Selective Permeability Function of Lysenin Channels

The selective transport of ions and molecules across cell membranes is crucial for correct cell functioning and life sustaining. The membrane of every living cell acts as a controlled permeability barrier that contributes to fundamental physiological processes such as the creation and maintenance of electrochemical gradients, communication, nutrient transport, waste disposal, or energy production [1-3] . Although the bilayer lipid partition possesses a primary selectivity function, many of the complex transport processes are actually assured by specialized transmembrane transporters of proteic nature. Many of these transmembrane proteins are ion channels found in every living cell on earth. Their importance is underscored by the fact that a significant number of genes are devoted to encoding each kind of ion channel, all of which have very specific functional properties designed for specific physiological roles in the cells in which they are found [2, 3]. Consequently, the ion channels that a particular cell is equipped with provide the electrical signature that defines the behavior of that cell. Although their salient feature is to selectively transport ions in a highly regulated fashion, the consequences and the implications of such primary activity are much larger. All living cells create and maintain a transmembrane potential by the means of selective permeability. Any attempt of constructing an artificial cell-like structure must provide such capabilities for powering the cell. Reconstitution of various ion transporters (carriers, or ion channels) into artificial bilayer lipid membranes constitutes viable approaches for rendering

electrogenicity. However, the use of self-inserted ion carriers (e.g. valinomycin), although straightforward, leads to the lack of a fundamental feature, namely regulation. In contrast, reconstitution of ion channels into artificial BLMs preserves the regulation, but the process requires obtaining and purifying the ion channels, and a more complicated reconstitution protocol. Moreover, many potential applications of such artificial structures require transportation capabilities for molecules much larger than ions, and ion channels are not suitable for such functions.

In this respect, we started looking for a transmembrane transporter simultaneously characterized by several fundamental features: high transport rate, regulation, self-insertion into artificial BLMs, ability to transport large molecules, and selective permeability. In this line of inquiries, we focused our attention on lysenin, a 297 amino acid pore-forming protein extracted from the coelomic fluid of the earthworm *Eisenia foetida* [4, 5]. Lysenin inserts relatively large conductance channels (~ 3nm diameter) in artificial and natural lipid bilayers containing sphingomyelin, a main component of the mammalian cell membrane [6-9]. Lysenin has cytolytic and hemolytic activity [9-11], which indicates a pore-forming toxin. Nevertheless, lysenin channels share fundamental features with those of ion channels, such as high transport rate and regulation. Recent experiments have revealed that lysenin channels elicit voltage-induced gating and that positive transmembrane potentials larger than 20 mV close the channels [6, 7, 12]. The non-linear and non-symmetrical I-V characteristic of a population of lysenin channels set it apart from other pore

forming toxins [13, 14]. Moreover, the conducting state of lysenin channels is strongly modulated by multivalent cations [15, 16]. This ligand-induced gating process is very common for ion channels, but only rarely observed for pore forming toxins. However, for a complete functional similarity with ion channels, a selective permeability function more complex than molecular sieving must be observed. Unfortunately, this important physiological future has been overlooked in previous reports on lysenin transport properties. To make the quest more interesting, completely contradictory observations have been published. Asymmetrical ionic conditions have been reported to elicit no shift in the zero-current potential, indicating no significant selectivity between anions and cations [7]. In contrast, a later report suggests that lysenin channels assembled from native or recombinant proteins are cation-selective [12].

To conclusively add ionic selectivity to the list of intriguing features of lysenin channels, we carried out several experiments meant for measuring the transmembrane potentials in response to asymmetrical ionic conditions for NaCl and KCl electrolyte solutions. Our results indicate that lysenin channels have selectivity filters for cations in their structure, and a possible role played by electrostatic interactions for the observed selectivity is revealed.

