Phospholemman Inhibition of the Cardiac Na\(^+\)/Ca\(^{2+}\) Exchanger

**ROLE OF PHOSPHORYLATION**

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We have demonstrated previously that phospholemman (PLM), a 15-kDa integral sarcolemmal phosphoprotein, inhibits the cardiac Na\(^+\)/Ca\(^{2+}\) exchanger (NCX1). In addition, protein kinase A phosphorylates serine 68, whereas protein kinase C phosphorylates both serine 63 and serine 68 of PLM. Using human embryonic kidney 293 cells that are devoid of both endogenous PLM and NCX1, we first demonstrated that the exogenous NCX1 current (I\(_{\text{NCX1}}\)) was increased by phorbol 12-myristate 13-acetate (PMA) but not by forskolin. When co-expressed with NCX1, PLM resulted in: (i) decreases in I\(_{\text{NCX1}}\), (ii) attenuation of the increase in I\(_{\text{NCX1}}\) by PMA, and (iii) additional reduction in I\(_{\text{NCX1}}\) in cells treated with forskolin. Mutating serine 63 to alanine (S63A) preserved the sensitivity of PLM to forskolin in terms of suppression of I\(_{\text{NCX1}}\) whereas mutating serine 68 to alanine (S68A) abolished the inhibitory effect of PLM on I\(_{\text{NCX1}}\). Mutating serine 68 to glutamic acid (pathophosphimetic) resulted in additional suppression of I\(_{\text{NCX1}}\) as compared with wild-type PLM. These results suggest that PLM phosphorylated at serine 68 inhibited I\(_{\text{NCX1}}\). The physiological significance of inhibition of NCX1 by phosphorylated PLM was evaluated in PLM-knock-out (KO) mice. When compared with wild-type myocytes, I\(_{\text{NCX1}}\) was significantly larger in PLM-KO myocytes. In addition, the PMA-induced increase in I\(_{\text{NCX1}}\) was significantly higher in PLM-KO myocytes. By contrast, forskolin had no effect on I\(_{\text{NCX1}}\) in wild-type myocytes. We conclude that PLM, when phosphorylated at serine 68, inhibits Na\(^+\)/Ca\(^{2+}\) exchange in the heart.

Phospholemman (PLM), a 72-amino acid membrane phosphoprotein with a single transmembrane domain (1), belongs to the FXYD gene family of small ion transport regulators (2). With the exception of the γ-subunit of Na\(^+\)-K\(^+\)-ATPase (FXYD2), all other known members of the FXYD gene family have at least one serine or threonine within the cytoplasmic tail (2), indicating potential phosphorylation sites. In particular, PLM (FXYD1) is the only FXYD family member to have a consensus sequence for phosphorylation by PKA (RR\(\text{X}\)XXXSR), PKC (RXXXXQR), and NIMA (never in mitosis A) kinase (FRX(S/T)). Indeed PLM has been shown to be phosphorylated by PKA at serine 68 and PKC at both serine 63 and serine 68 (3).

To date, PLM has been demonstrated to modulate ion fluxes through both the Na\(^+\)/K\(^+\)-ATPase (4–8) and the cardiac Na\(^+\)/Ca\(^{2+}\) exchanger (NCX1) (9–11). Based on analogy of phospholamban inhibition of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2) (12) and experimental observation on the effects of PLMs (a 15-kDa homologue of PLM isolated from shark rectal glands) on shark Na\(^+\)-K\(^+\)-ATPase (13, 14), the current working hypothesis is that the Na\(^+\) pump is inhibited by phosphorylated PLM. On phosphorylation of PLM, inhibition of Na\(^+\)-K\(^+\)-ATPase is relieved. This hypothesis has been given strong support by the observation that the V\(_{\text{max}}\) of sarcloemmal Na\(^+\)-K\(^+\)-ATPase is increased 3-fold after acute cardiac ischemia in association with increased PLM phosphorylation by >300% (5). In addition, Na\(^+\) pump current has been demonstrated to directly increase in association with PLM phosphorylation in response to forskolin (6). More recently, comparison of β-adrenergic effects on Na\(^+\) pump function between wild-type and PLM-knock-out (KO) myocytes supports the notion that the inhibitory effects of PLM on Na\(^+\)-K\(^+\)-ATPase are relieved by phosphorylation (8). It is at present not clear whether dissociation of the phosphorylated PLM from Na\(^+\)-K\(^+\)-ATPase is required to relieve its inhibition on the Na\(^+\) pump (5, 6, 8, 13, 14). With respect to the cardiac Na\(^+\)/Ca\(^{2+}\) exchanger, previous studies demonstrated that overexpression of PLM inhibits Na\(^+\)/Ca\(^{2+}\) exchange activity (9, 10), whereas down-regulation of PLM enhances NCX1 current (I\(_{\text{NCX1}}\)) (11). The importance of PLM phosphorylation in mediating its modulatory effects on NCX1 was not addressed in these early studies except that serine 68 in PLM was found to be important (15).

