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Intramembrane congestion effects on lysenin channel voltage-induced gating

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Abstract

All cell membranes are packed with proteins. The ability to investigate the regulatory mechanisms of protein channels in experimental conditions mimicking their congested native environment is crucial for understanding the environmental physicochemical cues that may fundamentally contribute to their functionality in natural membranes. Here we report on investigations of the voltage-induced gating of lysenin channels in congested conditions experimentally achieved by increasing the number of channels inserted into planar lipid membranes. Typical electrophysiology measurements reveal congestion-induced changes to the voltage-induced gating, manifested as a significant reduction of the response to external voltage stimuli. Furthermore, we demonstrate a similar diminished voltage sensitivity for smaller populations of channels by reducing the amount of sphingomyelin in the membrane. Given lysenin's preference for targeting lipid rafts, this result indicates the potential role of the heterogeneous organization of the membrane in modulating channel functionality. Our work indicates that local congestion within membranes may alter the energy landscape and the kinetics of conformational changes of lysenin channels in response to voltage stimuli. This level of understanding may be extended to better characterize the role of the specific membrane environment in modulating the biological functionality of protein channels in health and disease.

Keywords: lysenin, crowding, lipid rafts, sphingomyelin, open probability, voltage-gating

Introduction

The conformational changes by which protein channels exercise precise control over membrane permeability are central to life-sustaining processes. The basic science of membranes has demonstrated relationships between protein channel functionality and multiple environmental cues unrelated to variations in their primary structure. All cell membranes are packed with proteins, which may occupy up to 50% of the membrane surface area (Dupuy and Engelman 2008; Linden et al. 2012; Zhou 2009). This congested environment may promote specific and non-specific interactions originating from molecular crowding (excluded-surface effects) and confinement (Zhou 2009), changes to membrane elasticity (Phillips et al. 2009; Ursell et al. 2007), hydrophobic mismatch (de Pianque and Killian 2003), membrane bending (Phillips et al.

2009) or direct interactions between membrane species (Dan et al. 1993; Dekker and Yellen 2006; Erdem and Aydiner 2009). The two-dimensional configuration of the membrane further restricts spatial arrangements, promoting congestion and self-congestion of identical molecular species (Aisenbrey et al. 2008; Zhou 2009). The heterogeneous organization of the membrane into lipid rafts (Dietrich et al. 2001; Lingwood and Simons 2010; Munro 2003) adds another level of complexity since many membrane components, including protein channels, have specificity for accumulation into lipid rafts (Barfod et al. 2007; Brady et al. 2004; Jin et al. 2008; Kulma et al. 2010; Maguy et al. 2006), consequently promoting local congestion with fewer molecules.

Excluded surface effects, electrostatic, and membrane-elasticity mediated interactions each has a dependency on local channel density, ultimately determining the distance between protein channels and allowing short or long-range interactions to manifest (Erdem and Aydiner 2009; Phillips et al. 2009; Zhou 2009). Although a synergetic contribution from all of of these environmental cues is expected to influence channel functionality, previous reports have limited their investigative scope to individual factors. Nonetheless, the common denominator of congestion is the change of the free energy of the system, which in turn may be experimentally observable as changes in the kinetics of the protein channel conformational transitions (Dekker and Yellen 2006; Erdem and Aydiner 2009; Keleshian et al. 2000; Linden et al. 2012; Ursell et al. 2007; Zhou 2009). The scarcity of experimental reports on the behavior of protein channels in congested conditions originates from the lack of an adequate experimental platform to control channel population densities. Ion channels are not readily available and are not easily reconstituted in artificial systems in controlled-density conditions. Pore-forming toxins may provide a better in vitro platform for experimental realization of congestion conditions. However, they often lack regulation and their biological activity is not necessarily determined by a specific response to external stimuli that induce conformational changes observable as alterations of their functionality. In this line of inquiries, we focused our investigations on the effects of congestion within a membrane by analyzing changes in the gating behavior of lysenin channels inserted into artificial planar bilayer lipid membranes (BLMs). Lysenin is a 297 amino acid pore-forming protein extracted from the coelomic fluid of *Eisenia foetida* that self-inserts to form ~3 nm diameter channels in membranes containing sphingomyelin (SM) (Fologea et al. 2010; Ide et al. 2006; Ishitsuka and Kobayashi 2004; Yamaji-Hasegawa et al. 2003). Although lysenin is not an ion channel, it constitutes an excellent experimental model for studying the effects of congestion on regulated protein channels irrespective of their structure and biological function. Lysenin channels exhibit salient features of ion channels such as high transport rate and regulation by voltage (Fologea et al. 2010; Ide et al. 2006). Their response to voltage stimuli has been well characterized within a two-state (open-close) model, and changes in the energy landscape can be identified through established relationships between channel gating and Boltzmann statistics (Fologea et al. 2010) similar to ion channels (Bezanilla 2008; Hille 2001; Latorre et al. 2007). Lysenin's ability to self-insert stable channels into artificial membranes facilitates establishing congested conditions by successively increasing the number of channels inserted into the BLM, which is expected to influence the voltage-induced gating. In addition, lysenin has been shown to favor insertion into SM-rich lipid rafts (Abe and Kobayashi 2014; Kulma et al. 2010; Yamaji-Hasegawa et al. 2003; Yamaji et al. 1998; Yilmaz and Kobayashi 2015; Yilmaz et al. 2013), which facilitates further self-congestion conditions by manipulating the surface area of the rafts through changes in the SM amount in the membrane (Abe and Kobayashi 2014; Jin et al. 2008; Mitsutake et al. 2011).

