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Cholesterol modulation of molecular activity of reconstituted shark Na^+,K^+-ATPase

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Abstract

The cholesterol content of liposome bilayers has been varied between 0–40 mol% to study the effects on reconstituted Na^+,K^+-ATPase. The maximum hydrolytic activity of reconstituted Na^+,K^+-ATPase was increased by cholesterol at concentrations above 10 mol% for both the physiological Na^+/K^+-exchange reactions, as well as for the partial reactions Na^+/Na^+-exchange and uncoupled Na^+ efflux. Omission of cholesterol from the liposome bilayer modified the activation by cytoplasmic Na^+, indicating effects on both V_{max} and on the Na^+-affinity. Several other kinetic parameters were found to be strongly influenced as well, most notable the steady-state phosphorylation level, and the characteristics of the phosphorylation/dephosphorylation reactions. These results indicate that cholesterol interacts directly with the Na^+,K^+-ATPase as an essential effector perhaps by affecting its conformational mobility or monomer interaction.

Keywords: ATPase, Na^+/K^+-; Phosphorylation; Dephosphorylation; Reconstitution; Cholesterol

1. Introduction

In the preceding paper cholesterol was noted to drastically effect the steady-state phosphorylation level of reconstituted Na^+,K^+-ATPase. These effects and others of cholesterol are investigated in more detail in the present study.

Cholesterol is an important constituent in the cell membrane and among the biological effects are its impact on membrane microviscosity where it decreases the membrane fluidity. Cholesterol induces an internal positive dipole potential, which enhance the permeability of anions and decrease it for cations [1–3]. Cholesterol increases the degree of motional order of the phospholipids in a membrane, decreasing their motional freedom [4,5]. These are all potential effects that may influence the molecular activity of an integral protein like the reconstituted Na^+,K^+-ATPase. Apart from such indirect effects cholesterol may interact more directly with the Na^+,K^+-ATPase, and it is, e.g., overrepresented in the annulus lipids as compared to the bulk [6]. The hydrolytic activity of Na^+,K^+-ATPase has been shown to be dependent on cholesterol content in the cell membrane [7] and the distribution of phosphoenzyme intermediates are apparently affected by the cholesterol content in bilayers of reconstituted Na^+,K^+-ATPase [8].

In the reconstituted system cholesterol interaction with shark Na^+,K^+-ATPase can be conveniently studied since in this system the bilayer cholesterol content can be easily controlled. Cholesterol is demonstrated to play a crucial role in the kinetics of Na^+,K^+-ATPase, affecting both its activation by Na^+, the phosphorylation/dephosphorylation characteristics, and the steady-state phosphorylation level.

2. Methods

In the present study shark Na^+,K^+-ATPase was reconstituted into liposomes with different cholesterol content (expressed as mol% = mol cholesterol/(mol cholesterol + mol phospholipid)) by varying the initial cholesterol content in the chloroform solution before drying down in the rotary evaporator. The protein/lipid ratio was kept constant at 1:20 [9,10]. The cholesterol content was varied between 0–40% of total lipid, the remaining phospholipid composition was in all cases a constant mixture of PC/PE/PI = 78:19:3. This maximum cholesterol content and phospholipid composition correspond to the cholesterol content and phospholipid composition of solubilized shark Na^+,K^+-ATPase [6].

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Two modes of Na^+,K^+-ATPase activity, apart from the physiological Na^+/K^+-exchange, were investigated and compared using the reconstituted system, the ATP-hydrolysing Na^+/Na^+-exchange [11,12], in which extracellular Na^+ acts as a K^+-congener, and the uncoupled Na^+-mode ($Na^+/0$-exchange) [13,14], in which extracellular alkali cations are absent. Accordingly, proteoliposomes were prepared either to contain 130 mM Na^+, or 260 mM sucrose, besides 2 mM $MgCl_2$ and 30 mM histidine (pH 7.0) that was common in the two preparations.

The methods to measure hydrolytic activity, phosphorylation, dephosphorylation, and distribution of phosphoenzyme intermediates in the reconstituted Na^+,K^+-ATPase have been described in the preceding paper. All measurements were calculated according to determined sidedness of the proteoliposomes by measuring the fraction of inside-out orientated enzyme after reconstitution at varying cholesterol content, determined by functional tests as previously described [15]. The orientation of reconstituted enzyme was independent of the cholesterol content, and this was also the case for the size-distribution of the proteoliposome population as measured using quasi-elastic laser light-scattering (Nicomp, USA).

In the assays only inside-out oriented enzyme is activated by external ATP since non-oriented enzyme is completely inhibited by ouabain in the presence of Mg^{2+} (5 mM) and Pi (1 mM), and the substrate site of right-side out oriented enzyme is shielded inside the proteoliposome. Proteoliposomes could be reopened if requested by addition of 1 mg $C_{12}E_8$ per mg of lipid.

