The Na,K-ATPase provides the driving force for many ion transport processes through control of Na⁺ and K⁺ concentration gradients across the plasma membranes of animal cells. It is composed of two subunits, α and β. In many tissues, predominantly in kidney, it is associated with a small ancillary component, the γ-subunit that plays a modulatory role. A novel 15-kDa protein, sharing considerable homology to the γ-subunit and to phospholemman (PLM) was identified in purified Na,K-ATPase preparations from rectal glands of the shark *Squalus acanthias*, but was absent in pig kidney preparations. This PLM-like protein from shark (PLMS) was found to be a substrate for both PKA and PKC. Antibodies to the Na,K-ATPase α-subunit communoprecipitated PLMS. Purified PLMS also communoprecipitated with the α-subunit of pig kidney Na,K-ATPase, indicating specific association with different α-isofoms. Finally, PLMS and the α-subunit were expressed in stoichiometric amounts in rectal gland membrane preparations. Incubation of membrane bound Na,K-ATPase with non-solubilizing concentrations of C₁₂E₈ resulted in functional dissociation of PLMS from Na,K-ATPase and increased the hydrolytic activity. The same effects were observed after PKC phosphorylation of Na,K-ATPase membrane preparations. Thus, PLMS may function as a modulator of shark Na,K-ATPase in a way resembling the phospholamban regulation of the Ca-ATPase. 

The Na,K-ATPase α-subunit is phosphorylated by a cAMP-dependent protein kinase (PKA) as well as a Ca²⁺- and phospholipid-dependent protein kinase (PKC) (1–9). PKA phosphorylates the conserved Ser-938 (2), while the main PKC site, Ser-18, is present only in rat α₁ and α₂ isoforms (3). Phosphorylation/dephosphorylation is believed to form the molecular basis of the rapid hormonal regulation of Na,K-ATPase in vivo. However, many aspects of the kinase-mediated regulation are still not well understood (see Ref. 4, for review).

The catalytic properties of the Na,K-ATPase is associated with the α-subunit, whereas the β-subunit regulates the maturation and expression of the enzyme (5). A small protein of apparent molecular mass ~8 kDa, named the γ-subunit, was originally suggested to be a third subunit of the Na,K-ATPase (6). The γ-subunit is predominantly expressed in kidney (7) where it is suggested to modulate the K⁺ activation of Na,K-ATPase (8, 9), and the apparent affinity of Na,K-ATPase for ATP (10). Recently, differences in the γ-subunit distribution along the kidney tubules have been inferred to explain the segment-specific Na⁺ affinity of the nephron (11).

The γ-subunit belongs to a family of low molecular weight membrane proteins containing a single transmembrane domain and a conserved extracellular motif FXDYD. Another member of this family, phospholemman (PLM) (12, 13) with apparent molecular mass ~15 kDa is the major substrate for PKA and PKC in myocardium. The proteins of this family have a wide range of expression patterns and some have been shown to serve as ion channels (9, 14–16). Except for the γ-subunit that specifically associates with the α-subunit, none of these proteins, however, have been shown to associate with other proteins. Here we report the identification of a new member of this family, termed phospholemman-like protein from shark (PLMS) since it has substantial homology to PLM, is about the same molecular mass, and is a substrate for both PKA and PKC. It is also homologous to the γ-subunit and like this, it associates specifically with the α-subunit, none of these proteins, however, have been shown to associate with other proteins. Here we report the identification of a new member of this family, termed phospholemman-like protein from shark (PLMS) since it has substantial homology to PLM, is about the same molecular mass, and is a substrate for both PKA and PKC. It is also homologous to the γ-subunit and like this, it associates specifically with the α-subunit, none of these proteins, however, have been shown to associate with other proteins. Here we report the identification of a new member of this family, termed phospholemman-like protein from shark (PLMS) since it has substantial homology to PLM, is about the same molecular mass, and is a substrate for both PKA and PKC.

**EXPERIMENTAL PROCEDURES**

**Materials**

The catalytic subunit of PKA was from Roche Molecular Biochemicals (Mannheim, Germany). L-α-Phosphatidylserine was obtained from Avanti (Alabaster, AL). Dioleoyl-1,2-sn-glycero-3-phosphorylcholine (DOPC) and dioleoyl-1,2-sn-glycerol was from Sigma. Purified PKC from rat brain was from CalBiochem (La Jolla, CA). [γ³²P]ATP, protein A-Sepharose, and the enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech. All other chemicals were of the highest analytical grade available.
METHODS  

**Na,K-ATPase Preparation, Solubilization, and Reconstitution—**
Na,K-ATPase from shark rectal glands and pig kidney was purified, solubilized, and reconstituted as described previously (17). The specific hydrolytic activities measured at 23 °C were 10.5 and 8 units/mg for membrane-bound shark rectal and pig renal enzyme, respectively. The specificity of solubilized shark enzyme was 11 units/mg at 23 °C. 

