time between the acid injection and the beginning of the decrease in fluorescence), and ΔF, the relative amplitude of the associated fluorescence decrease (i.e., the flow)[1] (Figure 1). The subphase was chosen to be pH-buffered by 1 mM TES at pH 7.4, and various ionic contents were added. These initial conditions were highly reproducible. The pH jump was obtained by adding 150 μL of 3 N HCl in the injection compartment, inducing a pH lateral gradient of about 3.5 units.[2] The phosphate subphase was not used as in previous studies to prevent the chelation of calcium.

Determination of the Apparent pK (pK_{app}). pK_{app} was taken as the subphase pH at which the fluorescence F emitted by the film for a given surface pressure obeyed the relationship:

$$\frac{F(\mathrm{p}K_{app}, \pi) - F(\mathrm{pH}\ 4, \pi)}{F(\mathrm{pH}\ 7.5, \pi) - F(\mathrm{p}K_{app}, \pi)} = 1$$

the indicated pHs being those of the subphase. This operational definition was based on the observation that the fluorescence was not related in any obvious way to the subphase pH for values of pH outside the range 4.5–7.2. It was obtained by compressing films on subphases with different values of pH and recording the fluorescence intensity and the surface pressure in relation to the molecular area.[4]

Results

Zwitterionic Phospholipids: DSPC and PE Monolayers. When DSPC was used as a host lipid, fast interfacial proton diffusion was present when the film was loosely packed (molecular area between 0.58 and 0.75 nm^2) but disappeared when the surface molecular area was smaller than 0.58 nm^2 (Figure 2). This observation is partly in agreement with the results of Menger and co-workers,[13] except that they did not observe the occurrence of

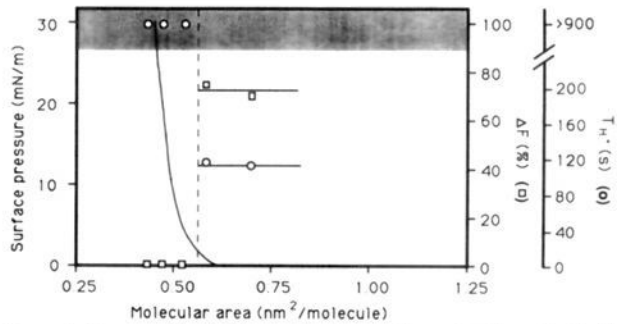

Figure 2. Compression isotherm, time lag, and associated amplitude of the FPE fluorescence change in the case of DSPC. FPE fluorescence is sensitive to the presence of protons and a sharp drop appears when the proton-facilitated conduction along the monolayer is present. The compression isotherm is characteristic of a condensed lipid with a very steep rise of the isotherm. Proton-facilitated movement is detected by the associated fluorescence change only as long as the film is fluid but is hindered when the film pressure is larger than 2 mN/m. The upper part of the graph (dash) is with the compressed scale in time (right ordinate). The vertical dotted line is indicative of the break in conduction and is associated with the induction of a gel state upon compression.

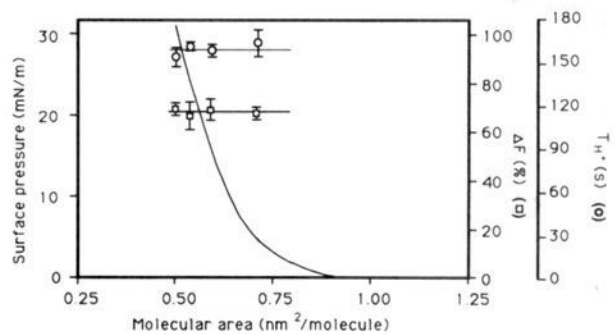

Figure 3. Compression isotherm, time lag, and associated amplitude of the FPE fluorescence change in the case of PE. The compression isotherm does not show any phase transition. The lipid remains in the LE state whatever its compression. Proton conduction is always detected.