Materials and methods

Bilayer Lipid Membrane (BLM) formation

The hand-made BLM chamber(Figure 1,2) consisted of two PTFE® reservoirs separated by a thin PTFE® (Teflon) film (~120 µM thickness) into which we “drilled” a small hole (~70 µm diameter) by using an electric spark (Figure 3). Each reservoir was filled with 1ml electrolyte solution (50 - 135mM NaCl or KCl, buffered with 20mM Hepes, pH7.2) and the BLM was formed by dabbing the hole[17, 18] with a minute amount of a decane-mixture of Asolectin, Sphingomyelin, and Cholesterol (all from Sigma, 4:2:2 weight ratios, ~20mg total lipids per ml). Alternatively, the preparation of neutral BLMs implied the use of DiPhytanoyl Phosphocoline (AvantiLipids) instead of Asolectin. The electric signal was recorded via two Ag/AgCl electrodes inserted into the aqueous solution in each of the reservoirs, and wired to the head-stage of an Axopatch 200B amplifier (Molecular Devices). The amplifier was connected to a DigiData 1440A digitizer (Molecular Devices) for real-time observation, recording, and further analysis (Figure 4). The solutions in the two reservoirs were continuously mixed by using a low-noise magnetic stirrer (Warner Instruments), and all the experiments were performed at room temperature (Figure 2). The BLM formation was monitored by estimating the BLM capacitance from the amplitude of the square-shaped capacitive current recorded in response to a triangle wave stimulus provided by a signal generator.

Planar Bilayer Lipid Membrane Setup for Lysenin Channels Studies

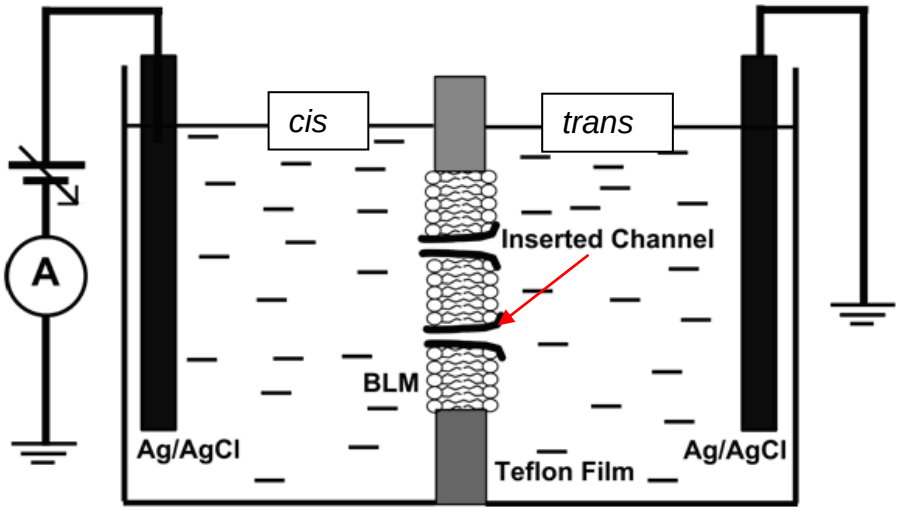

Figure 1 BLM Chamber Setup: PTFE® reservoirs setup

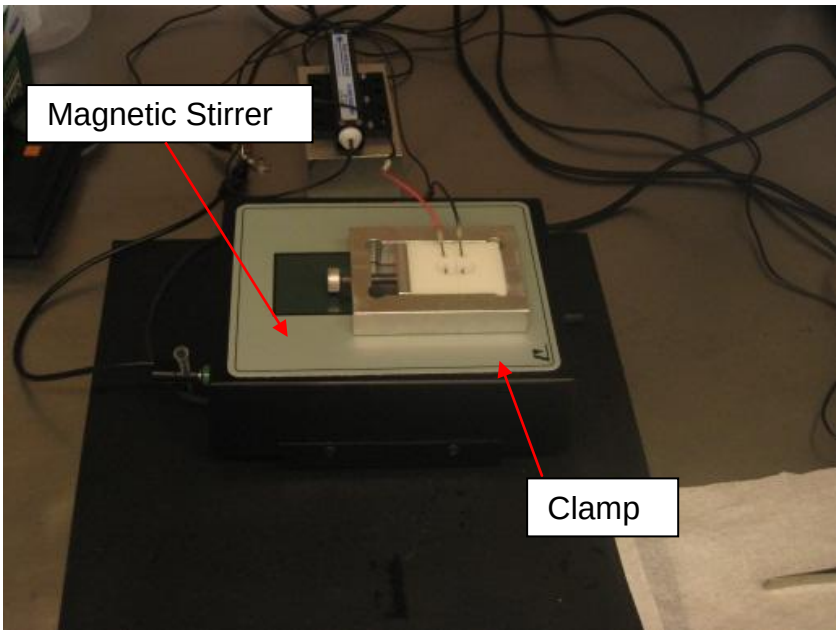

Figure 2 Picture of the BLM Chamber setup in addition to the magnetic stirrers and clamp

