Lipid packing stress and polypeptide aggregation: alamethicin channel probed by proton titration of lipid charge

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Lipid membranes are not passive, neutral scaffolds to hold membrane proteins. In order to examine the influence of lipid packing energetics on ion channel expression, we study the relative probabilities of alamethicin channel formation in dioleoylphosphatidylserine (DOPS) bilayers as a function of pH. The rationale for this strategy is our earlier finding that the higher-conductance states, corresponding to larger polypeptide aggregates, are more likely to occur in the presence of lipids prone to hexagonal H_II phase formation (specifically DOPE), than in the presence of lamellar Lα-forming lipids (DOPC). In low ionic strength NaCl solutions at neutral pH, the open channel in DOPS membranes spends most of its time in states of lower conductance and resembles alamethicin channels in DOPC; at lower pH, where the lipid polar groups are neutralized, the channel probability distribution resembles that in DOPE. X-Ray diffraction studies on DOPS show a progressive decrease in the intrinsic curvature of the constituent monolayers as well as a decreased probability of H_II-phase formation when the charged lipid fraction is increased. We explore how proton titration of DOPS affects lipid packing energetics, and how these energetics couple titration to channel formation.

Introduction

The evidence mounts. Membrane lipids are not just a filler or an inert solvent for membrane proteins; they are functionally involved. Interactions between lipids and embedded proteins control conformational equilibrium between different functional states of proteins. Among natural membrane proteins one clear example is the shift between the Meta-I and Meta-II forms of Rhodopsin with varied lipid species in the host membrane. The amount of the Meta-II form increases with the increase in phosphatidylcholine acyl chain unsaturation, thus demonstrating the important role of lipids in modulating membrane-signaling systems. Many other examples of the crucial role of lipid–protein interactions in enzymatic reactions and receptor regulation can be found in a recent review.

It is well known that the conductance, lifetime, and formation ‘on-rate’ of the channel-forming drug gramicidin A depend on host-lipid species. In addition to electrostatic effects of surface charge and poorly understood effects of neutral lipids on channel conductance, extensive systematic studies of the gramicidin channel in different host-lipid compositions have shown that
its properties can be controlled by purely mechanical parameters. It was shown that gramicidin channel life-time and free energy of dimerization are modified by bilayer curvature stress and membrane tension in a quantitatively predictable way.

Channels formed by the 20-amino acid peptide alamethicin also show properties that depend on membrane lipid composition or on tension applied to the bilayer. A clear correlation between the tendency of lipids to form the inverted hexagonal phase and the expression of higher-conductance states of alamethicin peptide channels in those lipids was demonstrated in studies with dioleoylphosphatidylethanolamine (DOPE)/dioleoylphosphatidylcholine (DOPC) mixtures. Alamethicin was inserted into bilayer membranes composed of lipids of empirically determined inverted hexagonal phase spontaneous radii. Lipids with different spontaneous radii form planar membranes with expectedly different degrees of stress of forcing lipids into a planar structure. It was found that this mechanical parameter of the host-lipid bilayer plays a crucial role in alamethicin channel formation. In particular, states of higher conductance were found to be much more probable in DOPE, a lipid of high curvature, than in DOPC, lipid of low curvature. In the case of mixtures, the relative probability of states was a monotonic function of the DOPE/DOPC ratio.

In this paper, whose preliminary version was reported elsewhere, we demonstrate that a continuous variation of factors that stress membrane structure can direct the conformational equilibrium of channel-forming peptides. Specifically, we insert alamethicin into DOPS bilayers and change the bilayer surface charge and lipid head group electrostatic interactions with varied pH and varied salt concentration. Dramatic changes in relative probabilities of channel conductance states observed as a result of such manipulations provide further evidence of the importance of host-membrane mechanical parameters for channel protein function.

By X-ray diffraction we show that the decreased electrostatic energy of the polar surface (that goes with the decreased charge at low pH values) shifts DOPS from the purely lamellar form seen at neutral pH to an H₄ phase of ever-higher spontaneous curvature. This shift agrees well with our transport measurements performed on the same lipids. In 0.1–0.3 M sodium chloride solutions at neutral pH, alamethicin channels exhibit the DOPC-like pattern expected for lamellar lipids; in acidic solutions they show the DOPE-like pattern expected for H₄-prone lipids of high spontaneous curvature. Specifically, we find that the higher conductance states of the channel are expressed much more at pH 2.0 than at pH 6.0. The corresponding observed 50-fold change in the relative probability of a particular state vs. the adjacent state suggests that there is a change of ~4 kT in the difference between free energies of adjacent states. As expected, a qualitatively similar change was observed when salt concentration was increased to 1 or 2 M.