3. Results

Fig. 1 compares how maximum hydrolytic activity of reconstituted Na^+,K^+-ATPase is affected by bilayer cholesterol content for three different modes of exchange reactions. The physiological Na^+/K^+-exchange can be probed if the closed proteoliposomes are reopened by addition of the detergent $C_{12}E_8$. The maximum specific hydrolytic capacity as a function of cholesterol can then be measured in the presence of optimal Na^+ and K^+ (130 mM Na^+, 20 mM K^+, 4 mM Mg^{2+}, and ATP 3 mM) as depicted in Fig. 1A. The activation is maximal for cholesterol values about 20 mol%, with a slight inhibition at 40 mol% cholesterol. Half-maximal activation is at about 4 mol% cholesterol. In Fig. 1B and C the activation curves for ATP-hydrolysis accompanying Na^+/Na^+-exchange and $Na^+/0$-exchange in closed detergent-free vesicles, respectively, are shown. Both modes of exchange show activation by cholesterol with a half-maximal activations of 8 mol% and 16 mol% cholesterol, respectively.

Fig. 2 depicts how the steady-state maximum phosphorylation (EP) varied in Na^+,K^+-ATPase reconstituted into liposomes with increasing cholesterol content during conditions of either Na^+/Na^+-exchange or uncoupled Na^+-efflux ($Na^+/0$-exchange), i.e., in the presence and absence of internal (extracellular) Na^+. In both modes of exchange the curve of steady-state EP level vs. cholesterol concentration is S-shaped (sigmoid). However, in the absence of extracellular Na^+ ($Na^+/0$-exchange) the S-shape of activation is much more pronounced than in the presence of extracellular Na^+ (Na^+/Na^+-exchange). In the case of $Na^+/0$-exchange fitting of a sigmoid curve to the data gives a half-saturation of 18.7 ± 1.0 mol% cholesterol and a Hill coefficient of 6.2 ± 1.4, whereas in the case of Na^+/Na^+-exchange K_m is 6.6 ± 1.2 mol% with a Hill coefficient of 4.6 ± 2.6.

The activation by cytoplasmic Na^+ of hydrolytic activity associated with Na^+/Na^+-exchange and $Na^+/0$-exchange at the two extremes, with either 40 mol% cholesterol, or without cholesterol, are depicted in Figs. 3 and 4. With 40 mol% cholesterol the activation curves both in the case of Na^+/Na^+-exchange and $Na^+/0$-exchange are well

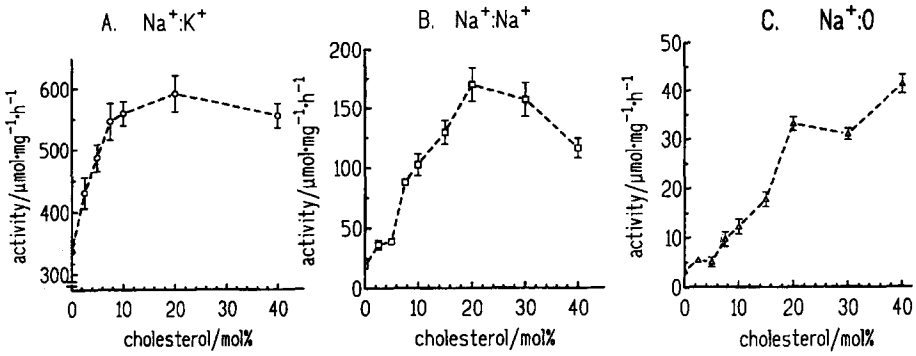

Fig. 1. Hydrolytic activity accompanying different modes of exchange as a function of bilayer cholesterol at 23° C. (A) Maximum Na^+,K^+-ATPase activity of reconstituted Na^+,K^+-ATPase after reopening of the proteoliposomes with $C_{12}E_8$ as a function of cholesterol concentration (mol%) in the liposome bilayer. Na^+,K^+-ATPase activity was measured at optimal conditions: Na^+ 130 mM, K^+ 20 mM, Mg^{2+} 4 mM, ATP 3 mM, and histidine 30 mM (pH 7.0). (B) ATPase activity accompanying Na^+/Na^+-exchange was measured on inside-out reconstituted Na^+,K^+-ATPase with 130 mM Na^+ inside (extracellular), 15 mM Na^+ and 230 sucrose outside (cytoplasmic), ATP 10 μM, and Mg^{2+} 1 mM. C. ATPase activity accompanying uncoupled Na^+-efflux was measured as in B, except internal Na^+ was replaced with 260 mM sucrose during liposome formation. In B and C the non-oriented fractions were inhibited by preincubation with Mg^{2+} (5 mM) and P_i (1 mM) and ouabain (1 mM).

