Phospholemman Phosphorylation Alters Its Fluorescence Resonance Energy Transfer with the Na/K-ATPase Pump

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¶ The abbreviations used are: NKA, Na/K-pump; FRET, fluorescence resonance energy transfer; PLM, phospholemman; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; GFP, green fluorescent protein; NCX, Na/Ca exchange; SERCA, sarcoplasmic reticulum Ca-ATPase; HEK293, human embryonic kidney 293; PKA, protein kinase A; PKC, protein kinase C; PDBu, phorbol dibutyrate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N'-tetraacetic acid.

Phospholemman (PLM) or FXYD1 is a major cardiac myocyte phosphorylation target upon adrenergic stimulation. Prior immunoprecipitation and functional studies suggest that phospholemman associates with the Na/K-pump (NKA) and mediates adrenergic Na/K-pump regulation. Here, we tested whether the NKA-PLM interaction is close enough to allow fluorescence resonance energy transfer (FRET) between cyan and yellow fluorescent (CFP/YFP) fusion proteins of Na/K pump and phospholemman and whether phospholemman phosphorylation alters such FRET. Co-expressed NKA-CFP and PLM-YFP in HEK293 cells co-localized in the plasma membrane and exhibited robust FRET. Selective acceptor photobleach increased donor fluorescence (plasma membrane and exhibited robust FRET. Selective NKA-CFP and PLM-YFP in HEK293 cells co-localized in the pholemman phosphorylation alters such FRET. Co-expressed proteins of Na/K pump and phospholemman and whether phospholemman phosphorylation alters such FRET. Co-expressed NKA-CFP and PLM-YFP in HEK293 cells co-localized in the plasma membrane and exhibited robust FRET. Selective acceptor photobleach increased donor fluorescence (FCFP) by 21.5 ± 4.1% (n = 13), an effect nearly abolished when co-expressing excess phospholemman lacking YFP. Activation of protein kinase C or A progressively and reversibly decreased FRET assessed by either the fluorescence ratio (F_{YFP}/F_{CFP}) or the enhancement of donor fluorescence after acceptor bleach. After protein kinase C activation, forskolin did not further reduce FRET, but after forskolin pretreatment, protein kinase C could still reduce FRET. This agreed with phospholemman phosphorylation measurements: by protein kinase C at both Ser-63 and Ser-68, but by protein kinase A only at Ser-68. Expression of PLM-YFP and PLM-CFP resulted in even stronger FRET than for NKA-PLM (F_{CFP} increased by 37 ± 1% upon YFP photobleach), and this FRET was enhanced by phospholemman phosphorylation, consistent with phospholemman multimerization. Co-expressed PLM-CFP and Na/Ca exchange-YFP were highly membrane co-localized, but FRET was undetectable. We conclude that phospholemman and Na/K-pump are in very close proximity (FRET occurs) and that phospholemman phosphorylation alters the interaction of Na/K-pump and phospholemman.

Myocyte sodium homeostasis is crucial to a plethora of cell functions, including excitability, excitation-contraction coupling, energy metabolism, pH regulation, and growth (1). The large [Na⁺] gradient maintained across the plasma membrane by the Na/K pump (NKA) drives numerous ionic and metabolic transport processes. Indeed, intracellular sodium can very directly influence intracellular calcium and pH via Na/Ca exchange (NCX), Na/H exchange, and sodium-bicarbonate co-transport systems (1, 2). Altered activity and regulation of the Na/K-pump can therefore profoundly affect normal cardiac function.

The Na/K-pump is regulated via numerous signaling pathways, including protein kinases, phospholipases, and phosphatases (3). For instance, sympathetic regulation is mediated by protein kinase A (PKA) and C (PKC) activation, but the detailed molecular mechanisms remain controversial (4–7). There are only a modest number of mammalian Na/K-pump isoforms, and it is increasingly clear that tissue-specific regulation of the Na/K-pump is mediated in part by associated FXYD proteins (8, 9). There are seven mammalian FXYD proteins (all with single transmembrane spans), and FXYD2 (γ-subunit) and FXYD4 (CHIF) are localized to different regions of the renal tubule and modulate Na/K-pump activity differently.

