Cholesterol-Dependent Interaction of Polyunsaturated Phospholipids with Na,K-ATPase[†]

Flemming Cornelius*

Institute of Physiology and Biophysics, Department of Biophysics, University of Aarhus, Ole Worms Allé 185, Aarhus C DK-8000, Denmark

Received October 23, 2007; Revised Manuscript Received December 5, 2007

ABSTRACT: Polyunsaturated phospholipids such as 16:0-22:6 PC and 22:6 PC both stabilized the E_1 conformation and inhibited turnover of Na,K-ATPase reconstituted into 18:1 PC or 18:1 PC/cholesterol liposomes. The inhibition increases in the order 22:6 PC > 16:0-22:6 PC both in the presence and in the absence of cholesterol, but is most pronounced in the absence of cholesterol. The inhibition of Na,K-ATPase turnover may thus correlate with the capability of polyunsaturated phospholipids and cholesterol to induce liquid-disordered and liquid-ordered lipid phases, respectively. In the presence of cholesterol 16:0-22:6 PC and 22:6 PC both increase the apparent Na^+ affinity and change the K^+ inhibition observed at low ATP concentration into activation. These effects on Na,K-ATPase kinetics can be explained by the ability of polyunsaturated phospholipids to induce lateral phase separation from cholesterol, which may be partially excluded from interaction with the Na,K-ATPase/lipid interface. Finally, inclusion of polyunsaturated phospholipids may induce changes in the bilayer hydrophobic thickness, which will increase the hydrophobic mismatch between lipids and protein.

The Na,K-ATPase is a P-type ion-transport ATPase present in animal plasma membranes required for cellular homeostasis. The Na,K-ATPase couples the free energy of ATP hydrolysis to the establishment of the electrochemical gradients for Na^+ and K^+ across the plasma membrane, which, e.g., are vital for uptake of substrates by cotransport.

The lipid composition of the plasma membrane of shark Na,K-ATPase preparations is characterized by a high cholesterol/phospholipid ratio with more than 50 mol % cholesterol, as well as a large fraction of polyunsaturated fatty acids (PUFAs)[1] (*1*). Thus, about 15 mol % of the fatty acids is docosahexaenoic acid (DHA). With 22 carbons and 6 double bonds DHA is the longest and most unsaturated *n* − 3 PUFA in membranes (the term *n* − 3 signifies that the first double bond exists as the third carbon−carbon bond from the terminal methyl end of the carbon chain). In mammals, organs such as the kidney and brain, both known for their high Na,K-ATPase content, have similar high cholesterol and DHA contents.

DHA has been reported to be beneficial for health in general, and a large number of neurological conditions are apparently improved by DHA (*4*, *5*). The fact that DHA affects a plethora of human health problems indicates that it affects fundamental cell membrane properties such as bilayer physical properties, interaction with rafts, lipid microdomain formations, and cell signaling. Previous evidence has suggested a marked incompatibility between DHA and cholesterol (*6*), and a bilayer rich in PUFA has a marked lower solubility to sterols (*7*). Thus, cholesterol is discriminated against in a highly unsaturated bilayer, and PUFA is important in the establishment of lateral lipid heterogeneity (*8*, *9*). The very complex lipid composition of the plasma membrane and formation of specific lipid microdomains such as rafts are almost certainly very important in the regulation of Na,K-ATPase both globally and specifically, e.g., by protein kinase regulation (*10*, *11*), and this has been further emphasized by the recent finding that Na,K-ATPase can be associated with caveolae/rafts (*12*), indicating an important function for cholesterol in Na,K-ATPase regulation and targeting (*13*).

The activities of a large number of proteins have been demonstrated to be affected by DHA, among which the rhodopsin DHA−membrane system is the best studied (*14*, *15*). Here the importance of PUFA and the interactions with cholesterol are investigated for Na,K-ATPase reconstituted into liposomes of defined lipid composition. We compare the kinetic properties of Na,K-ATPase in the presence or absence of cholesterol in combination with 22:6 PC or 16:0−22:6 PC. In this work we demonstrate that the molecular activity (turnover) and conformational state of the ubiquitous Na,K-ATPase are strongly dependent on the interaction of cholesterol and polyunsaturated phospholipids.

MATERIALS AND METHODS

Enzyme Preparation. Membrane-bound Na,K-ATPase (EC 3.6.1.37) from rectal glands of the shark *Squalus acanthia*s

[†] The work was supported by the Danish Medical Research Council and Aarhus University Research Foundation.

* Phone: +4589422926.Fax: +4586129599.E-mail: fc@biophys.au.dk.

[1] Abbreviations: Chol, cholesterol; 18:1 PC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; 22:6 PC, 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphocholine; 16:0-22:6 PC, 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; DHA, docosahexaenoyl acid; PUFAs, polyunsaturated fatty acids; E_1, Na,K-ATPase form with high affinity toward ATP and Na^+; E_2, Na,K-ATPase form with high affinity toward K^+ and low affinity toward ATP; EP, phosphoenzyme; l_o, liquid-ordered phase; l_d, liquid-disordered phase.

10.1021/bi702128x CCC: $40.75
Published on Web 01/15/2008

was prepared essentially as previously described (*16*). This involves isolation of well-defined membrane fragments by differential centrifugation following treatment of microsomes with low concentrations of DOC (~0.15%), which permeabilizes the microsomes and removes loosely attached proteins. The specific hydrolytic activity was 30−33 U/mg protein (37 °C) at standard conditions according to Ottolenghi (*17*). The protein content was determined according to Lowry et al. (*18*) using bovine serum albumin as the standard.

Reconstitution. Functional reconstitution of shark Na,K-ATPase was achieved as previously described (*19*, *20*): Initially membrane bound Na,K-ATPase was solubilized using the nonionic detergent $C_{12}E_8$. The lipids were solubilized using the same detergent, and the two solutions were mixed at a protein:lipid weight ratio estimated to give a final ratio of 1:20 in the proteoliposomes. The detergent was subsequently removed by addition of hydrophobic Bio-Beads, and liposomes containing reconstituted Na,K-ATPase spontaneously formed. Careful control during enzyme solubilization ensured that reconstitution took place without loss of catalytic activity or ion-transport capacity. Unless otherwise stated the proteoliposomes were produced in 30 mM NaCl, 2 mM $MgCl_2$, 200 mM sucrose, and 30 mM histidine, pH 7.0.

