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Review

Lipid modulation of ion channels through specific binding sites☆,☆☆

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ABSTRACT

Ion channel conformational changes within the lipid membrane are a key requirement to control ion passage. Thus, it seems reasonable to assume that lipid composition should modulate ion channel function. There is increasing evidence that this implicates not just an indirect consequence of the lipid influence on the physical properties of the membrane, but also specific binding of selected lipids to certain protein domains. The result is that channel function and its consequences on excitability, contractility, intracellular signaling or any other process mediated by such channel proteins, could be subjected to modulation by membrane lipids. From this it follows that development, age, diet or diseases that alter lipid composition should also have an influence on those cellular properties. The wealth of data on the non-annular lipid binding sites in potassium channel from *Streptomyces lividans* (KcsA) makes this protein a good model to study the modulation of ion channel structure and function by lipids. The fact that this protein is able to assemble into clusters through the same non-annular sites, resulting in large changes in channel activity, makes these sites even more interesting as a potential target to develop lead compounds able to disrupt such interactions and hopefully, to modulate ion channel function. This Article is Part of a Special Issue Entitled: Membrane Structure and Function: Relevance in the Cell's Physiology, Pathology and Therapy.

Contents

Abbreviations: BK, big potassium channel; BN-PAGE, blue native polyacrylamide gel electrophoresis; Ca_v, voltage-gated calcium; CNG, cyclic nucleotide-gated channels; CRAC, cholesterol binding sites; CTD, cytoplasmic domain; ENaC, epithelial sodium channels; FRET, Förster resonance energy transfer; $GABA_A$, γ-aminobutyric acid-gated channel; GIRK, G protein-coupled inwardly-rectifying potassium channel; HCN, hyperpolarization-activated cyclic nucleotide-gated channel; hERG, human Ether-à-go-go-Related Gene potassium channel; HOP, high opening probability pattern; IP_3R, IP_3-gated calcium release channels; K_{ATP}, potassium channel activated by intracellular ATP binding; KCNQ, potassium voltage-gated channel subfamily KQT; KcsA, potassium channel from *Streptomyces lividans*; Kir, inward-rectifier potassium channel; K_v, voltage-gated potassium channel; LOP, low opening probability pattern; MscL, large-conductance mechanosensitive channel; nAChR, nicotinic acetylcholine receptor; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PUFAs, polyunsaturated fatty acids; RyR, ryanodine-sensitive calcium release channels; SUR, sulfonylurea receptor; TMD, transmembrane domain; TRP, transient receptor potential channels; TRPL, transient receptor potential-like *Drosophila* phototransduction channels; VDAC, voltage-dependent anion channel.

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1. Introduction

Ion channels play important physiological roles in all living organisms, from prokaryotes to higher animals. Cell excitability, muscle contraction, synaptic transmission and cell signaling are among the processes mediated by this large family of proteins [1,2]. This is the reason why they have become a central target for developing new drugs against pathologies related to those processes, referred as channelopathies [3].

Ion channels are integral membrane proteins surrounded by a complex milieu of lipid molecules that, far from being just a passive barrier, play an active role in their structural and functional modulation. These effects are especially relevant for those proteins undergoing conformational changes/movements at their membrane-embedded domains, as it is the case of ion channels. However, the mechanism through which lipids exert such modulation remains elusive. Basically two different but not mutually exclusive modes of interaction between lipids and membrane proteins have been suggested. On one hand, there are non-specific interactions through which general physical properties of the membrane would influence protein structure and function. These include the hydrophobic mismatch between lipids and proteins that elicit lipid and/or protein deformations in order to avoid the energetic cost of exposing hydrophobic areas to water; interfacial curvature that determines the capacity of the membrane to deform as to avoid the mentioned hydrophobic mismatch; as well as membrane surface tension, lipid free volume, viscosity and lateral pressure profile, which establish the resistance of the lipid ensemble to the conformational movements of the protein (for a review see [4–6]). Basically all these parameters influence the packing and/or the conformational movements of membrane proteins within the lipid membrane, especially those that involve a considerable change in the protein volume or lipid-exposed surface, as it is the case in the mechanosensitive ion channels [7,8]. On the other hand, there is increasing evidence for the modulation of the structure and function of ion channels through a direct interaction with certain lipids, which is the scope of this chapter. Two different classes of bound lipids for such direct interactions have been defined, the annular ones, which would correspond to the first layer of lipids surrounding the transmembrane portion of the membrane protein; and the non-annular ones, that would be bound to certain grooves especially between protein subunits.

2. Lipid modulation of ion channel activity

Although there is a large heterogeneity in the structure of ion channels, all of them share a common feature: their structure contains transmembrane domain (TMD) which can move within the membrane bilayer to adopt diverse conformations associated to different functional states. This fact makes ion channels ideal candidates to be modulated by its lipid ensemble. Several modes have been proposed to explain how lipids bound to ion channels could modify their function [5].

