Cholesterol depletion inhibits Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in a near-native membrane environment

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Running title: \textit{Inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase by cholesterol depletion}

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ABSTRACT

Cholesterol’s effects on Na\textsuperscript{+},K\textsuperscript{+}-ATPase reconstituted in phospholipid vesicles have been extensively studied. However, previous studies have reported both cholesterol-mediated stimulation and inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity. Here, using partial reaction kinetics determined via stopped-flow experiments, we studied cholesterol’s effect on Na\textsuperscript{+},K\textsuperscript{+}-ATPase in a near-native environment in which purified membrane fragments were depleted of cholesterol with methyl-β-cyclodextrin (mβCD). The mβCD-treated Na\textsuperscript{+},K\textsuperscript{+}-ATPase had significantly reduced overall activity and exhibited decreased observed rate constants for ATP phosphorylation (ENa\textsuperscript{+}3 → E2P, i.e. phosphorylation by ATP and Na\textsuperscript{+} occlusion from the cytoplasm) and K\textsuperscript{+} deocclusion with subsequent intracellular Na\textsuperscript{+} binding (E2K\textsuperscript{+}2 → E1Na\textsuperscript{+}3). However, cholesterol depletion did not affect the observed rate constant for K\textsuperscript{+} occlusion by phosphorylated Na\textsuperscript{+},K\textsuperscript{+}-ATPase on the extracellular face and subsequent dephosphorylation (E2P → E2K\textsuperscript{+}2). Thus, partial reactions involving cation binding and release at the protein’s intracellular side were most dependent on cholesterol. Fluorescence measurements with the probe eosin indicated that cholesterol depletion stabilizes the unphosphorylated E2 state relative to E1, and the cholesterol depletion-induced slowing of ATP phosphorylation kinetics was consistent with partial conversion of Na\textsuperscript{+},K\textsuperscript{+}-ATPase into the E2 state, requiring a slow E2 → E1 transition before the phosphorylation. Molecular dynamics simulations of Na\textsuperscript{+},K\textsuperscript{+}-ATPase in membranes with 40 mol% cholesterol revealed cholesterol interaction sites that differ markedly among protein conformations. They further disclosed state-dependent effects on membrane shape, with the E2 state being likely disfavored in cholesterol-rich bilayers relative to the E1P state because of a greater hydrophobic mismatch. In summary, cholesterol extraction from membranes significantly decreases Na\textsuperscript{+},K\textsuperscript{+}-ATPase steady-state activity.
Inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase by cholesterol depletion

enzyme’s activity on membrane cholesterol composition is still not clear. For example, Lucio et al. (8) reported that cholesterol depletion from red blood cells had a biphasic effect, with a reduction in the cholesterol level by 5-25% causing Na\textsuperscript{+},K\textsuperscript{+}-ATPase activation, but with a reduction in the cholesterol level by 35-50% causing inhibition, thus suggesting an optimum membrane cholesterol content for Na\textsuperscript{+},K\textsuperscript{+}-ATPase function lower than the physiological level. Clarett et al. (5) reported, also in red blood cells, that cholesterol depletion can cause either an increase or a decrease in Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity depending on the cytoplasmic Na\textsuperscript{+} concentration. In contrast, in vesicles extracted from kidney tissue Yeagle et al. (7) found that cholesterol depletion of the membrane only caused a decrease in Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity. The identification of cholesterol’s intrinsic effect on Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in its native membrane environment and the elucidation of its mode of action are far from trivial. Much valuable information has been gained from studies of Na\textsuperscript{+},K\textsuperscript{+}-ATPase reconstituted into synthetic vesicles (11, 12, 15, 16, 19). The control of the enzyme surroundings such systems allow has enabled lipid-protein interactions which may be important in native cell membranes to be identified. Habeck et al. (17) carried out an analysis of membranes via mass spectrometry to postulate which effects may be operating under physiological conditions. However, the results of studies on reconstituted or detergent-solubilized protein require confirmation from measurements in real cell membranes. It is known from studies on model membrane systems that cholesterol’s effects vary with phospholipid composition. Results obtained by a variety of techniques indicate that cholesterol interacts more strongly with saturated than unsaturated hydrocarbon chains (2, 20-23). Thus, the magnitude of cholesterol’s effect on membrane thickness and chain order depend on phospholipid composition. The same applies to cholesterol’s effect on membrane dipole potential (23), suggested to modulate the kinetics of ion occlusion reactions of ion pumps (24). Even detergent molecules used to solubilize membrane proteins produce dipole potentials (25, 26) and could influence pump kinetics. To show that cholesterol has physiologically relevant effects on the Na\textsuperscript{+},K\textsuperscript{+}-ATPase, experiments must be done on protein embedded in a membrane with a phospholipid composition closer to that of its native membrane environment.

Measurements have been performed on the Na\textsuperscript{+},K\textsuperscript{+}-ATPase in cells before and after partial extraction of cholesterol (5, 8, 14). However, as pointed out by Lucio et al. (8), a difficulty associated with cell studies is maintenance of intracellular Na\textsuperscript{+} concentration. Membrane cholesterol depletion is expected to increase membrane passive permeability to Na\textsuperscript{+} (27). This allows Na\textsuperscript{+} to flow into the cell, increasing the cytoplasmic Na\textsuperscript{+} concentration. Because under physiological conditions cytoplasmic Na\textsuperscript{+}-stimulated phosphorylation by ATP is a major rate-determining step of the enzymatic cycle (28), via its effect on cytoplasmic Na\textsuperscript{+} level, cholesterol depletion should lead to Na\textsuperscript{+},K\textsuperscript{+}-ATPase stimulation. But this is an indirect effect via a substrate level, not an intrinsic effect of cholesterol acting on the Na\textsuperscript{+},K\textsuperscript{+}-ATPase from within the membrane. The same problem applies to any closed vesicular system, whether the Na\textsuperscript{+},K\textsuperscript{+}-ATPase is reconstituted into synthetic vesicles or present in vesicles of the native lipid composition. It is difficult in any closed vesicular or cellular system to separate out the intramembrane effects of cholesterol on the Na\textsuperscript{+},K\textsuperscript{+}-ATPase from its effects via the intracellular or intravesicular Na\textsuperscript{+} level.

