Lipid Electropore Stabilization

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Abstract

The stabilization of pores can be studied by different approaches such as simulations in silico or experimental procedures in vivo or in vitro. The energy to open a pore in a lipid membrane can be delivered by different external stimuli. To disrupt the membrane and initiate the pore opening, this energy has to reach a threshold. Then, once the pore is open, the external stimulus can be modulated to maintain the pore stable in time. This chapter first describes the basics of electropermeabilization, a process also called electroporation, and the basics of molecular dynamics in electropermeabilization. The chapter then describes in detail the molecular changes that lead to the pore opening and evolution by molecular dynamics. The chapter focuses on molecular dynamics because this technique allows the study of pore stabilization at molecular level, the interpretation of the lipid and water molecule rearrangements that are behind this phenomenon, and the visualization of the pore at the scale of size and time, in the order of nanometers and nanoseconds, respectively. Finally, the chapter also describes other approaches where pores remain open or the permeabilized state remains stable for a period of time, such as continuum modeling, experiments in planar membranes, and experiments in cells. The objective of this selection is to

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relate the results obtained by molecular dynamics with those obtained experimentally, or by other types of modeling, aiming to connect the mechanisms of pore stabilization by molecular dynamics at different scales.

Keywords

Electropermeabilization • Electroporation • Pore stabilization • Molecul dynamics

Contents

Introduction

The plasma membrane defines the boundaries of all living cells. Cell membranes are composed of lipid bilayers and proteins. The lipid bilayers of animal cell membranes are mainly composed of phospholipids and sterols, such as cholesterol. Both of these molecules are amphiphilic and have two defined parts: a hydrophilic one, consisting of a polar head group, and a hydrophobic one, composed of two hydrocarbon tails in phospholipids or four carbon rings and a hydrocarbon tail shorter than that of phospholipids in cholesterol. Due to their amphiphilicity and shape, when phospholipids are placed in water, they are spontaneously organized to form a bilayer with the polar head interacting with the water molecules, and the nonpolar tails of each layer facing each other in the inner part of the bilayer, preventing the contact of this hydrophobic region with the water molecules. This lipid bilayer separates the inner medium of the cell, called cytosol, from the extracellular fluid. The bilayer thus acts as a barrier with a selective permeability to small polar molecules (Fig. 1a). Another characteristic of cell membranes is the asymmetry in lipid composition of both leaflets. Under physiological conditions, the membranes are flexible and can break and spontaneously reseal. The disruption of the membrane

exposes the hydrophobic surface formed by the lipid tails, hidden in the core of the bilayer, to the water molecules, creating an energetically unfavorable condition. This induces a rapid rearrangement of the lipids, aimed to repair the rupture in the membrane.