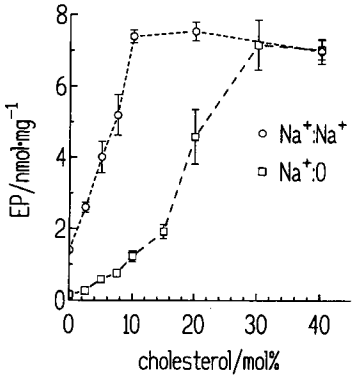

Fig. 2. Steady-state phosphorylation level (EP) for reconstituted Na^+,K^+-ATPase as a function of cholesterol fraction (mol%) in the liposome bilayer. The EP-level was determined after phosphorylation by 25 μM ATP in a medium containing 65 mM Na^+, 4 mM Mg^{2+}, and 30 mM histidine, pH 7.0. The proteoliposomes contained either (Na^+/Na^+-exchange) 130 mM Na^+, 4 mM Mg^{2+}, and 30 mM histidine, or ($Na^+/0$-exchange) 260 mM sucrose, 4 mM Mg^{2+}, and 30 mM histidine. Means $\pm$ S.E. ($n = 3$) are indicated. In both conditions the data could be fitted to a sigmoid curve with K_m, EP_{max}, and n_H of 6.6 $\pm$ 1.2 mol% cholesterol 7.4 $\pm$ 0.5 nmol/mg, and 4.65 $\pm$ 2.6 in the presence of extracellular Na^+, and 18.8 $\pm$ 1.0 mol% cholesterol, 7.3 $\pm$ 0.3 nmol/mg, and 6.2 $\pm$ 1.4. In both cases the Hill equation fitted the data with $r^2 = 0.996$.

fitted to a sigmoid curves with, for Na^+/Na^+-exchange: $K_m = 6.8 \pm 1.0$ mM, $V_{max} = 36.2 \pm 0.5$ μmol/mg per h, and Hill coefficient, $n_H = 2.2 \pm 0.2$; and for $Na^+/0$-exchange: $K_m = 3.8 \pm 1.1$ mM, $V_{max} = 29.5 \pm 0.5$ μmol/mg per h, and Hill coefficient, $n_H = 1.9 \pm 0.2$. In both cases omission of cholesterol in the liposome bilayer induces a progressive inhibition at high Na^+. The effect is most pronounced for $Na^+/0$-exchange (Fig. 3B), which is almost completely inhibited at 100 mM cytoplasmic Na^+ in the absence of cholesterol. The initial part of the activation curves is, however still sigmoid (disregarding the later part with inhibition). The fitting parameters are, Na^+/Na^+-exchange: $K_m = 4.7 \pm 1.1$ mM, $V_{max} = 6.3 \pm 0.3$ μmol/mg per h, and Hill coefficient, $n_H = 1.6 \pm 0.2$; and for $Na^+/0$-exchange: $K_m = 1.2 \pm 1.1$ mM, $V_{max} = 5.5 \pm 0.5$ μmol/mg per h, and Hill coefficient, $n_H = 2.3 \pm 0.6$.

The phosphorylation reaction was also drastically different in the presence and absence of cholesterol. In Fig. 5 the initial rate of phosphorylation in the absence of K^+ is measured on a rapid mixing device for Na^+/Na^+-exchange (Fig. 5A). In the presence of cholesterol the phosphorylation could be described by the sum of two exponentials with slopes indicated by λ and fractions indicated by f. However, in the absence of cholesterol the phosphorylation could be adequately described by a single exponential. Cholesterol apparently induced a significant fraction with an almost 40-times faster phosphorylation rate than for the slower fraction, which has an exponential slope similar to the monoexponential phosphorylation in the absence of cholesterol. The same general picture was found for $Na^+/0$-exchange (Fig. 5B) where 40 mol% cholesterol induced biphasic phosphorylation, whereas in

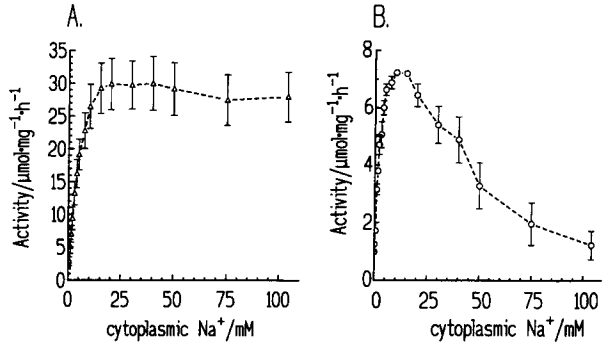

Fig. 3. Cytoplasmic Na^+-activation of reconstituted Na^+,K^+-ATPase in the absence of extracellular alkali cations ($Na^+/0$ exchange) and at 23° C. (A) With 40 mol% cholesterol in the bilayer. (B) Without cholesterol in the bilayer. Activity was measured at varying Na^+ by isosmotic replacement of sucrose, 25 μM ATP, and 1 mM Mg^{2+}. Non-oriented Na^+,K^+-ATPase was inhibited by preincubation with ouabain in the presence of MgP_i.

the absence of cholesterol is was monoexponential, with an exponential slope similar to the slower phase in the presence of cholesterol.

