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Modulation of Na,K-ATPase by Associated Small Transmembrane Regulatory Proteins and by Lipids[1]

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The effects of phospholipid acyl chain length (n_c) and cholesterol on Na,K-ATPase reconstituted into liposomes of defined lipid composition are described. The optimal hydrophobic thickness of the lipid bilayer decreases from $n_c = 22$ to 18 in the presence of 40 mol% cholesterol. Hydrophobic matching as well as specific interactions of cholesterol with the phosphorylation/dephosphorylation reactions is found to be important. A novel regulatory protein has been identified in Na,K-ATPase membrane preparations from the shark (phospholemmanlike protein from shark, PLMS) with significant homology to phospholemman (PLM), the major protein kinase substrate in myocardium. Both are members of the FXYD gene family. Another member of this family is the Na,K-ATPase γ subunit indicating that these proteins may be specific regulators of the Na,K-ATPase. A regulatory mechanism is described in which association/dissociation of PLMS with the Na,K-ATPase is governed by its phosphorylation by protein kinases.

KEY WORDS: Protein/lipid interaction; hydrophobic coupling; FXYD family; phospholemmanlike protein from shark (PLMS); acute Na,K-ATPase regulation, protein kinase A; protein kinase C; single transmembrane regulatory proteins (STRP).

INTRODUCTION

Although investigations on the Na,K-ATPase have been intense for more than 40 years since it was first isolated from crab nerves by Skou (1957) there are still important aspects of this remarkable ionpump that are largely unknown to us, namely, the interaction between the protein and the plasma membrane lipids into which it is suspended and the molecular mechanisms for acute hormonal regulation, which is also membrane dependent. In this minireview, these issues are addressed and some of our recent results concerning the two aspects presented.

It has been known for more than a decade that specific motifs exist for protein kinase phosphorylation in the α subunit of the Na,K-ATPase, suggesting that the molecular mechanism underlying the acute hormonal regulation could be via direct phosphorylation by protein kinases. However, the effects of protein kinase phosphorylation on Na,K-ATPase activity has been a matter of controversy and apparently inconsistent results have been obtained employing different preparations and cell systems (Therien and Blostein, 2000). This probably reflects the very complex nature of the regulation processes within the cell, including, e.g., cross-talk between different signaling pathways. However, numerous methodological problems have also been encountered.

The C-terminal part of the Na,K-ATPase α subunit contains the highly conserved PKA phosphorylation site, Ser-938 located in the small cytoplasmic loop between the M8/M9 transmembrane segments. Whether this site has any physiological significance is questioned, however, since its phosphorylation *in vitro* takes place only in the presence of detergents (Bertorello *et al.*, 1991) or after reconstitution (Cornelius and Logvinenko, 1996), a fact that has lead to misinterpretations of functional effects after PKA phosphorylation. Furthermore, *in vivo* studies have demonstrated that activation of the PKA signaling pathway apparently not always leads to Na,K-ATPase

[1] Key to abbreviations: di-n_c:1 PC and di-n_c:0 PC, phosphatidylcholine with unsaturated (*cis*-9) or saturated lipid acyl chains, respectively, of lengths n_c.

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phosphorylation except after transfection, which may lead to misfolding (Feschenco *et al.*, 2000).

The N-terminal segment of the Na,K-ATPase α-subunit contains two PKC phosphorylation sites, one of which, Ser-18, is present only in rat α1 and α3 isoforms. The other, Ser-11, is conserved but phosphorylation of this site takes place only to a very low stoichiometry. In rat, PKC phosphorylation of Ser-18 triggers Na,K-ATPase internalization (Chibalin *et al.*, 1998) whereas phosphorylation of both Ser-18 and Ser-11 leads to its cell membrane recruitment (Efendiev *et al.*, 2000). This leaves open the question if protein kinase phosphorylation of the Na,K-ATPase α subunit has any effects on Na,K-ATPase activity *per se*. One obvious possibility is that phosphorylation of the α subunit is a targeting signal rather than a conformational switching signal to change catalytic capacity. We still lack answers to the fundamental question on how individual hormones activate the specific pool of soluble protein kinases/phosphatases and target them to the membrane-bound substrate—the Na,K-ATPase. Which internal receptors are involved in this process? Further investigations are also needed to clarify the importance of the dynamic formation of lipid domains associated with this targeting in connection with protein kinase activation.

The latter question touches upon another unresolved and complicated issue concerning the significance of the very complex lipid composition of the plasma membrane for optimal ion-pump activity, i.e., the importance and molecular nature of the protein/lipid interaction. The activity of the Na,K-ATPase is known to be influenced by the lipid composition of the plasma membrane: The lipid acyl chain length, certain acidic phospholipids, and especially cholesterol are important for optimal catalytic activity of Na,K-ATPase (Johannsson *et al.*, 1981; Cornelius, 1995). A fluid lipid phase certainly seems to be a requirement common to all ion pumps, but is any specific lipid composition necessary?