Protein determination of reconstituted ATPase was performed according to Peterson modification (*21*). In this, the reconstituted protein was quantitatively precipitated with sodium deoxycholate and trichloroacetic acid followed by resuspension in water. SDS was included in the copper tartrate solution to leave the lipid transparent and noninterfering. Bovine serum albumin was run as the standard.

Phosphorylation Site Number. The maximum steady-state phosphorylation level of reconstituted Na,K-ATPase in the inside-out (i/o) orientation was measured as the amount of acid-stable phosphoenzyme in the presence of Na^+ and in the absence of K^+ (*22*). At these conditions the dephosphorylation step is slower by at least an order of magnitude compared to the other steps in the reaction, and the main state populated is the phosphoenzyme (EP). Liposomes were preincubated with 1 mM ouabain as described above to prevent phosphorylation of nonoriented enzyme, and phosphorylation was conducted by addition of 25 μM [^{32}P]ATP in a mixture of 30 mM imidazole, pH 7.4, 2.5 mM $MgCl_2$, 0.5 mM P_i, 72.5 mM NaCl, 115 mM sucrose, and 1 mM ouabain. The reaction time was 20 s at 0 °C. Phosphorylation was terminated by addition of acid stop solution containing 10% TCA, 100 mM phosphoric acid, and 10 mM sodium pyrophosphate. Radioactivity and protein were determined in the precipitate after resuspension in 1 M NaOH at 55 °C.

ATPase Activity. The rate of ATP hydrolysis of reconstituted i/o-oriented Na,K-ATPase was measured using [^{32}P]-ATP as described in ref *23* after preincubation of the proteoliposomes with Mg^{2+} (5 mM), P_i (1 mM), and ouabain (1 mM) to inhibit enzyme that was reconstituted in a nonoriented fashion. Right-side-out-oriented enzyme was inactive in the analysis since their substrate site was shielded inside the liposome that was impermeable to ATP. The hydrolytic activity was determined in a test medium containing 50 μM ATP, 130 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, and 0.7 μM nigericin to ensure rapid equilibration of K^+ across the proteoliposomes (*22*). The turnover number (k_{cat}) of inside-out-oriented enzyme is calculated by dividing the specific hydrolytic activity by the site number.

Data Analysis. Results are expressed as the mean ± SEM. Comparison between best-fit values was performed using an *F*-test, and $p < 0.05$ was considered significant.

Materials. Highly purified phospholipids were obtained from Avanti Polar Lipids. Cholesterol was from Sigma. ATP, purchased as the sodium salt from Boehringer Mannheim, was converted to the Tris salt by chromatography on a Dowex 1 column (Sigma). [γ-^{32}P]ATP was from Amersham. Nigericin was from Molecular Probes.

RESULTS

Reconstitution. As previously described in detail reconstitution of Na,K-ATPase by cosolubilization of protein and lipids in the detergent $C_{12}E_8$ followed by detergent elimination by Bio-Beads leads to asymmetric incorporation of fully active Na,K-ATPase (*19*, *20*). Functional analysis shows that ~45% of the enzyme is incorporated with an orientation as in the cell (right side out), whereas only ~15% has the opposite orientation (inside out). The rest is incorporated in a way where it is accessible to both ATP (intracellular side) and ouabain (extracellular side). The latter fraction (nonoriented) probably represents Na,K-ATPase adsorbed to liposomes.

The function of inside-out Na,K-ATPase can be specifically assessed in the combined presence of ATP and ouabain, since the ATP binding site of right-side-out enzyme is shielded inside the proteoliposomes, and nonoriented enzyme is inhibited by external ouabain, which cannot inhibit inside-out enzyme. To calculate the turnover of inside-out Na pumps, the hydrolytic activity measured in the presence of ATP and ouabain is related to the fraction of pumps with an inside-out orientation. To this end the site number of inside-out-oriented enzyme was determined at each lipid composition by phosphorylation in the presence of Na^+ and ouabain as described in the Materials and Methods. This is essential since the orientation of Na,K-ATPase varies at the different lipid compositions used (cf. Figure 2).

In cells the polyunsaturated fatty acids of phospholipids are primarily found in the *sn*-2 chain with the *sn*-1 chain mainly composed of the fatty acids palmitic (16:0) and stearic (18:0) acids. In a few tissues such as retinal membranes *sn*-1, *sn*-2 dipolyunsaturation such as 22:6 PC is found (*24*). In the present study proteoliposomes were prepared from either pure 18:1 PC or 18:1 PC with the addition of 40 mol % cholesterol, and in both proteoliposome preparations 10 mol % polyunsaturated phospholipids (22:6 PC or 16:0-22:6 PC) replaced 18:1 PC. All six proteoliposome preparations were prepared to have the same protein:lipid weight ratio of 1:20. Figure 1 shows the structure of the different phospholipids used.

Figure 2 demonstrates how the site number of inside-out Na,K-ATPase varies in proteoliposomes of different lipid compositions as measured by the maximum EP level. As seen, in the absence of cholesterol inclusion of 10 mol % 22:6 PC or 16:0-22:6 PC did not significantly change the EP levels, which are about 0.1 nmol of inside-out sites/mg of total protein in all cases. The presence of 40 mol % cholesterol increases significantly the EP level to about 0.7 nmol/mg, but again substitution of 10 mol % 18:1 PC with either 22:6 PC or 16:0-22:6 PC did not change this EP level.

