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A study of the membrane association and regulatory effect of the phospholemman cytoplasmic domain

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ABSTRACT

Phospholemman (PLM) is a single-span transmembrane protein belonging to the FXYD family of proteins. PLM (or FXYD1) regulates the Na,K-ATPase (NKA) ion pump by altering its affinity for K^+ and Na^+ and by reducing its hydrolytic activity. Structural studies of PLM in anionic detergent micelles have suggested that the cytoplasmic domain, which alone can regulate NKA, forms a partial helix which is stabilized by interactions with the charged membrane surface. This work examines the membrane affinity and regulatory function of a 35-amino acid peptide ($PLM_{38–72}$) representing the PLM cytoplasmic domain. Isothermal titration calorimetry and solid-state NMR measurements confirm that $PLM_{38–72}$ associates strongly with highly anionic phospholipid membranes, but the association is weakened substantially when the negative surface charge is reduced to a more physiologically relevant environment. Membrane interactions are also weakened when the peptide is phosphorylated at S68, one of the substrate sites for protein kinases. $PLM_{38–72}$ also lowers the maximal velocity of ATP hydrolysis (V_{max}) by NKA, and phosphorylation of the peptide at S68 gives rise to a partial recovery of V_{max}. These results suggest that the PLM cytoplasmic domain populates NKA-associated and membrane-associated states in dynamic equilibrium and that phosphorylation may alter the position of the equilibrium. Interestingly, peptides representing the cytoplasmic domains of two other FXYD proteins, Mat-8 (FXYD3) and CHIF (FXYD4), have little or no interaction with highly anionic phospholipid membranes and have no effect on NKA function. This suggests that the functional and physical properties of PLM are not conserved across the entire FXYD family.

1. Introduction

Regulation of ion transport, and in particular control of intracellular calcium levels ($[Ca^{2+}]_i$), is essential to muscle function and maintenance of normal patterns of contraction and relaxation. Calcium influx and efflux is coupled to Na^+ and K^+ transport into and out of myocytes, and the entire calcium cycling process is regulated synergistically by several proteins including sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), Na^+,K^+-ATPase (NKA) and the Na^+/Ca^{2+} exchanger (NCX1) [1,2]. NKA and NCX1 are both regulated independently by the 72-residue transmembrane (TM) protein phospholemman (PLM), which is widely distributed in human tissues with expression highest in cardiac and skeletal muscle [3–8]. Disruption of ionic fluxes in cardiac muscle is a contributing factor in heart failure (HF), and myocardial infarction, cardiomyopathy and cardiac hypertrophy all invoke changes in the expression and functions of proteins that maintain the homeostatic norm.

PLM belongs to a group of single-span membrane proteins known as the FXYD family, named in recognition of their invariant Phe-X-Tyr-Asp signature motif close to the N-terminus [9]. FXYD proteins appear to regulate the rate of ion transport across cell membranes via an association with ion pumps. PLM (or FXYD1) is one of at least four members of the family believed to act as a tissue specific regulator of NKA [6,10–12]. PLM associates specifically with the α1/β and α2/β isoforms of NKA, resulting in a small decrease in external K^+ affinity and a nearly two-fold decrease in the internal Na^+ affinity of the enzyme [6], although the precise regulatory effect remains a matter of debate. Regulation of NKA appears to involve contacts between the hydrophobic transmembrane domains of NKA and PLM, with additional interactions in the cytoplasmic regions [13,14]. Measurement of whole-cell NKA currents in the presence of water-soluble peptides representing the 19C-terminal residues of the cytoplasmic domain ($PLM_{54–72}$) indicates that the cytoplasmic domain alone is sufficient to regulate NKA [15]. The cytoplasmic domain of PLM is the major plasma membrane substrate for protein kinase A (PKA) and protein kinase C (PKC) [16,17]. In cardiac muscle, phosphorylation of PLM in the cytoplasmic domain, at S68 alone or at both S68 and S63, occurs following activation of either α or β adrenergic receptors and correlates with an increase in contractility