PROTEIN/LIPID INTERACTION

Less than 30 years ago, Singer and Nicolson (1972) changed the view of the cell membrane with the fluid mosaic membrane concept. They emphasized, for the first time, that the proteins partitioned into the lipid bilayer floating in a pseudo two-dimensional sea of fluid lipids. Transmembrane ion transport, assisted by proteins, was, therefore, conceived before the idea of integral membrane proteins was realized. The bilayer fluidity is the crucial property of the molecular assembly of the cell membrane and assures any necessary lateral and conformational mobility of the proteins. However, fluidity does not necessarily imply randomness. Membranes are, in fact, highly structured fluids separated into lipid domains important for the differential functions of the various proteins (Edidin, 1990).

The first question to address is if protein/lipid interaction is indirect via, e.g., fluidity or more specific, implying a closer interaction, or even binding between certain lipids and the Na,K-ATPase. The question is addressed using reconstitution of highly purified Na,K-ATPase into liposomes of defined lipid composition (Cornelius and Skou, 1984). In the following, the effects of phospholipid acyl chain length and saturation and of cholesterol on the overall catalytic reaction, as well as on some partial reactions of reconstituted Na,K-ATPase purified from shark rectal glands, will be described.

RECONSTITUTION

Functional reconstitution is achieved in the following way (Cornelius, 1988): Initially Na,K-ATPase from the rectal gland of the shark (*Squalus achantias*) is prepared in a membrane-bound form by homogenization and differential centrifugation. This Na,K-ATPase has the advantage of being very stable in certain detergents, like $C_{12}E_8$, which is, therefore, used to specifically extract the Na,K-ATPase from the membranes, i.e., to solubilize the protein. Next, the lipid of choice is solubilized in the same detergent and the two solutions are mixed. When the detergent is subsequently removed by addition of hydrophobic biobeads, liposomes are spontaneously formed that contain reconstituted Na,K-ATPase. Control experiments demonstrate that this reconstitution takes place without loss of catalytic activity or ion-transport capacity.

The proteoliposomes produced are unilamellar and large with a diameter of about 100 nm, as seen from freeze-fracture EM pictures (Cornelius and Møller, 1996). Before they can be used for quantitative experiments, they have to be thoroughly characterized, including determination of the pump density and symmetry of protein insertion since several of these parameters may depend on the lipid composition. Such a characterization is imperative in order to ascribe the measured hydrolytic activity to the population of active pumps (i.e., the specific activity or turnover), since only Na,K-ATPase incorporated with an inside-out orientation are activated upon ATP addition to the medium. Right-side out pumps are devoid of substrate (ATP is impermeable) and incubation of the proteoliposomes with MgP_i and ouabain is used to inhibit any enzyme that is not fully incorporated, or only adsorbed to

the liposomes. The enzyme orientation after this reconstitution is asymmetric, as measured by functional tests with inside-out fractions amounting to about 10% (Cornelius, 1988).

HYDROPHOBIC THICKNESS AND FLUIDITY

In a lipid bilayer of phosphatidylcholine (PC), the effective hydrophobic thickness, $\langle d \rangle$ is determined approximately by the acyl chain length, n_c. For saturated PC, the relation $\langle d \rangle \cong 1.75(n_c\text{-}1)$ holds. Double bonds in the acyl chains reduce the hydrophobic thickness so that for monounsaturated PC, $\langle d \rangle \cong 1.75(n_c\text{-}2.6)$, i.e., di-14:0 PC and di-16:1 PC have about the same hydrophobic thickness. This parameter is important because the membrane hydrophobic thickness must accommodate the hydrophobic stretch of the integral protein to avoid hydrophobic mismatch. If the energetic penalty for exposing hydrophobic groups to water is much larger than the change in bending stress of the elastic bilayer, the change in bending energy will be proportional to the square of the hydrophobic mismatch (Lundbæk *et al.*, 1996). Therefore, changing the hydrophobic thickness of the membrane can change the activation energy for certain protein reactions, e.g., associated with conformational changes. An integral protein incorporated into a lipid bilayer may influence the lipid domain formation reflecting the protein/lipid interaction. On the other hand, the domain structure may change the tension on the protein, which may lead to conformational changes, e.g., by rearrangement or tilting of transmembrane segments. Hydrophobic matching of the lipid bilayer thickness and the hydrophobic area of the protein could, therefore, be an important driving force for the coupling between lipid structure and integral membrane proteins—hydrophobic coupling (Mouritsen and Bloom, 1984).

