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Abstract

The pore-forming toxin lysenin self-inserts to form conductance channels in natural and artificial lipid membranes containing sphingomyelin. The inserted channels exhibit voltage regulation and hysteresis of the macroscopic current during the application of positive periodic voltages stimuli. We explored the bi-stable behavior of lysenin channels and present a theoretical approach for the mechanism of the hysteresis to explain its static and dynamic components. This investigation develops a model to incorporate the role of charge accumulation on the bilayer lipid membrane plays in influencing the channel conduction state. Our model is supported by experimental results and also provides insight into the temperature dependence of lysenin channel hysteresis. Through this work we gain perspective into the mechanism of how the response of a channel protein is determined by previous stimuli.

Keywords: Lysenin, Voltage gated channels, Hysteresis

1. Introduction

Pore-forming proteins are a class of membrane transporter that provides an aqueous pathway through the hydrophobic region of a Bilayer Lipid Membrane (BLM). A number of these channels possess the ability to regulate ion and molecular transport by restricting the channel lumen by way of a gating mechanism. In this paper we investigate the mechanisms responsible for the voltage-induced gating and consequential hysteresis exhibited by lysenin channels. We present a model that addresses the static and dynamic nature of lysenin channel hysteresis and is able to recognize the mechanisms involved that contribute to the observed phenomena. Our experimental observations suggest an interaction between the protein channel and the bilayer membrane which are supported by our model.

Lysenin is a 297 amino acid pore-forming protein that self-assembles ~3 nm diameter channels in BLMs containing sphingomyelin [1-4]. Experimental investigations with lysenin channels inserted into a vertical planar BLM have shown that the channels responded to an applied voltage by blocking the ionic current when the electric field was oriented opposite to the

direction of protein insertion [1]. Recently, the structure of lysenin has been uncovered and has provided some insight into the charged regions of the protein which we believe to be responsible for the gating [5]. Using this information, we assembled a model that describes a mechanism for the origins of lysenin gating and provided an explanation for the dynamic and static hysteresis.

2. Channel Gating and Hysteresis

The voltage-induced gating observed by lysenin channels is thought to be the result a change in the channel conformation due to the interaction between a voltage-sensitive element on the protein and the applied electric field. The channel inactivation is observed as a decrease in macroscopic ionic current as the channel transitions from a conducting state to a non-conducting state as shown in black in Fig 1a. The current increases linearly with increasing voltage until the channels begin to transition into the closed state where the I-V curve breaks its linearity. A more detailed explanation of the channel gating has been previously published [1].

The observed channel behavior shown in Fig 1a is the result of a population of channels responding to applied voltages. On an individual channel level, the transition of a channel's conduction state is a dynamic equilibrium governed by a voltage-dependent probability. To examine the dynamic equilibrium, we investigated the gating of lysenin channels over time in response to a constant 25 mV applied voltage. We observed that the channels continuously closed and reopened as shown in Fig 1b, characteristic of a dynamic equilibrium.

Even though channel reactivation during a voltage stimulus was observed, that did not explicitly imply a dynamic equilibrium. If the channel reactivation occurred as a result of random thermal fluctuations, it would be expected to be independent of voltage. To further analyze the dynamic behavior of channel gating, the reopening events were cataloged at different applied voltages shown in Fig 1c.

The histogram of the reopening events recorded for individual channels shows a strong correlation with the applied voltage. The channels continued to close and reopen as the result of thermal energy, but the applied voltage reduces the probability of a channel reopening.

The dynamic equilibrium between the two states of the protein give rise to the voltage-dependent probability of finding the channel in the open (conducting) state, which can be described by Boltzmann's statistics as:

$$(1) P_{open} = \frac{1}{1 + e^{\frac{-\Delta E + qV}{kT}}}$$

Where ΔE is the difference in the energies between the open and closed states, q is the gate charge on the protein, V is the applied voltage, k is the Boltzmann constant and T is the temperature. The observed current in Fig 1a produced by the applied voltage across the BLM yields an ionic current given by Ohm's law as:

$$(2) I = VgNP_{open}$$

Where g is the conductance of a single channel, N is the total number of channels. It follows that the observed current for increasing voltages can therefore be described as:

$$(3) I = \frac{gVN}{1 + e^{\frac{-\Delta E + qV}{kT}}} + G_L V$$

Where G_L is added as the leakage conductance observed when the open probability approaches zero. Equation 3 produces an excellent fit to the lysenin channel inactivation data for a population of channels shown in green in Fig 1a. Additionally, the model accurately describes

single channel behavior, also shown in green in Fig 1c, verifying that the observed behavior is the result of a dynamic equilibrium.