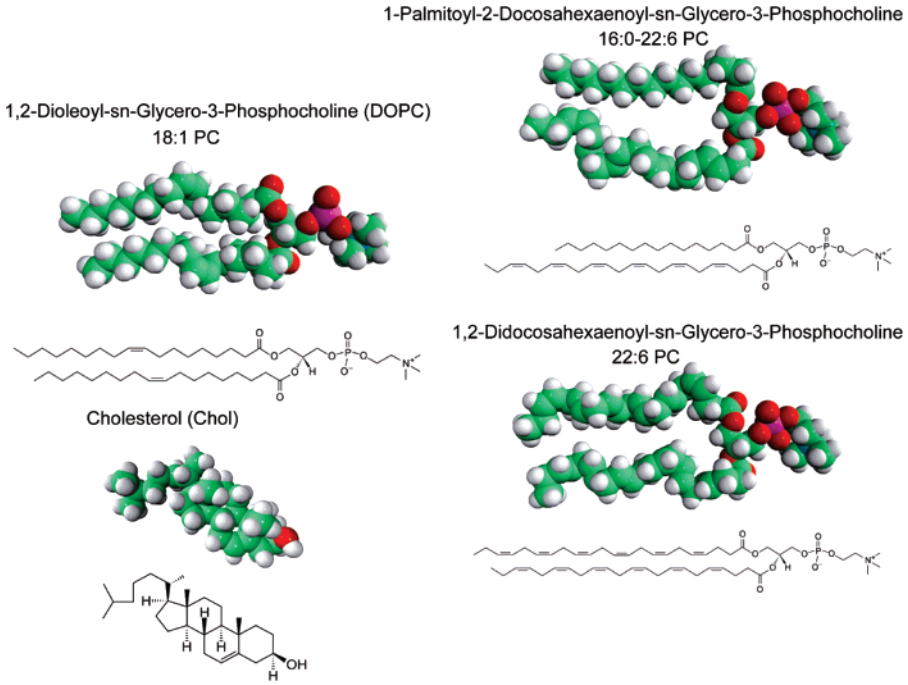

FIGURE 1: Lipids used. Structure of the monounsaturated 18:1 PC compared to the polyunsaturated 16:0-22:6 PC and 22:6 PC.

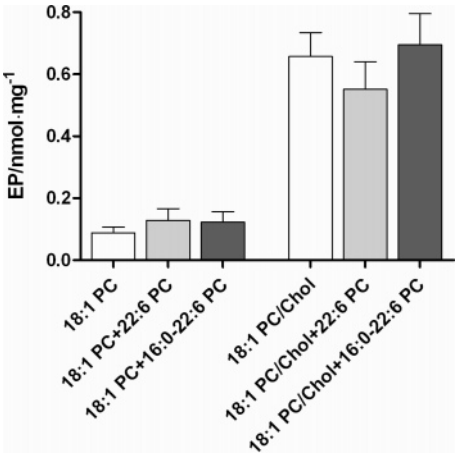

FIGURE 2: Effects of lipid composition on the phosphorylation site number (EP) of Na,K-ATPase reconstituted with an inside-out orientation. The EP level is significantly larger in the presence of 40 mol % cholesterol, whereas the presence of 10 mol % polyunsaturated phospholipids does not influence the enzyme phosphorylation site number significantly.

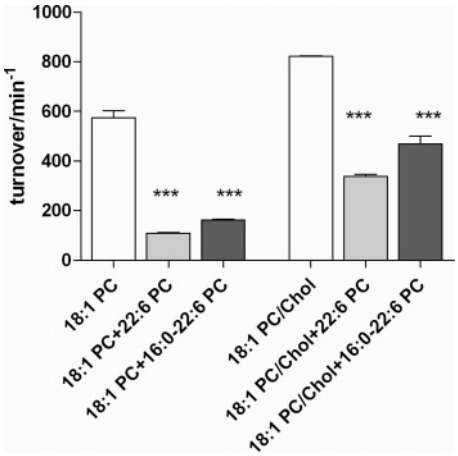

FIGURE 3: Turnover of Na,K-ATPase reconstituted in liposomes of different lipid compositions measured at 23 °C. In the absence of cholesterol the turnover is 575 ± 49, 109 ± 4, and 163 ± 4 min^{-1} without polyunsaturates (control) and with 10 mol % 22:6 PC or 16:0−22:6 PC, respectively. The values in the presence of cholesterol are 823 ± 4, 338 ± 15, and 470 ± 53 min^{-1}. The latter two figures differ significantly from the controls both in the absence and in the presence of cholesterol ($p < 0.0001$). Also, the turnover is significantly higher in the presence of cholesterol.

When the turnover of inside-out enzyme is measured at suboptimal ATP conditions (25 μM), it is obvious that cholesterol significantly activates Na,K-ATPase turnover (from 575 to 823 min^{-1}), as also previously demonstrated (*3*). As seen from Figure 3 polyunsaturated phospholipids inhibit Na,K-ATPase turnover significantly both in the presence and in the absence of cholesterol. However, the level of inhibition is different in the two conditions: in the absence of cholesterol inclusion of 22:6 PC and 16:1-22:6 PC decreased turnover to about 19% and 28% of the control values, respectively, whereas in the presence of cholesterol the turnover was decreased to 41% and 57%, respectively.

Activation of Na,K-ATPase by cytoplasmic Na^+ is one of the key parameters in cells for regulation of Na,K-ATPase turnover. Thus, the apparent Na^+ affinity is pivotal in Na,K-ATPase regulation. In Figure 4 Na^+ activation at a constant K^+ concentration of 10 mM of reconstituted inside-out Na,K-ATPase is shown at the six different lipid conditions used. In the absence of cholesterol (Figure 4A) the activation curve is sigmoid with an apparent Na^+ affinity ($K_{0.5}$) of about 11 mM and a Hill coefficient $n_H \approx 2.2$. Inclusion of either 22:6 PC or 16:0-22:6 PC did not change the Na^+ activation of the Na,K-ATPase. However, in the presence of 40 mol % cholesterol the picture is different. First, cholesterol in itself decreases the apparent Na^+ affinity; i.e., $K_{0.5}$ increases to about 30 mM, as previously found (*2*). Second, both 22:6 PC and 16:0-22:6 PC increase this apparent Na^+ affinity since $K_{0.5}$ decreases to about 15 and 16 mM, respectively, i.e., close to the values found in the absence of cholesterol. Finally, the n_H value is smaller in the presence of cholesterol ($n_H \approx 1.6$) than in its absence ($n_H \approx 2.2$).