1) Charged lipids alter the concentration of charged species close to the membrane that could affect ion channel activity. For instance, anionic lipids increase the negative charge at the membrane surface and therefore enhance the concentration of positively charged molecules close to the membrane. For a cation channel this could lead to an increased current and selectivity for cations over anions. Moreover, the higher local concentration of protons, Ca^{2+}, etc. could also modulate the channel activity in an allosteric-like manner. In the case of protons, for example, they would neutralize acid residues close to the membrane, thus increasing the relative hydrophobicity at that region of the protein.
2) Lipid headgroups can stabilize the α-helical ends of membrane proteins, so also their helix packing. This depends on their capacity to form hydrogen bonds with the amino acids at the ends of such helixes.
3) The acyl chain length and intrinsic interfacial curvature of lipids bound to the protein can also be determinant for channel structure and function. If there is a large hydrophobic mismatch between the lipids and the protein, this could force the adoption of a non-functional protein structure, or else favor the conformation state where that mismatch is reduced. These changes include the tilting or alterations in the packing of protein domains.
4) Specific lipids are often bound to intersubunit protein grooves, enhancing the stability of the protein and probably facilitating their movements, acting as a "lubricant".

There are several studies that have contributed to an increasingly refined model of this complex issue. The role of lipids on the function of voltage-gated channels seems particularly interesting. These proteins have voltage-sensor domains formed by the transmembrane helix S4 and part of the helix S3 (Fig. 1), which are thought to undergo a considerable movement through the membrane in response to a change in the membrane potential. Some authors suggest that negatively-charged phosphate groups in membrane lipids would help to stabilize specific positively-charged, voltage-sensing residues during the voltage-sensor gating process [9]. It is believed that lipid binding to such domains could explain their influence on the activation of these ion channels [10–12]. Alternatively, other authors propose that instead of specific lipid–protein interactions, annular lipids as a body would act as stabilizers of the voltage-sensor paddle in an active conformation. Thus, in this respect, the annular lipids and the protein would form a functional unit. Moreover, the lipid headgroup capacity to form hydrogen bonds has been pointed out as a key factor for the lipid to exert such effect. As a corollary, any molecule able to disturb the lipid annulus, such as cholesterol, would also be expected to affect the activation of these channels [13].

Other lipids such as certain polyunsaturated fatty acids (PUFAs) would exert their direct effect on ion channels through domains that do not involve the voltage sensor but undergo also rearrangements during the inactivation process. PUFAs inhibit most of the voltage-gated ion channels, although in some cases the opposite effect has also been reported [14]. In addition to the effects on the magnitude of the ionic currents, it has been shown that arachidonic acid (a 20-carbon omega-6 polyunsaturated fatty) converts the K_v delayed rectifiers into A-type rectifier channels. In general, PUFAs seem to act as open-channel blockers of K_v channels, eliciting an increase in the rate of inactivation. Thus, the observed process might be analogous to the N-type inactivation by the Kvβ1 auxiliary subunits. From studies on the time- and voltage-dependent interaction with K_v11.1 channels, it has been suggested that PUFAs preferentially bind to the open state of these channels [15]. Oliver and collaborators [16] explain these results, in the case of the arachidonic acid, by proposing that it inserts into the cell membrane from either side, interacts with the channel protein

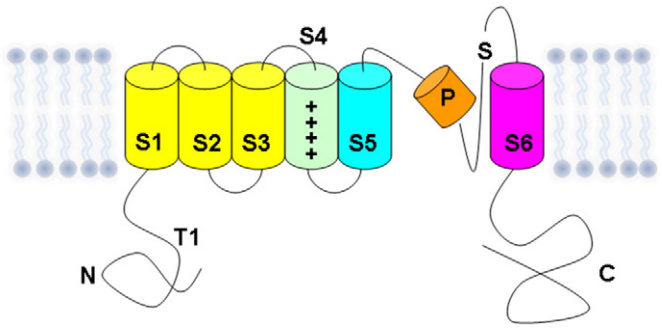

Fig. 1. Schematic representation of the membrane topology of K_V channels. S1 to S6 represent the six transmembrane segments, where S4 corresponds to the voltage sensor. S5 and S6 form the pore domain that includes the pore helix (P) and the signature sequence (S) N- and C-terminal domains are indicated as N and C, respectively. T1 corresponds to the tetramerization domain.

and allosterically induces a rapid closure of the open K_v channel pore through conformational modifications in the selectivity filter. This would also explain why these molecules modulate the function of many other types of ion channels, including the ligand-gated ones [17]. In general, it seems that the length of the lipophilic acyl chain, the number of double bonds and the polar head are critical for channel modulation. This is likely related to the degree of flexibility of the fatty acid, which allows it to adapt to the ragged surface of the protein transmembrane α-helices and to penetrate between them at little or no energy cost. However, there is not a detailed description of the structural requirements for binding, neither for the fatty acids acting as ligands, nor for the channel binding sites [18,19].

