

## Voltage-Induced Nonconductive Pre-Pores and Metastable Single Pores in Unmodified Planar Lipid Bilayer

Kamran C. Melikov,<sup>\*†</sup> Vadim A. Frolov,<sup>\*</sup> Arseniy Shcherbakov,<sup>\*</sup> Andrey V. Samsonov,<sup>\*</sup> Yury A. Chizmadzhev,<sup>\*</sup> and Leonid V. Chernomordik<sup>†</sup>

<sup>\*</sup>A. N. Frumkin Institute of Electrochemistry, Russian Academy of Sciences, Moscow, 117071 Russia; and <sup>†</sup>Section on Membrane Biology, Laboratory of Cellular and Molecular Biophysics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892 USA

**ABSTRACT** Electric fields promote pore formation in both biological and model membranes. We clamped unmodified planar bilayers at 150–550 mV to monitor transient single pores for a long period of time. We observed fast transitions between different conductance levels reflecting opening and closing of metastable lipid pores. Although mean lifetime of the pores was  $3 \pm 0.8$  ms (250 mV), some pores remained open for up to  $\sim 1$  s. The mean amplitude of conductance fluctuations ( $\sim 500$  pS) was independent of voltage and close for bilayers of different area (40,000 and  $10 \mu\text{m}^2$ ), indicating the local nature of the conductive defects. The distribution of pore conductance was rather broad (dispersion of  $\sim 250$  pS). Based on the conductance value and its dependence of the ion size, the radius of the average pore was estimated as  $\sim 1$  nm. Short bursts of conductance spikes (opening and closing of pores) were often separated by periods of background conductance. Within the same burst the conductance between spikes was indistinguishable from the background. The mean time interval between spikes in the burst was much smaller than that between adjacent bursts. These data indicate that opening and closing of lipidic pores proceed through some electrically invisible (silent) pre-pores. Similar pre-pore defects and metastable conductive pores might be involved in remodeling of cell membranes in different biologically relevant processes.

### INTRODUCTION

Barrier function, one of the most important functions of cell membranes, is based on the continuity of membrane lipid bilayers. However, diverse physiological processes including cell lysis and membrane rearrangements in endocytosis and exocytosis require transient breaking of bilayer structure and apparently involve formation of non-bilayer intermediates (Bechinger, 1999; Chernomordik, 1996; Nanavati et al., 1992; Schmidt et al., 1999). A local through-going pore in a membrane lipid bilayer is among the simplest examples of these hypothetical intermediates. In contrast to proteinaceous ionic channels, the edge of the lipidic pores is formed mainly by lipid molecules. Proteins involved in membrane fusion (Chernomordik et al., 1994, 1998; Melikyan et al., 1997), and apoptosis (Basanez et al., 1999), and cytolytic peptides (Bechinger, 1999; Matsuzaki et al., 1998; Miteva et al., 1999) were hypothesized to facilitate formation and expansion of these pores.

To better understand the mechanisms of the protein-mediated disruption of the continuity of lipid bilayers one may study the properties of the lipidic pores in a well-defined experimental system of protein-free bilayer lipid membranes (BLMs). Local disruption of BLMs can be readily achieved by applying high electric fields. Opening and expansion of lipidic pores is thought to underlie the

well-known phenomenon of permeabilization of protein-free bilayers (BLMs and liposomes) under high electric field (also referred to as electroporation and electrical breakdown) (Abidor et al., 1979; Weaver and Chizmadzhev, 1996). Two types of membrane behavior under electrical stress, irreversible and reversible electrical breakdown, are distinguished in literature. In the case of irreversible membrane breakdown observed for BLMs of any lipid composition, a measurable increase in membrane conductance rapidly leads to mechanical rupture of the membrane (Abidor et al., 1979; Genco et al., 1993; Wilhelm et al., 1993). On the other hand, BLMs of some specific compositions (for instance, bilayers formed from oxidized cholesterol) exposed to a short pulse of high electric field demonstrate so-called reversible breakdown. In this case even after five to six orders of magnitude increase, BLM conductance quickly drops to the initial level upon voltage decrease (Benz et al., 1979; Glaser et al., 1988). It has been hypothesized that in the case of irreversible breakdown few pores are formed before the first one of them reaches a critical radius and starts irreversible expansion leading to membrane rupture. In contrast, in the case of reversible breakdown a large population of pores accumulates under high voltage before the onset of BLM rupture. These studies have indicated that voltage application to BLMs of any lipid composition provides a convenient way to promote pore formation and simultaneously offers a very fast and sensitive assay for pore detection by the conductance measurements.