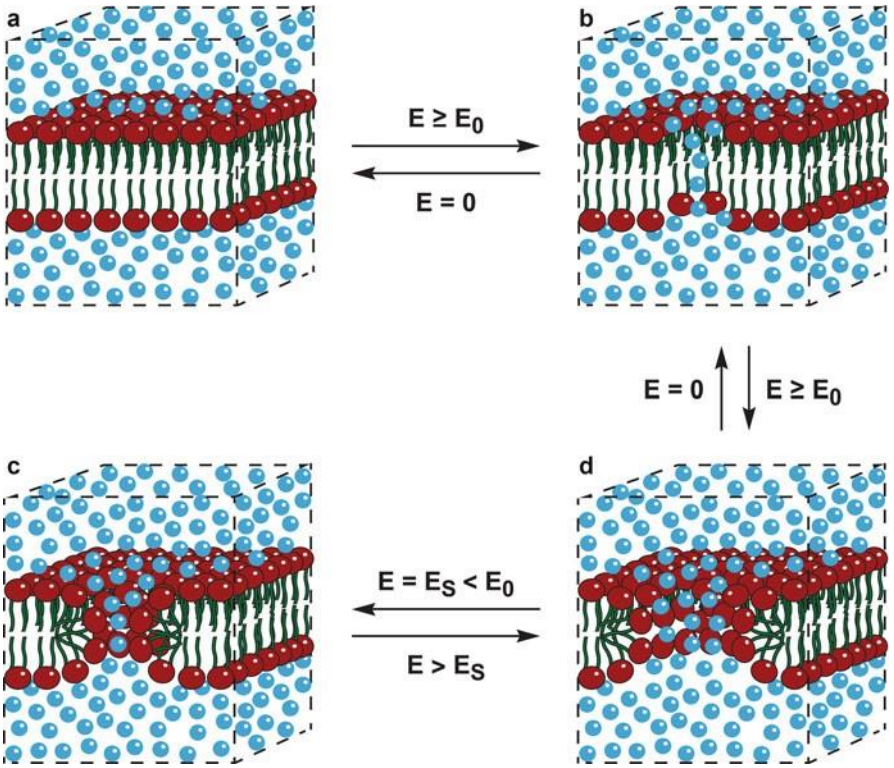

Fig. 1 Evolution of the pore: Schematic three-dimensional representation of a bilayer in a simulation box. The red beads are the phospholipid polar head groups, the green sticks are the hydrocarbon lipid tails, and the light blue beads are the water molecules. The sizes of the components are not in scale. (a) Intact bilayer before the application of the electric field; (b) Beginning of the pore formation with a hydrophobic pore after the application of an external electric field with an intensity equal to or greater than the minimum porating electric field (E_0); (c) Stage of expanded pore; (d) Stabilized pore at a value of electric field lower than E_0. If the electric field applied is equal to or greater than E_0, the pore begins to be formed and evolves to an expanded pore, from a to c. If the electric field applied is removed, the pore disappears, from c to a. If the electric field applied decreases to a low sustaining value (E_s) lower than E_0, the pore shrinks, from c to d. If the electric field applied increases to a value higher than the selected E_s, the pore expands, from d to c

The disruption in the continuity of the membrane can be artificially induced by different stimuli such as electrical or mechanical stress or chemical agents. If the intensity of the external electric field or the force applied to the membrane is high enough to exceed the respective membrane thresholds, the bilayer starts to break down due to the formation of artificial aqueous pores, which increase its

permeability and even allow the diffusion of ions and molecules to which the membrane is normally impermeable. Thus, once the pore is open, water molecules, ions, metabolites, and other molecules can cross the membrane either from the inside to the outside of the cell or in the opposite direction, depending on where they were confined, following their own gradient. The molecules that are allowed to go through the membrane can be normal cellular metabolites as exogenous molecules, such as drugs, which can be placed outside the cell during an experimental procedure.

Electropermeabilization

Electropermeabilization, also called electroporation, is a methodology of transient disruption of the membrane induced by pores due to the exposure of artificial bilayers or cell membranes to an external electric field in vitro or in vivo (Tsong 1991). The reversible or irreversible effects of this process on cell membranes are linked to the intensity (which can be high or low), the repetition, the duration (which can range from nanoseconds to microseconds), and the number of electric pulses applied. Artificial bilayers, cells or tissues, can be successfully electroporated by means of several protocols, which depend on the nature of the object of study selected. For instance, to electroporate cells in a reversible way, a field magnitude in the order of kV/cm can be applied in microsecond pulses, while higher field magnitudes in the order of MV/m can be applied in nanosecond pulses (Silve et al. 2014; Pakhomova et al. 2011; Vernier et al. 2006).

Molecular Dynamics and Electropermeabilization

The molecular events produced at atomic or molecular level that lead to pore formation cannot be visualized experimentally. So, the molecular structures of the induced pores during their formation, maturation, and closure can only be studied and described by molecular dynamics simulations.

Molecular dynamics is a method that allows the modeling of a biological structure such as the lipid bilayer at atomic level. In this method, the structure of interest (lipid bilayers, proteins, nucleic acids, etc.) is placed in a tridimensional box where the initial position of each atom is known. The structure in the box can be solvated by different approaches, such as adding implicit or explicit solvents, thus giving the biomolecule, or the biological structure, an adequate aqueous environment, including ions. Each atom in the simulation box is related to and interacts with the others and has a defined initial position and an assigned velocity. Then, by solving the Newton′s equation of motion, the forces on each particle due to the interactions are calculated and the movement of the atoms is simulated at each time step, in the order of femtoseconds. At each time step, the new position or velocity of each atom is generated. Following the movement of each atom at each time step, a trajectory is generated and the changes of the whole simulated system can be evaluated along the time.

The molecular dynamics method works with force fields, which allow the calculation of the potential energy at each step. The force field is constituted by a functional form to calculate the potential energy and all the parameters needed for this calculation. With the functional form and the parameters, the potential energies and the resulting forces can be calculated as a function of the position of each interacting atom. In the simplest force field, the calculation of the energy is the sum of terms that model the interactions.

The following methods have been developed for molecular dynamics simulations to induce the formation of pores and study their further evolution.

External Electric Field Molecular dynamics procedures allow simulating the application of an external electric field in the z-direction (transversal to the membrane) as a new force in the system, exerted over all atoms with partial charges, in the calculations of the potential energy. By means of this simulation, the transmembrane potential rises until the membrane threshold is reached, causing the formation of the pore (▶ Chap. 2, "Critical Electric Field and Transmembrane Voltage for Lipid Pore Formation in Experiments"). This method allows the comparison of results obtained by molecular dynamics with those obtained experimentally, where high-intensity nanosecond pulsed electric fields are applied to a system (Tieleman 2004; Tarek 2005).

