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# Controlled Gating of Lysenin Pores

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ABSTRACT

Lysenin forms unitary large conductance pores in artificial bilayer membranes containing sphingomyelin. A population of lysenin pores inserted into such a bilayer membrane exhibited a dynamic negative conductance region, as predicted by a simple two-state model for voltage-gated channels. The recorded I-V curves demonstrated that lysenin pores inserted into the bilayer are uniformly oriented. Additionally, the transition between the two-states was affected by changes in the monovalent ion concentration and pH, pointing towards an electrostatic interaction governing the gating mechanism.



## 1. Introduction

Pore forming protein toxins, one of the most potent biological weapons, share the ability of altering cell homeostasis simply by inserting an aqueous channel in the membrane, disrupting the permeability barrier, and leading to cell death. Lysenin is a 297 amino acid protein found in the coelomic fluid of the earthworm *Eisenia foetida* [1–3]. The interest in lysenin stems from its cytolytic and bacteriostatic activities [3–5] as well as its ability to disturb smooth muscle contraction [3,6]. This biologically active protein selectively recognizes and exerts its lytic activity on membranes containing sphingomyelin [1,2,5,7,8], a major plasma membrane lipid found in animal cells. The lytic action relies on formation of large conductance channels (water permeable nanopores) in the cell membrane, dramatically disturbing the ion balance and causing cell death [4]. Upon binding to sphingomyelin, lysenin forms hexagonal oligomers in the lipid bilayer that define a large pore (3 nm diameter) in the center of the complex [5,7,9]. Electrophysiological studies have shown that the conductance of lysenin pores in a sphingomyelin-enriched lipid bilayer membrane significantly decreases at positive voltages due to gating [9,10]. To further explore this gating behavior, we employed electrophysiological methods to understand the influence of electric field, ion concentration, and pH on the lysenin pore gating function. We assumed that the probability for lysenin pores to be opened or closed is described by a simple two-state model adapted from voltage-gated channels [11,12]. Our data showed that above a critical potential ($V_c$), the population of lysenin pores exhibited a region of negative dynamic conductance, followed by rectification, as predicted by the theoretical model. In addition we found that $V_c$ has an appreciable shift toward more positive voltages upon the addition of monovalent ions or at higher pH, suggesting an electrostatic interaction between the voltage domain sensor and the charged lipids in the bilayer as governing the gating mechanism. The conducting properties suggested that the mechanism of lysenin protein insertion and assembly leads to uniform channel orientation in lipid bilayers, and we proved this assumption by analyzing the I-V curves after inserting the channels successively on both sides of the bilayer.

These results provide further insight into how lysenin pores function as a gate and form the basis for a more general gating mechanism model of voltage-controlled pore forming proteins. In addition, the remarkable electrical properties of the lysenin pores can be useful in developing future bio-electronic devices capable of self-oscillation, amplification, or to control the transport across membranes.

## 2. Materials and Methods

Lysenin, asolectin, cholesterol, and sphingomyelin were purchased from Sigma-Aldrich, all other chemicals being commercial products of analytical grade. A vertical artificial bilayer membrane (20 mg asolectin, 10 mg sphingomyelin, and 5 mg cholesterol dissolved in 400 µl n-decane) was formed across a 70 µm aperture in a Teflon sheet separating two reservoirs filled with buffered electrolyte. Lysenin was added to the *trans* (virtual ground) side of the bilayer and a population of pores created. The voltage was defined as the voltage applied to the *cis* side. The currents were recorded using an Axopatch 200B amplifier (Molecular Devices), filtered at 1 kHz , feeding a Digidata 1440A digitizer (Molecular Devices), and further analyzed using the pClamp 10.02 (Molecular Devices) and Origin (OriginLab) software packages. The voltage ramps (scan rate 0.5 mV/s) were created using the embedded Episodic Stimulation protocol (pClamp10.02), and the current sampling rate was set to 0.1 s. The low scan rate was required because of the slow closing kinetics [10]; scanning rates faster than

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2 mV/s changed the I-V curve shape to a more linear one, while slower rates (down to 0.1 mV/s) preserved the shape proving that equilibrium was reached before sampling. Each point on the full lines in the graphs represents actual data points, with the symbols added solely to discriminate between the various curves, and the dashed lines represent added drawings through the experimental data points.