The energies of the different functional states are the most important factors in protein regulation. For membrane proteins these energies depend on the lipid molecules outside the protein. We see now that it is possible to regulate the energies of these lipids themselves to modulate their influence on proteins.

Materials and methods

Alamethicin channels were inserted into ‘solvent-free’ planar lipid bilayer membranes that had been formed by apposition of two phospholipid monolayers spread on aqueous solutions of sodium chloride (Baker Analyzed grade, Baker, Phillipsburg, NJ, USA). The monolayers were prepared from 10% DOPS or DOPE (Avanti Polar Lipids, Alabaster, AL, USA) in pentane (HPLC grade, Burdick and Jackson, Muskegon, MI, USA). The Teflon chamber (after Montal and Mueller) with two compartments of 1 ml was divided by 15 μm thick Teflon partition (CHEMFAB, Merrimack, NH) with a 60 μm diameter aperture. The aperture was pretreated with 1% solution of hexadecane (Aldrich, Milwaukee, WI, USA) in pentane and dried during 10 min prior to monolayer opposition. The same partition was used throughout all measurements reported in this paper.

Natural alamethicin (Sigma, St. Louis, MO, USA) was added only to one side of a membrane from 10⁻⁵ M stock solution in ethanol to a final concentration of (1–3) × 10⁻⁵ M. All experiments were done at 150 mV, positive from the side of alamethicin addition, and at a room temperature of (23 ± 1)°C. Alamethicin concentration was adjusted to a concentration that gave first current bursts in about 20 min after peptide addition; in this way we were able to monitor single-
channel activity (no channel overlapping) for about 10 min. Ion currents, amplified with an Axopatch 200A integrating patch clamp amplifier (Axon Instruments, Foster City, CA, USA), were recorded with a sampling rate of 50 kHz into computer memory and, simultaneously, onto recordable compact discs.

Statistical analysis of state probabilities was performed using direct comparison of the time spent by a channel at different conductance states (levels). First, current histograms were plotted and appropriate windows around each state were determined. Second, the total numbers of points within each such window were calculated and their ratios were taken to represent the relative probabilities of corresponding states. Each point in a relative probability graph represents averaging over more than 100 channels that were obtained, typically, from one membrane. A new membrane was formed for every pH or salt concentration.

Results

Typical recordings of alamethicin-induced currents in DOPS bilayers at different pH are shown in Fig. 1. They demonstrate that the probabilistic character of a conductance burst, corresponding to a single alamethicin channel, is very sensitive to membrane-bathing solution acidity. At relatively high acidity (pH 2.0), when lipid charge of the membrane is mostly neutralized by protons, a typical channel undergoes many transitions between different conductance states. Higher conductance states (labeled 4, 5) are well-expressed and are typically observed in every current burst. Increased pH and, presumably, increased lipid charge progressively suppress higher states. At pH 5.0 a typical channel goes only to Level 0 and back to the closed state. Note that the current burst at pH 2.0 represents a single ion channel of fluctuating size. Conductance increments, corresponding to channel transition to the next higher-conductive state increase with the level number from 0.104 nS (background to Level 0 transition) to 0.51 nS (Level 4 to Level 5 transition). If the burst were representing several identical channels occurring at the same time, the increments would be equal or would decrease with level number due to interference of ion currents in access areas.

While the probabilistic character of a single-channel burst changes dramatically with pH (Fig. 1), the acidity of the medium only slightly influences the channel conductance itself. Fig. 2 shows that at pH 2.0 all levels exhibit a conductance increase. This conductance increase is several times higher than the corresponding increase in solution specific conductivity (data not shown). The

Fig. 1 Typical current bursts representing alamethicin channels in DOPS membranes bathed by 0.3 M NaCl at three different pH values. Current is displayed with a 50 μs resolution. Horizontal dotted lines with numbers show conductance states' (levels') notation used in this paper. The pH-dependent character of current bursts corresponding to single alamethicin channels is clearly seen. At pH 2.0, the current burst always appears through the lowest conductance state, Level 0, fluctuates between several higher conductance states (Level 1 to Level 5, or even higher), and then disappears. At pH 5.0, a typical channel is seen at Level 0 only.
disparity is probably related to preferential transport of protons vs. sodium cations. At pH 3.0, lower levels show a small dip that reflects titration of the membrane surface charge and corresponding depletion in counterion concentration. This effect is similar to the recently reported titration of gramicidin channel conductance.  

