Ion Channel Fluctuations in Pure Lipid Bilayer Membranes: Control by voltage

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dedicated to Robert Graham

Abstract:
Lipid bilayer membranes are shown to represent a dielectric barrier, which due to the thermodynamic fluctuations in membrane thickness, reversibly breaks down forming ion channels of discrete conductivity. The probability of the phenomenon is controlled by the electrostatic membrane potential (voltage) as well as by pH, pCa, surface pressure and temperature. Ion channel probability increases with the isothermal monolayer compressibilities. The thermodynamics theory is used to explain voltage- as well as surface pressure induced ion channels observed in pure synthetic lipid bilayers in the absence of protein, polypeptide, or detergent. Ion channel localisation in the bilayer is demonstrated by variation of bilayer area and boundary conditions. Patch-clamp and Montal-Mueller techniques gave the same results. Many of the discrete steps in membrane current look indistinguishable from protein induced ion channels. It is concluded that ion channels are hydrophilic defects produced as a consequence of the thermodynamic fluctuations in the two-dimensional bimolecular phospholipid lattice.

This manuscript was newly typesetted in 2004 but is otherwise
identical to the original manuscript from 1989.

Contents

Chapter 1

Introduction

It is an established phenomenon that lipid bilayer membranes represent a barrier of very high electrical resistance in most conditions (Danielli and Davson, 1935; Mueller et al., 1962; Montal and Mueller, 1972). Still, the barrier becomes permeable, for instance, if a sufficiently high electrostatic membrane potential is applied. This phenomenon has been explained assuming a deterministic non-fluctuating barrier, which is broken down by electrostriction (Crowley, 1973) or pore formation (Miller, 1981; Zimmermann, 1982). However, opening and closing of ion channels has been assumed to reside in channel inducing polypeptide chains (Urry, 1968; Hladky and Haydon, 1972; Singer and Nicholson, 1972) although it has been also suggested that protein activities can increase the permeability of the lipid component of the membrane (Danielli and Davson, 1935; Robertson, 1958; Kaufmann, 1977).

Ion channel fluctuations, i.e. discrete steps in membrane current with a statistical distribution of life times, have recently been shown to represent an elementary event in pure lipid bilayers at the phase transition temperature (Antonov et al., 1980; Boheim et al., 1980), at the protonation pK (Kaufmann and Silman, 1983), and above a threshold of the applied voltage (Corcia and Babila, 1985). There was also an earlier report of voltage-induced ion channels in oxidized cholesterol (Yafuso et al., 1974). The establishment of the physical mechanism is indispensable for the experimental control of the

phenomenon in vitro and in vivo.

It is demonstrated in this article that the evident thermal motion of the lipid monolayers implies reversible membrane thickness fluctuations which can be controlled specifically and may achieve considerable magnitude under certain conditions. The strength of these fluctuations increases in states of large isothermal compressibility and is generally determined by the thermodynamic variables of the lipid bilayer, such as temperature, surface pressure, electrostatic potential, and the electrochemical potentials of protons, calcium, and other ions. The reversible thermal motion is thus responsible for the reversible opening and closing of aqueous defects in the lipid lattice.

The theory is used to explain ion channel fluctuations observed in pure lipid bilayers. The existence of a threshold for ion channel induction is predicted from lipid monolayer phase diagrams. The threshold voltage is obtained in order of magnitude only, since a quantitative correlation of monolayer and bilayer states is difficult at present.

Contamination artifacts are excluded by using synthetic lipids controlled by amino acid analysis and by solvent-free bilayer preparations. Boundary artifacts are excluded by varying bilayer area at constant boundary.

The theory is free of adjustable parameters. No specific model assumptions are required. Nevertheless, it is capable of crucial experimental predictions which in part have been experimentally investigated:

1. the appearance of ion channels in any lipid bilayer in the absence of any further membrane component, and the reversible opening and closing of such bilayer channels;

2. the statistical nature of the ion channel lifetime and the quantitative correspondence to lipid relaxation times; the discreteness of ion channels and the appearance of both very small and very large conductivities;

3. the control of ion channel probability by all lipid surface variables. The thresholds are also predicted, in correct order of magnitude.

The evident thermodynamic fluctuations therefore provide a physical mechanism which controls ion channel induction in lipid bilayers in vitro and in vivo.

According to the Boltzmann theorem, the probability W of a certain state n_i in any macroscopic system in thermal equilibrium is determined by the entropy function S of the system:

$$W = N \exp\left(\frac{S}{k}\right) \tag{1.1}$$

k is Boltzmann's constant an N is a normalization factor. The entropy is an analytical function of the state of the system. The state is characterized by the extensive thermodynamic variables n_i. Therefore, any allowed state occurs with a finite probability in equilibrium. In other words: the macroscopic state fluctuations among all allowed states. The probability of each state is determined by its entropy value. It is crucial to note that, in thermodynamic equilibrium, any allowed state will by necessity appear.

For sufficiently small fluctuations δn_i, the fluctuation strength can be quantified by expanding the entropy function up to second order according to Taylor:

$$S = S_0 + \sum_i \frac{\partial S}{\partial n_i}\delta n_i + \sum_i \sum_j \frac{1}{2}\frac{\partial^2 S}{\partial n_i \partial n_j}\delta n_i \delta n_j \tag{1.2}$$

In equilibrium the entropy is maximal with respect to all variables,

$$\frac{\partial S}{\partial n_i} = 0 \tag{1.3}$$

and the matrix $\partial^2 S/\partial n_i \partial n_j$ is therefore negative definite.

Quantitatively, the strength of the fluctuations is given by the second moment $\langle \delta n_i \delta n_j \rangle$, $\langle\ \rangle$ being the statistical mean. These moments are directly related to the second order derivative of the entropy, by virtue of eqs.1.1, 1.2 and

1.3:

$$\begin{aligned} \langle \delta n_i \delta n_j \rangle &= \int\int \dots \int dn_1 dn_2 \dots dn_k W \delta n_i \delta n_j \\ &= -k \left(\frac{\partial^2 S}{\partial n_i \partial n_j} \right)^{-1} \\ &\equiv -k \left(\frac{\partial n_i}{\partial X_j} \right)_{X_{l \neq j}} \end{aligned} \tag{1.4}$$

X_j is the independent thermodynamic force $\frac{\partial S}{\partial n_j}$.
For an ideal system with extensive variables energy U, volume V, area A and numbers of particles n_i these forces are, respectively

$$X_j = \frac{1}{T} \quad , \quad \frac{P}{T} \quad , \quad \frac{\Pi}{T} \quad , \quad -\frac{\mu_i}{T} \tag{1.5}$$

T is absolute temperature, P is pressure, Π is surface pressure, μ_i are the electrochemical potentials.