Ion Charge Imbalance Another approach to describe the generation of electropores is the induction of a transmembrane potential by the method of ion charge imbalance, where the transmembrane potential is generated by imposing an ion charge imbalance across the bilayer. This method simulates the effects on the transmembrane potential due to the charge imbalance in the membrane, which acts as a capacitor. The results obtained from these molecular dynamics experiments have been previously compared with those obtained experimentally (Sachs et al. 2004; Gurtovenko and Vattulainen 2005; Delemotte et al. 2008).

Mechanical Stress Another way to induce pores in membranes, in the absence of an electric field, is by mechanical stress. This method is based on the application of a surface tension, which exceeds the bilayer threshold tension needed to maintain the continuity of the membrane and initiates the process of pore opening (Leontiadou et al. 2004).

Chemical Agents The addition of amphiphilic molecules to the simulated system can affect the membrane stability and induce the pore formation. The presence of these molecules can also lower the electroporation threshold (Fernández and Reigada 2014; Polak et al. 2015).

The following sections in this chapter describe the principal methods to obtain and maintain stable electropores.

Pore Stability by Molecular Dynamics

As mentioned above, the formation of pores in bilayers may be induced by several methods by molecular dynamics. This chapter describes some of these methods, including the application of an external electric field, the modification of the transmembrane potential due to charge imbalance, and the poration process by mechanical stress or by chemical agents, in order to analyze the effects of these methods on the stability of pores.

Stable Pores Obtained by Electric Field Application

When an external electric field is applied perpendicularly to the membrane (z-direction) and the intensity of the field reaches the electroporation threshold, the pore begins to be formed.

The only way to analyze the molecular events involved in the initiation of the formation and evolution of an induced pore in the membrane during electroporation is through in silico simulations. The method of molecular dynamics simulation has revealed that the application of an external electric field that reaches a threshold, the minimum porating electric field (E_0), in an intact membrane (Fig. 1a) induces and then stabilizes water defects. These defects, which are also called fingers, are formed in the interface between the water molecules and the hydrophobic part of the bilayer membrane. These water molecules forming the defects are reorganized by the alignment of their dipole moments with the applied electric field starting the pathway to the formation of the pore. These fingers are extended across the hydrophobic core of the bilayer membrane, connecting to another defect formed in the opposite leaflet, finally resulting in a continuous path of water wires. These structures are known as hydrophobic pores because the water molecules are in contact with the hydrophobic part of the lipids, which constitutes the inner part of the bilayer (Fig. 1b). The new contacts between the water molecules and the hydrophobic tails of the lipids are energetically unfavorable. As mentioned previously, in a physiological situation, the disruption of the membrane continuity is immediately resealed, but, in this situation, the stimulus that causes the disruption continues exerting its effect over the membrane, preventing the resealing and inducing more water molecules to penetrate into the hydrophobic core, thus thickening the water wires. Immediately after, the lipids start a structural rearrangement to surround the water wires. This rearrangement begins with the movement of the polar lipid head group, in order to reach the inner part of the bilayer, and therefore ensuring the complete lining of the water molecules. This stage of the pore formation process is called hydrophilic pore. Then, this process restores the prepore surface contact, where the water molecules are located only around the polar lipid head groups and are not allowed to be in contact with the hydrophobic region of the bilayer (Fig. 1c).

Now, at this point of the formation of the pore, and under the influence of the external electric field, a new structure of an aqueous pore in the bilayer membrane is created, where there is a sort of continuity between the lipids of the upper leaflet and those of the lower leaflet, which allows the movement of lipids from one layer to the other. Prior to the formation of the pore, this arrangement was nonexistent because the migration of the lipids from one leaflet to the other, known as flip-flop process, is a very infrequent event in normal conditions, so the external electric field stabilizes this pore configuration. If the stimulus of the external electric field applied continues in time, the hydrophilic pore keeps growing to form an expanded pore (Fig. 1c), but if the stimulus is not removed, the growth of the pore ends up in the destabilization of the lipid bilayer and thus leads to the rupture of the membrane. Finally, removal of the field during the stage of expanded pore leads to the destabilization of the pore and later to pore annihilation, returning the membrane to the ground state (Tieleman 2004; Tarek 2005; Levine and Vernier 2010; Tokman et al. 2013) (▶Chap. 7, "Lipid Electropore Lifetime in Molecular Models").

Molecular dynamics studies show that electropores can be stabilized in two steps. In the first step, high electric field strength is applied in the z-direction. This has been tested, for instance, in the simulation of a bilayer formed by only one lipid, such as palmitoyl-oleoyl-phosphatidylcholine, and of bilayers with a mixture of lipids mimicking the membranes of bacteria, such as Staphylococcus aureus and Escherichia coli. In all the simulations, the pore evolution is the same as that depicted in Fig. 1a–c. The simulations are stopped when an expanded pore is obtained (Fig. 1c). In the second step, the structure of the expanded pore obtained in the first step is used as the initial configuration in a second simulation, where the field magnitude is reduced to obtain low field stabilized electropores for several nanoseconds, as depicted in Fig. 1d (Böckmann et al. 2008; Piggot et al. 2011).