Materials and methods

Dry asolectin (Aso) from soy bean (Sigma-Aldrich), powder brain SM (Avanti Polar Lipids) , and powder cholesterol (Chol) from Sigma-Aldrich were dissolved in n-decane in a 10:1:5 weight ratio for the 10% SM solution, and a 10:5:5 weight ratio for the 50% SM solution. The percentage indicates SM weight relative to Aso. Lyophilized lysenin (Sigma-Aldrich) was prepared as a 0.3 μM stock solution by dissolving it in a solution containing 100 mM KCl, 20 mM HEPES (pH 7) and 50% glycerol and used without further purification.

The experimental setup consisted of two 1 ml PTFE reservoirs separated by a thin PTFE film with a ~70 μm diameter aperture acting as a hydrophobic frame for BLM formation. Each reservoir was filled with buffered electrolyte (50 mM KCl, 20 mM HEPES, pH 7.2) and a planar BLM was formed by painting small amounts of one of the lipid mixtures over the aperture. The electrical connections were established via two Ag/AgCl electrodes embedded in the electrolyte solution on each side of the BLM, and connected to the headstage of an Axopatch 200B amplifier (Molecular Devices). The data was digitized and recorded through a DigiData 1440A Digitizer (Molecular Devices), and further analyzed by using Clampfit 10.2 (Molecular Devices) and Origin 8.5.1 (OriginLab) software packages. After a stable BLM was achieved, small amounts of lysenin (~0.3 nM final concentration in the reservoir) were added to the ground side of the BLM under continuous stirring with a low-noise magnetic stirrer (Dual Dipole Stirplate, Warner Instruments). Channel insertion was monitored by measuring the ionic currents through the BLM in voltage clamp conditions at negative transmembrane potentials and a 1 kHz low-pass hardware filter (Electronic Supplementary Material Fig. S1 and Fig. S2). Successive addition of increased amounts of lysenin to the ground side of the BLM provided additional channels to facilitate congested conditions (Electronic Supplementary Material Fig. S3), and ensured consistent channel orientation (Fologea et al. 2010). After each lysenin addition, the system was monitored to ensure completion of channel insertion before each data measurement, as indicated by a steady-state open current usually achievable in less than 1 hour (Electronic Supplementary Material Fig. S3) (Fologea et al. 2011b). All of the experiments were performed at room temperature (22.5 ± 0.5° C).

Results and discussion

We hypothesized that experimental congestion conditions may be achieved by increasing the number of lysenin channels inserted into planar BLMs. However, large channel populations in this study were expected to yield ionic currents exceeding the 200 nA limit of the instrumentation at practical transmembrane voltages. To circumvent this limitation, we decreased the conductance of the support electrolyte by using 50 mM KCl and characterized the insertion and functionality of individual lysenin channels to ensure consistent behavior among experimental conditions. Using BLMs comprised of 50% SM weight ratio relative to Aso, the addition of lysenin to the ground reservoir at -30 mV transmembrane potential produced discrete changes in the open current (Electronic Supplementary Material Fig. S1) indicative of individual channel insertions (Ide et al. 2006). The stepwise open current distribution for four inserted channels yielded a unitary conductance of 0.391 ± 0.025 nS/channel, consistent with previous reports (Fologea et al. 2011a; Ide et al. 2006; Kwiatkowska et al. 2007). The I-V curves recorded in response to voltage ramps ranging from -100 to 100 mV at 0.2 mV/s demonstrated the linear

behavior in the negative voltage region (hence absence of conformational changes and a constant conductance in the negative voltage range), followed by voltage induced gating at positive potentials (Fologea et al. 2011b; Ide et al. 2006) (Electronic Supplementary Material Fig. S1). Therefore, we concluded that the low ionic concentration did not affect the insertion, the transport capabilities, and the voltage regulation of lysenin channels.

