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Regulation of Na,K-ATPase by PLMS, the Phospholemman-Like Protein From Shark: Molecular Cloning, Expression, Cellular Distribution and Functional Effects of PLMS

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Regulation of Na,K-ATPase by PLMS, the Phospholemman-like Protein from Shark

MOLECULAR CLONING, SEQUENCE, EXPRESSION, CELLULAR DISTRIBUTION, AND FUNCTIONAL EFFECTS OF PLMS*

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In Na,K-ATPase membrane preparations from shark rectal glands, we have previously identified an FXYD domain-containing protein, phospholemman-like protein from shark, PLMS. This protein was shown to associate and modulate shark Na,K-ATPase activity in vitro. Here we describe the complete coding sequence, expression, and cellular localization of PLMS in the rectal gland of the shark Squaleus acanthias. The mature protein contained 74 amino acids, including the N-terminal FXYD motif and a C-terminal protein kinase multisite phosphorylation motif. The sequence is preceded by a 20 amino acid candidate cleavable signal sequence. Immunogold labeling of the Na,K-ATPase α-subunit and PLMS showed the presence of α and PLMS in the basolateral membranes of the rectal gland cells and suggested their partial colocalization. Furthermore, through controlled proteolysis, the C terminus of PLMS containing the protein kinase phosphorylation domain can be specifically cleaved. Removal of this domain resulted in stimulation of maximal Na,K-ATPase activity, as well as several partial reactions. Both the $E_2 - P \rightarrow E_3 - P$ reaction, which is partially rate-limiting in shark, and the $K^+$ deocclusion reaction, $E_3(K) \rightarrow E_4$, are accelerated. The latter may explain the finding that the apparent Na$^+$ affinity was increased by the specific C-terminal PLMS truncation. Thus, these data are consistent with a model where interaction of the phosphorylation domain of PLMS with the Na,K-ATPase α-subunit is important for the modulation of shark Na,K-ATPase activity.

The Na,K-ATPase is the enzyme responsible for active transport of Na$^+$ and K$^+$ across the plasma membranes of animal cells (for recent review, see Ref. 1). It establishes and maintains the electrochemical gradients for Na$^+$ and K$^+$ responsible for generation of a resting membrane potential necessary for excitability of muscle and nerve cells, co- and counter-transport of ions and nutrient molecules across the cell membrane, as well as the regulation of cell volume. The enzyme is composed of two essential subunits; a catalytic α-subunit, which undergoes conformational changes that couple ATP hydrolysis to ion transport, and the heavily glycosylated β-subunit responsible for maturation, assembly, and membrane targeting of the enzyme. Different isoforms of the α- and β-subunit have been identified, and these have unique kinetic properties and tissue distributions.

As a housekeeping enzyme the regulation of the Na,K-ATPase is very complex and occurs at many different levels, including both rapid (short term) and sustained (long term) hormonal control. Recently, considerable interest have been directed at studying the role of protein-protein interactions in the acute hormonal regulation of Na,K-ATPase activity. Indeed, regulation of transport ATPases by interaction with small regulatory proteins is a well known mechanism to achieve modulation of ATPase activity in vivo (for review see Ref. 2). Such interactions are especially well described for the regulation of SERCA1 by phospholamban (PLN) and sarcolipin (3–7).

The small protein called the γ-subunit is the first example of a small single transmembrane protein interacting with and regulating Na,K-ATPase (8–10). The γ-subunit has been shown to modulate Na,K-ATPase activity in the kidney by affecting the $E_2/E_1$ equilibrium toward $E_1$, thus regulating the affinity for ATP at its low affinity site and the cytoplasmic Na$^+$ and K$^+$ competition (11). The γ-subunit has a highly distinct distribution along different parts of the nephron allowing differential regulation of ion transport along different nephron segments (12).

The γ-subunit is a member of a family of small hydrophobic proteins, now termed the FXYD domain-containing protein family (13). This family includes phospholemman (PLM or FXYD1) (14), the γ-subunit (FXYD2) (15), mammary tumor protein of 8-kDa molecular mass (MAT-8 or FXYD3) (16), channel-inducing factor (CHIF or FXYD4) (17), related to ion channel (RIC or FYXD5) (18), as well as FXYD6 and FXYD7. Until recently the physiological functions of the FXYD pro-

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1 The abbreviations used are: SERCA, sarcoplasmic-endoplasmic reticulum calcium ATPase; PLN, phospholamban, PKA, protein kinase A; PKC, protein kinase C; DTT, dithiothreitol; RACE, rapid amplification of cDNA ends; MOPS, 4-morpholinoethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid; EST, expressed sequence tag.
The regulatory interaction of Na,K-ATPase with FXYD proteins seems to play an important role in cellular physiology and pathophysiology. For instance, the physiological relevance of the γ-subunit has recently been substantiated by identification of a point mutation of a glycine residue, which is highly conserved among all FXYD proteins, which correlates with a renal magnesium deficiency (25). In addition, phenotypic analysis of CHIF knockout mice indicated that CHIF plays a vital role in the tolerance to high K⁺ loading (26). Thus, characterization and localization of FXYD proteins in different tissues represents an important aim in identifying regulatory mechanisms of ion transport under physiological and pathophysiological states.

Little is known about the three-dimensional functional interactions leading to regulation of the Na,K-ATPase by FXYD proteins. Spatial localization of the γ-subunit has been indirectly inferred from cryo-electron microscopy of two-dimensional crystals (27) or from thermal denaturation experiments (28). They seem to indicate that the γ-subunit is associated with the C terminus of the α-subunit being located either between the M2/M9 or the M9/M10 transmembrane segments. Recently, the kinetic effects of γ on Na,K-ATPase were allocated to distinct domains within the γ-subunit (29). Also, mutagenesis studies of both the γ-subunit and CHIF indicated that the FXYD motif was important for long term and stable association with the α-subunit, whereas the basic residues located at the C terminus of CHIF are not necessary for association but are important determinants for the functional effects of CHIF on Na,K-ATPase (23). Recently it was demonstrated that residues in the transmembrane segment of γ and CHIF are important for their association with and regulation of the Na,K-ATPase (30).