This two-step protocol for the development and stabilization of the pore may allow understanding the effect on a preformed expanded pore by modulating the external electric field to several sustaining values (E_S) lower than the minimum porating electric field (E_0) (Fernández et al. 2012).

The initial configuration for an expanded pore can be obtained in a first-step simulation performed at the minimum porating electric field for a bilayer composed of dioleoylphosphatidylcholine and cholesterol. Then, in the second step, several simulations can be performed by applying different field values lower than the minimum porating electric field to analyze the temporal evolution of the pore volume. Three different E_S values have been found to stabilize the pore indefinitely at different final volumes. One of these results is shown in Fig. 1d, where the sustaining value for the field maintains the pore open in a volume lower than that obtained for the expanded pore.

Figure 2 represents the E_s values obtained in the above-described simulation and shows that the higher the E_s, the higher the volume of the pore (Fig. 2a–c). The whole volume of the pore is estimated dividing the inner part of the pore, located into the hydrophobic core of the bilayer, into bins. In each bin, a cylindroid that includes all the water molecules present in the pore is constructed. So, the whole

volume of the pore is calculated as the sum of the volumes of stacked cylindroids inside the pore (Fig. 3 and Fig. 2d–g).

These results show the three field sustaining values and determine two nonequilibrium conditions for the stability of the pore:

$E < E_S$: If the field applied is less than the minimum sustaining value, it leads to a decrease in the pore volume and later to the closure of the pore, as it occurs when the field is removed ($E = 0$), thus allowing the complete resealing of the membrane (Fig. 1 from 1c to 1a). So, fields lower than the minimum E_s are not high enough to maintain the pore open in a constant volume.