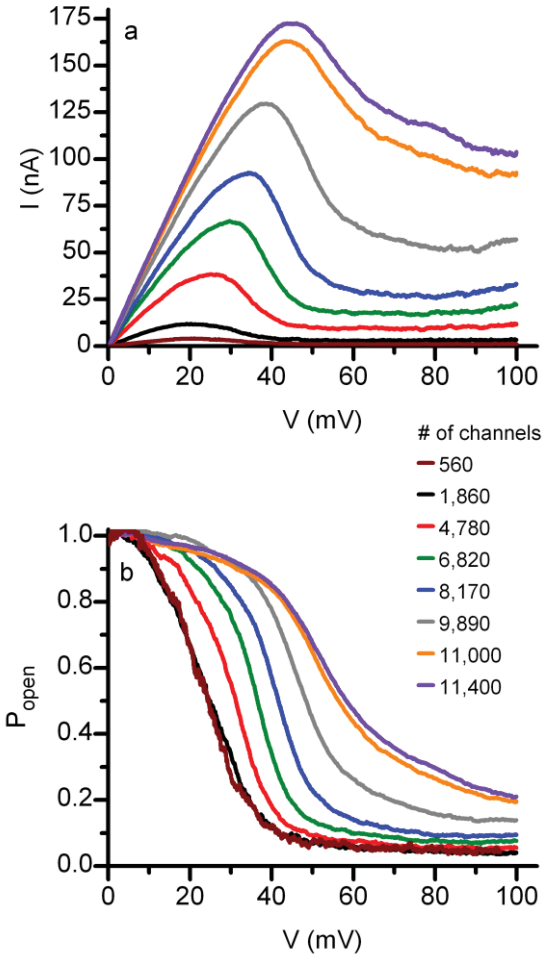

Fig. 1 The number of lysenin channels inserted into a planar lipid membrane containing 50% SM affects the I-V characteristics (a) and induces a rightward shift of the experimental open probability P_{open} (b). Each I-V and P_{open} plot is the average of three individual recordings for the same experimental setup and all data points in the graphs are experimental values with standard errors < 5%

After the insertion of lysenin channels into the 50% SM BLMs, the gating behavior was assessed by measuring the ionic current in response to a slow voltage ramp (0.16 mV/s) ranging from 0 mV to 100 mV. Incorporating more lysenin from the stock into the grounded bathing solution facilitated additional channel insertions into the BLM, increasing the density of channels within the membrane (Electronic Supplementary Material Fig. S3). The number of channels in the population was approximated from the ratio of the total membrane conductance determined from the ionic current measured at -60 mV (when all of the channels were open) to the unitary single channel conductance measured during the initial insertion events (~0.39 nS/channel). .

Effects due to the increased population were identified from the analysis of the I-V curves recorded for different numbers of inserted channels. As shown in Fig. 1a, the I-V curves revealed a significant increase in the voltage required for the channels to gate as the number of inserted channels increased. Each I-V plot shown in Fig. 1 is the average of three consecutive runs for the same experimental setup. Although multiple parallel experiments indicated an identical behavior (i.e., increase of voltage required to initiate gating), assuring identical experimental conditions by exact control of the number of inserted channels was elusive.

To further analyze the gating behavior, linear fits of the I-V curves in the low positive voltage range (when all of the channels were in the open state (Fologea et al. 2011b)) were used to determine a theoretical maximum current I_{max} for each voltage as if channel gating was not observed, and the probability of finding a channel in open state (P_{open}) was determined from (Bezanilla 2005; Tao et al. 2010):

$$P_{open} = \frac{I}{I_{\max}} \quad (1)$$

where I is the actual measured current at each voltage in the I-V curve. The plot of the open probabilities, shown in Fig. 1b, demonstrated the rightward shift of P_{open} with increased channel population. Ionic currents larger than the instrument's limit recorded for increased channel populations consequently limited the maximum number of channels that could be investigated. Analysis of the I-V curves shown in Fig. 1a indicated that subsequent increases to the channel population would yield currents outside of the measurable range of the instrument, making the open probability calculations unfeasible.