The ‘smooth’ dependence of channel conductance on the acidity of the medium is helpful for statistical analysis of relative probabilities. Fig. 3 demonstrates the results of such an analysis of relative probabilities to quantify the pH-dependence clearly seen ‘by naked eye’ in Fig. 1. The change in pH from 2.0 to 6.3 changes the relative probability of Level 1 vs. Level 0 observation (filled symbols) by a factor of $e^4 \approx 50$. Most of the probability change occurs between pH 2.0 and 4.0, that is, within the range that includes the lipid’s hydroxy group pKₐ.  

Relative probability of Level 2 vs. Level 1 (open symbols) changes similarly and, for some reason, is very close to that of Level 1 vs. Level 0 in its absolute value.  

Fig. 4 shows that substituting 0.1 M NaCl for 0.3 M NaCl does not have any statistically significant effect on the structure of channel probabilities. Within error bars the quantitative
Fig. 4 Relative probability of channel levels in DOPS membranes in 0.1 M NaCl shows behavior much like that in 0.3 M NaCl (Fig. 3). Filled symbols, probability of Level 1 vs. Level 0; open symbols, probability of Level 2 vs. Level 1.

behaviour of levels' relative probability as a function of pH is the same at these salt concentrations. As shown, the relative probability of Level 2 vs. Level 1 (open symbols) in 0.1 M NaCl closely follows the relative probability of Level 1 vs. Level 0 (closed symbols).

To check for a possible direct influence of acidity or salt concentration on the probabilistic character of the alamethicin channel, we ran a series of control measurements with neutral DOPE bilayers. Fig. 5 displays a typical channel in 2 M NaCl exhibiting well-defined conductance levels. It is seen that the higher levels are quite probable. This result agrees with earlier observations\textsuperscript{42} though in the present study we use a neutral form of alamethicin that, by Glu\textsuperscript{18} to Gln\textsuperscript{18} substitution,\textsuperscript{15,16} differs from peptide used earlier.

Fig. 6 shows that conductance of channel levels is a monotonic function of salt concentration. Comparison to solution specific conductivity (solid line) indicates that channel conductance grows slower than does the solution conductivity. Similar to alamethicin channels in DOPS (Figs. 1 and 2), differences between conductance levels diverge with level number (although the levels themselves are sublinear in salt concentration). In particular, for 2 M NaCl these increments (Level 0 through Level 5, measured in nS) are 0.27, 1.10, 1.59, 1.80, 2.13. This means that the current burst, shown in Fig. 5, does represent a single ion channel of fluctuating size and not a random overlap of several identical channels.

Fig. 7 demonstrates that the probabilistic character of alamethicin channel reconstituted into uncharged lipid does not depend on salt concentration or on the shift of pH from a neutral to acidic value. Within experimental error, changing sodium chloride concentration from 0.1 to 2.0 M or acidity from pH 6.2 to 2.5 does not influence levels' relative probabilities. This suggests that

Fig. 5 Typical current burst of alamethicin-induced conductance obtained from a DOPE bilayer bathed by 2.0 M NaCl at pH 6.3. Channel 'switches on' through Level 0 and then fluctuates between different well-defined conductance states reaching Level 5 several times during its lifetime. The probabilistic character of the channel is very close to that of the alamethicin channel in DOPS at pH 2.0 (Fig. 1).


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Fig. 6  Conductance of alamethicin channel states as a function of sodium chloride concentration measured on DOPE bilayers at pH 6.3. The solid line gives bulk solution specific conductivity scaled in such a way that Level 5 conductance and solution conductivity coincide at 0.1 M concentration to facilitate comparison. The comparison shows that conductance of Level 5 is a weaker function of salt concentration than bulk conductivity.

