Modulation of Pig Kidney Na^+/K^+-ATPase Activity by Cholesterol: Role of Hydration†

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ABSTRACT: Cholesterol is known to affect the activity of membrane-bound enzymes, including Na^+/K^+-ATPase. To gain insight into the mechanism of cholesterol's effect, we have used various hydrophobic fluorescent probes which insert into different regions of the membrane bilayer and report on the degree of hydration of their environment. Specifically, we have measured the generalized polarization of Laurdan and the lifetime of DPH and derivatives of DPH inserted into membranes from pig kidneys enriched in Na^+/K^+-ATPase. Spectral measurements were also carried out on these membranes after modification of their cholesterol content. The generalized polarization of Laurdan increased with increasing cholesterol, showing an abrupt modification at the native cholesterol content. The fluorescence lifetimes of DPH and the DPH derivatives were analyzed using a distribution model. The center value of these lifetime distributions and their widths also changed with increasing cholesterol. One DPH derivative, DPH-PC, showed a minimum value for the lifetime center at the native cholesterol concentration, whereas the other derivatives showed a maximum value for the lifetime center at that cholesterol concentration. DPH-PC is known to sense the protein−lipid interface, whereas the other derivatives sense the bulk lipid phase. These data suggest that hydration at the protein−lipid interface is maximal at the native cholesterol concentration as is the enzymatic activity. Hydration at the protein−lipid interface is therefore proposed to be required for activity. These results are in agreement with current models of membrane dynamics and thermodynamics of protein function.

The activities of a number of membrane associated enzymes are known to be modulated by the physical state of the membrane lipid matrix (*1*−*5*). Na^+/K^+-ATPase[1] is one such enzyme, its activity being directly related to membrane fluidity (*1*, *6*−*8*). Sutherland et al. (*9*) have shown that "cryptic" Na^+/K^+-ATPase could be uncovered in the apical membranes of hepatocytes by fluidizing with the membrane mobility agent A_2C [2-(2-methoxyethoxy)ethyl-8-(*cis*-2-*n*-octyl-cyclopropyl)octanoate]. Similar results were found using the chemical fluidizers benzyl alcohol and Triton WR-1339.

Together with phospholipids, cholesterol is ubiquitous in animal cell membranes and is one of the major modifiers of membrane structure and dynamics. Changes in the cholesterol content of biological membranes are known to alter the properties of the lipid lamella and hence influence the function of a variety of membrane-associated enzymes (*10*, *11*). The role of membrane cholesterol in modulating the physical properties of the bilayer has been examined mainly in phospholipid:cholesterol model systems using physical methods such as ^2H NMR spectroscopy (*12*−*15*), EPR (*16*−*19*), neutron scattering (*12*, *20*, *21*), and steady state and time-resolved fluorescence spectroscopy (*22*−*30*). Abrupt variations of several structural and dynamic parameters have been found at specific cholesterol concentrations. These variations have been interpreted as arising from specific complexes between phospholipids and cholesterol in molar ratios of 3:1, 2:1, and 1:1. Ipsen et al. (*31*, *32*) proposed an alternative model that does not require specific complexes between cholesterol and phospholipids. The essential aspect of this model is the different interaction free energies between all possible pair combinations of chain-ordered phospholipids, chain-disordered phospholipids, and cholesterol.

Phase diagrams of cholesterol in different phospholipids have been constructed from the aforementioned experimental techniques and from theoretical studies (*31*). To a first approximation, there is almost ideal mixing between cholesterol and phospholipids up to 6 mol % cholesterol. Cholesterol levels higher than 6 mol % have a disordering effect on the gel phase and an ordering effect on the liquid

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[1] Abbreviations: Na^+/K^+-ATPase, sodium and potassium activated adenosine triphosphatase (EC 3.6.1.37); BSA, bovine serum albumin; PC, Egg phosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluenesulfonate; TMAP-DPH, *N*-*p*-(6-phenyl-(1,3,5-hexatrienyl(phenyl(propyl))))trimethylammonium,*p*-toluene sulfonate; DPH-propionic acid, 3-(*p*-(6-phenyl-9-1,3,5-hexatrienyl)phenyl-propionic acid; DPH-PC, 2-3-(diphenyl hexa trienyl) propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phospho-choline; Laurdan, 2-(dimethylamino)-6-lauroylnaphthalene; pyrene-PC, 1-palmitoyl-2-(10-pyrenyl)decanoyl-*sn*-glycerol-3-phosphatidylcholine; A_2C, 2-(2-methoxyethoxy)ethyl-8-(*cis*-2-*n*-octyl-cyclopropyl)octanoate; SUV, small unilamellar vesicles; LUV, *Large* unilamellar vesicles; GP, generalized polarization.