The inverse $\partial n_i / \partial X_j$ of the second order entropy derivative is called the corresponding susceptibility. It is large if the variable n_i of the system responds strongly to a perturbation of the force X_j.

This result does not apply to unstable states, e.g. within a first order phase transition. The integral in eq.1.5 only converges in a stable regime of maximal entropy where the matrix $\partial^2 S / \partial n_i \partial n_j$ is negative definite. This property implies the obvious fact that the mean square is always positive: $\langle (\delta n_i)^2 \rangle > 0$.

The strength of the fluctuations of any independent variable obeys quantitatively

$$\left\langle (\delta n_i)^2 \right\rangle = -k \left(\frac{\partial n_i}{\partial X_i} \right)_{X_{j \neq i}} \tag{1.6}$$

It follows that the strength of the fluctuations around a given state is quantitatively predicted from the corresponding susceptibilities. These can be independently determined from the dependence of the extensive variables on

the forces. This dependence is measured in the phase diagram $n_i = n_i(X_j)$.

Let us now consider in detail the strength $\left\langle (\delta A)^2 \right\rangle$ of the fluctuations in lipid area, A. It is related after eqs.1.5 and 1.6 to the isothermal compressibility:

$$\left\langle (\delta A)^2 \right\rangle = -kT \left(\frac{\partial A}{\partial \Pi} \right)_{T,\mu_i,\ldots} \tag{1.7}$$

The thickness fluctuations can also be obtained since the lipid volume fluctuations are rather weak. The volume can therefore and in contrast to the area and thickness be considered rather constant. The relative thickness fluctuations $\frac{\delta B}{B}$ are approximately equal to the relative area fluctuations, because of the approximately constant volume

$$\frac{\delta B}{B} = -\frac{\delta A}{A} \tag{1.8}$$

The strength of the thickness fluctuations can therefore be calculated as well from the isothermal compressibility. Membrane thickness fluctuations, too, are large when the isothermal compressibility of the lipid monolayer is large.

Phase diagrams of lipid monolayers show a significant increase of compressibility, by more than one order of magnitude, in a "critical range" of the thermodynamic variables. For example, from Träuble (1977) weak relative phospholipid area fluctuations of only ca. 1% are predicted from the observed isothermal compressibility of dimyristoyl methyl phosphatidic acid; compressibility and consequent fluctuations increase, however, dramatically by order of magnitude at an electrochemical proton potential corresponding to the apparent pK. Since this effect occurs for pH changes of 1 or very few units, electrostatic threshold potentials for significant increase in order of fluctuations can be predicted to be of the order of the electrochemical unit, 60 mV at room temperature. In such critical ranges of increased compressibility, the thickness fluctuations likewise increase, and the phenomenon can be induced by adequately varying any of the extensive or intensive variables of the system.

As a consequence, the impermeable barrier represented by the macroscopic

lipid bilayer fluctuates reversibly in any state. If not forbidden by unknown microscopic constraints, the hydrophobic barrier of the flexible hydrocarbon chains must by necessity disappear eventually and locally with a certain, finite probability.

The question arises whether these fluctuations are merely uncontrolled stochastic events and therefore of little use in biological systems, or whether these stochastic fluctuations can be specifically determined.

Most remarkably, the strength of these fluctuations is controlled deterministically after eq.1.7. Above a given electrostatic membrane potential, for example, partial protonation or calcium binding induced by changes of the chemical or the electrical membrane potentials increase the compressibility deterministically, following the phase diagrams discussed already. This is so because the surface charge is affected in such states, altering the Coulombic interactions and the resulting area. In addition, more or less charged surface states are probable and appear as a consequence of the thermal motion in these deterministic, well-controlled states. At the apparent "pK", for instance, such a state is induced. This pK also depends on pCa, surface pressure, temperature, and the electrostatic surface potential. Any variable can by this effect increase the thickness fluctuations deterministically.

In conclusion, the membrane barrier is reversibly broken down spontaneously and in thermal equilibrium, without the presence of any deterministic Born activation energy for ion translocation, once a strongly fluctuating state is reached. The appearance of hydrophilic defects in the two-dimensional bilayer lattice after sufficient time becomes then unavoidable.

The interpretation of ion channel opening and closing by the thermodynamic theory of lipid bilayer fluctuations is therefore free of adjustable parameters. The susceptibilities are independently predicted from adequate phase diagrams. The thermodynamic fluctuations in the lipid bilayer state can also be easily controlled: One simply has to vary any lipid variable in such a way that the corresponding susceptibility increase, if the fluctuations are to be increased, or decrease if a rather non-fluctuating lipid bilayer state is

preferred.

Such defects, even though microscopic, allow for macroscopic trans-membrane currents. This peculiar property of two-dimensional molecular layers raises the possibility, so far unique in physical systems, to directly observe microscopic reversible thermodynamic defect fluctuations.

The thermodynamic theory is macroscopic and does not imply a detailed structural model of the defects. The hydration of the phospholipid surface groups, however, implies that defects produced in the flexible chain lattice by the thickness fluctuations are hydrophilic. No dehydration or hydrophobic barrier has to be involved. The defects per se do not represent a state, or phase, of the bilayer, and appear in any bilayer state with a given probability.

The bilayer lattice remains stable despite of the increased probability of formation of defects. Only if the compressibility is too large, the membrane should break. it can therefore be expected that discrete defect sizes (Kaufmann, 1985b) appear in the bilayer lattice. Large channel probabilities should destabilize the membrane eventually and irreversibly. In any stable state, however, the thermodynamic fluctuations imply by necessity the spontaneous and reversible formation of discrete conductive defects in the lipid bilayer lattice (Kaufmann and Silman, 1983).

The predictions of the theory have been summarized in the Introduction. They will now be tested experimentally. In particular, it will be shown that all lipid bilayers used eventually develop ion channel fluctuations. All thermodynamic lipid variables tested control the strength of the ion channel fluctuations: electrostatic voltage, surface pressure, as well as pH (Kaufmann and Silman, 1983) and temperature (Boheim et al., 1980; Antonov et al., 1980). Specific channel induction by any one of these variables is achieved if the others are kept constant. The reversibility, the statistical lifetime, and the threshold voltage are investigated quantitatively. More detailed studies on ion channel induction by surface pressure and calcium are still missing, however.

