High Transmembrane Voltage Raised by Close Contact Initiates Fusion Pore

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Membrane fusion lies at the heart of neuronal communication but the detailed mechanism of a critical step, fusion pore initiation, remains poorly understood. Here, through atomistic molecular dynamics simulations, a transient pore formation induced by a close contact of two apposed bilayers is firstly reported. Such a close contact gives rise to a high local transmembrane voltage that induces the transient pore formation. Through simulations on two apposed bilayers fixed at a series of given distances, the process in which two bilayers approaching to each other under the pulling force from fusion proteins for membrane fusion was mimicked. Of note, this close contact induced fusion pore formation is contrasted with previous reported electroporation under ad hoc applied external electric field or ionic charge in-balance. We show that the transmembrane voltage increases with the decrease of the distance between the bilayers. Below a critical distance, depending on the lipid composition, the local transmembrane voltage can be sufficiently high to induce the transient pores. The size of these pores is approximately 1~2 nm in diameter, which is large enough to allow passing of neurotransmitters. A resealing of the membrane pores resulting from the neutralization of the transmembrane voltage by ions through the pores was then observed. We also found that the membrane tension can either prolong the lifetime of transient pores or cause them to dilate for full collapse. This result provides a possible mechanism for fusion pore formation and regulation of pathway of fusion process.

Keywords: membrane fusion, transmembrane voltage, fusion pore formation, exocytosis, mechanical force

INTRODUCTION

Membrane fusion, as an important and ubiquitous cellular process, lies at the heart of neuronal communication wherein neurotransmitters are quickly released from synaptic vesicles following fusion with the presynaptic membrane. During this process, an initial fusion pore is assumed to be created between the two apposed membranes, and this tiny pore may either expand to a larger one or close back, which leads to two kinds of fusion mode (Alabi and Tsien, 2013). One type of fusion mode called full fusion (FF) requires fusion pore expand to the point where the vesicle membrane flattens into the plasma membrane surface, leading to complete luminal content release (Chernomordik and Kozlov, 2003; Jackson and Chapman, 2008); the other type of fusion mode proceeds without pore expansion, where vesicles transiently fuse at the plasma membrane to release a part of their neurotransmitters (Richards, 2009) without full collapse into the plasma.
membrane known as “kiss-and-run” fusion (KR) (He and Wu, 2007; Zhang et al., 2009). Although, the experimental results on membrane fusion at synapses are mounting (He and Wu, 2007), it is difficult to directly image the fusion process at sufficient resolution, thus the molecular mechanism of the fusion initiation is still unclear.

In addition, it is also unclear how the fusion process is regulated in vivo. For instance, there is a long standing debate over FF and KR fusion for the regulating mechanisms behind the synaptic release (Marx, 2014). One of the important issues in the debate is an incomplete understanding of the structural mechanics of the membrane under the force of fusogenic proteins (Jahn et al., 2003; Rizo and Rosenmund, 2008), and how these forces act on the membrane during the fusion process. It is known that when a small membrane pore is created (Chernomordik and Kozlov, 2008), it might either close or expand (Chanturiya et al., 1997; Wong et al., 2007; Diao et al., 2012). The reversible fusion pore modality, corresponding to KR fusion, enables a rapid and economical vesicle recycling compared to FF that may require ancillary proteins to retrieve fused vesicles (He and Wu, 2007). However, how the fusion pore is regulated for the fast synaptic transmission and the exact molecular picture of the pore evolution remains obscure.

Transient pore formation has been observed in many in vivo and in vitro systems, such as liposomes from yeast vacuoles (Starai et al., 2007; Zucchi and Zick, 2011), mating yeast pairs (Aguilar et al., 2007), cell pairs mediated by influenza fusase hemagglutinin (HA) (Blumenthal and Morris, 1999; Frolov et al., 2003), and protoliposomes reconstituted with neuronal SNAREs (SNAP [Soluble NSF attachment protein] Receptors) (Dennisson et al., 2006; Lai et al., 2013; Gong et al., 2015). And there are also many proposed mechanisms for the opening of the membrane pores through in silico simulations, such as transmembrane ionic charge imbalance, external electric and mechanical forces (Müller et al., 2003; Gurtovenko et al., 2010). However, the questions of how transient pores are produced in physiological conditions, and how they are regulated remain enigmatic. Here, through full atom molecular dynamics (MD) simulations, we discovered a fast and transient membrane pore formation when the distance of two apposed bilayers was set below a threshold value. The average size of these pores was of 1–2 nm in diameter, which is large enough for passing neurotransmitters. The initial pore could either shrink or expand depending on the magnitude of membrane tension. The high local transmembrane voltage caused by the close contact of apposed membranes was responsible for the formation of these fast and transient membrane pores. This study might thus shed promising light on the molecular mechanisms of pore formation and evolution.