The mechanosensitive channels are also modulated by phospholipids. In the case of the prokaryotic MscL, there is an increase in the amplitude and rate of calcein release depending on the presence of anionic phospholipids. This effect is compatible with an increase in the rate of the closed to open channel transition, and a decrease in the rate of the open to the subconductance state [20].

Other lipid found to be especially relevant in the direct modulation of ion channels is phosphatidylinositol 4,5-bisphosphate (PIP_2), a minor acidic membrane lipid found primarily in the inner leaflet of the plasma membrane. PIP_2 modulates the activity of many types of ion channels in a variety of modes [21]. For instance PIP_2 has been reported to act as an activator in (i) all inward rectifier potassium channels, (ii) epithelial sodium channels (ENaC), (iii) ryanodine-sensitive calcium release channels (RyR), and (iv) all the KCNQ channel family members. There are also reports of ion channels inhibited by PIP_2 including (i) cyclic nucleotide-gated channels (CNG), (ii) transient receptor potential-like (TRPL) *Drosophila* phototransduction channels, (iii) capsaicin-activated transient receptor potential (TRP) channels (VR1), and (iv) IP_3-gated calcium release channels (IP_3R) (for a complete list see [22,23]). There are even certain cases, such as those of voltage-gated calcium (Ca_v), voltage-gated potassium (K_v), and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels where PIP_2 has a dual effect on ion channel function, both as an activator and as an inhibitor, although it seems that distinct molecular determinants underlie the different effects [24]. In the case of the KCNQ1 and hERG channels this lipid also slows the deactivation kinetics [22]. However, for the KCNQ2/KCNQ3 complex, PIP_2 does not slow deactivation [25]. The KCNQ1/KCNE1 kinetic model shares similarities with the one of the Kir6.2/SUR1 channel [26] suggesting similar effects of PIP_2 on six- and two-transmembrane domain channels.

Cholesterol has also been reported to alter ion channel function. In some cases, it potentiates the activity of the channel, as it is the case of γ-aminobutyric acid-gated channel ($GABA_A$) or the nicotinic acetylcholine receptor (nAChR). However, for the voltage- and Ca^{2+}-gated K^+ potassium BK, and Kir channels, cholesterol diminishes their activity. This latter effect is achieved through a drop in the open probability of BK mediated currents, or through a change in the number of active Kir_2 channels [27]. In plants this role would be played by phytosterols, as it has been suggested for plant voltage-dependent anion channel (VDAC), which undergoes a reversible regulation in selectivity and voltage-dependence [28].

Lipid modulation of ion channels could be also mediated by inducing their segregation into specific lipid domains, as to ensure a defined milieu around the protein, different from the bulk membrane composition. In the case of lipid rafts enriched in cholesterol and sphingolipid, there are reports on a large number of channels associated to such domains. It seems that these raft domains regulate channel function in several different ways. Cholesterol and sphingolipids can do it either through direct protein–lipid interactions or by influencing the physical properties of the bilayer close to the channel [29]. The selective recruitment of other interacting signaling molecules or raft-associated scaffold proteins such as caveolin would be another way to influence channel function, by altering gating kinetics or by affecting trafficking and surface expression [29]. Other lipid domains different from rafts have also been reported to regulate ion channel function, as it is the case of the nAChR, one of the best studied ion channels in terms of lipid–protein interactions. Its structure and function are modulated by anionic phospholipids and cholesterol which bind with high affinity to the protein [30–33]. Although the mode of action of lipid regulation is not clear, it seems that it is linked to a change in the protein secondary structure [32]. Moreover, as if a bi-directional process would be taking place, the protein itself is able to induce the segregation of a lipid domain enriched in phosphatidic acid (PA) around it, which would assure a permanent contact with this particular anionic lipid, and thus a proper channel structure and function [34]. In fact, nAChR reconstituted in liposomes without PA shows no activity, but recovers partly when transplanted into oocytes, supporting the idea of a dynamic system; such activity is further increased if PA is preinjected in the oocyte. Thus, it seems that the channel is able to bind specifically this phospholipid in vivo as to recover its proper structure and activity [33]. This effect is not observed with other anionic phospholipids, possibly because of the unique properties of PA, such as its small headgroup or its high capacity to form hydrogen bonds.