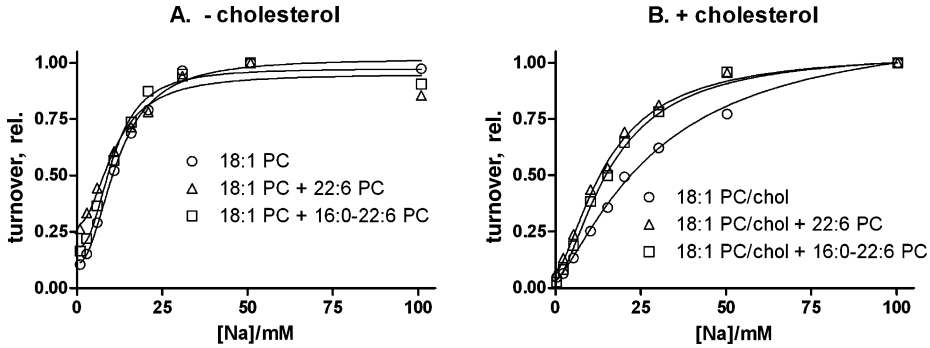

FIGURE 4: Activation of turnover (v) by cytoplasmic Na^+ of Na,K-ATPase reconstituted into liposomes of different lipid compositions as indicated on the graphs. The values have been scaled to a maximum turnover $k_{cat} = 1.00$. The curves represent the best fit to the data using the Hill equation $v = (k_{cat} - v_0)/(1 + 10^{[\log(K_{0.5} - X)]n_H}) + v_0$, where $K_{0.5}$ is the Na^+ concentration (X, mM) that gives a turnover (v) halfway between the baseline (v_0) and maximum response (k_{cat}) and n_H, the Hill coefficient, is the steepness of the curve. The fitting parameters used in panel A are (18:1 PC control) $K_{0.5} = 11.9 \pm 0.2$ mM and $n_H = 2.2 \pm 0.1$, (18:1 PC + 22:6 PC) $K_{0.5} = 11.0 \pm 0.5$ mM and $n_H = 2.1 \pm 0.3$, and (18:1 PC + 16:0-22:6 PC) $K_{0.5} = 10.6 \pm 0.2$ mM and $n_H = 2.4 \pm 0.2$. The fitting parameters used in panel B are (18:1 PC/Chol control) $K_{0.5} = 29.7 \pm 0.6$ mM and $n_H = 1.33 \pm 0.08$, (18:1 PC/Chol + 22:6 PC) $K_{0.5} = 15.3 \pm 0.4$ mM and $n_H = 1.57 \pm 0.09$, and (18:1 PC/Chol + 16:0-22:6 PC) $K_{0.5} = 16.4 \pm 0.2$ mM and $n_H = 1.59 \pm 0.07$.

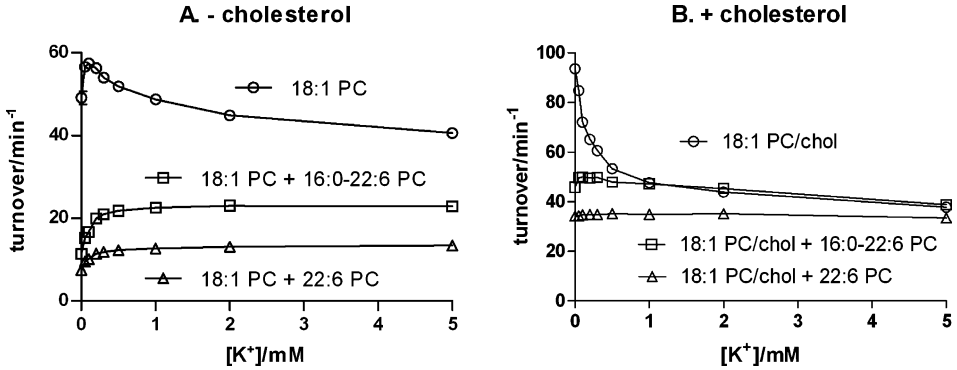

FIGURE 5: K^+ sensitivity of Na-ATPase activity at 1 μM ATP. Na,K-ATPase is reconstituted with 18:1 PC (A) or 18:1 PC + 40 mol % cholesterol (B) with inclusion of a 10 mol % concentration of either 22:6 PC or 16:0-22:6 PC. Reconstituted Na,K-ATPase was preincubated with ouabain in the presence of MgP_i to inhibit enzyme reconstituted as right side out or as nonoriented as described in the Materials and Methods. ATP hydrolysis and turnover of inside-out-oriented Na,K-ATPase were subsequently measured at 20 mM NaCl, 1 μM ATP, and varying K^+ concentrations between 0 and 5 mM at 23 °C. Nigericin (0.5 μg/mL) was present to allow K^+ access to the extracellular side of inside-out-oriented enzyme. Values for a typical experiment are shown as the mean ± SEM of triplicate determinations.

The main rate-determining step in the Na,K-ATPase reaction cycle is the $E_2 \rightarrow E_1$ reaction, even at physiological conditions with millimolar concentrations of ATP (*25*, *26*). This reaction is associated with the deocclusion of K^+ to the cytoplasmic side and is strongly accelerated by ATP: $E_2(K_2) + ATP \leftrightarrow E_1ATP + 2K^+$. At low (micromolar) ATP concentrations the forward reaction becomes so slow that addition of K^+ leads to accumulation of the $E_2(K_2)$ form, resulting in increasing inhibition of Na,K-ATPase turnover at increasing K^+ concentration (*1*, *27*). This effect is further enhanced by high-affinity binding of ATP to the E_1 form, stimulating the E_1ATP to E_2ATP backward reaction (*28*). This K^+ inhibition is demonstrated in Figure 5B for Na,K-ATPase reconstituted in 18:1 PC with 40 mol % cholesterol. As also seen, inclusion of a 10 mol % concentration of either 22:6 PC or 16:0-22:6 PC changes this pattern: The Na-ATPase activity is decreased, and K^+ addition no longer leads to inhibition of enzyme turnover. This indicates that the $E_2 \rightarrow E_1$ transition no longer rate limits the overall reaction mechanism, and PUFAs, therefore, seem to affect the deocclusion reactions associated with the $E_2 \rightarrow E_1$ transition. In the absence of cholesterol K^+ addition has a different effect (Figure 5A), as also previously demonstrated (*1*). Thus, without cholesterol addition of K^+ initially activates turnover, but this is soon followed by inhibition at higher K^+ concentrations. When PUFAs are included in cholesterol-free proteoliposomes, even the latter inhibition observed at the higher K^+ concentrations is absent, again indicating specific effects of PUFAs on the $E_2 \rightarrow E_1$ reaction.