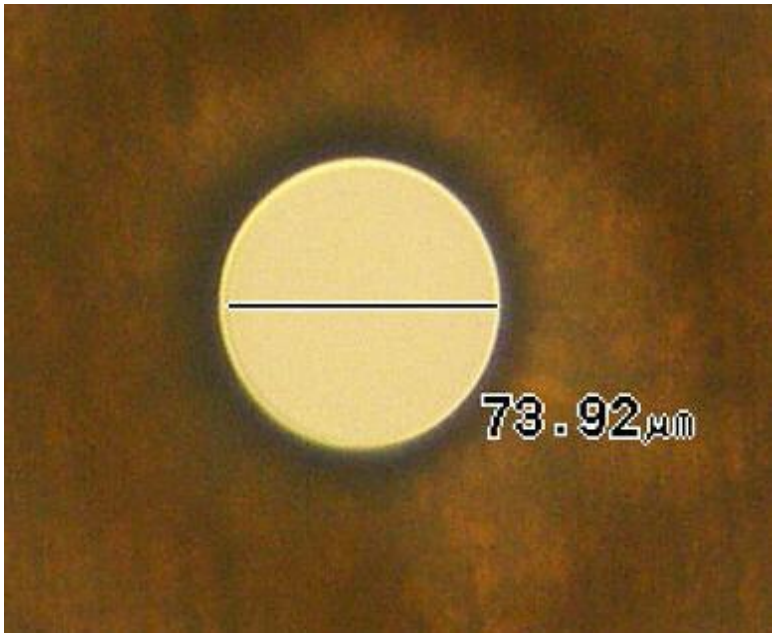

Figure 3 Microscope Picture of Teflon Film with Hole

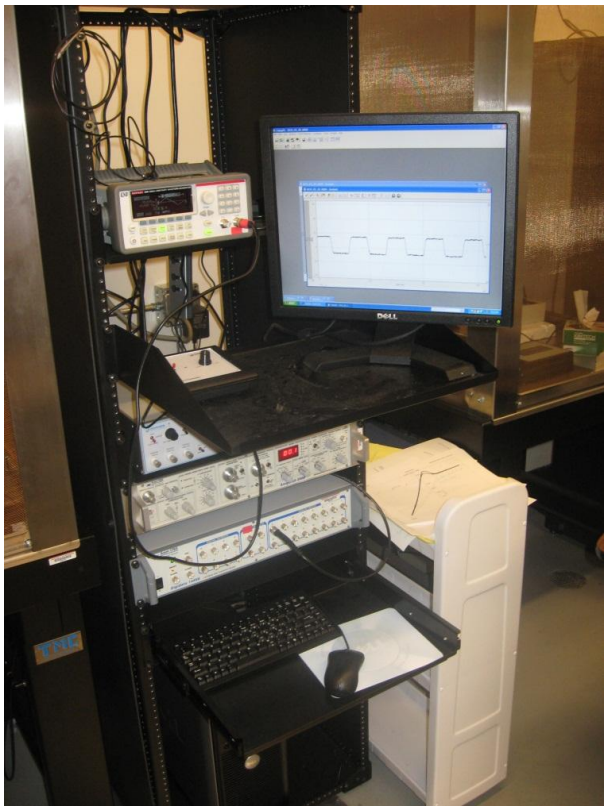

Figure 4 Recording Setup (Axopatch 200B Amplifier, DigiData 1440 Digitizer)

Channel insertion

After a stable BLM was achieved, channel insertion was performed by adding ~0.6 µl of 0.5 µM lysenin stock solution to the ground-wired (*trans*) reservoir under continuous stirring. Channel insertion was monitored by recording the ionic current in response to a constant -60 mV transmembrane potential applied to the head-stage (*cis*) wired reservoir. The insertion of individual lysenin channels was observed as discrete variations in the ionic current under voltage-clamp conditions. After the completion of the insertion process indicated by a steady value of the open current, asymmetrical ionic conditions were created by adding small amounts of concentrated electrolyte (2 – 4 M NaCl or KCl, buffered with 20 mM Hepes, pH7.2) to the *cis/trans* reservoir under continuous stirring.