**METHODS**

**MD Simulation**

To simulate the pore formation at the contact of a vesicle with the synaptic membrane, we created two apposed lipid bilayers set at various distances (see Figure 1). The lipid bilayers were generated by Membrane Builder online service (Jo et al., 2008, 2009). The simulation system was set up following a similar procedure in our previous studies (Li et al., 2010, 2011, 2015; Lai et al., 2015; Xu et al., 2015). The system was solvated in a \( 23 \times 23 \times 20 \text{ nm}^3 \) TIP3P (Jorgensen et al., 1983) water box, with \( 210,000 \) water molecules and potassium ions added to neutralize the system. All the simulations were performed using GROMACS package (Van Der Spoel et al., 2005) with CHARMM36 force field (Klauda et al., 2010). Periodic boundary condition was applied and the temperature was coupled to 300 K with V-rescale algorithm (Bussi et al., 2007). The pressure was coupled to 1 bar with Parrinello-Rahman approach (Parrinello and Rahman, 1981). The LINCS algorithm (Hess, 2008) was applied to constrain the covalent bonds with H-atoms. The time step of the simulations is 2.0 fs. The cut-off of the non-bonded interactions was set to 10 Å. The particle mesh Ewald (PME) (Essmann et al., 1995) method was used to calculate the long-range electrostatic interactions. The non-bonded pairs were updated in every 10 steps. Each MD simulation was performed independently for three replicates. The simulation system was validated by its reproducing the stalk-like structure in experiments under dehydration condition (Yang and Huang, 2002). All graphics and visualization analysis were processed using the VMD program (Humphrey et al., 1996).

Here the lipid composition was adopted as Chol:DOPC:POPE:POPS with different ratio of percentage as (20:45:20:15%), (20:48:20:12%), (20:51:20:9%), (20:54:20:6%), and (20:55:20:4.5%). Chol, DOPC, POPE, POPS are abbreviation of cholesterol, 1,2-dioleoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, and 1-palmitoyl-2-oleoyl-sn-glycero-3-phenylserine, respectively.

In the molecular dynamics simulations, we carried out simulations on two bilayers set at various membrane distances, where the distance \( D \) was chosen by reducing its value from 6 nm, in 0.5 nm steps (see Figure S1), for mimicking the approaching process of two apposed bilayer under the pulling force of fusion protein for membrane fusion. Each simulation was done at a given constant membrane distance.

The ions were uniformly distributed in the three cytosol/ﬂuid regions, i.e., the vesicle cytosol, cellular cytosol between the vesicle and presynaptic membranes, and the interstitial fluid around the synapse, as shown in Figure S1. The ionic concentration (potassium) was about 0.04–0.15 mol/L, depending on the lipid composition and their percentage. The density of water was 1 g/cm³. Note that for specific lipid composition, the density of water and ions was kept constant. In the simulation, we chose potassium ions as the only ions in the system without chloride ions. For double check, we also did simulations with both potassium and chloride ions. In the presence of chloride ions, we put more potassium ions to neutralize the system. Our results showed that they had similar results as those in the absence of chloride ions.