Chapter 2

Material And Methods

2.1 Materials

Synthetic diphytanoyl phosphatidylcholine, purchased from Avanti, was previously examined by amino acid analysis (Kaufmann and Silman, 1983). Synthetic 1 palmitoyl 2 oleoyl phosphatidylcholine from Avanti and dimyristoyl methyl phosphatidic acid, a gift from H. Eibl, were used without further control. Egg phosphatidylcholine, cholesterol, and soybean lecithin (Asolectin, type IV-S and type II-S) were from Sigma C. (St. Louis, Mo). Soybean lecithin was processed as described in Kagawa and Racker (1971). Salt and solvents were of analytical grade.

The experiments with micropipettes were performed either in unbuffered 1M NaCl, ca. pH 6.5, or with a solution containing 145 mM NaCl, 1.8 mM $CaCl_2$, buffered by 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid) to pH 7.6. The experiments with Teflon-supported bilayers were performed in an unbuffered solution of either 1M NaCl or 1 M KCl.

2.2 Preparation of solvent-free lipid vesicles

Two mg of lipids (either from a mixture of soybean lecithin, egg phosphatidylcholine and cholesterol in weight ration 8 : 11 : 1, or from pure diphytanoyl phosphatidylcholine) dissolved in chloroform:methanol 2 : 1 were dried under nitrogen, and then under vacuum for at least 12 hours. The dry film was sonicated in a volume of 770 μl of the experimental solution for a few seconds.

2.3 Formation of pipette-supported bilayers

Glass micropipettes were pulled in two steps (Hamill et al., 1981) from borosilicate glass capillary tubing (o.d. 1.5. mm; i.d. 0.86 mm) in a vertical pipette puller, and backfilled with the same solution used to resuspend the lipid film. These micropipettes had tip diameters between 1-1.5 μm and tip resistance from 10 to 50 $M\Omega$ when immersed in the experimental solution. Tip diameters of 5 μm or more were used for the experiments at variable bilayer area.

The method for lipid bilayer formation is a variation of Coronado and Latorre (1983); Hanke (1985). Briefly, a micropipette tip was introduced into the aqueous solution contained in a plastic well. The resistance of the micropipette tip was monitored continuously by applying pulses of 100 μV. Then, the solution containing the lipid vesicles was added. After up to 20 minutes, a lipid monolayer had spontaneously formed at the air-water interface. At this stage, the pipette was slowly pulled out of the solution, and reintroduced, using a three-dimensional hydraulic micromanipulator. Formation of a bilayer was monitored by measuring the increase in the micropipette resistance using an EPC-5 patch-clamp system (List Electronics, FRG).

Once a bilayer was formed, it was clamped at different trans-membrane potentials. The membrane current was monitored with an oscilloscope and recorded on FM tape.

2.4 Teflon supported bilayers

The formation of circular bilayers followed Montal and Mueller (1972) modified as described earlier (Hanke, 1985; Kaufmann and Silman, 1983). The formation of the bilayer and the bilayer area were monitored by both the resistance and the electrical capacitance, to values up to 500 $G\Omega$ and 0.8 $\mu F/cm^2$, respectively. The annulus in the Teflon partition was pretreated with 1:9 hexadecane:hexane. The lipid (either soybean phosphatidylcholine or diphytanoyl phosphatidylcholine) was dissolved in distilled n-hexane (5 mg/ml), before spreading the monolayers. Bilayers were broken and reconstituted several times to confirm reproducibility, and only bilayers stable for one or several hours were used for this study.

2.5 Variation of boundary and bilayer area

1. At constant glass pipette boundary of ca. $5\mu m$, the bilayer area of a given membrane was varied by suction (Guharay and Sachs, 1984). The area increase was followed on a video monitor and by measurement of the electrical capacitance of the system. Small steps in suction pressure were applied reversibly. Subsequently, increased steps were used until, at too high levels, the bilayer broke.

By this method, the surface pressure was altered together with the area.

2. An attempt was made to also vary the bilayer area at constant monolayer surface pressure. The method of "slit" apertures was used, instead of circular holes, in the Teflon window. The slit was introduced vertically with respect to the individual monolayers. The two monolayers on either side of the Teflon were raised to a variable level of the slit. Thus, bilayers of variable capacitance were formed at the lower part of the slit which was introduced into the aqueous medium.

Bilayer formation and bilayer area were controlled by measurement of the electrical capacitance and resistance. The slits were ca. $100\mu m$ wide and

several mm long. No Teflon boundary at all is present in this method at the site where the two monolayers fuse.

3. The bilayer area was also varied using a method already described previously by Miyamoto and Thompson (1967). Circular Teflon apertures of diameters between 50 and 200μm were produced as in Kaufmann and Silman (1983). Membranes of various lipids were compared at either diameter. Resting conductivity, capacitance, and electrochemical threshold for channel induction were determined.

Chapter 3

Results

Heterogeneous lipid membranes were investigated using a mixture of soybean lecithin, phosphatidylcholine, and cholesterol as described in Methods. Bilayers were formed at the tip of glass micropipettes. The background resistance was measured at the beginning and during the experiments. Resistances ranging from 1 to $50G\Omega$ or more were observed. Once the membrane was formed, the transmembrane potential was clamped at increasing values, starting at zero mV. Each potential was applied for 1 to 2 minutes. If the current trace looked stable, the potential was increased to a higher value, up to $200mV$. It is worth noting that no detergent was present in these experiments.

Above a certain threshold voltage, membranes of initial resistance below $20G\Omega$ presented random and transient changes of conductance. These stepwise conductance changes appear very similar to conventional channel activity. Fig. 3.1 shows current traces obtained from two experiments of this kind. The initial resistance of the membrane in Fig. 3.1A was $1.5G\Omega$. Reversible ion channel opening and closing can already be seen at 12 mV. The membrane in Fig.3.1B, with a resistance of 18 $G\Omega$, showed ion channel fluctuations at $200mV$. In both cases, the steps in conductance, plotted downwards, are quick, reversible, and of variable open times. Isolated single channels as well as bursts of channels can be observed. The voltage-induction of conductance