Transmembrane potential measurement and permeability estimation.

The transmembrane potential was measured from the I-V characteristics recorded in response to linear voltage ramps created with the ClampFit software package (Molecular Devices). Owing to the fact that lysenin channels show voltage-induced gating for positive applied voltages and a strong non-linearity of the I-V curve for such voltages [6, 7, 12], we took extra care in considering only the linear range in our analysis. While the linear-variable voltage stimulus spanned an ~ 40 mV range, the upper and lower limit were individually set for each run in such a way to intersect the x axis (corresponding to zero ionic current). The straight-line fit of the linear portion

of the I-V curves provided a direct way for estimating the transmembrane potentials from the fit parameters. The transmembrane potentials values, averaged from three independent runs, were plotted as a function of concentration using Origin 8.5.1 software package, and the permeability ratio extracted from the fit with the Goldman-Hodgkin-Katz (GHK) equation [19-22] adjusted for only two ions in the system:

$$V_m = -\frac{RT}{F}\ln\frac{\frac{P_{Na}}{P_{Cl}}[Na]_o + [Cl]_i}{\frac{P_{Na}}{P_{Cl}}[Na]_i + [Cl]_o}$$ where V_m is the transmembrane voltage, R is the gas constant, T is the absolute temperature, P denotes the permeability of that particular ion, and the index o and i indicates the side.

Results and discussions

Addition of lysenin to the *trans* chamber under continuous stirring and at a bias voltage of -60mV elicited the development of an ionic current, indicative of the fact that lysenin inserted conductance-channel into the Asolectin-based BLM and altered the permeability barrier function (Figure 5). The ion current stabilized after ~ 2 hours indicating the completion of the insertion process. The non-linear I-V curve (Figure 6) recorded in the voltage range -60mV - +60mV indicated that lysenin channels inserted into an Asolectin-based BLM are voltage gated, and that no miss-oriented channels are present in the channels' population [6, 7, 12, 14]. The ohmic behavior in the negative voltage range suggested that no

closures occur for such bias voltages, while the rapid decrease of the macroscopic current at positive voltages greater than ~ +20mV indicated the voltage-induced gating [6, 7, 12, 14].

Addition of NaCl to the *Cis* or *trans* reservoir shifted the IV curves in a concentration-dependent manner indicating the development of transmembrane voltages resulting from the non-symmetrical ionic conditions. Upon NaCl addition to the *cis* reservoir, the transmembrane voltages increased as the concentration ratio increased (Figure 7), as expected from the GHK equation. The fit of the experimental values of the transmembrane voltage versus concentration according to the GHK equation yields a permeability ratio of 6.38±0.16 for cations over anions. Although not as impressive as the permeability ratios currently encountered for ion channels (Maybe give a number for permeability of Ion Channels) [19, 21], this difference in permeability for sodium and potassium allowed development of transmembrane potentials around 50 mV when the concentration ratio between the *cis* and the *trans* reservoirs was about five.

Lysenin channels always insert into artificial BLMs in an oriented manner[6, 7], and we did not know if the selective transport relates to the channels' orientation. To answer this question, we prepared a similar BLM containing lysenin channels having exactly the same orientation as in the previous experiment. In contrast, the successive additions of ions were performed on the *trans* side of the bilayer. The plot of the developed transmembrane potential versus concentration ratios (Figure 8) indicated a transmembrane voltage of opposite sign but otherwise a similar evolution