One may argue that the actual transmembrane voltage decreased for large channel populations due to the increased voltage drop across the serial resistance of the bulk electrolyte. Each reservoir can be approximated as a cube of 1 cm side length, and the measured electrolyte solution was 6.5 mS/cm thus yielding a total conductance for the two reservoirs of ~13 mS. Since each channel contributes to the membrane conductance by ~ 0.39 nS, the total conductance of 10,000 inserted channels of ~3.9 μS is ~3300 times smaller than the electrolyte conductance. Therefore, any change in the actual transmembrane voltage due to increased voltage drop on the serial resistance of the electrolyte is negligible. Hence, we attribute the changes observed in the voltage response of lysenin channels to the increased population in the target membrane. Given the approximate surface area occupied by one channel (Yamaji-Hasegawa et al. 2003) (~10^{-4} μm^2) and the relatively large area of the BLM (~1,900 μm^2 for a 25 μm radius) it is difficult to link the observed shift in gating to excluded-surface effects (Zhou 2009) even for a population of 10,000 inserted channels. In addition, the average inter-channel distance for a uniform distribution of channels within the membrane (roughly estimated at ~ 400 nm for 10,000 channels inserted into a 1,900 μm^2 membrane) may be sufficiently large to prevent manifestation of any specific interactions between channels. However, lysenin channels prefer insertion into lipid rafts (Ishitsuka and Kobayashi 2004; Kulma et al. 2010), which promote localized accumulation and increased local channel density. Lysenin clustering into SM-rich domains and formation of 2-D arrays in supported lipid membranes has been clearly demonstrated by using high-speed Atomic Force Microscopy (Yilmaz and Kobayashi 2015; Yilmaz et al. 2013) and these studies revealed that rafts are the first target for oligomerization. However, at very high lysenin concentration, pore assembly has been shown to gradually expand into the disordered phase following exclusion of SM and Chol from the SM-rich domain. Nonetheless, our experiments comprised much lower amounts of lysenin added to the solutions and, implicitly, a significantly reduced number of inserted channels. Therefore, we may assume that our

experimental conditions describe the functionality of lysenin channels located into lipid rafts. Since SM is a major component of the lipid rafts and SM depletion may modulate the lipid raft size and distribution (Abe and Kobayashi 2014; Jin et al. 2008; Mitsutake et al. 2011), we expanded our investigations to a BLM with a lower SM concentration (10% weight ratio relative to Aso) in the same ionic conditions. Given the importance of SM for the lysenin pore formation and insertion (Ide et al. 2006), we questioned if the reduced SM concentration affects the functionality of individual lysenin channels. Similar to the 50% SM conditions, the open current recorded after addition of lysenin to the ground reservoir changed in discrete steps (Electronic Supplementary Material Fig. S2), and the stepwise distribution of the open current for the four inserted channels yielded a unitary conductance of ~0.394 ± 0.015 nS, comparable to the 50% SM conditions. The I-V plot recorded in response to voltage ramps from -100 to 100 mV also exhibited the familiar linear behavior in the negative voltage region, followed by voltage induced gating at positive potentials (Fologea et al. 2011b) (Electronic Supplementary Material Fig. S2). Therefore, we concluded that the reduced SM concentration did not affect the insertion, the conductance properties or the voltage regulation of lysenin channels.