the facilitated diffusion in the loosely packed film. In the case of phosphatidylethanolamine (PE) extracted from *E. Coli*, the fast interfacial proton diffusion was observed regardless of the packing of the film (molecular area less than 0.8 nm^2), as we had previously observed on a phosphate-buffered subphase[1] (Figure 3). As we showed that the nature of the polar headgroup did not prevent conduction,[3] this apparent contradiction can be explained by the induction of a gel state. Liquid-condensed (LC) and solid-condensed (SC) states can be induced upon compression in the case of DSPC where the fatty acid chains are saturated.[19] Such phase transition is not possible at room temperature with PE, which is a natural compound with a high degree of unsaturation.[20] This conclusion is supported by our previous study with dipalmitoylphosphatidylcholine (DPPC), where the facilitated conduction was inhibited when the lipid was compressed to the LC state.[3] The conclusion should then be that lipid films at the air/water interface have a special propensity to conduct protons as long as they are in the liquid-expanded (LE) state or even along the LE/LC plateau transition.

Acidic Phospholipids: DPPG Monolayers. We checked the validity of this prediction by inducing the phase transition by several methods in the case of the acidic dipalmitoylphosphatidylglycerol (DPPG). When the subphase ionic content is high (100 mM NaCl), the phase transition can be induced by compression of the film with the occurrence of a transition plateau (Figure 4). When the subphase contains a divalent ion (10 mM

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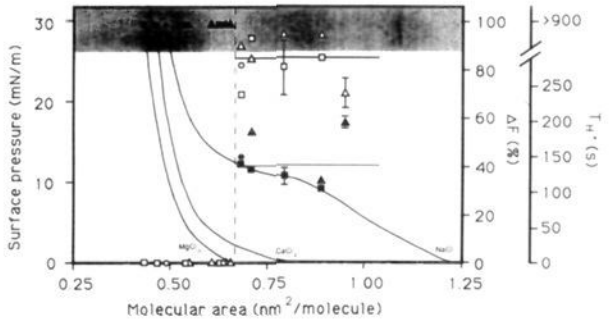

Figure 4. Compression isotherms, time lags, and associated amplitudes of the FPE fluorescence change in the case of DPPG spread on different subphases. The subphases were 100 mM NaCl (△,▲) 10 mM $CaCl_2$ (□,■), and 10 mM $MgCl_2$ (○,●). Open symbols are representative of T_{H^+} and closed symbols of ΔF. The LE/LC plateau transition is present only on the monovalent ion. The conduction is inhibited when the film is brought to the gel state, regardless of the composition of the subphase. The upper part of the graph (dash) is associated with the compressed scale in time. The vertical dotted line is indicative of the induction of the gel state and of the associated break in proton conduction.

Table I. Lateral Diffusion of 12-9-AS in the DPPG Monolayer in Relation to the Film Packing[a]

anthroylstearic acid, in:	molecular area (nm^2/molecule)	diffusion constant ($\times 10^6$ cm^2/s)
DPPG (subphase: 100 mM NaCl)	0.70	26.6
	0.51	0.6
	0.43	0.1
DPPG (subphase: 10 mM $CaCl_2$)	0.95	13
	0.80	24
	0.70	0.1
	0.57	0.1
DPPG (subphase: 10 mM $MgCl_2$)	0.64	3.8
	0.57	2.4
	0.51	0.1

[a] D was obtained by FRAP experiments as described in ref 17.

$MgCl_2$ or 10 mM $CaCl_2$), the gel state appears abruptly upon compression (Figure 4). The condensed organization of the film was observed by the very low value of the lateral diffusion coefficient of the lipids obtained by fluorescence recovery after photobleaching (Table I).[17] The fast proton diffusion was present as long as the monolayer was fluid and disappeared when the gel state was reached by compression of the film. This result is the experimental evidence that facilitated proton interfacial movement is present only as long as the monolayer is in the LE state but is suppressed when the film is brought to the gel state either by ionic effect and/or by compression.