A number of models were proposed to describe formation and development of pores under electric field (Krassowska and Neu, 1994; Moroz and Nelson, 1997; Needham and Hochmuth, 1989; Partenskii et al., 1998; Winterhalter and

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Address reprint requests to Dr. Leonid Chernomordik, Section on Membrane Biology, Laboratory of Cellular and Molecular Biophysics, NICHD, NIH, Bldg. 10, Rm. 10D-04, 10 Center Drive, Bethesda, MD 20892-1855. Tel.: 301-594-1128; Fax: 301-480-2916; E-mail: lchern@helix.nih.gov.

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Helfrich, 1987) and the properties of large irreversibly expanding pores (Needham and Hochmuth, 1989; Sukharev et al., 1983; Wilhelm et al., 1993; Zhelev and Needham, 1993). However the lack of experimental data on the properties of single pores of small, sub-critical radii limited the theoretical analysis of the early stages of pore formation.

In the present work we studied the conductance changes associated with the formation of the metastable transient single pores in unmodified BLMs under high electric field. Sizes of these pores and kinetic characteristics of the pore evolution were evaluated. The character of observed electrical activity suggests that opening and closing of the metastable lipidic pore proceed through a nonconductive pre-pore.

## MATERIALS AND METHODS

### Materials

Decane, octane, HEPES, and asolectin were purchased from Sigma (St. Louis, MO). All other lipids were purchased from Avanti Polar Lipids (Birmingham, AL). Squalene was purchased from Merck (Darmstadt, Germany) and ICN (Aurora, Ohio). Lipids and squalene were stored under argon at  $-18^{\circ}\text{C}$ .

### Experiments on BLMs

Planar bilayers were formed on a round aperture in a Teflon film of 40- or 100- $\mu\text{m}$  thickness, dividing two compartments of a special chamber. The aperture of  $\sim 100$ – $200$ - $\mu\text{m}$  radius was punctured by an injection needle. The aperture was pretreated by solution of the same lipid composition in a decane/octane (1:1) mixture. BLMs were painted using lipid solutions in squalene. Formation of a black BLMs was controlled visually and by capacitance measurements. Specific capacitance of membranes was  $0.9$ – $1$   $\mu\text{F}/\text{cm}^2$ . All experiments were performed in solution containing 100 mM KCl and 5 mM HEPES, buffered at pH 7.0. Ag/AgCl electrodes were immersed into both compartments of the chamber.

### Experiments on membrane patches in glass micropipette

In this experimental system measurements were conducted on small membrane patches placed on a tip of a glass micropipette prepared using P-80 micropipette puller (Sutter Instruments, Novato, CA). Micropipettes were filled with the solution containing 100 mM KCl and 5 mM HEPES, pH 7.0, filtered through  $0.22$ - $\mu\text{m}$  filters (Millipore, Bedford, MA), and had typical resistance of  $\sim 1$  M $\Omega$ . The micropipette was brought into contact with the BLM. The glass in the micropipette tip and the lipid bilayer within it established a very tight contact with seal resistance of  $\sim 20$ – $100$  G $\Omega$ , which remained constant during the experiment. This allowed us to measure currents through the small membrane patch inside the pipette with a very high shunting resistance. This membrane patch was mechanically separated from the BLM and remained stable after BLM rupture, and conversely rupture of the membrane patch under the pipette did not affect the stability of the BLM. In a special set of experiments we also showed that application of high voltage does not affect seal resistance in the time frame of our experiments (less than several minutes). Unless specially mentioned, all membranes were formed from diphytanoylphosphatidylcholine (DPhPC).

## Electrical setup

Measurements were conducted in the voltage-clamp mode of a patch-clamp amplifier. Voltage pulses were applied to the stimulus input of the amplifier. Current traces were observed on the screen of an oscilloscope and simultaneously recorded on the hard drive of the computer using an AD converter card with a time resolution of 1 ms.

## Single-pore analysis

Current recordings were analyzed using approaches developed for studying protein channels. To determine the amplitude and lifetime of every pore we first idealized the records by a specially developed computer program. This program is based on the algorithm that requires no beforehand knowledge on the number of the conductance levels and their amplitudes (VanDongen, 1996). The exact measurement of the pore's open-state duration was complicated by the fact that most of our recordings apparently represent activity of more than one pore. To find pore lifetime we took advantage of the rather wide distribution of the pore conductance and measured the duration of the pore's open state as the time between the closest (by time) upward and downward transition with similar amplitudes (less than 10% difference). An idealized record produced by the program was then used for statistical analysis.

## RESULTS

### Conductance recording of single pores

Fluctuations in membrane conductance were induced by applying a voltage step of sufficiently high amplitude (more than 100–150 mV). As described in earlier studies (Abidor et al., 1979), before the onset of conductance fluctuations, membrane current remained on the background level during some lag-time. We have chosen the voltage applied to be high enough to cause the conductance changes but low enough to provide us with minutes of the conductance recording before the onset of inevitable BLM rupture. For BLMs and membrane patches applied voltages were in the ranges of 150–400 mV and 250–550 mV, respectively. Fig. 1 presents the dependence of the lag-time on voltage amplitude applied to membrane patches.