compared to the previous experiment. However, the higher permeability ratio calculated from the fit with the GHK equation (P =18.3±1.34) suggested that the channels' orientation had a significant influence on the transmembrane potential. Although the permeability ratio was greater by a factor of ~3 when the NaCl addition was carried out on the *trans* side as opposite to the *cis* side, the non-linear relationship between transmembrane voltages and permeability ratio still allowed yielding transmembrane potentials of ~ 50mV for ionic concentration ratios of ~ 4. This result is extremely important when working with spherical bilayer membranes (liposomes), because it demonstrated the ability of creating and adjusting the transmembrane potentials whatever the orientation of the inserted lysenin channels, and by adjusting the ionic concentrations on either side of the membrane. For obvious reasons, facile insertion of lysenin channels into liposomes may be performed only for one orientation, and facile changes in concentrations would be performed from the outside (which is much easier than trying to adjust ionic concentrations from within the liposome). Next, we determined whether the chemical identity of the cations influences the values of the transmembrane potentials. In this respect, we performed an experiment in which we replaced NaCl with KCl, and added the extra ions to the *cis* side. The plot of the transmembrane potentials versus concentration ratios (Figure 9) indicated that the KCl-based electrolyte had a similar behavior to the NaCl-based electrolyte. However, the permeability ratio of 2.55±0.34 calculated for permeability of Potassium ions over permeability of Chlorine ions (P_{K+}/P_{Cl-}) from the fit with the GHK equation indicated a slightly reduced value of this parameter

compared with the NaCl case. This result is intriguing if we take into account that the K^+ ions have a higher mobility in solution, and the higher diffusion coefficient for K implies an increased permeability of those ions compared to those of Na^+. If we assume that the Cl^- mobility does not change when the solution contains different cations, it is natural to conclude that K^+ has a lower mobility than Na^+ when moves through the lysenin channel. This unusual result might be explained by supplementary electrostatic interactions between K^+ and the channel, and such interactions might be facilitated by the fact that the ions lose their hydration shell when threading the channel.

Lysenin channels possess a very intriguing voltage-induced gating feature that is not entirely understood. Several reports demonstrate that lysenin channels close at relatively low positive transmembrane voltages [6, 7, 12], and we used this salient feature to demonstrate the functionality and check the correct orientation of lysenin channels (*vide supra*). The strong voltage-induced gating suggests the presence of a charged voltage sensor domain in the channel's structure, which may influence the transport of ions by imposing electrostatic energy barriers or binding sites. However, the voltage-induced gating is practically abrogated when neutral lipids are exclusively used as BLM components [7, 15]. This behavior might be related to a specific interaction between the anionic lipids present in a BLM composed of Asolectin and the inserted channels. Since Asolectin is a complex mixture containing a large amount of anionic lipids, the selectivity might be a result of interactions between such lipids composing the BLM and the charged ions without requiring selectivity

filters to be present in the channel's structure. In this line of inquiry, we performed an experiment by replacing the Asolectin lipids with the neutral Diphytanoyl Phosphatidylcholine, while keeping Cholesterol and Sphingomyelin as BLM components. The BLM exclusively composed of neutral lipids completely abrogated the voltage-induced gating and rendered a rather linear I-V curve for a population of channels inserted in such BLM (Figure 10).

The transmembrane voltage was measured in response to addition of NaCl on the *trans* side, and its plot versus concentration (Figure 11) demonstrated that the transmembrane potential was not a consequence of interactions between anionic lipids and ions. Moreover, the permeability ratio estimated for the neutral lipids (P = 18.39± 0.38) was practically equal to the one recorded for Asolectin, suggesting that the presence of charged and uncharged lipids, in addition to their respective variations in voltage gating functions, had negligible influence on selectivity. By eliminating the charged lipids as a potential source for the selective filtering, we concluded that lysenin channels have an inherent selectivity filter for cations over anions.

Owing to the fact that multiple previous experiments [6, 7, 16] demonstrated that electrostatic interactions play a major role in the functionality of lysenin channels, we may assume that the selectivity filter consists of one or more electrostatic barriers placed within the channel's structure. The non-symmetrical response recorded upon addition of ions to the *cis* or *trans* reservoir suggests that the energetic barriers are not uniformly distributed along the channel's conducting pathway.