For simulating the pore evolution under membrane tension, we built membrane tension \( \Gamma \) by applying pressure on the simulation box, given by \( \Gamma = L_Z (P_Z - P_{Lat}) \), where \( L_Z \) is the length of the simulation box in the Z direction, \( P_Z \) is the pressure along the \( Z \) direction, and \( P_{Lat} \) is the pressure along lateral direction in the membrane plane (Leontiadou et al., 2004).
**Theoretical Model of Electric Potential**

In our model, we used five layers to consider the five regions at the contact between the vesicle and presynaptic membrane: the cytosol in vesicle, $R_1$, the vesicle membrane $R_{m1}$, the cellular cytosol between vesicle and presynaptic membrane, $R_2$, the presynaptic membrane $R_{m2}$, and the interstitial fluid around synapse, $R_3$ (see Figure 2). Since we considered the local contact region between vesicle and presynapse, we assumed that the distribution of charges was uniform in each layer. For a neutralized system, the charge density should satisfy

$$\rho_1 H_L + \rho_3 H_L + \rho_2 D + \rho_{m1} H_m + \rho_{m2} H_m = 0$$  \hspace{1cm} (1)$$

where $\rho_1$, $\rho_2$, $\rho_3$, $\rho_{m1}$, and $\rho_{m2}$ are charge density in layers $R_1$, $R_2$, $R_3$, $R_{m1}$, and $R_{m2}$, respectively. $H_L$ is the thickness of layers $R_1$ and $R_3$ (here we adopted that the thickness of the two domains is identical), $H_m$ is the thickness of membrane, and $D$ is the distance between the two apposed membranes. In this situation, the electric potential satisfies the one dimensional Poisson-Boltzmann equation,

$$\frac{d^2 \Phi(Z)}{dZ^2} = -\frac{\rho(Z)}{\varepsilon}$$  \hspace{1cm} (2)$$

where $\Phi(Z)$ is the electric potential along the $Z$ direction, $\rho(Z)$ is the charge density and $\varepsilon$ is the dielectric constant. We set $\varepsilon = 3$ according to experimental measurement by Gramse et al. (2013). And we obtained the electric potential as

$$\Phi(Z) = -\frac{1}{\varepsilon} \int_{0}^{Z} \int_{0}^{Z'} \rho(Z'') dZ'' dZ'$$  \hspace{1cm} (3)$$

The result of the integral in Equation (3) can be found in Supplementary Material. Thus the transmembrane voltage of the vesicle membrane can be calculated as

$$\Delta V_1 = -\frac{1}{\varepsilon} \left( \frac{1}{2} \rho_{m1} H_m + \rho_{m2} H_m + \rho_2 D + \rho_3 H_L \right) H_m$$  \hspace{1cm} (4)$$

and the corresponding voltage of the presynaptic membrane is

$$\Delta V_2 = -\frac{1}{\varepsilon} \left( \frac{1}{2} \rho_{m2} H_m + \rho_{m1} H_m + \rho_2 D + \rho_1 H_L \right) H_m$$  \hspace{1cm} (5)$$

As shown in Equations (4 and 5), because $\rho_{m1}$ and $\rho_{m2}$ are negative, $\Delta V_1$ and $\Delta V_2$ linearly increase with the reduction of the inter-membrane distance $D$. If we assume $\rho_1 = \rho_3$, and $\rho_{m1} = \rho_{m2} = \rho_m$, we have

$$\Delta V = \Delta V_1 = \Delta V_2 = -\frac{1}{2\varepsilon} \left( \rho_m H_m + \rho_2 D \right) H_m$$  \hspace{1cm} (6)$$
RESULTS

Close Contact Induces Transient Pore Formation

To understand the very early stage of membrane fusion when the vesicle and presynaptic membranes get close, we simulated two apposed membranes fixed at a series of given distances using MD simulations, mimicking the docking process pulled by SNAREs or other accessory proteins, as shown in Figure 1. By using a smaller distance between membranes below a critical value (discussed below), we found the formation of tiny pores on the membranes (Figure 3). When the two membranes were within ~5 nm, a water line first formed across the thickness of membrane in several nanoseconds, followed by the formation of a transient pore, like a water channel, spanning the membranes (Figure 3B). Then the transient pore grew rapidly, causing considerable redistribution of lipid headgroups close to the channel. At the same time, ions can transport through these pores, as shown in Figure 3B. The duration of the ion transportation was very short (approximately 10 ns). Once the ion transportation was finished, the pores healed automatically as the results of balancing charge difference (Figure 3C). More details of the pore formation, ions leakage and pore healing can be found in Figure S2 and Movie S1 in Supplementary Material. To examine the physiological relevance of our simulations, the membrane distance was further reduced to mimic a higher degree of dehydration, which induced a stalk-like structure for hemifusion. This result is consistent with previous experiments of Yang and Huang (Yang and Huang, 2002) (see Figure S3) and validates our MD simulations.