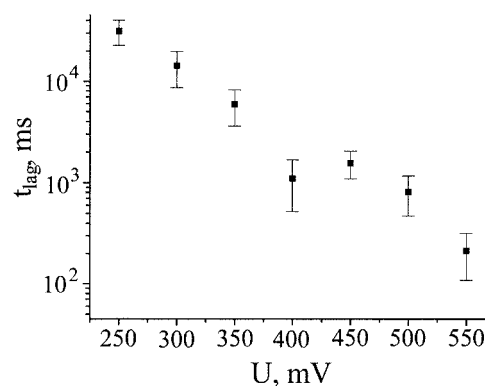
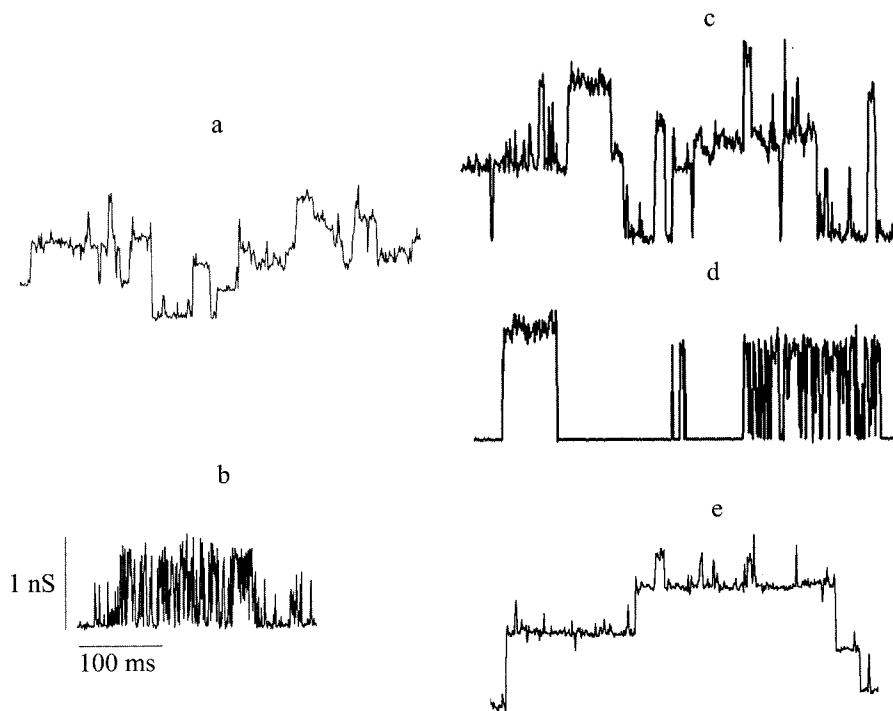


FIGURE 1 Dependence of lag-time before the onset of fluctuations on voltage applied. Experiments were on membrane patches. Bars represent SEM ( $n \geq 5$ ).

FIGURE 2 Conductance fluctuations induced by electric field. (*a* and *b*) Experiments on BLM;  $U = 400$  mV (*a*),  $U = 180$  mV (*b*). (*c–e*) Experiments on membrane patches;  $U = 300$  mV (*c*),  $U = 250$  mV (*d*),  $U = 350$  mV (*e*). Each recording represents a characteristic fragment of the recording obtained in an independent experiment.



Most of the observed changes in conductance were abrupt transitions from one conductance level to another. In Fig. 2 the representative recordings of conductance fluctuations are presented. The abruptness of the transitions and the closeness of the initial and final levels of conductance suggest that these fluctuations reflect opening and closure of single lipid pores. Besides the fluctuations presented above, in some experiments a slow drift of the mean conductance accompanied the abrupt transitions between different conductance levels. This slow drift of conductance, which complicated the analysis, was much less frequent for membrane patches than for BLMs. In addition, due to the small area, membrane patches allowed better amplitude and time resolution. Because of the mentioned advantages of membrane patches, most of the quantitative data presented below were obtained for this experimental model.