Illustrations

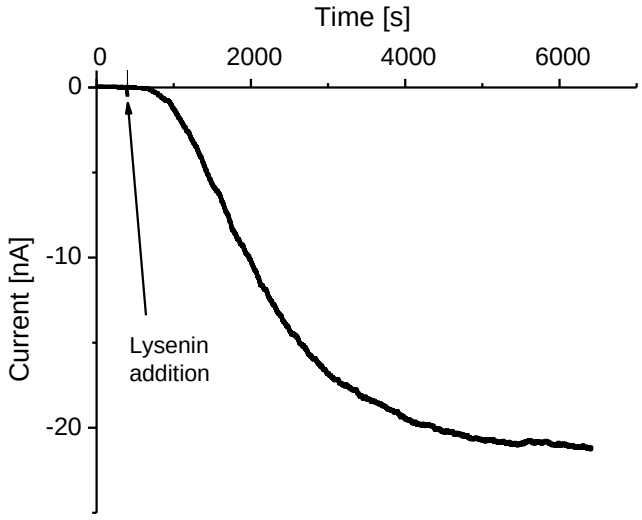

Figure 5 Addition of lysenin to the *trans* reservoir under voltage clamp conditions (V =-60mV) yielded an ionic current indicative of channel insertion. The macroscopic current stabilized after ~ 2 hours indicating the completion of the insertion process.

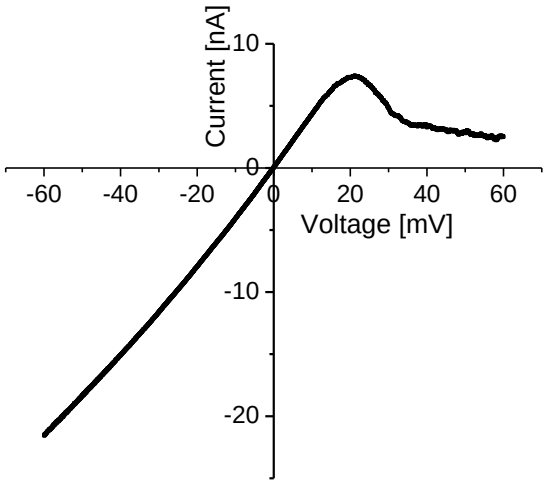

Figure 6 The strongly non-linear I-V curve recorded for a population of lysenin channels inserted into an Asolectin-based bilayer membrane indicated that positive voltages greater than ~20mV induced channels' closure, and that the lysenin channels were directionally inserted.

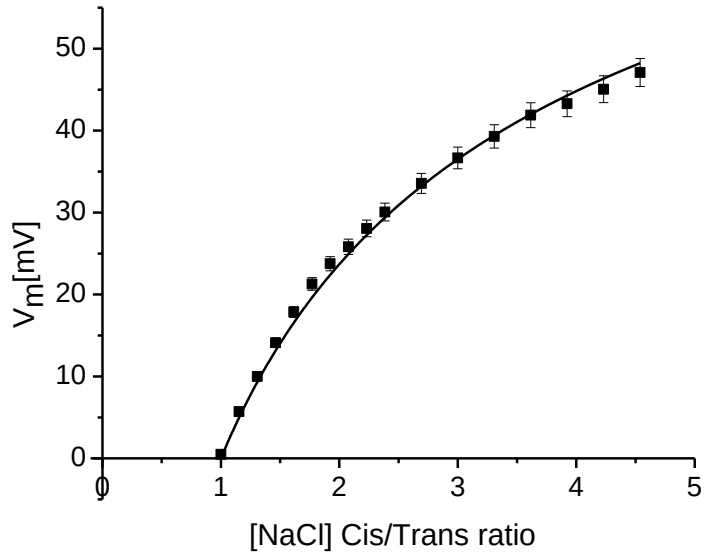

Figure 7 Addition of NaCl to the *cis* side elicited the development of transmembrane voltages (symbols) matching the GHK equation (full line) for a permeability ratio of 6.38±0.16 (n=3) for cations over anions.