Since inclusion of cholesterol is known to induce a significant internal positive dipole potential in the bilayer [2] it was tested whether or not changes in internal electrostatic potentials could explain the huge effects on the rate of phosphorylation seen with cholesterol. To elucidate such possible electrostatic interactions experiments were performed in which the hydrophobic cations (TPP^+) or anions (TPB^-) were included. These ions are known to adsorb a few tenths of a nm within the bilayer surfaces, producing significant internal potentials, which are either positive (TPP^+) or negative (TPB^-) [16,17]. As seen from Fig. 6A,B the hydrophobic ions did not, however, affect the phosphorylation characteristics significantly neither during $Na^+/0$-exchange (Fig. 6A), nor during Na^+/Na^+-exchange (Fig. 6B).

The spontaneous dephosphorylation was biexponential for all cholesterol contents tested (0–40 mol%) both in the presence of extracellular Na^+ (Na^+/Na^+-exchange, Fig.

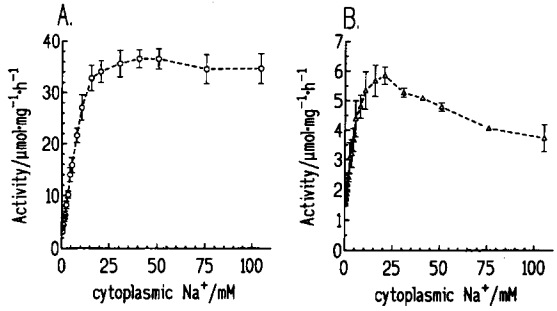

Fig. 4. Cytoplasmic Na^+-activation of reconstituted Na^+,K^+-ATPase in the presence of 130 mM extracellular Na^+, and no other alkali cations (Na^+/Na^+-exchange) and at 23° C. (A) With 40 mol% cholesterol in the bilayer. (B) Without cholesterol in the bilayer. Activity was measured as described in the legend to Fig. 3.

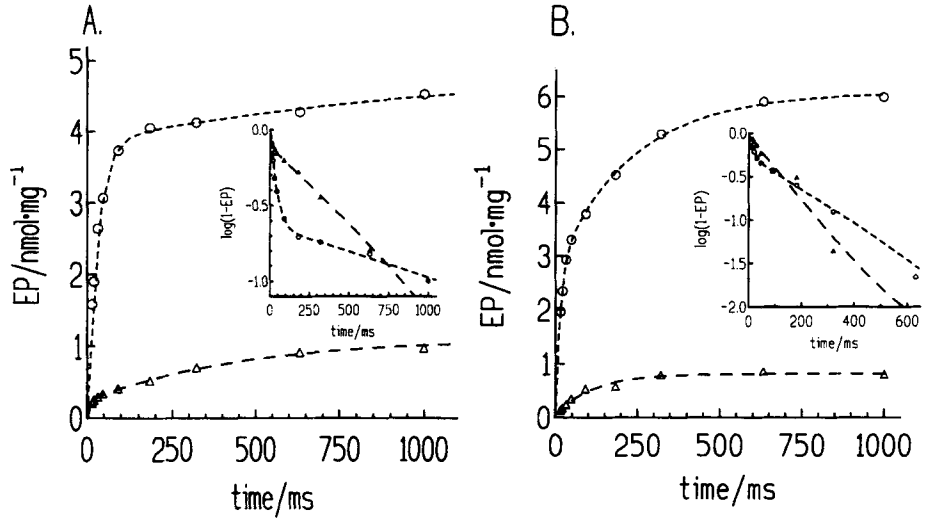

Fig. 5. Phosphorylation of Na^+,K^+-ATPase reconstituted into liposomes either containing 130 mM Na^+ (A), or 260 mM sucrose and no Na^+ (B). In each panel phosphorylation is performed either with 40 mol% cholesterol (○), or without cholesterol (△) in the liposome bilayer. The phosphorylation level was measured at 10° C using a rapid-mixing device, with varying ageing loops, allowing sampling times from 15 ms to 1 s. ATP-concentration was 10 μM. The curves are computer-fit of the data to exponential association curves. For the data with cholesterol biexponential fit was significantly better than monoexponentials as controlled by F-tests ($F = 94.7$, $P < 0.0001$), whereas without cholesterol the biexponential model does not fit the data significantly better than a simple monoexponential association curve ($F = 1.7$, $P = 0.27$). In panel A the fitted exponential slopes (λ) and fractions (f) for the data with cholesterol are: $\lambda_1 = 33.0 \pm 2.5$ s^{-1}, $f_1 = 3{,}79 \pm 0.16$ nmol/mg, $\lambda_2 = 0.77 \pm 0.14$ s^{-1}, $f_2 = 1.18 \pm 0.08$ nmol/mg. In the absence of cholesterol the fitted parameters are: $\lambda_1 = 2.20 \pm 0.37$ s^{-1}, $f_1 = 0.87 \pm 0.04$ nmol/mg. In panel B the fitted exponential slopes (λ) and fractions (f) for the data with cholesterol are: $\lambda_1 = 68.3 \pm 5.3$ s^{-1}, $f_1 = 2{,}78 \pm 0.11$ nmol/mg, $\lambda_2 = 4.3 \pm 0.3$ s^{-1}, $f_2 = 3.3 \pm 0.10$ nmol/mg. In the absence of cholesterol the fitted parameters are: $\lambda_1 = 8.6 \pm 1.7$ s^{-1}, $f_1 = 0.77 \pm 0.05$ nmol/mg. The insets show a semilogarithmic replot of the same data and curves expressed as residual relative phosphorylation level, $(1 - EP)$, vs. time.