PLM and its homologue PLMS are the only members of the FXYD family known to be phosphorylated by protein kinases. The C terminus of PLMS is heavily phosphorylated by PKC (21), as is the case for PLM (14). Co-immunoprecipitation experiments demonstrated that dephosphorylated PLMS associated more strongly with the α-subunit than PKC-phosphorylated PLMS (21). This suggests that the interaction between PLMS and the shark α-subunit could be controlled by protein kinase-mediated phosphorylation reactions in a similar way to that proposed for the phospholamban (PLN) regulation of the Na,K-ATPase in cardiac tissue in response to hormonal stimulation (3–7). Furthermore, PKC phosphorylation of the C-terminal cytoplasmic domain of PLMS, or disruption of interactions within the transmembrane domain by treatment with non-solubilizing concentrations of the non-ionic detergent C12,E8, have been shown to result in activation of the shark Na,K-ATPase by relieving the inhibitory effect of PLMS (21). This again emphasizes the implication of multiple domain interaction between FXYD regulatory proteins and Na,K-ATPase, as is the case for PLN regulation of Ca-ATPase.

In the present study we aim to further characterize the molecular interactions that result in the regulation of shark Na,K-ATPase by PLMS. To begin this, we have first cloned PLMS and determined its primary amino acid sequence from cDNA. In addition, we have characterized the cellular distribution of both PLMS and the α-subunit in rectal gland cells using immunocytochemical methods. Finally, through controlled proteolytic assay we have been able to preferentially cleave a 5-kDa fragment from the C terminus of PLMS, which contains the protein kinase phosphorylation sites. Using this approach, we have characterized the functional effect of the 3900 × g fraction between the C-terminal domain of PLMS and shark Na,K-ATPase. Some results of this study have been previously reported (31).

**EXPERIMENTAL PROCEDURES**

**Total RNA Extraction**—Total RNA was extracted using a modification of the Chomczynski and Sacchi method (32) as described previously (33). In brief, tissues were collected and rapidly frozen in liquid nitrogen before transfer and storage at −80 °C. The tissue was pulverized using a mortar and pestle and then homogenized in 10 volumes (v/w) of 4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% (vol/vol) Sarkosyl, and 90 mM 2-mercaptoethanol using a Polytorn PT 10 homogenizer (Kinematica Ltd.) set at position 5, for 2 × 20–30 s. Following homogenization, total RNA was extracted by the sequential addition of 0.1 volume of 2% sodium acetate, pH 4.0, 0.5 volume of water-saturated phenol, and finally 0.2 volume of 1- bromo-3-chloropropane. Tubes were vortexed briefly before the additions of each solution and then centrifuged at 3900 × g for 30 min at 4 °C in a Beckman J-6-MC centrifuge (Beckman Instruments Inc.). The upper aqueous phase was carefully transferred to a fresh tube, and then 2.5 volumes of 2-propanol and 0.2 volume of 1.2 M NaCl, 0.8 M sodium citrate, pH 7.0, was added sequentially with vortexing. The resulting solution was incubated at room temperature for 10 min, before centrifugation at 3900 × g for 30 min. The supernatant was aspirated, and the pellet was washed twice with 80% ethanol before drying under vacuum at room temperature for 5 min. After resuspension of the pellet in diethylpyrocarbonate-treated Milli-Q water, diluted samples (1:100) were prepared and the absorbance measured at 280 and 280 nm (Philips PU 8620 spectrophotometer) to estimate both the concentration and purity of the RNA samples. RNA samples from each extract were also run on denaturing formaldehyde gels and stained with ethidium bromide (as detailed below) to ensure that no degradation of the RNA had occurred.

**Cloning and Sequencing**—First strand cDNA synthesis was carried out in a total reaction volume of 20 μl containing 5 μg of total rectal gland RNA, 75 mM Tris-HCl, pH 8.3, 3 mM MgCl₂, 10 μM DTT, 10 μM oligo(dT)₁₂₋₁₅, 1 μM each of deoxyribonucleotide triphosphates (dNTPs; dATP, dGTP, dCTP, and dTTP), and 200 units of Superscript II (In-vitrogen, Paisley, UK). The reaction was incubated at 45 °C for 2 h and then stored frozen at −20 °C for use in PCR. This single strand cDNA template was used for the amplification and isolation of the initial 203-bp fragment with the sequences further 5' or 3' to this subsequently obtained by rapid amplification of cDNA ends using the Marathon RACE kit (Clontech, Basingstoke, UK) as described previously (33). All PCR reactions were carried out using 0.5 μl of cDNA template in a total volume of 20 μl comprising 10 μM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 4 pmol each of sense and antisense primers, and a 1:25 unit of Taq DNA polymerase (BioGene, C. Fos, UK). Primers used were as follows. Initial amplifications were carried out using Squ-1 sense (CGCCCTCAGGCGCTGAGGAGCG) and Squ-1 antisense (CCGGCTTGGGGTGGACAGCGC) primer pairs (X = any base). Subsequent nested 5' and 3' RACE reactions employed 5'-RACE-1 (CACACAGCTACTCGGCGACC), 5'-RACE-2 (CCACACTAGGTCCGCAACAGCC), 3'-RACE-1 (GCGTGTGTTGCGACTGTTGGT), and 3'-RACE-2 (GTTGGCCAGTGCTGTTGGT) primers in amplifications with the Marathon kit primers AP-1 (CATCCCTAATACGACGCTTATAGGGC) and AP-2 (ACTTACTATAGGGCTGAGGCG). PCR was performed using a hot start technique with an initial 2-min incubation at 92 °C, followed by 4 cycles of 94 °C for 5 s, 55 °C for 30 s, and 72 °C for 30 s, with a final incubation of 72 °C for 10 min.

