



General and specific interactions of the phospholipid bilayer with P-type ATPases

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Abstract

Protein structure and function are modulated via interactions with their environment, representing both the surrounding aqueous media and lipid membranes that have an active role in shaping the structural topology of membrane proteins. Compared to a decade ago, there is now an abundance of crystal structural data on membrane proteins, which together with their functional studies have enhanced our understanding of the salient features of lipid-protein interactions. It is now important to recognize that membrane proteins are regulated by both (1) *general lipid-protein interactions*, where the general physicochemical properties of the lipid environment affect the conformational flexibility of a membrane protein, and (2) *specific lipid-protein interactions*, where lipid molecules directly interact via chemical interactions with specific lipid-binding sites located on the protein. However, due to local differences in membrane composition, thickness, and lipid packing, local membrane physical properties and hence the associated lipid-protein interactions also differ due to membrane location, even for the same protein. Such a phenomenon has been shown to be true for one family of integral membrane ion pumps, the P2-type adenosine triphosphatases (ATPases). Despite being highly homologous, individual members of this family have distinct structural and functional activity and are an excellent candidate to highlight how the local membrane physical properties and specific lipid-protein interactions play a vital role in facilitating the structural rearrangements of these proteins necessary for their activity. Hence in this review, we focus on both the general and specific lipid-protein interactions and will mostly discuss the structure-function relationships of the following P2-type ATPases, Na⁺,K⁺-ATPase (NKA), gastric H⁺,K⁺-ATPase (HKA), and sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA), in concurrence with their lipid environment.

Keywords Na⁺,K⁺-ATPase · Gastric H⁺,K⁺-ATPase · Sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA) · Cholesterol · Phospholipids, lipid-protein interaction

Introduction

P-type ATPases are a large family of enzymes that are central to all forms of life, ranging from the simplest archaeobacteria to the much more complicated higher eukaryotes (Bublitz et al.

2011; Greie and Altendorf 2007). In general, P-type ATPases are integral membrane proteins located in various membrane types such as the plasma or cellular organelle membranes, where they are associated with the transport of cations, heavy metal ions, and lipids, thereby generating and maintaining crucial (electro-)chemical potential gradients across these membranes (Kaplan 2002; Møller et al. 1996; Skou 1957). The Na⁺,K⁺-ATPase was the first member of the family to be discovered (Skou 1957). It helps maintain the electrochemical potential gradients for Na⁺ and K⁺ across the plasma membrane of animal cells and also provides the basis for electrical excitation in neurons and muscle cells (Skou 1957). In plants and fungi, an equally important and analogous role to the NKA is played by the plasma membrane H⁺-ATPase (Serrano et al. 1986). Other important members of the family include Ca²⁺-ATPases of the sarco(endo)plasmic reticulum (SERCA), plasma membrane (PMCA), and secretory

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pathway (SPCA), where they play vital roles in muscle function and Ca^{2+} signaling and are equally crucial for animal viability. The same is true for the gastric H^+ , K^+ -ATPase (HKA) which causes stomach acidification and the heavy metal ATPases (HMA), which are required for trace metal homeostasis and detoxification in both prokaryotes and eukaryotes. In addition to these ion-specific ATPases, the P4-type ATPases or flippases are enzymes capable of transporting large molecules like lipids (Bublitz et al. 2011, 2010). Thus, all P-type ATPases are key players in maintaining the electrochemical potential gradients of a cell, constantly undergoing large conformational changes in protein structure to actively transport ions and lipids across the membrane during their catalytic cycle (Morth et al. 2011). The Albers–Post or E1–E2 model is the generally accepted working hypothesis of the overall mechanism of all P-type ATPases (Albers 1967; Post et al. 1972), as shown in Fig. 1.

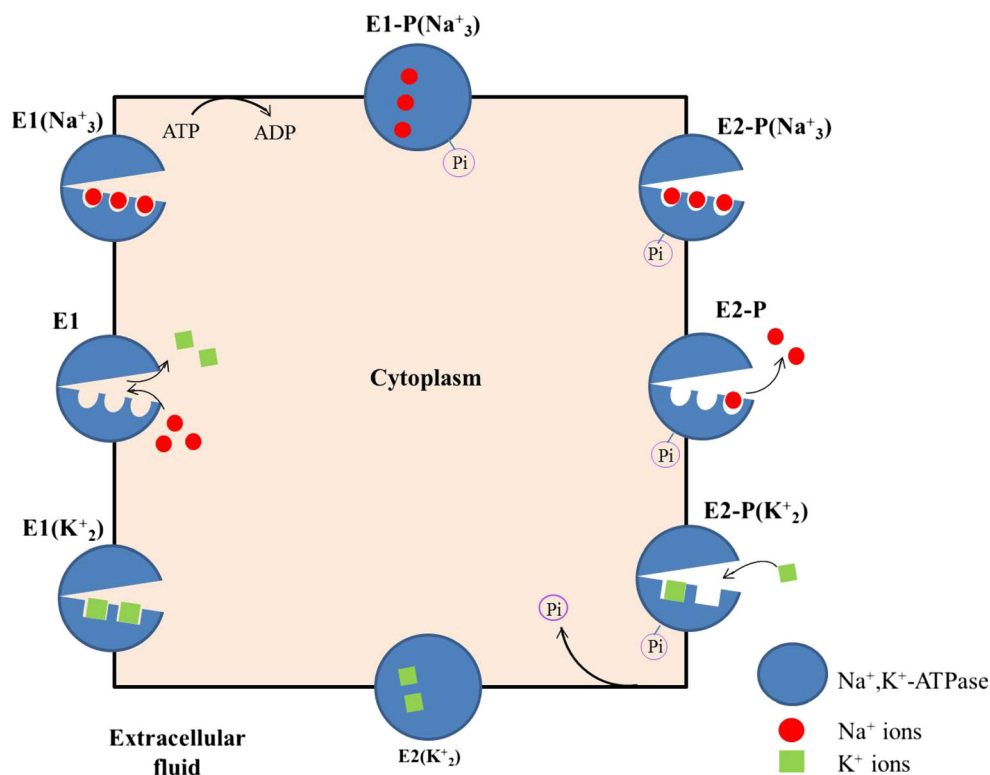
At the molecular level, ATPases derive their energy for ion pumping from ATP, oscillating between two main conformational states: E1, which is a high affinity state for the primary transported ions (Na^+ for NKA; H^+ for HKA; Ca^{2+} for SERCA), and E2, which is a low affinity state for the primary transported ions (Abe et al. 2018; Bublitz et al. 2011; Clarke et al. 2003; Jørgensen and Andersen 1988; Lüpfer et al. 2001; Morth et al. 2011, 2007; Olesen et al. 2007; Shinoda et al. 2009). The two states arise from the autophosphorylation of an aspartic acid residue of a conserved DKTGT motif in the E1 state, resulting in the formation and breakdown of