Discussion

The molecular mechanism responsible for facilitated interfacial proton movement was considered to be due to the occurrence of a hydrogen bond network between the polar phospholipid headgroups and the interfacial water molecules.[3,5,21] This was experimentally supported by the detection of a very steep pH gradient along the interface when the conduction was present[7] by the effect of magnetic field[10] and more directly by the behavior of deoxy derivatives of ether-linked phosphatidylglycerol.[22]

Changes affecting the polar head region of the phospholipids during their phase transition do not concern their conformation and orientation.[23] The modifications arise from differences in space requirement, intermolecular interactions, and hydration level. The expansion of the headgroup lattice in the liquid-expanded phase must result in the incorporation of water. This was confirmed by the evaluation of the volume of water per lipid molecule, which drops from 1053 Å^3 for DPPC in the LE state (50 °C) down to 405 Å^3 in the gel state (25 °C) in liposomes.[24] As a consequence of this expansion, direct hydrogen bonds between polar heads, which are prone to build infinite rigid ribbons in the gel state, are weakened in the LE phase, but at the same time more water molecules can approach the phosphate group and be hydrogen bonded to it.[25] The hydrogen bond network between polar heads and hydration water molecules, which is then present in the LE state, is very flexible. Fluctuations must occur because the intermolecular bonds between lipid headgroups cannot be long-lived.[26] In the case of PG, hydrogen bonds between neighbouring lipids face the repulsive character of the electrostatic interactions between the charged groups. They were shown to be present only in the LE phase where we detected the occurrence of facilitated proton conduction in the present work.[27]

Proton dissociation dynamics and the associated exchange between acceptors and donors were performed on lipid vesicles by use of very short laser flashes.[28,29] This methodology takes advantage of the differences in pK values of certain aromatics between their ground and excited electronic levels. Nanosecond-long high-power light flashes give access to the study of very fast transient proton escape out of the donor Coulomb cage and of its capture by the acceptor. When working with small unilamellar vesicles of different lipids, the conclusion was that repeated hopping of protons between nearby lipids would result in high surface mobility.[12] The lipids in a membrane would then be considered as a proton-collecting antenna.[12] This is in agreement with experiments on black lipid membranes where proton transmembrane conduction by ionophores may be explained by such high lateral conduction.[30] We should nevertheless mention that the authors of this contribution rejected such an interpretation arbitrarily. Analysis of transients in proton exchange showed that local electric fields would affect the process, but such an effect would of course dominate only very close to the membrane due to the very short range of the associated field.[31] Diffusion of protons along hydrogen bond networks would be facilitated only as long as the continuity of the network, i.e., of the overlapping of the Coulomb cages, would be present.[31] This conclusion was indeed present in our 1985 observations[1] where we insisted on the necessity of the continuity in the film. These authors pointed out that the hydrogen bond should be flexible to ensure very fast proton diffusion.[31] This was indeed one of the reasons why we proposed 4 years before this description that the conducting network should include interfacial water molecules.[3] Direct hydrogen bonds between polar heads as present in the gel state[1] are not suitable for proton transfer, as we have observed in the present work. We should mention that in more recent studies, the laser flash technique was used to detect proton diffusion between lipid layers in multilayered liposomes and to follow the effect of reducing their thickness by osmotic pressure.[31-33] The conclusion of the authors was that proton exchange rates were slowed down but that a key point was missing from their interpretation. Interbilayer hydrogen bond bridges are built when the bilayers are close to each other.[34,35] As a consequence, the interfacial regular water structure present along the free lipid surface is destroyed by this osmotic compression. We would then consider that their observations do not

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conflict with our results showing that proton transfer is facilitated along the normal lipid/water interface.[3,21,22]

Electrical conductivity in lipid multilayered systems is supposed to be supported by proton transfer.[8-11] It was shown to be dependent on the hydration level of the lipids and to strongly decrease when the lipids are brought to the gel state.[36]