Here we demonstrated that PKC but not PKA activation enhanced I\(_{\text{NCX1}}\) when NCX1 was expressed alone in HEK293 cells. Co-expression of PLM with NCX1 resulted in decreased I\(_{\text{NCX1}}\) in the basal state, an additional decrease in I\(_{\text{NCX1}}\) when stimulated with forskolin, and attenuation of the magnitude of increase in I\(_{\text{NCX1}}\) by PKC activation. Mutating serine 68 to glutamic acid (S68E) enhanced whereas substituting serine 68 with alanine (S68A) abolished the inhibitory effect of PLM on I\(_{\text{NCX1}}\). Mutating serine 63 to alanine (S63A) preserved the sensitivity of PLM to forskolin in terms of additional inhibition of I\(_{\text{NCX1}}\). Using a fundamentally different model system of murine cardiac myocytes, we...
first showed that endogenous $I_{\text{NaCa}}$ was larger in PLM-KO myocytes when compared with wild-type (WT) myocytes despite similar NCX1 protein levels. PKC but not PKA activation increased $I_{\text{NaCa}}$ in WT myocytes. PLM-KO myocytes exhibited significantly larger increases in $I_{\text{NaCa}}$ when stimulated with phorbol 12-myristate 13-acetate (PMA) as compared with WT myocytes. We conclude that PLM, when phosphorylated at serine 68, inhibits cardiac Na⁺/Ca²⁺ exchanger.

**EXPERIMENTAL PROCEDURES**

Construction of PLM Mutants and NCX1 Clones—PLM serine mutants (S63A, S68A, and S68E) were constructed with PLM in pAltered Sites II using Altered Sites II mutagenesis system (Promega, Madison, WI) as described previously (15). PLM and its serine mutants were authenticated by DNA sequencing and subcloned into the mammalian expression vector pAdTrack-CMV (16). Rat cardiac NCX1 clone in pcDNA3.1(+) was a generous gift from Dr. J. Lytton and subcloned into pAdTrack-CMV as previously described (17). We chose the pAdTrack shuttle vector because it allowed us to identify successfully transfected HEK293 cells through a separate cytomegalovirus (CMV) promoter present on the vector backbone that drives the expression of green fluorescent protein.

Transfection of HEK293 Cells—HEK293 cells (American Type Culture Collection, ATCC, Manassas, VA) were cultured and transfected with various combinations of NCX1 and PLM or its mutant clones as described previously (10). Briefly, cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 containing 10% heat-inactivated fetal bovine serum at a density of 1.2 × 10⁶ cells/100-mm dish. After 24 h, medium was changed, and cells were transfected with 25 μl of Lipofectamine and a total of 3 μg of plasmid DNA/dish: either pAdTrack-CMV alone (3 μg) or with PLM serine mutants (S63A, S68A, and S68E) were co-transfected with pcDNA3.1(+) (+) as a control. Cells were incubated for 48 h before experiments. For patch-clamp experiments, cells were trypsinized at 24 h post-transfection using trypsin/EDTA, transferred to 35-mm dishes containing sterile glass coverslips, and incubated a further 24 h prior to experiments. Transfection according to this protocol routinely yielded 30–50% transfection efficiency.

For brevity, HEK293 cells expressing NCX1 alone are referred in the text as NCX1 cells, whereas cells co-expressing NCX1 and PLM or its serine mutants are referred as PLM cells or SMx cells (where m is either 63 or 68, and X is either Ala or Glu).