Another important plasma membrane component is cholesterol. Most animal plasma membranes contain 30–40 mol% cholesterol. Cholesterol increases the acyl chain order of phospholipids. Cholesterol >25 mol% promotes the formation of a liquid-ordered phase, which is fluid from the point of view of lateral disorder and diffusion, but, at the same time, the acyl chains are highly ordered (Ipsen *et al.*, 1987). Therefore, cholesterol increases the thickness and the physical strength of the plasma membrane considerably, without limiting the lateral mobility of integral proteins.

In Fig. 1A, the optimal hydrolytic capacity (V_{max}) of shark Na,K-ATPase reconstituted into liposomes of monounsaturated PC of increasing acyl chain length from $n_c = 14$ to 24 with or without 40 mol% cholesterol is shown at 25°C. At this temperature, the phospholipids investigated are fluid. Three things are immediately apparent: For one, cholesterol shifts the optimal acyl chain length to shorter lengths, from $n_c = 22$ to 18. Second, cholesterol significantly increases the maximum specific activity or the turnover of the pump. Finally, the maximum specific activity obtained with di-18:1 PC + 40 mol% cholesterol matches the activity measured in natural membranes. The first observation is compatible with the effect of cholesterol to increase the bilayer thickness: a shift in optimal acyl chain lengths from 22 to 18 correspond to a

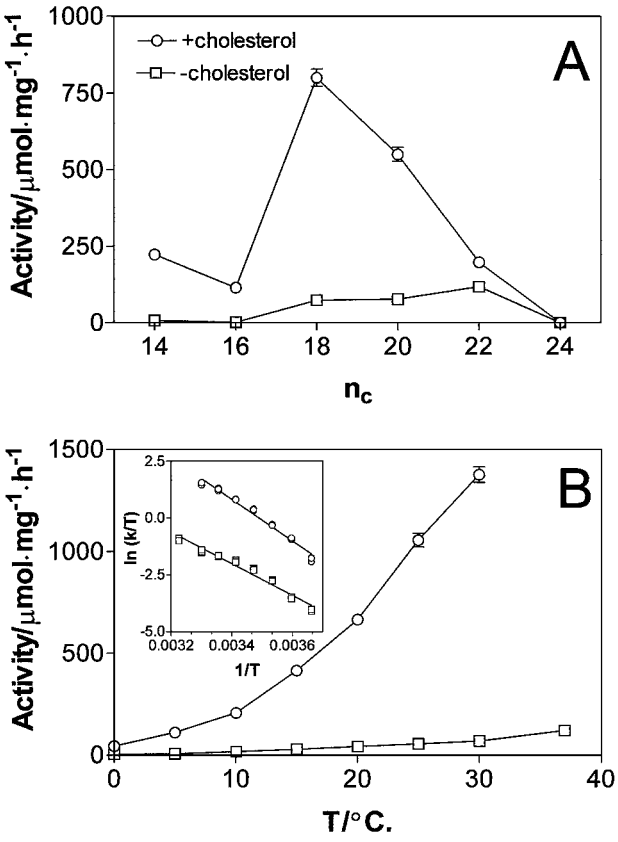

Fig. 1. (A) ATPase activity of Na,K-ATPase reconstituted with phosphatidylcholine-containing monounsaturated acyl chains of chain length (n_c) increasing from 14 to 24. The activity was measured at 25°C, pH 7.4 in the presence of MgATP = 4 mM, NaCl = 130 mM, and KCl = 20 mM. (B) Temperature dependence of ATPase activity of Na,K-ATPase reconstituted with di-18:1 PC with (○) and without (□) 40 mol% cholesterol. The inset shows Eyring plots of the data. From the slopes the enthalpy of activation with and without cholesterol can be calculated to, $\Delta H^{\ddagger} = 76 \pm 3$ kJ/mol and 58 ± 2 kJ/mol, respectively. $\Delta S^{\ddagger}$ is obtained from the intersect of the lines with the ordinate to 69 ± 2 J/mol · K and -17 ± 1 J/mol · K, respectively. Thus the free energy of activation, $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$ is 56 and 52 kJ/mol at 25°C, respectively.

shift in hydrophobic thickness of the bilayer from 34 to 27 Å, i.e., a 21% decrease. Such a decrease is in accordance with what cholesterol is expected to counterbalance by increasing the ordering of the acyl chains.

In Fig. 1B, the temperature dependence of Na,K-ATPase activity of enzyme reconstituted with di-18:1 PC is shown with and without cholesterol present. The Eyring plots shown in the inset appear to be linear over the temperature interval given. From the slope and intersect, the enthalpy ($\Delta H^{\ddagger}$) and entropy of activation ($\Delta S^{\ddagger}$) can be calculated. The presence of cholesterol increases $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, the latter attaining a positive value, leaving the free energy of activation, $\Delta G^{\ddagger}$ constant at about 54 kJ/mol. It could indicate that the Na,K-ATPase is slightly more unfolded without cholesterol.

