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Multivalent ions control the transport through lysenin channels

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ABSTRACT

We report the effect of different ions on the conducting properties of lysenin channels inserted into planar lipid bilayer membranes. Our observations indicated that multivalent ions inhibited the lysenin channels conductance in a concentration dependent manner. The analysis performed on single channels revealed that multivalent ions induced reversible sub-conducting or closed states depending on the ionic charge and size. Good agreement is reported between experimental results and a theoretical model that is proposed to describe the interaction between divalent ions and lysenin channels as a simple isothermal absorption process.

1. Introduction

Ion channels and porins are energy-independent transporters that play a central role in maintaining normal cell physiology [1–4]. Ion channels represent up to 50% of the total biological transporters within various philogenetic groups [1] and are characterized by high transport rate, specificity, and regulation [5–7]. Their gating induced by transmembrane voltages, ligands, pH or other stimuli is crucial for their functioning [5,8,9]. The ingenuity in the use of similar transporters to expand our knowledge of the structure–function relation for ion channels may prove extremely fruitful. A special class of transporters that preserve the characteristic high transport rate of ion channels is represented by pore forming toxins, which have the ability to form large conductance channels following insertion into cell membranes [10–13]. These unregulated pores provide open pathways that kill the host cells by disrupting the membrane chemical and electrochemical gradients [12]. Lysenin, a 297 amino acid pore forming toxin found in the coelomic fluid of the earthworm *Eisenia foetida* [14–18], forms large conductance channels (water permeable nanopores, ~3 nm diameter) [15,18] in bilayer lipid membranes (BLMs) containing sphingomyelin. Lysenin channels exhibit nonsymmetrical voltage gating at low positive potentials [19–21], which is affected by interactions with monovalent ions or by pH [21], most probably by modulation of the electrostatic forces governing the gating mechanism. To understand better the functioning of lysenin channels we addressed their interaction with different cations and analyzed the resulting conducting properties by monitoring the ionic current at non-gating negative voltages. We found that monovalent ions increased the macroscopic currents in a concentration dependent manner, while multivalent ions gradually and reversibly reduced the transport capabilities. Analyzing the effect of multivalent ions on single channels, we concluded that trivalent metal ions (Me^{3+}) completely block the open current by switching the pore to the closed state, and divalent ions (Me^{2+}) force the pore into a stable intermediate sub-conducting state. We described the interaction between lysenin channels and divalent ions as a Langmuir isothermal absorption and the predicted variation of the macroscopic current was observed experimentally. Furthermore, our findings strongly support a ligand gating mechanism influenced by the charge and size of the ions attached to a binding site.

2. Materials and methods

The BLM was formed across a small aperture (70 μm) created in a Teflon film separating two insulating reservoirs. A mixture of lipids (10 mg Asolectin, 5 mg Cholesterol, 5 mg Sphingomyelin – all from Sigma Aldrich – dissolved in 400 μl n-decane) was spread over the hole, followed by the addition of electrolyte to each reservoir (1 ml of 150 mM KCl, 20 mM Hepes buffer, pH = 7.2 — if not otherwise noted). The transmembrane voltage was applied via two Ag/AgCl electrodes embedded in each reservoir and connected to the headstage of an Axopatch 200B amplifier (Molecular Devices). The ionic current was recorded through a DigiData 1440A digitizer (Molecular Devices), and

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further analyzed using the pClamp10.2 (Molecular Devices) and Origin 8 (OriginLab) software.

Channel insertion was carried out by adding ~3 nM Lysenin (Sigma Aldrich) to the *trans* (grounded) side of the BLM while applying −80 mV to the *cis* (headstage) side under continuous stirring. After the open current reached a steady state (~40 min) and a population of stable inserted channels was achieved, the *trans* chamber was flushed with 20 ml of fresh electrolyte solution, and additional ions were added to both sides of the chamber as minute amounts of concentrated stock solutions of the chloride form. The influence of ions on the macroscopic current was estimated from the conductance values measured before (G_o) and after ion addition (G) as the relative variation G/G_o. The macroscopic conductance of the channel population was calculated as the average slope of six *I–V* curves recorded when a linear, symmetric voltage ramp (0 mV: −60 mV: 0 mV, scan rate 0.5 mV/s, 3 sweeps/run, created using the Episodic Stimulation Protocol in pClamp10.2) was applied to the BLM. For all conductance measurements the errors bars representing the standard deviations lie within the symbols. Full lines in the graphs represent experimental/fit data points, while dashed line connects the experimental points represented by symbols.