phosphoenzyme intermediates (E1P and E2P) that are coupled to the binding, occlusion, translocation, and release of ions (Fig. 1) (Post and Kume 1973). The E1 conformation has ion-binding sites open to the cytoplasm while the E2P sites open to the extracellular medium (or the lumen in the case of SERCA). The E1P and E2 conformations are termed occluded states in which the ions are enclosed within the protein and have no access to the aqueous medium on either side of the membrane. An example of the E1–E2 model for NKA is shown in Fig. 1. In summary, the NKA moves 3Na^+ ions out of, and 2K^+ ions into, the cell for each ATP hydrolyzed (Bublitz et al. 2011; Clarke et al. 2003; Morth et al. 2011; Shinoda et al. 2009). Similarly, for each ATP hydrolyzed, HKA results in the transport of H^+ ions out of the cell in exchange for K^+ ions into the cell in a 1:1 or 2:2 ratio (Abe et al. 2018), while SERCA results in the transport of 2Ca^{2+} ions into the sarcoplasmic reticulum lumen in exchange for 2–3 protons (Drachmann et al. 2014; Olesen et al. 2007).

Structural overview

Crystallographic studies of SERCA in several functional states and the more recently determined crystal structures of NKA, HKA, and plant H^+ -ATPase have provided a wealth of information on the specific and extensive structural rearrangements that accompany the different stages of the catalytic cycle, across the different subfamilies of

Fig. 1 The Albers–Post or E1–E2 model of P-type ATPases catalytic cycle, represented by the Na^+ , K^+ -ATPase. The binding of 3Na^+ ions to the E1(Na^+_3) state on the cytoplasmic side triggers phosphorylation by ATP. This leads to the formation of the occluded E1-P(Na^+_3) state (represented by the closed form) and a subsequent transition to the E2P state. The E2P state has reduced affinity for Na^+ ions, thus leading to the exchange of 3Na^+ ions for 2K^+ ions from the extracellular fluid. This results in the closure of the E2P state that stimulates dephosphorylation and formation of the occluded E2(K^+_2) state. The E2(K^+_2) state then relaxes back to the E1 state with the subsequent release of the 2K^+ ions into the cytoplasm and binding of Na^+ ions and the cycle continues



P-type ATPases, and also shed new light on the molecular details of both general and specific lipid-protein interactions. Figure 2 provides an overview of a topological model and crystal structures of rabbit SERCA (PDB: 3B9B) (Olesen et al. 2007), pig renal NKA (PDB: 3KDP) (Morth et al. 2007), and pig gastric HKA (PDB: 5YLU) (Abe et al. 2018) with indications of their functional states in the classical E1–E2 cycle. The plasma membrane NKA and the gastric HKA consists of a catalytic α -subunit and a glycosylated β -subunit, whereas SERCA is composed of a homologous α -subunit alone. The α -subunit comprises ten transmembrane helices (α TM1–10), which contain the cation-binding sites,

linked to three cytoplasmic domains, nucleotide-binding (N), phosphorylation (P), and actuator (A) domains, which are primarily responsible for ATP hydrolysis. In comparison to the α -subunit, the β -subunit acts as a chaperone that influences the packing of the cytoplasmic domains and hence is necessary for the correct folding and structural stability of the NKA and HKA in the different catalytic states and thus is crucial for their function (Bublitz et al. 2010; Morth et al. 2011). In addition to these domains, many ATPases also have regulatory (R) domains at the N- or C-terminus of the α -subunit, which are autoinhibitory and function when phosphorylated or when bound to regulating factors (Ekberg et al. 2010;