3. Location of lipid binding sites in ion-channels

There is still a lack of precise data on the localization of lipid binding sites on most ion channels and on how this binding modulates their structure and function. In the case of cholesterol, the use of steroid enantiomers and mutagenesis studies predicted the existence of specific steroid-recognition protein regions in the potassium BK channels [27], Kir2 [35], Kir2.1 [36], and $GABA_A$ channels [37]. These predictions have been seemingly confirmed by mutagenesis or computational modeling [38,39]. Cholesterol binding sites (CRAC) at the C-terminal domains of the BK channels [40], the transmembrane segments of the nAChR [41] or the C-terminal regions in the $Kir2.1K^+$ channels [36] are examples of proposed sites, although the exact location and structure of such cholesterol binding sites are still poorly defined.

As indicated in the Introduction, membrane phospholipids could be classified as annular or non-annular regarding to their site of action on membrane proteins. The first shell of lipids around transmembrane proteins is called the annular lipid shell. They are not believed to bind strongly to the protein, although their conformation is usually altered compared to that of bulk lipids. Annular lipids can exert a modulation of transmembrane proteins depending on their acyl chain length, the phospholipid headgroup charge or their capacity to form hydrogen bonds with the protein [5,42–44]. A subtle variation of this concept is the proposed "lipid shells". These would consist of a more stable "lipid annulus", where long-term interactions take place between specific lipids and selected proteins. The lipid shells and the protein they surround would exist as mobile entities in the plane of the membrane and would be thermodynamically stable structures with large affinity for pre-existing caveolae/rafts, targeting the protein they encase to these membrane structures [45].

Within the transmembrane protein surface, there have occasionally been found the so-called "hot spots" or groups of amino acids where particular annular lipids bind with a fairly high specificity. In some instances, this has enabled to solve its structure at atomic resolution, since they co-crystallize with the protein [5]. Among ion channels, a clear example is that of the mechanosensitive channel MscL: there is a cluster of three positively charged amino acid located at the cytoplasmic side that strongly binds anionic lipids which can alter the channel function [46]. In fact, the affinity for the binding of PA is nine times that of phosphatidylcholine (PC) [20]. Another similar case is that of potassium channels and the lipid PIP_2, which binds membrane proteins through electrostatic interactions with a group of basic amino acid residues and establishes additional hydrophobic and hydrogen-bonding interactions. Usually those residues are situated at intracellular protein domains [47–51]. For the mammalian K_v channels, PIP_2 would interact with the S4–S5 linker (see Fig. 1), that is, the linker between the voltage

sensor and the pore domain [49]. Li and collaborators [52] identified 4 basic residues (R67, K69, K70, and H73) located on an α-helix, following the TMD in KCNE1, that seem to play a critical role in this PIP_2 sensitivity. For the Kir2.2 and $GIRK_2$ channels PIP_2 binds at the interface between the TMD and the cytoplasmic domain (CTD). This site is conformed by a specific phosphatidylinositol-binding region in the CTD domain and a non-specific phospholipid-binding region in the TMD. For the Kir2.2, the binding of the lipid induces a large conformational change that finally results in the opening of the inner helix gate of the channel, an effect analogous to the activation of ion channels by ligands [53,54].

Opposite to annular lipids, the non-annular ones are bound to buried sites of the protein, usually at intersubunit clefts. Such sites are located at the same level as the bulk lipids, so it is supposed that the appropriate lipids enter these sites through simple diffusion from the bilayer. The role of these non-annular lipids is still unclear but they are believed to be important for proper folding and stability of membrane proteins, helping transmembrane helices to pack correctly [5]. Non-annular lipids could also act as a "lubricant" to facilitate the subtle movements of transmembrane helices, such as tilting, bending or unwinding, required to perform the conformational changes associated to protein function. The strong binding of non-annular lipids to their corresponding sites on the channels has occasionally enabled them to remain bound after harsh protein purification and crystallization processes. From those crystals, a detailed picture at atomic resolution has been obtained for the interaction of such lipids with their corresponding sites [55]. The idea emerging from such structures is that positive charges at the protein anchor the lipid headgroup through electrostatic interactions or hydrogen bonds, mainly through the phosphate carbonyl esters. Additionally, the acyl chains make multiple van der Waals contacts with the transmembrane hydrophobic residues of the protein, usually adapting its shape to that of the transmembrane portion of the protein, which in such regions usually conforms as grooves or clefts.

4. KcsA: a model potassium channel to study lipid–protein interactions

Since the publication of its structure at atomic resolution [56], the prokaryotic potassium channel KcsA has become a model membrane protein to decipher the structure and function of the superfamily of potassium channels, including the role of lipid–protein interactions on their modulation. KcsA is a prokaryotic potassium channel composed of four identical subunits around a central pore. Each subunit encompasses an N-terminal amphipathic helix, followed by a transmembrane helix (TM1), a short pore helix, the selectivity filter and a second transmembrane helix (TM2) ending in a soluble C-terminal domain which forms an intracellular helical bundle with the C-termini from the other three subunits (Fig. 2). Functionally, the passage of ions is controlled by two coordinated gates [57]: i) the intracellular or inner gate, which opens at acidic pH through a movement of the TM2 helixes and ii) the extracellular or outer gate, situated at the selective filter, which tend to adopt a non-conductive state when the inner gate is open, leading the channel to an inactivated state.