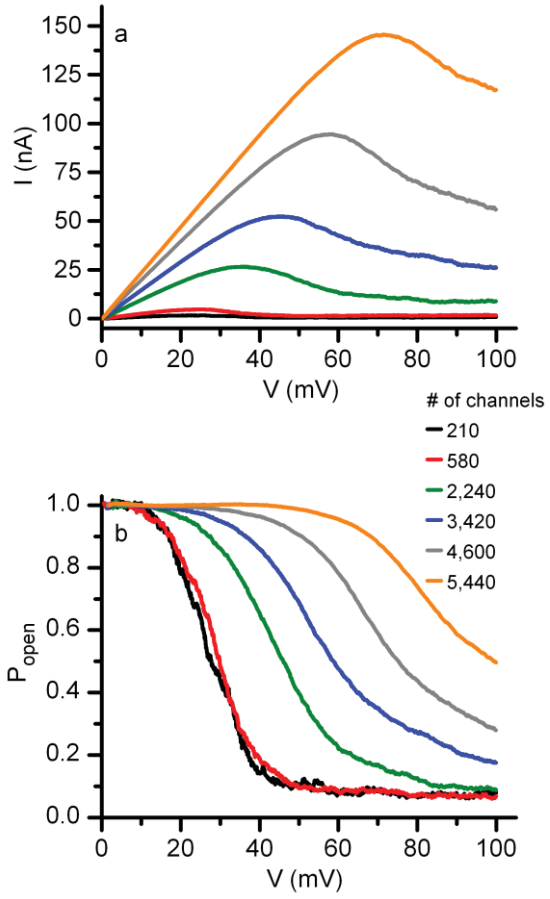

Fig. 2 (a) The I-V plots for increasing numbers of lysenin channels inserted into planar lipid membranes containing 10% SM. (b) A more significant rightward shift in the open probability is observed for the reduced SM concentration compared to the 50% SM condition shown in Fig. 1. Each I-V and P_{open} plot is the average of three individual recordings for the same experimental setup and all data points are experimental values with standard errors < 5%

We then introduced additional lysenin from the stock into the grounded bathing solution, which permitted further channel insertions into the BLM, consequently increasing the density of channels within the membrane. Experimentally, the conditions and methods were identical to the higher SM concentration BLMs, and the open probability was calculated in the same manner. The I-V curves and the open probability for increasing numbers of channels in the lower SM concentration membranes are shown in Fig. 2a and 2b, respectively.

As with the higher SM concentration membranes, increases in channel population shifted the gating toward higher voltages. However, the shift was more significant for the 10% SM conditions compared with the 50% SM conditions. To gain further insight into the effect of SM ratio, we considered a simple biophysical model of gating. The conformational changes of lysenin channels in response to transmembrane voltages are considered transitions between two conduction states, i.e open and close (Fologea et al. 2010). Accordingly, the theoretical probability of finding a channel in the open state at equilibrium is described by Boltzmann statistics as (Latorre et al. 2007; Phillips et al. 2009):

$$P_{open} = \frac{1}{1+K} = \frac{1}{1+e^{\frac{-\Delta G}{k_B T}}} = \frac{1}{1+e^{\frac{-q}{k_B T}(V-V_{0.5})}} \quad (2)$$

where K is the equilibrium constant of the open-close transition, ΔG is the difference in free energy between the open and closed state, q is the gating charge, k_B is the Boltzmann constant, T is the absolute temperature, and $V_{0.5}$ is the half-activation voltage, i.e. the voltage where P_{open} = 0.5 (Reeves et al. 2008; Tao et al. 2010). Equation (2) establishes the relationship between kinetics and free energy; the changes in P_{open} reflect changes in kinetics which are indicative of changes in the free energy due to all of the specific and non-specific interactions arising from local congestion, which may include excluded surface effects originating in crowding, electrostatic interactions, or changes in the mechanical properties of the membrane (Erdem and Aydiner 2009; Phillips et al. 2009; Reeves et al. 2008; Zhou 2009). Next, our analysis focused on the changes of the half-activation voltage, indicative of the balance between the energy supplied by the external electric field and all the other energies in the system (Reeves et al. 2008). The experimental $V_{0.5}$ values for 50% SM BLMs increased by ~30 mV over the approximately 20-fold increase in channel population (Fig. 3). The smaller populations of channels (less than 2,000 channels) exhibited negligible changes of the $V_{0.5}$ values, and indicated that smaller numbers of channels did not establish a congested environment to significantly alter channel functionality. The shift toward higher voltages for the increasing population of channels suggested that the congested conditions make the channels less sensitive to voltage.

For 10% SM, the values of $V_{0.5}$ induced by an approximately 25-fold increase in channel population shifted by ~72 mV (Fig. 3). Again, smaller populations of channels (~210 and ~580) did not exhibit significant changes in the $V_{0.5}$ values of the open probability, indicating that small numbers of channels did not constitute a congested environment in which significant changes in functionality manifest. Consistent with these observations, a parallel experiment comprising lysenin channels inserted into BLMs containing 20% SM (weight ratio relative to the amount of Aso) shows that for similar number of inserted channels the $V_{0.5}$ values are situated between the extreme values determined for the 10% SM and 50% SM (Fig. 3). A reasonable explanation for such behavior may consider the role played by lipid rafts as a target for lysenin channel insertion (Abe and Kobayashi 2014; Ishitsuka and Kobayashi 2004; Ishitsuka and Kobayashi 2007; Kulma et al. 2010). A BLM containing a smaller concentration of SM was theorized to create smaller

lipid rafts where lysenin channels will insert. This, therefore, led to a greater local density of channels from the decreased raft area. Comparing the lower-concentration to the higher-concentration SM membranes, we observed that for a given number of channels, the bilayers containing the lower SM concentration yielded greater values of $V_{0.5}$. However, for low numbers of channels, $V_{0.5}$ was approximately the same for both concentrations. As the number of channels in the populations increased, the BLMs containing a lower concentration of SM exhibited a more significant increase in $V_{0.5}$. Once again, the higher density of channels, presumed to originate in the reduced surface area of the lipid rafts, produced a prominent congestion effect manifested as a significant change in the voltage gating.