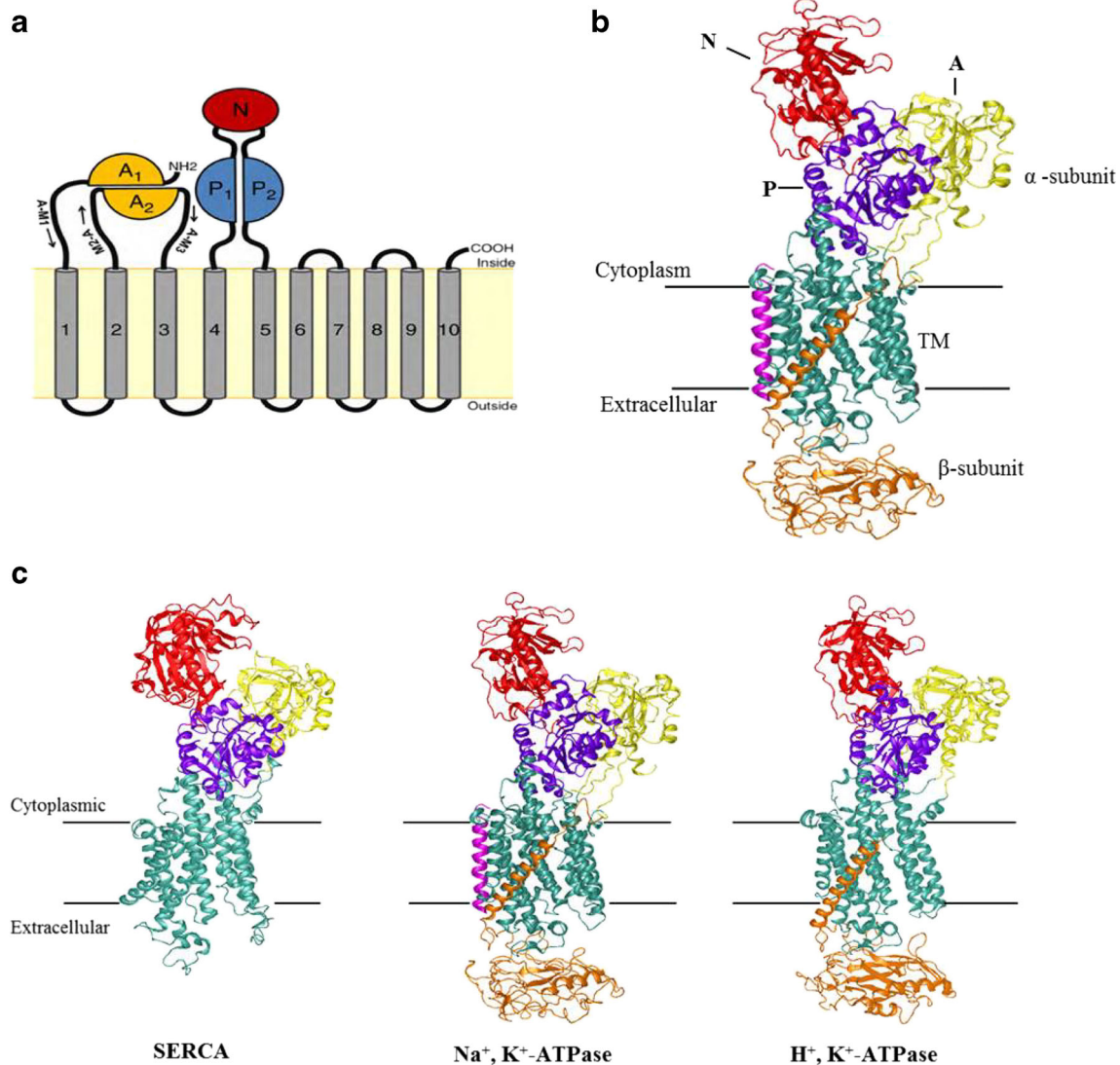


Fig. 2 Structural insights into P2-type ATPases. **a** Topology diagram of a typical P2-type ATPase α -subunit showing the 10 TM helices linked to the three cytoplasmic domains. **b** The crystal structure of Na^+, K^+ -ATPase at the E2-P state (generated using the DNASTAR LaserGene software based on PDB: 3KDP). **c** Comparisons of the P2-type ATPases as depicted by the crystals structures of rabbit SERCA (PDB: 3B9B), pig

renal NKA (PDB: 3KDP), and pig gastric HKA (PDB: 5YLU) in E2-P state. The cytoplasmic N-, P-, and A-domains are shown in red, purple, and yellow, respectively, and TM1–10 helices are shown in green for the three ATPases. The β -subunit of NKA and HKA is shown in orange while the γ -subunit for NKA is shown in pink

Morth et al. 2011; Zhou et al. 2013). Several allosteric modulators and scaffolding proteins have been identified as regulators; some examples include phospholamban and sarcolipin, which regulate SERCA (Traaseth et al. 2008), and the regulatory γ -subunit of NKA, which belongs to the family of FXYD proteins (Morth et al. 2011).

Phospholipid composition of P2-type ATPase membranes

To better understand the structural and functional properties of NKA, HKA, and SERCA, it is important to recognize that due to differences in their tissue and subcellular localization, the phospholipid environment surrounding these ATPases varies and so do their lipid-protein interactions (Cornelius et al. 2015). In general, the total lipid content of NKA, which is confined to the plasma membrane of animals, contains 51% phospholipids (PL), 36% cholesterol (CHL), 5% acylglycerols, and 9% free fatty acids on a molar basis. Of the phospholipids present, 36% were determined to be phosphatidylcholine (PC), 28% phosphatidylethanolamine (PE), 18% sphingomyelin (SM), 13% phosphatidylserine (PS), and 6% phosphatidylinositol (PI) (Nguyen et al. 2018; Peters et al. 1981). The lipid composition of gradient-purified gastric microsomes from different species have also shown HKA to have a similar total lipid molar composition of approximately 58% PL, 38% CHL, and 4% glycosphingolipids and to lack cardiolipin (Bailey et al. 1986; Olaisson et al. 1985). Although the total lipid content of HKA-containing membranes from different species vary widely (Sen and Ray 1979a, b), their phospholipid molar compositions are closely related, with PE being the dominant phospholipid (31%), followed by 27% SM, 22% PC, 12.8% PS, 4.2% PI, and 3% lyso-PE/PC (Bailey et al. 1986; Olaisson et al. 1985). On the other hand, SERCA, which is located in the endoplasmic reticulum inside the cell, is predominantly surrounded by phospholipids (89%) with significantly smaller molar concentrations of cholesterol (7%) and acylglycerols (4%) in comparison to NKA and HKA. Of the phospholipids present 65% were determined to be PC, 18.8% PE, 11.4% PS, 4.6% SM, and 0.3% cardiolipin (Borchman et al. 1982; MacLennan et al. 1971). As a result, the NKA, HKA, and SERCA have adapted differently to the different membrane environments they reside in. This difference in lipid composition was also demonstrated by the difference in the lipids found in crystal structures. The NKA was found to co-crystallize with molecules of PC and CHL with a few molecules of PS, whereas SERCA co-crystallized with PC and PE but no molecules of CHL (Cornelius et al. 2015), and in the case of the HKA, only PC molecules

have been shown bound within the crystal structure of HKA (Abe et al. 2018).

General lipid-protein interactions

The effects of different membrane properties, including membrane hydrophobic thickness, on the activity of these ATPases have been studied on several occasions by reconstituting the enzymes into liposomes produced from phospholipids of varying acyl chain lengths (n_c), head-groups, and saturation levels (Cornelius et al. 2015). The optimal activity of NKA was observed in proteoliposomes with a PL acyl chain (n_c) value of 18 in the presence of 40 mol% CHL, whereas in the absence of cholesterol, the maximum activity was found at an $n_c \geq 22$. This fact that there is a lower optimal n_c value in the presence of cholesterol, which also happens to be the average length of acyl chains in native membranes, was attributed to the presence of cholesterol increasing acyl chain order and bilayer thickness (Cornelius 2001; Cornelius et al. 2003). The importance of bilayer thickness in protein function was also further highlighted when NKA activity was inhibited or reduced by the presence of n-3 polyunsaturated PL in the absence or presence of CHL, respectively (Cornelius 2008). Cholesterol and polyunsaturated PLs exhibit different curvature (lateral pressure) profiles throughout the bilayer and have the ability to laterally phase separate into microdomains, thereby inducing changes in the lipid composition, thickness, and cross-sectional area at the protein-membrane interface (Cantor 1999; Mitchell and Litman 1998). The cross-sectional area of NKA near the membrane interface at the cytoplasmic side has also been shown to differ significantly in E1P and E2P conformations (Cornelius et al. 2003; Yoda and Yoda 1987). Similar experiments have been performed using HKA, where PC molecules enriched in mono- or di-unsaturated fatty acids (predominantly PC 18:1 and 18:2) and extremely poorly enriched in polyunsaturated fatty acids have been shown to be necessary in maintaining the activity and stability of the gastric microsomal HKA system (Nandi et al. 1983; Sen and Ray 1980). In contrast to the plasma membrane, the endoplasmic reticulum membrane, where SERCA is located, contains much less cholesterol, thus reducing the membrane's hydrophobic thickness (Sonntag et al. 2011). This results in SERCA matching thinner membranes with an $n_c > 12$ required for the retention of Ca^{2+} transport activity and PC 18:1 being the optimal chain length. Another remarkable effect of bilayer thickness on SERCA has been observed. Reducing the membrane thickness was found to induce a structural rearrangement of α -helix packing in the protein, disrupting one of the two Ca^{2+} -binding sites in the protein (Cornelius et al. 2015; Starling et al. 1993). It is now well-established that the function of SERCA is highly dependent on lipids through indirect, non-specific membrane