The Na,K-ATPase can adopt two different main conformations: the E_1 conformation is characterized by having high-affinity sites for Na^+, which are exposed to the cytoplasmic side, and binds ATP with high affinity. The E_2 conformation, however, has high-affinity sites for K^+, which are exposed to the extracellular side, and has low-affinity ATP binding sites.

The E_1/E_2 equilibrium can be estimated from vanadate binding experiments (*27*), since orthovanadate, a transition-state homologue of inorganic phosphate, binds preferentially to the enzyme in the E_2 conformation. Thus, the half-maximal inhibitor concentration (K_I) is a measure of the E_1/E_2 poise: the higher the apparent inhibitor affinity (the lower the K_I value), the more the E_1/E_2 equilibrium is poised toward the E_2 conformation.

Figure 6 demonstrates vanadate inhibition results for Na,K-ATPase reconstituted into liposomes of different lipid compositions. In the absence of cholesterol (Figure 6A) the half-maximal vanadate concentration for inhibition of Na,K-ATPase reconstituted in 18:1 PC proteoliposomes is about 0.9 μM, and both 22:6 PC and 16:0-22:6 PC shift the

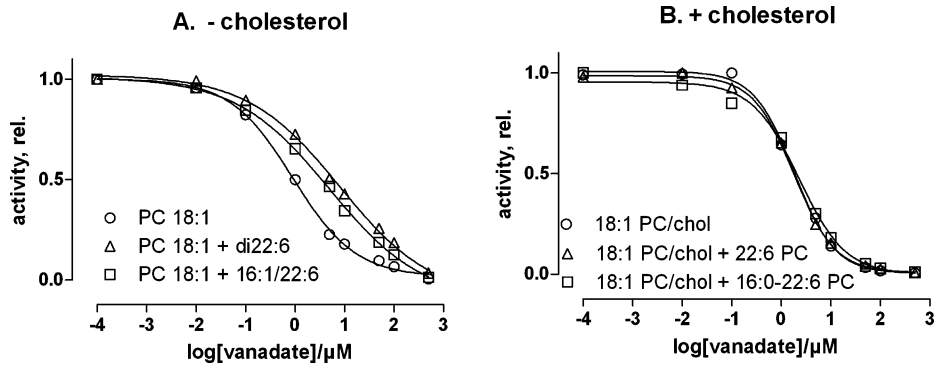

FIGURE 6: Vanadate sensitivity of Na,K-ATPase reconstituted with 18:1 PC (A) or 18:1 PC + 40 mol % cholesterol (B) with inclusion of a 10 mol % concentration of either 22:6 PC or 16:0-22:6 PC. ATP hydrolytic activity of inside-out Na,K-ATPase was measured at 100 mM NaCl, 10 mM KCl, 50 mM sucrose, 1 mM MgI_2, 10 μM ATP, 0.5 μg/mL nigericin, and 30 mM histidine, pH 7.0. Data are presented as fractions of the activity measured in the absence of vanadate. Means ± SEM of triplicate determinations are shown for a typical experiment. The curves represent the best fit to the data using the Hill equation $v = (k_{cat} - v_0)/(1 + 10^{[\log(K_I - [VO_3])]n_H}) + v_0$, where K_I is the inhibitor concentration ($[VO_3]$, μM) that gives a turnover (v) halfway between the baseline (v_0) and maximum (k_{cat}) values, and n_H, the Hill coefficient, is the steepness of the curve. The fitting parameters used in panel A are (18:1 PC control) $K_I = 0.90 \pm 0.1$ μM and $n_H = -0.90 \pm 0.02$, (18:1 PC + 22:6 PC) $K_I = 8.0 \pm 0.5$ μM and $n_H = -0.47 \pm 0.03$, and (18:1 PC + 16:0-22:6 PC) $K_I = 4.9 \pm 0.2$ μM and $n_H = -0.46 \pm 0.02$. The fitting parameters used in panel B are (18:1 PC/Chol control) $K_I = 1.8 \pm 0.1$ μM and $n_H = -1.06 \pm 0.05$, (18:1 PC/Chol + 22:6 PC) $K_I = 1.8 \pm 0.1$ μM and $n_H = -1.05 \pm 0.03$, and (18:1 PC/Chol + 16:0-22:6 PC) $K_I = 2.3 \pm 0.2$ μM and $n_H = -0.92 \pm 0.06$.

inhibition curve toward higher vanadate concentrations. Thus, in the presence of 10 mol % 16:0-22:6 PC K_I increases to about 4.9 μM, whereas in the presence of 22:6 PC K_I is further increased to about 8 μM. In the presence of 40 mol % cholesterol (Figure 6B) the pattern is different: K_I is increased to about 2 μM in 18:1/Chol proteoliposomes and does not change after inclusion of either 22:6 PC or 16:0-22:6 PC. Therefore, cholesterol in itself shifts the E_1/E_2 poise slightly toward the E_1 conformation, as also previously found. Polyunsaturates also stabilized the E_1 conformation, at least in the absence of cholesterol (Figure 6A). Another difference noted between proteoliposomes with or without cholesterol is that the Hill coefficient (n_H) is lower and significantly different from −1 in the absence of cholesterol.

DISCUSSION

The molecular mechanisms of protein/lipid interactions have been demonstrated to involve both global effects such as hydrophobic matching between the hydrophobic domain of the integral protein and the bilayer core (*29*, *30*) and changes in bilayer curvature stress (*31*) and more specific interactions such as direct binding of lipids to the protein. We have previously demonstrated that hydrophobic matching of the lipid bilayer with the integral Na pump is important to support optimal enzyme activity. Indeed, a specific phospholipid acyl chain length and the presence of cholesterol (*2*, *3*, *32*, *33*) are essential to support optimal hydrolytic activity of Na,K-ATPase. Thus, monounsaturated acyl chain lengths of 22 in the absence and 18 in the presence of 40 mol % cholesterol, which give about the same hydrophobic thickness, are optimal for hydrolytic activity and turnover of reconstituted Na,K-ATPase (*3*). Furthermore, several reaction steps in the reaction cycle were affected by cholesterol including the main rate-determining $E_2 \rightarrow E_1$ reaction (*1*). However, adjusting the hydrophobic bilayer thickness by cholesterol is more favorable than by long-chain phospholipids (*3*). It is likely, therefore, that cholesterol has effects on the Na,K-ATPase other than by adjusting the hydrophobic thickness of the bilayer. Indeed, cholesterol is important for the formation of the so-called liquid-ordered (l_o) lipid phase, which may be important to support optimal Na,K-ATPase activity.