To avoid any effects from variation in Na\textsuperscript{+} membrane permeability, here we utilize open membrane fragments containing Na\textsuperscript{+},K\textsuperscript{+}-ATPase. The experimental procedure for their purification was developed by Jorgensen (29). A crucial step in the procedure is treatment with sodium dodecyl sulfate (SDS), which removes surface-bound proteins and disrupts closed microsomal preparations of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase, leading to a final preparation 90-100% pure in Na\textsuperscript{+},K\textsuperscript{+}-ATPase with respect to protein and open on both sides, providing free access for both cytoplasmic and extracellular substrates (30, 31). Since the development of the open membrane Na\textsuperscript{+},K\textsuperscript{+}-ATPase system, it has been widely used, particularly to determine the kinetics of the enzyme’s partial reactions (32-36) and as an initial stage in protein crystallization (37-39). To identify the effect of cholesterol content on Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in this system, cholesterol must be preferentially extracted from the membrane. The method by which this is achieved is an important consideration. Marin et al. (6) used cholesterol oxidase, which converts cholesterol to cholest-4-en-3-one. However, cholest-4-en-3-one is unlikely to be inert with respect to interaction with the Na\textsuperscript{+},K\textsuperscript{+}-ATPase. Experiments using phospholipid vesicles show it causes a greater increase in dipole potential than cholesterol (23). Other researchers have used phospholipid vesicles as either cholesterol acceptors or carriers to perturb the binding equilibrium of
cholesterol to Na\textsuperscript{+},K\textsuperscript{+}-ATPase-containing membranes and to either decrease or increase the cholesterol content (5, 7, 10). More recently, the use of methyl-\(\beta\)-cyclodextrin (mβCD), which forms an inclusion complex with cholesterol, has gained in popularity as a tool for manipulation of membrane cholesterol content (14, 23, 40, 41). The advantage of mβCD over the use of phospholipid vesicles is that the mβCD binding cavity is of an appropriate size to bind cholesterol preferentially over phospholipids (40). Therefore, it can be used to manipulate membrane cholesterol content while minimizing perturbation of the membrane’s phospholipid composition.

Here we describe experiments in which we depleted open Na\textsuperscript{+},K\textsuperscript{+}-ATPase-containing membrane fragments of cholesterol by incubation with mβCD. Apart from avoiding any effects from changes in Na\textsuperscript{+} membrane permeability, this system has the crucial advantage that the phospholipid composition of the membrane fragments is close to that of the native membrane. Analysis of the lipid composition before and after treatment of the initial microsomal preparation with SDS (42) has shown no significant change in the percentage of phospholipid content comprising sphingomyelin, phosphatidylinositol or phosphatidyl-ethanolamine. The phosphatidylerserine (PS) content was found to increase from 7.9% to 13.1% at the expense of phosphatidylcholine (PC), which decreased from 43.6% to 35.6% after SDS treatment. These changes could be explained by a stronger interaction between the Na\textsuperscript{+},K\textsuperscript{+}-ATPase and the negatively charged PS molecule relative to the zwitterionic PC molecule (15, 42).

Considering the close similarity between the lipid composition of the membrane fragments and the native membrane, changes in Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity seen on cholesterol depletion from the membrane fragments can be expected to reflect changes that would most likely be observed in the native environment.

To complement the experimental studies, we have carried out molecular dynamics (MD) simulations of Na\textsuperscript{+},K\textsuperscript{+}-ATPase embedded in membranes containing 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 40 mol% cholesterol. Most MD simulations on the Na\textsuperscript{+},K\textsuperscript{+}-ATPase have concentrated on understanding the basis for Na\textsuperscript{+} or K\textsuperscript{+} selectivity of the protein (44-46). Here we focus on interaction of the protein with its surrounding membrane. Recently (47), simulations were performed in a pure DOPC membrane, i.e., in the absence of cholesterol. Comparison of the present simulations with those of Garcia et al. (47) has allowed us to identify changes in lipid packing and interaction with the Na\textsuperscript{+},K\textsuperscript{+}-ATPase induced by cholesterol, and how these changes depend on the protein conformational state. The MD results also allow comparison with Na\textsuperscript{+},K\textsuperscript{+}-ATPase crystal structures in which cholesterol was found to co-crystallize (38, 48) and prediction of other binding sites that may contribute to cholesterol-dependent Na\textsuperscript{+},K\textsuperscript{+}-ATPase function.

### Results

#### Steady-state activity

Removal of cholesterol through incubation of Na\textsuperscript{+},K\textsuperscript{+}-ATPase-containing membrane fragments with mβCD significantly reduces the enzyme’s steady-state activity (Fig. 1). The cholesterol content was measured relative to the protein concentration and was normalized to the cholesterol:protein weight ratio prior to cholesterol extraction, which was measured to be 1.1 (± 0.1) µg cholesterol/µg protein. Based on a molecular weight of an αβ unit of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase of 147,000 g mol\(^{-1}\) and the molecular weight of cholesterol of 387 g mol\(^{-1}\), this corresponds to a molar ratio of approximately 400 cholesterol molecules per protein unit. According to recent X-ray crystallographic data (16), only 13 of these molecules may be directly in contact with the protein as annular lipids, and just 3 are specifically bound within the protein. Thus, the majority of the cholesterol molecules in the membrane preparation are present in the bulk membrane phase, i.e., not in direct contact with the protein. The relationship between normalized cholesterol content (Chol/Prot) and the normalized ATPase activity was fitted arbitrarily to an exponential decay function, i.e., an exponential decay in the difference between the normalized activity of untreated membranes and membranes with zero cholesterol with increasing Chol/Prot values. This function was found to provide a better fit to the experimental data than a hyperbolic curve. The half-maximal Chol/Prot value was determined to be 0.15 (± 0.10). The cholesterol content of membrane fragments of enzyme prepared from pig kidney has been reported to be approximately 40 mol% of total membrane lipids (13). Thus, half-maximal activity of Na\textsuperscript{+},K\textsuperscript{+}-ATPase is predicted from the data shown in Fig. 1 to occur at a membrane cholesterol content of ~6 mol%.

When the enzyme is reconstituted into cholesterol-free phospholipid liposomes, an activity of ~5% of its maximal activity has been reported (11). Because it is very difficult or impossible to deplete membrane fragments totally of cholesterol, this value of 5% was used...
in the analysis of our experimental data as the y-intercept.

To test for any perturbation of the phospholipid content of the membranes (41), we measured the total phosphate content of our samples. After treatment of membrane fragments with 60 mM mβCD for 2 hrs at 37°C, the total phosphate content of the sample was still at 91% (± 8%, N = 3) relative to untreated membrane fragments. This agrees well with total phosphate measurements reported elsewhere using similar mβCD concentrations (49, 50), where it was found that treatment of plasma membranes with 40 mM mβCD reduced the cholesterol content to 12 – 26% of the native level with negligible change in phospholipid content.

**Reintroduction of cholesterol**

As just described, we investigated the effect of mβCD on the phospholipid population of Na⁺,K⁺-ATPase membrane fragments and observed <10% reduction in total phosphate. However, it has been postulated that mβCD binds within membrane interfaces and/or protein hydrophobic domains (51, 52). This could impact on ATPase activity. However, significant changes in cholesterol content of the membrane begin to occur before changes in ATPase activity are observed, suggesting that mβCD has a significantly higher affinity for cholesterol than for protein hydrophobic domains. If at higher mβCD concentrations the changes in ATPase activity were dominated by mβCD binding to the membrane interface or hydrophobic protein domains, then reintroduction of cholesterol via a cholesterol:mβCD inclusion complex should yield little recovery in activity. This was not the case.

We treated cholesterol-depleted membrane fragments with a range of concentrations of cholesterol:mβCD complex and observed recovery in activity in all cases (Fig. 2). This suggests that interactions of mβCD with non-cholesterol targets are not the dominant cause for the change in activity, and that it is in fact due to the change in cholesterol composition.

