Phosphorylation of Phospholemman (FXYD1) by Protein Kinases A and C Modulates Distinct Na,K-ATPase Isozymes*

Phospholemman (FXYD1), mainly expressed in heart and skeletal muscle, is a member of the FXYD protein family, which has been shown to decrease the apparent K⁺ and Na⁺ affinity of Na,K-ATPase (Crambert, G., Fuzesi, M., Garty, H., Karlish, S., and Geering, K. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 11476–11481). In this study, we use the Xenopus oocyte expression system to study the role of phospholemman phosphorylation by protein kinases A and C in the modulation of different Na,K-ATPase isozymes present in the heart. Phosphorylation of phospholemman by protein kinase A has no effect on the maximal transport activity or on the apparent K⁺ affinity of Na,K-ATPase α1/β1 and α2/β1 isoforms but increases their apparent Na⁺ affinity, dependent on phospholemman phosphorylation at Ser⁶⁸. Phosphorylation of phospholemman by protein kinase C affects neither the maximal transport activity of α1/β1 isoforms nor the K⁺ affinity of α1/β1 and α2/β1 isoforms. However, protein kinase C phosphorylation of phospholemman increases the maximal Na⁺,pump current of α2/β1 isoforms by an increase in their turnover number. Thus, our results indicate that protein kinase A phosphorylation of phospholemman has similar functional effects on Na,K-ATPase α1/β and α2/β isoforms and increases their apparent Na⁺ affinity, whereas protein kinase C phosphorylation of phospholemman modulates the transport activity of Na,K-ATPase α2/β but not of α1/β isoforms. The complex and distinct regulation of Na,K-ATPase isoforms by phosphorylation of phospholemman may be important for the efficient control of heart contractility and excitability.

Phospholemman (PLM)² or FXYD1 is one of the seven members of the FXYD protein family (1), which are small single span membrane proteins exposing their C terminus to the cytoplasmic side. Based on the observation that PLM can form ion-selective channels, it was suggested that PLM might be implicated in cell volume regulation (2). More recently, however, PLM, similar to other FXYD proteins, was identified as a tissue-specific regulator of Na,K-ATPase (3, 4), a ubiquitous cation specific Na,K-ATPase activity in cardiac myocytes (9). Similarly, isoproterenol treatment phosphorylates PLM and increases Na,K-ATPase activity in cardiac myocytes of wild type mice but not of PLM-deficient mice (13). The increase in Na,K-ATPase activity is mainly attributed to an increase in Na,K-pump rates (Vₘₐₓ) of Na,K-ATPase induced by PLM in certain expression systems indicates an inhibitory effect on Na,K-ATPase. This is supported by the observation that Na,K-ATPase activity increases in myocytes of PLM knock-out mice (11–13) and decreases in cardiac myocytes overexpressing PLM (14). In some reports, however, inhibition of Na,K-ATPase by PLM is attributed to an effect of PLM on the apparent Na⁺ affinity (13), whereas in other studies, PLM produced no effect on the apparent Na⁺ affinity but only on maximal pump rates (Vₘₐₓ) of Na,K-ATPase (14). Moreover, when expressed in Pichia pastoris together with Na,K-ATPase, PLM increases the apparent Na⁺ affinity of Na,K-ATPase (10). The matter is complicated further by reports that show a reduced rather than increased Na,K-ATPase activity in PLM-deficient cardiac sarcolemma (15).

A possible explanation for the apparently conflicting results on the effect of PLM on Na,K-ATPase activity may lie in the fact that PLM is subjected to phosphorylation by different protein kinases, which may differ in different experimental conditions. PLM has long been known as the main sarcolemmal substrate for protein kinases A (PKA) and C (PKC) in heart and skeletal muscle (16). PKA phosphorylates Ser⁶⁸, and PKC phosphorylates both Ser⁶³ and Ser⁶⁸ in the cytoplasmic domain of PLM (17). In myocytes, PLM becomes phosphorylated on Ser⁶⁸ upon forskolin treatment (9, 13) and on Ser⁶³ and Ser⁶⁸ upon PKC activation (11).

Regulation of Na,K-ATPase activity by activation of PKA and PKC has been described in various tissues (18), but it has remained unclear whether this occurs by direct phosphorylation of Na,K-ATPase or of an accessory protein. Recent experimental evidence suggests that PKA-dependent phosphorylation of PLM rather than of Na,K-ATPase α1 subunits stimulates α1-specific Na,K-ATPase activity in cardiac myocytes (9). Similarly, isoproterenol treatment phosphorylates PLM and increases Na,K-ATPase activity in cardiac myocytes of wild type mice but not of PLM-deficient mice (13). The increase in Na,K-ATPase activity is mainly attributed to an increase

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2 The abbreviations used are: PLM, phospholemman; PKA, protein kinase A; PKC, protein kinase C; IBMX, isobutylmethylxanthine; PDBu, 4-β-phorbol-12,13-dibutyrate.
increase in the apparent Na\textsuperscript{+} affinity of Na,K-ATPase with little effect on \( V_{\text{max}} \) (13). On the other hand, Pavlovic et al. (12) reported that phosphorylation of PLM by PKA stimulates Na,K-ATPase to a level above that seen in the absence of PLM, suggesting an important effect on \( V_{\text{max}} \).

Sympathetic stimulation of cardiac myocytes not only involves PKA activation via \( \beta \)-adrenergic receptors but also PKC activation via \( \alpha \)-adrenergic receptors. A recent study reports that PKC activation of myocytes leads to phosphorylation of PLM at Ser\textsuperscript{68} and Ser\textsuperscript{68} and increases the maximal Na,K-ATPase-mediated Na\textsuperscript{+} extrusion rate without changing the apparent Na\textsuperscript{+} affinity of Na,K-ATPase (11). PKC activation of myocytes from PLM-deficient mice had no effect on Na,K-ATPase activity, suggesting that PKC-dependent effects are due to PLM rather than phosphorylation of Na,K-ATPase. Interestingly, the effects on Na,K-ATPase of PKA and PKC phosphorylation of PLM are additive (11), opening the possibility that PKA and PKC phosphorylation of PLM regulates different Na,K-ATPase isoforms. Finally, studies on a shark phospholemman-like protein (FXYD10) also show that its phosphorylation by PKC stimulates Na,K-ATPase activity (19).