Lipids have a strong influence on KcsA structure and function and anionic lipids have been reported as necessary for the channel to function properly [58,59], to enhance its stability [60,61] and to help the protein to refold in vitro with full recovery of channel function [59,61,62]. The protein binding sites through which lipids exert such variety of functions still lack a sufficient definition, although three different protein regions have been identified so far. One of such regions is located close to the membrane, at the cytoplasmic side of the protein, formed by a cluster of arginines (R27, R117, R121 and R122). This protein site could be defined as an annular lipid "hot spot" and it has been associated to an enhancement in the affinity for anionic phospholipids at the intracellular leaflet, as well as to an increased KcsA tetramer stability [63,64]. A second protein region of interest in this regard is the N-terminus of KcsA, which is also rich in basic residues, containing two

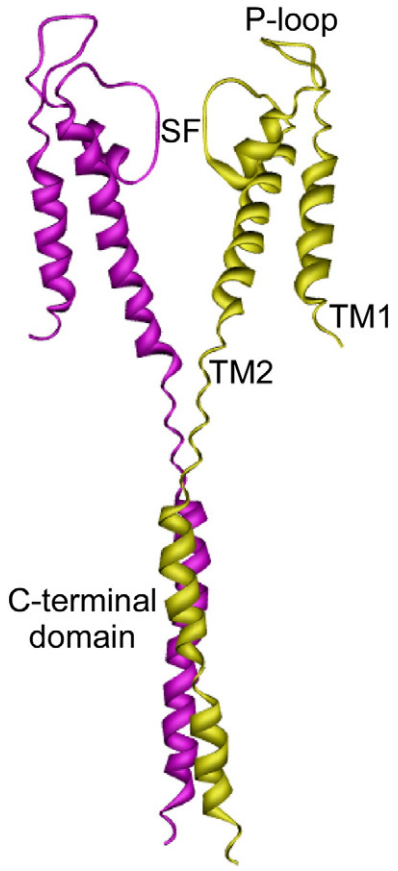

Fig. 2. Ribbon-type representation of the structure of the K^+ channel KcsA ("The Crystal Structure of Full-Length KcsA in its Closed Conformation PDB ID: 3EFF"). Only two out of the four identical subunits have been drawn for clarity. TM1 and TM2 correspond to the transmembrane segments, linked through the P-loop. Within the pore we find the selectivity filter (SF), responsible for K^+ selection over Na^+. N- and C-terminal domains are found in the intracellular side, but only the last structure has been solved so far by X-ray crystallography.

Arg residues at positions 11 and 19 and a Lys residue at position 14. Some authors have proposed a role for this domain in the stabilization of the channel structure by anionic lipids [64], while others have proposed that the N-terminus acts as a charge-sensitive "antenna" interacting with anionic lipids to stabilize the open conformation of the intracellular gate of the channel [65]. Finally, a third protein site has been reported, which is representative of the so-called non-annular lipid binding sites. This site is located at the extracellular side of the protein, between the TM1 and TM2 of two adjacent KcsA subunits and was found to contain a tightly but non-covalently bound lipid in the crystal structure of KcsA. Such lipid was identified as phosphatidylglycerol (PG), which binds its *sn*-1 acyl chain into a deep cleft on the protein transmembrane surface, while the *sn*-2 chain binds more peripherally [66]. Although the polar head-group of the phospholipid is not solved in this structure, it is expected to interact with nearby positively charged residues, such as R64 or R89, as supported by NMR and simulation studies [67–69]. The electrostatic nature of such interaction would explain the low affinity exhibited by this site for zwitterionic phospholipids and its high affinity for binding any anionic phospholipid [63,70,71].

The consequences of the occupancy of these sites in KcsA by non-annular lipids are beginning to be unveiled. Some authors have suggested a role in the modulation of channel activity, although there is a certain controversy between the relative relevance of such sites to channel function versus that of the N-terminal "antenna" referred above [65,69,72]. The role of the non-annular lipid binding sites on the stability of the KcsA tetrameric structure is more clearly established. Anionic lipids or detergents such as SDS greatly enhance the tetrameric protein

resistance against irreversible, heat-induced unfolding and dissociation into subunits. This occurs in a phospholipid or detergent concentration-dependent manner, as far as the acyl chains are longer than 10 carbon atoms [67]. Similar results have also been obtained from studying the chemical resistance of KcsA against TFE unfolding and denaturation [73]. As to the mechanism for such stabilizing effect, modeling the KcsA–phospholipid complex suggests that the increased protein stability originates from the intersubunit nature of the non-annular binding sites (Fig. 3A). Arg-64 from one subunit, and Arg-89 from the other would sandwich the phospholipid, neutralizing their positive charges and holding together the adjacent subunits [67]. In support of such hypothesis, a similar effect in enhancing protein stability has been observed when Arg-64 is mutated to Asp, which then forms a salt bridge with Arg-89 [68,74].