The Na⁺,K⁺-ATPase activity of membranes pretreated with 30 mM mβCD could be completely recovered after incubation with 25 μg of chol:mβCD complex per μg protein. Treatment with higher concentrations of chol:mβCD complex resulted in an activity drop below the maximum level. In comparison, the activity of membranes pretreated with either 45 or 60 mM mβCD could not be completely recovered, although recoveries to ~75% and ~50% of the initial untreated control activity were achieved. Regardless of the mβCD concentration used for cholesterol extraction, an activity increase was observed on treatment with low concentrations of chol:mβCD complex followed by a drop in activity at higher complex concentrations. Thus, reintroduction of too much cholesterol inhibits the Na⁺,K⁺-ATPase, and there appears to be an optimal cholesterol level within the membrane required to maximize Na⁺,K⁺-ATPase activity. This is in agreement with other observations (7, 53). Yeagle (53) suggested that inhibition at high cholesterol levels could be due to a specific interaction between cholesterol and the protein. In a later paper from the same group (7) it was suggested that it could be due to a cholesterol-induced increase in motional order of lipid hydrocarbon chains in the membrane which might decrease the conformational flexibility of the enzyme. However, regardless of the mechanism, whereas there is clear evidence that membrane cholesterol levels below the normal physiological level are medically relevant (e.g. in desmosterolosis and Smith-Lemli-Opitz syndrome), whether cholesterol levels above the normal physiological level ever occur in animals is unclear. It could be the case that in animal cells regulatory cholesterol homeostatic mechanisms maintain the cholesterol membrane composition at a constant level, so that excess cholesterol (e.g. from the diet) accumulates rather in the bloodstream, leading to atherosclerosis and eventually heart disease.

**Partial reaction kinetics**

The Na⁺,K⁺-ATPase reaction cycle consists of a series of partial reactions summarized by the Albers-Post cycle. Here we divided the cycle into three steps: ENa⁺³ → E2P (phosphorylation by ATP and occlusion of Na⁺ from the cytoplasm), E2P → E2K⁺² (dephosphorylation and occlusion of K⁺ from the extracellular fluid) and E2K⁺² → E1Na⁺³ (K⁺ release to and Na⁺ uptake from the cytoplasm). To isolate reactions steps influenced by cholesterol removal, we carried out stopped-flow measurements using the fluorescent probe RH421 (32, 34, 36, 54-57), which responds to local electric field strength changes associated with protein conformational changes. Experimental conditions used for isolating individual partial reactions are described under Materials and Methods.

The effects of removal of ~90% of the cholesterol content of Na⁺,K⁺-ATPase membrane fragments on the kinetics of the ENa⁺³ → E2P and E2K⁺² → E1Na⁺³ transitions are shown in Fig. 3. The observed rate constant, kobs, for the ENa⁺³ → E2P transition decreased from 138 (± 2) s⁻¹ prior to cholesterol extraction to 57 (± 10) s⁻¹ afterwards. In the case of the E2P → E2K⁺² transition the values of kobs were 248
Thus, the observed eosin fluorescence excitation spectrum is a sensitive indicator of changes in Na\textsuperscript{+},K\textsuperscript{+}-ATPase conformation.

The stopped-flow kinetic experiments on the ENa\textsubscript{3} \to E2P and E2K\textsubscript{2} \to E1Na\textsubscript{3} were carried out by initially equilibrating the enzyme in buffers which, based on experiments with native untreated enzyme, normally initially stabilize the E1Na\textsubscript{3} and E2K\textsubscript{2} states (see Experimental Procedures). However, there is no reason to believe that after cholesterol extraction the enzyme remains in the E1Na\textsubscript{3} or E2K\textsubscript{2} state, respectively, in either of these buffers. Analysis of the enzyme with eosin before and after cholesterol treatment allowed us to determine if cholesterol extraction caused any shift in the enzyme’s conformational equilibrium. The eosin excitation spectra obtained are shown in Fig. 4. In the upper panel of Fig. 4 it can be seen that if the enzyme is pre-equilibrated in a buffer containing 10 mM KCl, which is expected to stabilize the E2K\textsubscript{2} state, no significant change in the spectrum after cholesterol extraction is observed, indicating that the enzyme stays in the E2K\textsubscript{2} state. However, if the enzyme is pre-equilibrated in a buffer containing 130 mM NaCl, which is normally expected to stabilize the E1Na\textsubscript{3} state, a clear blue shift of the eosin spectrum and a significant drop in fluorescence intensity after cholesterol extraction are observed (see Fig. 4, lower panel). The spectrum observed after cholesterol extraction is characteristic of an E2 state, rather than an E1 state (58-60). This result indicates that cholesterol extraction is causing some of the enzyme to shift from the E1 back into the E2 state, i.e. cholesterol extraction favours the E2 state over E1. As explained in detail in the Discussion, the shift towards the E2 state after cholesterol extraction is consistent with the slowing of the ENa\textsubscript{3} \to E2P and E2K\textsubscript{2} \to E1Na\textsubscript{3} transitions observed in the stopped-flow experiments.

**MD simulations of membrane thickness and cholesterol sites**

Analysis of membrane perturbations around the Na\textsuperscript{+},K\textsuperscript{+}-ATPase in states E1P·ADP·3Na\textsuperscript{+} and E2·P\textsubscript{i}·2K\textsuperscript{+} is shown in Fig. 5. Cholesterol causes increases in lipid bilayer thickness away from the protein from 29.4 ± 0.2 Å (average values based on simulations of both states). This is due to an increase in order of chains packed around cholesterol. Cholesterol increased order parameters (Fig. S4) for lipids by as much as 0.1, consistent with studies of pure bilayers with 40 mol% cholesterol (61, 62). The close packing
causes a drop in area per lipid from 59.8 ± 6.4 Å² without cholesterol to 45.1 ± 0.7 Å² (Fig. S5), in agreement with previous membrane studies (61). Another consequence of the increased membrane thickness is the greater negative deflection adjacent to the protein relative to the distant bilayer, as seen in Fig. 5b-c (previously 2 – 3 Å without cholesterol (47), now as much as 6 Å), as the membrane responds to increased hydrophobic mismatch. Fig.5b reveals significant negative deflection in the annulus region of the protein, with the exception of one site of interest, with positive deviation near helices βM and M3 for both states, particularly in E1P·ADP·3Na⁺. This region (site M in Fig. 6b; to be discussed below) has high cholesterol density and strong phospholipid packing.

Overall, the radial profile for axially-averaged deflections in Fig. 5c shows a defined maximum negative deflection at ~20 Å from the protein, after a small increase close to the center, arising from effects of the positive deflection site near helices βM, M3 and M7. The deflection is large in both states, -4.7 ± 2.2 and -6.0 ± 1.2 Å in E1P·ADP·3Na⁺ and E2·Pi·2K⁺, with the difference between the states being maintained, but reduced compared to previous results in membranes without cholesterol (-0.7 ± 1.0 and -2.7 ± 1.3 Å for E1P·ADP·3Na⁺ and E2·Pi·2K⁺; (47)). Effects of cholesterol on local bilayer shape have the potential to modulate pump function. The 2-3 Å thinning of the cholesterol-free lipid bilayer around the E2 state, relative to the E1 state (Fig.5c of (47)) would be associated with increased strain energy that should disfavor the E2 state. Overall this relative thinning is maintained (although reduced to ~1 Å, owing to specific interactions with annular cholesterol molecules; see below).