To study the molecular mechanisms of the pore formation, we calculated the electric potential in our system. Our results showed that the distribution of electric potential is highly dependent on inter-membrane distance. As the distance between the two membranes decreased, the difference of electric potential across the lipid bilayer, i.e., the transmembrane voltage, rose significantly (Figure 3D). This transmembrane voltage formed the driving force for the pore formation, consistent with earlier reports that the transmembrane voltage caused by ionic charge imbalance between the two sides of the membrane (Gurtovenko and Vattulainen, 2005), or by external electric field (Tieleman, 2004; Sun et al., 2011), induces fusion pore and ion trafficking. Meanwhile, in previous studies, the transmembrane voltage was ad-hoc applied on the membrane (Tieleman, 2004; Gurtovenko and Vattulainen, 2005; Sun et al., 2011). Here we found that without the above ad hoc conditions, reducing the membrane distance alone was sufficient to cause a dramatic increase in transmembrane voltage capable of producing the driving force necessary for the fusion pore formation. Our simulation showed that the transmembrane voltage for pore formation produced by the close contact is in the range of 0.7–1.2 V, consistent with the value of 0.5–1.5 V used in electroporation experiments (Weaver and Chizmadzhev, 1996). The mean size of the pores was round 1–2 nm (see Figures 3G–I), which is large enough for the passing of small neurotransmitters (Zhang et al., 2009).

Transmembrane Voltage vs. the Membrane Distance

To understand the mechanism of transmembrane voltage rising with the decrease of membrane distance, we employed a physical model to derive the field of electric potential in the apposed membranes as a function of the membrane distance (Figure 2). For a given ion distribution corresponding our MD simulations, we obtained the analytical solution of the field of electric potential and in particular, a linear relationship between the transmembrane voltage and the membrane distance for a close contact (see Methods). We found that when the membrane distance was less than ~5 nm, the profile of the electric potential in each membrane became severely asymmetric with respect to the central line of the lipid bilayer thus producing a high transmembrane voltage (Figure 4A). However, after the ion leakage, the profile of the electric potential almost resumed its symmetry, resulting in a reduced transmembrane voltage (Figures 3F, 4A). This prediction is consistent with observations from our MD simulations (Figure S4). Moreover, it clearly indicates that the transmembrane voltage, produced by the close contact of the apposed membranes, was the driving force for the transient pore formation.

Threshold Values of Membrane Distance

To find the threshold value of membrane distance for the pore formation, we performed a series of MD simulations at different membrane distances. We defined the critical distance as the
distance above which the pore formation will not happen in up to 20 ns in our simulation (Figure 4B). When the distance, D, was larger than a critical value (~5 nm), the voltage was nearly zero because of the symmetry of the field of electric potential in the membrane. But as the distance decreased, the voltage increased, being negatively proportional to the distance (see Equation 6 in Methods). Referring to our MD simulations, a series of membrane distance D was chosen by reducing its value from 6 nm, in 0.5 nm steps, for the lipid composition Chol:DOPC:POPE:POPS (20:45:20:15%). In these settings, we found that the transmembrane voltage produced by the close contact could be up to >3 V, consistent with previous results produced by the transmembrane ionic charge imbalance (Gurtovenko and Vattulainen, 2005). We found that when the distance was larger than 5 nm, there was no transient pore formation. But when D became smaller, pore formation could be observed. In addition, the smaller the distance is, the faster the pore could form (Figure 4B, blue line). These results suggest there is a threshold value for the inter-membrane distance required for the transient pore formation.