Fig. 7  Relative probability of channel conductance states in DOPE bilayers at different pH and sodium chloride concentrations. Within experimental error, the relative probability of higher states does not depend on salt concentration or acidity of the medium if the channel is inserted into neutral lipid. Whatever pH or salt concentration, relative probabilities in DOPE stay close to those in DOPS at pH 2.0–2.5 (Figs. 3 and 4).

Fig. 8  Typical current burst of alamethicin-induced conductance obtained from a DOPS bilayer bathed by 2.0 M NaCl at pH 6.2. It is clearly seen that the high salt concentration increases the probability of higher conductance states (compare to the current recording of Fig. 1 for pH 5.0). However, due to the appearance of strange conductance substrates (shown by tilted arrows), it was impossible to quantify the salt effect in the alamethicin/DOPS system as done for DOPE (Fig. 7).
the dramatic pH effects shown in Figs. 1, 3, and 4 are related to lipid charge titration that affects channel behavior via lipid–channel interactions.

In addition to lipid charge titration by proton one can think about the analogous action of high salt concentration. As clearly seen in Fig. 8, illustrating a typical current burst obtained from a DOPS bilayer bathed by 2 M NaCl at neutral pH, we do observe a strong increase in higher level probabilities at high sodium chloride concentrations (compare to Fig. 1 recording for pH 5.0). Unfortunately, the effect of salt could not be quantified reliably because of additional, strange (compared to the channel in Fig. 5) sublevels in the alamethicin/DOPS system at high salt concentrations. Three of these sublevels are marked by tilted arrows (Fig. 8). Appearance of sublevels prohibited clear determination of level positions that is crucial to the subsequent statistical analysis. It should be noted that the bursts, an example of which is presented in Fig. 8, were rare enough (separated, on average, by 10-fold longer periods of ‘silent’ background recording) to exclude a trivial reason of several channels overlap.

Discussion

Many parameters influence the conformational equilibrium of membrane proteins. It is well known that a conformational transition can be triggered by a pH shift or by ligand and multivalent cation binding. The number of appropriate examples is overwhelming since these reactions constitute a basis for diverse physiological regulation at the cellular level.24–27

Much less studied are mechanisms of protein regulation by membrane lipids that act either directly or via the mechanical properties of host bilayers. Recent progress in this field demonstrates the possible ubiquity of such regulation. It has been shown that lipids modulate catalytic activity and binding properties of integral membrane proteins that include Insulin-R, Na+/K+-ATPase, Ca2+-ATPase, GABA transporter, acetylcholine receptor (Table 1, in ref. 2), and Rhodopsin,1 to name just a few. Nevertheless, the nature of the physical forces underlying lipid action is still a subject of considerable controversy.28

Membrane protein function can be modified by changes in the mechanical properties of a host membrane, e.g., by changes in spontaneous curvature of membrane lipids.12,22,29–33 There has been corresponding theoretical effort (e.g., ref. 11, 34, 35). The conformational equilibrium of a protein between different functional states is governed by the total free energy differences between these states. Clearly, if a conformational transition between states involves a change in the shape or length of the protein surface that is exposed to lipids, this transition has to be sensitive to membrane mechanics. Although the idea is general, the particular approaches permitting a quantitative description are model-specific.

A correlation between packing stress and conformational equilibrium of a single membrane-bound polypeptide structure was first observed with alamethicin channels in planar bilayer membranes made from DOPE/DOPC mixtures.22 It was shown that an increase in the mole fraction of DOPE, which favors a highly curved H2-phase, shifts the distribution of conductance levels towards those of higher conductance. The following relationship between spontaneous curvature of the lipid and polypeptide aggregation in the membrane was established: higher curvature stress promotes larger alamethicin aggregates.