Figure 2A demonstrates that cholesterol increased the phosphorylation rate constant as well as the EP level by factors between 4–5 at 10°C. The K^+-dependent dephosphorylation reaction is shown in Fig. 2B also at 10°C. In the presence of 40 mol% cholesterol, the dephosphorylation rate constant is increased by a factor of 1.7 compared to the rate constant in the absence of cholesterol. From the measured V_{max} at 10°C and maximum EP levels, the turnover number, k_{cat} of the Na,K-ATPase reaction with and without cholesterol can be calculated to 8.5 and 3.7 s^{-1}, respectively, demonstrating that phosphorylation and dephosphorylation rate constants as shown in Fig. 2 could contribute to rate determination. However, cholesterol effects on these rate constants alone cannot explain the activation of turnover seen in Fig. 1. Rather some major rate-limiting step must be cholesterol sensitive like the $E_2 \rightarrow E_1$ transition, which is considered rate limiting during the Na,K-ATPase reaction cycle.

The increase in the maximum level of EP measured in the absence of K^+ must mean either that the dephosphorylation rate constant that is rate limiting in the absence of K^+ is decreased by cholesterol and/or that some rate constants downstream of this reaction have increased. Since the rate constant of the spontaneous dephosphorylation in the absence of K^+ is independent of cholesterol (Cornelius, 1995) the latter must be the case. The increased EP level could, therefore, be the result of the increase in the phosphorylation reactions as seen in Fig. 2A. According to the following simplified Albers–Post scheme: $E_2 \overset{1}{\rightarrow} E_1ATP \overset{2}{\rightarrow} E_1P \overset{3}{\rightarrow} E_2P \overset{4}{\rightarrow} E_2$, cholesterol, as a minimum, is suggested to accelerate step 1, to increase V_{max}, and step 2 relative to step 4, to increase the EP level.

The marked effects of phospholipid chain length and cholesterol on the Na,K-ATPase reaction presumably follow from changes in the conformational mobility of the Na,K-ATPase. The Na,K-ATPase is surrounded by about 60 lipid molecules in the membrane and their cooperative action could be sufficient to result in significant conformational effects. This presumably follows from changes in the packing of the transmembrane segments embedded in the bilayer. This is in accord with the crystal structure of the Ca-ATPase where ATP binding, phosphorylation, and dephosphorylation result in substantial movement of the N and A domains (Toyoshima *et al.*, 2000), which is finally transmitted to the transmembrane segments in association with the E_1/E_2 conformational change.

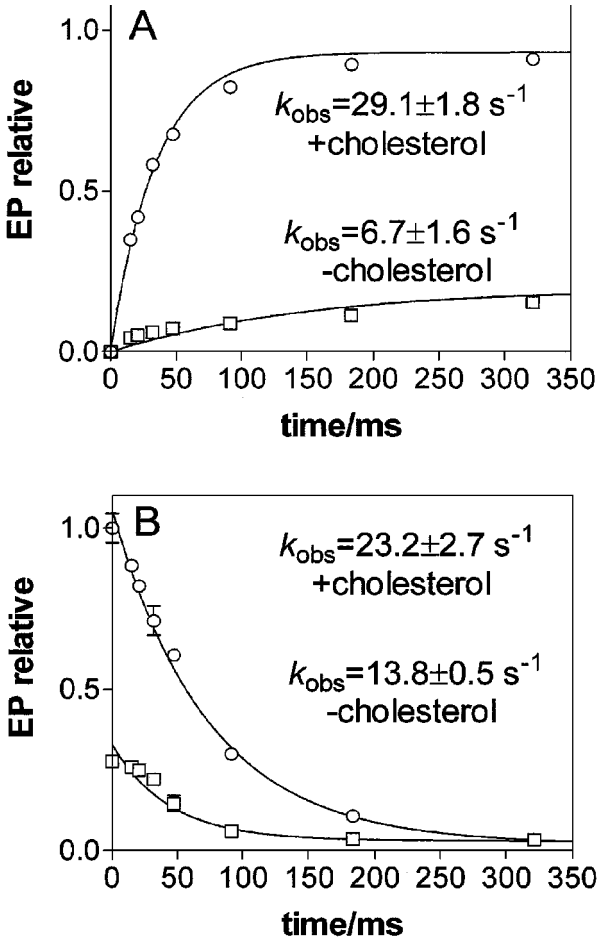

Fig. 2. Effect of cholesterol (40 mol%) on the rate of phosphorylation (A) and dephosphorylation (B) at 10°C using Na,K-ATPase reconstituted with phosphatidylcholine ± 40 mol% cholesterol. In (A) chemical quench experiments are shown, which indicates measured relative EP levels after addition of ATP (25 μM) and acid quench. In (B), the Na,K-ATPase is first phosphorylated until steady state is reached. Hot ATP is then chased with cold ATP in a chase solution containing 20 mM KCl in the presence of nigericin (K^+ ionophore) and the reaction quenched after different time intervals. The maximum EP level corresponded to 6.5 nmoles/mg protein, close to the theoretical value for one site per Na,K-ATPase of molecular mass 147 kDa. The curves are fit of monoexponentials to the data with indicated observed rate constants (k_{obs}).