As a result of the stiffening of the bilayer due to cholesterol, it would be expected that the presence of high cholesterol content would increase the energetic cost of incorporating the more highly mismatched E2 state (or alternatively that cholesterol extraction would favor the E2 state), consistent with our experimental findings.

The cholesterol density around the protein is shown in Fig. 6 for both states and membrane leaflets. Regions corresponding to density peaks have been classified with mean packing scores (Table S1), with analysis of key protein residues (Table S2 and Fig. S3). High scoring residues are mostly nonpolar, suggesting hydrophobic interactions and geometric factors cause cholesterol-protein interaction. Some sites have high cholesterol packing in both E1P·ADP·3Na⁺ and E2·Pi·2K⁺ states; in others packing is reduced or eliminated due to protein conformation changes.

Site A in Fig. 6 represents binding in a crevice made by 3 helices, αM10, αM7 and βM. The structure 3WGU (38) for this site shows interaction with the same helices, and includes interactions identified in simulations, except for interaction with G52 on helix βM. Site B involves interactions with helices γM, αM9, αM10 and connecting loop L9/10. But the interacting residues differ markedly between protein states, with high scoring residues from αM9 in the E2·Pi·2K⁺ state absent in E1P·ADP·3Na⁺. This shows the hydrophobic interactions responsible for high cholesterol density are dynamic in nature. Sites A and B correspond to cholesterols seen in PDB 3WGU (E1P·ADP·3Na⁺) (38), but seen here in both E1P·ADP·3Na⁺ and E2·Pi·2K⁺ states. Interacting residues differ markedly between these states, with high scoring residues from αM9 absent in the E1, showing dynamic hydrophobic interactions.

Site C corresponds to a crevice lined by αM2 and αM6 helices, and the L9/10 loop. The site marked D in Fig. 5a is found in both E1·ADP·3Na⁺ and E2·Pi·2K⁺, but only with a high score (0.34) in E2·Pi·2K⁺. Cholesterol is located in a gap between the αM1, αM3 and αM4 helices. Site E is located between helices αM3 and αM5. However, the interacting residues differ between protein states, revealing a dynamic site, and are mostly nonpolar, with the exception of an interfacial serine (S303), which could interact with the cholesterol OH group via H-bonding. Site F is only found with high packing in E1·ADP·3Na⁺, with cholesterol in contact with helices βM and αM7, a few residues from L7/8 and a single residue from αM5. We postulate that the absence of high cholesterol density in E2·Pi·2K⁺ for site F may be due to conformational changes involving βM movement. Site G is located between helices βM and αM10, forming a hydrophobic pocket, with some interactions with the interfacial K34 of the β-subunit. Site H has high score (0.44) in E1·ADP·3Na⁺ only, and is formed by hydrophobic interactions with αM8 and αM9, the connecting loop, and a residue from αM10. There is evidence for a low scoring site below H in E2·Pi·2K⁺ (Fig. 6), but this is further from the protein. Site I is formed by residues from γM, L8/9 and αM9. Most residues lining this site are hydrophobic, with an interfacial K945 of the α-subunit perhaps forming an H-bond with the cholesterol hydroxyl O. Site J has strong cholesterol packing in both E1·ADP·3Na⁺ and E2·Pi·2K⁺. Interestingly, in E2·Pi·2K⁺ two cholesterols interact, each with high scores. Site J is in a wide pocket between γM and αM2.
helices, with some interactions with αM6 and αM9 helices in E1·ADP·3Na+, and with αM4, αM6 and αM9 helices and the L5/6 loop in E2·P·2K+. This site is very dynamic, with changing pose and lists of interacting residues, which we attribute to helical movements, most notably γM, which moves towards αM2, effectively reducing the binding pocket size in E1·ADP·3Na+ (likely why only one cholesterol molecule binds in that state). Site K exists in both E1·ADP·3Na+ and E2·P·2K+, but with significant packing score (0.27) only in E2·P·2K+. This site involves hydrophobic interactions with the αM1 helix alone. Site L is also only high scoring (0.28) in E2·P·2K+, with cholesterol located in a hydrophobic pocket formed by αM1, αM3 and αM4 helices.

Site M is a crystallographically identified cholesterol site, and has high packing scores in E2·P·2K+. While no high scores are found in E1·ADP·3Na+ at this location, similar binding is seen in a region further from the protein (Fig. 6), with a lower packing score. This binding pocket resides between helices βM and αM3, and involves interactions that change greatly and includes residues from αM3, αM4, αM5, αM7 and βM helices. All interactions are hydrophobic, with the exception of the interfacial E840 and N324 of the α-subunit, which interact with the cholesterol OH. Helix βM changes position significantly between E1·ADP·3Na+ and E2·P·2K+, with βM and αM3 helices moving away from αM4, αM5 and αM7, altering the cholesterol pocket in E2·P·2K+. Interestingly, a crystallographic cholesterol is observed in site M in the 3WGU structure (E1·ADP·3Na+) (38), though our data suggest more defined and stronger interaction should occur in E2·P·2K+. The crystallographic cholesterol suggests interactions with helices βM, αM5 and αM7, while no interactions with either αM3 or αM4 are evident. Interactions in the crystal structure also involve R27 on βM, suggesting a cholesterol site close to the membrane interface, while the position of cholesterol in our identified site varies. The interacting residues from αM5 are not all the same in the crystal structure and simulations, but both consist of hydrophobic residues. Site M is in a region where the difference in bilayer thickness deviation is most pronounced between states, with a large 7 Å positive deflection. Given this is the region where the N-terminus is likely to be located (with its conserved LKKE motif in the vicinity of sites K, L and M), it may help explain state-dependent interactions. The dramatic increase in membrane thickness near this site is associated with increased lipid packing (Fig. S5), that may also involve increased charge density driving state-dependent interactions with the N-terminus.

Fig.6c shows a comparison of radial free energy profiles, obtained from axially-symmetrized 2D radial distribution functions for cholesterol around the protein center of mass in each bilayer leaflet. Differences in the energy of interaction of cholesterol between the E1·ADP·3Na+ and E2·P·2K+ states are at most 0.1-0.2 kcal/mol, revealing a small state-dependent binding affinity for a cholesterol molecule to the protein, albeit with statistically significant changes visible in the lipid annulus around the protein that favor either the E1·ADP·3Na+ or E2·P·2K+ state depending on the membrane side and the distance from the center of the protein. Because several cholesterol molecules bind to the protein, the total energy difference between the E1·ADP·3Na+ and E2·P·2K+ states would be amplified. However, based on the data shown in Fig. 6c there is no clear systematic preference for stabilisation of E1·ADP·3Na+ over E2·P·2K+ or vice versa due to protein interaction with cholesterol.