5. Lipid–protein vs. protein–protein interactions in the modulation of KcsA channel function

The characterization of the KcsA single channel properties has been surrounded by certain controversy. For instance, Schrempf's group, discoverers of KcsA, reported a dependence of channel opening on acidic pH, multiple conductance states with fairly high opening probabilities and unusual permeabilities to different cations besides K^+ [75–77]. In contrast, Miller's group [78,79] using KcsA reconstituted into planar lipid bilayers, found a single conductance state with a much lower opening probability, as well as orthodox ion selectivity and other properties to validate KcsA as a *bona fide* K^+ channel model. The above discrepancies were never fully explained but still, it became accepted that KcsA behaves as a moderately voltage-dependent, K^+-selective channel with low opening probability and the peculiar property of opening in response to acidic pH at the intracellular side of the membrane. Later on, however, different modes of activity of KcsA have also been reported, which differ greatly on their opening probability and channel kinetics [80]. In our laboratory, when reconstituting purified KcsA into giant liposomes made from asolectin phospholipids above a certain protein to lipid ratio, KcsA exhibits two major patterns of activity in patch-clamp recordings from excised, inside-out membrane patches: i) a Low Opening Probability (LOP) pattern seen in approximately 55% of the recordings, in which channel openings are scarce and result primarily from individual single channel events or from coupled gating of a few channels, and ii) a High Opening Probability (HOP) pattern observed in the remaining 45% of the patches, in which the channels are opened most of the time and exhibit a high degree of cooperativity through coupled gating of a larger number of channels [81]. Because abundant channel clusters in the giant liposomes were also detected, it was hypothesized that the different functional patterns could arise from "non-clustered" or "clustered" KcsA channels and that the assembly of such supramolecular entities would somehow determine the changes observed in the integrated behavior of the channels involved. KcsA clusters have been detected through analytical ultracentrifugation, FRET, fluorescence images in supported bilayers and giant vesicles, and BN-PAGE [81,82]. Because of its simplicity, this latter technique is particularly well suited to study experimental variables involved in clustering. From those studies it has been deduced that anionic lipids and SDS-like molecules larger than 10 carbon atoms break KcsA clusters rendering tetramers and dimers of tetramers which seem to be the building blocks for the larger clusters (Fig. 4). These are the same lipids or amphipathic "effectors" found to stabilize the individual channels through the non-annular sites (see above), so it is reasonable to assume that there is an overlap between those non-annular sites and the channel–channel interface involved in clustering. Interestingly, KcsA clusters have also been detected in vivo by immune electron microscopy, both in *Streptomyces lividans* and in *Escherichia coli*, suggesting that clusters may represent the native form of KcsA in the bacterial membranes [83].

In an attempt to explain how the individual channels could assemble into clusters to give raise to channel activity with a high opening probability, molecular docking between two adjacent KcsA molecules was performed using an open channel model of KcsA based on the X-ray open channel structure of MthK as a template [84]. The reason for choosing MthK for these studies is that it still remains as the only crystal structure of a potassium channel having open both, the inner and outer channel gates, as it is thought to happen in clustered KcsA [81]. Presence