Analysis of records shows that the amplitude of transition varied in a rather broad interval from 150 to 1500 pS. A histogram of amplitude distribution for the experiments on membrane patches at 450 mV is shown in Fig. 3. It can be fitted by a Gaussian distribution function with a mean value of  $\sim 450$  pS and dispersion of  $\sim 250$  pS. Both mean value and dispersion did not significantly depend on membrane voltage in the range of 250–450 mV (Fig. 4). An all-points amplitude histogram of conductance fluctuations observed on BLMs (Fig. 5) is similar to those obtained for experiments on membrane patches (Fig. 3). Importantly, mean amplitudes of the conductance fluctuations were similar for membrane patches and BLMs. Because the area of a BLM was  $\sim 10,000$  times larger than that of a membrane patch,

observed fluctuations most probably reflected opening and closure of single lipid pores, rather than some changes in the integral conductivity of the membrane (see also Chernomordik and Abidor, 1980). The existence of the distinct maximum in the amplitude distribution implies that lipid pores formed in the membrane by electric field are metastable. The existence of long-lived conductance steps with duration of up to hundreds of milliseconds further confirmed the metastable character of lipid pores.

Conductance of 450 pS corresponds to  $\sim 1$ -nm radius of the cylindrical pore as estimated by taking into account the

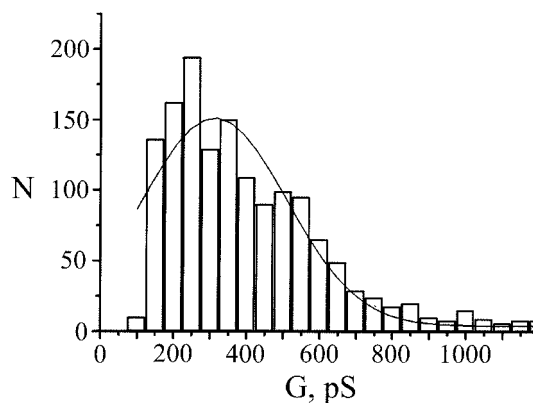


FIGURE 3 Histogram of the distribution of the pore conductance. Gaussian fit is shown as a solid curve. Histogram is based on the records obtained on the membrane patches clamped at 450 mV. Total number of analyzed pores was 1475.

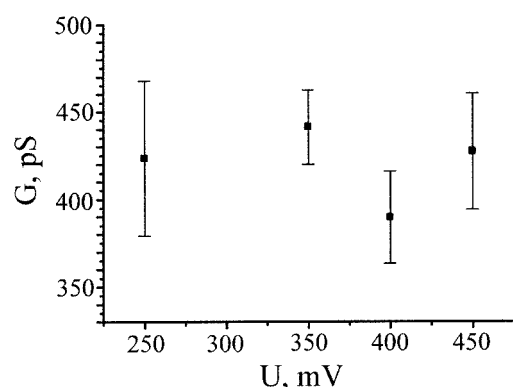


FIGURE 4 Dependence of the mean pore conductance on voltage for membrane patches. Points and error bars present mean conductance and its dispersion, respectively.

access resistance of the pore and assuming bilayer thickness and conductance of the solution in the pore lumen to be 5 nm and 0.01 S/cm, respectively. A pore of such radius can be expected to restrict passage of large ions such as *N*-methyl-D-glucamine (NMDG<sup>+</sup>, 1.1 × 0.5-nm rod) and glutamate ion (0.9 × 0.4-nm rod). (The sizes of the ions were estimated using ChemWindow 6.0, BioRad (Richmond, CA) software.) Indeed, the mean amplitude of conductance

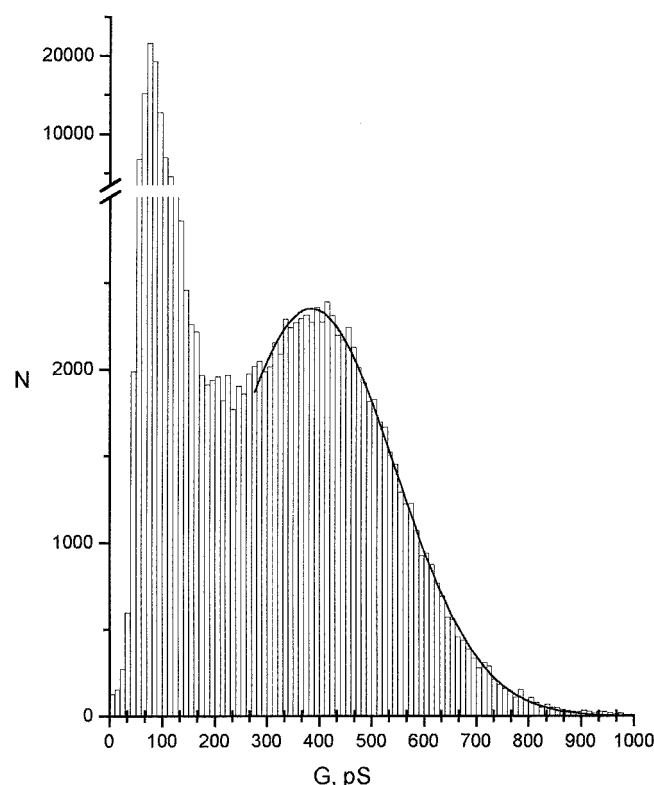


FIGURE 5 All-points amplitude histogram of the conductance fluctuations. Gaussian fit is shown as a solid curve. Histogram is based on the records obtained on the BLMs clamped at 180 mV.