Via integration of the data shown in Fig. 6c across both sides of the membrane to distances from the center of the protein which include the specifically bound cholesterol molecules. This may suggest that cholesterol’s effect on the protein’s thermodynamics could instead be primarily via its modulation of local bilayer shape, rather than relative stabilization through preferential binding.

Discussion

The effect of cholesterol on steady-state and partial reaction kinetics of Na+,K+-ATPase were investigated here using membrane fragments with a lipid composition close to that of the native environment. The results obtained confirm suggestions from previous studies using Na+,K+-ATPase reconstituted into lipid vesicles (9, 11, 12, 15, 16), in living cells (5, 8, 14), or after detergent-solubilization (4, 17) that cholesterol is crucial to enzyme activity. Cholesterol extraction from the membrane...
Inhibition of Na\(^+\),K\(^+\)-ATPase by cholesterol depletion

fragments causes a significant drop in steady-state activity.

Cholesterol removal from cell membranes or membrane fragments using cyclodextrin has been reported to have significant secondary effects on phospholipid content and non-specific binding to hydrophobic protein segments (41, 51, 52). That reintroduction of cholesterol through a cholesterol:m\(\beta\)CD inclusion complex was able to recover a significant amount of ATPase activity in cholesterol-depleted membrane fragments (Fig. 2) strongly suggests that the effects reported here are predominantly due to cholesterol depletion. The reason that incubation with the inclusion complex is not able to recover complete activity when the initial m\(\beta\)CD concentration used for extraction is \(\geq 45\) mM is not clear at this stage, but could be due to the removal of a population of cholesterol molecules bound strongly to the protein whose loss may lead to protein folding changes that are difficult to completely reverse. The presence of cholesterol molecules associated directly with the protein has been shown by X-ray crystallography (38, 48) and supported by the MD simulations presented here. The results shown in Fig. 1 indicate that Na\(^+\),K\(^+\)-ATPase activity only drops sharply after the cholesterol content of the membrane is reduced to below 30% of its initial level. This seems to indicate that removal of cholesterol molecules which are less strongly bound to the membrane have little effect on Na\(^+\),K\(^+\)-ATPase activity.

To isolate the cause of the drop in Na\(^+\),K\(^+\)-ATPase activity on cholesterol removal, we performed measurements of the enzyme’s partial reaction kinetics via the RH421 stopped-flow technique. The results indicate that cholesterol is necessary for efficient function of reactions involved with intracellular deocclusion and release of K\(^+\) and the binding and occlusion of Na\(^+\). In contrast, cholesterol appears to play a limited role in extracellular binding and occlusion of K\(^+\) ions. The results presented from the stopped-flow data suggest an approximate 50–70% reduction in the rate constants of the ENa\(_{3}\) → E2P and E2K\(_{2}\) → E1Na\(_{3}\) reactions on removal of \(\sim90\) % of cholesterol in the membrane. These results are similar to observations of Cornelius et al. (12), who studied the effect of cholesterol composition on Na\(^+\),K\(^+\)-ATPase reconstituted into di-18:1-PC vesicles. They found that the rate constants of the ENa\(_{3}\) → E2P and E2K\(_{2}\) → E1Na\(_{3}\) reactions both decreased to \(\sim50\) % of their optimum level on decreasing cholesterol content from 40 to 0 mol%.

Fluorescence measurements using the environmentally-sensitive probe eosin indicate that cholesterol extraction causes a stabilisation of the unphosphorylated E2 state relative to unphosphorylated E1. This is consistent with the slowing of the E2K\(_{2}\) → E1Na\(_{3}\) transition observed via stopped-flow. It is also consistent with the observed slowing of the ENa\(_{3}\) → E2P transition. The kinetics of this reaction depends critically on the enzyme’s initial conformation. If the enzyme is initially in the E1 state, then the sequence of reactions followed is E1 → E1P → E2P and at 24°C the observed rate constant is approximately 150 s\(^{-1}\) (63, 64), consistent with the results obtained here on untreated enzyme at the same temperature. However, if the enzyme is initially in the E2 state, the enzyme must first undergo a transition to the E1 state before phosphorylation by ATP can occur. Under these circumstances the sequence of reactions would be E2 → E1 → E1P → E2P. In the presence of ATP, previous experiments have shown that the E2 → E1 transition occurs with a rate constant of approximately 65 s\(^{-1}\) for pig kidney Na\(^+\),K\(^+\)-ATPase (63). Thus, it is the rate-limiting reaction in the reaction sequence. The drop in the \(k_{\text{obs}}\) for ATP phosphorylation from 138 (±2) to 57 (±10) s\(^{-1}\) after cholesterol extraction is thus consistent with phosphorylation starting in E2.

The decreases in the change in RH421 fluorescence after cholesterol extraction (see Fig. 3) are also in agreement with a shift towards E2. The fluorescence intensity of RH421 associated with Na\(^+\),K\(^+\)-ATPase-containing membrane fragments is known to increase in the order E1 < E2 < E2P (63, 64). Thus, if cholesterol extraction favours the E2 state, then this should lead to a lower degree of conversion into the E1 state on mixing with NaCl and a smaller fluorescence drop, as experimentally observed (see Fig 3CD). Furthermore, because of the sequence of fluorescence amplitudes just given, ATP phosphorylation should also give a smaller fluorescence increase for enzyme starting in E2 after cholesterol extraction compared to enzyme starting in E1, also as experimentally observed (see Fig. 3 AB).

Our MD simulations have revealed state-dependent cholesterol binding and its effects on local bilayer shape. While overall binding of cholesterol to the protein was similar between the states, we suggest that its primary influence could be on the mechanical energies to incorporate the different states. High cholesterol content is expected to raise the strain energies associated with increased mismatch for incorporation of the E2 state. This would suggest cholesterol extraction would favour the E2 state. However, the origin of the stabilization of the E2 state relative to E1 caused by cholesterol removal requires further
Inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase by cholesterol depletion

Investigation. Another possibility is that this stabilization could be associated with the protein’s lysine-rich N-terminus. A large body of biochemical data obtained on both the Na\textsuperscript{+},K\textsuperscript{+}-ATPase and the related H\textsuperscript{+},K\textsuperscript{+}-ATPase in their near-native membrane environments indicates that the N-terminus is intimately involved in enzyme activity (65-71). In contrast, experimental data on the Na\textsuperscript{+},K\textsuperscript{+}-ATPase and H\textsuperscript{+},K\textsuperscript{+}-ATPase after exogenous expression in cell lines do not support a role of the N-terminus in determination of ion pumping activity (72, 73). A possible explanation for these apparently conflicting findings is that the effect of the N-terminus on ATPase activity is mediated by the membrane surroundings and is thus only observed when the protein is present in either its native membrane environment or one which closely approximates it. If this is true, then changing the lipid environment by cholesterol extraction could lead to a modulation of the effect the N-terminus has on pump activity. We are currently carrying out further studies to explore this possibility.

In conclusion, the results obtained here confirm the importance of cholesterol for efficient function of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase in its native membrane environment. The effect of cholesterol depletion on Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity within the near-native membrane environment studied here is profound. Partial reactions which depend on the distribution of the enzyme between the unphosphorylated E1 and E2 states are sensitive to cholesterol depletion. Thus, the rate limiting partial reactions E2K\textsuperscript{+2} → E1Na\textsuperscript{+3} and subsequent phosphorylation by ATP still determine overall activity in cholesterol-depleted membrane preparations.