3. Results and discussions

The macroscopic conductance of the channel population increased monotonically upon NaCl addition to the 50 mM NaCl starting electrolyte (Fig. 1a) as might be expected due to a monotonic increase in bulk ionic conductivity with concentration, which is consistent with previous observations using KCl [21]. However, the observed linear increase in conductance indicated that neither Na^+ nor Cl^- affected the pore geometry, and any further changes induced by multivalent chlorides must be due to interaction with multivalent cations. A completely different behavior was recorded after Me^{2+} (Ca^{2+} and Mg^{2+}) were added to the support electrolyte (Fig. 1b). The recordings indicated a clear decrease in conductance and demonstrated that divalent metal ions interacted in a specific way with the lysenin channels and altered their ability to sustain the initial ion transport rate. A more dramatic decrease in conductance was obtained for Me^{3+} (Fig. 1c). While 20 mM Me^{2+} decreased the conductance by about 35%, the Me^{3+} examined caused a dramatic decrease in conductance and inhibited the ionic transport almost completely in the micromolar range (50 μM–250 μM). In fact, visible conductance decrease occurred when the concentration of Me^{3+} was less than 10 nM and demonstrated their effectiveness as current blockers. Furthermore, the *I–V* curves maintained linear for all ions and all concentrations used (see Supplementary data), thus the current inhibition by multivalent cations was not voltage dependent as reported for wild type α-hemolysin or bacterial porins [22–24].

To understand the mechanism underlying the diminished ion transport capabilities we investigated the reversibility of the conductance changes induced by trivalent ions. The inhibition induced by 10 μM La^{3+} ions was reversed rapidly to the initial open current after excess EDTA (1 mM) addition (Fig. 2). The fast recovery observed upon EDTA addition suggests that multivalent ions do not dramatically affect the pore assembly, for example by deoligomerization of the protein complex or, as an extreme case, by exclusion from the supporting BLM. Multivalent ions can inhibit the currents through pore forming toxins and porins [22–24] or can stabilize the open conformation of certain transport complexes [25]. However, it is well documented that multivalent ions affect the function of ion channels by electrostatic screening, physical blockage, or conformational changes that produce partial or total blockage of the conducting pathway [26–32]. To discriminate between these different potential mechanisms and to further understand the nature of the interaction between lysenin channels and multivalent ions we investigated the response from single channels (Fig. 3). The depicted recordings are