**PKA and PKC Phosphorylation of Na,K-ATPase—** PKA phosphorylation was performed in a mixture containing: 50 mM Hapes, pH 7.4, 10 mM MgCl₂, 0.5 mM CaCl₂, 10% glycerol, 0.02 mM phosphatidylerine, 0.01 mM dioleoyl-1,2-sn-glycerol, 0.1 mM ATP (Tris salt), 4 μg of protein, and 0.13 μg of PKA. PKC phosphorylation was performed in a 50-μl reaction mixture containing: 50 mM Hapes, pH 7.4, 10 mM MgCl₂, 0.5 mM CaCl₂, 10% glycerol, 0.02 mM phosphatidylerine, 0.01 mM dioleoyl-1,2-sn-glycerol, 0.1 mM ATP (Tris salt), 4 μg of protein, and 0.13 μg of PKC. For PKC phosphorylation in mixed micelles, the mixture contained the same ligands plus 8 μg of C₅E₅. The phosphorylation reaction for both kinases was initiated by the addition of 100 μM ATP containing 2 μCi of [³²P]ATP, proceeded for 30 min at 24 °C, and then terminated by the addition of 16 μl of sample buffer (18).

**Measurement of Hydrolytic Activity—** The hydrolytic activity of membrane-bound Na,K-ATPase in the presence of nonsolubilizing concentrations of C₅E₅, and after PKC phosphorylation was measured by preincubating the Na,K-ATPase (4 μg) with C₅E₅ (values indicated in figure legends) for 5 min on ice in a typical PKC phosphorylation mixture followed by a further incubation for 30 min at 24 °C in the presence or absence of PKC, as described above. The hydrolytic activity was subsequently measured by addition of 20 μl of this mixture to 500 μl of ATPase test medium containing: 30 mM histidine, pH 7.4, 3 mM MgCl₂, 1 mM EGTA, 0.06% bovine serum albumin, plus C₅E₅ and ATP concentrations as indicated in figure legends. The ³²P liberated from [³²P]ATP was measured as described previously (19). The ATP added during PKC phosphorylation was almost completely hydrolyzed during the 30-min incubation; this and the subsequent 25 times dilution in the ATPase test medium ensured that the [ATP] in the test medium was not affected.

**Gel Electrophoresis, Immunoblotting, and Immunoprecipitation—** The phosphorylated proteins were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 3% stacking gel, 9% intermediate gel, and 16% resolving gel). The gels were stained with Coomassie Blue, destained and dried, then analyzed by autoradiography. The bands corresponding to the α-subunit were excised from the gel and the phosphorylation stoichiometry calculated from the measured radioactivity and the calculated content of Na,K-ATPase α-subunit (17). To detect oligomeric structures of membrane proteins, we used the detergent perfluoro-octanoic acid (PFO). The PFO-PAGE was performed essentially as described in Ref. 20.

For immunoblotting, proteins were transferred to polyvinylidene difluoride membranes, washed three times for 20 min with phosphate-buffered saline buffer, and incubated with primary antibody overnight at 24 °C. The blots were then incubated with secondary antibody followed by washing, proteins were detected by ECL reagents.

Immunoprecipitation was performed by ligation of the α-subunit to protein A through C-terminal specific α-antibody (C1002–1016, kindly provided by J. V. Møller). About 500 μl of protein A-Sepharose was prewashed five times with the immunoprecipitation buffer containing: 300 mM NaCl, 10 mM EDTA, 40 mM methionine, 0.04% Na₂-azide, 2% Triton X-100, 0.2% bovine serum albumin, and 100 mM Tris-HCl, pH 7.4. After prewashing, the protein A-Sepharose beads were suspended in 300 μl of the immunoprecipitation buffer. About 20 μg of ³²P-labeled, PKA-phosphorylated Na,K-ATPase was added and the mixture incubated for 30 min at 24 °C with head-over-head shaking. The antibody (100 μl) was added and the mixture incubated for further 30 min. The mixture was then washed five times with RIPA buffer (650 mM NaCl, 20 mM EDTA, 1% Na deoxycholate, 0.1% SDS, 1% Triton X-100, and 50 mM Tris-HCl, pH 7.4) to remove nonspecific binding. Finally, the protein A-Sepharose beads were suspended in 200 μl of sample buffer and boiled for 5 min, then 30 μl was loaded onto 14% Laemmli gel (18). A control was run in parallel in which the anti-α serum was replaced by serum preadsorbed with proteins from shark preparations (non-immune rabbit serum). In immunoprecipitation experiments using pig kidney α-subunit, 40 μg of purified membrane-bound pig kidney enzyme was incubated with about 10 μg of the 15-kDa shark protein purified by electrophoresis as described below. Both α- and β-antibodies are used for immunoprecipitation of pig α, and a non-immune rabbit serum was used as control. The β antibody (β, 59–70) was a gift of J. V. Møller.

**Two-dimensional Gel Electrophoresis and Silver Staining—** Horizontal first-dimensional focusing was performed according to Görg et al. (21) using immobilized pH gradients from Amersham Pharmacia Biotech. Re-hydration of the immobilized pH gradients strips was performed in a solution containing 8% urea, 2% CHAPS, 2% immobilized pH gradients buffer (pH 3–10NL), 0.3% dithiothreitol. Approximately 80 μg of protein was included in the re-hydration solution and loaded to the immobilized pH gradients strip in a re-swelling tray. Vertical second-dimension gels were run in equipment designed as described by Cefis et al. (22). Silver staining was carried out as described in Ref. 23.