Experimental procedures

**Enzyme**

Na\textsuperscript{+},K\textsuperscript{+}-ATPase-containing membrane fragments from pig kidney outer medulla were purified as described by Klodos et al. (74). Detailed lipid analyses of membrane fragments from mammalian kidney prepared via the same procedure are reported elsewhere (42, 75). The fragments have radii of 100 – 300 nm, as determined by cryoelectronmicroscopy (see Fig. S1). The protein concentration of the preparations used was 6.1 and 4.0 mg mL\textsuperscript{-1}, determined by the Peterson (76) modification of the Lowry (77) method using bovine serum albumin as a standard.

**Materials**

N-(4-Sulfobutyl)-4-(4-(diphenylamino)phenyl)butadienyl)-pyridinium inner salt (RH421) was obtained from Molecular Probes (Eugene, OR) and was used without further purification. RH421 was added to Na\textsuperscript{+},K\textsuperscript{+}-ATPase-containing membrane fragments from an ethanolic stock solution. RH421 spontaneously partitions into the membrane fragments.

The origins of the other reagents used were: imidazole (≥99%, Sigma, Castle Hill, Australia), mβCD (cell-culture-tested, 1.5-2.1 methyl per mol glucose, Sigma), cholesterol (≥99%, Sigma), eosin Y (C.I. 45380, BDH, Kilsyth, Australia), NaCl (suprapure, Merck, Kilsyth, Australia), KCl (analytical grade, Merck), L-histidine (99%, Sigma), MgCl\textsubscript{2}·6H\textsubscript{2}O (analytical grade, Merck), EDTA (99%, Sigma), ATP disodium-3H\textsubscript{2}O (special quality, Roche, Castle Hill, Australia), NADH disodium salt (approx. 100%, grade 1, Roche), phospho(enol)pyruvic acid cyclohexylammonium salt (≥97%, Sigma), pyruvate kinase (PK)/lactate dehydrogenase (LDH) from rabbit muscle (900-1400 units/ml LDH, 600-1,000 units/ml PK, Sigma), NaOH (analytical grade, Merck) and HCl (0.1 N Titrisol solution, Merck).

**Cholesterol extraction**

Cholesterol was extracted by incubating 200 μg mL\textsuperscript{-1} of Na\textsuperscript{+},K\textsuperscript{+}-ATPase-containing membrane fragments with mβCD (10, 20, 30, 45 or 60 mM) for 2 hrs at 37°C in an E1Na\textsuperscript{+3} buffer (30 mM imidazole, 130 mM NaCl, 5 mM MgCl\textsubscript{2}, 1 mM EDTA, pH 7.2), an E2K\textsuperscript{+2} buffer (30 mM imidazole, 10 mM KCl, 1 mM EDTA, pH 7.4) or, in the case of experiments for which eosin fluorescence measurements were subsequently carried out, in a general buffer (30 mM imidazole, 1 mM EDTA, pH 7.4). Previously published data from a number of laboratories (41) indicate that mβCD concentrations in the tens of millimolar range are necessary to extract cholesterol from both raft and non-raft domains of plasma membranes. Because the membrane fragments which we used in our experiments are derived from the plasma membrane and we wished to extract cholesterol from the entire membrane, the mβCD concentration range utilised here was also in the tens of millimolar range. To perform subsequent cholesterol assays and fluorescence experiments the enzyme was removed from mβCD by ultracentrifugation (75,000 g for 1 hr at 24°C; details in the following section). The supernatant was then removed and the pellet gently resuspended in the required buffer. The removal of mβCD was required before any fluorescence measurements with RH421 or eosin because both probes are able to form an inclusion complex with mβCD which produces fluorescence changes unrelated to their
interaction with the Na⁺,K⁺-ATPase. After centrifugation the protein concentration in the resuspended pellet was determined using the Peterson (76) modification of the Lowry (77) method.

**Reintroduction of cholesterol**

The cholesterol:mβCD complex was made as previously described (78). Briefly, a solution of cholesterol in methanol-chloroform (2:1 v/v) was added in small aliquots to a stirred solution of mβCD (5% w/v) on a water bath (80 °C). The mixtures were stirred at 80 °C until complete dissolution of the initially precipitating steroid. Quantities of substrates were 30 mg of cholesterol to 1 g of mβCD. The solutions were freeze-dried and stored at -20 °C.

Reintroduction of cholesterol to the membrane fragments was performed after ultracentrifugation, described above. A stock concentration of the cholesterol:mβCD inclusion complex was dissolved in E1Na⁺₃ buffer. Concentrations varied from 5 µg of inclusion complex per µg protein to 400 µg of inclusion complex per µg protein. Membrane fragments with the inclusion complex and the control sample were incubated for 2 hrs at 37 °C prior to measurement of ATPase activity.

**Cholesterol assay**

Cholesterol levels of Na⁺,K⁺-ATPase-containing membrane fragments were determined before and after treatment with mβCD via the Amplex Red cholesterol assay kit (Molecular Probes, Eugene, OR). Membrane fragments treated with mβCD were first separated from mβCD via ultracentrifugation (75,000 g for 1 hr at 24ºC) using an Optima XE-100 ultracentrifuge and SW 32 Ti Swinging-Bucket Rotor (Beckman Coulter, Lane Cove, Australia) to prevent contamination from cholesterol present in the aqueous solution, e.g. complexed with mβCD. Cholesterol standards (0.25 to 16 µg/ml) and membrane fragments (75 µl) were pipetted into microfuge tubes and incubated with reagent (300 µM Amplex Red, 2 units/ml horse radish peroxidase, 2 units/ml cholesterol oxidase, 0.2 units/ml cholesterol esterase) at 37°C for 30 min in the dark. Fluorescence intensity measurements were then performed in duplicate with an RF-5301 PC spectrofluorophotometer (Shimadzu, Kyoto, Japan) using 1 cm pathlength quartz microcuvettes. Temperature was maintained at 37°C via a circulating water bath. The values of λeo and λem were 545 nm and 590 nm (bandwidth 5 nm) with an OG570 filter (Schott, Mainz, Germany) in front of the photomultiplier.

**Steady-state activity measurements**

The specific ATPase activity of Na⁺,K⁺-ATPase was determined by the PK/LDH coupled assay at 37°C (79) in buffer containing 25 mM imidazole, 100 mM NaCl, 10 mM KCl, 5 mM MgCl₂, 3 mM Na₂ATP, 2 mM phospho(enol)pyruvate and an approximate final concentration of 5 U/ml of PK and 7 U/ml of LDH. Buffer pH was adjusted to 7.2 with HCl. At the beginning of each experiment, i.e. prior to initiating the reaction by adding Na⁺,K⁺-ATPase-containing membrane fragments, 80 µM of NADH was added directly to the solution in a 1 cm pathlength cuvette. The time course of the drop in NADH absorbance caused by ATPase activity was monitored at 340 nm using a UV-2450 UV-visible spectrophotometer (Shimadzu, Kyoto, Japan). At least 99% of the activity could be blocked by addition of 1 µM ouabain, a specific Na⁺,K⁺-ATPase inhibitor.