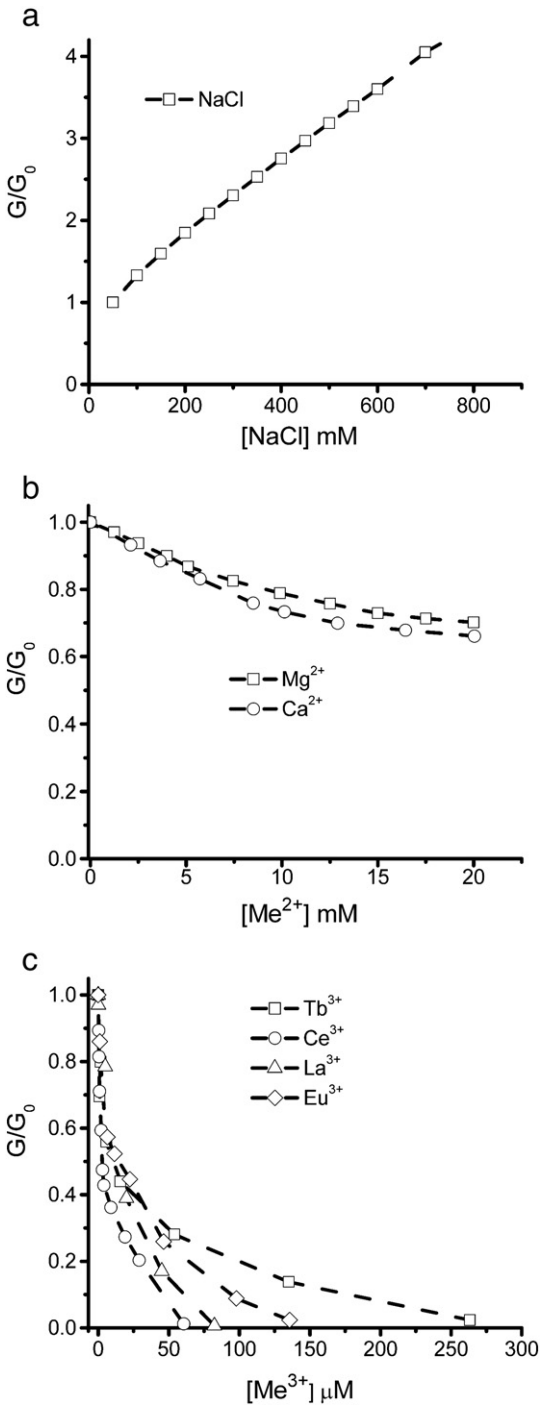

Fig. 1. Metal ions effect on the conductance of the lysenin channels. (a) The addition of NaCl yielded a monotonically increased conductance in the range of 50 mM–800 mM. b) The divalent metal ions Ca^{2+} and Mg^{2+} decreased the conductance in the range of 0 mM–20 mM and indicated that the ions interacted with lysenin channels and reduced the ionic transport rate. c) Trivalent metals Ce^{3+}, Eu^{3+}, La^{3+}, and Tb^{3+} acted as the most effective conductance reducers and annihilated almost completely the conductance in the range of 50 μM–250 μM.

not a continuous trace because of the required manipulations like chamber flushing, ions or chelator addition, and changes in the signal sampling to appropriate values for each experiment time-scale. The step-wise current variations for three inserted channels (Fig. 3a) were characterized by an individual current decrease of ~−47 pA at −80 mV bias voltage. After the addition of La^{3+} (100 μM), the step-wise variation of the recorded current was reversed (Fig. 3b), and the changes were characterized by the same discrete and uniform current

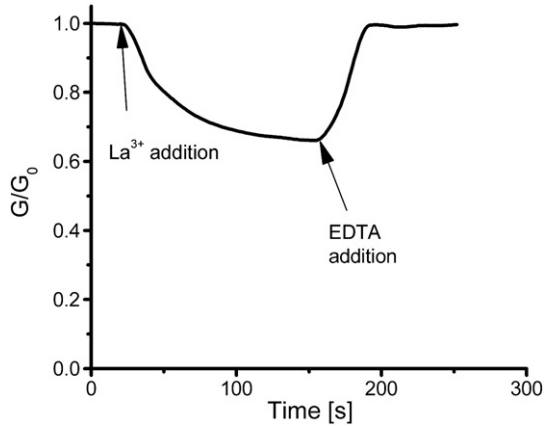

Fig. 2. The conductance changes induced by La^{3+} were reversible. The addition of 10 μM La^{3+} decreased the open current (at −80 mV holding potential, 0.1 s sampling rate, and 1 kHz hardware filter), but the addition of excess EDTA (1 mM) quickly reinstated the conducting properties.