7A) and in the absence (uncoupled Na^+-efflux, Fig. 7B). The dependence of the fitted exponential slopes and fractions on cholesterol content is shown in Fig. 8A,B. Both exponential slopes (λ_1 and λ_2) show a bell-shaped dependence on cholesterol content, with a maximum around 20 mol% cholesterol.

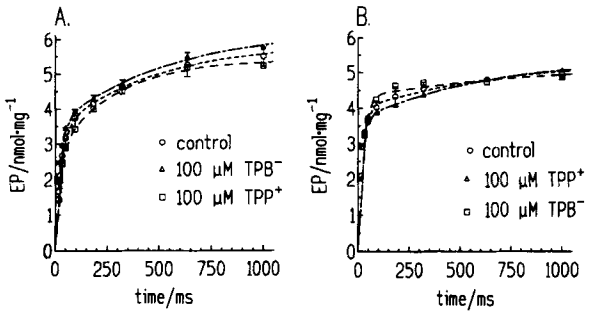

Fig. 6. (A) Effects of hydrophobic ions TPB^- and TPP^+ on phosphorylation reaction of Na^+,K^+-ATPase engaged in uncoupled Na^+-efflux. Phosphorylation was measured at 10° C as described in Fig. 5 but except the Na^+,K^+-ATPase was reconstituted into liposomes without Na^+. 100 μM of either TPB^- or TPP^+ was included in the reaction. (B) Effects of hydrophobic ions TPB^- and TPP^+ on phosphorylation reaction of Na^+,K^+-ATPase engaged in Na^+/Na^+-exchange.

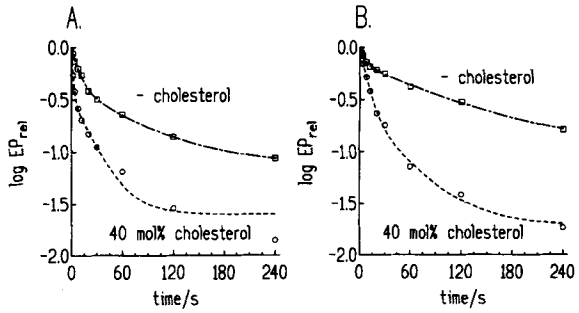

Fig. 7. Spontaneous dephosphorylation at 10° C of reconstituted (i/o)-oriented Na^+,K^+-ATPase with 40 mol% cholesterol and without cholesterol in the liposome bilayer in the presence of extracellular Na^+ (Na^+/Na^+-exchange, panel A), or in the absence of extracellular alkali ions (uncoupled Na^+-efflux, panel B). The values are normalized EP-values on a logarithmic ordinate to facilitate comparison. The curves are computer-fit using nonlinear regression to the data using exponential decay curves. In the case of Na^+/Na^+-exchange (panel A) and in the absence of cholesterol: $\lambda_1 = 0.30 \pm 0.03$ s^{-1}, $f_1 = 1.7 \pm 0.1$ nmol/mg, $\lambda_2 = 0.03 \pm 0.01$ s^{-1}, $f_2 = 0.54 \pm 0.10$ nmol/mg. For Na^+/Na^+-exchange in the presence of 40 mol% cholesterol: $\lambda_1 = 0.30 \pm 0.05$ s^{-1}, $f_1 = 6.1 \pm 0.6$ nmol/mg, $\lambda_2 = 0.023 \pm 0.013$ s^{-1}, $f_2 = 1.1 \pm 0.5$ nmol/mg. For $Na^+/0$-exchange (panel B) in the absence of cholesterol: $\lambda_1 = 0.18 \pm 0.03$ s^{-1}, $f_1 = 0.063 \pm 0.006$ nmol/mg, $\lambda_2 = 0.009 \pm 0.002$ s^{-1}, $f_2 = 0.123 \pm 0.006$ nmol/mg. In the presence of 40 mol% cholesterol: $\lambda_1 = 0.12 \pm 0.02$ s^{-1}, $f_1 = 4.8 \pm 0.8$ nmol/mg, $\lambda_2 = 0.023 \pm 0.013$ s^{-1}, $f_2 = 1.6 \pm 0.8$.