One of the prominent characteristics of lipids is their phase property: A fluid, liquid-crystalline phase is necessary for enzyme function. Therefore fluidity has often been assumed to play the key role in lipid/protein interaction. However, according to transition state theory, fluidity cannot account for changes in equilibrium property of a system since fluidity only changes the height of an energy barrier over which the reaction takes place and not the energy of the individual enzyme states (Lee, 1991).

This is in accord with the work of reconstitution presented here that seems to exclude any specific importance of the very complex lipid composition of biological membranes on Na,K-ATPase reaction: A fluid membrane with PC chain length about 18 and cholesterol is sufficient to support optimal Na,K-ATPase activity. The exact fluidity of the liquid–crystalline phase seems to be unimportant. This is only true from the point of view of optimizing turnover rate, however; other factors like protein regulation may still be very dependent on the specific lipid composition. Actually, this situation is different from what is found for the sarcoplasmic reticulum Ca-ATPase (SERCA) where optimal catalytic activity is supported at shorter acyl chain length ($n_c = 18$) and independent of cholesterol (Lee, 1991). The SERCA ATPase is presumably fine tuned for optimal function in the cholesterol-poor sarcoplasmic/endoplasmic membranes.

SINGLE TRANSMEMBRANE REGULATORY PROTEINS (STRP)

The Na,K-ATPase is a hetero-oligomeric protein consisting of the catalytic α subunit of $M_r \sim 112$ kDa and the smaller β-subunit of $M_r \sim 35$ kDa . The β subunit is essential for the maturation, expression, and membrane targeting of the Na,K-ATPase. A γ subunit of apparent $M_r \sim 8$ kDa is a third, mainly kidney specific subunit. This small protein was first noticed by Rivas *et al.* (1972) and later Forbush *et al.* (1978) provided evidence that the γ subunit is part of the Na,K-ATPase. The γ subunit modulates the ATP and K^+affinity of the Na,K-ATPase probably due to an effect in supporting the E_1 conformation of the Na, K-ATPase (Therien *et al.*, 1999).

As seen from Table I, association of STRP with P-type ATPases seems to be a general phenomenon and their mode of regulation appears to be either via protein kinase phosphorylation [phospholamban (PLN)-type], or for those that are not protein kinase substrates, via other mechanisms (γ-type). Several STRP are known to form oligomers in the membrane, e.g., PLN and PLM (Simmerman and Jones, 1998) and to induce channel activity. The γ subunit has also been shown to induce ion channel activity when expressed in *Xenopus* oocytes (Minor *et al.*, 1998) but it is still unresolved if this has any physiological bearings.

Table I. Small Transmembrane Regulatory Proteins (STRP) of P-Type ATPases[a]

ATPase/type	STRP	Apparent M_r (kDa)	Mechanism
Ca-ATPase	PLN	~6	Protein kinase-regulated
(type IIA)	SLN	~4	γ type
Na,K-ATPase	γ	~8	Nonprotein kinase regulation
	PLMS	~15	PLN type
(type IIC)	PLM?	~15	PLN type
	CHIF?	~15	γ Type
H,K-ATPase (type IIC)	?	?	?
H-ATPase	PMP1	~4	γ Type
(type IIIA)	PMP2	~4	γ Type
	PMP3	~6	γ Type
Kdp-ATPase (type IA)	KdpC	~21	PLN Type?
Cu-ATPase (type IB)	?	?	?

[a] Some known single transmembrane proteins with presumed or known regulatory functions are listed. SLN is sarcolipin (Odermatt *et al.*, 1998). PMP1, PMP2, and PMP3 (Navarro and Goffeau, 2000) are associated with yeast plasma membrane H-ATPases. KdpC is a subunit of the bacterial Kdp-ATPase (Altendorf *et al.*, 1992). No STRP has yet been associated with the H,K-ATPase or the Cu-ATPases. The STRP seem functionally to be grouped into two: One works by protein kinase phosphorylation (PLN type), whereas another lacks a protein kinase motif (γ type).