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crystalline phase. Specifically, in the gel phase, lateral diffusion and axial rotation are increased by cholesterol. In the liquid crystalline phase, axial rotation is decreased. At least three spectroscopic techniques with quite different characteristic time scales (NMR, ESR, and fluorescence) have failed to detect distinct environments in a region of the phase diagram where separated domains are expected, and hence the early hypothesis of the coexisting domains seems unsupported (*28*). However, the possibility remains that these theoretical domains are very small, composed of a few phospholipid molecules, and/or are fluctuating more rapidly than previously anticipated (*28*).

Studies performed with two guest molecules in phospholipid bilayers indicated a peculiar behavior of the excimer to monomer fluorescence ratio of pyrene-PC and of the fluorescence intensity of dehydroergosterol when these probes were added to model membranes in the liquid-crystalline state (*33*−*35*). At some specific concentrations of pyrene-PC and dehydroergosterol, the excimer-to-monomer ratio of the former and the fluorescence intensity of the latter are drastically decreased. These results were interpreted in terms of the hexagonal super-lattice theory proposed by Somerharju and co-workers (*36*, *37*). This theory proposes that (1) the acyl chains of phospholipids form a hexagonal host lattice; (2) pyrene-PC and dehydroergosterol, being bulky guest elements, cause steric hindrance in the host lattice; and (3) at certain mole fractions, the guest molecules maximally separate in order to minimize the total free energy and as a result are regularly distributed into hexagonal super-lattices within the hexagonal lattice of the bilayer matrix.

The above model proposes that the lateral organization of pyrene-PC/phospholipid and of sterol/phospholipid mixtures are modulated by minute changes in lipid composition on either side of a critical mole fraction. Slight concentration deviations cause an increase in the ratio of irregular to regular areas and an increase in membrane free volume. Consequently, certain membrane functions, especially those requiring free volume for their normal activity, should be modulated by slight variations of bulky molecules such as cholesterol (*35*).

Fluorescence studies performed by Parasassi et al. (*29*, *30*) using Laurdan (2-dimethyl-amino-6-lauroylnaphthalene) in binary and ternary systems composed of vesicles of phospholipids and cholesterol showed that specific cholesterol concentrations induced abrupt dynamic changes in the membrane bilayer. Some incremental additions of cholesterol cause a much larger (or smaller) effect than the equivalent incremental addition when the absolute cholesterol concentration is different. Their hypothesis is that ordered microdomains are formed in the hydrophobic matrix at critical cholesterol concentrations. In these microdomains, water content is changed and the diffusional properties are altered. The critical cholesterol concentrations, identified by analysis of Laurdan's emission and excitation spectral shifts, are in close agreement with the particular concentrations of pyrene-PC that yield discontinuities in the excimer-to-monomer ratio (*33*, *34*).

Some information has appeared concerning the effects of cholesterol on mammalian cell membrane enzymes. Particularly in Na^+/K^+-ATPase, early experiments showed inhibition of activity by high levels of membrane cholesterol (*38*, *39*). In a study of cholesterol's effect on Na^+/K^+-ATPase activity in bovine kidney basolateral membranes, Yeagle et al. (*7*) reported that maximal activity is observed at the native membrane cholesterol content. When the cholesterol content of the membranes was above or below that found in the native membrane, the enzyme activity was decreased.

To further study the effect of cholesterol on structural and dynamic properties of the membrane in which Na^+/K^+-ATPase resides and its correlation with the enzyme activity, we have utilized a series of fluorescent probes that localize the fluorescent moiety at different locations in the bilayer and/or yield information on different physical events or processes. Specifically, we have employed DPH (1,6-diphenyl-1,3,5-hexatriene), derivatives of DPH and Laurdan. The lifetime of DPH and the spectral properties of Laurdan (as judged by the generalized polarization function) are sensitive to the dielectric constant of their environments and are therefore sensitive to the presence of water. Both steady-state and time-resolved fluorescence methods were utilized.