$Na⁺/Ca²⁺$ Exchange Current ($I_{\text{NaCa}}$) Measurements—Whole cell patch-clamp recordings were performed at 30 °C as described previously (10, 11, 18, 19). Briefly, fire-polished pipettes (tip diameter, 2–3 μm) were filled with a buffered Ca²⁺ solution containing 100 mM Cs⁺-glutamate, 7.25 mM Na⁺-HEPES, 1 mM MgCl₂, 12.75 mM HEPES, 2.5 mM Na₃ATP, 10 mM EGTA, and 6 mM CaCl₂, pH 7.2. Free Ca²⁺ in the pipette solution was 205 nM, measured fluorometrically with fura 2. Cells were bathed in an external solution containing 130 mM NaCl, 5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM NaH₂PO₄, 5 mM CaCl₂, 10 mM HEPES, 10 mM Na⁺-HEPES, and 10 mM glucose, pH 7.4. Verapamil (1 μM), ouabain (1 mM), and niflumic acid were added to the bath as before. Holding potential was −73 mV. $I_{\text{NaCa}}$ was defined as the difference current measured during the descending voltage ramp in the absence and presence of Cd²⁺ (5 mM).

In a second series of experiments, the effects of PMA on $I_{\text{NaCa}}$ were measured under Cl⁻-free conditions. Pipette solutions consisted of 100 mM Cs⁺-glutamate, 7.25 mM Na⁺-HEPES, 1 mM MgCl₂, 12.75 mM HEPES, 2.5 mM Na₃ATP, 10 mM EGTA, and 6 mM CaCl₂, pH 7.2. External solutions consisted of 130 mM NaCl, 5 mM CsCl, 1.2 mM MgSO₄, 1.2 mM NaH₂PO₄, 5 mM Ca(OH)₂, 10 mM HEPES, 10 mM Na⁺-HEPES, and 10 mM glucose, pH 7.4. Verapamil, ouabain, and niflumic acid were added to the bath as before. Holding potential was −73 mV. $I_{\text{NaCa}}$ was defined as the difference current measured during the descending voltage ramp in the absence and presence of Cd²⁺ (1 mM) or Ni²⁺ (5 mM).

In a third series of experiments, the effects of PMA on $I_{\text{NaCa}}$ were measured under high [Na⁺]i conditions. Pipette solutions contained 60 mM Cs⁺-glutamate, 40 mM Na⁺-glutamate, 7.25 mM Na⁺-HEPES, 1 mM MgCl₂, 12.75 mM HEPES, 2.5 mM Na₃ATP, 10 mM EGTA, and 6 mM CaCl₂, pH 7.2. External solution consisted of 130 mM NaCl, 5 mM CsCl, 1.2 mM MgSO₄, 1.2 mM NaH₂PO₄, 0.2 mM CaCl₂, 10 mM HEPES, 10 mM Na⁺-HEPES, and 10 mM glucose, pH 7.4, and the usual inhibitors. [Ca²⁺]o was deliberately lowered to 0.2 mM so that the calculated reversal potential of $I_{\text{NaCa}}$ (−103 mV), and thus the holding potential, was closer to the holding potential of −73 mV used in other experiments. Keeping [Ca²⁺]o at 5 mM would have resulted in a very negative holding potential of −188 mV. $I_{\text{NaCa}}$ was defined as the difference current measured during the descending voltage ramp in the absence and presence of Ni²⁺ (5 mM).

**Generation of PLM-KO Mice**—A mouse line deficient in PLM was generated by replacing exons 3–5 of the PLM gene with lacZ and neomycin resistance genes as described in detail previously (20). These mice grow to adulthood and are fertile. Studies were performed using mice backcrossed to a pure congenic C57BL/6 background. Homozygous adult littermates that were 3–6 months old were used in the experiments. Mice were housed in ventilated racks in a barrier facility supervised by the Department of Comparative Medicine at the Pennsylvania State University College of Medicine. Standard care was provided to all mice used for experiments.