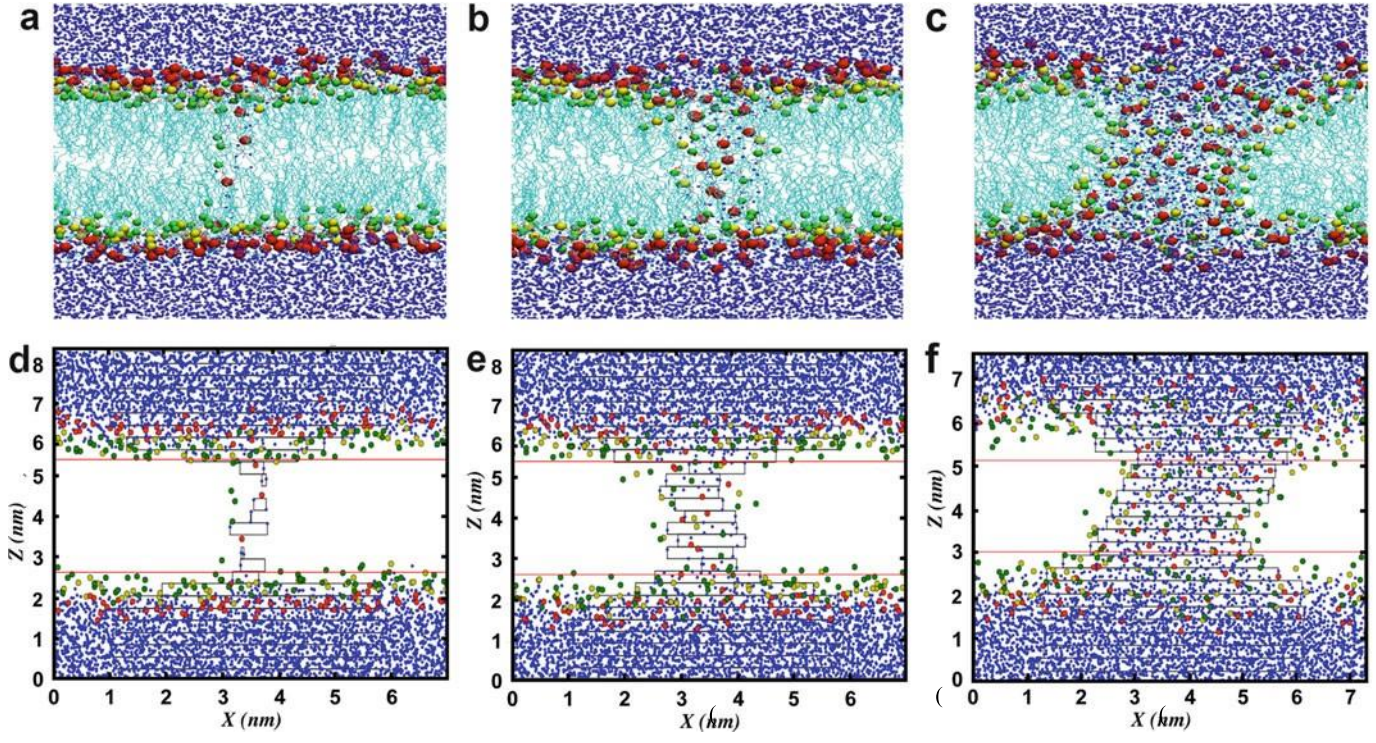

Fig.2 Snapshotsforthestabilizedporestructuresafter100nsat(a) E_s = 0.125 V/m; (b) E_s = 0.150 V/m;and (c) E_s = 0.175 V/m. (d–f) Plotsofthe(x, z)viewofthecylindroidsthatformthestabilizedpores(FigurefromFernándezetal. 2012,takenwithpermissionfromElsevier). Blue spheresarewateroxygen atoms, red spheresarethephosphorusatomsofdioleoylphosphatidylcholine, yellow spheresarethehydroxyloxygenatomsofcholesterol, green spheresarethe firstsn-1carbonatomsofdioleoylphosphatidylcholine,and turquoise stickstructuresarethelipidhydrocarbontails.Allthesnapshotswereperformedwith VisualMolecularDynamics(Humphreyetal. 1996)

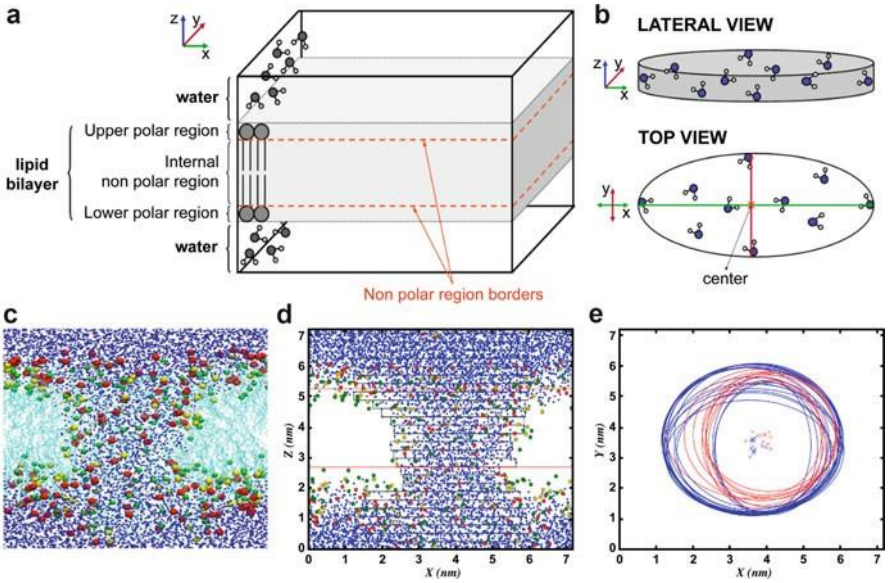

Fig. 3 Schematic representation of (a) the simulation box, indicating the water region, the upper and the lower polar regions of the bilayer and the internal nonpolar region of the bilayer and (b) the cylindroids that constitute the pore (lateral and top view). (c) Snapshot of the initial pore structure. (d) Plot of the (x, z) view of the cylindroids that form the starting pore. The blue spheres are the water oxygen atoms, the red spheres are the phosphorus atoms of dioleoylphosphatidylcholine, the yellow spheres are the hydroxyl oxygen atoms of cholesterol, and the green spheres are the first sn-1 carbon atoms of dioleoylphosphatidylcholine. (e) Plot of the (x, y) view of the cylindroids that characterize the water pore. Crosses correspond to the center of the cylindroids. The cylindroids of the nonpolar region that contribute to the pore volume are in red; the "external" cylindroids excluded from the calculation are in blue (Figure from Fernández et al. 2012, taken with permission from Elsevier)

$E > E_S$: If the field applied is higher than the maximum sustaining value, it leads to a continued growth of the pore volume.

These results show a direct association between the magnitude of E_S and the volume of the stable pore, indicating that the pore size can be controlled and sustained in time (Fernández et al. 2012).

The method of controlling the pore size by adjusting the value of the sustaining field can also be used to measure the ion conductance through nanopores (Ho et al. 2013). The simulations performed using this method have been developed in two systems: one in the presence of sodium chloride and the other in the presence of potassium chloride, showing that the high interaction of the sodium ions with the interface between water and phospholipids induces a slower conductance than potassium ions. This slowing effect can be compensated by increasing the field intensity and as a consequence increasing the pore radius, since at higher pore radii, the interaction of the sodium ions with the pore wall diminishes as the bulk water predominates inside the pore.