To contribute to the clarification of the role of PKA and PKC phosphorylation of PLM in the regulation of Na,K-ATPase activity, we have used, in this study, the Xenopus oocyte expression system to address some open questions, which have not been or cannot easily be answered in cardiac myocytes or have produced contradictory results. In particular, we have investigated the effect of PKA and PKC phosphorylation of PLM on the apparent Na\textsuperscript{+} and/or K\textsuperscript{+} affinity and on \( V_{\text{max}} \) of Na,K-ATPase and have explored the possibility that PLM phosphorylation might have differential effects on different Na,K-ATPase isoforms.

**EXPERIMENTAL PROCEDURES**

**Materials**—Forskolin and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Calbiochem, and epinephrine and 4-\( \beta \)-phorbol-12,13-dibutyrate (PDBu) were from Sigma. Phorbol-12,13-dibutyrate (PDBu) were from Sigma. Epinephrine and 4-\( \beta \)-phorbol-12,13-dibutyrate (PDBu) were purchased from Calbiochem, and epinephrine and 4-\( \beta \)-phorbol-12,13-dibutyrate (PDBu) were from Sigma.

**Mutagenesis and cDNAs**—Point mutations were introduced into cDNAs of dog PLM (7) by the PCR-based method. The mutated PLM cDNAs were amplified from dog PLM using a sense primer tagged with an EcoRI site (5'-GCCGAATTTCAG-GACCTTCCACCAATCTTTGGTCTC-3') and antisense primers tagged with a NotI restriction site. The single S68A mutation and double mutations S63A/S68A were introduced using the antisense mutagenic synthetic oligonucleotide primers (5'-GGGGGGGCCTCTCCCGCTGGTGGC CAGACGCGATGGAATCCTGGCTCG-3'/5'-GGGGGGGCGGCCTCGTCCCGCTGGTGGC CAGACGCGATGGAATCCTGGCTCG-3'). The double mutations S63D/S68D were introduced using the antisense mutagenic synthetic oligonucleotide primers (5'-GGGGGGGCCTCTCCCGCTGGTGGC CAGACGCGATGGAATCCTGGCTCG-3'/5'-GGGGGGGCGGCCTCGTCCCGCTGGTGGC CAGACGCGATGGAATCCTGGCTCG-3'). Mutated nucleic acids are underlined. All products were cloned into a pSD5 vector and sequenced. cDNAs of rat Na,K-ATPase \( \alpha 1 \) (20), rat ouabain-resistant \( \alpha 2 \) (21), and rat \( \beta 1 \) subunits (22) (all kindly provided by J. Lingrel; University of Cincinnati, Cincinnati, OH); human Na,K-ATPase \( \alpha 1, \alpha 2, \) and \( \beta 1 \) subunits (23); human \( \beta 2 \) adrenergic receptor (24) (kindly provided by S. Cotecchia, University of Lausanne, Switzerland); and dog PLM (23) were subcloned into a pSD5 vector. cRNAs were prepared by in vitro translation (25).

**Expression and Association of Wild Type and Mutant PLM in Xenopus Oocytes**—Stage V-VI oocytes were obtained from Xenopus laevis as described (26). Oocytes were injected with rat \( \alpha 1 \) or \( \alpha 2 \) subunit cRNA (10 ng) and \( \beta 1 \) subunit cRNA (1 ng) alone or together with wild type or mutant phospholemman cRNAs (3 ng). To study protein expression and PLM association, cRNA-injected oocytes were incubated in modified Barth’s solution in the presence of 1 mCi/ml \[^{35}\text{S}\text{]methionine (Easy Tag Express [\[^{35}\text{S}\text{]Protein Labeling Kit; PerkinElmer Life Sciences) for 6 h and subjected to 24- and 48-h chase periods in modified Barth’s solution containing 10 mCi/ml methionine. After the pulse and chase periods, microsomes were prepared and subjected to immunoprecipitations under nondenaturing conditions as described (26) with an antibody against the Na,K-ATPase \( \alpha \) subunit (27), and proteins were resolved by SDS-PAGE and revealed by fluorography.

**Phosphorylation by PKA and PKC and Detection of PLM**—Phosphorylation of wild type and mutant PLM after PKA or PKC activation was studied after incubation of cRNA-injected oocytes for 48 h at 19 °C. PKA was activated by incubation of oocytes in modified Barth’s solution containing 10 \( \mu \text{M} \) forskolin and 1 \( \mu \text{M} \) IBMX for 4 h or by incubation of oocytes expressing the \( \beta 2 \)-adrenergic receptor in modified Barth’s solution containing 0.1 \( \mu \text{M} \) epinephrine for 2 min. Preliminary experiments showed that removal of PKA activators led to dephosphorylation of PLM within 1 h (data not shown). Therefore, long term experiments, such as measurements of the apparent Na\textsuperscript{+} affinity (see below), were performed in the continuous presence of epinephrine, which maintained phosphorylation. PKC was activated by incubation of oocytes in modified Barth’s solution containing 100 \( \mu \text{M} \) PDBu for 3 or 10 min. In some experiments, oocytes were continuously incubated with PDBu over a period of 15 min.

The phosphorylation state of PLM was assessed before and after PKA or PKC activation and at the end of electrophysiological measurements by preparing oocyte microsomes with buffers containing 100 mM NaF, 10 mM sodium pyrophosphate, and 10 mM sodium orthovanadate to inhibit phosphatases. The protein content was determined by the method of Lowry. Microsomal proteins were subjected to SDS-PAGE (10–50 \( \mu \text{g} \)) to or immunoprecipitations (100 \( \mu \text{g} \)) under nondenaturing conditions with a Na,K-ATPase \( \alpha \) subunit antibody and then transferred overnight at 40 V to nitrocellulose membranes. Membranes were blocked with 10% nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with primary antibody. The following primary antibodies were used: 1) a PLM antibody recognizing PLM, which is not phosphorylated at Ser\textsuperscript{68} (10) (1:500) (kindly provided by H. Garty and S. Karlish, Weizmann Institute, Israel) (we show in this study that this antibody recognizes the S68A and S68D PLM mutants, suggesting that it is the phosphate moiety that impedes recognition of PLM by this antibody); 2) a PLM antibody CP68 recognizing PLM phosphorylated at Ser\textsuperscript{68} (28) (1:10’000) (kindly provided by D. M. Bers (Loyola University, Chicago, IL)); and 3)
PLM Phosphorylation Affects Different Na,K-ATPase Isozymes

a Na,K-ATPase α subunit antibody (1:10,000) (27). After binding of the primary antibody, peroxidase-coupled secondary antibodies (1:10,000; Amersham Biosciences) were bound, and the complex was revealed with the ECL chemiluminescence kit (Amersham Biosciences) according to the manufacturer’s protocol. Quantification of nonphosphorylated and phosphorylated PLM was performed with an ultrasonic laser densitometer (LKB Bromma).