MATERIALS AND METHODS

Egg phosphatidylcholine (PC), cholesterol, ATP, EDTA, histidine, sucrose, BSA, NADH, pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, HEPES, and sodium dodecyl sulfate were obtained from Sigma (St. Louis, MO). 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-(4trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluenesulfonate (TMA-DPH), *N*-*p*-(6-phenyl-(1,3,5-hexatrienyl(phenyl(propyl))))trimethylammonium,*p*-toluenesulfonate (TMAP-DPH), 3-*p*-(6-phenyl-9-1,3,5-hexatrienyl)phenylpropionic acid, (DPH-propionic acid), 2−3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phospho-choline (DPH-PC), and 2-(dimethylamino)-6-lauroylnaphthalene (Laurdan) were obtained from Molecular Probes (Eugene, OR). All other chemicals used were of analytical or spectroscopic grade and obtained from commercial sources.

Membrane Preparation. Na^+/K^+-ATPase-rich membranes were prepared from the outer medulla of pig kidney using the procedure of Jørgensen (*40*). This method yields partially purified enzyme in the form of membrane fragments rich in Na^+/K^+-ATPase, containing about 0.6 mg of phospholipid and 0.2 mg of cholesterol/mg of protein (*40*, *41*). The specific ATPase activity was determined by the pyruvate kinase/lactate dehydrogenase assay (*42*) and the protein concentration by the method of Lowry et al. (*43*) using bovine serum albumin as a standard. Phospholipid content was determined as inorganic phosphate using a kit from Boehringer (Mannheim, Germany) based on the method of Zilversmith and Davis (*44*). The suspension of Na^+/K^+-ATPase-rich membrane fragments (about 5 mg/mL protein) in buffer (30 mM histidine, pH 7.4, 1 mM EDTA, and 3 mM ATP) was quickly frozen in liquid nitrogen in samples of 500 uL and stored at −80 °C.

Membrane Lipids Extraction. Total membrane lipids were extracted from membrane preparations according to the method of Bligh and Dyer (*45*).

Preparation of Phospholipids Vesicles. Small unilamellar vesicles (SUV) were prepared according to the procedure of Yeagle and Young (*46*). A multilamellar liposome suspension of egg phosphatidylcholine (PC) at a concentra-

tion of 4 mg/mL was sonicated at 5 °C for 20 min in cycles of 30 s with a Braun model 1550 Labsonic tip sonicator. Metal dust and large lipid aggregates were separated by centrifugation at 40 000 rpm for 30 min in a Beckman ultracentrifuge (Ti 42.1 rotor). When cholesterol was added to SUVs, it was done at a 1:1 molar ratio with PC or at the native cholesterol content found in the actual membrane preparation. Large unilamellar vesicles (LUVs) were prepared by extrusion of a frozen and thawed multilamellar liposome suspension of membrane total lipids at a concentration of 4 mg/mL through two stacked polycarbonate filters of 400 nm pore size (Costar, Nucleopore) employing nitrogen pressure at 37 °C.

Membrane Cholesterol Modification. The procedure of Yeagle et al. (*7*) was used to alter the cholesterol content of Na^+/K^+-ATPase-rich membrane fragments. Mixtures of equal volumes of PC SUVs (4 mg/mL phospholipid) and of membrane suspension (3.5 mg/mL protein) were incubated at 37 °C, under nitrogen, in a shaking water bath for different periods according to the desired cholesterol modification; however, in every set of experiments all samples were kept at 37 °C for the same time. SUVs of pure PC and with 1:1 PC:cholesterol ratio were used to decrease and increase the cholesterol level, respectively. Control samples were incubated with SUVs containing the native cholesterol level found in the actual membrane preparation, to account for changes in the activity due solely to the incubation. SUVs were separated from membranes by centrifugation at 40 000 rpm for 30 min in a Beckman ultracentrifuge (Ti 42.1 rotor). The supernatant solution containing the SUVs was discarded. Membrane cholesterol content was determined by a cholesterol oxidase assay as previously described (*47*).

Steady-State Fluorescence Measurements. Fluorescence emission spectra were obtained with a Spex Fluorolog photon counting spectrofluorometer interfaced to a personal computer for data collection. The sample temperature was controlled using an external circulating water bath and measured at the sample cell prior to and after each measurement using a digital thermometer. A xenon arc lamp was utilized as light source.

Steady-state polarization measurements were performed in the "L" configuration with an ISS GREG-200 spectrofluorometer interfaced to a personal computer for data collection, using Glan Thompson prism polarizers in both the excitation and emission paths. The emission was measured using Schott KV-399 and WG-420 band-pass filters, which themselves showed negligible fluorescence. Blank subtraction was performed using unlabeled samples.