Another way to modify the pore size is by periodically modulating the intensity of the field (Kohler et al. 2015). This can be simulated by means of a time-varying external electric field. Then, using a unipolar sinusoidal function, the variations in the intensity of the field are associated with variations in the same direction of the pore size. Therefore, a modulation in the expansion and shrinkage of the pore can be achieved in a single simulation following the same frequency applied to the electric field. This is schematically shown in Fig. 1 (Fig. 1c–d and d–c).

Stable Pores Obtained by Charge Imbalance

This method generates an artificial charge imbalance by simulating bilayers that separate two water regions where different ion concentrations are imposed. This ion imbalance generates a transmembrane potential able to induce pores as in simulations where an external electric field is applied. There are two proposed setups:

1. In the first one, two bilayers are modeled in the same simulation box, separated by a water region. So, one leaflet of each bilayer is in contact with the middle water slab, and the other faces a second aqueous region. This second water slab is connected by the upper and lower borders of the box in the z-direction due to the periodic boundary conditions.
2. In the second one, a single bilayer separating two water slabs is modeled, butthese aqueous regions are not connected because two additional vacuum/air slabs are inserted in the upper and the lower borders of the box in the z-direction, generating a water/vacuum interface that impedes the ionic movements between both water reservoirs (Kirsch and Böckmann 2015).

In both methods, the ion diffusion through the pore dissipates the charge imbalance, leading to a drop in the transmembrane potential.

Molecular dynamics simulations have shown that in a double bilayer of dimyristoylphosphatidylcholine system with a sodium charge imbalance, a pore begins to be formed within the first nanoseconds in one of the two bilayers. As the ions cross the bilayer, the field decreases and the permeation of the sodium ions and the pore radius decrease, leading to a stable pore for several nanoseconds, with a remaining charge imbalance of only one sodium ion. If this residual charge imbalance is removed while the pore is still stable, the pore remains open for a few additional nanoseconds and then closes in less than 10 ns. This indicates that if the transmembrane voltage is lower than a certain threshold, the pore becomes metastable and independent of the fall of the transmembrane potential. In this condition where the transmembrane voltage is below the threshold, the closure is associated with the pore size fluctuations, rather than with the disappearance of the charge imbalance (Gurtovenko and Vattulainen 2005). This is comparable with the simulations performed in size-controlled pores by electric field, where if the field

applied is lower than the E_s threshold, the pore shrinks and finally closes even in the presence of an external electric field (Fernández et al. 2012).

In the simulations performed to evaluate the charge imbalance effect on the transmembrane potential and the pore induction using either the method of the double bilayer or the vacuum method, as described in the previous example, when the pore opens, the ion gradient dissipates. When this happens, the transmembrane potential decreases and the pore cannot be longer sustained in time.

This drop in the transmembrane potential can be avoided by maintaining the initial imposed charge imbalance, by the swapping method (Kutzner et al. 2011). In this approach, if one ion crosses the membrane from its initial water reservoir to the other, this ion and a water molecule from the initial reservoir are swapped and the charge imbalance is restored. This method allows obtaining stable pores at different transmembrane potentials and a pore radius that is linearly associated with the transmembrane potential (Casciola et al. 2016).

Stable Pores Obtained by Mechanical Methods

The previous two sections of this chapter described the methods to open a pore and maintain it open in a stable way by molecular dynamics, which is by using external electric fields or the charge imbalance method. As previously mentioned, another method that can be used to form pores is that based on mechanical stress. This mechanical stress may be induced experimentally by the aspiration of the lipid structures of a membrane, a vesicle or cells into a micropipette. This mechanical stress produced by the aspiration, in other words, by a negative pressure, makes possible the rupture of the membrane in the form of a pore, equivalent to the electropores obtained by exposure to electric fields (Kirsch and Böckmann 2015).

Both the opening and closure of a pore can be considered to be due to the interaction of two tensions: one aiming to maintain the pore closed (the so-called line tension) and the other aiming to open the pore (the so-called surface tension). The latter causes a stretching of the membrane. Then, the aspiration of the micropipette causes a negative pressure, causing a surface tension at the level of the membrane. The energy of formation of the pore (E_{pf}), according to the theory of pore formation, is defined by the following equation:

$$E_{pf} = 2\pi R T_L \pi R^2 T_S$$

where R is the radius of the pore, T_L is the line tension, and T_S is the surface tension (Barnett and Weaver 1991).

This equation of pore formation shows the energy necessary to open a pore, which is the difference between the energy produced by the line tension and the surface tension components, in a way that, if the line tension is maintained, an increase in surface tension will result in a decrease in the energy needed to open or maintain a pore open.