Phospholemman (PLM or FXYD1) is highly expressed in the heart, where it is among the most prominent substrates for PKA and PKC (10). Early studies showed that phospholemman expression induced chloride currents in Xenopus oocytes (presumably through oligomers) (11), and taurine efflux and regulatory volume decrease in mammalian cells. More recently, however, functional interactions of phospholemman and Na/K-pump and Na/Ca exchange have been described in the heart. In cardiac myocytes, phospholemman phosphorylation at the PKA site (Ser-68) was found to mediate β-adrenergic effects on the Na/K-pump (12, 13). In other words, phospholemman inhibits sodium pump function by decreasing its affinity for internal sodium, and this effect is relieved upon phospholemman phosphorylation. Phospholemman may also interact with and modulate Na/Ca exchange in myocytes or when co-expressed in HEK cells (14, 15).

Fluorescence resonance energy transfer (FRET) is a sensitive indicator of the physical distance between and relative orientation of two fluorophores (a donor and acceptor). Intra- and intermolecular FRET has been used extensively to assess protein interactions and as molecularly coded biosensors (16–18). Variants of green fluorescent protein (e.g. cyan and yellow flu-
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orescent proteins (CFPs and YFPs)) that have suitable fluorescence spectra have been especially useful (16–18), because they can be incorporated into fusion proteins for proteins of interest.

Although the functional effects of phospholemman and its phosphorylation on the Na/K-pump are quite compelling, the only data regarding physical association is from co-immunoprecipitation of phospholemman with cardiac Na/K-pump, which is unaltered by phospholemman phosphorylation (13, 19–21). Our goal here was to test the hypothesis that the Na/K-pump-phospholemman interaction is close enough to allow intermolecular FRET between CFP and YFP fusion proteins of the Na/K-pump and phospholemman and that phospholemman phosphorylation could alter such FRET in cells. The results are consistent with this hypothesis and with the possibility that phospholemman-phospholemman complexes may occur, but we did not detect FRET between phospholemman-Na/Ca exchange constructs.

EXPERIMENTAL PROCEDURES

Construct Generation—Cyan and yellow fluorescent fusion proteins were made of phospholemman. The coding sequence for PLM (279 bp) was inserted into the pECFP N1 (Clontech, Palo Alto, CA) and the pEYFP N1 vector with the citrine modification (QuikChangeXL, Stratagene, La Jolla, CA) using the primers 5′-CTGTTCCACCCGACGCGGAAGAGATCTCGAGCTCAA-GCT-3′ and 5′-AGCTTGAGCTCGATCTTCCGCCCT-GCGGTTGACAG-3′. This was followed by a SacI and AgeI restriction digest. The generation of PLM-YFP and PLM-CFP fusion proteins (where YFP and CFP were linked to the intracellular loop at residue 358 in the full-length NCX1), were isoform-specific anti-NKA (where CFP and YFP were linked to the intracellular loop at residue 358 in the full-length NCX1), and the pipette solution contained 30 mmol/liter NaCl, 70 mmol/liter NaOH, 70 mmol/liter aspartic acid, 20 mmol/liter potassium aspartate, 20 mmol/liter triethanolamine-Cl, 10 mmol/liter HEPES, 5 mmol/liter MgCl2-ATP, 0.7 mmol/liter MgCl2 (~1 mmol/liter free Mg), 3 mmol/liter BAPTA, 1.15 mmol/liter CaCl2 (~100 nm free Ca), pH 7.2. The potassium-free external solution contained 136 mmol/liter NaCl, 5 mmol/liter NiCl2, 2 mmol/liter BaCl2, 1 mmol/liter MgCl2, 5 mmol/liter HEPES, 10 mmol/liter glucose, Tris-Cl, pH 7.4. In the potassium-containing solution, 15 mmol/liter NaCl was replaced with KCl.

FRET Measurements—We used two different approaches to measure FRET (Fig. 1A) (19–21). As a first approach (emission ratio), we excited the samples at 430 nm and measured fluorescence intensities of both the donor (CFP) and acceptor (YFP) using appropriate wavelengths (F535 and F485, respectively) as in Fig. 5 or emission spectra as in Fig. 3. The occurrence of FRET reduces the donor intensity and increases the acceptor intensity upon donor excitation, such that dynamic changes in FRET can be followed upon phospholemman phosphorylation as changes in F535/F485. Utility of these ratio measurements is limited by 1) direct acceptor excitation at donor excitation and 2) some donor emission at the acceptor peak wavelength. These can be partially corrected for (see discussion of Fig. 3 below). Alternatively, we used the acceptor photobleach method (Fig. 1A, right). When FRET is present, bleach of the acceptor prevents FRET with consequent increase in direct emission from the donor (F485). The FRET efficiency, E, is inversely proportional to the 6th power of the donor-acceptor distance, R, as follows,