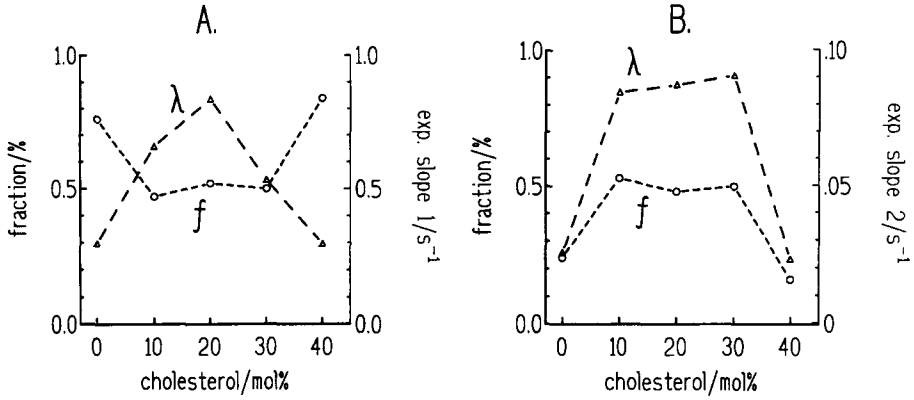

Fig. 8. Computed parameters (exponential slopes, λ, and relative fractions, f) used in the fitting of biexponential functions to the dephosphorylation data of reconstituted Na^+,K^+-ATPase in the presence of extracellular Na^+ (Na^+/Na^+-exchange) at increasing cholesterol content from 0 to 40 mol%. Fitting parameters for each of the two exponentials are shown separately in the two panels (A and B).

The distribution of phospho-intermediates at different cholesterol content was determined as described in details in the preceding paper [18] from the ADP-sensitive and K^+-sensitive dephosphorylation curves as depicted in Fig. 9A,B for 0 mol% and 40 mol% cholesterol. The calculated EP-distributions at 10° C as a function of cholesterol content are shown in Fig. 10. As seen from the figure the fraction of E_1P decreases whereas the fraction of E_2P increases as cholesterol increases from 0–40 mol%.

4. Discussion

These data indicate an activation of the hydrolytic activity of Na^+,K^+-ATPase both during the physiological Na^+/K^+-exchange and during Na^+/Na^+-exchange and uncoupled Na^+-efflux (Figs. 1A–C) by bilayer cholesterol in the range of 0–40 mol% cholesterol. A similar modulating effect of membrane cholesterol in the same concentration range of both ATP-hydrolysis associated with Na^+/K^+-exchange and K^+-activated phosphatase activity has previously been demonstrated for human erythrocytes and bovine kidney basolateral membranes [7,19].

A direct corollary of the cholesterol effect on hydrolytic activity is the observed increase of steady-state phosphorylation level both in the presence and absence of extracellular Na^+ (Fig. 2). It is tempting to ask if the lower maximum steady-state phosphorylation level observed in membrane-bound and solubilized preparations as compared to reconstituted preparations could be due to a