DNA fragments within PCR reactions were either purified directly using an Edge BioSystems Quick-Step PCR purification kit (VHI Bio Ltd., Gosforth, UK) or separated by Tris acetate-EDTA-agarose gel electrophoresis (34) and bands of interest were purified using a Gene-clean II DNA purification kit (Anachem Ltd., Luton, UK).
Northern Blotting—Northern blotting was performed as described previously (33). The probe used for Northern analysis was a full-length cDNA containing the complete sequence shown in Fig. 1. Total RNA (5 μg, as measured by absorbance at 260 nm) extracted from various Squalus tissues was resuspended in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.8) containing 12.5 mM formamide and 2.2 M formaldehyde and then denatured at 65°C for 15 min and snap-cooled on ice before adding 0.1 volume of 5% “Loading Dye” (0.025% bromophenol blue, 0.025% xylene cyanol, and 50% glycerol; all w/v). Samples (30–100 μl) were then loaded onto the agarose gel (1.2% agarose w/v/Biogene Ltd.), MOPS buffer containing 6.7% (w/v) formaldehyde and 125 μM from MOPS buffer. After electrophoresis, gels were stained for 30 min in 0.1 mM ammonium acetate, 5 μg/ml ethidium bromide before destaining for 1–2 h in several changes of 0.1 x ammonium acetate before viewing on a UV transilluminator. The integrity and relative amounts of RNA loaded onto each lane were qualitatively assessed by viewing the sharpness and intensity levels of ethidium bromide-stained 18 S and 28 S ribosomal RNA bands as quantified using a gel documentation and analysis system (Syngene, Cambridge, UK). The staining intensities of the tissue RNA bands were compared with a known standard, and the amount of total RNA loaded on each lane was re-determined. The separated RNAs were electrophoretically run using TAE (40 mM Tris base, 80 mM sodium acetate, 1 mM EDTA, pH 8.3) and 18% resolving gels, unless indicated elsewhere. Molecular weight standards were from Bio-Rad ( Hercules, CA). For the detection of 32P-labeled kinase phosphorolysis, the gels were stained with Coomassie Blue, destained, dried, and then analyzed by autoradiography overnight at ~80°C. For immunoblotting, proteins were transferred to polyvinylidene difluoride membranes, then washed for 1 h with PBS buffer containing 5% Tween 20, and incubated over-night at room temperature with primary antibody. The membranes were washed again with PBS and incubated with goat anti-rabbit antibody for 2 h. After washing, the proteins were detected using ECL reagents (Amersham Biosciences). For the detection of the α-subunit from shark rectal gland and pig kidney, the antibody NKA1002 anti-PLMS (Amersham Biosciences) was used. Preparation of Tryptsinized PLMS—To obtain cleavage of the C terminus of PLMS, membrane-bound enzyme was incubated with trypsin (w/v trypsin to protein 1:1000) for 0–10 min on ice in the presence of 130 mM NaCl or 20 mM KCl, plus 1 mM EDTA. The trypsinization reaction was started by the addition of trypsin and stopped by adding a 10-fold excess of soybean trypsin inhibitor. The mixtures were diluted 10-fold with imidazole buffer (25 mM) and centrifuged at 170,000 × g for 1 h. The membranes were washed with imidazole and centrifuged again, then finally suspended in a 30 mM histidine buffer, pH 7.4, containing 25% glycerol, and stored at −20°C. All procedures were carefully performed on ice. Production of an Antibody to PLMS—Anti-PLMS antisera was prepared by injection of rabbits with PLMS resolved by SDS-PAGE together with Freund’s adjuvant as previously described (40). Characterization and epitope mapping of this antibody will be described elsewhere.


**RESULTS**

**Cloning and Sequencing of PLMS**—The initial *Squalus* PLMS cDNA fragment to be cloned was amplified using oligonucleotide primers based on the known PLMS amino acid sequence around the FXYD motif (21) and a C-terminal consensus sequence based on information from all known mammalian phospholemman proteins (43). Initial amplifications using a specific sense primer (Squ-1 sense) to the known RFTYDYY motif within the previously isolated peptide and a specific antisense primer (Squ-1 antisense) based on the RRLSTRRR motif found at the C terminus of the known mammalian proteins resulted in the amplification of a 203-bp fragment. When analyzed, this fragment encoded the expected amino acid sequence resulting from the deletion of the peptide (with the exception of leucine replacing lysines at positions 19 and 24) plus 18 other amino acids before a premature stop codon was encountered. Because the 3'-end of the amplicon exhibited no homology to any known FXYD gene, specific sense (3'RACE, 1; 3'RACE, 2) and antisense (3'RACE, 1; 3'RACE, 2) primers were synthesized based on the RRLSTRRR motif found at the C terminus of the known mammalian proteins. The final sequence based on information from all known mammalian members of this family, is altered to FXYD in the amino acid sequence in the transmembrane signature motif (21), and a C-terminal consensus sequence around the 3'-end of the amplicon exhibited no homology to any known FXYD gene. Specific sense (3'RACE, 1; 3'RACE, 2) and antisense (5'RACE, 1; 5'RACE, 2) primers were synthesized based on the RRLSTRRR motif found at the C terminus of the known mammalian proteins. Nested RACE reactions incorporating PLMS-specific primers in combination with AP1 and AP2 primers were then used along with the Marathon cDNA template to amplify 5'- and 3'-ends of the cDNA. When the 3'RACE products were cloned and sequenced, it was clear that the amplification of the original 203-bp fragment was due to mispriming of the antisense primer with the last 107 nucleotides having no homology with any FXYD genes. The final nucleotide sequence based on the consensus sequence of all cloned fragments is shown in Fig. 1. Following the results of the initial Northern analyses (see below) repeated attempts using the 3'RACE procedure failed to amplify any fragment larger than 318 bp. Although a short poly(A) sequence was present at the 3'-end, there was no obvious signs of a polyadenylation signal in the short 3'-untranslated region.

The cDNA sequencing showed that the protein consisted of 94 amino acids (Fig. 1) and contained a putative 20-amino acid N-terminal signal sequence (44), giving a mature protein with a length of 74 amino acids. The hydrophy plot (not shown) indicates the precursor protein has a hydrophobic amino terminus with one putative transmembrane domain approximately extending between amino acids 40 and 60. The hydrophobic transmembrane domain contains a leucine-isoleucine zipper motif conserved in many FXYD proteins and probably responsible for the strong tendency of some FXYD proteins to form oligomeric structures (43). The protein is thus a typical type I protein that is initially targeted to the endoplasmic reticulum by a cleavable N-terminal signal sequence and subsequently anchored in the plasma membrane by a stop-transfer sequence. The cytoplasmic domain contains several basic residues, two conventional protein kinase phosphorylation sites (Thr-53 and Ser-55), and several additional putative phosphorylation sites. There are six potential trypsin cleavage sites at the C-terminal end (positions 42, 44, 46, 51, 52, and 54) just in front and partially overlapping the conventional phosphorylation sites.