**Determination of the Apparent K+ and Na+ Affinity of Na,K-ATPase**—Electrophysiological measurements were performed 2 days after oocyte injections with rat α1 (10 ng) or ouabain-resistant α2 (12 ng) and β1 (1 ng) cRNAs with or without wild type (3 ng) or mutant PLM (3 ng) cRNAs by using the two-electrode voltage clamp technique. Oocytes were loaded with Na+ by overnight incubation in a K+-free medium (30). Measurements of the apparent external K+ affinity were carried out as described (29) in the presence of 1 μM ouabain that inhibits the endogenous oocyte Na,K-ATPase but not the expressed ouabain-resistant rat Na,K-ATPase. The apparent K+ affinity (Kₐ,K+) measured in the presence of external Na+ (100 mM) was obtained by fitting the Hill equation to the data using a Hill coefficient of 1.6 (30). Measurements of the apparent Na+ affinity of Na,K-ATPase were performed as described (31) after co-expressing rat α1 or α2 and β1 cRNAs together with rat epithelial Na+ channel α, β, and γ subunit (0.5 μg each) cRNAs in the presence or absence of PLM cRNAs. The apparent Na+ affinity (Kₐ,Na+) was obtained by fitting the Hill equation by using a Hill coefficient of 3 (31). Statistical analysis was performed by unpaired Student’s t test.

For the determination of effects of PKA stimulation on the apparent external K+ affinity of Na,K-ATPase, oocytes were incubated for 4 h in modified Barth’s solution containing 10 μM forskolin and 1 mM IBMX before measurements.

For the determination of effects of PKA stimulation on the apparent K+ and Na+ affinities of Na,K-ATPase, oocytes were injected with Na,K-ATPase cRNA alone or together with wild type or mutant PLM plus β2 adrenergic receptor cRNA, and the β2 adrenergic receptor signaling pathway was activated by incubation of oocytes for 2 min with 0.1 mM epinephrine before electrophysiological measurements. Epinephrine was present during the duration of electrophysiological measurements (e.g. for about 3 min for K+ affinity measurements and about 1 h for Na+ affinity measurements).

For the determination of effects of PKC stimulation on the apparent external K+ affinity of Na,K-ATPase, oocytes were incubated for 3 min in modified Barth’s solution containing 100 nM PDBu before measurements.

**Maximal Na,K-ATPase Current Measurements**—Measurements of maximal Na,K-pump currents (Iₘₐₓ) were performed by the two-electrode voltage clamp technique 2 days after oocyte injection with human Na,K-ATPase α1 or α2 cRNAs and β1 cRNA in the presence or absence of wild type or mutant PLM cRNAs. Oocytes were loaded with Na+ by overnight incubation in a K+-free medium (30). The membrane potential was set at −50 mV, and the Na,K-pump current was measured at room temperature as the outward current induced by the addition of 10 mM K+.

For the determination of effects of PKA stimulation on Iₘₐₓ of Na,K-ATPase, oocytes were treated for 4 h with 10 μM forskolin and 1 mM IBMX before measurements. Iₘₐₓ measurements were performed within less than 1 min. Moreover, the effect of PKA activation on Iₘₐₓ of Na,K-ATPase α1/β1 isozymes was determined by the following protocol. Iₘₐₓ was determined on oocytes by the addition of 10 mM K+ until plateau values were reached. K+ was removed, 0.1 mM epinephrine was added, and K+-induced currents were measured after 2, 5, 10, and 15 min in the continuous presence of epinephrine.

For the determination of effects of PKC stimulation on Iₘₐₓ of Na,K-ATPase, oocytes were treated for 3 or 10 min with 100 nM PDBu before measurements or in the continuous presence of PDBu over a 15-min time period. In separate experiments, it was verified that the K+-induced currents corresponded to ouabain-inhibitable Na,K-pump currents (data not shown).

**[3H]OuabainBinding on Intact Oocytes**—Two days after injection of oocytes with human α1 or α2 cRNAs and β1 cRNA in the presence or absence of wild type or mutant PLM cRNAs, the total number of Na,K-ATPase isoforms expressed at the cell surface was determined by [3H]ouabain binding essentially as described (26). Briefly, oocytes were loaded with Na+ in a K+-free solution containing 90 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.4, for 2 h at 19 °C. Oocytes were then incubated at room temperature in a K+-free solution containing 0.3 μM [21,22,3H]ouabain (Amersham Biosciences; specific activity 15 Ci/mmol) and 0.7 μM of cold ouabain for 30 min. Oocytes were extensively washed with a buffer containing 90 mM NaCl, 30 mM imidazole, pH 7.4, individually transferred to scintillation tubes, and solubilized with 100 ml of 5% SDS. Solubilized oocytes were counted after the addition of 2 ml of Scintillator 299 (Packard). Ouabain binding measured in non-injected oocytes was subtracted from values obtained in CRNA-injected oocytes. Previous experiments have shown that nonspecific binding determined in the presence of a 1000-fold excess of cold ouabain amounts to 3–7% of total [3H]ouabain binding (26). For the determination of effects of PKA stimulation on cell surface expression of Na,K-ATPase, oocytes were treated for 4 h with 10 μM forskolin and 1 mM IBMX before incubation with [3H]ouabain. For the determination of effects of PKC stimulation on cell surface expression of Na,K-ATPase, oocytes were treated for 3 or 10 min before incubation with [3H]ouabain.

**Turnover Number of Na,K-ATPase**—The turnover number of Na,K-ATPase α1/β1 and α2/β1 was calculated as the ratio between the maximal Na,K-pump current and the number of ouabain binding sites.