For the ionic concentration study we performed the adjustments by adding small amounts of 4M KCl stock solution in both reservoirs, while maintaining the 20 mM Hepes buffering. The pH study comprised KOH addition to a 150 mM KCl, 2 mM Hepes support electrolyte, and pH measurements with an Orion microelectrode (Thermo Electron Corporation). The orientation study was performed in 150 mM KCl and 20 mM Hepes solution.

## 3. Results and discussions

We considered a lipid bilayer membrane containing N water permeable pores which can be either in the opened or closed state depending on the polarity and magnitude of an applied voltage. The ionic current passing through the pores when applying a voltage V across the membrane is given by $I = G \times V$, where G is the membrane conductance. We assumed that the pore probability to be in the open state when a voltage V is applied is described by Boltzmann statistics, $P_{open} = 1/(1 + e^{k(V-V_0)})$ [11–13] . Consequently, the number of pores contributing to the ionic current at equilibrium is given by $N_{open} = NP_{open}$ (we assumed no current passes through completely closed pores), and the expression of the ionic current becomes [13,14]:

$$I = \frac{NgV}{1 + e^{k(V-V_0)}} + VG_L \quad (1)$$

where g is the unitary conductance of a single opened pore, and the added second term denotes a leaking current which depends linearly on the leakage conductance $G_L$. The I-V plot of Eq. (1) (Fig. 1), showed a non linear behavior for positive applied voltages, while negative voltages yielded a linear dependency. Within the limits of the model a population of voltage gated pores can switch from opened to closed by undergoing a negative dynamic conductance region, which starts at a critical potential $V_c$, consistent with earlier observations on ion channels inserted into artificial lipid bilayers [11,15] or intracellular recordings [16]. Additionally, the model described by Eq. (1) predicted a rectification effect at increased voltages and a diminished negative conductance region for small values of $V_0$ and k (Fig. 1). The conductance of the large lysenin pores is voltage dependent and ion passage is obstructed at positive applied voltages due to gating [9,10]. According to the above model, the strongest non linearity of the I-V curve and the dynamic negative conductance may occur over a limited voltage range due to the steep exponential dependency. Previous I-V measurements showed that at equilibrium the current amplitudes decreased dramatically when positive voltage was applied, proving the rectifying effect, but a region of negative conductance was not observed [10].

In order to study more closely the behavior of the population of pores as a function of voltage, we recorded the ionic current for lysenin pores in an I-V curve in the range -100 mV to +100 mV when applying a linear ramp voltage, and fitted the experimental data to Eq. (1) (Fig. 2A). The results provided an insight of the gating behavior and of the transition between states. Furthermore, the conducting properties of the pores were analyzed by plotting the pores resistance (Fig. 2B), defined by R = V/I, and the pores dynamic conductance (Fig. 2C), defined by dI/dV, as a function of applied voltage. We chose to analyze the dynamic conductance as opposed to dynamic resistance to avoid the inflection points where the dynamic resistance will tend to infinity.

The ionic current for applied voltages from -100 mV to about +10 mV showed a linear increase with applied voltage. The ohmic behavior over this region, where the current is proportional to the applied voltage, demonstrated that within this range all pores remained open without being affected by the applied electric field.

However, for applied voltages above +10 mV, although the ionic current continued to increase, the slope of the I-V curve decreased progressively indicating that some pores were likewise, progressively closing with higher applied voltage. At an applied voltage of +22 mV the slope of the I-V curve (the dynamic conductance) became zero and was defined as the critical voltage, $V_C$. As the applied voltage increased further, the ionic current decreased reaching a near zero value at about +60 mV. This region, (+22 mV<V< +60 mV) is referred to as the negative conductance region, and indicates that the gated pores reached their maximum closure at about V = +60 mV.