**Comparison with Mammalian and Teleost FXYD Proteins**—Fig. 2 compares the PLMS sequence with the sequences of the known seven FXYD proteins found in humans as an example of mammalian species. In addition to the mammalian species, three gene products from the zebrafish, *Danio rerio*, are homologues of the FXYD proteins (FXYD6dr, FXYD8dr, and FXYD9dr in the provisional terminology suggested by Sweedner and Raedt; Ref. 13). Although there is high conservation of the amino acid sequence in the transmembrane signature motif, there are certain differences between the shark and teleost transcripts and most other mammalian FXYD containing proteins. The FXYD amino acid motif, which is common to all mammalian members of this family, is altered to FXYD in FXYD8dr and one of the phospholemman-like proteins cloned from the Japanese Medaka, FXYD.b (Fig. 2). However, as

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**Fig. 1. Squalus PLMS interleaved nucleotide and amino acid sequence.** The double lines indicate the putative leader sequence, and the putative transmembrane domain is indicated by the single solid line. Potential phosphorylation sites for PKA and PKC are indicated in boldface and framed. Possible trypsin cleavage sites are indicated by ▲.
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Fig. 2. The amino acid sequence of _Squalus_ PLMS aligned with known human FXD family members and published sequences from teleost fish. The amino acid sequence of _Squalus_ PLMS aligned with known human FXD family members and related EST sequences from various teleost fish. It should be noted that these sequences should be considered to be provisional, because they were based on single unverified sequence runs. Variant sequences have been reported for the human gamma (F YD2) and for a number of teleost ESTs and various teleost fish. It should be noted that the sequences based on the teleost ESTs should be considered to be provisional, because they are based on single unverified sequence runs. Variant sequences have been reported for the human gamma (F YD2) and for a number of teleost ESTs and various teleost fish.

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</tr>
<tr>
<td>Fugu GeneBank - FXD.b</td>
<td>MTIT</td>
<td>DQ023580</td>
<td>1.00</td>
<td>0.97</td>
<td>0.95</td>
<td>0.93</td>
<td>0.91</td>
<td>0.89</td>
<td>0.87</td>
<td>0.85</td>
</tr>
</tbody>
</table>

In_ PLMS_, other putative homologues of phospholemman retain the FXD motif in Medaka (FXD.a), Fugu (FXD.a and .b), and the Japanese flounder. The normally conserved proline residue found immediately before the FXD motif in all mammalian proteins is replaced either by a positively charged arginine (_Squalus_ PLMS) or a negatively charged aspartic acid residue (Medaka FXD.a, Fugu FXD.a, and Japanese flounder FXD). In addition, the invariant serine, which is ubiquitously found within the signature sequence and is considered to signal the end of the transmembrane domain (Swedner and Rael (13)), is replaced by either an alanine in both PLMS and Japanese flounder FXD or cysteine in Fugu FXD.a (Fig. 2).

The two invariant glycines at positions 46 and 57 in the consensus sequence in Fig. 2 are retained in all PLMS homologues with the exception of Japanese flounder FXD where the second invariant glycine is replaced by a serine. Because ESTs are the result of single sequencing runs, they often contain errors, and thus the sequences for all of the teleost fish in Fig. 2 are tentative.

The derived amino acid sequence of PLMS shares closest homology with Mat-8/FXD3 with 39–45% amino acid identity and 59–67% amino acid similarity to the known mammalian isoforms. The sequence also exhibits high homology (45% identity and 69% similarity) to the zebrafish protein designated FXD9dr (Swedner and Rael (13)) and to the Medaka FXD.b sequence (48% identity and 66% similarity). Homologies (measured as percent identity/percent similarity) are then reduced when the putative amino acid sequence is compared with mammalian FXD1 (32–39/60–65%), FXD4 (31/65%), FXD5 (32–33/53–54%), FXD6 (29–32/57%), FXD2 (19–29/41–47%), and FXD7 (17–18/48%). Likewise, low homologies were also found when comparison were made to the EST sequences for Medaka FXD.a (29/54%), Fugu FXD.a (28/49%), and FXD.b (20/38%) and the Japanese flounder FXD (28/47%) proteins (Fig. 2).

**Immunolocalization of PLMS and Na,K-ATPase a-Subunit**

The rectal gland is composed of winding tubules formed by tall columnar cells arranged around a central duct or lumen as previously described (45–47). The luminal cell surface exhibits only a few small microvilli (Fig. 4A), but the basolateral cell surface is greatly amplified through slender lateral and basal cytoplasmic folds (Fig. 4B). Notably, almost the entire base of the cells consists of such thin cytoplasmic folds, which are devoid of mitochondria and abut the basal lamina (Fig. 4C), thus comparable to parts of the peritubular surface of amphibian kidney tubules (48).

Immunolocalization of PLMS showed distinct labeling of the entire basolateral cell membrane (Figs. 5A and 6A), except the most apical part at the tight junction and around the apical...
desmosomes, where labeling was sparse. Importantly, no labeling was associated with the luminal cell membrane (Fig. 5A).

The cell membrane of the extensively interdigitating lateral folds was strongly labeled (Figs. 5A and 6A). Most colloidal gold particles were associated with the cytoplasmic face of the cell membrane, which is consistent with an intracellular localization of the epitope. No label was associated with mitochondria or accumulated in any other part of the cytoplasm. Labeling with pre-immune serum was negative (Fig. 5B).

The localization of the Na,K-ATPase α-subunit was similar to that of PLMS. Notably, the luminal membrane was unlabeled (Fig. 5C) and again much of the label was associated with the cytoplasmic face of the cell membrane (Figs. 5C and 6B).

Cleavage of the C Terminus of PLMS by Trypsin—As seen in Fig. 1 the sequence of PLMS contains several potential tryptic cleavage sites in the C-terminal cytoplasmic domain upstream of the phosphorylation motif. We have previously described how PKC phosphorylation of PLMS leads to Na,K-ATPase activation by impairing of the protein-protein interaction (21). To investigate the nature of such interaction between the PLMS C terminus and the Na,K-ATPase in further details we have undertaken studies to preferentially cleave the PLMS C terminus while keeping the α-subunit intact. This was achieved by using an extremely low concentration of trypsin and incubation for short periods on ice in the presence of the Na,K-ATPase physiological ligands.

**Fig. 4.** Apical (A), middle (B), and basal (C) regions of shark gland cells. A, the apical cell membrane facing the gland lumen (L) shows a few short microvilli, but the lateral cell surface exhibits numerous folds projecting into the lateral intercellular space (*). B, the lateral intercellular space (*) at the level of the nuclei (N) contains extensively interdigitating folds of adjacent cells. C, also the basal cell surface is very much amplified through basal folds of the cell membrane. Magnification, ×7000.

**Fig. 5.** Apical parts of shark gland cells. A, immunoelectron microscopy shows extensive PLMS labeling of the lateral cell membrane. Most gold particles are associated with the inner surface of the cell membrane, which is consistent with an intracellular localization of the epitope. No label was associated with mitochondria or accumulated in any other part of the cytoplasm. Labeling with pre-immune serum was negative (Fig. 5B).