**RESULTS**

To study the role of PLM phosphorylation on Na,K-ATPase expressed in Xenopus oocytes, we have produced the following PLM mutants: 1) a S68A mutant in which the PKA phosphorylation site (17) was abolished; 2) a S63A/S68A mutant in which the two PKC phosphorylation sites (17) were abolished; and 3) a S63D/S68D mutant that mimics constitutive phosphorylation of PLM. To test whether these mutants associate with Na,K-ATPase, they were expressed in Xenopus oocytes together with Na,K-ATPase and metabolically labeled. Oocyte microsomes
PKA Phosphorylation of PLM Affects Different Na,K-ATPase Isozymes

PLM Phosphorylation Affects Different Na,K-ATPase Isozymes

PKA Phosphorylation of PLM Does Not Influence the Apparent K⁺ Affinity of Na,K-ATPase α1/β1 and α2/β1 Isozymes—We next tested whether PKA phosphorylation of PLM affects the effect of PKA on the Na⁺ affinity of Na,K-ATPase α1/β1 isozymes. Measurements of the apparent Na⁺ affinity of Na,K-ATPase were performed in the absence of epinephrine (Fig. 2A). Analysis of the phosphorylation state of PLM performed with antibodies recognizing PLM, which are not phosphorylated at Ser68 (10), confirmed that 1) wild type PLM, but not the double S63A/S68A and the single S68A mutant did not influence the apparent Na⁺ affinity of Na,K-ATPase. 

Similar experiments performed in oocytes expressing Na,K-ATPase α2/β1 isozymes with PLM showed that phosphorylation of PLM by PKA also did not influence the effect of PLM on the Na⁺ affinity of α2/β1 isozymes (Table 1). PKA Phosphorylation of PLM Influences the Apparent Na⁺ Affinity of Na,K-ATPase α1/β1 and α2/β1 Isozymes—We next tested whether PKA phosphorylation of PLM affects the effect of PKA on the Na⁺ affinity of Na,K-ATPase α1/β1 isozymes with PLM significantly increased the K⁺ value for Na⁺. PKA stimulation of oocytes nearly completely abolished the effect of PLM on the Na⁺ affinity of Na,K-ATPase. In agreement with an effect of PKA phosphorylation of PLM on the apparent Na⁺ affinity of Na,K-ATPase, the double S63A/S68A and the single S68A mutant did not produce an effect on the apparent Na⁺ affinity upon epinephrine treatment, whereas the S63D/S68D mutant increased the apparent Na⁺ affinity even in the absence of epinephrine (Fig. 3A). Analysis of the phosphorylation state of PLM performed with antibodies recognizing PLM, which is not phosphorylated at Ser68 (10), confirmed that 1) wild type PLM, but not the double S63A/S68A, the S68A, or the S63D/S68D mutant, was partially phosphorylated after superfusion with epinephrine during the 1-h duration of the experiment, and 2) phosphorylation of Ser68 of PLM is responsible for the effect of epinephrine on the apparent Na⁺ affinity of Na,K-ATPase.

Similar experiments performed in oocytes expressing Na,K-ATPase α2/β1 isozymes with PLM showed that phosphorylation of PLM by PKA also abolished the effect of PLM on the Na⁺ affinity of α2/β1 isozymes (Table 1). PKA Phosphorylation of PLM Does Not Influence the Maximal Na,K-ATPase Activity—Contradictory results have been reported concerning the mechanism by which PKA phosphorylation of PLM increases Na,K-ATPase activity in cardiac myocytes. Increased Na,K-ATPase activity has either been attributed to an increase in its apparent Na⁺ affinity (13) or to
PLM phosphorylation affects different Na,K-ATPase isozymes

FIGURE 2. PLM phosphorylation by PKA does not affect the apparent K$^+$ affinity of Na,K-ATPase α1/β1 isozymes. A, phosphorylated PLM is associated with Na,K-ATPase. Two days after injection of rat Na,K-ATPase α1 plus β1 subunit cRNAs together with 3 ng of wild type PLM cRNA, oocytes were incubated for 4 h with forskolin. Microsomal proteins were either directly loaded on a gel (50 μg) (lanes 1 and 2) or immunoprecipitated (IP) (100 μg) under nondenaturing conditions with antibodies against Na,K-ATPase α subunit (lanes 3 and 4). Proteins were transferred onto nitrocellulose membranes and immunodetected with an antibody recognizing PLM phosphorylated on Ser$^{68}$ (CP68). After stripping of nitrocellulose membranes, PLM was revealed by an antibody recognizing PLM not phosphorylated at Ser$^{68}$ (CP68). After stripping of nitrocellulose membranes, PLM was revealed by an antibody recognizing PLM not phosphorylated at Ser$^{68}$ (CP68). After stripping of nitrocellulose membranes, PLM was revealed by an antibody recognizing PLM not phosphorylated at Ser$^{68}$ (CP68).

Effects of PKC Phosphorylation of PLM on Na,K-ATPase Activity

PKC phosphorylation of PLM does not influence the maximal activity of Na,K-ATPase α1/β1 isozymes—Phorbol ester treatment for 3 min of oocytes expressing human Na,K-ATPase α1/β1 isozymes and PLM led to an increase in the population of phosphorylated PLM (Fig. 5A, lanes 3 and 4 and lanes 7 and 8, middle). Both nonphosphorylated and phosphorylated PLM were associated with Na,K-ATPase (Fig. 5A, lanes 5 and 6). For unknown reasons, phosphorylation by PKC increases not only the apparent Na$^+$ affinity of Na,K-ATPase, as confirmed in this study, but also its V$^{\text{max}}$, we investigated whether PLM phosphorylation by PKA influences the maximal Na,K-pump currents (I$^{\text{max}}$) in Xenopus oocytes. Forskolin treatment of oocytes had no effect on I$^{\text{max}}$ of human Na,K-ATPase α1/β1 isozymes alone or together with wild type PLM or the S63A/S68A mutant (Fig. 4A). Analysis of the phosphorylation state confirmed that wild type PLM but not the S63A/S68A mutant was phosphorylated after the electrophysiological measurements (Fig. 4D). The pool of nonphosphorylated, wild type PLM decreased by about 88% after forskolin treatment (Fig. 4E). Since I$^{\text{max}}$ is determined either by the cell surface expression or by the turnover number of Na,K-pumps, we performed $[^{3}H]$ouabain binding on intact oocytes to measure the number of Na,K-ATPase isozymes at the cell surface. Forskolin treatment did not influence the cell surface expression of Na,K-ATPase expressed alone or together with wild type PLM or the S63A/S68A mutant (Fig. 4B). Thus, PLM phosphorylation by PKA has no effect on the cell surface expression or the turnover number (Fig. 4C) of Na,K-pumps. The lack of an effect of PKA phosphorylation of PLM on I$^{\text{max}}$ of Na,K-ATPase was confirmed by continuous incubation of oocytes with epinephrine over a 15-min time period (Fig. 4F).
PLM Phosphorylation Affects Different Na,K-ATPase Isozymes