\[ E = R_0^6 / (R_0^6 + R^6) \]  

where \( R_0 \) is the Förster distance (5.3 nm for the CFP-YFP pair) (23). In acceptor photobleach experiments, \( E \) is calculated as the ratio between the increase in the CFP emission upon YFP photobleach and the CFP emission after YFP photobleach. Thus, according to Equation 1, detectable FRET occurs if the CFP and YFP are at a distance of 9 nm or less of each other (\( E \) higher than 4%).

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**In Vitro Fluorescence Measurement**—The fluorescence spectral characterization of the phospholemman, NKAα1, and
NCX fusion proteins was performed on a SLM Aminco spectrofluorimeter (SLM Instruments). Monochromator excitation and emission slit widths were set at 2 nm. Excitation was at 430 and 510 nm, respectively, for CFP and YFP. Fluorescence measurements were recorded (at 22 °C) in polystyrene cuvettes containing a suspension of transiently transfected cells in phosphate-buffered saline.

Dynamic fluorescence changes were also obtained using standard epifluorescence dual emission microscopy and commercially available filters (Chroma); CFP and YFP excitation was at 430 and 510 nm, respectively, and emitted fluorescence was recorded at 535 ± 30 nm and 470 ± 20 nm. To minimize photobleaching, measurements were obtained for 5 s at 1-min intervals. Corrections for photobleaching and direct activation of acceptor were applied.

**Confocal Imaging**—A laser-scanning unit (Bio-Rad Microradiance 2000) attached to an inverted microscope was used. CFP and YFP excitation was at 457 and 514 nm, respectively, and emitted fluorescence was measured at 485 ± 30 nm (for CFP) and 545 ± 40 nm (for YFP). Acceptor photobleaching was performed by excitation at 514 nm. Acceptor (YFP) emission at donor (CFP) excitation was corrected in some cases for direct activation of the acceptor (using YFP emission at 514 nm excitation and the measured relative YFP fluorescence for 457 versus 514 nm excitation where only YFP was present).

**Statistical Analysis**—Pooled data are represented as mean ± S.E. Statistical comparisons were made using Student’s t test; p < 0.05 indicated statistical significance.

**RESULTS**

**Characterization of the Constructs**—To validate the CFP and YFP fusion protein constructs, cell lysates from transiently transfected HEK293 cells were examined by Western blotting using PLM-α1, GFP-α1, and NKAα1-specific antibodies. Fig. 2A illustrates that all of the constructs express at high levels (albeit less so for CFP-NKAα1) and can be recognized by antibodies against both GFP and the original protein. Also shown is the
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Figure 3. Detectable FRET between CFP-NKα1 and PLM-YFP. A, co-immunoprecipitation of CFP-NKα1 and PLM-YFP. An NKα1-specific antibody was used in cells expressing CFP-NKα1 and PLM-YFP or either protein alone. Shown is the YFP and CFP content in the bead-associated immune complexes, where the presence of both indicates interaction of CFP-NKα1 and PLM-YFP. B–G, corresponding fluorescence spectra normalized for B–D to maximal YFP fluorescence in C and for E–G to maximal CFP fluorescence in E. The lower trace in F is corrected for direct excitation of YFP fluorescence by 430 nm excitation (as described under “Experimental Procedures”).

Here an NKα1-specific antibody was used in cells co-expressing CFP-NKα1 and PLM-YFP or either protein alone. The CFP and YFP content of the bead-associated immune complexes is shown in Fig. 3A (n = 3). Immunoprecipitations with nonimmune IgG failed to pull down significant fluorescence. The substantial presence of CFP and YFP fluorescence in the immunoprecipitate from co-expressing cells (middle bars) indicates that CFP-NKα1 and PLM-YFP interact. Moreover, the YFP fluorescence in the immunoprecipitate from cells expressing PLM-YFP alone (right) indicates that PLM-YFP associates with endogenous Na/K-pump. Thus, both fusion proteins are functional and interact with each other at the level of co-immunoprecipitation.