**Fig. 7.** Shows the tryptic cleavage pattern of PLMS in the presence of either 130 mM Na⁺ or 20 mM K⁺. A proteolytic site separating a 5-kDa peptide of PLMS from the intact protein was split by trypsin. In the absence of trypsin, PLMS migrates...
Regulation of Na,K-ATPase by PLMS

increased apparent mobility of about 10 kDa, (Fig. 7A, lane 2). In the presence of 20 mM K⁺, the protein was highly sensitive to trypsin and virtually no intact PLMS could be detected (Fig. 7A, lane 3). Interestingly, increasing the ionic strength to 130 mM K⁺ partially protected PLMS from proteolysis by trypsin (not shown), suggesting that the partial protection produced by the ion is not specific.

From the autoradiogram (Fig. 7C) it is indicated that the 5-kDa proteolytic fragment contained the phosphorylation motifs, because PKA phosphorylation of the 15-kDa PLMS decreased in parallel with the degree of cleavage (Fig. 7C, lanes 2 and 3). In addition, no phosphorylation was observed of products migrating with a mobility of 10 kDa in the gel. Therefore, truncation is associated with the concomitant loss of the ability of the resulting 10-kDa fragment of PLMS to become phosphorylated by PKA. The 5-kDa trypsin cleavage product was not observed in the SDS-gel, because it was removed by the washing and centrifugation performed after the trypsin treatment. However, autoradiography of a 5-kDa proteolytic fragment isolated and concentrated after the mild trypsin treatment demonstrated the phosphorylation of this fragment by PKC (Fig. 7D), indicating that it arises from PLMS and contained the PLMS multisite phosphorylation motif, because the phosphorylation of the α-subunit, the only other protein demonstrating significant phosphorylation, was unchanged (not shown). As seen from the immunoblot in Fig. 7A the 10-kDa proteolytic fragment can still be probed by the anti-PLMS antibody demonstrating that the epitope for binding the antibody is located upstream of the C-terminal phosphorylation domain.

The mild trypsinization conditions used to split PLMS had no direct effect on the α-subunit as confirmed by SDS-PAGE of the α-subunit in these preparations. As seen from the immunoblot in Fig. 7B, the α-subunit before and after trypsin treatment has the same mobility on SDS-PAGE, and probing of the α-subunit by anti-α-antibody showed no decrease in the intensity. Therefore, these conditions seem not to result in the well known proteolysis of the α-subunit N terminus in the presence of Na⁺, nor was it sufficient for cleavage at the T1 position in the presence of K⁺. This is to be expected, because controlled trypsinolysis of the α-subunit has previously been performed using a much higher trypsin to protein ratios and higher temperatures (49) than those used in the present experiments.

Functional Effects of C-terminal Cleavage of PLMS—To investigate any functional effects caused by the interaction between the C-terminal domain of PLMS and the Na,K-ATPase, we characterized the overall Na,K-ATPase catalytic reaction as well as some partial reactions in enzyme preparations where the 5-kDa phosphorylation domain of PLMS is either partially (130 mM NaCl) or completely (20 mM K⁺) cleaved. The preparations were assayed using the following experimental approaches: (i) measurements of the Na⁺-, K⁺-, and ATP-activation of Na,K-ATPase catalytic activity at V₉₅ conditions, (ii) activation of the Na-ATPase reaction by K⁺ at low ATP concentration to measure the K⁺ deocclusion pathway, (iii) measurements of the Na⁺ activation curve at low ATP concentration to probe effects on the Na⁺-binding affinity, (iv) measuring the vanadate sensitivity of control and PLMS-truncated preparations to probe the E₃/E₄ equilibrium, and (v) measurements of the low affinity ATP-supported transition E₃(K) → E₃(Na₃)ATP and the following phosphorylation reaction pathway leading to E₅-P using time resolved fluorescence measurements. The latter reactions include the major rate-limiting steps of the Na,K-ATPase catalytic cycle under physiological conditions (42, 50, 51).

Cation and ATP Substrate Dependence of Hydrolysis—Fig. 8 shows the Na⁺ activation in the presence of 20 mM K⁺ (A), the
\textbf{Fig. 8.} Ion- and substrate-dependent Na,K-ATPase activity in control membranes and in membranes where the C terminus of was PLMS truncated. The hydrolytic activity of control (Cont., ◦), partially truncated PLMS (Tr-Na\textsuperscript{+}, ○), and completely truncated PLMS (Tr-K\textsuperscript{−}, □) membrane preparations. A, Na\textsuperscript{+}-stimulated ATP hydrolysis in the presence of 3 mM ATP, 3 mM MgCl\textsubscript{2}, and 20 mM KCl. Partial and complete truncation of PLMS resulted in about 25 and 50% stimulation of ATP hydrolysis, respectively ($p < 0.0001$). The apparent Na\textsuperscript{+} affinities were: 11.2 ± 1.0, 12.1 ± 1.0, and 13.0 ± 1.0 mM, respectively. B, K\textsuperscript{−}-stimulated ATP hydrolysis in the presence of 3 mM ATP, 3 mM MgCl\textsubscript{2}, and 130 mM NaCl. Partial and complete truncation of PLMS resulted in 30 and 60% stimulation of ATP hydrolysis, respectively ($p < 0.0001$). The apparent K\textsuperscript{−} affinities were: 0.66 ± 0.08, 0.82 ± 0.006, and 0.99 ± 0.09 mM, respectively. C, ATP-stimulated Na,K-ATPase activity in the presence of 3 mM MgCl\textsubscript{2}, 130 mM NaCl, and 20 mM KCl. Partial and complete truncation of PLMS resulted in about 15 and 40% stimulation of ATP hydrolysis, respectively ($p < 0.0005$ and 0.0001, respectively). The apparent ATP affinities were: 0.247 ± 0.017, 0.246 ± 0.016, and 0.253 ± 0.014 mM, respectively. All reactions were performed in 30 mM histidine buffer, pH 7.00, and 2 μg of protein. P\textsubscript{i} hydrolyzed from ATP was measured as described under “Experimental Procedures.”

K\textsuperscript{+} activation in the presence of 130 mM Na\textsuperscript{+} (B), as well as the low affinity ATP activation (C) of the Na,K-ATPase in controls, partially truncated, and completely truncated PLMS preparations. Truncation, partial or complete, of PLMS leads to a significant increase in the hydrolytic activity of the Na,K-ATPase in all three cases. However, the apparent affinity for Na\textsuperscript{+} in the presence of saturating K\textsuperscript{−} (20 mM) did not change significantly, and this was also the case for the apparent K\textsuperscript{−} affinity in the presence of saturating Na\textsuperscript{+} (40 mM), and for the ATP affinity. The fact that $V_{\text{max}}$ increases after PLMS truncation, however, indicates that some rate-determining steps of the Na,K-ATPase reaction must have been influenced.