Table 1

|                      | α1/β1 isozyme | α2/β1 isozyme |
|----------------------|---------------|---------------|
|                      | $K_a$ [M]     | $K_a$ [M]     | $I_{max}$ | $K_a$ [M]     | $I_{max}$ | $K_a$ [M]     | $I_{max}$ |
| Na,K-ATPase          | 0.61 ± 0.02   | 11.8 ± 0.61   | 133.4 ± 4.17 | 61.34 ± 4.46 | 1.09 ± 0.02 | 13.55 ± 0.52 | 245.3 ± 7.75 |
| With PKA             | 0.80 ± 0.01   | 18.6 ± 0.65   | 139.3 ± 6.60 | 62.64 ± 4.36 | 1.59 ± 0.06 | 20.1 ± 0.68 | 236.2 ± 8.46 |
| With PKC-phosphorylated PLM | 0.81 ± 0.04 | 13.9 ± 0.25$^*$ | 123.8 ± 4.06 | 59.02 ± 2.72 | 1.60 ± 0.05 | 15.5 ± 0.84$^*$ | 228.9 ± 5.08 |
| With PKC-phosphorylated PLM | 0.82 ± 0.06 | ND$^b$       | 142.3 ± 9.08 | 63.61 ± 4.61 | 1.72 ± 0.08 | ND$^b$       | 332.3 ± 6.47$^*$ |

$^a$ p < 0.01 phosphorylated PLM versus nonphosphorylated PLM.

$^b$ ND, not determined.

FIGURE 3. PLM phosphorylation by PKA increases the apparent Na$^+$ affinity of Na,K-ATPase α1/β1 isozymes—A, one day after injection of rat Na,K-ATPase α1 and β1 subunit cRNAs plus β2 adrenergic receptor cRNA, alone or together with wild type or mutant PLM cRNAs, the epithelial Na$^+$ channel subunit cRNAs (0.5 ng of α, β, and γ subunit cRNAs) were injected. The following day, the apparent Na$^+$ affinity of Na,K-ATPase (K$_a$Na$^+$) was determined at −50 mV in the presence or absence of 0.1 μM epinephrine. Shown are means ± S.E. of 10 oocytes from five different batches. p < 0.01, α1/β1, and PLM without epinephrine versus plus epinephrine and versus α1/β1 and S63D/S68D without and plus epinephrine. B, microsomes were prepared from oocytes treated with epinephrine for the duration of the electrophysiological measurements and 50 μg of proteins were loaded on a gel, transferred onto nitrocellulose membranes, and probed with an antibody recognizing PLM not phosphorylated at Ser$^{68}$ (bottom). After stripping of nitrocellulose membrane, the amount of Na,K-ATPase α subunit was revealed with a Na,K-ATPase α subunit antibody (top). C, quantification of PLM not phosphorylated at Ser$^{68}$ after epinephrine stimulation and electrophysiological measurements as shown in B and normalized by the amount of Na,K-ATPase α subunit. Shown are means ± S.E. of three independent experiments.

Recent experimental evidence suggests that PKC phosphorylation of PLM in myocytes increases the maximal Na,K-ATPase-mediated Na$^+$ extrusion rate without changing its apparent Na$^+$ affinity (11). We tested whether stimulation of the PKC pathway in Xenopus oocytes expressing human Na,K-ATPase α1/β1 together with PLM increases $I_{max}$ of Na,K-pumps. Phorbol ester treatment of oocytes did not influence $I_{max}$ of Na,K-ATPase α1/β1 isozymes expressed alone or together with wild type PLM or with the S63A/S68A mutant (Fig. 5B). Moreover, PKC activation did not affect the cell surface expression of Na,K-pumps (Fig. 5C) or its turnover number (Fig. 5D). In view of the transient phosphorylation of PLM (Fig. 5A), it was not possible to assess the effect of PKC phosphorylation of PLM on the apparent Na$^+$ affinity of Na,K-ATPase, since the experimental protocol to determine this parameter lasts up to 1 h.

PKC Phosphorylation of PLM Increases the Maximal Activity of Na,K-ATPase α2/β1 Isozymes—We next tested the effect of PKA and PKC phosphorylation of PLM on $I_{max}$ of human Na,K-ATPase α2/β1 isozymes. Neither forskolin nor phorbol ester treatment of oocytes had an effect on $I_{max}$ of Na,K-ATPase expressed without PLM (Fig. 6A, bars 1–4). Forskolin also did not affect $I_{max}$ of Na,K-ATPase α2/β1 isozymes expressed with PLM (bars 5 and 6). Interestingly, however, phorbol ester treatment for 3 min significantly increased $I_{max}$ of α2/β1 isozymes expressed with PLM by about 30% (bar 7), in parallel with PLM phosphorylation (Fig. 7A and B). After 10 min of phorbol ester treatment, the effect on $I_{max}$ was lost (Fig. 6A, bar 8), coinciding with a decrease in phosphorylation of PLM (Fig. 7C, lane 10). Consistent with an effect of PLM phosphorylation on $I_{max}$ of α2/β1 isozymes, the constitutive phosphorylation mutant S63D/S68D produced a similar increase in $I_{max}$ of α2/β1 isozymes in the absence and in the presence of phorbol ester for 3 min (Fig. 6A, bars 9 and 10). A slight decrease of $I_{max}$ of α2/β1 isoforms was observed after exposure of oocytes to phorbol esters for 10 min (bar 11), suggesting that compensatory mechanisms, independent of PLM phosphorylation, might operate during prolonged phorbol ester treatment. In any case, the increase in $I_{max}$ of α2/β1 isozymes after phorbol ester treatment for 3 min is entirely due to PLM phosphorylation, since the double S63A/S68A (bars 12 and 13) as well as the single S68A (bars 15 and 16) mutant did not produce an effect on $I_{max}$ of α2/β1 isozymes. This was also confirmed in an experiment of continuous exposure to PDBu, which showed that maximal activation of $I_{max}$ of α2/β1 isozymes associated with wild type PLM was reached after about 5 min, whereas that of α2/β1 isoforms associated with the S68A mutant was not affected. Moreover, $I_{max}$ of α2/β1 isozymes associated with the S63D/S68D mutant was already increased in the absence of PDBu to