With a further increase in voltage above +60 mV, the current remained near zero. That is, the ionic current in this region (V> +60 mV) actually increased linearly but with a very small slope, calling attention to the existence of a small leakage current. The origin of the leakage current most likely stems from the inability for some pores in the population to close completely. However, we cannot rule out the possibility of a small leakage current associated to each inserted pore. The experimental data of the I-V curve were fitted to Eq. (1) (N, g, and $G_L$ were estimated from the corresponding linear behavior regions) and an excellent fit was obtained for $V_0 = 31 \pm 0.1$ mV and $k = 0.21 \pm 0.004$ mV$^{-1}$ (Fig. 2A). $V_0$ can be interpreted as being the voltage where the opening and closing rates are equal [12], while $V_c$ should be considered as the voltage where the rate of increase in current with increasing voltage is balanced by a decrease in current due to the rate at which pores are closing.

Based on the observed behavior of the induced pores as a function of applied voltage, it seemed reasonable to further explore the

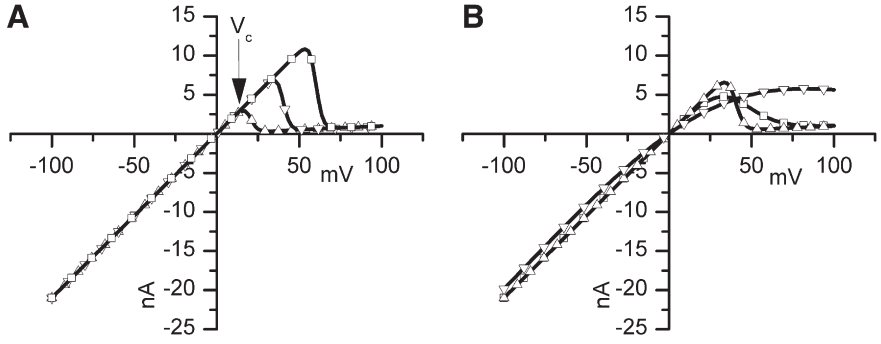


**Fig. 1.** Theoretical behavior of voltage gated channels predicted by Eq. (1). A) A population of oriented voltage-gated channels can exhibit a linear behavior at negative voltages, and a negative dynamic conductance region over a critical positive voltage, $V_c$, followed by rectification. The negative conductance region diminished as $V_0$ decreased ($V_0 = 60$ mV(□), 40 mV(▽), and 20 mV(Δ), $k = 0.5$ mV$^{-1}$), while the linear behavior at negative voltages and rectification at high positive voltages maintained. B) The negative conductance region diminished when k decreased ($k = 0.4$(Δ), 0.1(□), and 0.02 (▽) mV$^{-1}$, $V_0 = 40$ mV), maintaining the linear behavior at negative voltages and rectification at positive voltages.