Steady-state measurements of apparent ion and ATP affinities ($K_{m,\text{app}}$ or $K_{a}$) are not sensitive kinetic indications able to identify changes in single steps of the Na,K-ATPase reaction, because $K_{m}$ contains all the rate constants around the Na,K-ATPase reaction cycle. A more precise indication of changes in the Na\textsuperscript{+} binding affinity is provided by the ratio $K_{m,\text{app}}/V_{\text{max}}$, which will only contain rate constants of intermediates that are directly involved in the interaction with the ligand (52). Indeed, changes in the apparent affinity for cytoplasmic Na\textsuperscript{+} ($K_{m,\text{app}}$) can be the result of a change in the Na\textsuperscript{+} binding affinity or a change in the $E_{1}$/E\textsubscript{2} equilibrium. In the combined presence of Na\textsuperscript{−} and K\textsuperscript{+} changes in the competition between Na\textsuperscript{−} and K\textsuperscript{+} at the cytoplasmic face will also change the apparent Na\textsuperscript{+} affinity. The latter was demonstrated to be the case for α1-HeLa cells transfected with γ\textsubscript{c} or γ\textsubscript{e} splice variants (11). If the Na\textsuperscript{+} activation curve depicted in Fig. 8A is measured at different fixed K\textsuperscript{+} concentrations, both $V_{\text{max}}$ and $K_{m}$ changes. A detailed kinetic analysis of cytoplasmic Na\textsuperscript{−}/K\textsuperscript{+} competition of shark Na,K-ATPase has previously demonstrated that $K_{m}$ inhibition of $V_{\text{max}}$ activity is a mixed multisite type inhibition in which K\textsuperscript{+} competes with three similar site dissociation constants (53). In such models a linear relation between the calculated apparent Na\textsuperscript{−} affinities and the K\textsuperscript{−} concentration is expected, $K_{m,m} = K_{m} + (K_{\gamma}K_{K}/K_{\gamma}[K\textsuperscript{−}])$, as demonstrated by Garay and Garrahian (54). As seen from the results shown in Fig. 9A (note the $[K\textsuperscript{−}]$ is on a logarithmic scale) this linear relation apparently applies to the data giving a K\textsuperscript{−} inhibition dissociation constant, $K_{K} = 14$ mM, and an apparent Na\textsuperscript{−} affinity at zero $[K\textsuperscript{−}]$, $K_{m,m} = 4.4$ mM. As indicated, no obvious change in the Na\textsuperscript{−}/K\textsuperscript{−} competition is observed after PLMS truncation. Likewise, $K_{m,m}$ seems to change only slightly after PLMS truncation.

To investigate further if the Na\textsuperscript{−} binding affinity changed after PLMS truncation, we measured the Na\textsuperscript{−} activation in the presence of 130 mM Na\textsuperscript{+} (Fig. 9B). As indicated, no obvious change in the Na\textsuperscript{−} inhibition dissociation constant, $K_{K} = 14$ mM, and an apparent Na\textsuperscript{−} affinity at zero $[K\textsuperscript{−}]$, $K_{m,m} = 4.4$ mM. As indicated, no obvious change in the Na\textsuperscript{−}/K\textsuperscript{−} competition is observed after PLMS truncation. Likewise, $K_{m,m}$ seems to change only slightly after PLMS truncation.

$K^\textsuperscript{+}$ Deocclusion—The $K^\textsuperscript{+}$ activation of Na-ATPase activity at 1 μM ATP is a sensitive measure of the $K^\textsuperscript{+}$ deocclusion pathway, $E_{1}(K) \rightarrow E_{1}$, as previously described (55). Fig. 10A shows...
experiments where the K⁺ sensitivity of Na-ATPase at 1 μM ATP, which is only sufficient to saturate the high affinity ATP binding site, is measured. Addition of only 15 mM Na⁺ ensures that the enzyme conformation is not permanently locked in the E₂ conformation. Without K⁺ only Na-ATPase activity is measured, in which the deocclusion reaction (of Na⁺) is not rate limiting at low ATP concentrations (56). Addition of K⁺ to the medium at conditions where only the high affinity ATP site is occupied will induce dephosphorylation of the enzyme and production of the E₂(K) form. Without low affinity ATP-binding K⁺ deocclusion is rate limiting, and the hydrolytic activity can be inhibited (57). Fig. 10A shows that this is actually the case for the control enzyme. However, after truncation of PLMS, the hydrolytic activity is stimulated about 200% by the addition of low K⁺ concentrations, indicating that some of the steps in the K⁺ deocclusion pathway are accelerated, either the spontaneous and/or the ATP-supported K⁺ deocclusion, because truncation of PLMS had no effect on the ATP affinity at the low affinity site (see Fig. 8C).

That the former could be the case was indicated by measurements of the main rate-limiting E₂(β) → E₁NaATP reaction at physiological conditions, including the low affinity ATP binding (42, 50, 51). This reaction can be measured by stopped-flow fluorescence using the potential sensitive styryl dye RH421 (42). Initially, the enzyme is incubated in an E₂-supporting buffer like histidine in the absence of Na⁺ and with CDTA to bind any residual Mg²⁺. The enzyme is then rapidly mixed with Na⁺ and ATP to induce the transition to E₁NaATP, whereas the following phosphorylation step is prevented by the presence of Mg²⁺. The transition from E₂ to E₁ is followed by a small drop in fluorescence (∆FP0 ← 5%), which can be measured, as demonstrated in Fig 10B. As seen from the figure, PLMS-truncated preparations and controls gave identical fluorescence decays indicating no significant effects on this reaction.

**FIG. 10.** K⁺ activation of Na-ATPase activity and the E₂(β) → E₁NaATP transition. A, the ATP hydrolysis measured in the presence of 1 μM ATP (containing 0.2 pmol of [32P]ATP), 15 mM NaCl, 2 mM MgCl₂, and varying K⁺ concentrations as indicated in the figure, as described under "Experimental Procedures." ○, control; □, PLMS cleaved preparation; and ◊, N-terminal truncated Na,K-ATPase membrane preparations. Data are presented as percent of Na-ATPase activity measured in the absence of K⁺. A representative of three independent experiments is shown. Values are means ± S.E. B, the rapid mixing stopped-flow RH421 fluorescence response of control membranes and PLMS-truncated enzyme, 106 M ATP (containing 0.2 pmol of [32P]ATP), 15 mM NaCl, 2 mM MgCl₂, and varying K⁺ concentrations as indicated in the figure. Data are from double determinations and expressed as percentage of control measured in the absence of vanadate. The data were fitted with a sigmoid dose-response curve. The E₅₀ is 0.505 ± 0.001 μM for control and 1.609 ± 0.001 μM for the PLMS-truncated sample (significantly different, p < 0.0001). A representative of two independent measurements is shown.