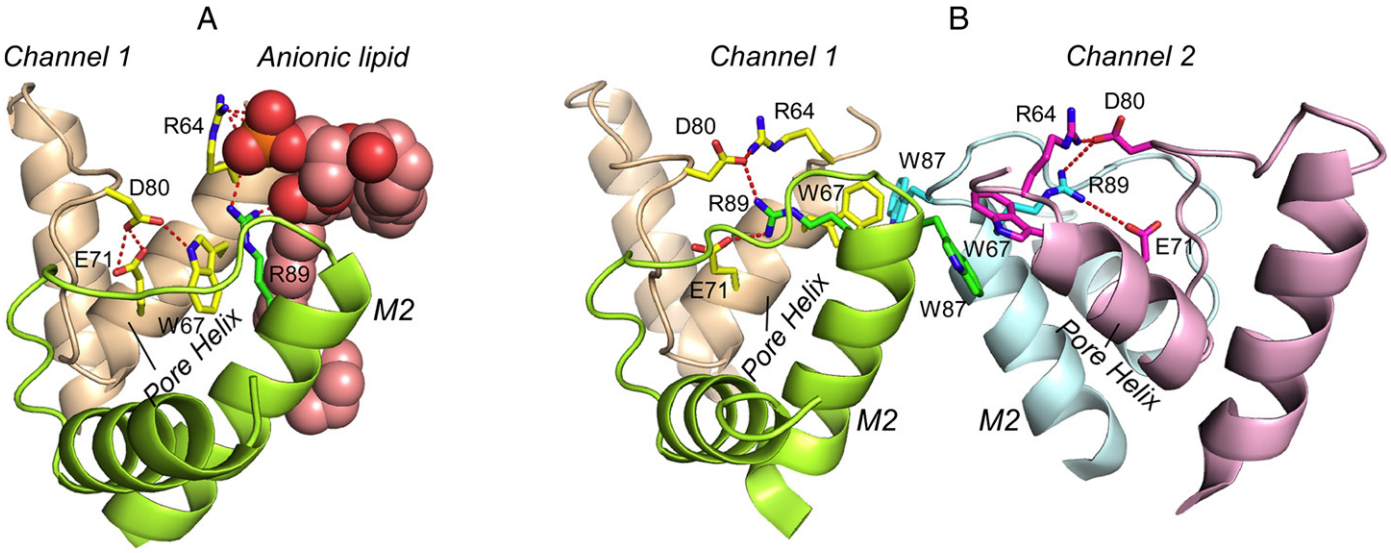

Fig. 3. Molecular models of the interaction between an anionic phospholipid and a non-annular site of KcsA (panel A), and that of the interaction between two KcsA tetramers (panel B). Panel A shows a side view of an extracellular portion in the crystallographic structure of KcsA ("Potassium Channel KcsA–Fab complex in high concentration of K^+ PDB ID: 1K4C") defining one of the four intersubunit crevices acting as non-annular lipid binding sites. A DPPA molecule (atoms represented as spheres) has been drawn bound to such site, using the partial lipid structure appearing in the protein crystal as a scaffold. Adjacent protein subunits defining the non-annular site are colored in light brown and green. The one letter code and numbering in the KcsA sequence for the main amino acid residues involved in the interactions have been included. Hydrogen bonds are indicated by red dashed lines. It should be noted that the W67–E71–D80 inactivation triad is fully configured and that the lipid polar head interacts primarily with R64 and R89. Panel B shows the results from computer docking two KcsA channels in an attempt to model a cluster. In this case, the KcsA sequence was previously modeled on the basis of homology onto the open structure of MthK ("Crystal structure of MthK at 3.3 Å PDB ID: 1LNQ"). The non-annular binding sites, flanked by the pore helix and M2 in each channel, interact now with the M2 from the adjacent channel (Channel 2; subunits colored in light blue and pink), while the inactivation triad has been disrupted. Molecules were edited and reconstructed with the general purpose molecular modeling software Yasara. The local docking procedure was accomplished with AutoDock 4. Figures were drawn with Pymol (DeLano Scientific, Palo Alto, CA).

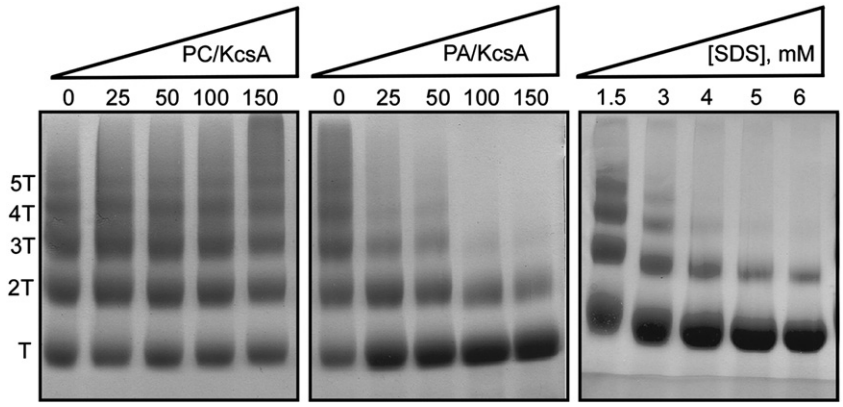

Fig. 4. BN-PAGE analysis of the modulation of KcsA supramolecular assembly by amphipathic agents. Increasing amounts of eggPC (left panel), eggPA (central panel) and SDS (right channel) were added to DDM-solubilized KcsA samples before being applied to BN-PAGE. The gels show up to five bands, corresponding to different supramolecular species formed by the auto-association of KcsA. It is observed that KcsA clusters dissociate in the presence of the anionic lipid eggPA or the alkylsulphate SDS, while the zwitterionic phospholipid eggPC does not result in cluster disassembly in a significant manner. T stands for the KcsA tetramer, while 2T, 3T, 4T, 5T refer to cluster species having 2, 3, 4, 5 times the molecular weight of the KcsA tetramer.