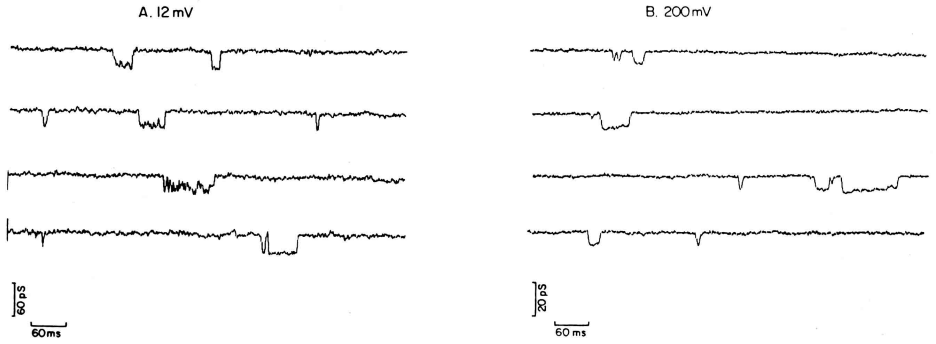

Figure 3.1: *Ion channels in heterogeneous lipid bilayers made of soybean lipids, egg phosphatidylcholine, and cholesterol. Bilayers formed at the tip of micropipettes. Membrane diameter ca. 1 μm. Symmetrical solutions containing 140 mM NaCl, 1.0 mM $CaCl_2$, 10mM Hepes, pH 7.6. A. Membrane of 1.5 GΩ initial resistance and -12 mV (negative inside the pipette) threshold potential. B. Membrane of 10 GΩ initial resistance and 200mV threshold. Opening and closing of ion channels demonstrates the reversible thermal motion in the lipid matrix.*

changes is completely reversible, too. The changes disappear if the transmembrane potential is clamped again below the threshold voltage required for induction.

Fig.3.2 shows the histograms of conductances and open times, derived from data obtained in the experiment of Fig.3.1A. During a period of 24 seconds of this particular experiment, 125 single channel events were detected. The majority of the conductance events appears in the histogram around $25-30\ pS$. Smaller and larger channel amplitudes can also be seen. Channels up to 180 pS occurred in this experiment, but in other situations, conductance values as low as 1 pS and as high as hundreds of pS could also be observed, although much less frequently. As can be seen from the histogram of channel open times, about 80 % of these 125 channels remained open for less than $10ms$. In this particular experiment, no channel was observed with an open time longer than 70 ms. In other experiments, the prevailing channel lifetimes were similar, but lifetimes as short as 1 ms (resolution limit) and as long as many seconds also appeared.

The threshold voltages of various heterogeneous membranes made as those

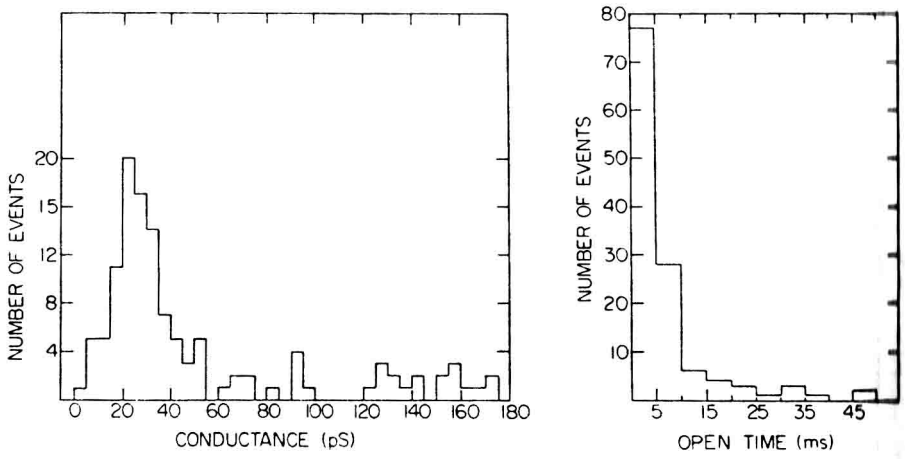

Figure 3.2: *Histogram of channel conductance and open times for 125 single channel events detected in the experiment of Fig.3.1A. The statistical lifetime demonstrates the thermodynamic fluctuations of the lipid system. The variable discrete conductivities are expected in the macroscopic ordered two-dimensional bilayer lattice.*

of Fig.3.1, are plotted in Fig.3.3. Values for the threshold range from ca. $10mV$ till $200mV$ (experimental limit). Apparently, the threshold increases with the initial membrane resistance. This is indicated by the regression line in $Fig.3.3$. The voltage threshold was reproducible in each membrane, and the ion channel induction was reversible.

The reversibility of induction is also evident for ion channels observed in pure homogeneous synthetic lipid. Fig.3.4 demonstrates the behavior of a membrane made of synthetic diphytanoyl phosphatidylcholine at the tip of a ca. 1 μm patch pipette. It has been mentioned already that no detergent was present in these experiments. This membrane had an initial resistance of 18 $G\Omega$, corresponding to ca. 55 pS resting conductivity. Channel fluctuations started to appear at a threshold value of ca. 140 mV. When the voltage was at 140 mV, the typical opening and closing of ion channels can be clearly discerned (upper trace, left). When the membrane was subsequently clamped at 100 mV, i.e. below threshold, the channel fluctuations disappeared completely after a period of relaxation and reappeared reversibly when the membrane potential was increased again to 140 mV threshold (upper trace, right). The lower trace expands some of the channels observed.

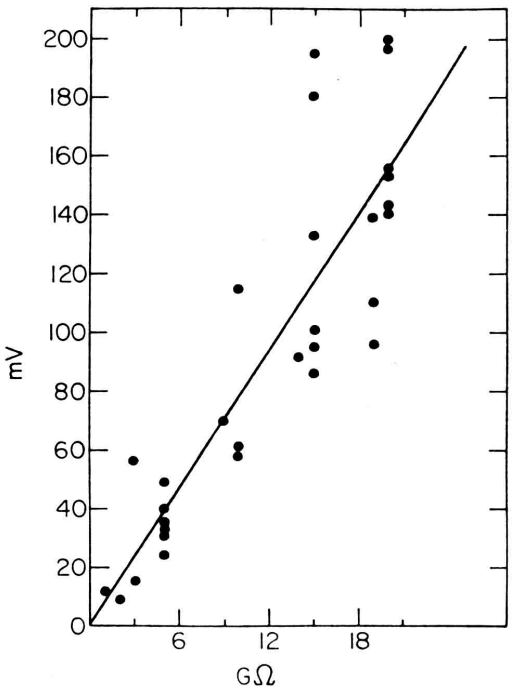

Figure 3.3: *Threshold voltages of 30 lipid membranes, as in Fig.3.1, plotted versus the initial membrane resistance. The straight line is the best fit by regression analysis. High threshold is equivalent to initial states of low conductivity fluctuations.*

It demonstrates that channel opening, as well as induction, is reversible, yet of statistical nature. This trace shows very long-lived ion channels of several seconds open time. But channels much shorter also appeared in the phospholipid system. Clearly, the phenomenon does not require the presence of any additional membrane component. It can be argued, however, that ion channel fluctuations are not physical a physical property of the lipid bilayer but, rather, some boundary artifact. In fact, if the assumption is maintained that the lipid bilayer were a non-fluctuating barrier, the phenomena observed could only be assigned to the boundary; for example, the background resistance had to be interpreted as a weak "seal" between the bilayer and the boundary. Ion channels would in this case occur in the thick boundary, rather than in the thin bimolecular layer. This seems a paradox since, in principle, resistance does increase and not decrease with thickness.