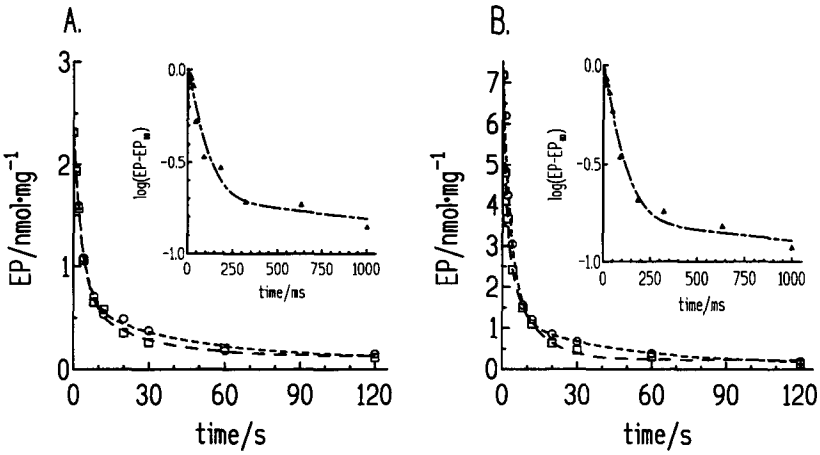

Fig. 9. Dephosphorylation at 10° C of reconstituted Na^+,K^+-ATPase in the presence of extracellular Na^+ (Na^+/Na^+-exchange) either without cholesterol (A), or with 40 mol% cholesterol present (B) in the liposome bilayer. In each panel are shown spontaneous dephosphorylations by chasing with cold ATP (○), ADP-activated dephosphorylations (□), and K^+-activated dephosphorylations (△). The latter is measured during the initial first second using a rapid mixing device and shown as log (EP) in the insets. The curves are computer-fit using nonlinear regression to the data using exponential decay curves. The fitting parameters for the spontaneous dephosphorylations ± cholesterol are given in the legend to Fig. 7. In the case of ADP- and K^+-chases the fitting parameters are: In panel A (−cholesterol) in the presence of ADP, $\lambda_1 = 0.30 \pm 0.05$ s^{-1}, $f_1 = 1.66 \pm 0.20$ nmol/mg, $\lambda_2 = 0.04 \pm 0.02$ s^{-1}, $f_2 = 0.56 \pm 0.18$ nmol/mg. In panel B (+cholesterol): $\lambda_1 = 0.66 \pm 0.07$ s^{-1}, $f_1 = 4.2 \pm 0.3$ nmol/mg, $\lambda_2 = 0.09 \pm 0.01$ s^{-1}, $f_2 = 2.7 \pm 0.3$ nmol/mg. In panel A in the presence of K^+: $\lambda_1 = 14.2 \pm 8$ s^{-1}, $f_1 = 1.7 \pm 0.7$ nmol/mg, $\lambda_2 = 0.3 \pm 0.04$ s^{-1}, $f_2 = 0.4 \pm 0.3$ nmol/mg. In panel B in the presence of K^+: $\lambda_1 = 14.4 \pm 1.7$ s^{-1}, $f_1 = 6.1 \pm 0.3$ nmol/mg, $\lambda_2 = 0.25 \pm 0.04$ s^{-1}, $f_2 = 1.2 \pm 0.4$ nmol/mg.

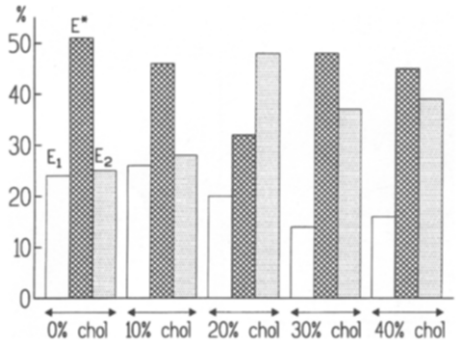

Fig. 10. Distribution of EP-intermediates calculated using the 3-pool model as described by Nørby et al. [38] as a function of cholesterol content of the proteoliposomes.

cholesterol deficiency (see preceding paper [18])? Apparently not, since incubation with excess cholesterol during $C_{12}E_8$ solubilization does not increase the measured EP-level (data not shown).

The stimulation of phosphorylation level along with hydrolytic activity suggests an essential role for the cholesterol in the Na^+,K^+-ATPase function. However, the mechanism of this stimulatory effect cannot be unambiguously related to any particular effects of cholesterol on the bilayer dynamics like fluidity, or lipid/lipid interaction [7]. It cannot, therefore, be excluded that the effect is by cholesterol per se [19–21]. This is in keeping with the observation for Ca^{2+}-ATPase that cholesterol is found both to be excluded from the annulus lipids [22] and to be without effects on catalytic activity [23,24].

The results shown in Fig. 1 demonstrate that cholesterol activate maximum turn-over, V_{max}, for all investigated different modes of exchange reactions accommodated by the Na^+/K^+-ATPase (Na^+/K^+-, Na^+/Na^+-, and Na^+/0-exchange). Furthermore, by comparing with the results depicted in Figs. 3 and 4 for Na^+/Na^+-exchange and uncoupled Na^+-efflux, cholesterol is demonstrated to effect not only V_{max}, but the entire shape of the curve for cytoplasmic Na^+-activation. Most notably is a pronounced inhibition showing up at increasing Na^+-concentrations in the absence of cholesterol (Figs. 3B and 4B). Such an inhibition is absent at 40 mol% cholesterol. For Na^+/Na^+-exchange the apparent K_m for cytoplasmic Na^+-activation of hydrolytic activity calculated by fitting the sigmoid activation curve to the data is 6.8 mM and V_{max} is 36.2 μmol/mg per h in the presence of 40 mol% cholesterol. In the absence of cholesterol, omitting the part of the activation curve showing inhibition, K_m decreases slightly to 4.7 mM, whereas V_{max} is reduced by a factor of 6 to 6.3 μmol/mg per h. For uncoupled Na^+-efflux K_m and V_{max} decreases from 3.8 mM and 29.5 μmol/mg per h in the presence of 40 mol% cholesterol to 1.2 mM and 5.5 μmol/mg per h, respectively, in its absence. In the consecutive ping-pong model for Na^+,K^+-ATPase K_m and V_{max} are expected to vary proportionally, when only V_{max} is affected [25]. However, in the present study K_m decreases by less than V_{max} in both modes of exchange, indicating that the affinity for cytoplasmic Na^+ decreases when cholesterol is omitted in the proteoliposome bilayer. This is in keeping with previous observations, using cholesterol depletion of erythrocytes, that cholesterol interacts with the cytoplasmic Na^+ binding sites of Na^+,K^+-ATPase [26], and there suggested as an indirect interaction of cholesterol, mediated through changes in phospholipid order [29]. The inhibition seen at increasing Na^+ in the absence of cholesterol (compare Figs. 3B and 4B), could indicate that the inner positive dipole potential created inside the bilayer by cholesterol [2] may be important to accelerate the electrogenic release of Na^+ to the extracellular side through the extracellular ion-well [27], a step that may well become rate limiting in the absence of cholesterol at high cytoplasmic Na^+.