THE FXYD GENE FAMILY

The γ subunit belongs to a family of proteins now termed the FXYD domain-containing ion-transport regulators (Sweadner and Rael, 2000). In contrast to the γ subunit that specifically associates with the kidney Na,K-ATPase, none of these proteins have been shown to associate with other proteins. Members of this family shares three common features: (1) They have a single transmembrane domain, (2) they have a conserved motif, FXYD, in the extracellular (N-terminal) domain, and (3) they are, presumably, able to associate to form oligomers in membranes and in detergent solution. Seven small proteins closely related to the γ-subunit (FXYD2) have been identified so far. Among them is phospholemman (PLM, FXYD1), the major sarcolemmal substrate for PKA and PKC (Palmer *et al.*, 1991) and the channel-inducing factor (CHIF, FXYD4) (Attali *et al.*, 1995).

The first evidence that another protein of the FXYD family, other than the γ, may regulate Na,K-ATPase activity came from the identification of an FXYD

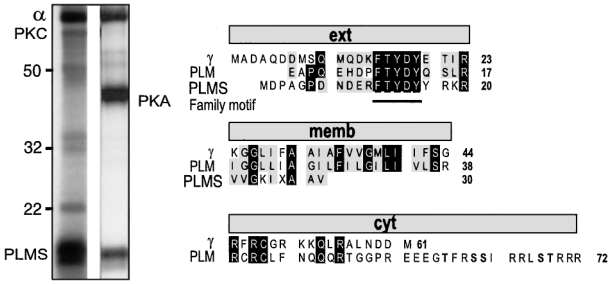

Fig. 3. The right panel shows alignment of the partial sequence of PLMS with that of the γ subunit from *Xenopus* (Minor *et al.*, 1998) and PLM from dog heart (Palmer *et al.*, 1991). Autoradigrams after SDS–PAGE of shark Na,K-ATPase phosphorylated by either PKC (left) or PKA in the presence of 0.1% Triton X-100 (right) is shown in the left panels. The bands corresponding to the α subunit, autophosphorylated protein kinases, and PLMS, as well as molecular weight markers (kDa), are indicated.

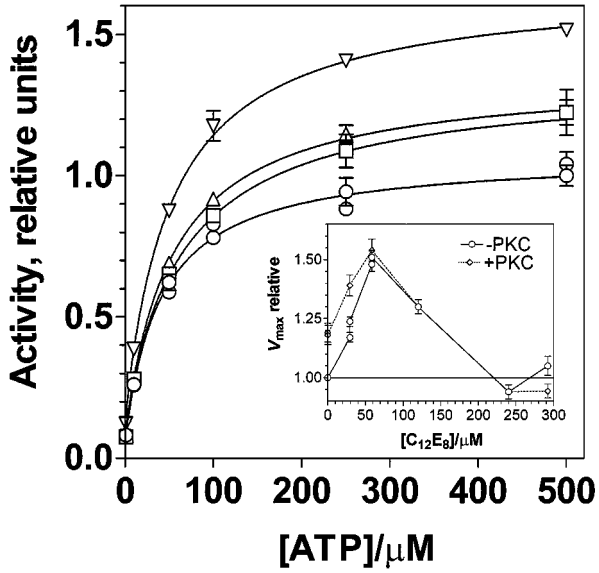

Fig. 4. Effects of nonsolubilizing $C_{12}E_8$ or PKC phosphorylation on the ATP activation curve of shark Na,K-ATPase. Control (○), 30 μM (□), and 60 μM (▽) $C_{12}E_8$, and after PKC phosphorylation (△). The curves are fit of a two-site binding equation to the data. The major low-affinity K_{ATP} increases in proportion to V_{max} from 41 (control) to about 60 μM ($C_{12}E_8$, or PKC). The inset shows the activation of V_{max} obtained from ATP-activation curves at increasing nonsolubilizing concentrations of $C_{12}E_8$ of nonphosphorylated (○) or PKC phosphorylated shark Na,K-ATPase (◇).

phosphoprotein in Na,K-ATPase membrane preparations from shark rectal glands (Mahmmoud *et al.*, 2000). A 15-kDa protein sharing homology to the γ subunit and to PLM was found to copurify with shark Na,K-ATPase in membrane preparations (Fig. 3). This protein is phosphorylated by cAMP-dependent (PKA) and Ca^{2+}/phospholipid-dependent protein kinase (PKC) (Fig. 3) and it was, therefore, named phospholemmanlike protein from shark (PLMS). Specific association of PLMS with the α subunit was demonstrated by the stoichiometric expression of this protein and the Na,K-ATPase in the rectal gland membranes and by its coimmunoprecipitation by antibodies to the α subunit. The fact that PLMS associates with the α subunit and is phosphorylated by protein kinases indicates that it is a component of the protein kinase signaling pathways leading to modulation of shark Na,K-ATPase activity.