**N-terminal Sequence Analysis of the 15-kDa Protein—** Polyvinylidene difluoride strips containing the 15-kDa proteins were excised and analyzed using an Applied Biosystems Procise sequencer. In two separate experiments initial yields were 7.8 and 2.9 pmol. The primary sequence of the N-terminal 30 amino acids was obtained.

**Purification of the 15-kDa Shark Protein by Electrophoresis from SDS Gels—** Membrane fragments were resolved using 15% Laemmli gels and the bands corresponding to the 15-kDa protein excised. The protein in the SDS gel strips was extracted using a Bio-Rad system as described in Ref. 24. The extracted protein was concentrated using Centricon tubes (3 kDa cut off). Protein determination was as described (25), using bovine serum albumin as a standard.

RESULTS  

**Protein Profiles of Different Enzyme Preparations—** The regulation of Na,K-ATPase transport activity by PKC phosphorylation exhibits different characteristics among different cell types as well as in purified preparations (for review, see Ref. 4). The latter observations could be accounted for by the presence of ancillary proteins important for mediating the PKC effects in Na,K-ATPase enriched preparations, which are removed to various extents during purification. Therefore, the effects of PKC in different enzyme preparations and in preparations purified to different degrees (membrane-bound enzyme and solubilized enzyme from shark rectal glands) were compared and correlated with their protein patterns using two-dimensional gel electrophoresis. Fig. 1 demonstrates that several low molecular weight proteins co-purifying with Na,K-ATPase-enriched membranes are removed to a significant degree following solubilization of Na,K-ATPase with the non-ionic detergent C₁₂E₅₄. Solubilization resulted in the removal of about 30% (w/w) of insoluble proteins that were separated by centrifugation.

**PKC and PKA Phosphorylation Profiles—** Fig. 2 shows autoradiograms of PKC-phosphorylated membrane-bound (lane 1) and solubilized (lane 2) Na,K-ATPase. In both preparations the α-subunit was phosphorylated to low stoichiometries (<0.1 mol of PІ/mol of α), as found previously in other species, except for the rat (3). PKC phosphorylation of the shark Na,K-ATPase preparations revealed the existence of several low molecular weight weight phosphoproteins migrating as bands with apparent molecular mass in the range 35–15 kDa, including a heavily phosphorylated 15-kDa band, showing that in addition to the α-subunit several proteins in the preparation are targets for PKC. The bands migrating at molecular mass of about 80 kDa (labeled PKC) and 50 kDa correspond to autophosphorylated PKC holoenzyme and its catalytic subunit, respectively (26). PKC phosphorylation of the low molecular weight proteins was abolished or significantly reduced after solubilization of the membrane fragments (lane 2).

The 15-kDa protein was also observed in autoradiograms after phosphorylation by PKA both in membrane preparations and, with less intensity, after solubilization (Fig. 2, lanes 5 and 6, respectively). As seen from Fig. 2, lane 7, this 15-kDa protein is completely absent in pig kidney Na,K-ATPase preparations.

**Detergent Effects on PKC Phosphorylation of the 15-kDa Protein—** The absence of a PKC-phosphorylated 15-kDa protein band in the autoradiograms of solubilized Na,K-ATPase (Fig. 2,
The scattered light at 220 nm the solubilization of shark rectal preparations. Membrane-bound (A) and solubilized (B) shark Na,K-ATPase preparations were analyzed by two-dimensional gel electrophoresis and silver staining. Several low molecular weight proteins that were co-purified with membrane-bound Na,K-ATPase were removed after selective solubilization of Na,K-ATPase by C12E8 (indicated by arrows in panel A). Protein determination indicated that about 70% (w/w) of the total protein in membrane fragments was solubilized, whereas 30% was separated by centrifugation. The Na,K-ATPase α- and β-subunits are detected by Western blot analysis using specific antibodies. The β-subunits are recognized as multiple spots at about 53 kDa probably representing different modifications (glycosylations) of the protein. Bracketed spots are recognized by the PLM antibody and signify PLMS. As indicated by panel B the amount of PLMS is significantly decreased after solubilization. Molecular weight and pl are indicated on the figure.

lanes 2 and 3) suggested that solubilization either removed this component from the Na,K-ATPase preparation, or specifically inhibited its PKC phosphorylation, or both. Controls in which PKC phosphorylation of solubilized Na,K-ATPase was performed without separation of insoluble proteins demonstrated that phosphorylation of the 15-kDa band by PKC was specifically abolished by C12E8 (Fig. 2, lane 3). To test if PKC phosphorylation of this protein is dependent on its membrane association, Na,K-ATPase solubilized by C12E8 was incorporated into liposomes by complete removal of the detergent (17) followed by phosphorylation by PKC. As seen from Fig. 2, lane 4, the 15-kDa protein is phosphorylated by PKC after detergent removal, indicating that detergent inhibition of PKC phosphorylation of this protein was reversible.

Finally, in membrane-bound Na,K-ATPase phosphorylated by PKC in the presence of increasing concentrations of C12E8 it was found that PKC phosphorylation of the 15-kDa protein was abolished at a detergent/protein ratio of about 1.5, at a C12E8 concentration of ~200 μM (Fig. 3, upper panel). By measuring the scattered light at 220 nm the solubilization of shark rectal membrane fragments was followed at increasing C12E8 concentrations, performed in identical PKC phosphorylation medium without PKC. As indicated by the decrease in light scattering, membrane solubilization was found to take place at an identical detergent/protein ratio of 1.45 that abolished PKC phosphorylation of the 15-kDa protein (Fig. 3, lower panel). Taken together these results demonstrated that phosphorylation of the 15-kDa protein by PKC is dependent on its membrane association in contrast to its phosphorylation by PKA (see above).