The E₅₀/E₂ Equilibrium—Subsequently, the effect of PLMS cleavage on the vanadate sensitivity of Na,K-ATPase was studied. Orthovanadate is a transition state analogue of inorganic phosphate that binds preferentially to the E₂ conformation of P-type ATPases. Thus, the sensitivity of Na,K-ATPase to inhibition by vanadate reflects the proportion of the enzyme adopting an E₂ conformation. As can be seen from Fig. 11 truncation of PLMS produced an enzyme preparation more resistant to vanadate (K₁ = 1.60 ± 0.001 μM) when compared with control (K₁ = 0.50 ± 0.001 μM, p < 0.0001), suggesting that PLMS truncation stabilized the E₁ conformation of the enzyme. The vanadate sensitivity of the shark enzyme is comparable to the mammalian kidney α₁ enzyme (56, 57).

The Phosphorylation/Dephosphorylation Reactions—The phosphorylation reactions in Fig. 12 were investigated by stopped-flow measurements using the membrane probe RH421 (41, 58). This styryl dye partitions into the membrane containing Na,K-ATPase and is sensitive to the formation of E₂P.

The phosphorylation reactions in Fig. 12A show the formation of E₂P from mixing of the enzyme in the presence of 30 mM Na⁺ with 5 μM MgATP and thus represents the reactions E₁NaATP → E₂NaATP → E₂P. In shark Na,K-ATPase the initial phosphoryl transfer and formation of E₂P are faster than the formation of E₂P, at least at temperatures below 15 °C (58). Thus, the stopped-flow fluorescence reaction mainly measures the rate of the E₁P → E₂P reaction. As seen from Fig. 12A this rate increases after PLMS truncation, from about 60 to 80 s⁻¹ (p = 0.009).

The dephosphorylation reaction was measured using enzyme pre-treated with vanadate in the presence of Na⁺ and Mg²⁺ to induce a maximum steady-state level of the E₂P form, as described above for the phosphorylation reaction. The enzyme is then reacted with K⁺ in a rapid-mixing stopped-flow experiment using RH421 to detect the decrease of fluorescence as the E₂P phosphoenzyme is hydrolyzed. As seen from Fig. 12B, the rate of the K⁺-activated dephosphorylation is not significantly different from the control enzyme and PLMS-truncated enzyme.

**DISCUSSION**

PLMS Sequence—The nucleic acid sequence encoded a 94-amino acid coding sequence of PLMS (Fig. 1), which was identical at the N terminus to the partial sequence previously determined by protein sequencing of the purified protein (21), with the exception that leucine replaced lysine at positions 19

**FIG. 11.** Vanadate sensitivity of shark Na,K-ATPase membrane preparations. ATP hydrolysis was measured in the presence of 1.5 mM Tris-ATP, 100 mM NaCl, 20 mM KCl, 2 mM MgCl₂, and vanadate concentrations as denoted in the figure. Data are from double determinations and expressed as percentage of control measured in the absence of vanadate. The data were fitted with a sigmoid dose-response curve. The E₅₀ is 0.505 ± 0.001 μM for control and 1.609 ± 0.001 μM for the PLMS-truncated sample (significantly different, p < 0.0001). A representative of two independent measurements is shown.
and 24. The reason for this difference in sequence is unknown but may relate to errors in reading the protein sequence as sequencing progresses to the C-terminal end of the peptide. Alternatively, it is possible that the cloned cDNA is a closely related isoform of the gel-extracted protein. Further molecular studies will be required to examine the possibility of other PLMS isoforms. *Squalus* PLMS exhibits highest homology to MAT-8 or FXYD3 (39–45% amino acid identity and 59–67% amino acid similarity). Because the amino acid sequence homology between the zebrafish FXYD9(d) and the human FXYD6 genes (43% identity and 64% similarity) is not considered close enough for these two proteins to be identified as orthologues, we suggest that, until more information is available regarding the evolution of the FXYD family genes, the PLMS gene should be designated as FXD10(sa).

Although the amplified and cloned cDNA, which contains the entire coding sequence of PLMS, was only 445 bp long, Northern blot analyses revealed the presence of a major 3.8-kb transcript. In all tissues that expressed this 3.8-kb mRNA, additional minor transcripts of 0.8, 1.1, 1.3, and 2 kb were also detected, which were most apparent in the rectal gland that exhibited the highest overall levels of expression. Further 3′- and 5′-RACE amplifications using a range of annealing temperatures (52–62 °C) with extension times up to 4 min failed to reveal any larger amplicons. Although there is no experimental evidence explaining these observations, it is possible that the Marathon oligo(dT) cDNA synthesis primer has primed on the PLMS mRNA at a short poly(A) tract some 100 bp to the 3′ of the coding sequence (Fig. 1).

The first 20 amino acid residues are assumed to form a cleavable signal sequence as found for other FXYD proteins except the γ and FXYD7 resulting in a mature protein containing 74 amino acid residues with a calculated molecular mass of 8216 Da. Thus, the electrophoretic mobility (15 kDa) of this small protein differs significantly from the protein mass as for the other hydrophobic FXYD proteins. The previous finding that PLMS is resolved as a doublet on two-dimensional SDS-PAGE (21) could suggest that PLMS may also undergo co- or post-translational processing, as is the case for the γ (12, 59) and possibly also for CHIP (23) and FXYD7 (24).

As noted in the sequence the serine immediately adjacent to the plasma membrane on the cytoplasmic side, which is conserved in all known mammalian FXYD sequences, is changed to an alanine in PLMS. This serine is located inside a conventional PKC phosphorylation motif, and in γ it can be phosphorylated by PKC in vitro in the presence of detergent (60).