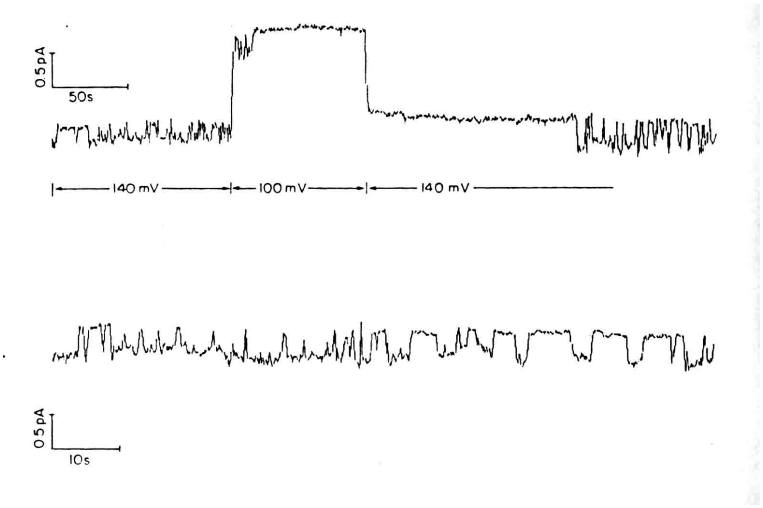

Figure 3.4: *Reversibility of voltage induction. Pipette supported membrane made of pure synthetic diphytanoyl phosphatidylcholine. Aqueous solution as in Fig.3.1. Upper panel: Continuous trace starting at the threshold voltage 140mV, continued at a subthreshold voltage of 100mV and, again, at threshold 140mV. Lower panel: Expanded trace from the latter state. No other membrane component was present. The Figure shows the reversible, voltage induced transitions between strongly and weakly fluctuating states of the lipid system.*

Moreover, the boundary is present in all studies on single ion channels so far. It is therefore of general interest to assess the location, whether in the bilayer or in the boundary, of the ion channel observed in any experiment.

Fig.3.5 demonstrates ion channels in a bilayer made on a 5 μm glass pipette tip. Bilayer area was varied by suction and observed on the monitor. This experiment was performed with soybean phosphatidylcholine. The reversible voltage-induction of ion channel fluctuations was at ca. 400 mV threshold in this case. Fig.3.5A shows weak channel fluctuations at 500 mV. No suction was applied yet. Fig.3.5B shows recordings of the same soybean lecithin membrane after application of suction, again at 500 mV (positive inside the pipette). Bilayer area variation was also observed by electrical capacitance which increased from 210 pF in panel A to 530 pF in panel B. The threshold for voltage-induction was still rather high in this membrane, but decreased from 400 mV in panel A to 200 mV in panel B.

Fig.3.5 shows traces at 500 mV only. It is to be emphasized, however, that

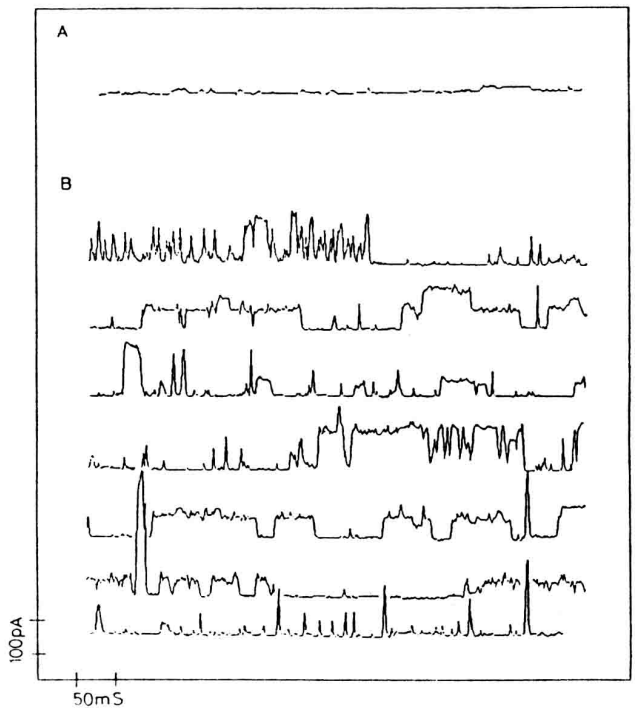

Figure 3.5: *Bilayer localization if ion channels. Membrane made of soybean phosphatidylcholine in 1M unbuffered NaCl, glass pipette tip ca. 5 µm, at 500 mV. A. Before area variation by suction. Total capacitance 260 pF. Ion channel fluctuations are shown above threshold. B. After area increase by suction. Total capacitance 510 pF. The symmetric curvature (ca. 10 µm diameter) and the area were also visually seen. The Figure demonstrates that channel frequency increases with bilayer capacitance with bilayer capacitance at constant boundary. Surface pressure and voltage induced ion channel fluctuations in the lipid bilayer cannot be a priori distinguished phenomenologically. Note that asymmetric surface pressure has similar effects as asymmetric electrostatic membrane potential. Note also the increase in channel current with bilayer asymmetry.*

the membrane was stable even at 1 V applied voltage, and that no irreversible break-down was observed in that state. Reversibly, ion channels up to ca. 200 pS opened and closed even at that elevated electrostatic membrane potential (not shown). Similar experiments as in Fig.3.5 were performed with synthetic lipid, too. The increase of channel frequency and amplitude with area at constant boundary was confirmed (not shown).