variation (~47 pA). The initial current reinstated in seconds following excess EDTA addition (Fig. 3c). Each recovery step changed the current by the same ~47 pA, demonstrating again that the process was reversible. Together, these results demonstrate that screening was not the main mechanism for the observed reduction in conductance since simple electrostatic screening would result in monotonic changes of the current through single channels [28,32]. Moreover, lysenin pores show no significant selectivity between K^+ and Cl^- [19], and electrostatic screening by multivalent cations cannot simultaneously annihilate the transport of monovalent anions and cations. Bound multivalent ions may cause an energy barrier along the permeation pathway, which might be overcome at higher voltage. Strong depolarization (+140 mV) partially relieved the blockage induced by Ce^{3+}, while hyperpolarization had no significant effect (Supplementary Fig. S2). Voltage dependence indicates that multivalent ions act by binding to a site in the channel pore [28]. Partial unblocking only in extreme depolarization conditions sustains the hypothesis of an energy barrier placed close to the pore entrance, while the simultaneous transport cancellation of monovalent anions and cations rules it out. In this context we hypothesize that a strong depolarizing field removes the multivalent ions bound to a binding site buried within the channel structure [6], while hyperpolarization stabilizes the bound ions. Hence, channel blocking by La^{3+} stems from either total occlusion by bound ions or induced conformational transitions to the closed state. Total current blocking by occlusion is not consistent with the physical properties of lysenin channels. The diameter of a lysenin pore is estimated to be approximately 3 nm [15,18], therefore it is too large to allow complete blocking by individual ions attached to a binding site inside the lumen. The possibility that multiple cations sequentially bound to sites inside the conducting pathway and blocked it completely is not supported by the observed single-level current variations in the recordings (Fig. 3b, c) examined at 100 μs sampling rate. Moreover, the step-wise ion-induced current blockages observed here are distinct from the small, rapid, and transient current blockages induced by binding of divalent metals to the multiple binding sites of engineered α-hemolysin pores [33–37]. Taken together, our data strongly suggest that trivalent metal ions induced single step conformational transitions that blocked the open pathway of lysenin channels by closing them.

Since macroscopic currents decreased to a much lesser extent in the presence of Me^{2+} (see Fig. 1b), we explored this interaction at single channel level to identify if the decreased sensitivity is attributable to a different mechanism of current blockage. The insertion of four channels into the BLM was performed as described for the trivalent ions, and each insertion yielded the same current variation (approximately −47 pA at −80 mV holding potential). When 20 mM Ca^{2+} was added to the support electrolyte, the current was inhibited in discrete steps (Fig. 3d) similar to the addition of trivalent ions. However, each step changed the current by approximately 22 pA, which is about one half of the open current for a single channel (as indicated in Fig. 3a). This particular variation of the

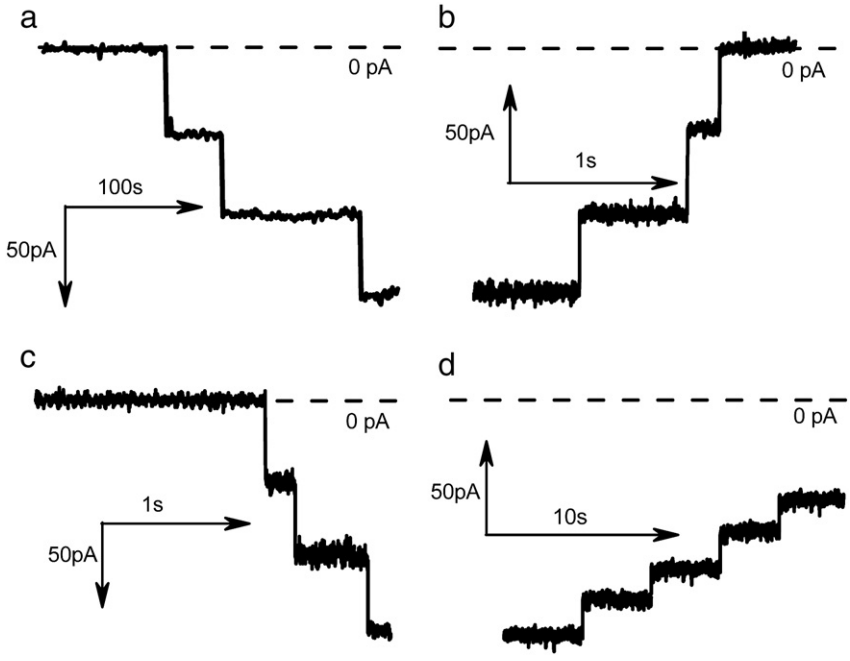

Fig. 3. The reversible interaction with multivalent ions resulted in full or partial closing of channels in the presence of multivalent ions. (a) Single channels insertion was sensed as discrete and uniform changes in the open current (−80 mV, sampling rate 1 s, and 1 kHz hardware filter). (b) The channels closed completely and fast upon the addition of 0.1 mM La^{3+} (−80 mV, and 100 μs sampling rate). (c) Addition of excess EDTA (1 mM) completely reopened the channels and reinstated the open current (−80 mV, and 100 μs sampling rate). (d) The discrete current changes upon Ca^{2+} addition (20 mM) counted for about one half of the fully open current, and suggested that a stable sub-conducting state was induced by the divalent ions (−80 mV, and 100 μs sampling rate).

current may be explained by considering the possibility of a partially closed channel that is sub-conducting [38]. The experiment did not reveal if each of the four channels went through a single conformational transition from the open state to a sub-conducting state, or if some closed completely in multiple steps while others remained open. The number of transitions induced by Ca^{2+} was equal to the number of inserted channels, and no further transitions were observed when [Ca^{2+}] increased, suggesting that the divalent ions switched each of the channels to an intermediate stable sub-conductance state, with no further ability to promote full closing.