effects such as the hydrophobic thickness and membrane fluidity. Furthermore, the lipid dipole potential surrounding an ion pump has also been proposed to play an important role in the structural and functional activity of these proteins (Clarke 2015). A detailed mechanism has been suggested explaining how changes in membrane dipole potential by modifiers such as cholesterol could induce preferential stabilization or destabilization of occluded versus non-occluded states of different P-type ATPases. Dipole potential changes would be expected to accompany changes in lipid packing necessitated by changes in membrane hydrophobic thickness and protein cross-sectional area caused by membrane protein conformational changes (Clarke 2015). MD simulations of SERCA showed that during the catalytic cycle, as the TM helices move, the membrane thickness and the amino acid residues interacting with the PL head-groups change and so does the orientation of the whole protein. This would result in large changes in the cross-sectional area of the TM region as well as in the distribution of lipids surrounding the pump which in turn could affect pump activity (Cantor 1999; Cornelius et al. 2015). In addition to the strong evidence indicating general lipid-protein interaction effects on ATPase activity, there are compelling data showing specific and selective lipid-binding sites in the NKA, HKA, and SERCA.

Lipid-protein interactions in Na⁺,K⁺-ATPase

Structural and functional studies with NKA reconstituted into lipid vesicles, in living cells, or after detergent-solubilization demonstrated that phospholipids and cholesterol play a crucial role in enzyme activity (Hayashi et al. 1988; Cornelius 2001; Cornelius et al. 2015; Esmann and Marsh 2006). In addition to these overall effects of PLs and CHL, advanced structural studies have recently shown three specific lipid-binding sites, A–C, for NKA (Habeck et al. 2017; Kanai et al. 2013).

Effects of different phospholipids on NKA activity

Lipid-protein interaction was evident from the structural selectivity of acyl chain lengths and saturation level of PS for optimal NKA activity. PS having only saturated fatty acyl chains was found to induce a rapid loss in NKA activity, with at least one double bond required to maintain activity (Cornelius et al. 2015; Habeck et al. 2017; Mimura et al. 1993; Mishra et al. 2011; Wheeler and Whittam 1970). In an extensive study using spin labeled lipids, PS was shown to be superior to PI in supporting NKA activity, while PC supports activity initially, but the effect is rapidly lost, and PE does not support activity at all (Esmann and Marsh 2006). Binding of PS was also preserved in extensively trypsinized NKA, in which the major cytoplasmic segments were removed while retaining the TM segments and extracellular loops of the

protein (Shinji et al. 2003). In addition to PS interaction with NKA, PE interaction with the enzyme was shown to be associated with thermostability (Cornelius et al. 2015). NKA reconstituted into PS/CHL/brain PE proteoliposomes reduced the overall thermostability of the protein and was found to be more stable in the E2(K⁺₂) state (in KCl) than in the E1(Na⁺₃) state (in NaCl) (Habeck et al. 2015; Jørgensen and Andersen 1986). This is in contrast to enzyme reconstituted in PS/cholesterol, which is more stable in NaCl solution compared to KCl (Mishra et al. 2011). This specific activity of NKA prepared with PS/CHL/brain PE lipids was found to be similar to the activity observed for highly purified native renal NKA (Habeck et al. 2015). Furthermore, unsaturated neutral PE/PC has also been shown to increase the specific activity of NKA by increasing the molar turnover rate and the stimulation is structurally selective for neutral PLs with polyunsaturated acyl chains (Cohen et al. 2005; Haviv et al. 2013). The order of selectivity is asymmetric, with mixed saturated and polyunsaturated acyl chains of PE/PC being more effective than mono- or di-unsaturated PLs. Asymmetric 18:0–20:4 and 18:0–22:6 PE or PC were shown to be optimal for stimulating human $\alpha\beta$ FXD complexes resulting in a 1.6-fold increase in NKA activity (Habeck et al. 2015).

Effects of cholesterol on NKA activity

The activity of NKA was also shown to increase with increasing concentration of CHL (Cornelius et al. 2015; Lambropoulos et al. 2016; Garcia et al. 2019), with the optimal activity achieved at 20 mol% concentration followed by a slight decrease at 40 mol% CHL (Cornelius 1995). This increase in activity seen at 20 mol% CHL was attributed to a CHL-induced increase in the rate determining E2 → E1 reaction step due to a stabilization of the E1 state (Cornelius 1995; Cornelius et al. 2003). Comparable observations were published in a recent study, where cholesterol was extracted from pig renal NKA-containing membrane fragments using methyl- β -cyclodextrin (Garcia et al. 2019). This approach allowed the effect of cholesterol in a membrane environment much closer to that of the native membrane to be studied, i.e., much closer than in reconstituted vesicles. It was found that in these near-native membranes cholesterol extraction also resulted in a significantly reduced overall activity. From stopped-flow kinetic studies of the enzyme's partial reactions, significant reductions in the rate constants for the E1(Na⁺₃) → E2P and E2(K⁺₂) → E1(Na⁺₃) steps were observed. Thus, partial reactions involving binding and release of cations at the protein's intracellular side are most dependent on cholesterol. Furthermore, MD simulations of the NKA in membranes with 40 mol% CHL have revealed CHL interaction sites to differ markedly between protein conformations and based on membrane shape, with the E2 state likely disfavored in cholesterol-rich bilayers relative to the E1P state due to a

greater hydrophobic mismatch (Garcia et al. 2019). In addition to these, other studies have also shown CHL to be associated with increasing NKA phosphoenzyme level, ATP affinity, the rate of phosphorylation, and K^+ -activated dephosphorylation, thus affecting almost every step that one could measure in the NKA reaction cycle (Cornelius et al. 2015).