Detection of FRET from CFP-NKα1 and PLM-YFP—Fig. 3, B–G, shows spectral scans of immunoprecipitated samples from Fig. 3A. Ideally, upon CFP excitation, any signal detected at YFP emission would originate from energy transfer from CFP to YFP and represent FRET. However, the excitation and emission spectra of CFP and YFP partially overlap. Fig. 3B shows that CFP does not produce a complicating fluorescence signal when excitation is ≥490 nm, which is used to detect YFP (despite strong fluorescence upon excitation at 430 nm) (Fig. 3E). Thus, Fig. 3, B–D, can be used to assess the relative amount of YFP expressed (regardless of CFP). Fig. 3G shows that excitation at 430 nm (intended for CFP) produces measurable direct YFP fluorescence between 510 and 570 nm. This direct YFP excitation complicates Fig. 3F, where both CFP and YFP are present and where FRET is to be examined. Using the data in Fig. 3C and D, we can correct for the amount of YFP and then subtract the scaled direct YFP spectrum (Fig. 3G) from the spectrum in Fig. 3F. This gives the lower curve in Fig. 3F, where the increase at 535 nm and decrease at 485 nm (versus 3E) reflect substantial FRET. The corrected $F_{485}/F_{485}$ increased 4-fold from 0.33 to 1.34 when phospholemman was present.

FRET was also determined by measuring the increase in donor fluorescence upon acceptor (YFP) photobleach (Fig. 1A, right) (25). In other words, without FRET, more energy from the donor is released as direct fluorescence (proportional to the FRET efficiency). Control YFP photobleach experiments were done with CFP-NKα1 expression alone. The same YFP photobleach protocols as used below did not cause any increase in CFP fluorescence (not shown). This assures that any enhance-
PLM Phosphorylation Alters FRET from CFP-NKA\alpha{1} and PLM-YFP—Fig. 5A shows the influence of PKA and PKC activation on the crude FRET fluorescence ratio \( (F_{535}/F_{485}) \). Both forskolin (1 \( \mu \)M) and PDBu (100 nM) progressively reduced FRET. After 30 min of washout, the ratio almost completely recovered, indicating that the effect is reversible. H-89, a PKA inhibitor, prevented the forskolin effect (not shown). Similarly, 20 min of forskolin, PDBu, or 8-bromo-cAMP significantly depressed the YFP photobleach-induced enhancement of CFP fluorescence (Fig. 5B). Despite this 80–90% reduction in FRET caused by activation of PKA and PKC, the residual 2–3% \( F_{485} \) enhancement upon photobleach was significantly different from CFP-NKA\alpha{1} alone.

Since phospholemman has partially overlapping phosphorylation sites for PKA (Ser-68) and PKC (Ser-63 and Ser-68), we also looked at potential cross-talk between the pathways (Fig. 5C). After a 30-min exposure to forskolin, PDBu caused a further decrease in the YFP/CFP ratio. This was less so for forskolin after an initial PDBu treatment (mean data in Fig. 5D). The PDBu potentiation of the forskolin effect may reflect the extra PKC phosphorylation site (Ser-63). Overall, these findings indicate that not only does the CFP-NKA\alpha{1} and PLM-YFP interaction result in detectable FRET, but the latter is altered by the phosphorylation state of phospholemman. These results are also consistent with phospholemman binding to (and inhibiting) the Na/K-pump, with less close interaction (and relief of inhibition) upon phospholemman phosphorylation (12, 13).

Detection and Modulation of FRET from PLM-YFP and PLM-CFP—Early studies suggested that phospholemman expression in bilayers induces ion channels (26). Therefore, the potential of phospholemman to oligomerize was assessed by measuring FRET between PLM-CFP and PLM-YFP. A typical acceptor photobleach experiment is shown in Fig. 6A. The basal PLM-CFP and PLM-YFP (left panels) were obtained with the 457 and 514 nm laser lines. The right panels show the increase in PLM-CFP fluorescence upon bleaching of PLM-YFP with the 514 nm argon laser line. Mean data from these experiments (Fig. 6B) show that for a comparable extent of YFP photobleach for PLM-PLM (70 ± 9%) versus PLM-NKA\alpha{1} (74 ± 8%), there was a considerably larger increase in CFP (donor) fluorescence for the interaction of PLM-CFP and PLM-YFP (36.9 ± 5.8% \( n = 22 \) versus 21.5 ± 4.1% \( n = 13 \)). Unlabeled phospholemman could again largely prevent the increase in PLM-CFP upon acceptor photobleach (Fig. 6B). Furthermore, phospholemman phosphorylation by activation of PKA or PKC caused increased FRET seen as a significant increase in \( F_{535}/F_{485} \) ratio (Fig. 6C) and a larger increase in donor fluorescence upon acceptor photobleach (Fig. 6D). These findings suggest that phospholemman may form oligomers and that phospholemman phosphorylation causes a closer interaction.