The current response to divalent ions observed in the single channel analysis can be integrated in a model for the macroscopic current. This current is determined not only by external measurable parameters like applied voltage and conductivity, but by the individual status of each channel in the population. To develop the model, we assumed that the interaction between channels and divalent ions is a single step, all ions are permeant, the solutions are perfectly mixed at all times, the concentration of the divalent ions in the bulk can be approximated as constant, and electro-diffusion does not affect the interactions. Within the limits of these assumptions, the interaction with Me^{2+} can be described in its simplest approach as a Langmuir isothermal absorption [39,40]. In this case, the fraction of channels interacting one to one with Me^{2+} and characterized as sub-conducting is given by:

$$\theta = \frac{\alpha\left[Me^{2+}\right]}{1 + \alpha\left[Me^{2+}\right]} \tag{1}$$

where the fraction θ take values from zero (no Me^{2+} added, no sub-conducting states) to one (all channels are sub-conducting), and α is the equilibrium constant. After Me^{2+} addition some of the channels sub-conduct (N_2) and some stay open (N_1), while at any time the total number of channels in the population is constant, $N_0 = N_1 + N_2$. An open channel sustains a current I_1, and a sub-conducting one sustains a current I_2 (in the same external conditions). We consider here that $I_2 = f I_1$, $f<1$. The total current through all channels after Me^{2+} addition is:

$$I = N_1 I_1 + N_2 I_2 = N_0(1-\theta)I_1 + N_0\theta I_2 = N_0 I_1(1-\theta(1-f)) \tag{2}$$

By replacing θ from the Langmuir relation (Eq. 1), we find the expression of the total current in the presence of divalent cations:

$$I = N_0 I_1\left(1-(1-f)\left(\frac{1}{1+\frac{1}{\alpha\left[Me^{2+}\right]}}\right)\right) \tag{3}$$

In the absence of any divalent ions, the total current through the N_0 fully opened channels is given by:

$$I_t = N_0 I_0 = N_0 A K_0 V \tag{4}$$

where I_0 is the current through a single open channel in absence of multivalent ions, A is a constant depending on the pore geometry, K_0 is the specific conductivity of the bulk in the same conditions, and V is the bias voltage. By assuming that the presence of Me^{2+} does not affect the geometry of the remaining open pores, the current I_1 in the presence of divalent ions is given by:

$$I_1 = A K_1 V \tag{5}$$

where K_1 stands for the specific conductivity of the bulk after ion addition. When we measured the conductivities of the bulk before (K_0) and after (K_1) divalent ion addition (Ca^{2+} and Mg^{2+}) we found the expected linear variation:

$$K_1 = K_0 + b\left[Me^{2+}\right] \tag{6}$$

The relative changes of the current/conductance in the presence of divalent ions become:

$$\frac{I}{I_t} = \left(\frac{\left(K_0 + b\left[Me^{2+}\right]\right)}{K_0}\right)\left(1-(1-f)\left(\frac{1}{1+\left(\frac{1}{\alpha\left[Me^{2+}\right]}\right)}\right)\right) \tag{7}$$

The expression (Eq. 7) was used for the numerical simulation of the current response in the presence of divalent ions with parameters $f = 0.2$, $K_0 = 15$ mS cm^{-1}, $b = 0.15$, $\alpha = 0.05$ mM^{-1}, and the estimated relative current variation versus [Me^{2+}] (Fig. 4a) was interpreted in terms of changes induced by divalent ions. If the divalent ions produce only partial closing, the predicted macroscopic current decreases at first on addition of a small amount of divalent ions (<50 mM) until all the channels are partially closed and sub-conducting. In this region, the decrease in current because of closing is not compensated by the increase in conductivity upon divalent cations addition. Additional divalent ions (>50 mM) increase the electrolyte conductivity (we assumed the divalent cations are permeant) but do not produce further closing (the second term in Eq. (7) remains approximately constant), and the predicted macroscopic current increase with ion concentration (the first linear term in Eq. (7) become dominant).