Binding of phospholipids and cholesterol to specific sites on NKA

In addition to the strong evidence on how lipid-protein interactions affect NKA activity, three individual lipid-binding sites, designated as A, B, and C, bound to different PLs and CHL have been shown to regulate the stability and molecular activity of Na^+,K^+ -ATPase (Habeck et al. 2017; Kanai et al. 2013). Several PLs and three cholesterol molecules (CHL1–3) bound to NKA structure (PDB: 3WGV) are shown in Fig. 3. The binding of PL and CHL molecules to each of the different

lipid-binding sites (discussed below) have been shown to exhibit a separate functional effect based on the specific lipid-protein interaction. For example, binding of PLs, particularly anionic lipids like PS or cardiolipin, and CHL2 and CHL3 at site A is associated with stabilizing the protein. Neutral polyunsaturated PLs bound at lipid-binding site B have been shown to stimulate protein activity, while PC or SM interaction with CHL1 at lipid site C has an inhibitory effect on NKA activity (Habeck et al. 2015, 2017; Kanai et al. 2013). Two lipid-binding sites, A and C, are shown in Fig. 3b, c, respectively; however, due to the location of site B within the protein, it was not feasible to structurally include this site in Fig. 3.

Lipid-protein interactions at site A

The lipid-binding site A is positioned between TM segments $\alpha M8$ -M10 and FXYB and is divided into subsites A1–A4

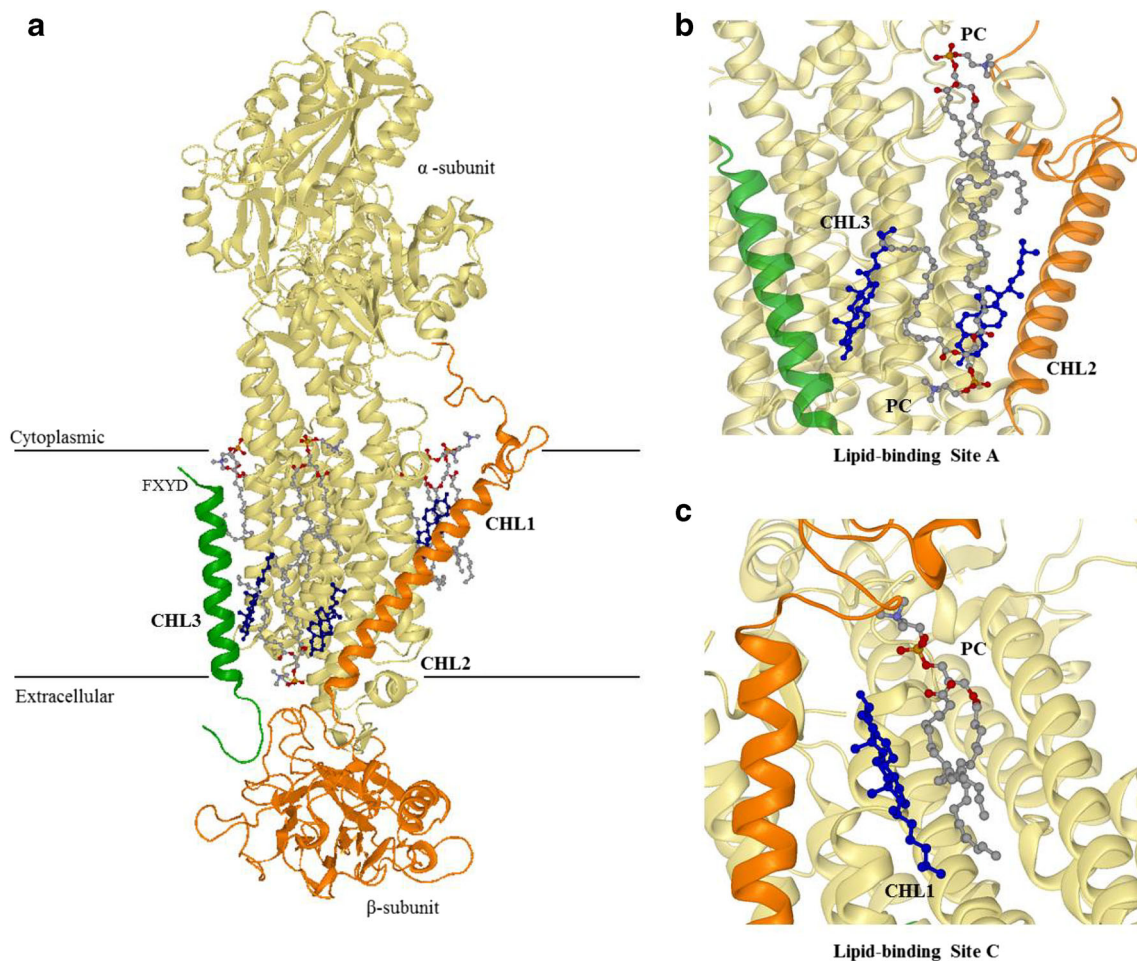


Fig. 3 Na^+,K^+ -ATPase interaction with phospholipid and cholesterol molecules. **a** NKA in the E2-P state (PDB: 3WGV) with the α -, β -, and γ -subunits shown in yellow, orange, and green, respectively; the three cholesterol molecules (CHL1–3) shown in blue and the phospholipids shown in stick representation, with the phosphorous atom shown in yellow, oxygen atoms in red and nitrogen in blue. **b** Enlargement of the

structure in **a** showing only the TM part that corresponds to the lipid-binding site A. Two PC lipids and CHL2 and CHL3 are shown bound to site A. **c** Enlargement of the structure in **a** showing the lipid-binding site C bound to CHL1 and PC. **a** Rotated towards the right and by 90° towards the left to better display lipid-binding site A in **b** and site C in **c**, respectively