Detection of FRET from PLM-CFP and NCX358YFP—Several reports have suggested that phospholemman could function as an endogenous inhibitor of Na/Ca exchange function (15, 27, 28). Thus, we assessed FRET occurrence in HEK293 cells co-expressing PLM-CFP and NCX358YFP (YFP was inserted into the large cytoplasmic loop of the full-length exchanger). Both proteins target to the plasma membrane as expected (Fig. 7A) and appear highly co-localized when expressed in the same cell.
The left panels again show basal fluorescence obtained with the 457 and 514 nm argon laser line. However, upon acceptor photobleach, no significant increase in PLM-CFP was detected (right panels and mean data in Fig. 7B). In addition, PDBu exposure did not result in detectable FRET for this pair.

**DISCUSSION**

We show for the first time that phospholemman and the Na/K-pump are sufficiently close to each other in the plasma membrane that CFP/YFP fusion proteins exhibit specific and robust FRET, which can be largely inhibited by either excess phospholemman without fluorophores or by phospholemman phosphorylation. PLM-CFP and PLM-YFP also produce substantial FRET (which is enhanced by phospholemman phosphorylation and inhibited by nonfluorescent phospholemman). In contrast, we could not detect FRET between phospholemman and another putative plasma membrane partner Na/Ca exchange, despite confocal co-localization.

Phospholemman is a member of the FXYD protein family that has recently emerged as tissue-specific regulators of the Na/K-pump (8, 9). Other members include the Na/K-pump γ-subunit (FXYD2), the regulator of renal Na/K-pump (FXYD4, or CHIF), and the phospholemman-like shark rectal gland protein (29–31). All FXYDs tested co-immunoprecipitate with Na/K-pump α subunits and modulate Na/K-pump function. Phospholemman is unique among mammalian FXYD proteins in having multiple phosphorylation sites. In the heart, phospholemman is a major target for phosphorylation by PKA and PKC (32, 33). How phospholemman regulates the Na/K-pump is only starting to become clearer. Our work and others has indicated that phospholemman inhibits Na/K-pump activity (by reducing [Na]i affinity) and that PKA- and PKC-dependent stimulation of Na/K-pump activity in ventricular myocytes is mediated mainly by phospholemman phosphorylation and consequent relief of phospholemman-dependent Na/K-pump inhibition (12, 13, 19, 34). Part of our working hypothesis is that phospholemman and Na/K-pump physically interact and that upon phosphorylation interaction is modified to enhance Na/K-pump activity. This parallels closely how phospholamban is thought to inhibit the sarcoplasmic reticulum Ca-ATPase (SERCA) and how phosphorylation relieves that inhibition (1, 35). In the SERCA-phospholamban system, it was long thought that phospholamban phosphorylation caused dissociation from SERCA. However, Asahi et al. (36) showed that phospholamban remains bound to SERCA after phosphorylation, despite a loss of SERCA inhibition.

**NKA-PLM Interaction**—Previous work on Na/K-pump-phospholemman interaction has come mainly from co-immunoprecipitation experiments. These studies showed that phospholemman associates with Na/K-pump α1 and α2 isoforms and that the association may be more robust for α1 than α2 (19–21). As valuable as these data are, there are intrinsic limitations. For example, co-immunoprecipitated proteins might be distant members of very large macromolecular complexes, and these may not be highly specific. Moreover, if there are subtle changes in interaction between the proteins that influence function (e.g. in our case with phospholemman phosphorylation), immunoprecipitations may not be sensitive enough to detect them. FRET can provide information on a more molecular scale in living cells and is sensitive to small changes in distance and/or orientation between the fluorophores. Detect-
able FRET requires the molecules to be physically very close (<9 nm when using the CFP-YFP pair), and for the FRET we see here (~30%) that they are likely to be ≤5 nm apart (i.e. molecular neighbors), according to Equation 1. This is likely to be an overestimate for two reasons. First, CFP and YFP fluorophores are in the center of barrel structures that add 20–30 nm as a lower limit. Second, CFP and YFP are attached to the intracellular ends of the proteins of interest. These proteins are likely to be physically closer together, based on cross-linking studies of Na/K-ATPase α1 and the phospholemman-related FYXD2 protein (or γ-subunit) (37) and infrared spectroscopy measurements which show that phospholemman can form tetramers in proteoliposomes (38). Thus, whereas our direct measurements of dynamic FRET changes in live cells is useful as a sensor of proximity and/or orientation changes, more quantitative intermolecular distance measurements would require smaller fluorophores incorporated into known sites in Na/K-ATPase and phospholemman, as has been done for the potentially analogous SERCA-phospholamban pair (39). The fact that nonfluorescent phospholemman could compete away FRET between CFP-NKAα1 and PLM-YFP suggests that the apparent phospholemman-Na/K-pump interaction is specific.