Channel reactivation was observed upon decreasing the applied voltage and measuring the macroscopic current as shown in red in Fig 2a. However, the reactivation curve takes a different reaction pathway than inactivation until all of the channels return to the open state. The two distinct I-V curves result in an observed hysteresis in the macroscopic current as previously reported [6].

This hysteresis behavior was observed when the voltage sweep rate exceeded the characteristic time of the channels and is not observed or predicted for ion channels [7,8]. Initial speculation into the mechanism of “static” hysteresis pointed towards an analogous mechanism to that of a ferroelectric in which dipoles flip in direction and are locked into a polarization state by dipole-dipole interactions that must be overcome in order to flip the dipoles back. However, protein-protein interactions were ruled out because hysteresis was observed in single channel measurements indicating that the hysteresis is not due to neighbor-neighbor interactions [6]. Another possibility is that the hysteresis is due to a conformational change. However, given the clear evidence that the channel opening and closing is a probability processes, reopening of the channel would follow the same equilibrium probability distribution and closing the channel for a given applied electric field.

Deviating from the hypothesis that changes in the protein occur during the state transition, we focused on the BLM itself for origins of the hysteresis. To examine this possibility, the open probability given by Eq. 1 can be plotted for the inactivation and reactivation I-V curves shown in Fig 2b.

Analysis of the channel open probability for increasing and decreasing voltage ramps shows that the open probability of the decreasing voltage follows the same profile as the increasing voltage ramp, but it is shifted to lower voltages by δV . This suggests that the channels do not respond immediately to the decrease in voltage. To investigate this behavior, we turned our attention to the electric fields present in the BLM.

3. Role of BLM in Hysteresis

The BLM is bathed in an electrolyte solution, so the voltage applied by the electrodes is transferred to the membrane by the ions in the solution. The voltage drop from the electrolyte solution was measured to be approximately 10,000 times less than that of a single channel. Therefore, the electrode voltage was approximately equal to the voltage across the membrane. The ions in the solution provide the free charge which produces an electric field through the dielectric BLM as shown in Fig 3.

The electric field external to the BLM from the ions is $\mathbf{E}_{\text{ext}} = (\sigma_f/\epsilon)$, where σ_f is the surface charge density of the ions surrounding the BLM. The external field results in a polarization of the BLM given by $\mathbf{P} = \epsilon_0\chi\mathbf{E}_{\text{ext}}$, where ϵ_0 is the permittivity and χ is the electrical susceptibility of the BLM. The induced electric field therefore is $\mathbf{E}_{\text{BLM}} = -(\sigma_b/\epsilon)$ where σ_b is the bound surface charge density of the induced dipoles in the membrane. The electric field inside the BLM can be given as the sum of the external and dipole electric fields which can be written as a function of the external field

$$(4) \mathbf{E}_{\text{int}} = \mathbf{E}_{\text{ext}}/(1 + \chi)$$

According to Eq. 4, the external electric field is greater than the internal field by a factor of the dielectric constant of the BLM. The internal electric field is the net field that interacts with

a charged component on the protein to close the channels. Reversal of the external field produces a reversed internal field, but does not induce gating as shown for the negative voltages [1]. The induced dipoles inside the membrane provide the fixed charge in the BLM that interact with the free charge in solution. It is the coulombic interaction between the free charge and fixed charge that we hypothesize to be the origin of the observed hysteresis behavior. Additionally, since the value of δV was found to be independent of the number of channels we rule out the possibility that the polarization of the protein plays a significant role in the hysteresis.

Starting at low voltages, the increasing voltage applied across the electrodes is equal to the voltage across the BLM. The free charge in solution collected along the electrolyte/membrane interface, while the electric field polarized the BLM. An electric field arises at the interface between the free charge and fixed charge, E_i , as shown in Fig 4a. The total charge on the electrode, Q , is equal to the total charge built up on the BLM surface. Since the electrolyte resistance was measured to be negligible, the voltage across the electrodes is nearly equal to the voltage across the BLM and the electric field in the bulk solution is 0.

Upon decreasing the voltage across the electrodes, the charge in the electrodes is reduced by ΔQ . The free charge, however, is not decreased due to the coulombic interaction with the fixed charge. An electric field is produced due to the difference in voltages, $E(\Delta Q)$, as shown in Fig 4b. The magnitude of $E(\Delta Q)$ is less than E_i , so the subsequent force pulling the free charge away from the membrane is too small to separate the charges. Consequently, the internal field acting on the channels remains constant.