Now we demonstrate that with respect to the probabilistic character of the alamethicin channel, the same charged lipid species can be made equivalent to DOPC or DOPE by changing bathing solution pH or salt concentration. Again, we correlate ion channel function with the stress of forcing lipids of a given spontaneous curvature into a planar membrane form. The cartoon in Fig. 9 illustrates how a change in the charge of lipid head groups is able to change lipid spontaneous curvature. A useful notion in description of mechanical properties of a bilayer is the effective ‘shape’ of the membrane molecules. A cylindrical molecular shape (when the cross-sectional area of the polar head group is similar to the cross-sectional area of the acyl chains) will correspond to lamellar phases and stress-free packing into the bilayer form. At pH 6 and small enough salt concentration, DOPS molecules have an approximately cylindrical effective shape because of repulsion of fully charged neighboring head groups. At pH 2 proton binding titrates out the head group charge; lipid shape is conical. Correspondingly, DOPS hexagonal phase can go from a rather low spontaneous curvature at neutral pH to a high spontaneous curvature in an acidic environment.
Fig. 9 Cartoon illustrating the proton-induced increase in spontaneous curvature of DOPS. Neutralization of the lipid head group charge by proton binding reduces head–head repulsion; it thus effectively changes lipid molecule 'shape'. At a fully deprotonated, charged state, repulsion between DOPS head groups drives system into a lamellar structure; however, at high proton concentrations this repulsion is 'switched off' so that a preferred packing is an Hₐ-phase of high curvature.

Strong X-ray diffraction evidence, Fig. 10, supports this interpretation. Samples with an excess of water solution were used in diffraction measurements and solution pH was adjusted before, and checked after, sample equilibration. It is seen that the Bragg repeat spacing for the DOPS hexagonal phase changes sharply between pH 2.5 and 4.0, in excellent agreement with transport measurements (Figs. 3 and 4). For samples above pH 4, X-ray scattering showed disorder characteristic of highly and irregularly separated bilayer membranes. The common but puzzling coexistence of hexagonal and lamellar phases at low pH was probably related to the small free energy difference between these two lipid assemblies.⁵⁶,⁵⁷

A general thermodynamic analysis permits us to quantify the energetics of alamethicin channel regulation. Indeed, because the different conductance levels of the channel are well defined states,
it is possible to speak of the chemical potentials (or free energies) of the individual states, \( \mu_i = \mu_i(pH, \muNaCl) \). The relative probability of two adjacent levels, \( p(i, i-1) = P_i/P_{i-1} \), is given then by

\[
p(i, i-1) = \exp\left[-(\mu_i - \mu_{i-1})/kT\right]
\]

where \( k \) and \( T \) have their usual meaning of Boltzmann constant and absolute temperature. A change in the relative probability induced by a pH shift can be expressed as the ratio of relative probabilities at the two pH values and equals \( \exp[-\Delta(\mu_i - \mu_{i-1})/kT] \). Here \( \Delta(\mu_i - \mu_{i-1}) \) is the pH-induced change in the states’ chemical potential difference. This ratio, reflecting total change in relative probability caused by acidity shift from pH 2 to 6 (Figs. 3 and 4), is measured to be close to 50. Thus

\[
\Delta(\mu_i - \mu_{i-1})|_{\text{pH } 2 \to \text{pH } 6} \approx 4 \ kT
\]

The rate of change in relative probability with pH is an indication of difference in the number of protons associated with the surface. By standard Gibbs–Duhem reasoning, assuming that the active factors are proton and sodium activity, the changes in state energy go as

\[
d\mu_i = -n_{\text{NaCl}}^i d\mu_{\text{NaCl}} - n_H^i d\mu_H
\]

where the functions \( n_{\text{NaCl}}^i \) and \( n_H^i \) themselves depend on NaCl and H activity. These are the Gibbs excess numbers of sodium or protons that are associated with the membrane–channel system when the channel is in state \( i \). The change in the relative probability of two conductance levels \( i \) and \( i-1 \) depends on the difference in these \( n_s \)

\[
d(\mu_i - \mu_{i-1}) = -(n_{\text{NaCl}}^i - n_{\text{NaCl}}^{i-1})d\mu_{\text{NaCl}} - (n_H^i - n_H^{i-1})d\mu_H
\]

When salt concentration is kept fixed but pH varied, the change in relative probabilities gives us \( n_H^i - n_H^{i-1} \), a difference in the number of protons associated with the system upon a transition from conductance state \( i-1 \) to conductance state \( i \). From these equations and \( \mu_H = kT \ln[H] \), pH = \( -\log[H] \) we obtain:

\[
n_H^i - n_H^{i-1} = \frac{\partial (\mu_i - \mu_{i-1})}{\partial \mu_H} = \frac{\partial \ln p(i, i-1)}{\partial \ln[H]} = \frac{\partial \ln p(i, i-1)}{\ln 10 \ \partial (\text{pH})}
\]

Analysis of the data for the Level 0 to Level 1 and Level 1 to Level 2 transitions around pH 3.0 (which corresponds to the maximum slope of the relative probability dependence on pH, Fig. 3) gives \( n_H^i - n_H^{i-1} = 1.1 \pm 0.1 \). The change in the number of associated protons upon transition to a higher conductance state is thus positive. Higher conductance states by rearranging the whole channel/bilayer system accommodate more protons so that the increase in proton chemical potential makes these states more favorable. As a result, higher conductance states are more expressed at high proton concentrations.