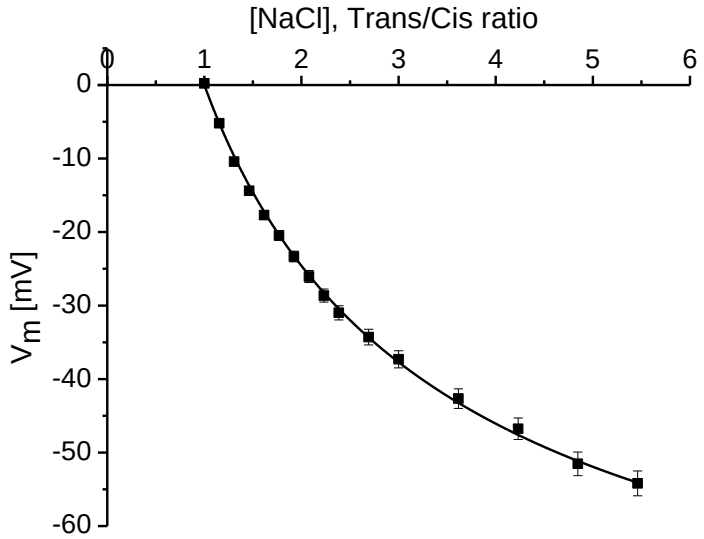

Figure 8 NaCl addition to the *trans* side elicited the development of transmembrane voltages (symbols) matching the GHK equation (full line) for a permeability ratio of 18.3±1.34 (n=3) for cations over anions .

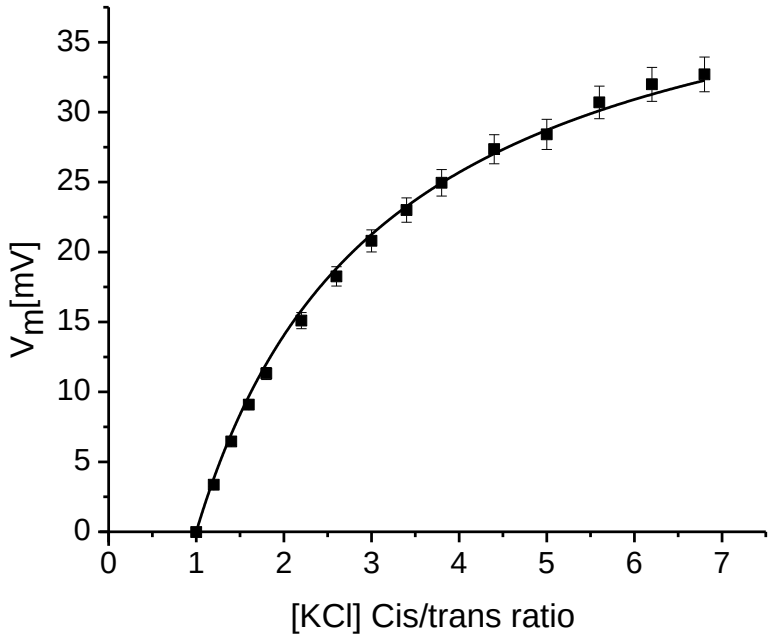

Figure 9 KCl addition to the *cis* side elicited the development of transmembrane voltages (symbols) matching the GHK equation (full line) for a permeability ratio of 2.55±0.34 (n=3) for cations over anions.

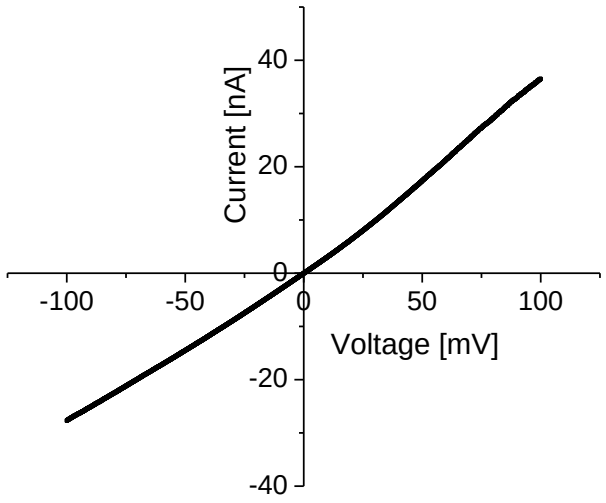

Figure 10 The linear I-V curve recorded when working with neutral lipids shows no abrupt drop in the macroscopic current and indicates the absence of a voltage gating mechanism in play.