The high cholesterol content of the shark membrane preparation and the mean phospholipid acyl chain length of about 18 are remarkably close to the conditions shown to be optimal for supporting maximal turnover of the Na,K-ATPase (*1*). Previous comparisons of fatty acids from microsomal membranes of mammals and ectotherms reveal greater levels of polyunsaturation in mammalian tissues, with the brain having a high content of $n - 3$ fatty acids, while the kidney has high $n - 6$ content (*34*). The brain is generally the most polyunsaturated organ in most species, and the rectal gland has a composition (unsaturation index and chain length) that is quite similar to that of the rat brain.

The present findings demonstrate that DHA-containing phospholipids significantly inhibit Na,K-ATPase activity especially in the absence of cholesterol, indicating effects of some global membrane structure properties such as hydrophobic thickness and "fluidity" on intrinsic enzyme functions. In keeping with this, it is known that PUFA-containing phospholipids have an increased cross-sectional area due to their very flexible structure and thus should decrease bilayer thickness. The effect of PUFAs on bilayer hydrophobic thickness could be important per se for the inhibitory effects of polyunsaturated phospholipids on Na,K-ATPase turnover (Figure 3), since quite dramatic decreases in enzyme activity were previously observed by decreasing the monounsaturated acyl chain length from 18:1 (∼27 Å) to 16:1 (∼23 Å) (*3*). It is, however, difficult to predict how addition of 10 mol % polyunsaturated phospholipids will affect the bilayer hydrophobic thickness locally around the Na,K-ATPase.

In a bilayer system consisting primarily of monounsaturated phospholipids such as 18:1 PC or 18:1 PC plus cholesterol, addition of DHA-containing phospholipids is likely to induce liquid-disordered phases (l_d). As seen from Figure 3 the inhibitory effect of polyunsaturated phospholipids such as 16:0-22:6 PC and 22:6 PC is much more

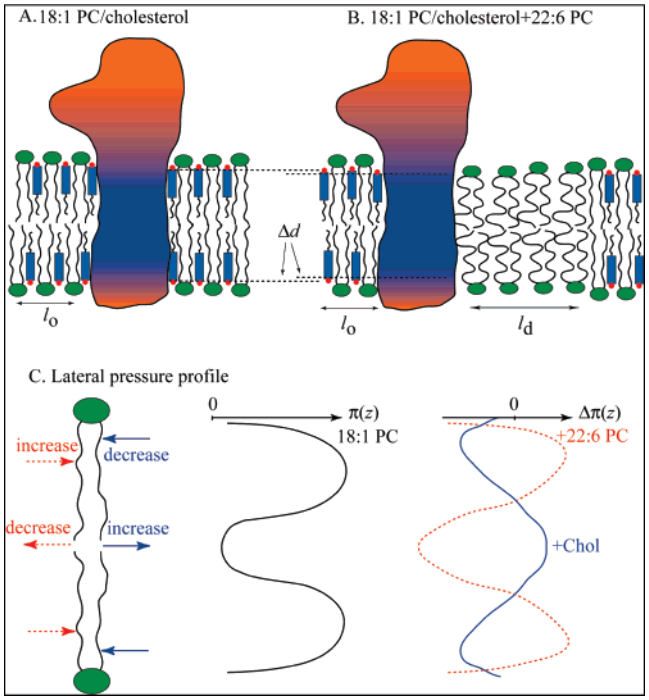

FIGURE 7: Cartoon showing the lateral phase separation of cholesterol and 22:6 PC for reconstituted Na,K-ATPase (A, B) and the effects of cholesterol and 22:6 PC on the lateral pressure profiles (C). (A) Liposome bilayer composed of 18:1 PC/cholesterol where the l_o domain facilitates interaction of cholesterol and protein. (B) Liposome bilayer after inclusion of 22:6 PC where the DHA-rich l_d domain excludes cholesterol and decreases the bilayer hydrophobic thickness (Δd). (C) Lateral pressure profile ($\pi(z)$) through an 18:1 PC bilayer (black profile) and the (much smaller) change in pressure ($\Delta\pi$) when DHA-rich phospholipids such as 22:6 PC (red stippled profile) or cholesterol (blue profile) are added (*31*, *37*).

pronounced in the absence of cholesterol than in the presence of 40 mol % cholesterol. This might suggest that an l_o phase is essential for optimal Na,K-ATPase activity and that cholesterol counteracts DHA inhibition by promoting the l_o phase instead of the l_d phase initiated by DHA. 16:0-22:6 PC and especially 22:6 PC are expected to pack poorly with other phospholipids and especially with the rigid steroid moiety of cholesterol due to the large packing free volume of DHA owing to the high disorder of the DHA chains. This probably explains the propensity of cholesterol and DHA to laterally phase separate into l_o domains of DHA-poor/cholesterol-rich and l_d domains of DHA-rich/cholesterol-poor microdomains (*10*, *35*). Such phase separation could partially exclude cholesterol from the Na,K-ATPase and thus lead to inhibition (Figure 7). Indeed, in a recent investigation Yethiraj and Weisshaar (*36*) suggested a model by which the distribution of proteins relative to lipid microdomains may be regulated. In this, integral membrane proteins may help to stabilize l_o/l_d domain interfaces by their localization preferentially at interdomain boundaries. Such a propensity for interdomain localization of proteins results when the interaction of the l_o-domain (cholesterol)- and l_d-domain (22:6 PC)-forming lipids with the protein is more favorable than that with each other. This situation is illustrated in Figure 7A,B, where the localization of Na,K-ATPase is shifted to the l_o/l_d domain interface by addition of polyunsaturated phospholipids. The spatial position of the Na,K-ATPase need not be permanently confined to such interdomain localization; rather panels A and B are "snapshots" of two extreme equilibrium states of the multidomain structure.