fluctuations decreased from ~450 pS to ~100 pS when K<sup>+</sup> and Cl<sup>-</sup> were replaced by NMDG<sup>+</sup> and glutamate ion. This decrease was significantly more profound than the 1.5 times decrease in the bulk solution conductance assayed as conductivity of micropipettes filled with different solutes. Assuming that the change of solute does not affect pore size, these data can be interpreted as an indication that the radius of the voltage-induced pore is close to the size of the large ions and thus restricts their passage.

Analysis of conductance recordings allowed us to evaluate not only the size but also lifetimes of the voltage-induced pores. The transition to a new level of conductance was often followed by a return back to the initial conductance within a few milliseconds. The mean duration of such conductance spikes appeared to be independent of the voltage ( $3.0 \pm 0.8$ ,  $1.2 \pm 0.1$ ,  $2.5 \pm 0.3$ , and  $5.7 \pm 0.8$  ms for 250, 350, 400, and 450 mV, respectively;  $n > 1000$  for each voltage). As already mentioned, besides short-lived spikes, we also observed another type of electrical activity, conductance steps. In this case, after abrupt establishing of a new conductance level, this level was stable for up to several hundred milliseconds.

Reversible changes in conductance induced by electric field were observed not only on DPhPC membranes but also on membranes made of azolectin, DPhPC/diphytanoyl phosphatidylethanolamine mixtures 2:1 and 1:1, and bacterial phosphatidylethanolamine with 3 mol % lauroyl lysophosphatidylcholine. However, the thorough investigation of the influence of the lipid composition on the properties of the pores was complicated by the differences in voltage amplitudes required for inducing pore formation in membranes of different lipid compositions.

### Nonconductive pre-pores

One of the important features of conductance records under high voltage was the existence of the bursts of activity with multiple consecutive spikes coming one after another separated by short gaps,  $t_g \approx 1$  ms (mean value for 250 mV is  $2.0 \pm 0.4$ ;  $n > 100$ ), with the background conductance. The mean value of  $t_g$  did not depend on the voltage applied. In many records, the bursts of electrical activity were separated by rather long (compared with  $t_g$ ) intervals during which membrane conductance remained at the background level (Fig. 6).  $t_g$  was also much shorter than the lag-time before the onset of electrical activity, which in the experiments with BLMs had a mean value of  $1.9 \pm 3.8$  s ( $n = 15$ ) for 300 mV and decreased with the voltage increase.

Our observation that conductance spikes usually come in bursts rather than singly implies that there is more than one closed or shut state. This important conclusion was substantiated by statistical analysis. We tested whether apparent clustering of short-lived closed states in bursts of activity occurs by chance in a random series of closed states, or indeed reflects a correlation between adjacent closed states.



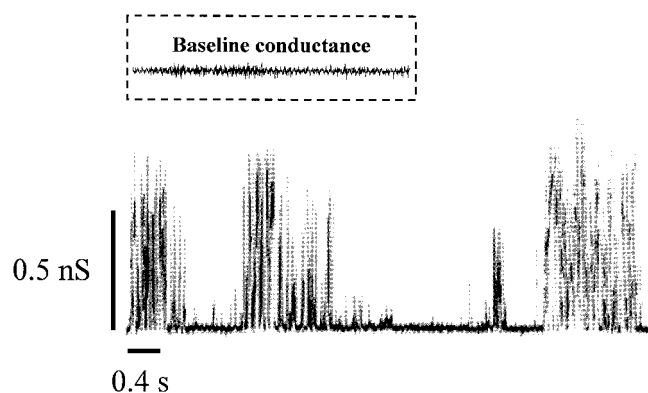


FIGURE 6 Bursts of electrical activity are separated by long intervals with background conductance. This recording represents a characteristic fragment of the conductance recording obtained for BLM clamped at 180 mV. Inset shows baseline conductance of membrane before onset of the first fluctuation. Temporal and amplitude resolution on the inset is the same as on the figure.