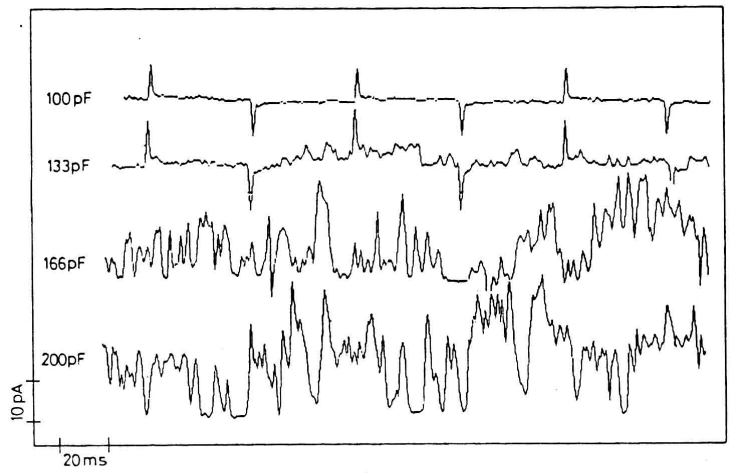

Figure 3.6: *Area dependence of ion channel fluctuations at constant surface pressure. Same as Fig.3.5, but bilayer was formed using a 'split' aperture made of hydrophobic Teflon. The two monolayers constituted the bilayer up to variable levels of the split. No boundary was present at the monolayer/bilayer interface. The variable bilayer capacitance is indicated at each trace. All at 10 mV. The current fluctuations increase in frequency and amplitude with bilayer area.*

It may be argued that the surface pressure was not kept constant and that the ion channels in Fig.3.5B were actually induced by surface pressure rather than by the area increase per se. This argument cannot question the bilayer localization of the ion channels, since both area and surface pressure are lipid bilayer variables. As a matter of fact, Fig.3.5 does establish the localization of the ion channels in the lipid bilayer as well as the mechanical induction of ion channels by surface pressure. Clearly, ion channels can be induced by surface pressure as well as by voltage, and the threshold values depend mutually on each other.

A new technique has been developed in order to vary the area of the bilayer at constant monolayer surface pressures. A hydrophobic "slit" boundary made of Teflon was used. The slit for the formation of the bilayer was ca. 100 to 200 μ m wide, several mm long, and was introduced vertically into the aqueous solution. In contrast to standard techniques (Montal and Mueller, 1972; Boheim et al., 1980), part of the aperture slit was in the air. A boundary-

free continuum between the bilayer and the constituting monolayers appears in this technique at presumably constant surface pressure (see Methods). Some asymmetry due to hydrostatic pressure between the two chambers has, however, not been excluded.

Under these conditions, too, both the frequency and amplitude of the fluctuations increases with the electrical capacitance of the system. The membrane current fluctuations were always reversible. In one membrane made of soybean lipid (Fig.3.6), voltage-induced channels could already be observed at 10 mV for increasing values of capacitance. Increase and decrease of the capacitance increased again the fluctuations reversibly, although the dependency was not exactly linear presumably due to the variable asymmetry of monolayer surface pressures. The slit boundary was hydrophobic in this case, instead of the hydrophilic glass boundary used previously.

Fig.3.7 demonstrates single ion channels in a membrane made of synthetic diphytanoyl phosphatidylcholine using a regular hydrophobic Montal-Müller Teflon boundary of circular diameter 200 μ m. The area of the lipid membrane formed in this way is bigger by more than 4 orders of magnitude than the circular pipette boundaries, and the ratio between the boundary and the bilayer area is smaller by at least two orders of magnitude. The traces do not only show single channels of different amplitudes and open times, but also situations with more than one channel opening simultaneously. Panel B of Fig.3.7 represents a current histogram of 2 minutes of the same experiment. The histogram clearly demonstrates the existence of, at least, three distinct conductivity levels.

No significant effect of the hydrophobic boundary on the lifetimes or amplitudes can be discerned, both being distributed within the same orders of magnitude (compare examples in Fig.3.4 and 3.7). It is interesting to note that this particular membrane only developed ion channel activity after the background conductivity has increased, within 20 minutes, to ca. 200 pS. This observation again stresses the relation of background and ion channel conductivity in the lipid bilayer, noted already in Fig.3.3.

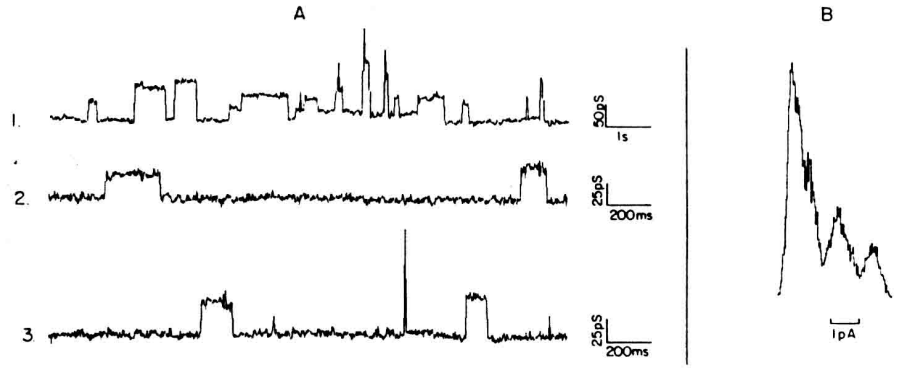

Figure 3.7: *Same synthetic lipid as in Fig.3.4, but hydrophobic Teflon boundary of circular diameter ca. 200 μm. 1M KCl unbuffered, pH ca. 6.5. A. Current traces at an applied potential of 77 mV showing different conductance levels. B. Current histogram over a period of two minutes.*

The origin of the background conductivity and its relationship to the voltage threshold have been further analyzed, using the method of Miyamoto and Thompson (1967). In this method, regular Montal-Müller apertures of different diameter are used, in our case 50, 100, and 200 μm. The surface pressure cannot be kept constant in this case since a new membrane has to be produced for each diameter, and the surface pressure is almost certainly different in each case. Therefore, it is not surprising that a large variability of background conductivities from membrane to membrane is observed.

We have essentially confirmed these experimental results for various lipid bilayers, made of heterogeneous soybean lecithin, synthetic diphytanoyl phosphatidylcholine, or dimyristoyl methyl phosphatidic acid (not shown). This latter lipid was identical to the one used in the monolayer phase diagrams (Träuble (1977), for bilayer diagrams see also Träuble and Eibl (1974)) employed for the quantitative predictions of the theory. We note in passing that in this lipid, too, ion channel fluctuations were observed if the electrochemical proton potential was sufficiently increased, by lowering the pH, in the order of 50 to 200 mV (1 to 3 pH units).

It is most remarkable that, despite of the highly variable background con-

ductivity, membrane capacitance was rather constant, between ca. 0.5 and 0.8 $\mu F/cm^2$. This situation is well established (Henn and Thompson, 1967; Montal and Mueller, 1972; Hamill et al., 1981) and has been used by some investigators to support the hypothesis of a constant, impermeable capacitive bilayer and a leaky, conductive boundary artifact. This hypothesis does, however, not explain the values of the background conductivity. It also does not explain the increase of variability with area (Miyamoto and Thompson, 1967). We have observed, on the other hand, that the lowest values for the background conductivity are always of the same magnitude, 1 or a few pS, no matter what boundary, area, or lipid was used. The highest conductivities of stable lipid bilayers are of magnitude 1000 pS. It is crucial to note that the same range of values for background conductivities is observed with patch pipettes ("Gigaseal"), and that this is exactly the range of conductivities of the most prominent ion channels (c.f., e.g., Fig.3.2).