**Cell and Tissue Distribution**—The co-localization of PLMS with Na,K-ATPase on basolateral membranes of rectal gland cells (Figs. 5 (A and C) and 6 (A and B)) fits the physiological role of this FXYD protein as a specific regulator of Na,K-ATPase. The shark rectal gland is the organ responsible for the extra-renal salt secretion and has served as an epithelial transport model for secretory epithelial organs where chloride secretion relies on the coordination and polarized localization of at least five transport pathways: Cl− is initially concentrated inside the cell by the basolateral Na-K-2Cl co-transporter and diffuses to the lumen across the apical membrane via the chloride channels (cystic fibrosis transmembrane conductance regulator). The K+ ions are re-circulated by basolateral K+ channels and the energy for the Cl− uptake is provided by the Na,K-ATPase that is also located on basolateral membranes (61). The transcellular Cl− transport is accompanied by a paracellular Na+ transport across the tight junctions (Fig. 5, A–C). In accordance with this model, the present results have indicated co-localization of Na,K-ATPase and PLMS on basolateral membranes in the rectal gland cells. By the recognition of the new functional role of FXYD proteins in regulation of the Na,K-ATPase the transport pathways responsible for Cl− secretion seem all to be controlled by protein kinase/phosphatase regulation (62–64).

**Functional Effects**—In the present study we demonstrate that in Na,K-ATPase membrane preparations the C-terminal protein kinase phosphorylation domain of PLMS can be cleaved by controlled trypsin treatment. Specific PLMS cleavage without cleavage of the N-terminal Na,K-ATPase α-subunit can be obtained at low trypsin to Na,K-ATPase ratio, low temperature, short incubation time, and 20 mM K+ (Fig. 7). It is well known that, in the presence of K+, trypsin treatment leads to cleavage of the mammalian α-subunit near the middle but does not cleave off the small N terminus (49). Nevertheless, the possibility that the N terminus of the Na,K-ATPase α-subunit may itself be cleaved by the trypsin treatment used to cleave PLMS was excluded by several controls. On the one hand, cleavage of the N terminus of the Na,K-ATPase α-subunit decreases the hydrolytic activity significantly (49, 65), whereas the mild trypsin treatment used to specifically cleave PLMS leads to a significant increase in maximum hydrolytic activity (Fig. 8). Furthermore, after PLMS truncation the α-subunit is still phosphorylated by PKC at the N-terminal site (data not shown). Finally, at conditions used for PLMS cleavage the well known increase in mobility of the N-terminal truncated α-subunit on SDS-PAGE is absent (Fig. 7B). It is interesting to note, however, that the opposite is not true, i.e. at conditions where the N-terminal domain of the α-subunit is cleaved by trypsin, the C terminus of PLMS also becomes cleaved. In contrast, controlled N-terminal truncation of kidney Na,K-ATPase α-subunit leaves the γ intact (not shown).

Specific cleavage of the C terminus of PLMS activates the Na,K-ATPase at $V_{\text{max}}$ conditions indicating effects on rate-determining steps. At saturating substrate concentrations the rate-limiting steps are the $E_1 \rightarrow E_2$ transition associated with...
by the increase in RH421 fluorescence was increased, indicating dephosphorylation, whereas the ATP-phosphorylation reaction measured at 30°C decreased (Fig. 10B). The dephosphorylation supported by low affinity ATP binding (Fig. 10C), as well as the apparent ATP affinity (Fig. 8C) was unchanged. PLMS truncation did not change the K\(^{+}\) supported dephosphorylation, whereas the ATP-phosphorylation reaction measured by the increase in RH421 fluorescence was increased, indicating that the partially rate-limiting reaction \(E_1 - P \rightarrow E_2P\) is accelerated. Thus, the increased \(V_{\text{max}}\) induced by specific PLMS truncation is probably an effect mainly on the catalytic site phosphorylation reaction and not on the main rate-limiting step, the K\(^{+}\) dephosphorylation supported by low affinity ATP binding.

A major effect of FYXD1 proteins hitherto investigated relates to modulation of apparent Na\(^{+}\) or K\(^{+}\) affinities (19, 20). A similar situation seems to exist for the PLMS/Na,K-ATPase system where PLMS truncation increases the apparent cytoplasmic Na\(^{+}\) affinity (Fig. 9B) without affecting the Na\(^{+}\)/K\(^{+}\) competition at the cytoplasmic site (Fig. 9A). Thus PLMS association decreases the Na\(^{+}\) affinity. This is in agreement with recent studies on mammalian PLM where co-expression of the α-subunit and PLM in Xenopus oocytes resulted in a decrease in the apparent cytoplasmic Na\(^{+}\) affinity of Na,K-ATPase (22). Thus, association of FYXD1 proteins with the Na,K-ATPase leads in both cases to a decreased Na\(^{+}\) affinity and inhibition of enzyme activity, whereas dissociation of the FYXD1 proteins results in stimulation of hydrolytic activity. Other FYXD proteins have different patterns of effects: association of γ (FYXD2) with Na,K-ATPase has been shown to increase the apparent ATP affinity by supporting the \(E_1\) conformation of the enzyme and to decrease the apparent affinity for cytoplasmic Na\(^{+}\), which apparently is an effect secondary to an increased antagonism of cytoplasmic K\(^{+}\) to activation by Na\(^{+}\) (10, 11). In other investigations the change in apparent affinity for Na\(^{+}\) could not be unambiguously assigned to such increased Na\(^{+}\)/K\(^{+}\) competition alone but also indicated γ induced changes in the intrinsic binding affinity for Na\(^{+}\) (59). Effects on the extracellular K\(^{+}\) affinity of both splice variants of γ have also been reported (11, 59). Co-expression of the FYXD4 protein CHIP with rat a1 increased the apparent affinity for cytoplasmic Na\(^{+}\), without affecting the K\(^{+}\) affinity or \(V_{\text{max}}\) (23, 66). It should be noted, however, that assignment of changes in apparent ion affinities from steady-state measurements to specific steps in the reaction mechanism might be difficult. In co-expression experiments where changes in \(V_{\text{max}}\) are not usually controlled, this can be important, because variations in \(V_{\text{max}}\) in itself are expected to change the apparent ion affinities in a ping-pong kinetic model (67).

It is interesting to note that the functional effects following cleavage of the C-terminal domain of PLMS resemble in some aspects the effects reported after truncation of the N-terminal domain of the rat kidney α-subunit. Thus, in mutagenesis studies deletion of up to 40 N-terminal residues of the α-subunit accelerates K\(^{+}\) deocclusion at limited ATP concentrations and shifts the \(E_1/E_2\) conformation of the enzyme toward the \(E_1\) form, whereas further N-terminal deletions reversed this effect (57). From these experiments it was suggested that the N-terminal domain of the α-subunit play an autoregulatory role in controlling the \(E_1/E_2\) conformation of the enzyme by inter-
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Regulation of Na,K-ATPase by PLMS, the Phospholemman-like Protein from Shark: MOLECULAR CLONING, SEQUENCE, EXPRESSION, CELLULAR DISTRIBUTION, AND FUNCTIONAL EFFECTS OF PLMS

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