The reduction in the electrode voltage is observed in the I-V curve shown in Fig 4c. The decreasing voltage curve (red) deviates from the increasing voltage curve (black) where the leakage current term dominates in Eq. 3. The channels have not started to reopen because the internal electric field has not decreased. This is the beginning of the lag between the applied electrode voltage and the voltage producing the internal field. Upon further reduction in the electrode voltage, shown in Fig 4d, the charge in the electrode is reduced by some factor $2\Delta Q$. The electric field in the bulk solution becomes large enough that the force produced can overcome the force between the free charge and the fixed charge. Reducing the number of free charges at the interface reduces the internal electric field acting on the lysenin channel. Consequently, the reduction in E_{int} allows the reactivation of channels which is observed as an increase in the ionic current shown in Fig 4e. Continuing to decrease the electrode voltage increases the open probability, shown in Fig 2, resulting in two values of voltage which produce the same current. The true voltage, V_T , shown in Fig 4e is the voltage across the BLM which is responsible for the change in the open probability indicated in Eq. 3. The apparent voltage, V_A , in Fig 4e is the voltage across the electrodes that was recorded in the I-V curve. As the voltage decreases further, channel reactivation causes the red dashed current response shown in Fig 4e. Reversal of the current to increasing once again will cause the I-V curve to retrace the inactivation (black) pathway.

The model used to describe the inactivation given by Eq. 3 does not describe the observed hysteresis. Equation 3 assumes that the applied electrode voltage and the BLM voltage are equal throughout the experiment. Since the electrode voltage and the BLM voltage lag one another resulting in a delayed reactivation response, the model should reflect that behavior. The voltage lag, δV , causes a delay in the open probability of the lysenin channels, not the current measured in the I-V curve. Therefore, the open probability given in Eq. 1 becomes:

$$(5) P_{open} = \frac{1}{1 + e^{\frac{-\Delta E + q(V + \delta V)}{kT}}}$$

The voltage dependent term has been adjusted for the lag in voltage. Likewise, the current described by Eq. 3 changes to:

$$(6) I = \frac{gVN}{1 + e^{\frac{-\Delta E + q(V + \delta V)}{kT}}} + G_L V$$

Equation 6 now describes the reactivation current of the hysteresis accounting for the shift in the open probability due to the voltage lag.

We have shown that Eq. 3 produces an excellent fit to the inactivation part of the hysteresis curve, and using the same parameters for the open channel conductance, ΔE , and q we can fit the reactivation curve to the model.

4. Temperature

As further verification of our model, we examined the temperature dependence of the hysteresis. Previous work has demonstrated that the voltage gating behavior is temperature dependent, as would be expected in a dynamic equilibrium [6]. Our model predicts that the δV should decrease at higher temperatures, and at a critical temperature (T_c), the hysteresis should disappear altogether ($\delta V = 0$). Using the experimental procedures described elsewhere, the temperature was varied over the range of 10°C to 40°C after BLM formation and lysenin channel insertion [6].

As described, the free charge in solution is held at the BLM/electrolyte interface by the coulombic interaction with the fixed charge. Taking into account the free charge's thermal energy, we assume there is a temperature at which the thermal energy of the free charge is high enough to overcome the coulombic interaction without help from the electrodes. Hence, no force from the electrodes ($F_{\delta V}$) would be required for the free charge to escape from the interface. Assuming a spring-like restoring force proportional to the average motion due to temperature, we find that the force required to pull the free charge away from the BLM is directly proportional to δV . The thermal energy of the free charge allowing it to escape the BLM interface is related to δV by the square root of the temperature. Therefore, a plot of δV versus $T^{1/2}$ is predicted to be linear, with T_c being the point where $\delta V = 0$, as shown in Fig 6.

As expected, the difference between the inactivation and reactivation decreases with increasing temperature. Using this model, we expect the hysteresis to completely vanish at 321.61 ± 0.001 K as indicated in Fig 6. The complete disappearance of the hysteresis was not experimentally observed since it is outside the range of temperatures examined.

Our model is in agreement with experimental observations made with lysenin channels and provides an explanation as to the origins of channel gating and hysteresis. Further research will be required to corroborate our findings with measurements of the membrane polarization and by pinpointing specific regions within the channels that are responsible for channel gating. In the meantime, it is hoped that this work will bring about new discussions as to the origins and mechanisms of cellular memory to further our understanding of the basic functioning of biological systems.