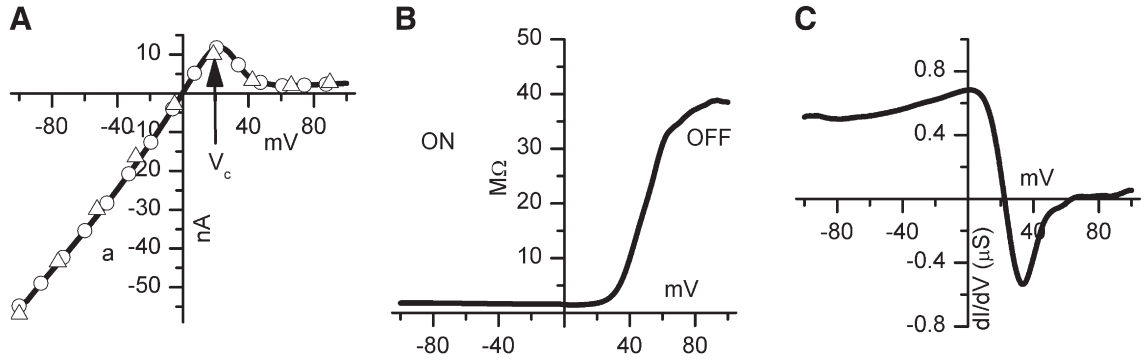


**Fig. 2.** The lysenin pore population showed the non linear I-V curve predicted by the two-state model. A) The experimental I-V curve (○) maintained linear at negative voltages but showed dynamic negative conductance and rectification at positive potentials. The experimental data fit of Eq. (1) (Δ) returned $V_0 = 31 \pm 0.1$ mV, and $k = 0.21 \pm 0.004$ mV$^{-1}$. B) The pore population switched gradually from its ON state (opened pores, low resistance) to an OFF state (closed pores, high resistance) when the applied voltage increased over +20 mV. The resistance was calculated from the experimental data as V/I. C) The dynamic conductance (dI/dV) curve shows clearly the negative conductance region from +22 mV to +60 mV, and the ON-OFF states.

electrostatic nature of the transition. For example, we examined the influence of the ionic concentration on pore population behavior by analyzing the I-V curves as a function of different KCl concentrations (50 mM - 640 mM) in the electrolyte (Fig. 3A). In this case, the ionic current and the conductance of the pore population at -100 mV, when they are fully open, depended linearly on the KCl concentration (Fig. 3B). This was expected since the conductivity of the electrolyte also behaves quite linearly with the salt concentration in this range. We also observed that the critical voltage increased with higher concentrations of KCl. For example, between 50 mM and 640 mM KCl, the critical voltage increased from +17 mV to +60 mV (Fig. 3A and C). All traces in Fig. 3A continue to show the negative resistance and rectification behavior although shifted along with the critical voltage. Meanwhile, the open current (measured at -100 mV) increased as the KCl concentration increased, from 15 nA to 45 nA, as one might expect since the concentration of ionic charge carriers increased. However, the leakage current (measured at +100 mV) increased more than 50 times, from 0.38 nA to 20 nA, suggesting that the high salt concentration affected the gating by keeping the pores open. These results are consistent with the fact that high ionic concentrations can hinder the closing of the pores by a 'foot in the door' effect [17,18].

A salient feature of the changes induced by KCl addition was the reversibility of the I-V curve upon reduction of the ionic concentration. The observed reversible shift in critical voltage upon changes in