Sequencing of the 15-kDa Protein—N-terminal sequencing of the 15-kDa band indicated the presence of only one protein with the partial sequence shown in Fig. 4. Comparison of the partial sequence by BLAST scanning against protein data bases revealed that the protein is novel and has a substantial homology to 13 other sequences. The closest homology (about 33% identity) was to PLM (15) which has a similar mobility on SDS-PAGE of 15 kDa and to the Na,K-ATPase γ-subunit with mobility of 8–11 kDa. PLM has a highly basic C-terminal sequence with phosphorylation motifs for one PKA and three PKC phosphorylation sites (13). This C-terminal sequence is absent in the γ-subunit. Due to the close homology to PLM this protein was termed PLMS. These proteins belong to a family of small hydrophobic proteins with a conserved signature pattern (DNS)(X)(XY)(X2/3)(ST/IVLM)(RQ)(XY)/2G (27). This family of proteins has a single transmembrane domain and has been suggested to form ion channels (9), or serve as channel regulators (mammary tumor, Mat-8 (16); channel inducing factor, CHIF (28); and related ion channel, RIC (29)). The partial sequence of PLMS includes a region highly homologous to PLM that represents in part a transmembrane domain. An antibody raised against the TYDY motif of phospholemman (kindly provided by L. R. Jones) reacted with PLMS, and recognized two

![Fig. 1. Two-dimensional gel analysis of shark Na,K-ATPase preparations.](image)

![Fig. 2. PKA and PKC phosphorylation profiles of Na,K-ATPase preparations.](image)
The partial sequence of PLMS is a man-like protein from shark Na,K-ATPase.

The fact that PLMS specifically associates with shark Na,K-ATPase—indicated by immunoprecipitation using a specific antibody to the α-subunit (Fig. 5, lanes 1–3)—demonstrated by immunoprecipitation using a C-terminal specific antibody to the α-subunit antibody linked to Protein A-Sepharose beads. After extensive preincubation, untreated and PKC-phosphorylated membrane-bound Na,K-ATPase were incubated with anti-α-subunit (Fig. 5, lanes 1–3). From reactions with isoform-specific antibodies (kindly provided by T. A. Pressley) the main α isoform of shark Na,K-ATPase was found to be α3, in accordance with previous findings (30). Furthermore, PLMS electroeluted from SDS gels and incubated with pig kidney Na,K-ATPase preparations, that lack a 15-kDa phospholemman-like protein, also immunoprecipitated with rabbit α1-subunit (Fig. 5, lanes 4 and 5), indicating that PLMS associates with at least two different α isoforms.

Further indications of the specific association of the two proteins came from protein determination of the amount of α-subunit and PLMS extracted from SDS gels. Here a stoichiometry of 1.1 mol PLMS/mol of α was calculated using a molecular mass of 112 kDa for the α-subunit and assuming a molecular mass of 8.4 kDa for PLMS, the same as found for PLM. This indicated stoichiometric expression of the Na,K-ATPase and PLMS.

Effects of C

PKC phosphorylation of PLMS on membrane association. Upper panel shows autoradiograms of 32P-labeled proteins after SDS-gel electrophoresis on 9–16% Tricine gradient gels after PKC phosphorylation of membrane-bound Na,K-ATPase in the presence of increasing C

PKC phosphorylation stoichiometry of the α-subunit was identical at the concentrations used. However, the band migrating at 15 kDa corresponding to phosphorylated PLMS is seen to disappear at concentrations of C

in the light absorption at 220 nm is achieved at the same C

phosphorylated PLMS is seen to disappear gradually at concentrations above 50 μM. In the lower panel solubilization of membrane fragments by C

Complete membrane solubilization, as indicated by a decrease in the light absorption at 220 nm, is achieved at the same C

spots in (Fig. 1). Association of PLMS with Na,K-ATPase—That PLMS specifically associated with shark Na,K-ATPase α-subunit was demonstrated by immunoprecipitation using a C-terminal specific antibody to the α-subunit antibody linked to Protein A-Sepharose beads. After extensive preincubation, untreated and PKC-phosphorylated membrane-bound shark Na,K-ATPase were incubated with anti-α antibody linked to Protein A-Sepharose beads. After extensive

Further indications of the specific association of the two proteins came from protein determination of the amount of α-subunit and PLMS extracted from SDS gels. Here a stoichiometry of 1.1 mol PLMS/mol of α was calculated using a molecular mass of 112 kDa for the α-subunit and assuming a molecular mass of 8.4 kDa for PLMS, the same as found for PLM. This indicated stoichiometric expression of the Na,K-ATPase and PLMS.