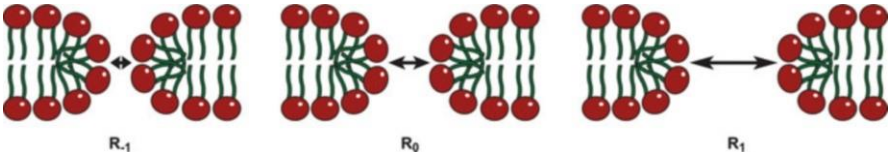

Fig. 4 Three scenarios where the radius R of the pore increases as the surface tension increases: R_1 when the surface tension is small and increasing, $R_0 > R_1$ when the surface tension reaches the critical threshold for tension, and finally R_1 when the surface tension is beyond the critical threshold for tension

In molecular dynamics simulations of a dipalmitoylphosphatidylcholine lipid bilayer membrane, a surface tension is applied, causing the formation of stable hydrophilic pores (Leontiadou et al. 2004). This mechanical stress on the membrane, under the form of a surface tension, was the result of the application of a lateral pressure, in this way simulating a pressure produced by the aspiration of a micropipette. Molecular dynamics simulations were performed in two different initial states: one using a bilayer membrane with a metastable pore, and the other using an equilibrated bilayer membrane without a pore. In both states, a threshold of critical surface tension was reported. Below this threshold, the pores remain stable, reaching a minimum radius in the order of 1 nm, at the center of a toroidal-shaped pore. If the critical threshold is exceeded, the pore results unstable, thus expanding, and finally causing the rupture of the membrane. Figure 4 shows three scenarios of the opening of the pore due to mechanical stress, in a transversal plane view, where the radius R of the pore is increased as the surface tension increases: R_1 when the surface tension is small and increasing, $R_0 > R_1$ when the surface tension reachesthecritical threshold fortension, andfinally R_1 when the surface tension is beyond the critical threshold for tension.

In another work, the study of the transport of ions across pores in the bilayer is combined with mechanical stress to stabilize pores with different radii. The presence of ions seems to induce a reduction of the stability of the pores, mainly because of the increase in the line tension. As stated before, this tension maintains the pore closed, and therefore, an extra mechanical stress is needed to stabilize the pore. This can be attributed to the binding of sodium ions at the interface between the lipids and water, in other words, the surface of the bilayer membrane (Leontiadou et al. 2007).

Pore Stability and Chemical Methods

The addition of amphiphilic agents to the system can affect the integrity of the membrane due to the chemical nature of these compounds. A small amphiphilic molecule such as dimethyl sulfoxide added to a dipalmitoylphosphatidylcholine bilayer can alter the normal structure of the bilayer. Three dimethyl sulfoxide concentration regimes are described to have different effects on the bilayer (Gurtovenko and Anwar 2007):

- At a low concentration, the molecules of dimethyl sulfoxide locate in the interface between the lipids and water molecules, inducing a lateral expansion of the bilayer and a decrease in the bilayer thickness.
- An intermediate concentration induces a decrease in the bilayer thickness and the dimethyl sulfoxide molecules locate in the interface as well as in the hydrophobic core of the bilayer, favoring the intrusion of water molecules to the inner part of the bilayer and leading to the stabilization of hydrophobic pores and then allowing the formation of hydrophilic pores.
- A high concentration leads to the rupture of the bilayer.

A molecular dynamics simulation work performed in dioleoylphosphatidylcholine bilayers in the presence of different amounts of cholesterol combined the effect of different concentrations of dimethyl sulfoxide with the presence of an external electric field. The analysis of the results showed that the dimethyl sulfoxide can facilitate the stability of the hydrophobic pores and diminishes the electroporation threshold at very low concentrations of dimethyl sulfoxide even in the presence of cholesterol (Fernández and Reigada 2014).

The effects of another amphiphilic molecule, the polyoxyethylene glycol, on membranes can be analyzed by molecular dynamics and compared with experimental data. The results obtained using the method of charge imbalance to induce pores showed that polyoxyethylene glycol seems to favor the formation and stabilization of the water columns, even allowing the ion transport across the bilayer through these stabilized pores. In a later stage, a few lipid molecules migrate to increase the pore stabilization. The presence of this molecule lowers the electroporation threshold (Polak et al. 2015). This finding is in line with the results described in previous experiments performed in planar lipid bilayers electroporated in the presence of polyoxyethylene glycol (Troiano et al. 1998).

Pore Stability by Continuum Modeling

An approach different from molecular dynamics to simulate the effects of electroporation is the continuum modeling of a cell exposed to an electric field (Son et al. 2014). Two different pulses are included in the model: a low-intensity long pulse, in the order of microseconds, and a high-intensity nanosecond pulse. In this model, the lifetime of the pore is a parameter in the order of seconds. The results obtained using this approach show that the long pulses lead to populations of pores with a wide range of sizes distributed in two major subpopulations with radius of approximately 1–10 nm and that the short pulses produce only one population of small size pores but in high number. This model indicates that the differences in the size of the radii of the pores obtained for each pulse type are related to the association of the radius with the duration of the pulse, because the pores can only grow during the application of the pulse. This is consistent with the results obtained in molecular dynamics simulations where, if the electric field applied exceeds the threshold of the sustaining electric field, the pore keeps growing. On the other hand,

the model also explains the differences in the number of pores formed by each pulse, associating the dependence of the pore creation on the transmembrane voltage. So, in this model, high-intensity pulses induce a greater number of pores (Son et al. 2014).