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Figures

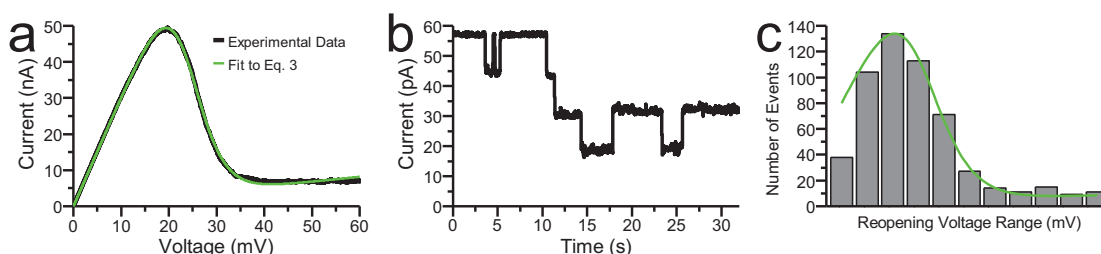


Fig 1. The behavior of lysenin channels resulting from applied positive voltages. (a) An I-V curve showing the measured current for increasing applied voltages (black) and the fit of Eq. 3 (green) to the experimental data where $\Delta E = 0.20 \pm 0.001 \text{ eV}$ and $q = 8.13 \pm 0.02e$ (g , N , and G_L were fit from the linear sections of the I-V curve) (b) Individual channel inactivation and reactivation is observed with an applied voltage of 25 mV. (c) A histogram of the number of channel reopening events as a function of applied voltage. The model given by Eq. 3 provides an excellent fit to single channel events as well (green).

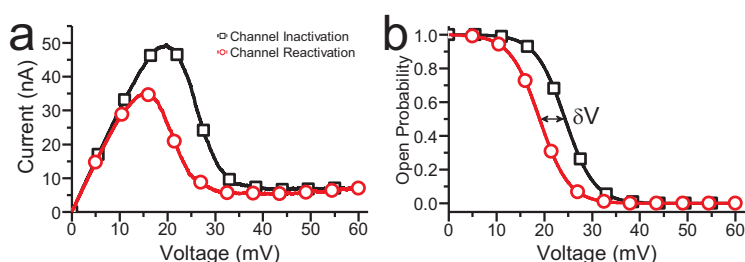


Fig 2. Lysenin channel inactivation (black) and reactivation (red) due to applied voltages. (a) Decreasing voltages initiates channel reactivation along a different pathway than inactivation which produces a hysteresis in the current. (b) The open probabilities from the inactivation and reactivation I-V curves are separated by a difference in voltage δV .

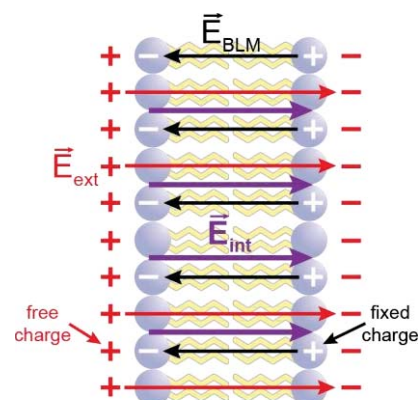


Fig 3. The internal electric field in a BLM is the result of an external field caused by the free charge and the field caused by the polarization of the BLM. The charges shown on the lipid headgroups indicate polarization not the net charge of the lipid.