$\textit{JANUARY 4, 2008 • VOLUME 283 • NUMBER 1}$

$\textit{JOURNAL OF BIOLOGICAL CHEMISTRY}$

481
FIGURE 4. PLM phosphorylation by PKA has no effect on the turnover number of Na,K-ATPase \( \alpha_1/\beta_1 \) isozymes. Two days after injection of human Na,K-ATPase \( \alpha_1 \) (10 ng) and \( \beta_1 \) (1 ng), subunit cRNAs alone or together with 3 ng of wild type or mutant PLM cRNAs, oocytes were incubated or not for 4 h with forskolin. A, maximal Na,K-pump currents \( I_{\text{max}} \) at a membrane potential of \(-50\) mV. The pump currents activated by 10 mM \( K^+ \) were measured by the two-electrode voltage-clamp technique in \( Na^+ \)-loaded oocytes. Na,K-ATPase currents measured in noninjected oocytes (35–55 nA) were subtracted. Data are means ± S.E. of 20 oocytes from four different batches. B, \([3H]\)ouabain binding to intact oocytes. Values obtained in noninjected oocytes (without forskolin, 150–203 dpm; with forskolin, 154–227 dpm) were subtracted. Data are means ± S.E. of 30 oocytes from four different batches. C, turnover number of Na,K-ATPase. The turnover number \( (\text{charges transported/s/molecule}) \) was calculated as the ratio between the level of \( I_{\text{max}} \) of \( \alpha_2/\beta_1 \) isozymes associated with wild type PLM treated for 5 min with PDBu (Fig. 6D).

In order to distinguish whether the increase in \( I_{\text{max}} \) of Na,K-ATPase \( \alpha_2/\beta_1 \) isozymes after PKC phosphorylation of PLM is due to an increased number of Na,K-pumps at the cell surface or to an increase in their turnover number, we measured the cell surface expression of Na,K-ATPase by \([3H]\)ouabain binding. The cell surface expression of \( \alpha_2/\beta_1 \) isozymes associated with wild type PLM or with S36D/S68D, S63A/S68A, or S68A mutants was similar in the absence or presence of phorbol ester (Fig. 6B). In consequence, it is likely that wild type PLM phosphorylated by PKC (Fig. 6C, bar 7) and the S63D/S68D mutant (bars 9 and 10) increase the turnover number of \( \alpha_2/\beta_1 \) isozymes, leading to the observed increase in \( I_{\text{max}} \).

Phosphorylation analysis confirmed that wild type PLM was phosphorylated at Ser\(^{68} \) and that the S63A/S68A, the S68A and the S63D/S68D mutants were not phosphorylated at the point of functional measurements (Fig. 7, A and B). Moreover, Fig. 7C shows that both nonphosphorylated and PKC-phosphorylated PLM are associated with Na,K-ATPase \( \alpha_2/\beta_1 \) isozymes.
PLM Phosphorylation Affects Different Na,K-ATPase Isozymes

Since, due to the transient phosphorylation of PLM by PKC, it was not possible to measure the apparent Na\(^+\) affinity of α2/β1-PLM complexes exposed to phorbol ester, we cannot entirely exclude the possibility that the increased \(I_{\text{max}}\) may be due to an increased apparent Na\(^+\) affinity rather than to an effect on the turnover number of Na,K-ATPase α2/β1 isozymes. This is, however, unlikely, since intracellular Na\(^+\) concentrations in Na\(^+\)-loaded oocytes used for \(I_{\text{max}}\) measurements reach values up to 80 mM (32). Under these conditions, it is improbable that an increase in the apparent Na\(^+\) affinity would be translated into an increased \(I_{\text{max}}\), considering a \(K_{1/2}\) Na\(^+\) of about 12 mM for human Na,K-ATPase α2/β1 isoforms (23).

PKC Phosphorylation of PLM Does Not Influence the Apparent K\(^+\) Affinity of Na,K-ATPase α1/β1 or α2/β1 Isozymes—We finally tested whether PKC phosphorylation of PLM might influence the effect of PLM on the K\(^+\) affinity of Na,K-ATPase α1/β1 or α2/β1 isozymes. Phorbol ester treatment of oocytes had no effect on the apparent K\(^+\) affinity of α1/β1 isoforms expressed without PLM or on that of α2/β1 isoforms expressed with PLM (Fig. 8A). As reported previously (7), α2/β1 isoforms have a lower apparent K\(^+\) affinity than α1/β1 isoforms over a large range of membrane potentials, and PLM increases the \(K_{1/2}\) value for K\(^+\) to a greater extent (Fig. 8B). Phorbol ester treatment of oocytes did not influence the apparent K\(^+\) affinity of α2/β1 isoforms expressed without PLM or that of α2/β1 isoforms expressed with PLM (Fig. 8B).

DISCUSSION

Using the Xenopus oocyte as an expression system, our studies on the functional effects of PLM phosphorylation on Na,K-ATPase show that PKA phosphorylation of PLM increases the apparent Na\(^+\) affinity of Na,K-ATPase α1/β as well as α2/β isoforms but has no effect on the maximal Na,K-pump activity. Moreover, we provide evidence that PKC phosphorylation of PLM produces a distinct functional effect on Na,K-ATPase α2/β isoforms.

Regulation of Na,K-ATPase by PLM and its phosphorylation has extensively been studied in cardiac myocytes of wild type and PLM-deficient mice (8, 9, 11–13, 33) as well as in some expression systems (7, 10) and on a shark phospholemman-like protein (19). These studies, in particular those performed in myocytes, have provided some controversial results and have left several open questions with respect to the mechanism by which PKA and PKC phosphorylation of PLM might influence Na,K-ATPase activity. In the present study, we have used the Xenopus oocyte expression system to address certain unanswered questions, which are difficult to assess in cardiac myocytes. The Xenopus oocyte expression system, even if it may not be as physiologically relevant as cardiac myocytes, permits dissection of the intrinsic effects of PLM phosphorylation on Na,K-ATPase activity, independently of the complex physiological conditions existing in cardiac myocytes and the presence of different Na,K-ATPase isoforms, which may determine the outcome of the overall response to PLM phosphorylation. In this respect, it has been proposed that the contradictory results obtained in different laboratories on PLM action may be due to comparison of myocytes of knock-out mice with myocytes of wild type mice with different proportions of phosphorylated and nonphosphorylated PLM determined by the adrenergic state of the heart upon preparation (35).