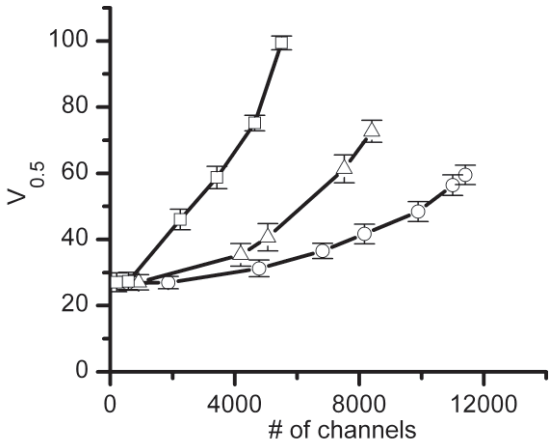

Fig. 3 The half-activation voltage $V_{0.5}$ (mean ± SD, n=3) as a function of the number of lysenin channels inserted into similar-size planar lipid membranes undergoes more pronounced changes for bilayers containing 10% SM (open squares), 20% SM (open triangles), and 50% SM (open circles)

The behavioral changes for channels in congested conditions, observed as changes in the voltage gating profile, indicates an altered kinetics of the conformational transitions. Our analysis implies that the measured open probability was independent of time, thus was estimated from open currents measured at equilibrium. However, the slow equilibration of lysenin channels in response to transmembrane voltages (Fologea et al. 2011b) may prevent accurate estimations of the open probability as a representative of the steady state for each of the experimental conditions. For small populations of inserted channels, the low voltage ramp rate used to plot the I-V characteristics (0.16 mV/s) suffices to approximate each current as descriptive of a true steady state (Fologea et al. 2011b). A more thorough analysis would require either using extremely slow voltage rates (Fologea et al. 2011b) or determining the time evolution of the ionic currents in response to step voltages. Neither of these approaches is feasible owing to membrane lifetime and technical limitations. Irrespective of the steady-state approximation, our results suggest that high density conditions alter channel functionality by changing the free energy landscape of conformational transitions.

The open probabilities of the small populations show the presence of a minimum threshold in the number of channels required to initiate observable changes in gating, consistent with congestion effects dependent on inter-channel distance. We also observed that low SM ratio conditions exhibit a more abrupt relationship between $V_{0.5}$ and the number of channels, providing further evidence that the behavioral effects could be attributed to the more congested

conditions. Since only minor changes in gating were observed for low numbers of channels irrespective of SM concentration, this suggests that the congested conditions were dependent on the local density of the channels in the BLM. We assumed that as the channels were inserted into the membrane, smaller rafts produced more noticeable effects on the gating than when the channels were allowed to spread out in the higher SM concentration membrane. To further analyze the behavior of lysenin channels at low densities, we estimated $V_{0.5}$ for populations ranging from 3 to 943 channels inserted into 50% SM BLMs (Fig. 4). Although each $V_{0.5}$ represents the average value determined for a particular number of inserted channels, each point was determined from an independent experiment. Very low number of inserted channels (< 20) yielded a spurious signal owing to the large contribution to the open current of individual closing events. In order to avoid the large experimental errors achievable in such conditions, at least ten I-V plots were averaged for each analysis, while for larger number of channels the average was calculated from at least three I-V plots. We found no change in the values of $V_{0.5}$ (within the errors) for the channel populations in this range, reinforcing our assertion that suggests a minimum inter-channel distance is required to manifest changes in the energy landscape.