PLM, NCX1, and Calsequestrin Immunoblotting—Mouse left ventricles were excised, rinsed in ice-cold phosphate-buffered saline, and cut into small pieces. Approximately 60 mg of tissue were suspended in 700 μl of ice-cold lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM Na⁺-orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 100 μM NaF, 1 mM EGTA, and 0.5% Nonidet P-40. A Complete Mini protease inhibitor mixture tablet (Roche Applied Science) was also added to 10 ml of lysis buffer. The tissue was homogenized with a glass Dounce homogenizer (15–20 strokes) and placed on ice for 15 min before centrifugation at 20,800 × g for 10 min at 4 °C. The supernatant was snap frozen with dry ice-ethanol and stored at −80 °C.
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Proteins in heart homogenates were subjected to 7.5% (NCX1 and calsequestrin) or 12% (PLM) SDS-PAGE under either non-reducing (10 mM N-ethylmaleimide for NCX1 and calsequestrin) or reducing (5% β-mercaptoethanol for PLM) conditions. The fractionated proteins were transferred to ImmunBlot polyvinylidene difluoride membranes. Primary antibodies were used polyclonal antibody C2Ab (1:10,000) for PLM (21), polyclonal antibody π11-13 (1:500; Swant, Bellinzona, Switzerland) for NCX1, and rabbit anti-calsequestrin antibody (1:5,000; Swant). The secondary antibodies used were donkey anti-rabbit IgG (Amersham Biosciences). Immunoreactive proteins were detected with an enhanced chemiluminescence Western blotting system. Protein band signal intensities were quantitated by scanning autoradiograms of the blots with a PhosphorImager (Amersham Biosciences). Because calsequestrin expression has been shown to be unchanged during ontogenic development, aging, cardiac hypertrophy, and failing human myocardium (22), we used calsequestrin as an internal control for protein loading.

Isolation of Marine Myocytes and Measurement of I_{NaCa}—Cardiac myocytes were isolated from the septum and left ventricular free wall of WT and PLM-KO mice (25–37 g) according to the protocol of Zhou et al. (23). Briefly mice were heparinized (1500 units/kg intraperitoneally) and anesthetized (pentobarbital sodium, 50 mg/kg intraperitoneally). The heart was excised, mounted on a steel cannula, and retrograde perfused (100 cm of H₂O at 37 °C) with Ca²⁺-free bicarbonate buffer followed by enzymatic digestion (collagenases B and D and protease XIV) as described previously (23). Isolated myocytes were plated on laminin-coated glass coverslips in a Petri dish, and the Ca²⁺ concentration of the buffer was progressively increased from 0.05 to 0.125 to 0.25 to 0.5 mM in three steps (10-min interval for each). The 0.5 mM Ca²⁺ buffer was then aspirated and replaced with minimal essential medium (Sigma catalogue number M1018) containing 1.2 mM Ca²⁺, 2.5% fetal bovine serum, and antibiotics (1% penicillin/streptomycin). After 1 h (in 5% CO₂ at 37 °C), medium was replaced with fetal bovine serum-free minimal essential medium. Myocytes were used within 2–8 h of isolation. The protocol for heart excision for myocyte isolation was approved by the Institutional Animal Care and Usage Committee. The protocol for heart excision for myocyte isolation was approved by the Institutional Animal Care and Usage Committee. The protocol for heart excision for myocyte isolation was approved by the Institutional Animal Care and Usage Committee.