To test for correlation we analyzed the representative conductance record obtained for BLMs clamped at 180 mV using a runs test (Colquhoun and Sigworth, 1995). First we divided all closed states (which were determined as states with conductance within 1.5 times of background variance) by their duration into two groups containing either short-lived (duration  $< 20$  ms) or long-lived (duration  $\geq 20$  ms) states. The number of runs  $N_r$  (i.e., uninterrupted sequences of the closed states of the same type) was counted. We then asked whether runs occur with the frequency expected for independent events or, for instance, short-lived closed states tend to follow each other, as expected if the closed state between the bursts of spikes is different from the closed state within the burst. The test statistic, which characterizes the randomness of the series of the runs, is  $z = [N_r - E(N_r)]/[var(N_r)]^{1/2}$ , where  $E(N_r)$  and  $var(N_r)$  stand for the mean and variance of  $N_r$  (Colquhoun and Sigworth, 1995). The value of  $z$  for our record,  $|z| = 60$ , was much higher than the value of  $\leq 2$  expected for the random distribution, indicating that the probability for clustered closed states in the analyzed recording to occur by chance is less than 0.001. Replacing the 20-ms threshold duration of the closed state used in the analysis with values within the range from 5 to 50 ms did not change the conclusion:  $|z|$  remained much higher than 2.

The histogram of the number of closed states in bursts presented in Fig. 7 confirms that the number of longer bursts containing many consecutive short-lived closed states is increased in comparison with that generated by computer simulation for independent events (shown by solid line).

These results indicated that the series of the conductance spikes within a burst reflect the transitions between 1) a conductive, open pore and 2) a closed precursor or pre-pore, which differs from the intact membrane by an increased probability to form open pores. Note that our operational

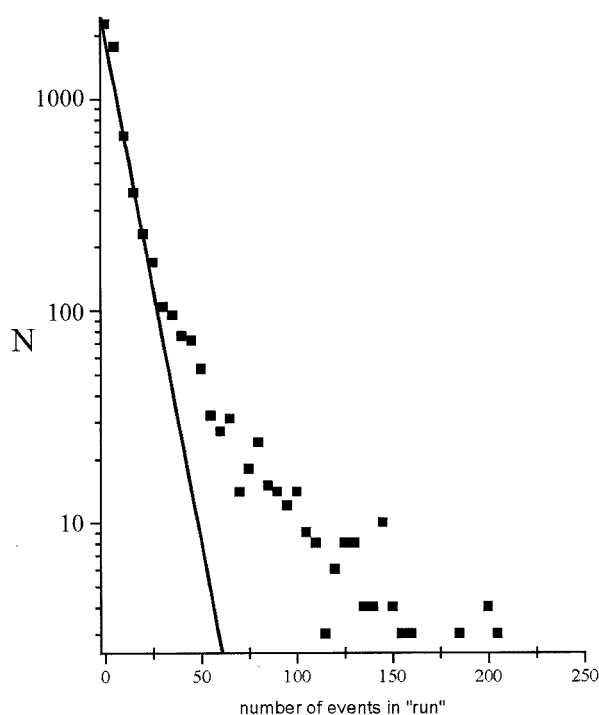


FIGURE 7 Histogram of the distribution of the number of short-lived closed states (conductance staying within 1.5 times of background variance for less than 20 ms) within the same burst. Histogram is based on the 12-min record obtained for BLM clamped at 180 mV. Total number of closed states in the record was 9032; the number of short closed states was 7023. Solid line represents the distribution expected, based on computer simulation assuming that short closed states were independent events.

definition of the pre-pore state involves reopening of the pore within the same burst of electric activity. Thus, we cannot exclude the possibility that the first opening of a pore in the burst does not proceed through the pre-pore state, which in this case forms only upon resealing of the existing pore.

The existence of the pre-pore state was further substantiated by the experiments with two consecutive pulses of high voltage (250–500 mV) separated by an inter-pulse interval (varied from 20 ms to 5 s) at 50 mV. Changes in the conductance for different inter-pulse intervals (50 ms and 1 s) are shown on Fig. 8. A voltage drop at the end of the first step resulted in a rapid ( $< 33\text{-}\mu\text{s}$ ) relaxation of membrane conductance toward background level. During the inter-pulse interval, conductance remained at the background level. The conductance behavior during the second voltage step depended on the duration of the inter-pulse interval. In the case of rather short intervals ( $< 250$  ms), application of the second voltage pulse resulted in an immediate upsurge in conductance; i.e., there was no lag-time detectable with our 1-ms time resolution (Fig. 8 *a*). In the case of longer ( $> 500$ -ms) inter-pulse intervals, lag-time observed in the beginning of the second voltage step was similar to the response observed during the first step (Fig. 8