In a previous study [20] the activation of turnover by cholesterol could not be mimicked by other fluidizing agents, suggesting direct interaction of cholesterol on the Na^+,K^+-ATPase apart from the indirect ones, probably mediated through changes in the order of the phospholipids surrounding the Na^+-sites. In accordance with this suggestion, cholesterol is found in the present study to accelerate the overall rate of phosphorylation by inducing a major initial rapid phase, which is not observed in the absence of cholesterol (Fig. 5). Biphasic phosphorylation has previously been observed under a variety of experimental conditions [28–30]. The interaction of cholesterol on the phosphorylation reaction is probably not due to electrostatic interaction from the cholesterol dipole potential, since inner electrostatic potentials induced by hydrophobic ions like TPP^+ or TPB^- were without gross effects on the phosphorylation kinetics (Fig. 6). This is also expected from previous results demonstrating that the phosphorylation reaction in itself is electroneutral [31]. It is interesting that cholesterol potentiates biphasicity of phosphorylation, since this could indicate that the membrane microenvironment can induce kinetic heterogeneity [30,32]. Such effects could be produced via subunit interactions in an oligomer, a process that could be facilitated by cholesterol which is known to produce lateral segregation inducing membrane areas with aggregated membrane proteins [33]. This would ascribe an effect of cholesterol on monomer/dimer interaction of Na^+,K^+-ATPase, a process that could be an essential element in the reaction cycle of this enzyme [34–36].

The dephosphorylation reaction is also affected by the presence of cholesterol, which accelerated dephosphorylation both during Na^+/Na^+-exchange and during uncoupled Na^+-efflux. This is clearly seen from the logarithmic transforms depicted in Fig. 7A,B. The fractional activation by adding 40 mol% cholesterol is most prominent in the case of Na^+/0-exchange. The activation of maximum hydrolytic activity accompanying Na^+/Na^+-exchange and Na^+/0-exchange by cholesterol can be accounted for by its activation of the dephosphorylation reaction, which is

considered rate-determining during conditions where extracellular K^+ is absent. The activation of maximum hydrolytic activity accompanying Na^+,K^+-ATPase activity is in accord with its acceleration of the phosphorylation reaction, which may be the rate-determining step during Na^+/K^+-exchange at saturating ATP [37].

The spontaneous dephosphorylation of the phosphorylated enzyme is the result of a parallel dephosphorylation of at least three specific phosphointermediates: E_1P, E^*P, and E_2P [38–40]. In principle, therefore, the dephosphorylation observed should as a maximum be characterized by the sum of three exponentials. It should be emphasized, however, that neither of the observed exponential slopes (λ) in the phosphorylation/dephosphorylation curves are directly equivalent numerically to any rate constant of interflow or outflow between the three EP-species. The present data do not allow a decision as to the maximum number of exponentials in the overall EP-decay, but there are at least two at all experimental conditions tested at 10° C.

The rate of the spontaneous dephosphorylation reactions calculated from the fitting parameters λ (the exponential slope) and f (the fraction) of the spontaneous dephosphorylation as $d(EP)/dt = \lambda_1 \cdot f_1 + \lambda_2 \cdot f_2$, which is considered rate-limiting both during Na^+/Na^+-exchange and during uncoupled Na^+-efflux, is more than adequate to account for the observed hydrolytic activity during Na^+/Na^+-exchange and uncoupled Na^+-efflux both in the presence and absence of cholesterol. This is in contrast to results comparing hydrolytic Na^+,K^+-ATPase activity with the K^+-activated dephosphorylations performed at exactly identical conditions: The initial rate of the K^+-dephosphorylation exceeds by far the Na^+,K^+-ATPase activity, however, if it is assumed that the lowest exponential slope (λ_2) in the K^+-dephosphorylation (the residual rate when all E_2P and E^*P have dissipated) represents the forward rate-constant during steady-state turnover, then the calculated turnover, $v = E_1P \cdot \lambda_2$, can far from account for the hydrolytic activity measured during exactly identical conditions, as also previously found for broken preparations of both Na^+,K^+-ATPase (for references see [41]) and Ca^{2+}-ATPase [42].

Cholesterol has previously been shown to modify the distribution of phosphointermediates in the EP-pool depending on temperature and Na^+-content [8,40]. An effect of cholesterol on the EP-distribution, somewhat smaller than previously found by Yoda and Yoda [8], is also noted in the present investigation (Fig. 10). Most notably is a decrease in ADP-sensitivity of the dephosphorylations in the absence of cholesterol, indicating decreasing E_1P at low cholesterol, in accord with Yoda and Yoda [8], However, in the present investigation E_2P increases at increasing cholesterol content (10° C), in contrast to the eel Na^+,K^+-ATPase at 15° C [8]. These differences could well be the result of the different preparations employed, or the different methods applied for calculating the EP-distribution, since Yoda and Yoda [8] use only 1 point (1 s) in the dephosphorylation curve to calculate the EP-distribution.

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