Effects of PKA and PKC Phosphorylation of PLM on Association with Na,K-ATPase—Co-immunoprecipitation experiments performed on Xenopus oocyte extracts suggest that PLM remains associated with Na,K-ATPase after PKA and PKC
PLM Phosphorylation Affects Different Na,K-ATPase Isozymes

**A**

![Western blot antibody](image)

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|----|
| Na,K-ATPase a subunit | Western blot antibody | non-phosph PLM |
| cRNA a2β1 | PDBu | PLM | PLM S6BD | PLM S6SA | PLM S6BD | PLM S6SA | PLM S6BD | PLM S6SA |

**B**

![Flowchart](image)

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|----|
| cRNA a2β1 | PDBu | 3' 5' 10' |

**C**

![Flowchart](image)

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|----|
| cRNA | PDBu | Western blot antibody |
| a2β1 | non-phosph PLM |

**FIGURE 7.** Phosphorylation and association of wild type and mutant PLM was determined. Two days after injection of rat Na,K-ATPase a2 (12 ng) and b1 (1 ng) subunit cRNAs alone (lanes 1 and 2) or together with 3 ng of wild type (lanes 3 and 4) or mutant (lanes 5–10) PLM cRNAs, oocytes were incubated for 3 min with PDBu. Fifty µg of proteins from Xenopus oocyte microsomes were directly loaded on a gel, transferred onto nitrocellulose membranes, and probed with an antibody recognizing PLM not phosphorylated at Ser68 or with a Na,K-ATPase a subunit antibody. Western blot analysis of the amount of PLM not phosphorylated at Ser68 remained after PDBu stimulation normalized for Na,K-ATPase a subunit expression. Shown are means ± S.E. of three independent experiments. A, rat Na,K-ATPase a2 subunit and b1 subunit cRNAs were injected in Xenopus oocytes in the absence (lanes 1 and 2) or presence of wild type (lanes 3–10) PLM cRNA. Two days after injection, oocytes were incubated for 3 min (lanes 2, 4, 6, and 8), 5 min (lane 9), or 10 min (lane 10) with PDBu. Microsomal proteins were either directly loaded on a gel (lanes 1–4 and 7–10) or immunoprecipitated (IP) under non-denaturing conditions with a Na,K-ATPase a subunit antibody (lanes 5 and 6). Proteins were transferred onto nitrocellulose membranes and probed with an antibody recognizing PLM phosphorylated at Ser68 or an antibody recognizing PLM not phosphorylated at Ser68.

**FIGURE 8.** PLM phosphorylation by PKC has no effect on the apparent K+ affinity of Na,K-ATPase a1β1 and a2β1 isozymes. Two days after injection of rat Na,K-ATPase a1 (A) or ouabain-resistant a2 (B) and b1 subunit cRNAs alone or together with wild type PLM cRNA, oocytes were incubated or not for 3 min with PDBu, and the apparent K+ affinity of Na,K-ATPase (Km) was determined. Closed diamonds, nonactivated oocytes expressing Na,K-ATPase; open diamonds, PDBu-activated oocytes expressing Na,K-ATPase; closed squares, nonactivated oocytes expressing Na,K-ATPase plus PDBu; open squares, PDBu-activated oocytes expressing Na,K-ATPase plus PDBu.

PLM phosphorylation. This result is in line with observations made in cardiac myocytes (8, 11, 33). Moreover, recent experimental evidence shows that PKA and PKC phosphorylation of PLM reduces but does not abolish fluorescence resonance energy transfer between Na,K-ATPase and PLM (36). Together, these data favor the hypothesis that PLM regulates Na,K-ATPase in a manner, which is similar to regulation of sarcoendoplasmic reticulum Ca2+-ATPase by phospholamban (37). PKA- and PKC-mediated phosphorylation of PLM may change interaction with Na,K-ATPase but does not result in a complete dissociation.

**Effects of PKA and PKC Phosphorylation of PLM on the Apparent K+ Affinity of Na,K-ATPase Isozymes**—According to our previous observation, PLM slightly but significantly decreases the apparent K+ affinity of Na,K-ATPase a1β1 and a2β1 isozymes over a large range of membrane potentials after co-expression in Xenopus oocytes (7). As shown in this study, phosphorylation of PLM by PKA or PKC does not influence the effect of PLM on the K+ affinity of Na,K-ATPase a1β1 and a2β1 isozymes. The lack of an effect of PLM phosphorylation on the apparent K+ affinity of Na,K-ATPase is consistent with our previous observation that the effect of several FXYD proteins on the K+ affinity is mainly determined by interactions in the transmembrane domains of the two proteins (38, 39), which may not be influenced by modifications in the cytoplasmic domain of PLM.

**Effects of PKA Phosphorylation of PLM on the Apparent Na+ Affinity of Na,K-ATPase Isozymes**—As we have previously shown, PLM not only has an effect on the K+ affinity of Na,K-ATPase but also decreases the apparent Na+ affinity of a1β1 and a2β1 isozymes (7). The effect of PLM on the Na+ affinity was later confirmed in cardiac myocytes by showing that the Km of Na,K-ATPase was lower in myocytes from PLM knock-out mice than in myocytes from wild type mice (13). It remains unexplained why Zhang et al. (14) observed inhibition of Na,K-ATPase after overexpression of PLM in cardiac myocytes but no effect on its apparent Na+ affinity. In the present study, we confirm the conclusions drawn by Despa et al. (13) from comparative studies in cardiac myocytes of wild type and knock-out mice that PKA phosphorylation of PLM increases the apparent Na+ affinity of Na,K-ATPase and thus abolishes the inhibitory effect of PLM. Notably, our results show that PLM phosphorylation by PKA influences the effect of PLM on the Na+ affinity of both Na,K-ATPase a1β1 and a2β1 isozymes. Consistently, our results show that PLM mutated in its phosphorylation site Ser68 has no effect on the apparent Na+ affinity of Na,K-ATPase upon PKA activation, whereas a constitutive phosphorylation mutant produces an effect even in the absence of PKA activation (Fig. 3A). These latter results, together with the observation that PKA activation has no effect on the apparent Na+ affinity of Na,K-ATPase expressed without PLM, also indicate that PKA activation produces its functional effect on PLM-associated Na,K-ATPase by phosphorylation of PLM rather than by phosphorylation of the Na,K-ATPase α subunit. This conclusion is in line with results obtained in cardiac myocytes (13) and with studies showing that forskolin treatment of cardiac myocytes...
PLM Phosphorylation Affects Different Na,K-ATPase Isozymes

leads to phosphorylation of PLM at Ser68 but not of the PKA consensus site in Na,K-ATPase α1 subunits (9).