**Estimation of phospholipids**

Lipid phosphate was assayed after total digestion by nitric acid-calcium carbonate solution with Na₂HPO₄ as a standard using the method of Baginski et al. (80). Samples treated without nitric acid-calcium carbonate solution produced negligible absorbance changes.

**Stopped-flow fluorimetry**

Stopped-flow experiments were performed using an SF-61SX2 stopped-flow spectrofluorimeter (TgK Scientific, Bradford on Avon, UK) as described previously (32, 54, 81). All solutions were equilibrated to a temperature of 24°C before measuring the kinetics. The methods used to study different enzyme partial reaction kinetics are explained briefly below.

For the ENa⁺₃ → E2P reaction, Na⁺,K⁺-ATPase-containing membrane fragments (10 µg/ml of enzyme), labelled with RH421 (250 nM), were pre-mixed in one drive syringe with 130 mM NaCl to stabilize the ENa⁺₃ state. The enzyme was then mixed with an equal volume of 2 mM Na₂ATP solution from the other drive syringe. Both enzyme suspension and ATP solution were prepared in the same buffer (30 mM imidazole, 130 mM NaCl, 5 mM MgCl₂, 1 mM EDTA). The Mg²⁺ ions allow ATP to phosphorylate the protein after the solutions are mixed (81). Buffer pH was adjusted to 7.2 with HCl.

The kinetics of the E2P → E2K⁺₂ transition were determined by first adding 1 mM of Na₂ATP to a suspension of Na⁺,K⁺-ATPase-containing membrane fragments (30 µg/ml of enzyme), labelled with 250 nM RH421, in one of the stopped-flow drive syringes. The enzyme
suspension was in buffer containing 30 mM imidazole, 130 mM NaCl, 5 mM MgCl₂ and 1 mM EDTA (as in ENa⁺₃ → E2P experiments). Thus, the enzyme is already phosphorylated by ATP in the drive syringe. The enzyme suspension was then mixed with an equal volume of 20 mM KCl solution. The KCl solution was prepared in the same buffer as the enzyme suspension and also contained 1 mM Na₂ATP. Buffer pH was adjusted to 7.2 with HCl.

To determine the kinetics of the E2K⁺₂ → E1Na⁺₃ transition, Na⁺,K⁺-ATPase-containing membrane fragments (30 μg/ml of enzyme), labelled with RH421 (250 nM) were pre-mixed in one of the drive syringes with 10 mM KCl to stabilize the E2K⁺₂ state. The enzyme was then mixed with an equal volume of solution containing 260 mM NaCl and 2 mM Na₂ATP from the other drive syringe. To avoid any changes in K⁺ concentration on mixing, solutions in both drive syringes were prepared in 30 mM histidine, 10 mM KCl and 1 mM EDTA. The pH was adjusted to 7.2 with HCl. The absence of Mg²⁺ ions prevents phosphorylation by ATP, but still allows ATP to act in an allosteric fashion, accelerating the E2K⁺₂ → E1Na⁺₃ transition.

To improve the signal/noise ratio, typically between 9 and 21 experimental traces were averaged before observed rate constants, k_{obs}, and relative fluorescence changes, ΔF/F₀, were evaluated. This was done by fitting a sum of either 1 or 2 exponential functions to the averaged trace. The choice between a single- or double-exponential fit was based on residual plots and values of the X² parameter.

Eosin fluorescence measurements

All fluorescence measurements using eosin were carried out using an RF-5301 PC spectrofluorophotometer (Shimadzu, Kyoto, Japan) with 1 cm pathlength quartz semicuvettes. 100 μL of either E1-stabilizing buffer (30 mM imidazole, 130 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, pH 7.2) or E2-stabilizing buffer (30 mM imidazole, 10 mM KCl and 1 mM EDTA, pH 7.2), 7.4 μL of Na⁺,K⁺-ATPase-containing membrane fragments (3.2 mg ml⁻¹ in 30 mM imidazole, 1 mM EDTA, pH 7.2) and 2.9 μL of eosin (1.1 μM in water) were consecutively added to the cuvette. The temperature was maintained at 24°C via a circulating water bath. The value of emission wavelength used was 550 nm (bandwidth 5 nm) with an OG530 cut-off filter (Schott, Mainz, Germany) in front of the photomultiplier.

The final Na⁺,K⁺-ATPase concentration in the cuvette was 230 μg/ml. This concentration was chosen based on previous studies by Skou and Esmann (59, 60) in order to saturate the eosin with protein and ensure that all of the measured fluorescence derives from protein-bound eosin and any fluorescence from eosin in the neighboring aqueous solution can be neglected. In the case of membranes from which cholesterol had been extracted using mβCD, the percentage of cholesterol extracted after treatment was determined to be 99.8% based on cholesterol assays before and after mβCD treatment.

Statistical analysis

Statistical analysis was performed in Graphpad Prism 7. Comparisons were performed using an unpaired t-test with statistical significance defined as P < 0.05.

MD simulations

Available structures of pig Na⁺,K⁺-ATPase in E1 and E2 states (PDB codes 3WGU (38) and 3B8E (39) were embedded in bilayers of DOPC (264 and 261 lipids, respectively) and 40 mol% cholesterol (176 and 178 molecules, respectively) immersed in 150 mM NaCl solution using explicit TIP3P water molecules, with the E1 system containing 267,757 atoms and E2 containing 226,973 atoms. Four copies of each system were built with random distributions of cholesterol using CHARMM-GUI (82), and equilibrated using NAMD 2.9 (83) with the CHARMM36 force field (84, 85). Temperature was maintained at 303.15 K via a Langevin thermostat, with the pressure set at 1 atm with the Nose-Hoover Langevin piston method (86) using rectangular periodic boundary conditions. The temperature was chosen to match standard conditions in previous experimental studies of pure DOPC bilayers (87). A further increase in temperature by 7°C to body temperature is expected to cause only a minimal change in membrane hydrophobic thickness of 0.35 Å (88). Similar systems without cholesterol (47) were used as reference. The lipid composition of DOPC and 40 mol% cholesterol was chosen based on experimental reconstitution studies indicating that this composition allows the enzyme to regain full activity (11).

For 2-D maps of carbonyl C to C membrane deflection, the lipid bilayer thickness was computed on a 2 × 2 Å xy grid, offset by removing the average away from the protein to obtain a measure of deflection. Error bars are the standard error of means obtained from the 4 independent runs. All other simulation procedures were as described previously (47). Perturbations far from the protein in Fig.5b are
Inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase by cholesterol depletion

due to the influence of the protein from the neighboring periodic image.