Effects of C

PKC phosphorylation of PLMS on membrane association—The fact that PLMS specifically associates with the Na,K-ATPase α-subunit and is phosphorylated by protein kinases could indicate that it is a component of the protein kinase regulation of Na,K-ATPase. The ability of C

to dissociate PLMS from the shark Na,K-ATPase membrane preparations was investigated by measuring PLMS recovered in the pellet after incubation of the membranes with increasing concentrations of C

As seen in Fig. 6 A, dissociation of PLMS from the Na,K-ATPase membrane preparations is evident at a C

concentration lower than needed for complete membrane solubilization (∼200 μM, cf., Fig. 3).

In order to test if PKC phosphorylation of PLMS affects its binding to the α-subunit of shark Na,K-ATPase, as shown above for nonsolubilizing concentrations of C

The partial sequence of PLMS is a 15-kDa phospholemman-like protein from shark Na,K-ATPase.

The partial sequence of PLMS is aligned with the γ-subunit from Xenopus (9) and the mature PLM from dog heart (13). In the γ-subunit and PLM the N-terminal parts are extracellular while the C-terminal parts are intracellular. Bold letters mark the C-terminal phosphorylation sites in PLM. The conserved family motif is underlined.
wash with RIPA buffer (see “Experimental Procedures”) the two preparations were resolved by SDS-PAGE, immunoblotted, and probed with the anti-TYDY antibody to detect PLMS. As seen from Fig. 6B the fraction of PLMS associated with the Na,K-ATPase α-subunit was substantially decreased after PKC phosphorylation. PKC phosphorylation and nonsolubilizing concentrations of C12E8 thus seem to act in parallel both inducing dissociation of the PLMS-α-subunit membrane complex.

Functional Effects of C12E8 and PKC Phosphorylation on Shark Na,K-ATPase—Whether dissociation of PLMS from the Na,K-ATPase α-subunit has any functional effects on Na,K-ATPase activity was investigated using mild treatment of nonionic detergents to dissociate PLMS from the α-subunit. This is a similar approach as previously employed to impair the phospholamban/Ca-ATPase interaction in cardiac sarcoplasmic reticulum (31, 32) and also to dissociate PLM from sarcomemmal membranes (33). In the following the effects of the C12E8 concentrations in the range between 0 and 300 μM and PKC phosphorylation on the kinetics of several partial reactions of the Na,K-ATPase were investigated.

The ATP-substrate Curve—In Fig. 7 the activating effect of nonsolubilizing concentrations of C12E8 and PKC phosphorylation on the ATP substrate curves of shark Na,K-ATPase is compared. As previously found (34), and as seen from the curved Eadie plots (inset to Fig. 7), the ATP substrate curves are biphasic in the presence of K+ with a major fraction with a low affinity for ATP and a smaller fraction with a high affinity for ATP. PKC phosphorylation affected the ATP substrate curve of shark Na,K-ATPase in exactly the same way as nonsolubilizing C12E8 at otherwise strictly comparable conditions (Fig. 7).

In Fig. 8A the maximum Na,K-ATPase activity (Vmax) derived from ATP-substrate curves, as depicted in Fig. 7, are plotted for various concentrations of C12E8. As seen Vmax increases significantly in a dose-dependent manner at C12E8 concentrations below CMC (~80 μM). The activation was maximal at C12E8 concentrations close to the CMC and above 300 μM inhibition sets in. Nonsolubilizing concentrations of C12E8 increased Vmax and K0.5 in parallel whereas above the detergent CMC K0.5 for ATP increased slightly above that of Vmax (Fig. 8B).

Similar activation by nonsolubilizing concentrations of C12E8 was previously observed in dog kidney Na,K-ATPase only at suboptimal ATP concentrations (100 μM), and was related to an increase in the ATP affinity at the low affinity site, measured as a decreased slope (K0.5/Vmax) in the double-reciprocal plot of the ATP substrate curve (35). As in the present study K0.5 for the low affinity ATP site increased slightly with increasing concentra-
The effects on Na,K-ATPase activity as observed with nonsolubilizing concentrations of detergents (Fig. 7) is further supported by the results given in Fig. 8. Here PKC phosphorylation is found to enhance the detergent-induced increase in $V_{\max}$ only at $[C_{12}E_8]$ below CMC, whereas above CMC PKC phosphorylation does not cause further increase in the catalytic activity of the enzyme.

It was found that the detergent-induced increase in maximum catalytic activity was not accompanied by an increased level of phosphoencezyme, which was constant and about 2.7 nmol/mg protein within the detergent range investigated. The increased catalytic activity is therefore due to an increase in maximal turnover.