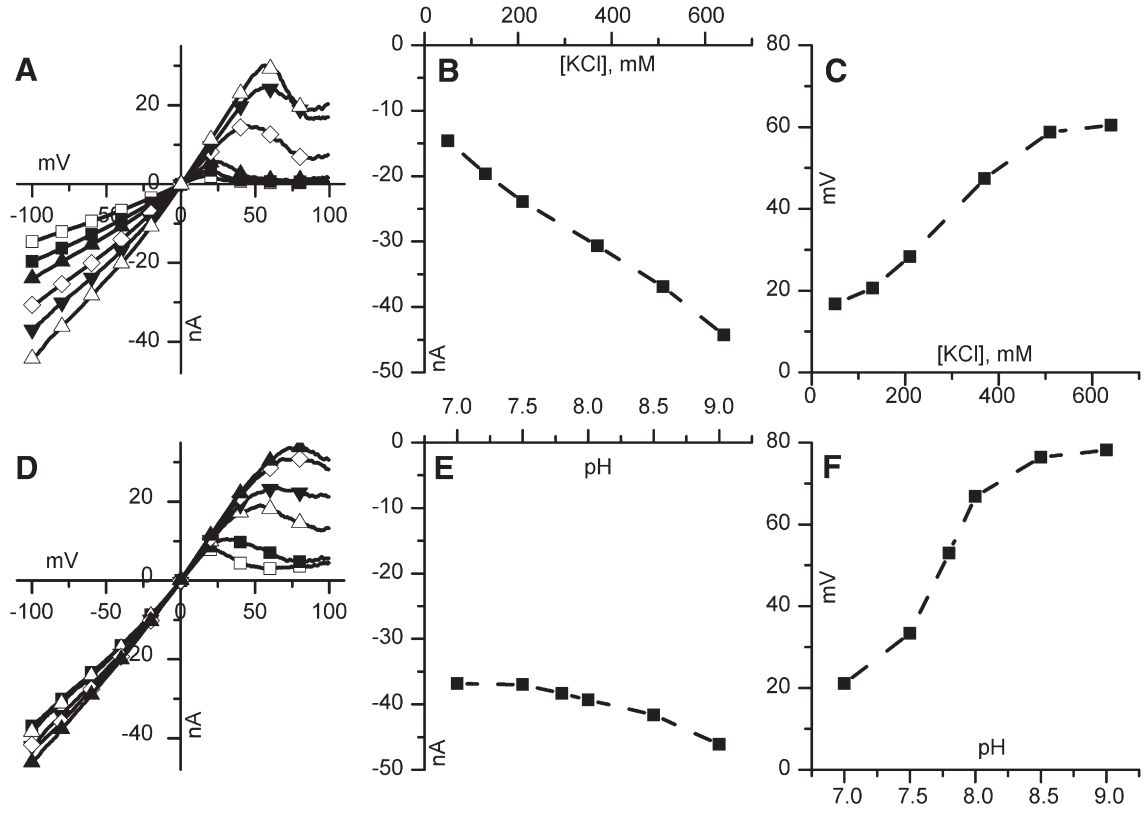


**Fig. 3.** KCl concentration and pH influence on the conducting properties of lysenin pore population. A) The I-V curve in response to KCl addition of 50 mM(□), 130 mM (■), 210 mM (▲), 370 mM (◊), 510 mM (▼), and 670 mM (Δ), 50 mM Hepes buffer. B) The open current (measured at -100 mV) changed linearly with KCl concentration due to the increased conductivity of the support electrolyte. C) The critical voltage shifted towards more positive values as the KCl concentration increased. D) The I-V characteristic changed when pH increased from pH = 7 (□) to pH = 7.5 (■), pH = 7.8 (Δ), pH = 8 (▼), pH = 8.5 (◊), and pH = 9 (▲). E) The open current (measured at -100 mV) dependency on pH. F) The critical voltage shifted towards more positive values as pH increased, similar to increased KCl concentration.

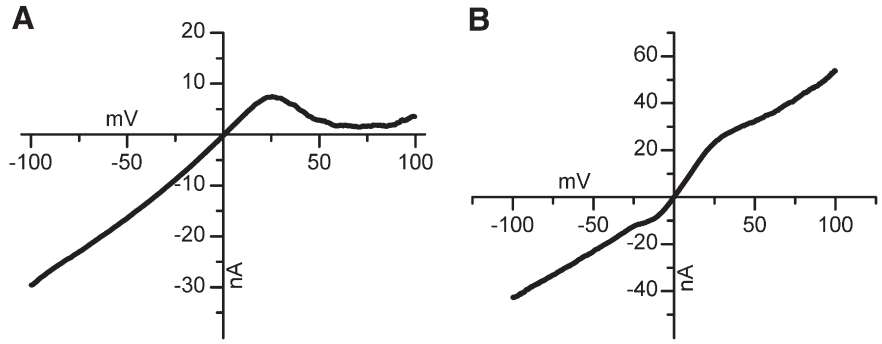


**Fig. 4.** The nanopore forming proteins are inserted oriented (rectification ready) into the bilayer (150 mM KCl, 20 mM Hepes). A) When proteins were added only on one side the I-V curve showed negative conductance and rectification. B) When the proteins were added sequentially on both sides of the bilayer, the lysenin pore population lacked the negative conductance and rectification features.