**Effect of Lipid Composition on Fusion Pore Formation Threshold**

To investigate the contribution of the lipid composition in fusion pore formation, we modified our simulations to adopt various lipid compositions by changing the percentage of POPS, which modulates the charge density on the membrane. Our results showed that the threshold value of membrane distance for pore formation was highly dependent on the percentage of POPS (Figure 5). For example, we found that reducing the percentage of POPS in both membranes generally reduced the threshold value due to dissipated charge density of the membrane. The lower the percentage of the POPS, the smaller the critical distance needed, and thus the more difficult the pore formation. This result also emphasizes why two membranes should be brought close together for fusion: only a membrane
distance smaller than the threshold value can produce a sufficiently high transmembrane voltage that can induce pore formation.

Note that we did not consider the asymmetry of lipid composition between the inner and outer leaflet of cell membrane (Svennerholm, 1968; Devaux, 1991). Although, the asymmetry may affect the process of fusion pore formation, it is a secondary factor in the membrane fusion in comparison with the close contact between the membranes. Heterogeneity of lipid composition may also be an influencing factor in the fusion process. For instance, phosphatidylinositol-4,5-bisphosphate (PIP2), a lipid with -5e negative charges, is an important composition that accumulates at the location of fusion site (McLaughlin and Murray, 2005; Graber et al., 2014). But the localized PIP2 mainly interacts with syntaxin proteins during the course of membrane fusion. And the function of fusion protein syntaxin is to pull the two apposed membranes to be close contact, which we had effectively considered by applying pulling force on the membranes. To test the influence of the asymmetric lipid composition and localized PIP2, we performed an additional simulation with 5% PIP2 in the cytoplasmic leaflet which make the two membranes asymmetric, as shown in Figure S5. It shows that the close contact of the two membranes containing localized PIP2 could still give rise to a high local transmembrane voltage which induced the pore formation.

**Force Regulates the Pore Evolution**

During membrane fusion, fusion proteins apply not only vertical force to pull the vesicle close to the presynaptic membrane, but also lateral force along the membrane which produces a local membrane tension. To mimic this behavior of fusion proteins, we imposed a lateral force on the membrane once the pores were initiated, to study how the local membrane tension regulates its structural evolution. We found that when the tension was small (20.6 pN/nm), the membrane pore shrank and resealed quickly in less than 20 ns. However, when the tension was increased (24.9 pN/nm), the resealing process of
The opening of the fusion pore, initiated from a voltage-dependent perturbation of lipid organization at the contact of apposed bilayers, begins with a lateral parting of headgroups on two membranes for allowing water molecules to enter the hydrophobic regions (Helm et al., 1992; Fesce et al., 1994). Although, the transmembrane voltage can be produced by an ad hoc ionic charge imbalance across membranes (Gurtovenko and Vattulainen, 2005) or external electric field (Tieleman, 2004; Sun et al., 2011), how cells modulate the ionic charge imbalance or electric fields in order to produce the required voltage is not known. In this study, we showed that the transmembrane voltage can be generated by reducing the distance between two membranes, an action that could be carried out by fusion proteins, such as SNAREs or the calcium sensor, synaptotagmin, etc., (Diao et al., 2009, 2013; Kyoung et al., 2013; Lai et al., 2014). Our data agrees with a growing body of work suggesting that pore formation, stability, and evolution can be subject to regulation by cellular processes rather than a stochastic thermodynamic process (Wang et al., 2009; Risselada and Grubmüller, 2012).

In agreement with previous studies, our results further support the notion that native proteins are capable of modulating fusion pore dynamics with additional helps from other proteins producing differential layers of modulation (Alabi and Tsien, 2013; Lai et al., 2015). We found that the fusion pore dynamics appeared to be finely modulated by the type and magnitude of mechanical force exerted on membrane. A vertical force pulls the vesicle close to the plasma membrane, increasing the local voltage difference resulting in more pore formation. A further increase of the vertical force produces a closer contact for to a stalk-like structure for hemifusion (Figure S3), as observed in previous experiments (Yang and Huang, 2002; Zhao et al., 2016). In contrast, we found that the membrane tension at the contact site induced by the lateral force stabilizes pore formation and increases pore duration, while a larger force could induce pore expansion (Figure 6). These results suggest that the fusion dynamics could be regulated by the force of fusion proteins, and by extension, so as the rate of neurotransmitter diffusion out of the synaptic vesicle (Richards, 2009; Zhang et al., 2009; Alabi and Tsien, 2013). Since KR fusion and hemifusion are intermediate states between prime (un-fused) state and FF, the mechanical forces produced by fusion machinery proteins could select among KR, hemifusion, and FF (Figure 7), to modify the kinetics of neurotransmitter release which could ultimately provide a mechanism to regulate synaptic strength and achieve synaptic plasticity (He and Wu, 2007).