The $K^+$ and Na$^+$ Activation—Further evidence of the synergistic effects of detergent and PKC is given by the $K^+$- and Na$^+$-activation curves in Fig. 9, A and B. The effects of $C_{12}E_8$ (20 µM) and $C_{12}E_8$ plus phosphorylation by PKC on the $K^+$-activation curve of Na,K-ATPase is shown in Fig. 9B. The intercept on the ordinate ($[K^+] = 0$ mM) is a measure of the Na,K-ATPase activity, which is shown in Fig. 9A. As indicated, nonsolubilizing concentrations of $C_{12}E_8$ (20 µM) and PKC phosphorylation both increased $V_{\max}$ for Na-ATPase activity. Fitting of the Hill equation to the data showed no effect on either $K_{0.5}$ or the Hill number, $n_H$. Also in case of the Na,K-ATPase activity both $C_{12}E_8$ and PKC significantly stimulated the catalytic activity (Fig. 9B). Evaluation of the $K^+$-activation curves showed that nonsolubilizing concentrations of $C_{12}E_8$ decreased the $K_{0.5}$ for $K^+$ for non-phosphorylated enzyme whereas it was unchanged for the PKC-phosphorylated enzyme (Fig. 10B). However, the $K_{0.5}$ value alone is a poor indicator of whether a ligand interacts with the same enzyme species as the substrate when $V_{\max}$ changes (36–38). Such interaction is, however, uniquely signified by the ratio $K_{0.5}/V_{\max}$, which is equivalent to the slope in double-reciprocal plots. As seen from Fig. 10B the ratio $K_{0.5}/V_{\max}$ decreases with increasing concentrations of $C_{12}E_8$ both for PKC-phosphorylated and non-phosphorylated enzyme. A similar decrease in the $K_{0.5}/V_{\max}$ ratio was also observed after PKC phosphorylation of the enzyme in the absence of detergent. Therefore, both $C_{12}E_8$ and PKC interacted with the $K^+$-activation of Na,K-ATPase. When the $K^+$-activation was investigated at increasing nonsolubilizing $[C_{12}E_8]$ for membrane-bound Na,K-ATPase with or without PKC phosphorylation (not shown) it was found that the activation in the presence of $C_{12}E_8$ below the detergent CMC was further increased by PKC phosphorylation (Fig. 10A). The PKC-induced enhancement of the $C_{12}E_8$ activation was only observed for the Na,K-ATPase activity, whereas the $C_{12}E_8$-induced increase in the Na activity was independent of PKC phosphorylation (Fig. 10A).

Comparison of Detergent Effects on Different Enzyme Sources—In order to test whether the effects of nonsolubilizing concentrations of detergents on shark Na,K-ATPase activity were specifically correlated with dissociation of PLMS from the $\alpha$-subunit or due to a direct effect on the enzyme, the effects of nonsolubilizing concentrations of $C_{12}E_8$ and CHAPS on the maximum Na,K-ATPase activity of pig renal enzyme were investigated. This enzyme preparation lacks a 15-kDa phopholemman-like protein comparable to PLMS (Fig. 2, lane 7). As seen from Fig. 11A a different pattern from that of shark enzyme was observed: nonsolubilizing concentrations of both $C_{12}E_8$ and CHAPS increased the Na,K-ATPase activity at a suboptimal ATP concentration of 250 µM in shark enzyme. However, with pig kidney enzyme no or very small activating effects of nonsolubilizing concentrations of CHAPS were observed in contrast to the activating effect of $C_{12}E_8$. This may relate to the fact that the pig renal Na,K-ATPase contains another small ancillary protein, the $\gamma$, which was previously shown to dissociate from the Na,K-ATPase $\alpha$-subunit (9). Interestingly, however, treatment with anti-$\gamma$ antiserum (kindly provided by S. J. D. Karlish) was assayed. As seen from Fig. 11B low concentrations of anti-$\gamma$ antiserum activated Na,K-ATPase whereas inhibition was observed at higher anti-$\gamma$ concentrations as also previously demonstrated (10). Interestingly, however, treatment with anti-$\gamma$ at activating concentrations (1:200 Ab:protein ratio) was found to completely abolish the activation by nonsolubilizing concentrations of $C_{12}E_8$ (Fig. 11B, inset). This suggested that the activating effect of nonsolubilizing $C_{12}E_8$ previously found in dog renal enzyme preparations (35) and in the present investigation using pig kidney enzyme could be caused by dissociation of $\gamma$ from the $\alpha$-subunit. Such a mechanism could conceivably also explain the effects of nonsolubilizing concentrations of detergents on the ATP affinity and $K_{0.5}$ for $K^+$ as found by Huang et al. (35) since the $\gamma$ has been demonstrated to modulate both the ATP affinity and the $K^+$-activation (8–11).
Detergent is related to the ability of the detergent to dissociate the γ-subunit from the enzyme. A comparison of the effects of C₄E₈ and CHAPS on pig kidney (open symbols) and shark rectal (closed symbols) Na,K-ATPase. To compare the two detergents their concentrations are given relative to their CMC (87 µM for C₄E₈ and 4 mM for CHAPS). For shark enzyme CHAPS concentrations below CMC activated the Na,K-ATPase activity measured at a suboptimal ATP concentration of 250 µM as observed with C₄E₈. For pig kidney enzyme, nonsolubilizing concentrations of C₄E₈ activated the Na,K-ATPase activity whereas no activation was observed with CHAPS. B, concentrations of anti-γ antiserum to the hydrolytic activity of pig kidney enzyme treated with increasing volumes of anti-γ antiserum. Na,K-ATPase was incubated with different volumes of anti-γ antiserum for 20 min at 24 °C. Maximum stimulation was obtained at an antibody to protein ratio of 1:200, which is comparable to the effect of nonsolubilizing C₄E₈. Treatment with anti-γ antibody prevented activation by nonsolubilizing concentrations of C₄E₈ as seen from the inset where the effects of C₄E₈ on the catalytic activity of pig kidney enzyme before and after treatment with anti-γ antiserum are compared.