When comparing the conductance results on DSPC monolayers[14] with the fluorescence measurements reported in Figure 2, the drop in conductance takes place in the region of the surface pressure uptake, i.e., where the loss of proton-facilitated conduction is observed by fluorescence. Due to the higher resolution in molecular areas of our data collection with the fluorescence approach, we are able to correlate this loss of conduction with the occurrence of the gel state. From Figure 2, we can conclude that this transition occurs with a change in packing of less than 0.06 nm^2. This observation was missed in the conductance experiments when the phase transition was not mentioned.[14]

All of these observations showing that this conductive network is no longer effective when the film is in the condensed state can be explained by a dehydration of the interface when the lipids are brought to the LC state or by a conformational change of the polar headgroup region. These changes cause a disruption of the hydrogen bond network between the interfacial water and polar headgroups. But this inhibition is specific to the gel state, and as biological membranes are always fluid, we may conclude that lipid domains at their surface are putative conduits for proton movement.

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Registry No. DSPC, 4539-70-2; DPPG, 4537-77-3; H_2O, 7732-18-5; H^+, 12408-02-5.

Carbon Isotope Effect Studies of the Mechanism of the Hofmann Elimination Reaction of Para-Substituted (2-Phenylethyl-*1*-^{14}C)- and (2-Phenylethyl-*2*-^{14}C)trimethylammonium Bromides[1]

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Abstract: Carbon-14 kinetic isotope effects (KIE) were measured for the NaOEt-promoted elimination reaction of para-substituted (2-phenylethyl)trimethylammonium bromides successively labeled at the α- and β-carbons in ethanol at 40 °C. The substantial KIE observed for labeling at both carbons ($k/^{\alpha}k$ = 1.050, 1.044, 1.040, 1.032, 1.019 and $k/^{\beta}k$ = 1.040, 1.040, 1.044, 1.044, 1.042 for *p*-CH_3O, H, *p*-Cl, *p*-CF_3, and *p*-NO_2, respectively) indicates an E2 rather than an E1 or E1cB-irrev mechanism, which has substantial E1cB character in all systems studied. Since both primary β-deuterium and nitrogen-15 leaving group isotope effects have been determined by others employing similar aromatic substituents under these same reaction conditions, this system affords the best test to date of the present theories used to predict structural changes in transition states for elimination reactions and of the successive labeling approach.

"Transition state structures for elimination reactions can be examined by looking for trends in experimental data—a trend being a quantity with a specified direction but an unspecified magnitude. Given enough data on trends, it should be possible to do the sort of curve-fitting or mapping suggested for E2 reactions, in which transition states are located qualitatively on a potential-energy surface by requiring that the predictions for the given transition state structure fit the observed trends." This quotation is taken from Winey and Thornton's paper[2] in which they present a revised formulation of Thornton's original technique[3] for analyzing the effects of substituent changes on transition-state geometry. While this statement applies to many of the methods used to investigate E2 and other elimination reaction mechanisms, measurement of kinetic isotope effects (KIE) provides one of the best methods for studying transition-state structures and reaction-coordinate motions. The importance of the "successive labeling" technique[4,5] for isotope-effect studies lies in the specification of which atoms are undergoing bonding changes in going from the reactants to the transition state. Elimination reactions are especially susceptible to KIE mechanistic studies using the successive labeling approach, since there are so many atoms at which there are bonding changes in the transformation from reactants to products. In order to exploit this KIE method even further, trends in the isotope effects for each atom undergoing bonding changes should be measured as a function of systematic changes in substrate and/or reaction conditions. The present research involves such a KIE study of the NaOEt-promoted Hofmann elimination reactions of successively α- and β-carbon-14 labeled para-substituted (2-phenylethyl)trimethylammonium ions, $ZC_6H_4{}^{\beta}CH_2{}^{\alpha}CH_2N^+Me_3$. We have made measurements in KIE trends as the substituent, Z, at the para position of the aromatic ring in the ions is changed. Such substituent changes have the potential to alter the transition-state structure, the timing of bonding changes, and the detailed nature of the reaction coor-

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