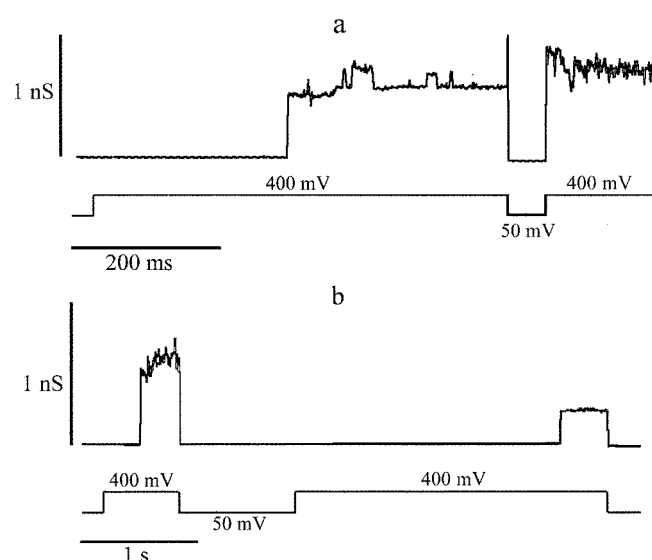


FIGURE 8 Membrane response to the second step of voltage depends on the time interval after the end of the first step. BLM was treated with two successive voltage steps of 400 mV each. Between the steps BLM was clamped at 50 mV for 50 ms (a) or 1 s (b).

b). Thus, although conductance between pulses was the same as in the initial state, the membrane “remembered” for some time the previous pulse. Taking into account the size of our pores ( $\sim 1.0$  nm, see above), we do not expect them to demonstrate the non-ohmic behavior described in Glaser et al. (1988). Thus, our data indicate that after the end of the first pulse the open pore quickly turns into some non-conductive but activated state (pre-pore), which is ready to reopen in response to the second pulse. The pre-pore is a metastable structure and without a second pulse it reseals with a relaxation time of the order of 100–1000 ms. Note that this type of experiment (Fig. 8) is feasible only for relatively long-lived conductive pores (conductance steps, see above).

## DISCUSSION

Numerous biologically relevant processes, including membrane fusion, lysis, and apoptosis of cells, were hypothesized to involve an opening of a lipidic pore to join the volumes initially separated by membranes. To study the properties of such a lipidic pore and the mechanisms of its opening and closing, we focused on a relatively simple experimental system: unmodified planar lipid bilayer under high electric field. In the earlier work the structure of the voltage-induced pores was evaluated by following either huge populations of small transient pores (Benz et al., 1979; Glaser et al., 1988) or just a few very large and irreversibly expanding pores (Abidor et al., 1979; Sukharev et al., 1983; Wilhelm et al., 1993). In this study we analyzed the electrical activity of single transient pores and found that open-

ing and closing of these pores proceed through some electrically invisible (silent) pre-pores.

Conductance spikes reflect evolution of the voltage-induced pores. Our finding that the amplitude of changes in the membrane conductance was independent of the membrane area supports the hypothesis that conductance rise under electrical stress is caused by the formation of local conductive defects (lipid pores) rather than by an increase in average conductive properties of membrane. In the latter case the amplitude of electrical activity observed under voltage would increase with the area.

A pore of 0.5-nS conductance is expected to be of  $\sim 1$ -nm radius and thus might involve only  $\sim 100$  lipid molecules, which corresponds to less than  $10^{-8}\%$  of all lipids in the BLMs of  $1\text{-mm}^2$  area. Because such amounts of contaminants are undetectable by usual biochemical techniques, one may hypothesize that the conductive pores in bilayers are formed by some minor contaminants rather than by the major lipid components. If so, the organic solvents used to form the bilayers should not introduce these contaminants because the observed electrical activity was sensitive to the lipid composition. On the other hand, the voltage-induced pores were observed for bilayers of all studied compositions, indicating that these hypothetical pore-forming contaminants have to be present in all used lipids, both natural and synthetic.

Another possible source of contamination is small amounts of lipid oxidation products. They can form clusters in which formation of pores actually takes place. However, in this case one should expect the number of clusters to be proportional to membrane area, as is the amount of contaminant in membrane. In fact, the number of observed pores was not proportional to the lipid bilayer area (data not shown). It is also feasible that after formation of a lipidic pore, the hypothetical contaminants gradually replace the background lipids at the pore edge to lower its energy. If so, one may expect the properties of the pores to change with time under voltage. The lack of any changes in the mean amplitude and lifetime of the open state argues against this scenario. Finally, qualitatively similar conductance spikes under similar voltages were reported for biological membranes (Chernomordik et al., 1987; Stampfli, 1958), suggesting that if these conductance changes reflect the traces of hypothetical impurities rather than the general properties of membrane bilayer, this contaminant has to be present in biological membranes. To conclude, although we can rule out some specific mechanisms by which the hypothetical contaminants can affect formation and properties of the pores, it still remains possible that some minor impurities, present in protein-free lipid bilayers of diverse compositions and in biological membranes, play some role in pore formation. Future systematic studies on the dependence of the pore properties on the composition of lipid bilayers will hopefully characterize the molecular components of the pore edge.