with each having been shown to bind PLs (Habeck et al. 2015, 2017; Kanai et al. 2013). A1 and A4 sites are associated with PLs binding only, while sites A2 and A3 are bound to CHL3 at the $\alpha\gamma$ interface and CHL2 at the $\alpha\beta$ interface on the extracellular side of the membrane, respectively (see Fig. 3b). Several studies showed the PS, located at lipid site A at the cytoplasmic surface, to interact via the acyl chains with the CHL3 bound near the extracellular membrane and stabilize the protein (reviewed in (Cornelius et al. 2015)). Recently, an ion mobility mass spectrometric study provided direct evidence of binding of one molecule of 18:0–18:1PS to the purified recombinant $\alpha 1\beta$ 1FXDYD1 NKA complex which further suggested that CHL and PS interact directly and that this specific lipid-protein interaction leads to stronger additional stabilization of the protein (Mayan et al. 2018). Several structural and functional studies support the notion that cholesterol association with PS, FXDYD1, and the α -subunit at the cytoplasmic side, together with the $\alpha\beta$ -subunit interactions at the extracellular side, helps maintain the topological stability of α M8–M10 and the unique configuration of the sodium site III (Habeck et al. 2017; Kanai et al. 2013; Mayan et al. 2018; Mishra et al. 2011). Although there is a difference in PL binding to site A, with pig kidney NKA bound to PC (PDB: 3WGU) (Kanai et al. 2013) and shark rectal gland NKA to PS (PDB: 2ZZXE) (Shinoda et al. 2009), there is a wealth of evidence that shows anionic lipids affect several kinetic properties of NKA including ion-activation, K^+ -deocclusion, and the E1/E2 conformational equilibrium of the protein (Cornelius et al. 2015).

Lipid-protein interactions at site B

Stimulation of NKA activity by neutral polyunsaturated PE or PC is independent of cholesterol and the FXDYD protein, in contrast to stabilization by PS/cholesterol (Habeck et al. 2015; Haviv et al. 2013). This was made evident from structural and functional studies of PE/PC bound to lipid-binding site B (Kanai et al. 2013). In the E1P conformation, the neutral PE/PC bound to site B is located in a very narrow pocket between TM helices α M2, 4, 6, and 9, whereas in the E2P structure, due to the different positions, especially of α M1–M2 helices, site B expands considerably and hence the PL expands to change its own conformation. Hence, it is speculated that the PL facilitates the E1P \rightarrow E2P conformational transition by lowering the activation energy, while in the E2 \rightarrow E1 transition, the conformation of the PL readjusts to fit back into its binding site in the E1 conformation (Habeck et al. 2015; Kanai et al. 2013).

Lipid-protein interactions at site C

Apart from the PS/CHL stabilizing and polyunsaturated PE/PC stimulatory effects on NKA activity, specific lipid-protein

interactions at lipid site C (see Fig. 3c) results in the inhibition of protein activity (Habeck et al. 2015; Kanai et al. 2013). A CHL molecule (CHL1) is positioned in the lipid-binding site C, bounded by α M3, 5, 7, and β -subunit, at the $\alpha\beta$ interface on the cytoplasmic side of the membrane. Analysis of the kinetic mechanism revealed that when this pocket is occupied by saturated PC and SM but not saturated PE or PS, NKA activity was inhibited by 70–80% due to the inhibition of the rate determining E2(K^+_2) \rightarrow E1(Na^+_3) reaction step, thus stabilizing the enzyme in the E2 conformation. This inhibition by PLs was also shown to be dependent on the presence of CHL at site C and the order of lipid effectiveness was found to be PC > SM > PE > PS with acyl chain lengths 22:0 < 20:0–18:0 > 16:0 > 14:0 respectively (Habeck et al. 2015). Thus, PL and cholesterol interactions with NKA result in three separate functional effects based on the specific lipid-protein interactions at different lipid-binding sites (A, B, or C) on the protein.

Lipid-protein interactions in H^+,K^+ -ATPase

Although specific lipid-binding sites in gastric HKA have not yet been fully elucidated, the crystal structure of HKA in the E2P state (PDB 5YLU), resolved at 2.8 Å, showed the presence of several phospholipids and detergent molecules being associated with the protein (Abe et al. 2018). Two PC molecules have been shown to bind to the protein, as seen in Fig. 4. The role of annular and non-annular lipids on the activity of pig gastric microsomal HKA in their in situ membrane environment has been systematically investigated employing several approaches (reviewed in (Ray et al. 2008)). Studies have shown that extraction of PC or PE from HKA microsomes resulted in the inhibition of ATPase activity which, upon the addition of PC, was fully restored. PC molecules enriched in high amounts of mono- and di-unsaturated fatty acid chains (predominantly PC 18:1 and 18:2) were found to be optimal for activity, while polyunsaturated and fully saturated PC were about 50% less effective (Abe et al. 2018; Nandi et al. 1983; Sen and Ray 1979a, b, 1980). HKA also showed selectivity for the PL head-group, with PC found to produce twice the activity of PE, whereas SM had little or no effect on HKA activity. It was speculated that PC molecules correctly embedded in the protein hydrophobic surface are necessary for the optimal functional freedom needed for pump activity, including its subtle regulation (Nandi et al. 1983). Furthermore, the asymmetric orientation of PE across the phospholipid bilayer and its interaction with the HKA complex in microsomes is considered to play an important role in the topological layout and structural stability of HKA across the membrane. In gastric HKA microsomes, the asymmetric distribution of unsaturated PE molecules (70% of the total PE facing the inner microsomal membrane) have been shown to dictate the