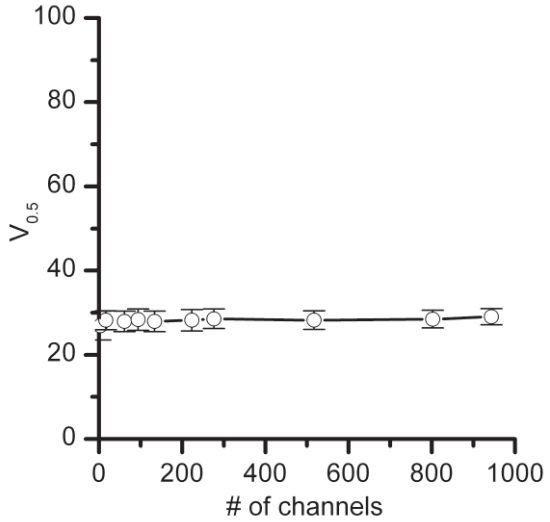

Fig. 4 The half-activation voltage $V_{0.5}$ undergoes negligible changes for low number of lysenin channels inserted into 50% SM lipid membranes. Each $V_{0.5}$ was determined from independent parallel experiments and the data are reported as mean ± SD, n=3-10

A better analysis of the experimental results should include the average distance between channels, instead of number of inserted channels. However, such calculations are seriously impeded by the current lack of knowledge with regards to the size of the rafts as a function of lipid composition. Lipid rafts are not exclusively composed of SM therefore the area occupied by rafts for a given SM concentration may not be accurately determined. It is estimated that only 20-30% of the SM is actually included into lipid rafts (Kulma et al. 2010; Pike et al. 2002), which may significantly contribute to a reduced raft area, higher channel density and shorter inter-channel distance.

We reported on changes in lysenin channel voltage-induced gating exclusively prompted by congestion effects within a planar BLM. The origins of these effects are hypothesized to stem from changes in the energy landscape, which resulted in modified channel-state kinetics and/or equilibrium. Our work illustrates how congested conditions typical of cell membranes influence

the performance and behavior of voltage-gated channels. Although the precise physiological role of lysenin remains unknown, our work revealed functional relationships between micro-environmental conditions and regulated protein channels. Lysenin is not an ion channel but its response to external voltage stimuli is satisfactorily described by two-state models commonly used for ion channels. This mechanistic similarity may prove crucial for deeper insights into ion channel physiology since all ion channels are crowded proteins in their native environments and many are known to have specificity for accumulation in lipid rafts (Barfod et al. 2007; Brady et al. 2004; Jin et al. 2008; Maguy et al. 2006). This extra layer of complexity and the connection between biological functionality and local environment may be expanded to explain the biological activity of porins, malfunctioning of ion channels, impaired signaling, or the development of serious diseases (Jin et al. 2008; Mitsutake et al. 2011).

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Conflict of interest

The authors declare that they have no conflict of interest.

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Electronic supplementary material

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Intramembrane congestion effects on lysenin channel voltage-induced gating

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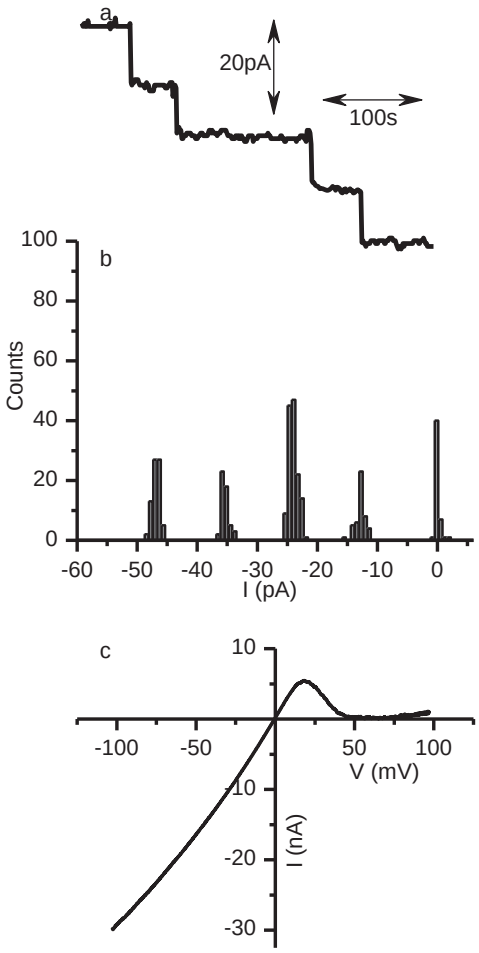

Fig. S1 Low ionic concentration does not affect the functionality of lysenin channels. (a) Insertion of identical lysenin channels in a lipid membrane containing 50% sphingomyelin (weight ratio relative to the amount of asolectin) and bathed by 50 mM KCl is indicated by the stepwise increase of the open current. (b) The current distribution of the open current indicates a unitary conductance increase of ~0.391 ± 0.025 nS/channel. (c) The I-V plot recorded at 0.2mV/s indicates the voltage-induced gating at positive transmembrane potentials and demonstrates the functionality of lysenin channels in low ionic concentration conditions