A similar mechanism could explain the increased apparent Na^+ affinity induced by DHA-containing phospholipids in 18:1 PC/cholesterol proteoliposomes (Figure 4), as well as the effects on the K^+ inhibition observed at low ATP concentrations (Figure 5), properties that are both affected by cholesterol interaction with Na,K-ATPase (*1*−*3*). Indeed, DHA-induced phase separation could decrease cholesterol interaction with Na,K-ATPase and as a result increase the apparent Na^+ affinity (Figure 4B) as well as prevent the K^+ inhibition observed at low ATP-concentrations in the presence of cholesterol (Figure 5B).

Such an explanation does not necessary imply direct interaction of cholesterol or polyunsaturated phospholipids by actual binding to the Na,K-ATPase. The effect could also be indirect, exerted, e.g., via changes in the lateral pressure profile ($\pi(z)$) in the lipid domains (*31*) (Figure 7C). The lateral pressure, which varies by several hundreds of atmospheres through the bilayer, can couple to protein function via the stress it exerts on the protein transmembrane domain. Indeed, DHA with its six double bonds supports curved bilayer structures, and addition of polyunsaturated phospholipids to the 18:1 PC bilayer changes the curvature stress by redistributing and increasing the pressure from a broad region near the center of the bilayer to regions closer to the aqueous interfaces near the head groups (*31*, *37*). The effect of cholesterol is opposite. Here the pressure decreases (by tenths of an atmosphere) near the interfaces with a compensating pressure increase in the bilayer center (*31*, *37*) (cf. Figure 7C).

Cholesterol has previously been demonstrated to stabilize the E_1 conformation of Na,K-ATPase (*1*). As seen from Figure 6 this is also observed in the present investigation. Furthermore, DHA-containing phospholipids have the same or even stronger tendency of stabilizing E_1 (Figure 6). That both l_o-domain- and l_d-domain-forming lipids stabilize the same E_1 conformation seems to indicate that this effect is linked to bilayer properties other than "fluidity", e.g., effects on the bilayer hydrophobic thickness. DHA has previously been demonstrated to influence the conformation and activity of proteins such as the light-sensitive rhodopsin in the rod outer segment and explained by a model proposed by Mitchel and Litman where PUFAs induce lateral phase separation into l_o domains of DHA-poor/cholesterol-rich and l_d domains of DHA-rich/cholesterol-poor microdomains (*14*, *35*). The retinal rod outer segment membranes are known to have almost 50% DHA. Other non-lamellar-forming lipids, such as phospholipids with PE head groups, have similar effects on rhodopsin conformations, suggesting that effects other than direct binding of these lipids are the important mechanism. Finally, it was recently found that PUFAs and cholesterol both decrease the orientational polarizability of membrane surfaces due to reorientation of lipid dipolar groups (*38*). It is feasible that such changes in membrane electrical properties could lead to stabilization of certain pump conformations, as also previously suggested (*1*).

In conclusion, DHA-containing phospholipids such as 16:0-22:6 PC and 22:6 PC inhibit the hydrolytic activity of Na,K-ATPase, especially in the absence of cholesterol. This inhibition of Na,K-ATPase turnover thus seems to be associated with the capability of PUFA to induce lateral

phase separation of l_d domains comprising DHA-containing phospholipids from l_o domains comprising 18:1 PC/cholesterol, which may partially exclude cholesterol from the Na,K-ATPase interface. The inhibition increases in the order 22:6 PC > 16:0-22:6 PC both in the presence and in the absence of cholesterol. In the presence of cholesterol 16:0-22:6 PC and 22:6 PC both increase the apparent Na^+ affinity and prevent the K^+-inhibition observed at low ATP concentration, suggesting effects on the $E_2 \rightarrow E_1$ transition associated with K^+ deocclusion. Finally, DHA-containing phospholipids and cholesterol both stabilized the E_1 conformation of Na,K-ATPase compared to pure 18:1 PC bilayer, indicating that other changes in the bilayer properties such as changes in the hydrophobic matching or changes in membrane electrical properties are also important.

ACKNOWLEDGMENT

Hanne R. Z. Christensen is kindly acknowledged for expert technical assistance.