A prerequisite for observing the turning point of the relative current is to assure that the added divalent ions have a significant contribution to

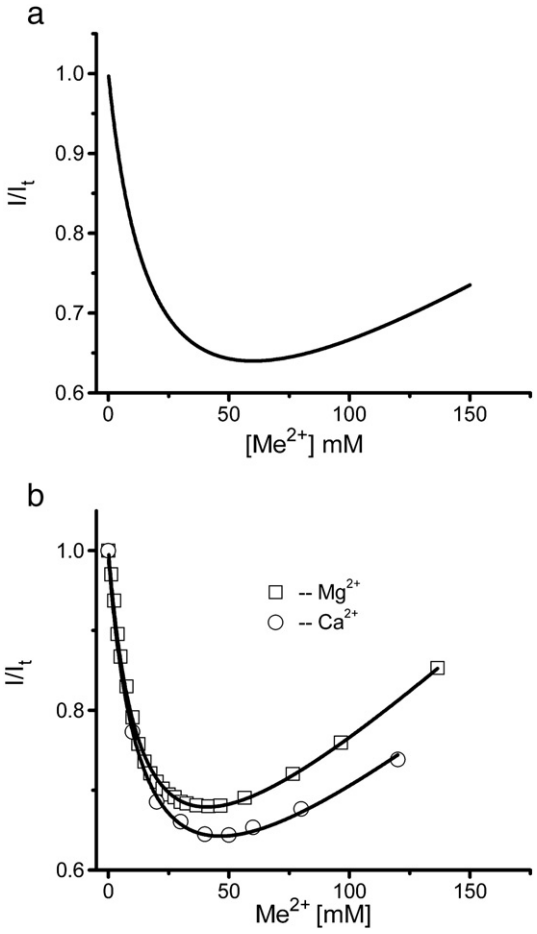

Fig. 4. The interaction with divalent ions described as a Langmuir isothermal absorption process. (a) The model predicted that a stable sub-conducting state should be observed as a decrease in the relative macroscopic current (I/I_t) at low divalent ion concentration, and an increase at high divalent ions concentrations. (b) The relative current measured in the presence of Mg^{2+} and Ca^{2+} satisfactorily matched the absorption model. The solid lines are the least square fit of the relative macroscopic current expression.

the electrolyte conductivity. To test the model, we increased in steps the divalent cation (Ca^{2+} and Mg^{2+}) concentration of a 150 mM KCl support electrolyte while performing macroscopic current measurements (at −60 mV holding potential) for a population of lysenin channels. The current was measured after a steady state was achieved for each concentration. The relative value (I/I_t) of the steady state current decreased in the low concentration range (Fig. 4b), as predicted by the model, indicating channel closing. There was a critical concentration (~40 mM), where the relative current reached its minimum indicating the completion of the closing process. An increase of the divalent ion concentration beyond this point produced a further increase of the relative current due to the increased bulk conductivity. The experimental results were in good agreement with the model for both divalent ions (Fig. 4b), and similar equilibrium constants were determined from the least square fit ($\alpha = 0.075\ mM^{-1}$ for Mg^{2+} and $\alpha = 0.08\ mM^{-1}$ for Ca^{2+}).

Lysenin channels responded to multivalent ions by partial or total closing and this ligand induced gating reduced the macroscopic currents. The trivalent ions demonstrated an increased effectiveness as current blockers versus divalent ions, partially explained by their capability to induce full closing as opposed to partial closing induced by divalent ions. Apparently, the net charge of the ions interacting with lysenin established the closing pathway. To test this assumption, we analyzed the conductance reduction for divalent and trivalent forms of the same metal, Fe, and observed that Fe^{2+} was far less effective than Fe^{3+} in reducing the current (Fig. 5a). While 0.25 mM Fe^{3+} nearly eliminated the conductance, resembling the lanthanides (decrease of ~97%), the same concentration of Fe^{2+} decreased the conductivity only by a few percent, confirming that the current blocking mechanism was influenced by charge, not chemical identity. The high sensitivity observed for Me^{3+} was not encountered for spermidine, a trivalent organic polyamine, which produced a decrease in conductance similar to divalent ions and the relative changes in conductance followed the pattern established by the Langmuir absorption model (Fig. 5b). While a trivalent ion, spermidine has a much larger volume than the trivalent metals. This divalent-like behavior suggests that ionic size and charge together are responsible for different conformational changes.