Time-Resolved Fluorescence Measurements. Measurements were performed at the Laboratory for Fluorescence Dynamics, Department of Physics, University of Illinois at Urbana-Champaign, IL, using a multifrequency phase and modulation fluorometer. In this instrument the frequency modulation of the excitation source is realized using the harmonic content approach (*48*, *49*). The exciting light was from a Coherent Nd:YAG mode-locked laser pumping a rhodamine dye laser. The dye laser was tuned to 690 nm, which was then frequency doubled to 345 nm. Emission was observed through a Schott KV-399 long band-pass filter and a Corning TB-400 band-pass filter to isolate emission from DPH (at 395−500 nm) and block scattered light. The exciting light was polarized parallel to the vertical laboratory axis,

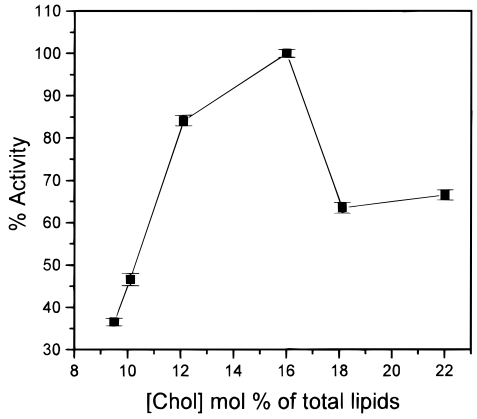

FIGURE 1: Relationship between Na^+/K^+-ATPase activity of pig kidney basolateral membrane and membrane cholesterol content. Each point represents an average of data in duplicate and standard error. Native membrane cholesterol content corresponds to 16 mol % of total lipids.

and the emission was viewed through a Glan-Thompson polarizer oriented at 55° (*50*). Phase and modulation values were obtained as previously described (*51*−*53*). In this study, the frequencies utilized ranged from 3 to 110 MHz. Dimethyl-POPOP in ethanol was used as a reference ($\tau = 1.45$ ns) for lifetime measurements.

Data Analysis. The phase and modulation data were analyzed using the Globals Unlimited software (Urbana, IL). The lifetime data were analyzed either by assuming a sum of discrete exponentials (*52*) or by using continuous distribution models which assumed either Lorentzian or Gaussian distributions (*54*−*56*). The goodness of fit of the data to a particular model was judged by the value of the reduced chi-square (χ^2). Analyses were performed using a constant, frequency-independent standard deviation of 0.2° for phase angles and 0.004 for modulation ratios. Correlated error analysis (i.e., one parameter is varied near the χ^2 minimum while the other parameters are all free) were performed on the lifetimes, and the rigorous 67% confidence limits are reported for each parameter.

Laurdan Fluorescence. The generalized polarization of Laurdan was measured as previously described (*57*−*59*). Briefly, the generalized polarization (GP) is defined as

$$\mathrm{GP} = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$$

where I_{440} and I_{490} are the fluorescence intensities at 440 nm (gel phase emission maximum) and 490 nm (liquid crystalline phase emission maximum), respectively, upon excitation at 360 nm.

RESULTS

Figure 1 shows that the Na^+/K^+-ATPase activity, at 37 °C, in sets of samples with different cholesterol contents were in agreement with the reported results of Yeagle et al. (*7*). That is, the enzyme activity was inhibited when the membrane cholesterol content was increased above that found in the native membrane and when it was decreased below that content. The phospholipid/ protein ratio of membranes was determined in order to detect incidental fusion between the SUV and membrane fragments, during cholesterol content

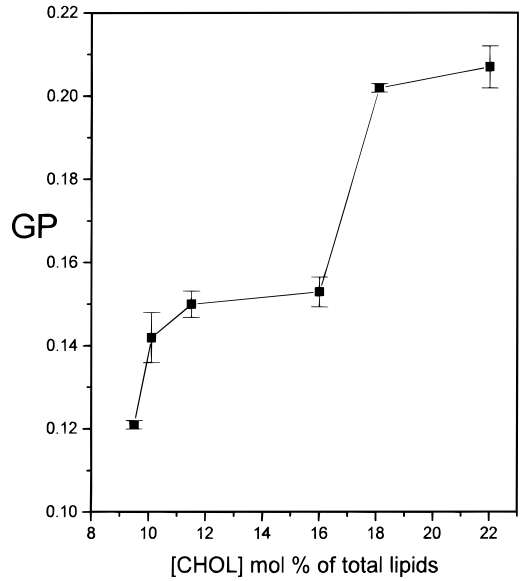

FIGURE 2: General polarization of Laurdan incorporated into Na^+/K^+-ATPase rich membranes as a function of membrane cholesterol content at 37 °C with excitation at 360 nm. Each point represents an average of data in duplicate and standard error. Native membrane cholesterol content corresponds to 16 mol % of total lipids.