We also found that phospholemman phosphorylation by either PKA or PKC reduced FRET substantially, suggesting changes the phospholemman-Na/K-pump interaction and thereby relieves the inhibitory functional effect of phospholemman on Na/K-pump. Of course, this working hypothesis will require further tests.

**PLM: Other Roles and Oligomerization**—Earlier work on the role of phospholemman suggested that phospholemman induced ion currents in lipid bilayers or in *Xenopus* oocytes (26, 40). This was thought to require channel formation by phospholemman oligomers, but clear evidence of phospholemman oligomerization has been elusive. Our present results do suggest that phospholemman can oligomerize, because considerable FRET was detected between PLM-YFP and PLM-CFP. Again this cannot be attributed to nonspecific CFP-YFP interaction, because unlabeled phospholemman could largely prevent the increase in PLM-CFP fluorescence upon acceptor photobleach (Fig. 6B). Moreover, Bevers and Kukol (38) recently suggested that phospholemman can form homotetramers upon reconstitution in proteoliposomes. It is unclear at this point whether phospholemman oligomers serve as a storage pool for non-Na/K-pump-associated phospholemman, have functional roles as multimers, or even exist in that form in normal cell membranes. In other words, the formation of oligomers may be an artifact of overexpression of phospholemman in large stoichiometric excess over Na/K-pump α.
Modulation of PLM-NKA Association

Remarkably, phospholemman phosphorylation enhanced FRET between PLM-CFP and PLM-YFP, the opposite direction of that seen for phospholemman-Na/K-pump. This may reflect stronger association among phospholemman monomers upon phosphorylation or simply a change in shape or orientation of the multimers. Of course, further study would be required to determine if this phosphorylation effect alters phospholemman multimer function, which at present is unclear.

PLM-NCX Interaction—Several studies have suggested that phospholemman is an inhibitor of Na/Ca exchange function (27, 28) and that phospholemman antibodies could co-immunoprecipitate Na/Ca exchange in co-transfected HEK293 cells and rat cardiac myocytes (15). However, in the present study, we failed to detect significant FRET between PLM-CFP and the full-length Na/Ca exchange with YFP inserted at position 358 (Fig. 7) despite co-localization at the plasma membrane. In addition, phospholemman phosphorylation still did not result in detectable FRET between NCX-YFP and PLM-CFP, despite reports that Na/Ca exchange inhibition by phospholemman was enhanced by phospholemman phosphorylation (14). Although we failed to find FRET evidence to support Na/Ca exchange-phospholemman interaction, our data do not rule it out either. This failure could be due to the specific distance or orientation between the fluorophores being nonconducive to FRET in this complex. Under conditions where we see robust co-immunoprecipitation of Na/K-pump α1 and α2 with phospholemman, we have not detected a phospholemman-Na/Ca exchange complex. It is possible that the affinity of phospholemman for Na/Ca exchange is simply lower than for Na/K-pump α.

In conclusion, we found that the CFP-NKAα1-PLM-YFP interaction resulted in detectable FRET and that phospholemman phosphorylation altered this interaction. We also found evidence for phospholemman oligomerization, which was modulated by phospholemman phosphorylation (in the opposite direction). However, we could not detect any FRET between phospholemman and Na/Ca exchange. Thus, our data are consistent with a role for phospholemman in Na/K-pump regulation similar to that of phospholamban for SERCA (an inhibition relieved upon phospholemman phosphorylation).

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