Cholesterol density was computed on a 2 \times 2 Å \( xy \) grid for each membrane leaflet separately. All values were computed relative to the average density away from protein, yielding maps of relative cholesterol density. A reproducibility test was performed by analysing all simulations independently in Fig. S2. Areas near the protein that exhibited peaks in density were visually inspected in VMD to identify residues that might facilitate favourable interactions with cholesterol (Fig. S3). The cholesterol density, relative to far from the protein, \( \rho(x, y) \), has been converted to a 2-D axially-averaged radial distribution function, \( g(r) \), normalized to converge to 1 far from the protein, from which we have calculated a radial free energy profile \( \Delta G(r) = -k_B T \ln g(r) \), where \( k_B \) is Boltzmann’s constant, and \( T \) is the absolute temperature. We offset \( \Delta G(r) \) so that it is 0 in the bulk (\( \Delta G_{\text{bulk}} = 0 \)), defined by the average in the region between 50 and 60 Å from the protein center-of-mass. We have calculated the net \( \Delta G \) for the relative binding of a cholesterol molecule to the E2 relative to the E1 P state, including both leaflets of the membrane, as

\[
\Delta G_{E1 \rightarrow E2} = -k_B T \ln \frac{K_{D,E2}}{K_{D,E1}},
\]

where \( K_{D,E1} \) and \( K_{D,E2} \) are dissociation constants, computed for states E1P and E2, respectively, as

\[
K_D^{-1} = \int_{r}^{\infty} e^{-\Delta G(r)/k_B T} r dr,
\]

where “bound” is defined as the region between \( r = 0 \) and 30 Å from the protein center-of-mass, corresponding to the lipid annulus with defined cholesterol binding sites in Fig.6b and c.

Packing scores for cholesterols around the protein were computed as the sum of all \( 1/r^6 \) terms for all atoms of a cholesterol molecule that exist within 5 Å of any atom of the protein, averaged over the simulations (88). Sites with at least one binding pose with a total packing score > 0.25 were considered high scoring (Table S1) and visualised in Fig. S3. Contributions from specific residues of the protein were computed as the sum of all \( 1/r^6 \) terms between any atom of a cholesterol molecule and any atom of a specific protein residue within 5 Å. Contributions from any residue were considered high and reported in Table S2 and in Fig. S3, if > 0.02.

Lipid chain order parameters as a function of carbon number for lipid tails, were computed using \( S_{CD} = \langle 3 \cos^2 \alpha - 1/2 \rangle \), where \( \alpha \) is the angle of each C-H vector to the bilayer normal.

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16
Inhibition of Na⁺,K⁺-ATPase by cholesterol depletion

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FOOTNOTES
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The abbreviations used are: mβCD, methyl-beta-cyclodextrin; DOPC, dioleoyl-sn-glycero-3-phosphocholine; MD, molecular dynamics; PK, pyruvate kinase; LDH, lactate dehydrogenase; PC, phosphatidylcholine; PLL, poly-L-lysine; PDB, protein data bank; PS, phosphatidylserine; SDS, sodium dodecyl sulfate
Figure 1. Dependence of relative Na⁺,K⁺-ATPase (NKA) activity on normalized relative membrane cholesterol content. Cholesterol extraction was performed using sequentially higher concentrations of mβCD, and ATPase activity was measured after mβCD removal. (Solid line) Nonlinear least-squares fit of an exponential decay curve to the data, i.e., the fit function was $y = y_0 + (y_{\text{max}} - y_0)(1 - e^{-kx})$. $k$ is here the reciprocal of the relative membrane cholesterol content at which the difference between the normalized Na⁺,K⁺-ATPase activity and its value at zero cholesterol content has dropped to 1/e of its initial value at zero cholesterol content. The half-saturating relative membrane cholesterol content was determined to be 0.15 (±0.10). The Na⁺,K⁺-ATPase activity of untreated membranes was 1671 (± 71) μmol ATP hydrolyzed (mg protein)⁻¹ hr⁻¹ (N = 7).
Figure 2. Effect of reintroduction of cholesterol through a cholesterol:mβCD complex to membrane fragments treated with different concentrations of mβCD (30, 45 and 60 mM). Measurements are given as relative activity to untreated controls and are an average of 3 measurements with the errors presented as standard deviations.
Figure 3. Stopped-flow fluorescence transients of Na^+\text{,}K^+-ATPase from pig kidney noncovalently labeled with RH421. Panels A and B show the kinetics of the E1Na^+3 → E2P transition of an untreated control (panel A) and membrane fragments treated with 60 mM mβCD (panel B). Panels C and D show the kinetics of the E2K^+2 → E1Na^+3 transition of an untreated control (panel C) and membrane fragments treated with 60 mM mβCD (panel D). All experimental conditions are described under Experimental Procedures.
Figure 4. Fluorescence excitation spectra of eosin. The fluorescence intensity is given in arbitrary units. The upper panel represents eosin in the presence of Na⁺,K⁺-ATPase membrane fragments from which cholesterol was either extracted (solid line) or an untreated control (dotted line) in E2K⁺₂ buffer (30 mM imidazole, 10 mM KCl, 1 mM EDTA, pH 7.2). The lower panel represents eosin in the presence of Na⁺,K⁺-ATPase membrane fragments from which cholesterol was either extracted with mβCD (solid line) or an untreated control (dotted line) in E1Na⁺₃ buffer (30 mM imidazole, 130 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, pH 7.2). The emission wavelength was 550 nm (+OG530 cutoff filter). The bandwidths for both excitation and emission were 5 nm. The eosin and Na⁺,K⁺-ATPase concentrations were 29 nM and 230 μg mL⁻¹ in every case. Cholesterol extraction was achieved by equilibrating the Na⁺,K⁺-ATPase membrane fragments with 60 mM mβCD for 2 hours at 37 °C. The spectra were recorded at a temperature of 24 °C.
Inhibition of Na⁺,K⁺-ATPase by cholesterol depletion

Figure 5. (a) Na⁺,K⁺-ATPase simulation systems: E1P·ADP·3Na⁺ (left, “E1”, PDB 3WGU) and E2-Pi·2K⁺ (right, “E2”, PDB 3B8E). (b) Bilayer thickness deviation is based on glycerol C to glycerol C distance between lipids in each leaflet, viewed from the cytoplasm. Deviation is relative to thickness far from the protein, with an average of 34.8 ± 0.2 Å for both states. (c) Axially-averaged bilayer thickness, defined as the glycerol-carbon-to-glycerol-carbon distance between lipids in each membrane leaflet. The error bars represent ± one standard error of means.
Figure 6. Cholesterol density for each membrane leaflet relative to the mean far from the protein. The left column shows the distribution for E1P·ADP·3Na⁺ (PDB 3WGU, “E1”) and the right column E2·Pi·2K⁺ (PDB 3B8E, “E2”). Density is viewed from the cytoplasm. Reproducibility of the 4 simulations for each state is shown in Fig. S1. Sites with a cholesterol packing score of ≥ 0.25 for at least one state are indicated with letters A – M, with green letters for sites unique to one state (packing scores in Table S1). a) Shows results for extracellular side, while b) shows results for cytoplasmic site. c) Radial free energy profile for cholesterol binding, shown in blue for extracellular leaflet and red for cytoplasmic leaflet. Darker colours represent the E1 state, while lighter ones represent the E2 state, as labelled.
Cholesterol depletion inhibits Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in a near-native membrane environment
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