Oligomeric State of PLMS and γ—Oligomeric assembly is an inherent property of certain small regulatory proteins like PLB (39) and PLM (40), which may explain their channel properties (15, 39). The presence of oligomeric states of PLMS and γ was investigated using PFO-PAGE (20). Fig. 12 shows that both PLMS and the γ could be isolated as oligomers when the detergent PFO is employed to stabilize the interactions within oligomers that is often disrupted using SDS-PAGE. On SDS-PAGE PLMS migrates as a band with apparent molecular mass of 15 kDa (monomeric PLMS, PLMSₘ) and the γ migrates as a doublet around 8 kDa (γₘ). These bands are present using PFO-PAGE as detected by PKA phosphorylation and immunoblots, respectively. However, an additional band with an apparent molecular mass of 22 kDa for shark rectal enzyme (PLMSₘ in Fig. 12, left panel) and of 18 and 21 kDa (γₘ in Fig. 12, right panel) for pig renal enzyme are also observed. This is consistent with the presence of oligomeric forms of both of these proteins in the membrane, the exact number of units cannot be deduced from the decrease in apparent mobility on SDS-PAGE. The oligomeric state of the γ may explain the recent finding that this protein exhibits cation channel activity (9).

Discussion

In the present investigation a novel phospholemman-like protein was identified in shark rectal Na,K-ATPase membrane preparations (Fig. 3). The protein has been termed PLMS since it exhibits the closest homology to PLM from myocardium. It is also closely homologous to the Na,K-ATPase γ-subunit and like this protein it is found to be specifically associated with the Na,K-ATPase. It is expressed in stoichiometric amounts to the Na,K-ATPase α-subunit and is immunoprecipitated by an antibody to the α-subunit (Fig. 4). Like PLM (19), both PKA and PKC phosphorylate PLMS (Fig. 2) whereas the γ-subunit lacks a C-terminal phosphorylation motif for PKA and PKC. PLM was first identified in cardiac tissue (12), and is mainly expressed in contractile tissues (13), liver and brain (41). Although the physiological function of PLM is elusive and it has not previously been reported to associate with other proteins (12–16), the close homology between PLM and PLMS could indicate that PLM is specifically associated with the Na,K-ATPase of heart cells, like PLMS in shark. In this respect it is interesting that PLM is mainly expressed in tissues where the expression of the γ-subunit is low. The γ-subunit is highly expressed in the kidney and stomach while its expression in the heart, brain, and liver is very low (8). Therefore, PLM could be a substitute for the γ in tissues like the heart. In pig kidney Na,K-ATPase preparations that contain the γ we could not detect a 15-kDa phosphoprotein (Fig. 2). Furthermore, in shark rectal enzyme preparations where PLMS is present we could not detect the γ, neither as the typical doublet migrating at 8 and 9 kDa on SDS-PAGE (7), nor by immunoblots probed by anti-γ antisera raised against rabbit γ. It remains, however, an open question whether significant expression of PLMS and the γ is mutually exclusive since a low molecular weight protein of 11 kDa with amino acid composition resembling that of mammalian γ has previously been isolated from shark rectal gland Na,K-ATPase preparations (42).

The fact that PLMS is found to be associated with the Na,K-ATPase and is phosphorylated by both PKA and PKC is consistent with a role as a modulator of the Na,K-ATPase. Several lines of evidence support this hypothesis. For one, it is demonstrated that C₄E₈ and PKC phosphorylation both dissociate PLMS from the Na,K-ATPase α-subunit (Fig. 6). Second, both cause an activation of shark Na,K-ATPase (Figs. 7–9). Finally, C₁₂E₈ and PKC both seem to modulate the K⁺ activation of the Na,K-ATPase.
Na,K-ATPase (Fig. 10B), whereas no interactions were observed on the apparent ATP affinity (Fig. 8B), or on the apparent Na$^+$ affinity associated with Na$^+$-ATPase activity (Fig. 10A). Therefore, the PKC regulation of shark Na,K-ATPase may be an indirect consequence of a phosphorylation-dependent dissociation of PLMS from the Na,K-ATPase α-subunit, rather than the result of a direct phosphorylation of the α-subunit. The low phosphorylation stoichiometry obtained by PKC phosphorylation of shark Na,K-ATPase seems to support this possibility.

Also, the mainly kidney-specific γ-subunit may serve a regulatory role (8, 10, 11) controlled by its association with the Na,K-ATPase. Nonsolubilizing C$_{12}$E$_8$ concentrations as well as anti-γ treatment was found to increase the Na,K-ATPase activity of pig kidney enzyme (Fig. 11, A and B). In dog kidney enzyme activation by nonsolubilizing C$_{12}$E$_8$ has previously been observed at suboptimal ATP concentrations and associated with an increase in the apparent affinity for ATP at its low affinity site (35). However, anti-γ treatment of rat kidney Na,K-ATPase, which may also act to dissociate the γ from the α-subunit of Na,K-ATPase, was previously shown to inhibit the catalytic activity and to decrease the apparent ATP affinity probably as a consequence of a shift in the $E_1/E_2$ conformational equilibrium (10), effects apparently opposite to those observed by nonsolubilizing concentrations of C$_{12}$E$_8$. However, in the present study using pig kidney enzyme, nonsolubilizing C$_{12}$E$_8$ concentrations increased the hydrolytic activity at saturating ATP (Fig. 11A) as observed in the shark rectal enzyme and this was found to be the case for anti-γ treatment at low antibody/protein ratios (1:200, Fig. 11B), which decreased the $K_{0.5}/V_{\text{max}}$ ratio for ATP activation (equivalent to the slope in double-reciprocal plots), in contrast to higher concentrations of antibody (1:50) that increased the $K_{0.5}/V_{\text{max}}$ ratio for ATP activation (not shown). A decreased slope in the double-reciprocal plots of the ATP-substrate curves has previously been found for rat renal membranes and γ-transfected HEK cells using a high anti-γ-protein ratio (10).