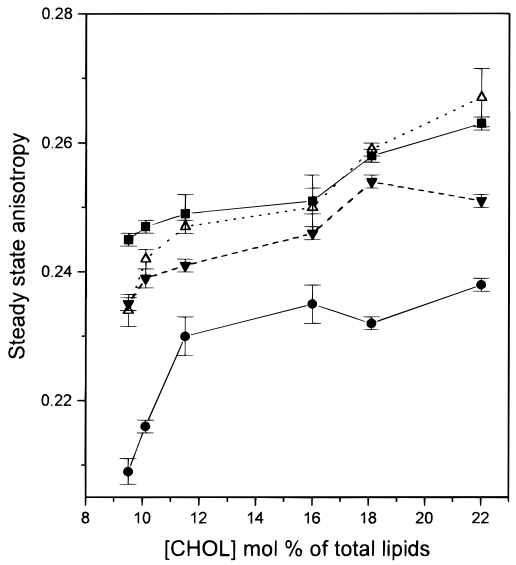

FIGURE 3: Steady-state fluorescence anisotropy of DPH derivates probes incorporated into Na^+/K^+-ATPase rich membranes as a function of membrane cholesterol content at 37 °C with excitation at 360 nm [(●) DPH; (■) TMA-DPH; (△) TMAP-DPH; (▼) DPH propionic acid]. Each point represents an average of data in duplicate and standard error. Native membrane cholesterol content corresponds to 16 mol % of total lipids.

modification, resulting in a change in phospholipid content. However, no significant changes were found under the conditions employed.

Laurdan emission spectra were measured at 37 °C in Na^+/K^+-ATPase-rich membranes with altered cholesterol content. In Figure 2, the generalized polarization (GP) of Laurdan at 37 °C is shown as a function of membrane cholesterol content. As shown, the increase of cholesterol concentration results in an increase of the GP value. Interestingly, the increase of GP values is not monotonic with the increase in cholesterol content. A plateau in the GP value is observed where the cholesterol concentration matches the native cholesterol content, followed by an abrupt increase in the GP value.

Figure 3 shows the steady-state polarization values of the various DPH derivatives with increasing cholesterol content. The polarization values increase with increasing cholesterol and show a slight plateau around the native cholesterol value. This plateau is similar to that seen for the Laurdan GP values shown in Figure 2, though not as dramatic.

The lifetimes of DPH derivative probes were measured at 37 °C in Na^+/K^+-ATPase-rich membrane preparations with different cholesterol contents. In all cases, a two-component model consisting of one discrete exponential and one Lorentzian distribution gave fits to the data comparable to those with two Lorentzian distributions (with or without fixed components) and superior to three-component models (either three Lorentzian distributions or three discrete exponentials). A fixed discrete component of 0.001 ns was used to account for scattered light. For all probes studied, the distributed component accounted for the majority ($\geq$99%) of the emission as judged by the fractional intensity contributions. The values recovered from the analysis are given in Table 1. The fluorescence lifetime obtained from the Lorentzian distribution center for free DPH and the ionic derivative probes showed a similar pattern of change with cholesterol content, i.e., the lifetimes decreased when the cholesterol content of the membrane was either increased or decreased from that found in native membranes. The contrary was found for DPH-PC, i.e., the lifetime of this probe showed a minimum value in membranes with the native cholesterol content, increasing when cholesterol content was decreased or increased. TMA-DPH, which localizes the DPH moiety in a shallow depth where water penetration is significant, showed the minimum change except at the lowest cholesterol concentration. The lifetime distribution widths of all probes also showed as a function of membrane cholesterol content a pattern of change with a maximum at the native cholesterol content. However, these changes were not significant for TMAP-DPH or DPH-PC when the cholesterol content was decreased.

The center lifetime values and widths of the distributions for all probes incorporated in Na^+/K^+-ATPase rich membranes and in large unilamellar vesicles (LUVs) prepared with total lipids extracted from these membranes are given in Table 2. The lifetime values obtained for these probes in LUVs showed the expected sequence according to the fluorophore moiety depths, but with small differences between the ionic probes. These results are in agreement with those of Kaiser and London (*60*) who suggested that the effect of derivatizing DPH with a charged anchoring group results in only a modest change in DPH depth. Interestingly, DPH-PC showed a lower lifetime value in membranes than in LUVs, contrary to DPH, TMAP-DPH and DPH-propionic acid, which showed higher lifetime values in membranes than in LUVs. (TMA-DPH showed only a minimal decrease.)