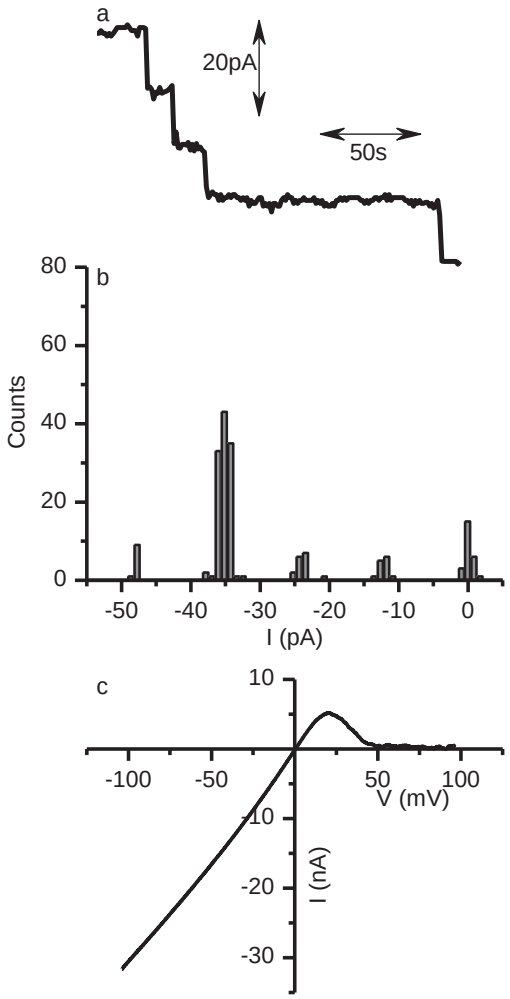

Fig. S2 Sphingomyelin depletion does not affect the functionality of lysenin channels. (a) Insertion of identical lysenin channels in a bilayer containing 10% sphingomyelin (weight ratio relative to asolectin) and bathed by 50 mM KCl. (b) The current distribution of the open current indicates a unitary conductance increase of ~0.394 ± 0.025 nS/channel. (c) The I-V plot recorded at 0.2mV/s indicates the voltage-induced gating at positive transmembrane potentials and demonstrates the functionality of lysenin channels in sphingomyelin-depleted conditions

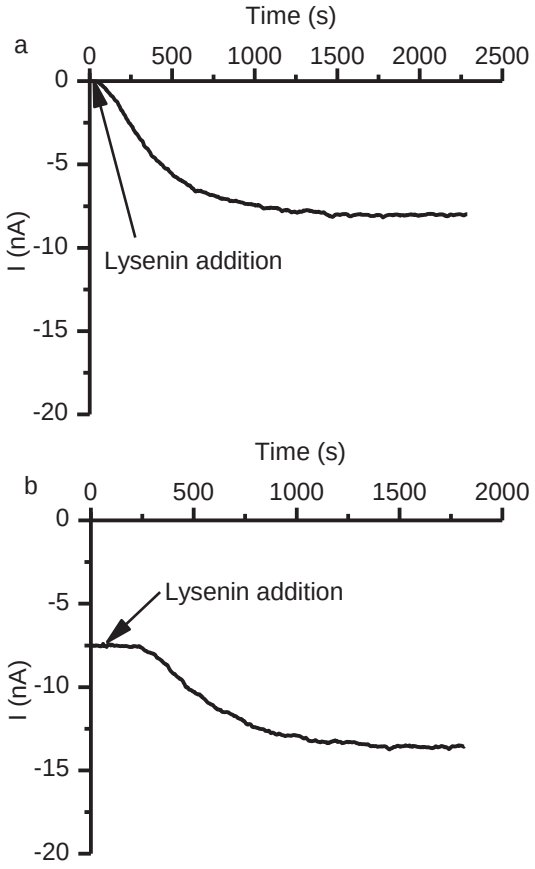

Fig. S3 Consecutive addition of lysenin for achieving increased number of inserted channels into the target lipid membrane. a) Channel insertion after addition of lysenin to a pristine membrane biased by -60 mV is indicated by changes in ionic current. The completion of insertion is indicated by a steady state is achieved in ~ 40 minutes. b) After recording the I-V plots, a new lysenin addition promotes further insertion and leads to an increased number of channels in the membrane