A very similar indirect regulatory mechanism accounts for the regulation of the Ca-ATPase via PKA or CaM kinase phosphorylation of PLB (43). In cardiac but not skeletal muscle, PLB regulates the Ca-ATPase by dephosphorylation/association and phosphorylation/dissociation processes. Low concentrations of C$_{12}$E$_8$ were found to activate the Ca-ATPase only in cardiac muscle due to dissociation of phospholamban from the Ca-ATPase (31, 32). In skeletal muscle, C$_{12}$E$_8$ had no effect on activity. Effect of PLB on the oligomeric state of the calcium pump has been reported (31). One feature of the PLB regulation of Ca-ATPase, which is not quite understood, is how the oligomeric structure of PLB as a pentamer is important, since both the monomeric and oligomeric forms of PLB are phosphorylated and both are inhibitory (44). PLM also forms oligomers (40) and in the present study this has now been shown to be the case for PLMS and the γ, too. Thus, oligomeric assembly appears to be an inherent property of these regulatory proteins, the significance of which remains to be established.

Whether or not association/dissociation of small regulatory protein has a physiological bearing in Na,K-ATPase regulation is still unclear. However, it may turn out that interactions of these proteins with the Na,K-ATPase could be a general mechanism for Na,K-ATPase regulation, as hypothesized in the model depicted in Fig. 13. In the case of PLMS the dissociation/association reaction could be controlled by protein kinase phosphorylation, probably through regulation of the electrostatic interaction between the proteins. In case of the γ, however, it is more difficult to envisage how its association with the Na,K-ATPase could be controlled, since the γ was found not to be phosphorylated by PKA or PKC (not shown (13)), unless its interaction is controlled via PKC phosphorylation of the Na,K-ATPase α-subunit.

That phosphorylation of some membrane components affects the Na,K-ATPase activity has in fact been observed earlier. In a study by Turu and Török (45) it was reported that Ca$^{2+}$ inhibited myometrial Na,K-ATPase from rat and that the inhibition was abolished by treating the membranes with SDS. In a subsequent study on the effects of Ca$^{2+}$ and SDS, it was found that Ca$^{2+}$ increased the phosphorylation level of substrate proteins and that phosphorylation of these membrane components was abolished by SDS (46).

An indirect PKC regulation of the Na,K-ATPase via small regulatory proteins could conceivably explain the heterogeneous effects of Na,K-ATPase regulation as found in different tissues, in purified preparations, and in transfected cells (47–49). It is, however, an open question if the observed effects seen by activation of PKC in vivo are due exclusively to direct phosphorylation of the α-subunit. Recent studies seem to indicate regulation of Na,K-ATPase by PKC by phosphorylation-independent mechanisms (50). One may ask what are the effects of the direct phosphorylation of the Na,K-ATPase α-subunit by PKC? In a previous study using solubilized Na,K-ATPase it was found that PKC phosphorylation of the Na,K-ATPase α-subunit under conditions where PLMS was dissociated from the membrane thereby preventing its phosphorylation, the catalytic activity of the Na,K-ATPase was inhibited (51). This could indicate that phosphorylation of the α-subunit per se could have functional importance maybe in the regulation of the interaction with the regulatory proteins, or phosphorylation of the N-terminal segment could regulate its association with lipid domains in the membrane formed by activation of PKC (52). The electrostatic profile around the PKC phosphorylation site situated in the N-terminal part of the α-subunit has previously been shown to be functionally important (53).

To conclude, this study raises the possibility of indirect kinase regulation of Na,K-ATPase through small regulatory proteins that are protein kinase substrates. In shark membrane-bound enzyme preparations a novel protein, PLMS, with such properties has been identified. PLM found in myocardium may be another example. Studies using nonsolubilizing concentrations of C$_{12}$E$_8$ and PKC phosphorylation indicate that dissociation from the Na,K-ATPase causes increase in the catalytic activity, a mechanism resembling the PLB regulation of the Ca-ATPase. This activation results from various changes in the detailed kinetic reactions, depending on the enzyme source, including the apparent ATP affinity and the K$^+$ activation, which may rely on the $E_1/E_2$ conformational equilibrium. In this respect it is interesting that PKC phosphorylation of shark enzyme have been reported to affect the $E_1/E_2$ conformational

![Fig. 13](image-url)
equilibrium (54), an effect resembling that seen by anti-γ-treatment of renal enzyme (10). Another common feature of these small proteins is their oligomeric structure, the functional significance of which is unknown but may account for their proposed channel properties. The concept of association of low molecular weight proteins with ion pumps, therefore, seems to be a general feature of regulation of these transport ATPases by protein kinases.

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