Abbreviations: PLM, phospholemman (FXYD1); $PLM_{38–72}$, a peptide representing residues 38–72 of phospholemman; Mat-8 (FXYD3), mammary tumor protein; CHIF (FXYD4), corticosteroid induced factor; REDOR, rotational-echo double-resonance; SSNMR, solid-state NMR; ITC, isothermal titration calorimetry; NKA, Na^+,K^+-ATPase; NCX, Na^+/Ca^{2+} exchanger; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DOPG, dimyristoylphosphatidylglycerol; DOPS, dioleoylphosphatidylserine

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[8,16]. Phosphorylation of PLM_{54-72} at S68 stimulates NKA activity to a level above that in the absence of the peptide [15], providing a link between kinase activation and pump modulation.

The three-dimensional structure of PLM in a lipid bilayer environment is not known and the precise nature of its association with NKA is also unclear, although structural models have been proposed [18–20]. NMR structural analysis of PLM in SDS micelles identified two helical segments in the cytoplasmic domain, including an amphipathic helix H4 from residues 59 to 70 which associates with the negatively charged micelle surface and orients approximately perpendicular to the transmembrane region [19,21,22]. Studies of a 35-amino acid peptide representing the PLM cytoplasmic domain (PLM_{38-72}) also revealed strong interactions with the headgroups of negatively charged phospholipids [23]. A recent study found that phosphorylation of PLM at S68 does not alter significantly the structure or dynamics of the cytoplasmic domain in SDS micelles, and increases only slightly the dynamics of the C-terminal helical segment [20]. In the same study it was speculated that the membrane location of H4 allows it to be positioned close to the end of transmembrane helix 10 of NKA, where it could contribute to the network of positive charges constituting a putative voltage-sensing module [20]. The extent to which the structure and dynamics of PLM in detergent micelles emulate its properties in a lipid bilayer has not been established. In view of the functional role of the PLM cytoplasmic domain, studies of its structural and dynamic properties in the presence of lipid bilayers would provide useful new insights into the properties of PLM.

Here we use isothermal titration calorimetry (ITC) and solid-state NMR to examine the interactions of the peptide PLM_{38-72} (sequence shown in Fig. 1) with phospholipid membranes of different head-group composition. We show that the membrane affinity of PLM_{38-72} is highly dependent on membrane surface charge and is reduced after phosphorylation of the peptide at S68. Both peptides lower the rate of ATP hydrolysis by NKA in a kidney membrane preparation, but the S68 phosphorylated peptide has less pronounced inhibitory effect than PLM_{38-72}. Both peptides also lower the affinity for ADP binding to the high-affinity nucleotide binding site. Peptides representing the cytoplasmic domains of two other FXYD family proteins, the 8 kDa mammary tumor protein Mat-8 (FXYD3) and the corticosteroid induced factor CHIF (FXYD4), have much lower membrane affinities than PLM_{38-72} and do not affect NKA activity, suggesting that there is variability in the physical and functional properties amongst the FXYD family members.

2. Methods and materials

2.1. Materials

Synthetic analogues of the human PLM, Mat-8 and CHIF cytoplasmic domains were purchased in pure form (>95%) from Peptide Protein Research Ltd (Fareham, U.K.). PLM_{38-72} and the S68-phosphorylated derivative ($pPLM_{38-72}$) were N-terminally acetylated in order to mimic the internal peptide bond. One batch of PLM_{38-72} was uniformly labeled with ^{13}C and ^{15}N at residues R61, R65, R66 and R67 ($[^{13}C,^{15}N]PLM_{38-72}$). In addition a shorter cytoplasmic fragment (PLM_{63-72}) and an N-terminal extracellular fragment (PLM_{1-12}) containing the FXYD sequence were also prepared. The C-terminus of PLM_{1-12} was amidated to mimic the internal peptide bond of full-length PLM. $pPLM_{38-72}$ was synthesized using the appropriate fmoc-protected phosphoserine precursor and 100% incorporation of the phosphate group was confirmed by electrospray mass spectroscopy and ^{1}H and ^{31}P NMR spectroscopy.