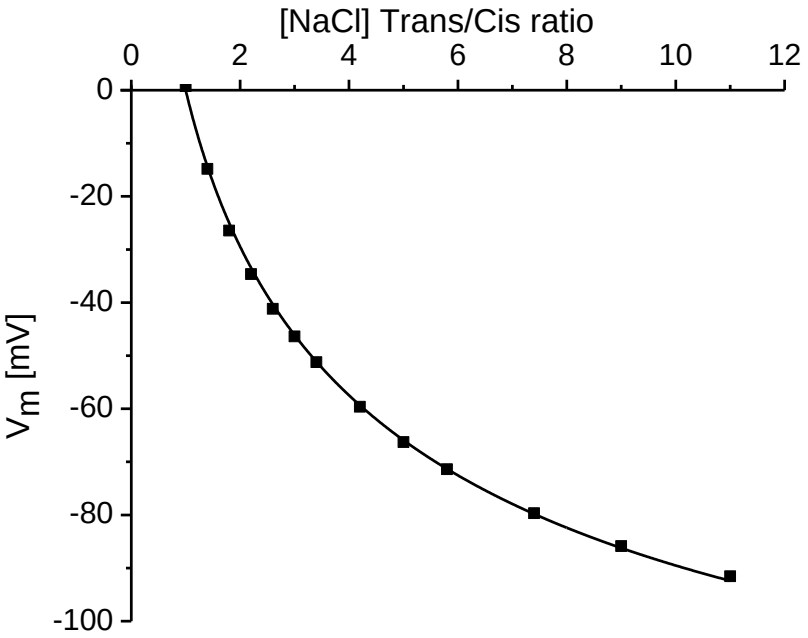

Figure 11 The use of neutral lipids preserved the ionic selectivity of the lysenin channels and indicated a permeability ratio of 18.39± 0.38 for cations over anions. The almost identical behavior of charged and neutral lipids suggests that the selectivity filter is part of the lysenin channel's structure.

Conclusions and Future Work

Our work clearly demonstrated an enhanced permeability of lysenin channels for cations over anions, which translated to yielding strong transmembrane voltages when the channels were exposed to asymmetrical ionic conditions. Such remarkable features suggest that lysenin, although considered a toxin, shares all the salient features of ion channels, and such an observation might be useful in deciphering its obscure physiological role. Beside the selectivity filter function, we found a non-symmetrical response of the transmembrane voltage upon addition of ions in the *cis* or *trans* compartment. We interpreted this behavior as stemming from a non-symmetrical distribution of the energy barriers within the channel's structure, without ruling out other possible interactions as being responsible for this effect. The secondary and tertiary structure of the lysenin channel is still unknown, and various constrictions within the lumen may impede the flow of ions and yield selectivity. The future use of voluminous organic ions as anions may help decipher the presence of other constrictions imposing a supplementary mechanical barrier for ions' movement. The unusual higher mobility of Na^+ over K^+ in the lysenin channels (totally opposite to their behavior in solutions) can be explained by considering the higher ionic radius of K+ compared to Na+. However, for accounting the smaller radius, one must assume that the ions are losing their hydration shells while threading the lysenin channel and we do not know the validity of this assumption for this particular case.

Our observations may have a large impact on future developments ranging from artificial cells to carriers for drug delivery. The electrochemical gradients across membranes have direct implications in energy production and storage, and the ability of lysenin to create such gradients might be used for powering up liposomes as a first step in producing artificial cells. These liposomes also have a proven potential for medical applications requiring localized drug delivery. In addition to the ability of lysenin channels to accommodate larger molecules, the channel itself is voltage-controlled. When combined with an external stimulus for controlling ionic concentrations, such liposomal carriers might be used for spatial and temporal control of drug release within a living organism.

We have further speculated as to the use of Lysenin in other functions even outside of biological processes. Lysenin's permeability function could be key in any ionic filter, especially those of salt water filtration. Similar to methods by which seagulls are able to desalinate their blood to survive for days at sea while only drinking seawater, desalination with artificial BLM's could exploit lysenin's selective permeability to create a desalination process more efficient than current yields in reverse osmosis desalination plants.

Potential applications exploiting not only this function, but also the versatility of the lysenin protein in general, could lead to advancements in the understanding of ion channels and permeability functions for use in modes of drug delivery, cell batteries, and any device that requires selective ion transport for functioning.

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