Another interesting difference shown by DPH-PC is related to the lifetime distribution width (Table 2). For the other probes, the analysis gave a significant distribution width both in membranes and in LUVs. For DPH-PC, a significant distribution width was obtained only in membranes, whereas in LUVs the distribution width was the mathematical lower limit of the model (0.005), suggesting discrete character, and

Table 1: Lifetimes of DPH Derivative Probes in Pig Kidney Na^+/K^+-ATPase Rich Membranes, Effect of Membrane Cholesterol Content Modification, and Two Component Analysis with One Lorentzian Distribution and One Discrete Component[a]

membrane cholesterol content	center (ns)	width (ns)	f_1	χ^2
DPH				
7.5	9.26 (±0.03)	1.06 (+0.13, −0.15)	0.99	0.42
7.8	9.40 (+0.03, −0.08)	1.24 (+0.14, −0.16)	0.99	0.27
15.0[b]	9.76 (+0.06, −0.05)	1.48 (+0.20, −0.25)	1.00	0.48
17.2	9.37 (±0.02)	1.18 (+0.09, −0.10)	0.99	0.15
21.0	9.53 (±0.02)	1.01 (+0.10, −0.11)	0.99	0.17
TMA-DPH				
7.5	5.58 (±0.03)	0.73 (+0.11, −0.12)	0.99	0.43
7.8	6.03 (±0.02)	0.65 (+0.09, −0.11)	0.99	0.29
15.0[b]	6.20 (±0.03)	1.01 (+0.11, −0.12)	0.99	0.44
17.2	6.04 (±0.03)	0.73 (+0.13, −0.15)	0.99	0.68
21.0	6.05 (±0.03)	0.69 (+0.12, −0.14)	0.99	0.63
TMAP-DPH				
7.5	7.23 (±0.02)	0.41 (+0.06, −0.07)	1.00	0.29
7.8	7.30 (±0.02)	0.39 (+0.06, −0.07)	1.00	0.24
15.0[b]	8.06 (±0.04)	0.44 (+0.13, −0.20)	1.00	0.17
17.2	7.38 (±0.01)	0.37 (+0.03, −0.06)	1.00	0.12
21.0	7.47 (±0.03)	0.31 (+0.06, −0.07)	1.00	0.19
DPH-propionic acid				
7.5	6.47 (±0.03)	0.73 (+0.11, −0.13)	0.99	0.48
7.8	6.47 (±0.03)	0.80 (+0.13, −0.14)	0.99	0.58
15.0[b]	7.03 (±0.03)	1.12 (+0.12, −0.14)	1.00	0.59
17.2	6.67 (±0.02)	0.67 (+0.11, −0.12)	0.99	0.29
21.0	6.51 (±0.03)	0.76 (+0.11, −0.12)	0.99	0.42
DPH-PC				
7.5	7.36 (±0.04)	0.85 (+0.15, −0.18)	0.99	0.62
7.8	7.26 (+0.03)	0.89 (+0.13, −0.13)	0.98	0.39
15.0[b]	7.11 (±0.04)	1.00 (+0.14, −0.16)	1.00	0.68
17.2	7.49 (±0.02)	0.52 (+0.06, −0.07)	0.99	0.07
21.0	7.65 (±0.03)	0.64 (+0.13, −0.16)	0.99	0.44

[a] Center refers to the center of the Lorentzian distribution; width refers to the full width at half-maximum for the distribution, and f_1 corresponds to the fractional contributions to the intensity of the distributed component. The minor discrete component was fixed at 0.001 ns to account for scattered light. The reduced chi-square (χ^2) value corresponds to the fit of the phase and modulation data to the model. The errors given in parentheses represent the correlated 67% confidence limits of the reduced χ^2. [b] Native membrane cholesterol content