Structures of no detectable conductance have also been observed by us in both pipette and Montal-Müller experiments of completely normal lipid bilayer capacitance. These structures are usually non-responsive, i.e. of very high threshold. This was expected from the threshold increase with initial resistance (Fig.3.3).

Chapter 4

Discussion

It has long been considered a fundamental problem in membrane theory how the dielectric barrier formed by the lipid bilayer can be broken down (Danielli and Davson, 1935). Proton conduction wires have been recently described (Nagle and Morowitz, 1978) and high proton mobilities were observed in phospholipid liposomes (Nichols and Deamer, 1980), as predicted earlier from the semiconductor-like proton mobilities in water and ice (Eigen and deMaeyer, 1958).

In order to describe the passage of ions through the dielectric barrier provided by the lipid membrane, different models have been investigated. Dielectric break-down, Born barriers, kink diffusion, as well as hexagonal phases, ion-specific pores and fluctuating pore structures have been assumed, but also static crystallographic molecular "channels" or three-dimensional reconstructions of amino sequences (for a review see Hille (1984)). At this stage, it seems fair to say that the physical mechanism for formation of the ion channels has not been unequivocally identified.

The problem to open hydrophilic pathways across the hydrophobic lipid lattice disappears when considering the thermodynamic lipid fluctuations, since the barrier thickness fluctuates. The probabilities for reversible opening and closing of channels cannot, with present techniques, be determined in quantitative detail, but are obviously very large in transitional thermodynamic

states. This follows from the observed monolayer compressibilities and is confirmed by the bilayer ion channel fluctuations.

The theory of the thermodynamic bilayer fluctuations presented above is free of adjustable parameters. Nevertheless, it makes testable predictions: the existence of ion channels in lipid bilayers, their reversible opening and closing, statistical lipid lifetimes, discreteness and variability of channel conductivities, reversible threshold induction by any lipid surface variable, and the magnitude of the threshold of voltage-, pH-, temperature-, and surface pressure-induced ion channel fluctuations.

The kinetics of the phenomenon is not described by the thermodynamic theory and is only resolved in our experiments for time scales longer than 1 ms. Still, the mechanism described allows to answer the crucial question: How can the lipid membrane represent both an electrical insulator as well as, under certain specific conditions, a fluctuating permeable system?

In order to form a barrier to hydrophilic ions, the lipid monolayers have to be in states of low compressibility, i.e., sufficiently far away from the critical ranges of states with large compressibility. Such states of small fluctuation strength are evident in particular at high monolayer surface pressure (Langmuir, 1917; Träuble, 1977; McConnell et al., 1984). Membranes can thus be stabilized by increasing the surface pressure of the lipid component. Alternatively, such states of low compressibility can also be achieved at neutral pH as well as by complete protonation protonation at very acidic pH (Träuble, 1977; Kaufmann and Silman, 1983). A convenient indication for choosing low initial compressibility, i.e. high threshold, following Fig.3.3, is the use of membranes of high initial resistance (Corcia and Babila, 1985; Corcia et al., 1986). In these cases, the thermal energy kT will only create weak fluctuations of the then impermeable lipid lattice.

On the other side, in order to induce permeability fluctuations, the lipid bilayer must be sufficiently close to the critical ranges of the thermodynamic variables, such that a local lipid perturbation will suffice to increase the monolayer compressibility significantly. Insulating membrane states near

critical ranges are therefore sensitive to an increase or decrease in permeability fluctuations induced by specific perturbation of the lipid surface.

Frequently, the term "critical range" is used to express the analogy to a critical point of second order phase transition where the fluctuations even diverge. However, no critical point and no phase transition at all is required, and even in monophasic regimes of the phase diagram, critical ranges of extremal compressibility are generally observed (e.g. Träuble (1977)).

Applying the theory to the voltage-induced channel fluctuations, the electrochemical control of the compressibility is crucial; the threshold voltage does appear due to the fact that the reversible fluctuations of the barrier are significantly increased in states where the surface charges are affected. The thermal motion of the lattice then becomes detectable as fluctuations in the conductivity of the lipid bilayer. Surface pressure, electrical or chemical surface potentials, and temperature control the monolayer states, their compressibilities, and consequently the ion channel fluctuations. The threshold of channel induction by voltage therefore depends on surface pressure, pH, pCa, temperature.

Whatever the microscopic structure of the ion channel in the lipid lattice may be (Cullis et al., 1980; Miller, 1981; Zimmermann, 1982; Kaufmann, 1985b), its formation does not require macroscopic free energy but unavoidably follows (from) the thermal motion. This motion is reversible and therefore causes opening and closing of ion channels.

The probabilities of channel formation could in principle be obtained quantitatively from adequate phase diagrams and increase with the isothermal monolayer compressibilities. Ion channel lifetimes are a property of the lipid bilayer. Ion channels fluctuate reversibly even in thermodynamic equilibrium. The strength of these fluctuations is deterministically controlled by the local surface variables of the lipid bilayer.

The theory is limited by the limits of macroscopic thermodynamics, by the linearization for not too large fluctuations, and by the assumption that lipid monolayer phase diagrams represent the physical properties of lipids in a

bilayer situation in the membrane.

THe experimental results are now compared with a number of crucial predictions of the theory, as outlined in the Introduction.

4.1 Lipid bilayer ion channels

The experiments described above clearly demonstrate that ion channels opening and closing is a consequence of the physical properties of the bimolecular lipid bilayer. No additional membrane component was required. The membrane boundary is not responsible for the phenomenon, since channel activity increases with area even at constant boundary. Contaminations have been excluded using pure synthetic lipids controlled by amino acid analysis. Furthermore, both pipette-supported membranes and Teflon-supported membranes were only prepared in chambers not used previously in any experiment involving polypeptides or proteins. Finally, no organic solvent or prepainting of the boundary was involved in the experiments of Figs.3.1 to 3.4.

A quantitative contribution of the unavoidable experimental membrane boundary has nevertheless to be expected since it influences the surface pressure. However, variations in the ratio of boundary to area by two orders of magnitude, and of the rim material from hydrophilic to hydrophobic, did not affect the channel phenomenology nor the lifetimes significantly (examples are shown in Figs.3.4 and 3.7). Whatever the rim material, the channel activity increases with the bilayer area reversibly (Figs.3.5 and 3.6).