of lipid at the non-annular binding sites prevented docking between the two KcsA channels to occur and therefore, lipids were eliminated from the model. In the absence of such competing lipids, the inter-subunit crevice defining the non-annular site in each channel becomes now occupied by the N-terminal end of transmembrane M2 from the other channel. The W67 side chain rotamer pointing away from the channel central core is now favored compared to that seen in the phospholipid-bound channel, thus, disrupting the interactions within the W67–E71–D80 triad which in turn, changes the interaction of the later residues with those configuring the selectivity filter (Fig. 3B). In other words, a "clustered" KcsA channel should have different dynamics and conformational stability at the selectivity filter (different gating and inactivation properties) than those seen when anionic phospholipids occupy the non-annular sites of the channel. To further test the above hypothesis, we examined the reported X-ray structure of the potassium transporter TrkH [85], which already crystallizes as a dimer and therefore, does not require docking procedures to be applied. In this case, it is the N-terminal end of the pore helix of one channel (instead of the M2, as mentioned above) what goes closer to the inter-subunit crevice in the adjacent channel. Nonetheless, the consequences of such interaction on the W67–E71–D80 triad are practically identical to those predicted from docking, as the side chain of W67 also rotates away from the channel core, thus, disrupting the interactions within the "inactivation" triad [86].

As commented above, the electrostatic interaction between the anionic phospholipids and positively-charged amino acid residues in the KcsA sequence constitutes the basis for lipid binding to the non-annular sites [63,71]. Therefore, substitution of the R64 or R89 residues by uncharged amino acids in KcsA mutants should disfavor the stabilization of anionic phospholipids at such sites and according to the working hypothesis from above, such mutants should be expected to display an altered pattern of channel clustering and gating. Such an expectation became fulfilled by the experimental observation that such mutants, more markedly the R64A one, are relatively insensitive to the effects of anionic phospholipids on the disassembly of channel clusters observed by BN-PAGE in the wild-type channel. Moreover, the rate of inactivation in these channel mutants was measured by monitoring the changes in the current mediated by macro-patches from KcsA reconstituted into asolectin giant liposomes. Results show that indeed all the arginine mutants examined had a much slower rate of inactivation than wild-type KcsA [86].

The conclusion from the above studies is that binding of the phospholipid to the inter-subunit, non-annular sites on the channel is a determinant factor in facilitating interactions within the W67–E71–D80 triad which in turn, causes a change in the conformation of the residues configuring the selectivity filter and leaves the phospholipid-bound channel prone to inactivate. Because KcsA is a homotetramer, there are four identical non-annular sites, each of them acting on just one out of the four rows of T75–V76–G77–Y78–G79 residues from each subunit, which contribute their carbonyl groups to configure the selectivity filter, and it is to be expected that more than one of such sites should become lipid-bound to affect sufficiently the selectivity filter conformation as to make inactivation a truly effective process, whose occurrence should be dependent on the phospholipid concentration.

The above evidence seemingly completes a scenario in which competing lipid–protein or protein–protein interactions at the non-annular sites of KcsA determine both, the occurrence of clustering and whether the W67–E71–D80 inactivation triad can be adequately configured, which in turn bears on the selectivity filter and on the gating properties of the channel. In other words, "clustered" KcsA channels have different dynamics and conformational stability at the selectivity filter than "single", phospholipid-bound KcsA, thus changing the gating behavior.

Similar to KcsA, supramolecular clustering has been reported as relevant for the activity of other membrane proteins. The first solid experimental evidence is that of the acetylcholine receptor [87], in which protein clustering induces a positive coupled gating of the channels. Since then, other reports have appeared for rhodopsin, gramicidin, the serotonin transporter, ryanodine receptors, potassium and sodium channels, the mechanosensitive MscL channel, as well as for receptors involved in bacterial chemotaxis [88–94]. It has been proposed that such concerted activity is crucial to control cellular sensitivity [94], in processes such as chemotaxis [95], or for shaping action potentials in neurons [96]. As expected, due to the lipid ensemble of these proteins, some of the reports on physiologically-relevant clustering also show a dependence on membrane lipid composition or other membrane properties [88,89,92,93]. All these results are consistent with a recently revised model of the membrane bilayer called the 'patchwork membrane'. In this model the complex interaction between lipids and membrane proteins leads to a membrane dynamically compartmentalized into domains, some of them small and short-lived, and others large and long-lived. The maintenance of such heterogeneity seems crucial for the proper function of cells [97]. Nonetheless, there is still a lack of information on the protein sites involved in those lipid–protein and protein–protein interactions. Due to the influence that such processes seem to have on cellular physiology, data obtained on KcsA, although a prokaryotic channel, is an important step toward the definition at the molecular level of new targets to develop drugs able to modulate ion channel function.

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