Table 2: Lifetimes of DPH Derivative Probes in Pig Kidney Na^+/K^+-ATPase Rich Membranes and in Large Unilamellar Vesicles (LUV) Prepared with Lipids Extracted from the Same Native Membranes and Two Component Analysis with One Lorentzian Distribution and One Discrete Component[a]

	center (ns)	width (ns)	f_1	χ^2
DPH				
membranes	9.76 (+0.06, −0.05)	1.48 (+0.20, −0.25)	1.00	0.48
LUV	8.56 (±0.11)	3.73 (+0.41, −0.47)	0.98	1.52
TMA-DPH				
membranes	6.20 (±0.03)	1.01 (+0.11, −0.12)	0.99	0.44
LUV	6.32 (±0.04)	0.92 (+0.17, −0.19)	0.99	1.07
TMAP-DPH				
membranes	8.06 (±0.04)	0.44 (+0.13, −0.20)	1.00	0.17
LUV	6.64 (+0.02, −0.01)	0.75 (+0.02, −0.05)	0.99	0.23
DPH-propionic acid				
membranes	7.03 (±0.03)	1.12 (+0.12, −0.14)	1.00	0.59
LUV	6.42 (±0.04)	0.67 (+0.14, −0.17)	0.98	0.69
DPH-PC				
membranes	7.11 (±0.04)	1.00 (+0.14, −0.16)	1.00	0.68
LUV	8.25 (±0.15)	0.00[b]	1.00	1.38

[a] See footnote to Table 1. [b] The best fit was obtained with a two discrete component model.

the best fit was obtained with a two discrete component model. This result is coincident with that of Ho et al. (*61*).

DISCUSSION

We have studied the relation between pig kidney basolateral membrane Na^+/K^+-ATPase activity modulation and the concomitant change in membrane physical properties produced by cholesterol modifications. Interestingly both types of changes showed biphasic patterns with abrupt modifications at the native cholesterol content. Specifically, the activity of the enzyme was found to have a maximum in membranes with the native cholesterol content, as shown in Figure 1 and in agreement with Yeagle et al. (*7*). In our results, this pattern was found irrespective of the native cholesterol content of a specific preparation, which varied from preparation to preparation.

Because of the wide variety of effects cholesterol has on the physical properties of membranes (*27*), we utilized different probes that sense distinct regions of the bilayer and respond to different physical events in order to study the

possible role of cholesterol in modulating Na^+/K^+-ATPase activity. In this context, it was found that alteration of the basolateral membrane cholesterol content modifies the hydration and dynamics of the lipid membrane matrix at the level of the hydrophobic−hydrophilic interface, i.e., the phospholipid glycerol backbone region, as sensed by Laurdan. The fluorescence spectral shift of this probe reflects its sensitivity to the polarity and dynamics of the environment of its naphthalene moiety during the dipolar relaxation that has been attributed to water molecules being present at the hydrophobic−hydrophilic interface of the membrane bilayer (*62*). The general pattern of Figure 2, showing an increase in the GP parameter with increasing membrane cholesterol, is in agreement with previous findings in artificial lipid vesicles by Parasassi et al. (*29*, *30*). Although the effect on the polarity cannot be isolated from the effect on the molecular dynamics, these authors reported evidence that cholesterol reduces the polarity of phospholipid bilayers and decreases the dipolar relaxation rate of water molecules present at the hydrophobic−hydrophilic interface (*28*). This decrease must be related to a decrease in the dynamics of phospholipid molecules due to the general effect of cholesterol of increasing the bilayer packing, particularly in the liquid-crystalline phase (*30*, *59*). What is significant about the data reported here is that the effect of cholesterol is not monotonic but biphasic, with a plateau in the vicinity of the native cholesterol content and an abrupt increase in the GP value as cholesterol content increases. This characteristic is consistent with the effect of cholesterol on Laurdan GP reported by the same authors (*30*), in binary and ternary systems of synthetic phospholipid vesicles with increasing cholesterol concentrations from 0 to 50 mol %, where several discontinuities were observed. According to their hypothesis, at each cholesterol concentration where a discontinuity was observed, ordered microdomains with different physical properties and water content were formed.

Five DPH derivative probes were utilized here in order to sense different depths within the hydrophobic region of the membrane. The comparison of the lifetime distribution widths of these probes incorporated into membranes and LUVs indicates that DPH-PC senses the protein−lipid interface (Table 2). In agreement with Ho et al. (*61*), the lifetime of DPH-PC was homogeneous (discrete) in LUVs, but heterogeneous (distributed) in membranes. Other probes were heterogeneous when incorporated into both systems. These authors suggested that since DPH-PC is unable to sample the bilayer vertically (the dielectric constant gradient), it only senses heterogeneity in the lateral direction. Therefore, DPH-PC's lifetime in a protein-free vesicle should be homogeneous (discrete), as we have found it to be. The broader width of DPH-PC's lifetime in a membrane is therefore attributed to the protein/lipid interface alone and hence this probe may be used to study this region. The other probes should provide information on the bulk lipid phase preferentially. Fluorescence lifetime measurements are suitable for this purpose considering that the time scale of the excited-state lifetime is at least 2 orders of magnitude faster than the lipid exchange between this interface and the bulk lipid phase (*63*).