Another model simulating cells in 3-dimensions takes into account the conducting and the permeable states separately in the calculation to simulate the effects on the membrane and the transport due to the application of an external electric field. These states depend, among other parameters, on poration characteristic time, permeabilization dynamic, and membrane recovery time, all of them inputs of the model. In this model also, the pores open when a voltage threshold is exceeded (Leguèbe et al. 2014).

Pore Stability in Planar Membranes

Pore stability can also be studied in planar membranes. In these experiments, a planar bilayer lipid membrane is formed, separating two water reservoirs, and then the effects of membrane poration are analyzed under current-constant conditions. In a work performed by Koronkiewicz et al., the constant current causes an increase in the transmembrane potential and the following membrane poration. Once the current applied reaches the membrane threshold, the potential increases up to 250 mV and then keeps varying around 150 mV for several nanoseconds. These potential variations can be attributable to the generation of a pore with a fluctuating size in the order of nanometers. In a different approach, the current is maintained for 10 ns and then interrupted for few nanoseconds during several on-off current cycles. In this approach, the pore keeps its radius stable for the whole experiment. If the interval of interruption of the current is increased up to 100 s, the pore still remains stable in size, but at higher interruption periods (300 s), the pore disappears and the continuity of the membrane is fully restored (Koronkiewicz et al. 2002). This indicates that the stability of the pore is sustained even in the absence of the stimulus for a period of time, but in the end the membrane restores its initial continuity, as it occurs in molecular dynamics pore simulations.

Permeabilized State in Cells

This section describes different electroporation protocols for pore stabilization in experimental procedures in cells (▶Chap. 20, "Different Cell Sensitivity to Pulsed Electric Field"). Early works performed in unfertilized sea urchin eggs in a low-calcium medium, similar to the calcium concentration of the intracellular medium, showed that cells can be loaded with radiolabeled low molecular weight metabolites for several minutes. These works indicated that, in these conditions, the permeabilized state remains stable for this period of time (Swezey and Epel 1989).

In experiments performed by Pakhomov et al. in CHO cells preloaded with a thallium-sensitive fluorophore and exposed to nanopulses, thallium was added at different times after the application of the pulse. The measure of thallium uptake due to electroporation indicates that the nanopores remain open for several minutes after the application of the electroporating stimulus (Pakhomov et al. 2009).

Conclusions

Molecular dynamics simulations show that pores in a lipid bilayer membrane can be obtained and stabilized either by electrical or mechanical stress, or by addition of chemical agents, such as amphiphilic molecules.

In either electrical or mechanical stress, the simulations show that the external stimulus has to reach a minimum electric field or mechanical stress, in order to initiate the pore opening, and then the stimulus has to be maintained lower than a critical value to stabilize the pore.

The addition of amphiphilic molecules to the simulated system facilitates the stabilization of the water columns, which initiate the pore formation and decrease the electroporation threshold.

The approaches using molecular dynamics establish that the modulation of the external stimulus in values below the threshold modifies the size and keeps the pores stable, while the stimulus is still present. In the case that the stimulus is ceased or the intensity is lower than a sustaining value, the pore closure begins.

The approach to simulate the effects of an electric field on cells with the continuum modeling is less detailed than molecular dynamics but more versatile in terms of incorporating parameters as inputs of the model to describe the electroporation process.

The experiments made on planar membranes show that the pore stability can also be sustained in time.

The experiments of electroporation in cells show that the permeabilized state can be detected for several minutes, thus indicating higher stability of this state when the experiments are performed in cells.

The stability of the pores or the permeabilized state is related to the complexity of the system and may range from nanoseconds in molecular dynamics simulations to seconds in the continuum modeling and the planar membrane experiments under constant current conditions, and to minutes in cell experiments with pulses of different intensity and duration. Finally, the stability of the pores is due to the modulation of the stimulus, for both molecular dynamics and planar membrane experiments. In cells, the mechanisms that stabilize the permeabilized state after electroporation remain unknown. This could be attributed to the complexity of the plasma membrane and to the interactions of the plasma membrane with other components either inside or outside the cell.

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Cross-References

▶Critical Electric Field and Transmembrane Voltage for Lipid Pore Formation in Experiments

▶Different Cell Sensitivity to Pulsed Electric Field ▶Lipid Electropore Lifetime in Molecular Models

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