PLMS MODULATION OF Na,K-ATPase FUNCTIONS

As mentioned in the Introduction, PKC phosphorylation of the α subunit *in vitro* takes place to a very low level, except in rat Na,K-ATPase, indicating that PKC regulation may be indirect via phosphorylation of other components of the Na,K-ATPase than the α. The association of PLMS with the Na,K-ATPase α subunit can be impaired by treatment with nonsolubilizing concentrations of $C_{12}E_8$. This treatment is found to result in a significant and dose-dependent increase in the turnover rate of the Na,K-ATPase (Fig. 4), suggesting that the association of PLMS with the shark α subunit leads to inhibition. Direct phosphorylation of PLMS by PKC also dissociates PLMS from the Na,K-ATPase α subunit and also results in an activation of the Na,K-ATPase (Fig. 4). The activation of Na,K-ATPase induced by either PKC phosphorylation of PLMS, or by treatment with nonsolubilizing concentrations of the detergent $C_{12}E_8$ is by interaction with the enzyme species that binds K^+, whereas the increase in the apparent ATP affinity is indirect, probably by an effect on the E_1/E_2 conformational equilibrium (Mahmmoud *et al.*, 2000). Thus, PLMS is proposed to regulate the shark Na,K-ATPase activity in a way resembling the PLN regulation of the Ca-ATPase (Simmerman and Jones, 1998).

INTERACTION BETWEEN PLMS AND Na,K-ATPase

The functional interaction between PLN and the Ca-ATPase has been proposed to be via a series of cytoplasmic and transmembrane interaction sites (Kimura *et al.*, 1996; Asahi *et al.*, 1999). Comparable data identifying the area of contact important for the PLMS/Na,K-ATPase interaction are not yet available. However, using controlled proteolysis, we have defined conditions at which

PLMS is truncated leaving the α subunit essentially intact (manuscript in preparation). Incubation with trypsin at very low trypsin/protein ratio resulted in the specific cleavage of a 5-kDa fragment from intact PLMS. The proteolysis was associated with a concomitant loss of the ability of the truncated 10-kDa PLMS fragment to become phosphorylated by protein kinases, indicating that the 5-kDa fragment is C-terminal containing the PKC/PKA phosphorylation motifs. This C-terminal truncation of PLMS resulted in a significant increase in the Na,K-ATPase activity. Furthermore, phosphorylation of PLMS resulted in a decreased sensitivity to proteolysis, as found previously for PLN (Kirchberger *et al.*, 1986). The C-terminal part of PLMS is, therefore, likely to be involved in the interaction with the shark Na,K-ATPase.

INTERACTIONS BETWEEN γ AND Na,K-ATPase

Nonsolubilizing concentrations of detergents have previously been shown to activate dog kidney Na,K-ATPase activity (Huang *et al.*, 1985). Using Na,K-ATPase purified from pig kidney, we studied if this detergent effect was mediated through interaction of kidney Na,K-ATPase with the γ subunit in a fashion resembling the PLMS/Na,K-ATPase interaction. Screening of different detergents showed that nonsolubilizing concentrations of the $C_{12}E_8$ specifically impaired the interaction between the γ subunit and kidney Na,K-ATPase. As with shark enzyme, this caused a stimulation of pig kidney Na,K-ATPase activity, suggesting that the γ and PLMS may act by a common regulatory mechanism. In contrast, nonsolubilizing concentrations of CHAPS, while activating shark rectal Na,K-ATPase activity, had no effect on the activity of kidney renal enzyme. Coimmunoprecipitation experiments have indicated that CHAPS does not impair the α/γ association, whereas $C_{12}E_8$ does (Mercer *et al.*, 1993). Thus, detergents which abrogate the α/γ interaction caused stimulation of Na,K-ATPase activity, while detergents that do not also fail to stimulate the Na,K-ATPase activity.

Other results characterizing the α/γ interaction come from the effects of γ-specific antibody on renal Na,K-ATPase: Treatment of pig kidney Na,K-ATPase membranes with a γ-specific antibody at a low antibody to Na,K-ATPase weight ratio (1:1000) resulted in stimulation of Na,K-ATPase activity and increased the apparent ATP affinity, while inhibition was observed at a higher antibody to Na,K-ATPase ratio (1:200) where the apparent ATP-affinity was decreased (cf., Therien *et al.*, 1999). The activation of Na,K-ATPase by low concentrations of the γ antibody was abolished if the membranes were pretreated with $C_{12}E_8$, indicating that the γ-antibody acted by impairing the γ/Na,K-ATPase interaction (Mahmmoud *et al.*, 2000). The graded functional effects induced by increasing the anti-γ antiserum concentration could be taken to indicate the successive breaking of functional α/γ interaction sites, as found for the PLN/Ca-ATPase interaction.