The lifetime of DPH is extremely sensitive to the value of the dielectric constant of the medium (*64*). Therefore, the significant decrease in the lifetime value of DPH-PC in the presence of membrane proteins compared to that in LUVs (without proteins) and the corresponding increase of the other probes (DPH, TMAP-DPH, and DPH-propionic acid) suggest that, in the native membranes, water molecules localize preferentially at the lipid−protein interface and away from the bulk lipid phase.

Modification of membrane cholesterol content significantly affects interchain hydration as shown by changes in the lifetimes of DPH derivatives. Importantly, these changes were also nonmonotonic with increasing cholesterol content (Table 1). A clear maximum is observed in the case of DPH, TMAP-DPH, and DPH-propionic acid and a minimum for DPH-PC precisely at the native cholesterol content. (TMA-DPH localizes the fluorophore at a shallow depth where the water penetration is significant and shows minimal change.) Considering the different domains sensed by each probe, the data suggest that membranes with the native cholesterol content have maximum hydration at the protein−lipid interface and minimum hydration in the bulk lipid phase. When the cholesterol content is either increased or decreased with respect to the native content, hydration of the protein−lipid interface decreases while that of the bulk lipid phase increases. Modification of the cholesterol content also seems to affect the heterogeneity of the acyl chain region in a nonmonotonic fashion, showing a maximum value at the native cholesterol level (with the exception of TMAP-DPH, which did not show significant changes).

Results regarding cholesterol's effect on the activity of different membrane proteins reconstituted in lipid vesicles showed abrupt changes in the activity of the protein (*37* and references therein, *65*). Notably, two studies, one on reconstituted shark Na^+/K^+-ATPase (*65*) and another on reconstituted γ-aminobutyric acid (GABA) transporter (*66*), reported similar changes in activity as the Na^+/K^+-ATPase reported here, i.e., a stimulatory effect with low cholesterol concentration and an inhibitory effect at high concentration. The characteristic nonmonotonic changes of these membrane proteins' activity has been rationalized in terms of the superlattice model (*36*, *37*), according to which regularly (superlattice) and irregularly distributed lipid domains coexist in a given membrane containing guest molecules (cholesterol). According to this model, there is an abrupt increase of ordered domains with respect to the more or less randomly organized domains that takes place when a critical guest (cholesterol) concentration is reached. The physical properties and water content of the ordered domains are thus drastically altered. Also according to the superlattice model, the membrane lipid composition would spontaneously tend to that which permits superlattice formation, since each superlattice is thought to represent a local minimum in free energy.

Applying these concepts to the Na^+/K^+-ATPase membranes, at a concentration of cholesterol below or above a critical value, a large fraction of the membrane is more or less randomly organized. Under this condition, a high number of packing defects will exist within the lipid phase where water molecules can fit, thus a high interchain hydration will be observed in the lipid phase. When the critical concentration is reached, an abrupt increase in ordered domains takes place. In this new condition, the lipid phase will have less packing defects and minimal hydration. This lipid arrange-

ment would result in a local enrichment of water molecules at the protein−lipid interface as a place of existing membrane packing defects.

The results of this work suggest that the major effects upon the physical properties of the membrane produced by the cholesterol content modification are the hydration changes in the hydrophobic−hydrophilic interface and the hydrophobic acyl chain region. Importantly, the hydration did not vary smoothly in either region: discontinuities or abrupt changes occurred at the native membrane cholesterol concentration, where the enzyme showed maximal activity.

The presence of water at the protein−lipid interface, first described by Ho and Stubbs (*67*), stressed the possible role of hydration as a factor influencing membrane protein structure and in turn its activity. Investigations on soluble proteins has led to the proposition that water molecules hydrating a protein can act thermodynamically as allosteric ligands, providing an essential energetic contribution to the protein's functional regulation *(68*, *69*). Although the energy change involved in association/dissociation of one water molecule is small, they can dominate interaction energies when many molecules are involved (*70*), suggesting that hydration/dehydration reactions contribute far more to the energetics of conformational changes than previously thought.

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