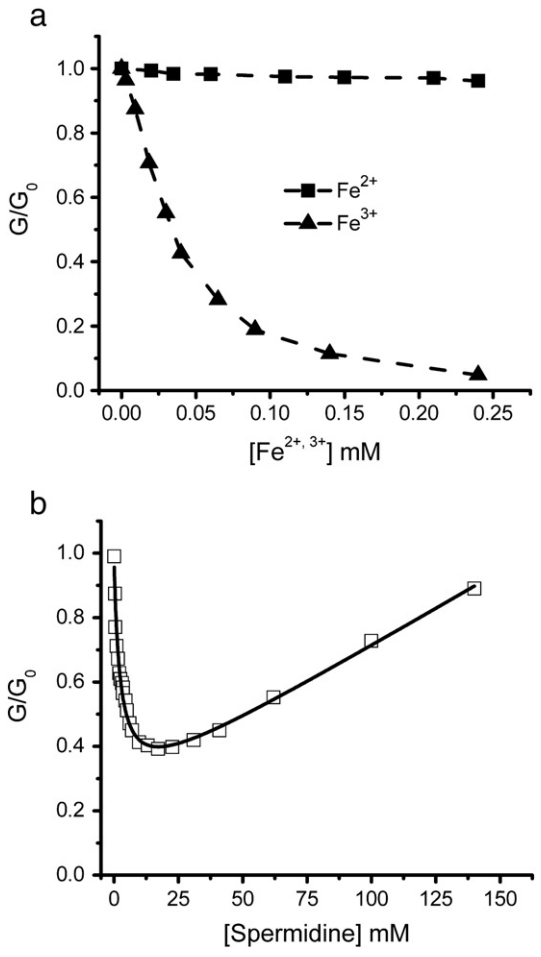

Fig. 5. The response to multivalent ions depended on ionic charge and size. (a) The relative decrease of the macroscopic conductance depended strongly on the valence of Fe; Fe^{3+} was a more effective conductance blocker than Fe^{2+}, suggesting that charge, not chemical identity, influenced the channels closing. (b) The low sensitivity toward spermidine, a trivalent ion, and the agreement of the experimental data with the mathematical model for sub-conducting (solid line) demonstrated a divalent-like behavior, and indicated that size and charge play a major role in establishing the closing pathway.

Electrical measurements along multivalent ions demonstrated the ability to modulate reversibly the transport properties of lysenin channels inserted into BLMs. The step-wise current variation using single channels demonstrated that the interaction with multivalent ions manifested as partial or total closing, suggesting that conformational changes accompanied the transition induced by the attachment of ions to a binding site. Although we observed a clear distinction between the closing induced by Me^{2+} and Me^{3+} the differences should not be understood as being exclusive. Our observations on larger populations of lysenin channels indicated that Me^{3+} may induce sub-conducting states and some channels may close completely in the presence of Me^{2+}, but these were rare exceptions. The sub-conducting states induced by the interactions with divalent ions open the possibility to control reversibly the pore size, which will allow their use as controlled molecular sieves. The studies also provided the phenomenological basis for a novel approach in ion sensing applications, especially for trivalent metals. The built-in ion sensing mechanism circumvents the labeling of targets or the use of engineered proteins for channels formation. The ability to trigger externally the opening and closing of large conductance channels has potential applicability for controlled transport of macromolecules across natural and artificial lipid membranes. Beyond these potential applications, our results suggest that further studies of lysenin channels may provide a detailed understanding of the gating mechanism and physiological role of pore forming toxins and ion channels.

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Appendix A. Supplementary data

Supplementary materials related to this article can be found online at doi:10.1016/j.bpc.2010.07.004.

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