L-α-Dioleoylphosphatidylcholine (DOPC), L-α-dimyristoylphosphatidyl-choline (DMPC), L-α-dioleoylphosphatidylserine (DOPS), L-α-dioleoyl-phosphatidylglycerol (DOPG), and all other chemicals were purchased from Sigma Chemicals Ltd (UK). [^{14}C]ADP with a specific radioactivity of about $2 \cdot 10^{9}$Bq/mmol was obtained from New England Nuclear (USA).

2.2. Preparation of Na,K-ATPase

Membranous NKA from pig kidney microsomal membranes was prepared using SDS and purified by differential centrifugation to a specific activity of about 30 μmol ATP hydrolyzed/mg protein per min at 37 °C [24,25]. Stock solutions of the enzyme were stored at about 5 mg protein/ml in 20 mM histidine, 1 mM EDTA and 250 mM sucrose (pH 7.0). Renal NKA co-purifies with the γ-subunit protein (FXYD2), which is homologous to PLM in the transmembrane domain but lacks the extended cytoplasmic region of PLM. For this latter reason it was assumed that functional effects of PLM_{38-72} would not be masked by the presence of the γ-subunit, although this possibility cannot be ruled out.

2.3. NKA activity measurements

Enzymatic activities and protein contents were determined as described previously [26]. Here, assays of Na^{+}-activation of steady-state NKA activities (at 37 °C, pH 7.4, with 20 mM KCl, 3 mM ATP and 4 mM $MgCl_2$ in the assay medium) were performed by measuring phosphate liberation from ATP with colorimetric methods [27,28]. Inclusion of 1 mM ouabain served as a background control. The NKA concentration was 0.4 μg/ml and the concentration of PLM-peptide was 0.1 mM. The incubation with ATP lasted 15 min. PLM-peptides were solubilized in 10 mM histidine (pH 7.0) and kept at 20 °C for 120 min before use. The pH of the assay medium was not altered after the addition of any of the PLM peptides to 0.1 mM.

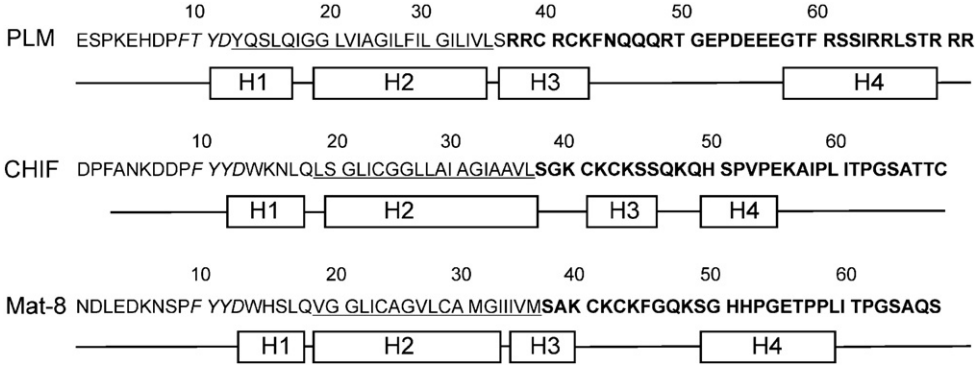

Fig. 1. Primary sequences of human PLM (FXYD1), Mat-8 (FXYD3) and CHIF (FXYD4). Highlighted are the FXYD sequence (italics), predicted transmembrane domain (underlined) and residues corresponding to the peptides studied here (bold). Below each sequence are secondary structure profiles showing the approximate length and position of helical regions (H1–H4) determined by solution-state NMR [20–22].