ionic concentration made it apparent that by holding the potential at around +60 mV, the pore population can potentially be switched between the open and closed states solely by changing the ionic concentration. In this case, for example, the monovalent ions play the role of gating modulator. Taking further the concept of electrostatic interactions, we found that the pH of the electrolyte affected the conducting properties of the lysenin pore population (Fig. 3D). For example, a change in pH from 7 to 9 was associated with a shift in the critical voltage from +22 mV to +75 mV (Fig. 3D and F), similar to KCl, while the open current measured at -100 mV suffered less changes (Fig. 3E). The behavior of the current was linear in all cases for negative voltages, and the critical voltage and the region of negative conductance, though shifted, were observed for all recorded traces. The changes induced by pH proved to be reversible, and the reversible shift in critical voltage upon pH changes indicated that at a certain voltage (+60 mV), the pore population can switch between the open and closed states due to pH variation, making pH another candidate for gating modulation. The higher pH and the higher ion concentration also increased the leakage current most probably by keeping the pores preponderantly in the open state.

Postulating that an electrostatic interaction between the voltage domain sensor and the charged lipids governs the voltage-gating mechanism, we can attempt to explain the effects of KCl and pH. Higher salt concentration neutralizes the surface potentials by screening or binding, reducing the electrostatic interactions. Higher pH increases the lysenin net negative charge ( pI = 5.7-5.8 [6]), while the phosphatidylinositol negative net charge (the main anionic lipid in asolectin) remains constant (pK = 2.5), somehow opposed to the KCl effect. Since lysenin contains positively charged aminoacids [9], the voltage-sensor domain may contain positively charged residues fully exposed to the surrounding lipid environment, similar to other ion channels [19,20]. The electrostatic interaction between the voltage-sensor and the lipid polar head may play a major role for gating, and this interaction is weakened by high pH (the positive charge decreases), similar to high salt concentration.

In addition to an indication that the pores have closed, the near zero current at applied voltages above +60 mV (low salt concentration and neutral pH) suggests that lysenin pores are inserted into the lipid bilayer mostly in an oriented manner (rectification-ready). In order to test this hypothesis we inserted lysenin pores by adding them first only to one side of the bilayer (*trans*) and observing the rectifying feature, including the negative conductance region (Fig. 4A). After this step, additional pores were inserted into the same bilayer by protein addition to the opposite side of the bilayer (*cis*). After equilibrium, the I-V curve showed that both rectification and negative conductance were diminished (Fig. 4B). The highest slope of the recorded trace in the range ±15 mV indicated that the conductance was sustained there by pores fully open on both sides. In this case either higher positive or higher negative voltages reduced the slope (or conductance), indicating closing only for pores with proper orientation. This behavior fully supports a model in which the protein pores are inserted in an oriented manner. Therefore the oriented pore population can be described as an ionic diode, which shows both rectification and negative conductance. When the protein is added on both sides, the pore population can be described as two anti-parallel diodes, each one with a serial resistor. We obtained the same oriented insertion with no voltage applied to the bilayer (open circuit) during pore insertion, indicating that orientation is related to a primary protein-lipid interaction, and not mediated by an external electric field.

The data presented demonstrate that lysenin forms large conductance pores in lipid bilayer membranes which are characterized by two important features of ion channels: the ability to sustain high transport rates and regulation of the ionic current. Our experiments show that the gating mechanism for the lysenin induced pores depends on applied voltage, the ionic strength, and the pH of the surrounding electrolyte. The observations of negative conductance, rectification, ionic and pH control over the pore gating behavior provided valuable clues for an electrostatic based gating mechanism that can help elucidate the physiological function of the toxin. Some of these features were predicted by a simple two-state model for voltage gated channels, proving the similarities between ion channels and lysenin pores. The validity of the model can be easily extended for multiple states, and a more sophisticated model should take into account the ionic concentration and pH effects on the interaction between the voltage sensor domain and charged lipids. The stability of the pores and their modulation by the external environment has potential applications for controlled transport across artificial or natural membranes.

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