Effects of PKA Phosphorylation of PLM on the Maximal Activity of Na,K-ATPase Isozymes—It remains controversial whether PKA phosphorylation of PLM influences the apparent Na\(^{+}\) affinity of the maximal Na,K-ATPase activity or both. In agreement with studies by Despa et al. (13) in cardiac myocytes, we find an effect of PKA phosphorylation of PLM on the apparent Na\(^{+}\) affinity but not on \(V_{\text{max}}\) of both Na,K-ATPase α1/β1 and α2/β1 isozymes. An increase in the apparent Na\(^{+}\) affinity without an effect on \(V_{\text{max}}\) was also correlated with PLM phosphorylation of Na,K-ATPase coexpressed in *P. pastoris* (10). This contrasts with studies that describe an increase in \(V_{\text{max}}\) of Na,K-ATPase after PKA phosphorylation of PLM (9, 12). The reasons for these discrepancies are not clear, but it is possible that experimental conditions (such as temperature), variations in the proportion of phosphorylated and unphosphorylated PLM in different cell types, or other undefined variables may be the cause for different results.

Effects of PKC Phosphorylation of PLM on Na,K-ATPase Activity—Recent studies by Han et al. (11) show that PKC phosphorylation of PLM does not influence the apparent Na\(^{+}\) affinity of Na,K-ATPase but increases its \(V_{\text{max}}\). Moreover, this study provides evidence that successive PKA and PKC activation leads to additive effects on PLM-associated Na,K-ATPase despite common phosphorylation sites on PLM. In the present study, we tested the interesting hypothesis that PKA and PKC may target different pools of PLM associated with different Na,K-ATPase isozymes. Our results indeed show that PKC phosphorylation of PLM has no effect on \(I_{\text{max}}\) of Na,K-ATPase α1/β1 isozymes but significantly increases \(I_{\text{max}}\) of α2/β1 isozymes.

The study by Han et al. (11) has not assessed the question of whether the increase in \(V_{\text{max}}\) of Na,K-ATPase upon PKC phosphorylation of PLM is due to an increase in the cell surface expression or an increase in the turnover number of Na,K-ATPase. We show in this study that the increase in \(I_{\text{max}}\) of α2/β isozymes observed after PKC phosphorylation of PLM cannot be attributed to an increase in cell surface expression but most likely to an increase in their turnover number. Since it was not possible to measure the apparent Na\(^{+}\) affinity of Na,K-ATPase after PKC treatment of *Xenopus* oocytes, we cannot entirely exclude the possibility that the increase in \(I_{\text{max}}\) of α2/β isozymes is due to an effect of PKC phosphorylation of PLM on the apparent Na\(^{+}\) affinity or that PKC phosphorylation of PLM may have an additional effect on the apparent Na\(^{+}\) affinity of either Na,K-ATPase α1/β or α2/β isozymes. However, since no effect on the apparent Na\(^{+}\) affinity of Na,K-ATPase has been observed in cardiac myocytes after PKC activation (11), this is unlikely and would probably be physiologically irrelevant.

Overall, our studies suggest that PKA can target PLM associated with Na,K-ATPase α1/β and α2/β isozymes to increase their apparent Na\(^{+}\) affinity. On the other hand, PKC can phosphorylate PLM associated either with α1/β or α2/β isozymes but produces a functional effect, namely an increased turnover number, only on α2/β isozymes. If we consider that PKA phosphorylates Ser68 and PKC phosphorylates Ser63 and Ser68 in PLM, and taking into account that mutation of Ser68 abolishes the effect of PKA phosphorylation of PLM on the apparent Na\(^{+}\) affinity as well as the effect of PKC phosphorylation of PLM on the turnover number of Na,K-ATPase, we may speculate that the effect of PKA-phosphorylated PLM on the Na\(^{+}\) affinity of α1/β and α2/β isozymes might be mediated by phosphorylation of Ser68, whereas the \(V_{\text{max}}\) effect of PKC phosphorylation of PLM associated with α2/β isozymes might necessitate phosphorylation of both Ser63 and Ser68.

Since both α1/β and α2/β isozymes are present in cardiac myocytes (40–42), the molecular basis that permit the differential effects of PKC-mediated phosphorylation of PLM on α1/β and α2/β isozymes remains to be determined. α2/β isozymes may only make up about 11% of the total Na,K-ATPase (43), but they may play a more important and different role in cardiac contractility than α1/β isozymes (43, 44) due to their preferential localization in T-tubules and their co-localization with the Na/Ca exchanger (34, 43).

Physiological Relevance—Our results support the hypothesis that PLM phosphorylation by PKA and PKC play differential roles in the regulation of different Na,K-ATPase isozymes. This result raises the interesting possibility that the additive effects of PKA and PKC phosphorylation of PLM on Na,K-ATPase, which are observed in cardiac myocytes, might reflect that 1) PKA activated by β-adrenergic receptor stimulation can target PLM associated with Na,K-ATPase α1/β and α2/β isozymes to increase their apparent sodium affinity, and 2) PKC activated by α-adrenergic receptor stimulation targets, in addition, PLM associated with α2/β isozymes to increase their turnover number. According to our results, PKC phosphorylation of PLM associated with Na,K-ATPase α1/β isozymes is possible but does not lead to an increase in their turnover number. Physiologically, the overall response to concomitant PKA and PKC activation would be an additive increase in sodium extrusion, which may limit sodium load during increased heart activity and hence may limit positive inotropy during sympathetic stimulation of the heart by favoring calcium extrusion through the sodium/calcium exchanger.

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Phosphorylation of Phospholemman (FXYD1) by Protein Kinases A and C Modulates Distinct Na,K-ATPase Isozymes
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