In conclusion, we provided a new mechanism for the initiation of fusion pore and its evolution through MD simulations of two membranes being set close together. Subsequent fusion pore initiation occurred within several nanoseconds once the membrane distance was smaller than ~5 nm. A high transmembrane voltage induced by close membrane proximity could cause these fast-forming transient membrane pores. The fusion pores are able to conduct ions across membrane and in doing so, alleviate the voltage difference which leads to

| Time (ns) | Membrane tension (pN/nm) |
|----------|--------------------------|
| 0        | 20.6                     |
| 10       | 24.9                     |
| 20       | 32.6                     |

The changing of the pore size as function of the membrane tension (right column) of 32.6 pN/nm leads to dilation of the pores. A large membrane tension (left column) of 20.6 pN/nm leads to dilation of the pores. The lipids of membrane are represented by cyan, while the fusion pores are represented by blue.

**FIGURE 6 | Evolution of fusion pores under membrane tension.**

(A) Snapshots of the evolution of fusion pores under various degrees of membrane tension. A small membrane tension (left column) of 20.6 pN/nm allows quick resealing of the pores in 20 ns, which leads to fast transient pores. A medium membrane tension (middle column) of 24.9 pN/nm stabilizes the pores and prolongs the duration of the transient pores. Finally, a large membrane tension (right column) of 32.6 pN/nm leads to dilation of the pores. The lipids of membrane are represented by cyan, while the fusion pores are represented by blue. (B) The changing of the pore size as function of the simulation time at different membrane tension. (C) The final size of fusion pore at t = 20 ns.

The fusion pore became slower. When the tension was further increased (32.6 pN/nm) the fusion pore started to expand and the vesicle began to collapse into the membrane (Figure 6). In our simulations, the first two cases, where the membranes resealed, are correspond to the scenarios of fast and slow transient pore for KR fusion, while the third case corresponds to the pore dilation of FF. This result clearly indicates that the stability and evolution of the fusion pore, and the selection of fusion pathways between KR fusion and FF, could be regulated by the membrane tension.

**DISCUSSION**

This study suggests a feasible mechanism for pore formation in membrane fusion: a close contact of membranes could generate a high transmembrane voltage, which in turn produces the membrane pores. Note that the close-contact induced voltage observed in our simulation is contrasted with the ad hoc applied voltage in previous electroporation experiments (Weaver and Chizmadzhev, 1996).
Bu et al. Membrane Fusion Pore Initiation and Regulation

FIGURE 7 | Illustration of pore formation, evolution and selection of fusion mode proposed by our MD simulation. (A) Close contact of vesicle with presynaptic membrane pulled by the fusion proteins. (B) Pore formation and associated release of neurotransmitters once the membrane distance, D, is smaller than a critical value. (C) Resealing of the fusion pores after the release of neurotransmitters for a KR fusion. (D) Formation of stalk-like structure for hemifusion appears when the membrane distance is further reduced. (E) Full fusion can be achieved by either expansion of the fusion pore (B under a larger lateral force), or following the hemifusion (D).

final resealing of the membranes within a few nanoseconds. These sequential processes of pore formation, ion exchange, and pore healing in our MD simulations are consistent with the features of KR fusion observed in experiments (Zhang et al., 2009). In addition, we showed that the applied force on the membrane from native proteins plays a crucial role in modulating the stability and lifetime of fusion pores and may regulate the ultimate fusion mode to either KR fusion or FF. Therefore, both the pore formation and evolution for membrane fusion are tightly controlled by fusion proteins in physiological conditions.

AUTHOR CONTRIBUTIONS
DL and BJ designed research; BB, ZT, and DL performed research; BB, ZT, DL, and BJ analyzed data; BB, ZT, DL, and BJ wrote the paper. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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