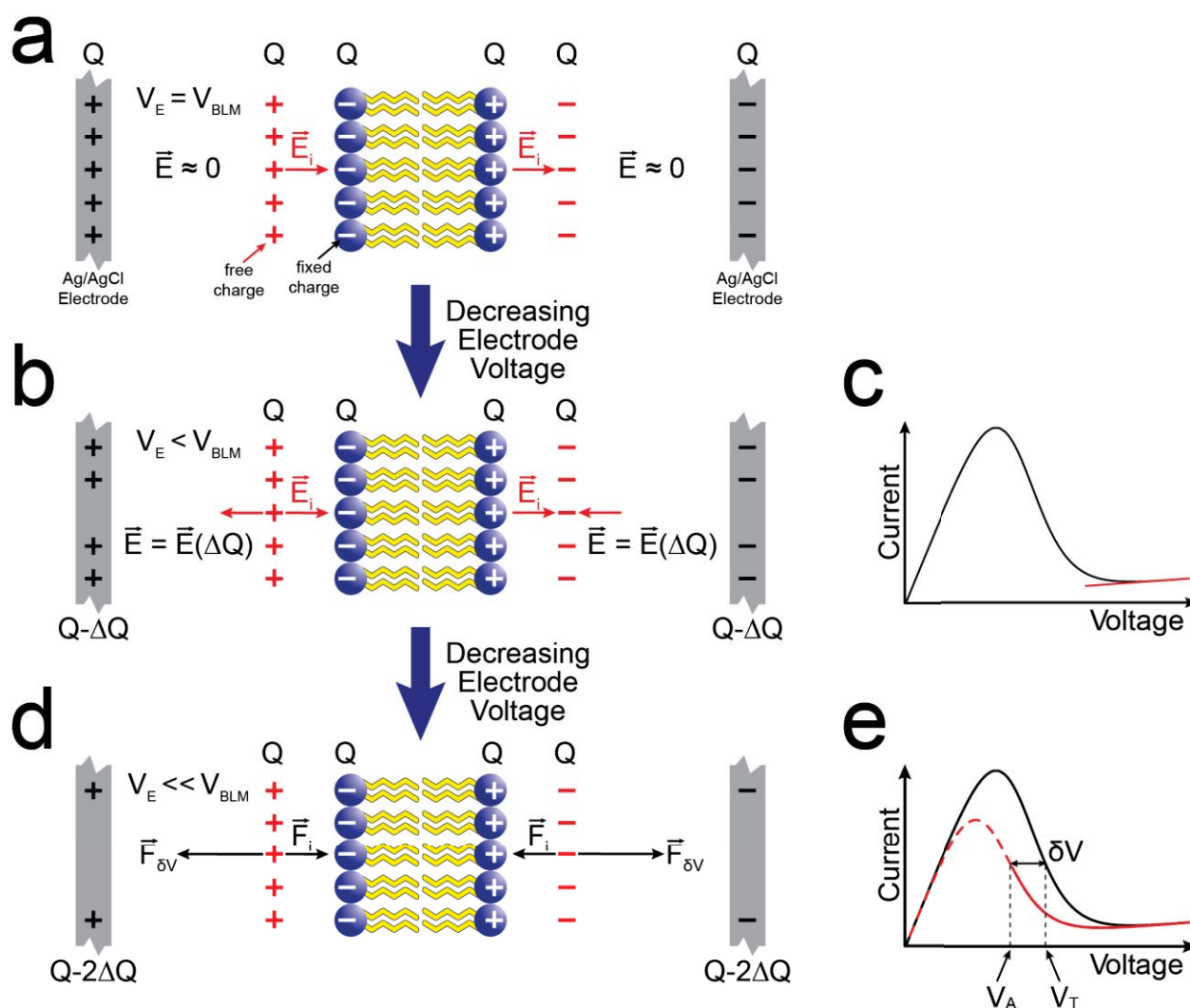


Fig 4. (a,b,d) Schematic of the charge interactions from the electrodes and BLM resulting in the observed hysteresis. (c,e) The theoretical observed I-V curve due to the coulomb interaction between free charges in the electrolyte solution and the fixed charges in a polarized BLM. The charges shown on the lipid headgroups indicate polarization not the net charge of the lipid.

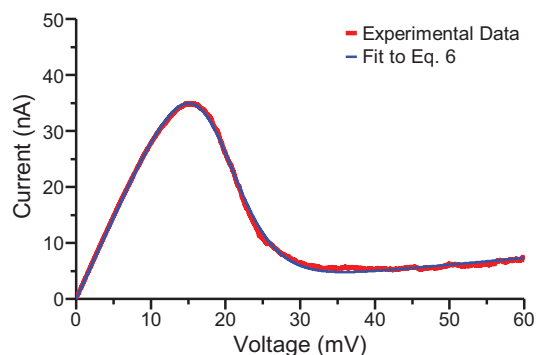


Fig 5. The fit using Eq. 6 (blue) provides an excellent fit for the experimental data for channel reactivation (red) where $\delta V = 5.34 \pm 0.01$ mV.

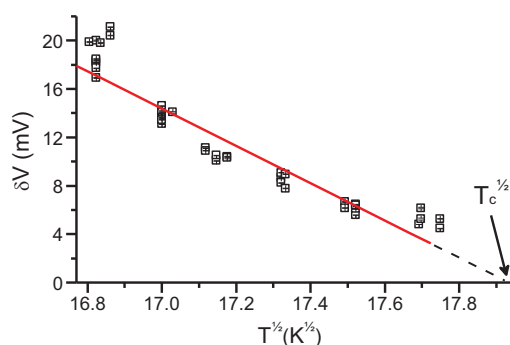


Fig 6. A plot of δV for different temperatures reveals a linear relationship between δV and $T^{1/2}$. The linear fit (red) determined that $T_c = 321.61 \pm 0.001$ K. The errors from fitting the hysteresis curves with Eq. 3 and Eq. 6 are represented by the error bars.