The thermodynamic description shown above is very useful for quantifying the effect of pH on channel function and for restricting the number of possible models. However, as usual, thermodynamics does not elucidate a specific physical mechanism. To approach the mechanism of channel regulation by pH, more structural knowledge of the conformation transformations of channel opening/closing and on lipid–peptide interactions is needed. Such knowledge is necessary to discriminate between stress of packing, surface tension, or other mechanical factor contributions.

As recently pointed out on the basis of a careful study of different mechanical contributions to the energetics of protein inclusions into lipid bilayers,\(^\text{11}\) the increase in hydrophobic mismatch between the protein and the lipid by a mere 0.3 Å can change the equilibrium distribution between the corresponding protein states by a factor of 10. Also, the energy of the protein-induced bilayer deformation can be as high as 2–3 \( kT \) per one lipid molecule. In this case, the 4 \( kT \) effect reported in the present study can be explained by perturbation of a few lipid molecules only.

It is worthwhile to compare the pH-shift-induced energy change found in our study to other characteristic energies in the system. The electrostatic energy of recharging of a single lipid head group in the fully charged DOPS bilayer (e.g., at pH 6.0) can be easily found as a product of the membrane surface potential \( \psi_0 \) (e.g., ref. 38) and the elementary charge \( e \)

\[
ev_0 = 2 \ kT \sinh^{-1}\left[8 \ kT e \psi_0(Na)^{-1/2}\sigma\right]
\]
Here \( \sigma \) is the lipid charge surface density, \( \varepsilon \) is the dielectric constant, and \( \varepsilon_0 \) is the permittivity of free space. Taking \( \sigma = 0.25 \text{ C m}^{-2} \) (one elementary charge per 64 \( \text{Å}^2 \)) and \([\text{Na}] = 6.02 \times 10^{-5} \text{ m}^{-3} \) (0.1 M NaCl), we obtain \( e\psi_0 = 5.2 \text{ kT} \). This energy compares well with the 4 \( kT \) change found for the energy of the alamethicin state to state transitions [eqn. (2)].

The work of forcing one lipid molecule from the hexagonal \( \text{H}_{12c} \) phase into the lamellar phase can be estimated as\(^{36,39} \)

\[
E = \frac{ak_s}{2R_0^2}
\]

where \( k_s \) is the monolayer bending modulus (about 10 \( kT \)), \( a \) is the area per lipid molecule, and \( R_0 \) is the radius of spontaneous curvature. Taking \( a = 64 \text{ Å}^2 \) and deducing the radius of spontaneous curvature from the Bragg spacing for the hexagonal phase at pH 2.0 (Fig. 10), we get \( E \approx 0.3 \text{ kT} \). This energy is about an order of magnitude less than the acidity-induced change in the energy of alamethicin state-to-state transitions. However, many lipid molecules are in direct contact with the alamethicin aggregate. If, in addition, the lipids that are to be perturbed by protein conformational change extend over distances of many lipid molecules,\(^{11} \) this estimate is reasonable.

To conclude, varying the pH changes the probabilistic character of alamethicin channels in a way that correlates with the pH-induced changes in the nonlamellar tendency of the host-lipid. This correlation, made for the charged lipid DOPS, agrees with the logic of our conclusions drawn from earlier observations on alamethicin in mixtures of neutral lipids.\(^{22} \) The channel's response to pH is all the more impressive given the fact that the present measurements were made on a neutral form of alamethicin that does not possess any residues that can be titrated in this pH range. Still, there is a pronounced, 50-fold effect on channel state-to-state transitions from a pH shift of about two units.

Whatever the mechanism, lipid charge titration modifies channel structural equilibrium. This finding probably unveils an additional, previously unrecognized way of pH regulation in membrane transport.

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References

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