The boundary conditions in these experiments are similar to those present in any observation of single ion channels. Therefore, our observations demonstrate that ion channels are not a property of the boundary but of the bimolecular lipid layer. Furthermore, membrane capacitance steps associated to ion channel fluctuations have been reported both biological (Neher and Marty, 1981) and synthetic lipid membranes (Kaufmann, 1985a), with similar amplitude and time scales. These directly demonstrate fluctuations in

membrane area and thickness.

Our experiments demonstrate clearly that the formation of ion channels is not due to dielectric break-down of the bilayer achieved by the increased transmembrane potential. Bilayers may be stable even at membrane potentials as high as 1 V. Ion channel formation is rather reversible, as predicted from the thermal motion.

The opening and closing therefore demonstrates the reversibility of the mechanism of ion channel formation. The persistence of such fluctuations for very long times (up to many hours) indicates the absence of any macroscopic activation energy, i.e. thermodynamic equilibrium.

The two-dimensional compressibilities in the membrane plane, rather than the membrane gradients in the third dimension, are crucial for explaining the reversible opening of ion channels.

4.2 Statistical lifetimes and discrete conductivities

Channel lifetimes are statistically distributed and apparently correspond to fast kinetic lipid relaxation times. For example, two relaxation times of 1 ms and 50 ms were earlier reported (Tsong, 1974) in phospholipids using T-jump techniques (Eigen and deMaeyer, 1963), but further relaxation times may appear in both bilayer and liposomal preparations. No correlation of specific relaxation to conductance lifetimes is established, however.

Lifetimes do not seem to depend on the hydrophilic or hydrophobic nature of the boundary significantly. Whether on glass pipette tips or at Teflon apertures, fast kinetic events as short as 1 ms (resolution limit) and discrete conductivity levels of 50 ms lifetime or more can be observed.

It is also difficult to identify any crucial dependence of lifetime on the lipid used. This seems reasonable, since the hydrocarbon chain lattice is approximately the same. This fact should also be considered in the interpretation

of lipid-independent ion channel lifetimes and amplitudes.

Lifetimes are used in reaction kinetics to identify the molecular basis and the physical nature of reversible and irreversible phenomena (Eigen and de-Maeyer, 1963). The most prominent time scale of lipid ion channels (Fig.3.2) is also that of biological membrane channels (Hille, 1984; Hanke, 1985). Clearly, biological ion channels occur with apparent lipid channel lifetimes.

Ion channel conductivities in the lipid bilayers are often discrete and most frequently appear in the range of 10-100 pS in most experiments (see, e.g., Fig.3.2). However, amplitudes in a wider range could also be observed occasionally. Whatever the microscopic structure of the ion channels may turn out to be, the conductivity is apparently related to conductive defects of discrete size in the impermeable lipid bilayer. Since the bilayer lattice is still present during ion channel fluctuations, as it is clear from the observed capacitance, any defect represents a lattice defect of probably discrete size. Several orders of magnitudes of discrete conductivities, between 1 and 10^4 pS, cannot be the properties of single molecules. Rather, some macroscopic ordered molecular lattice is required to account for so different and still discrete defect areas. The lipid bilayer represents the only known macroscopic lattice present in all experiments on ion channels.

The most prominent channel conductivities in lipid bilayers are of the magnitude 10 to 100 pS. Channel amplitudes are very similar to typical conductivities of biological membrane channels (Hille (1984); Hanke (1985), see also Ishii et al. (1986)).

The lipid channels do, however, not provide for absolute selectivity with respect to ion conduction. We have demonstrated their appearance in the presence or in the absence of either Na^+ or K^+.

4.3 Control of lipid bilayer ion channels

It is an important thermodynamic fact that the strength of the statistical fluctuations can be controlled in a deterministic fashion. Any of the ther-

modynamic lipid variables in our experiments did control the induction of ion channels. The threshold was deterministic and reversible (cf. Fig.3.4). The magnitude of that threshold had already been quantitatively observed in certain cases for temperature (Antonov et al., 1980; Boheim et al., 1980), pH (Kaufmann and Silman, 1983) and, in our experiments, for voltage. The predictions made from monolayer phase diagrams (e.g. from Träuble (1977)) are confirmed at least in order of magnitude: 10° C, 1 pH unit, 100 mV, 10 dynes/cm change to achieve a critical range from initial states of weak compressibility, or high resistance, in any bilayer membrane containing phospholipids of typical chain length. The voltage threshold for channel induction was in our experiments usually between 50 and 200 mV, although values as low as 10 mV or as high as 400 mV were also observed.

The voltage threshold did depend on the surface pressure produced by suction (Fig.3.5), as was to be expected. The threshold pressure was not quantitatively determined. Still, at fixed electrostatic membrane potential, ion channel fluctuations could be induced by surface pressure (cf. Fig.3.5B).

The threshold for voltage-induction of ion channels in lipid bilayers is not always of the same quantitative magnitude. The threshold voltage increases with the initial membrane resistance (Fig.3.3). The surface pressure may be responsible for the difference, since it represents an experimentally undetermined parameter of the membrane state. An attempt has been made (see Methods) to keep the monolayer surface pressures constant at a continuum free of any boundary between the bilayer and the constitutive monolayers (Fig.3.6). No quantitative determination of the surface pressure has however been presented.

The voltage threshold value lies in these experiments in the range of ca. 50 to 200 mV, comparable to an equivalent change of pH by ca. 1 to 4 units. In fact, membranes of diphytanoyl lecithin of below 10 pS resting conductivity, under the same conditions as in Fig.3.7, required a bulk pH of 4 units below neutrality (Kaufmann and Silman, 1983) to reproducibly induce ion channel fluctuations at constant voltage.

Clearly, the electrochemical ion potentials control ion channel fluctuations in the lipid bilayer. This property is reminiscent of the dependence of many protein-induced ion channels on voltage, calcium, or pH. Calcium and protons, but also sodium and potassium, are known to control the thermodynamic state of phospholipid bilayers (Träuble and Eibl, 1974).

Spontaneous ion channel fluctuations in lipid bilayer membranes are not artifacts, but indicate that the bilayer is already in a state of high isothermal compressibility. It has been shown that these statistical fluctuations can be easily controlled following the theory of the thermal motion in the lipid bilayer. Low threshold for channel induction and high initial conductivity are expected for bilayers close to a critical range. Spontaneous fluctuations are generally abolished by lowering voltage or increasing surface pressure sufficiently, or most conveniently by choosing an initial state of high resistance (Corcia et al., 1986).

The thermal motion in the lipid matrix is also present in protein-lipid bilayer membranes. Membrane proteins, if they locally alter any of the lipid surface variables, will therefore control the probability of the appearance of ion channels in the lipid bilayer lattice.

Chapter 5

Acknowledgments

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