OLIGOMERIZATON

A puzzling property observed for several of the FXDY proteins as well as for PLN is that they either modulate or induce Cl^-or K^+ channel activity (Minor *et al.*, 1998). Moreover, PLM and PLN are known to form oligomeric structures in the membrane, apparently stabilized by a leucine zipper (Simmerman *et al.*, 1996). Recently, we have demonstrated that both the γ subunit and PLMS, which also have large concentrations of Leu and Ile residues to one face of the transmembrane helix form oligomers in purified Na,K-ATPase membranes (Mahmmoud *et al.*, 2000). The oligomeric nature of the STRP can conceivably explain their channel-like properties, but the physiological function of oligomeric assembly of these proteins, if any, is still unclear.

CROSS-TALK BETWEEN PKA AND PKC SIGNALING PATHWAYS

The low level of PKC-phosphorylation of the α-subunit of shark rectal gland Na,K-ATPase was found to increase substantially if detergent was added to the phosphorylation medium (Mahmmoud and Cornelius, 2000). Proteolytic fingerprinting and N-terminal truncation indicate that this phosphorylation is occurring at a separate site located at the C-terminal segment of the Na,K-ATPase α-subunit. Furthermore, PKC phosphorylation of the 19-kDa membrane preparation can clearly be demonstrated at high detergent concentrations (Mahmmoud and Cornelius, 2000). The only PKC consensus site present in the 19-kDa membrane fragment that includes M7 to M10 is Thr-932, located four amino acids upstream the PKA site in the same cytoplasmic loop. Actually, phosphorylation of a threonine residue by PKC was reported previously (Bertorello *et al.*, 1991). It is possible, therefore, that phosphorylation at this part of the enzyme by PKA or PKC is structurally impeded and controlled. The M8/M9 loop is very flexible (Arystarkhova *et al.*, 1995) and it is possible that PKA phosphorylation requires targeting by tethering the AKAP (A Kinase-Anchoring Protein)-anchored PKA to the plasma membrane (Kurihara *et al.*, 2000).

Recently several reports have indicated interaction between the PKA and PKC pathways (Borghini *et al.*, 1994; Cheng *et al.*, 1997) and phosphorylation by the two kinases has been suggested to be affected in a reciprocal way by ligands that stabilize the E_1 and E_2 conformations, respectively (Feschenko and Sweadner, 1994). The close proximity of both PKA and PKC phosphorylation sites in the same M8/M9 cytoplasmic loop may indicate that this loop is the functional motif responsible for cross-talk between the PKA and PKC signaling pathways.

GENERAL MECHANISM FOR STRP INCLUDING FXYD PROTEINS

As seen from Table I, association of STRP with P-type ATPases seems to be a general feature. It is conceivable that the FXYD proteins have common regulatory functions. In case of PLMS, the regulation of Na,K-ATPase is achieved by an association/dissociation mechanism controlled by protein kinase phosphorylation in a way resembling the PLN/Ca-ATPase regulation (Fig. 5). Another Na,K-ATPase regulator may be CHIF that is found in kidney tissue (Attali *et al.*, 1995) and, like the γ, lacks a phosphorylation motif. It is conceivable that PLM in cardiac tissue is also a regulator of the Na,K-ATPase, which could work by a protein kinase-dependent mechanism similar to PLMS and PLN. Preliminary experiments have shown that PLM is coimmunoprecipitated with Na,K-ATPase using an α-specific antibody (manuscript in preparation).

As indicated by Table I, each P-type ATPase often has various regulatory proteins associated. It is possible that different P-type ATPase isoforms have tissue-specific regulatory proteins associated. For Na,K-ATPase, e.g., the kidney $\alpha 1$ is specifically associated with the γ, whereas shark Na,K-ATPase, which is mainly $\alpha 3$, is regulated by PLMS. In the heart, where γ is absent, the $\alpha 2$ Na,K-ATPase isoform may be regulated by a third small regulatory protein, PLM.

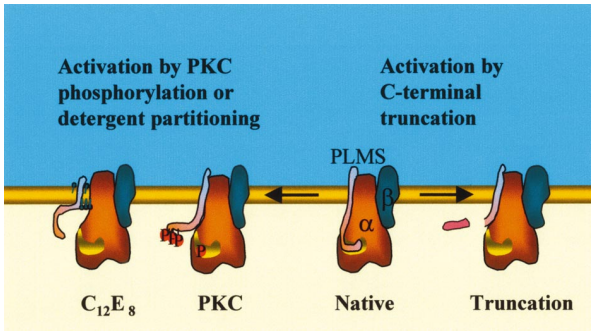

Fig. 5. Model for the proposed regulation of Na,K-ATPase by PLMS. Nonsolubilizing concentrations of detergent and PKC phosphorylation both cause dissociation of PLMS from the α subunit. This, together with C-terminal truncation of PLMS, all relieves the PLMS inhibition of Na,K-ATPase activity.

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