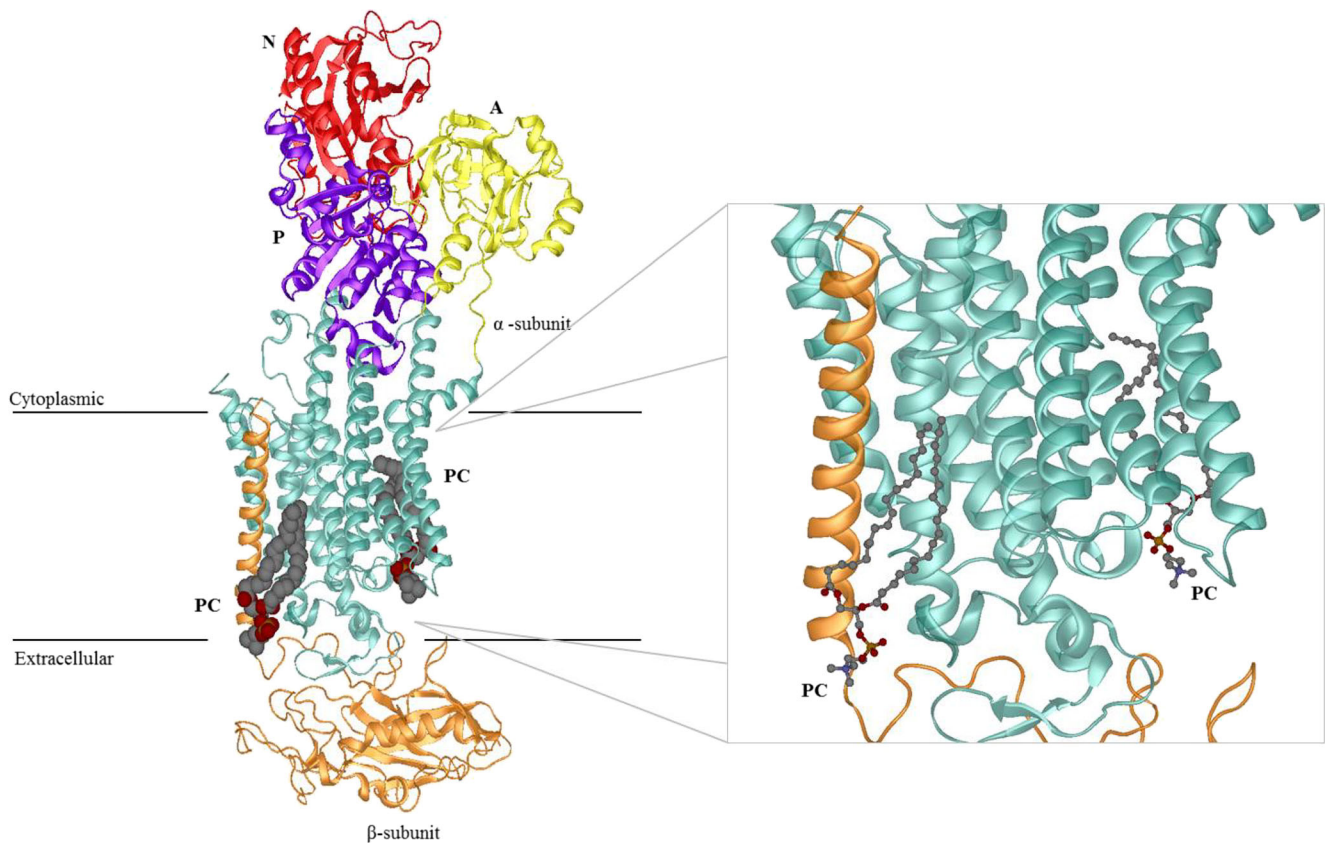


Fig. 4 H^+,K^+ -ATPase interaction with phospholipid molecules. HKA in the E2-P state (PBD: 5YLU) showing two PC lipids modeled in the structure (spheres). The cytoplasmic N-, P-, and A-domains are shown in red, purple, and yellow, respectively, TM1–10 helices are shown in

green and the β -subunit in orange. The box shows an enlargement of the TM structure of HKA with the two PC lipids (sticks), showing the phosphorous atom in yellow, oxygen atoms in red, and nitrogen in blue

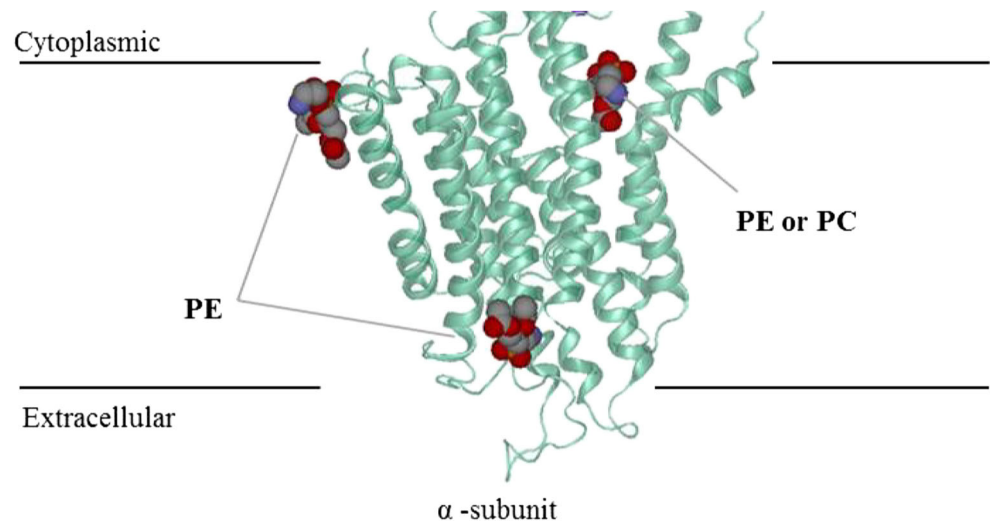
observed uniform orientation of the ATP catalytic site of native HKA to face the microsomal membrane exterior. This specific orientation is thought to be important in determining the correct topological layout of the protein, following the fusion of HKA to the apical plasma membrane of parietal cells upon stimulation (Ray et al. 2008). Analysis of gastric HKA reconstituted in PC/cholesterol proteoliposomes also showed cholesterol to have an inhibitory effect on enzyme activity in a dose-dependent manner. Depletion of membrane cholesterol by 80% reduced the activity by 35% and completely abolished the HKA-dependent H^+ uptake by the microsomes (Ray et al. 1983). This strongly suggests that CHL also plays a key role in the overall stability of gastric HKA, perhaps in a similar manner to that observed for NKA. However, the precise mechanisms involved in many of the processes discussed above remain to be elucidated.