Rapid transitions between different conductance levels for lipidic pores (see also Antonov et al., 1980; Yafuso et al., 1974) appear rather similar to activity observed in the case of protein channels. Furthermore, some lipidic pores (conductance steps in Fig. 1) had relatively long lifetimes (up to 100 ms), suggesting that their structure is rather stable and stressing the similarity between these electrical recordings and bona fide protein channels. However, lipidic pores in our experiments had much broader distributions of sizes and lifetimes than typical protein channels, suggesting that the specific structure and actual number of lipid molecules involved in pore formation may be different from pore to pore.

The existence of a nonconductive pre-pore state, evidenced by the bursts of pore flickering with background conductance between the bursts, was completely unexpected and apparently the most intriguing finding of this work. Our data show that the rate of the pre-pore formation is much slower than the rates of transition from pre-pore to pore and back from pore to pre-pore. This means that pre-pore and pore can be considered as two sub-states of a common structure. Distribution of the pore conductance within a single burst appears to be smaller than the overall pore amplitude distribution, indicating that the properties of the pore were determined by the structure of its pre-pore. The existence of the pre-pore state emphasizes the similarity between lipidic pores and proteinaceous channels. For example, a potassium channel in an excitable cell passes through three nonconductive states before opening (Hille, 1992).

The metastable lipidic pores identified and studied here most probably correspond to the small metastable lipidic pores whose existence was hypothesized earlier to explain the accumulation of very large numbers of pores during reversible electroporation of BLMs modified by uranyl ions (Glaser et al., 1988). Glaser and co-authors suggested that closing of these pores is hindered by the energy barrier related to hydration repulsion and the increase in bending energy for very narrow pores. Expansion of the pores leading to irreversible rupture of the BLM is hindered by the energy barrier related to the interplay between surface tension of the BLM and linear tension of the pore edge (Abidor et al., 1979).

The physical structure of nonconducting pre-pores remains puzzling. Opening and expansion of conductive hydrophilic pores, i.e., pores with the edges formed by polar heads of the lipids, is thought to be preceded by formation of very small and short-lived hydrophobic pores with the edge formed by hydrocarbon chains of the lipids (Abidor et al., 1979; Glaser et al., 1988). Evolution of a hydrophobic pore into a hydrophilic pore involves reorientation of the polar heads of the lipids from the surface of bilayer to the edge of the pore. One may hypothesize that newly identified pre-pores correspond to small clusters of lipids with their polar heads trapped inside the hydrophobic interior of the

membrane upon closing of the hydrophilic or partially hydrophilic pore. Interaction between the lipid polar heads in the same cluster can increase the lifetime of the cluster and, thus, stabilize the pre-pore state. Alternatively, the pre-pore state can correspond to a cluster of water molecules trapped inside a hydrophobic interior. Such clusters of lipid polar heads or water molecules will then transform back into a small hydrophilic pore.

## Lipidic pores and biologically relevant processes

Local and transient loss of the stability of the lipid bilayer with formation of the lipidic pores was studied here for the particular case of electroporation of protein-free bilayers. Electroporation of biological membranes is widely utilized to transfer nucleic acids and other macromolecules through membranes of different eukaryotic and prokaryotic cells (Neumann et al., 1982; Rols and Teissie, 1998). High electric fields are also used to fuse cells (Neil and Zimmermann, 1993; Ramos and Teissie, 2000). We hope that further development of these biomedical and biotechnological applications will eventually benefit from better understanding of the physical mechanisms underlying voltage-induced formation and evolution of the pores.

Importantly, application of external electric field is not the only way to initiate opening of lipidic pores. Pores in membranes can be induced by cytotoxic peptide antibiotics (Bechinger, 1999; Matsuzaki et al., 1998; Miteva et al., 1999) and by peptide fragments of viral glycoproteins (Soltész and Hammer, 1997). Some of the proteins involved in the apoptosis pathway, which leads to the release of cytochrome C from mitochondria, also promote formation of lipidic pores in planar bilayers and liposomes (Basanez et al., 1999). Structure of these pores and the mechanisms by which proteins and peptides form them remain to be understood. It has been suggested recently that some membrane-bound peptides and proteins can induce local electroporation (Miteva et al., 1999).

We hypothesize that development of lipidic pores under high electric field and/or strong tension (Akinlaja and Sachs, 1998; Zhelev and Needham, 1993) or at high local concentration of membrane-active peptide (Miteva et al., 1999) or non-bilayer lipids (Chernomordik et al., 1985) can all involve small metastable pores and pre-pores identified in this work. Future systematic work on the properties of lipid pores will also bring insights relevant to studies on the mechanism and applications of electroporation of protein-free bilayers and biological membranes.

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