REFERENCES

1. Cornelius, F., Turner, N., and Christensen, H. R. (2003) Modulation of Na,K-ATPase by phospholipids and cholesterol. II. Steady-state and presteady-state kinetics, *Biochemistry 42*, 8541−8549.
2. Cornelius, F. (1995) Cholesterol modulation of molecular activity of reconstituted shark Na^+,K^+-ATPase, *Biochim. Biophys. Acta 1235*, 205−212.
3. Cornelius, F. (2001) Modulation of Na,K-ATPase and Na-ATPase activity by phospholipids and cholesterol. I. Steady-state kinetics, *Biochemistry 40*, 8842−8851.
4. Stillwell, W., Shaikh, S. R., Zerouga, M., Siddiqui, R., and Wassall, S. R. (2005) Docosahexaenoic acid affects cell signaling by altering lipid rafts, *Reprod. Nutr. Dev. 45*, 559−579.
5. Stillwell, W., and Wassall, S. R. (2003) Docosahexaenoic acid: membrane properties of a unique fatty acid, *Chem. Phys. Lipids. 126*, 1−27.
6. Huster, D., Arnold, K., and Gawrisch, K. (1998) Influence of docosahexaenoic acid and cholesterol on lateral lipid organization in phospholipid mixtures, *Biochemistry 37*, 17299−17308.
7. Brzustowicz, M. R., Cherezov, V., Caffrey, M., Stillwell, W., and Wassall, S. R. (2002) Molecular organization of cholesterol in polyunsaturated membranes: microdomain formation, *Biophys. J. 82*, 285−298.
8. Brzustowicz, M. R., Cherezov, V., Zerouga, M., Caffrey, M., Stillwell, W., and Wassall, S. R. (2002) Controlling membrane cholesterol content. A role for polyunsaturated (docosahexaenoate) phospholipids, *Biochemistry 41*, 12509−12519.
9. Filippov, A., Orãdd, G., and Lindblom, G. (2007) Domain formation in model membranes studied by pulsed-field gradient-NMR: The role of lipid polyunsaturation, *Biophys. J. 93*, 3182−3190.
10. Zidovetzki, R. (1997) Membrane properties and the activation of protein kinase C and phospholipase A_2, *Curr. Top. Membr. 44*, 255−283.
11. Mahmmoud, Y. A., Vorum, H., and Cornelius, F. (2000) Identification of a phospholemman-like protein from shark rectal glands. Evidence for indirect regulation of Na,K-ATPase by protein kinase C via a novel member of the FXYDY family, *J. Biol. Chem. 275*, 35969−35977.
12. Liu, L., Mohammadi, K., Aynafshar, B., Wang, H., Li, D., Liu, J., Ivanov, A. V., Xie, Z., and Askari, A. (2003) Role of caveolae in signal-transducing function of cardiac Na^+/K^+-ATPase, *Am. J. Physiol. Cell Physiol. 284*, C1550−C1560.
13. Bretscher, M. S., and Munro, S. (1993) Cholesterol and the Golgi apparatus, *Science 261*, 1280−1281.
14. Litman, B. R., and Mitchell, D. C. (1996) A role for phospholipid polyunsaturation in modulating membrane protein function, *Lipids 31*, S193−S197.
15. Mitchell, D. C., Straume, M., and Litman, B. J. (1992) Role of sn-1-saturated,sn-2-polyunsaturated phospholipids in control of membrane receptor conformational equilibrium: effects of cholesterol and acyl chain unsaturation on the metarhodopsin I in equilibrium with metarhodopsin II equilibrium, *Biochemistry 31*, 662−670.
16. Skou, J. C., and Esmann, M. (1988) Preparation of membrane Na^+,K^+-ATPase from rectal glands of Squalus acanthias, *Methods Enzymol. 156*, 43−46.
17. Ottolenghi, P. (1975) The reversible delipidation of a solubilized sodium-plus-potassium ion-dependent adenosine triphosphatase from the salt gland of the spiny dogfish, *Biochem. J. 151*, 61−66.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent, *J. Biol. Chem. 193*, 265−275.
19. Cornelius, F., and Skou, J. C. (1984) Reconstitution of (Na^+ + K^+)-ATPase into phospholipid vesicles with full recovery of its specific activity, *Biochim. Biophys. Acta 772*, 357−373.
20. Cornelius, F. (1988) Incorporation of $C_{12}E_8$-solubilized Na^+,K^+-ATPase into liposomes: determination of sidedness and orientation, *Methods Enzymol. 156*, 156−167.
21. Peterson, G. L. (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicaple, *Anal. Biochem. 83*, 346−356.
22. Cornelius, F. (1995) Phosphorylation/dephosphorylation of reconstituted shark Na^+,K^+-ATPase: one phosphorylation site per alpha beta protomer, *Biochim. Biophys. Acta 1235*, 197−204.
23. Lindberg, O., and Ernster, L. (1956) Determination of organic phosphorus compounds by phosphate analysis, *Methods Biochem. Anal. 3*, 1−22.
24. Aveldano, M. I. (1989) Dipolyunsaturated species of retina phospholipids and their fatty acids, *Colloq. INSERM 195*, 87−96.
25. Lüpfert, C., Grell, E., Pintschovius, V., Apell, H.-J., Cornelius, F., and Clarke, R. J. (2001) Rate limitation of the Na^+,K^+-ATPase pump cycle, *Biophys. J. 81*, 2069−2081.
26. Humphrey, P. A., Lüpfert, C., Apell, H.-J., Cornelius, F., and Clarke, R. J. (2002) Mechanism of the rate-determining step of the Na^+,K^+-ATPase pump cycle, *Biochemistry 41*, 9496−9507.
27. Segall, L., Lane, L. K., and Blostein, R. (2002) New insights into the role of the N terminus in conformational transitions of the Na,K-ATPase, *J. Biol. Chem. 277*, 35202−35209.
28. Clarke, R. J., Apell, H. J., and Kong, B. Y. (2007) Allosteric effect of ATP on Na^+,K^+-ATPase conformational kinetics, *Biochemistry 46*, 7034−7044.
29. Mouritsen, O. G, and Bloom, M. (1993) Models of lipid-protein interactions in membranes, *Annu. Rev. Biophys. Biomol. Struct. 22*,145−71.
30. Jensen, M. Ø., and Mouritsen, O. G. (2004) Lipids do influence protein function-the hydrophobic matching hypothesis revisited, *Biochim. Biophys. Acta 1666*, 205−226.
31. Cantor, R. S. (1999) Lipid composition and the lateral pressure profile in bilayers, *Biophys. J. 76*, 2625−2639.
32. Johannsson, A., Smith, G. A., and Metcalfe, J. C. (1981) The effect of bilayer thickness on the activity of (Na^+ + K^+)-ATPase, *Biochim. Biophys. Acta 641*, 416−421.
33. Yeagle, P. L., Young, J., and Rice, D. (1988) Effects of cholesterol on (Na^+,K^+)-ATPase ATP hydrolyzing activity in bovine kidney, *Biochemistry 27*, 6449.
34. Wu, B. J., Else, P. L., Storlien, L. H., and Hulbert, A. J. (2001) Molecular activity of Na^+/K^+-ATPase from different sources is related to the packing of membrane lipids, *J. Exp. Biol. 204*, 4271−4280.
35. Mitchel, D. C., and Litman, B. J. (1998) Effect of cholesterol on molecular order and dynamics in highly polyunsaturated phospholipid bilayers, *Biophys. J. 75*, 896−908.
36. Yethiraj, A., and Weisshaar, J., C. (2007) Why are lipid rafts not observed in vivo? *Biophys. J. 93*, 3113−3139.
37. Gullingsrud, J., and Schulten, K. (2004) Lipid bilayer pressure profiles and mechanosensitive channel gating, *Biophys. J. 86*, 3496−3509.
38. Le Goff, G., Vitha, M. F., and Clarke, R. J. (2007) Orientational polarisability of lipid membrane surfaces, *Biochim. Biophys. Acta 1768*, 562−570.

BI702128X