Lipid-protein interactions in SERCA

X-ray diffraction studies and crystal structures of SERCA in different states revealed a number of cavities that may serve as non-annular binding sites for specific lipid-protein

interactions, as seen in Fig. 5. So far, a total of five lipid-binding sites for PLs have been identified in SERCA (Drachmann et al. 2014). Lipid-binding sites A and C are specific to certain conformational states of the enzyme while sites B and D are state independent. The fifth one is a crystal packing site where specific interaction with a PC molecule was found to be crucial for the formation of SERCA crystals (Drachmann et al. 2014). In contrast to NKA and HKA, SERCA has not been shown to be activated or inhibited by CHL, even though CHL does interact with the protein's TM surface and compete with PC for binding in reconstituted SERCA proteoliposomes (Warren et al. 1975; Starling et al. 1995; Cheng et al. 1986). Several fluorometric studies have proposed a model of direct and specific CHL inhibition of SERCA activity through lipid-binding sites located between TM α -helices, but MD simulations did not support this idea (Cornelius et al. 2015; Drachmann et al. 2014). However, numerous other studies supported an indirect and non-specific inhibitory effect of cholesterol, mainly through a decrease in membrane fluidity and other physicochemical properties that in turn affect SERCA activity (Drachmann et al. 2014). For instance, in advanced atherosclerotic lesions, enrichment of CHL in ER membranes of macrophages resulted

Fig. 5 SERCA interaction with phospholipid molecules. The TM segment (green) of SERCA in the E2 state (PDB: 2AGV) showing only the head-groups of two PE and one PE/PC lipids modeled in the structure (spheres). The oxygen atoms are shown in red, nitrogen in blue, and carbon atoms in gray for the PLs



in the inhibition of SERCA2b activity through changes in the physical properties of the membrane (Li et al. 2004). As previously mentioned, endoplasmic reticulum has a much lower CHL level than the plasma membrane and this may help explain why CHL is excluded from SERCA annulus lipids and has little effect on enzymatic activity, although PLs has been shown to influence SERCA activity.

Lipid-protein interactions at state-dependent sites

The lipid-binding site A is located in a relatively wide cleft between α M2 and α M4 at the cytoplasmic leaflet of the bilayer and is capable of binding PC lipids of varying acyl chain lengths. PC 18:1 was shown to have the greatest affinity for binding and the highest stimulatory effect on SERCA activity in comparison to PCs with shorter or longer acyl chains (Drachmann et al. 2014; Gustavsson et al. 2011; Starling et al. 1993). Several published crystal structures of SERCA have shown this site to be generally occupied by PC in the E2 state, where the lipid molecule was suggested to act like a wedge to keep α M2 and α M4 apart from each other in order to stabilize the E2 conformation. Therefore, site A is considered as an E2-dependent lipid-binding site, whereas site C has been shown to be an E1-dependent lipid-binding site (Drachmann et al. 2014; Gustavsson et al. 2011; Starling et al. 1993). Crystal structures of SERCA in E1(Ca^{2+}_2) and E1P states revealed PC molecules bound to site C similar to that observed for site A (Clausen et al. 2013; Toyoshima et al. 2004). Structural data shows that in the E1 state, the conformation of TM α M1–4 helices drastically changes, causing the disappearance of the site A pocket in the E1 conformation and the appearance of a wide groove (site C) between α M2, 6, and 9 that binds PC in order to stabilize the conformation. This groove has also been shown to bind regulatory proteins such as sarcolipin and phospholamban, and hence, PLs are also thought to either compete with these regulators and/or help

fine-tune the equilibrium of regulator binding (Drachmann et al. 2014).

Lipid-protein interactions at state-independent sites

Several crystal structures of SERCA in both E1 and E2 conformation have revealed state-independent lipid-binding sites B and D (Drachmann et al. 2014). Site B is located at the C-terminal end of α M10 and the loop between α M8–9 at the cytoplasmic leaflet, whereas site D forms a pocket deeper inside the hydrophobic core of the membrane between α M3, 5, and 7. Since the C-terminal domain of SERCA undergoes only small conformational changes throughout the catalytic cycle, site B is preserved in different states, where it shows a similar selectivity for PC 18:1 as site A (Drachmann et al. 2014). The crystal structure of the Mg^{2+} -bound E1 SERCA revealed a PE lipid in site B instead of PC (Toyoshima et al. 2013). Despite the difference in PL head-groups, both lipids were found to share the same position of the phosphate moiety and maintain the same key interactions with the surrounding residues. Thus, it was suggested that site B is integral to the C-terminal domain of SERCA where the specific lipid-protein interaction act as a stable anchor to the membrane. In comparison, site D is considered as a regulatory site for SERCA-membrane interactions because of the fact that in the presence of the SERCA inhibitor, thapsigargin (TG), the inhibitor was found bound at site D (Drachmann et al. 2014). Hence, this site is often known as the thapsigargin-groove or TG-binding site. TG binding has been shown to completely inhibit pump activity and, since in its absence a PE molecule occupies the same pocket (Obara et al. 2005), this strongly suggests some regulatory role for the lipid molecule that requires further elucidation.

Conclusion

It is evident now that both annular lipids and non-annular lipids specifically bound to NKA, HKA, and SERCA dictate the structural and functional properties of these enzymes. Interestingly, interaction with mixed mono- or di-unsaturated PLs (18:1 PC or 18:2 PC) showed optimal activity for all three enzymes and is preferred over fully saturated or polyunsaturated PLs. Both NKA and HKA, being plasma membrane proteins, showed structural and functional specificity and selectivity for CHL, whereas SERCA located in the endoplasmic reticulum showed no preference for the presence or absence of CHL in the membrane. Each P-type ATPase is likely to display individual patterns of lipid-protein interactions with large diversity based on the different conformational states of the protein and also on the specific composition of membranes of particular cells or organelles. Nevertheless, the concept of direct and specific interactions of particular lipids and the indirect effects of bilayer structure on P-type ATPases represents an avenue that still needs further discovery and explanation. It seems likely that these ATPases, despite being homologous, may have adopted differently based on the different membrane environments they reside in. This may well be true for other membrane proteins as well. It may even be the case that membrane composition and protein structure have evolved in parallel to provide optimal protein activity as previously suggested by Lambropoulos et al. (2016) for NKA; however, this warrants further investigation.

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Compliance with ethical standards

Conflict of interest Khondker R. Hossain declares that she has no conflict of interest. Ronald J. Clarke declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Abbreviations NKA, Na⁺,K⁺-ATPase; HKA, H⁺,K⁺-ATPase; SERCA, Sarco(endo)plasmic reticulum Ca²⁺-ATPase; TM, Transmembrane; CHL, Cholesterol; PL, Phospholipid; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PS, Phosphatidylserine; PI, Phosphatidylinositol; SM, Sphingomyelin

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