RESULTS

Effects of PMA or Forskolin on I_{NaCa} in HEK293 Cells Expressing NCX1 Alone—We have shown previously that HEK293 cells do not express NCX1 and demonstrate measurable I_{NaCa} or Na⁺-dependent Ca²⁺ uptake (10). When transfected with rat cardiac NCX1, HEK293 cells exhibited characteristic I_{NaCa} demonstrating both forward (inward current, 3 Na⁺ in:1 Ca²⁺ out) and reverse (outward current, 3 Na⁺ out:1 Ca²⁺ in) Na⁺/Ca²⁺ exchange (Fig. 2A). In addition, the reversal potential of I_{NaCa} was between −70 and −60 mV, close to its theoretical equilibrium potential of −73 mV under our experimental conditions (Fig. 2A). There were no significant (p < 0.076) differences in base-line I_{NaCa} measured with either Cd²⁺ or Ni²⁺ (data not shown). Treatment with PMA, which activates PKC, resulted in a large increase in I_{NaCa} in NCX1 cells (Fig. 2A; p < 0.0001). For example, at +100 mV, PKC stimulation resulted in an ~120% increase in I_{NaCa}. Control experiments performed in Cl⁻-free solutions demonstrated that the PMA-induced current increase was not due to an increase in Cl⁻ currents (Fig. 2B). In addition, PMA induced large increases in currents whether Cd²⁺ (~122% at +100 mV) (Fig. 2B) or Ni²⁺ (~81% at +100 mV) (data not shown) was used to define I_{NaCa} under Cl⁻-free conditions. To control for the possibility that the observed PMA-induced I_{NaCa} increase was due to small changes in [Na⁺], rather than enhancing intrinsic NCX1 activity, experiments were performed in high [Na⁺], conditions such that I_{NaCa} would not be so sensitive to small changes in [Na⁺]. In Fig. 2C shows that base-line I_{NaCa} was significantly (p < 0.0001) smaller in high [Na⁺], and low [Ca²⁺ₜ] (0.2 mM) when compared with normal [Na⁺], and high [Ca²⁺ₜ] (5 mM) conditions (Fig. 2A), likely due to the 25-fold reduction of [Ca²⁺ₜ]. However, addition of PMA increased I_{NaCa} (~84% at +100 mV) under high [Na⁺], conditions, similar to the observations obtained under lower but more physiological [Na⁺], conditions. In con-
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**FIGURE 2.** Effects of PMA on \(I_{\text{NaCa}}\) in transfected HEK293 cells. A, HEK293 cells were transfected with NCX1 (open circles, \(n = 14\)). At 48 h post-transfection, \(I_{\text{NaCa}}\) was measured at 30 °C using standard solutions and Ca\(^{2+}\) as described under “Experimental Procedures” and in Fig. 1. After base-line \(I_{\text{NaCa}}\) was obtained, PMA (0.1 μM) was added, and \(I_{\text{NaCa}}\) was measured 3–5 min after drug addition (open squares, \(n = 8\)). B, \(I_{\text{NaCa}}\) was measured in HEK293 cells transfected with NCX1 under Cl\(^-\)-free conditions (“Experimental Procedures”) both before (open circles, \(n = 4\)) and after (open squares, \(n = 4\)) addition of PMA. Ca\(^{2+}\) was used to define \(I_{\text{NaCa}}\). C, \(I_{\text{NaCa}}\) was measured in HEK293 cells transfected with NCX1 under high (Na\(^+\)) conditions (“Experimental Procedures”) both before (open circles, \(n = 6\)) and after (open squares, \(n = 7\)) addition of PMA. Ca\(^{2+}\) was 0.2 mM rather than 5.0 mM so that the calculated reversal potential for \(I_{\text{NaCa}}\) was −153 mV as compared with the holding potential of −73 mV used in other experiments. Ni\(^{2+}\) was used to define \(I_{\text{NaCa}}\). Error bars are not shown if they fall within boundaries of the symbols. pF, picofarad.

In contrast to results obtained with PMA stimulation, forskolin treatment did not affect \(I_{\text{NaCa}}\) in NCX1-expressing cells (Fig. 3B; \(p < 0.64\)).

**Effects of PMA or Forskolin on \(I_{\text{NaCa}}\) in Cells Expressing Both NCX1 and PLM—Co-expression of PLM with NCX1 in HEK293 cells resulted in a significant decrease in \(I_{\text{NaCa}}\) compared with cells expressing NCX1 alone (Fig. 3, A and B; \(p < 0.0005\)), consistent with our previous observations (10). At +100 mV, PLM inhibited \(I_{\text{NaCa}}\) by ~26%. PMA treatment of PLM cells resulted in a significant increase in \(I_{\text{NaCa}}\) when compared with unstimulated NCX1 or PLM cells (Fig. 3A; \(p < 0.0001\)). However, the magnitude of \(I_{\text{NaCa}}\) increase by PMA was much smaller in PLM cells when compared with NCX1 cells (39 vs 120% at +100 mV).

Despite the absence of an effect of forskolin on \(I_{\text{NaCa}}\) in cells expressing NCX1 alone, PKA stimulation in PLM cells resulted in a significant decrease in \(I_{\text{NaCa}}\) compared with unstimulated PLM cells (Fig. 3B; \(p < 0.0001\)). For example, at +100 mV, forskolin effected an ~49% decrease in \(I_{\text{NaCa}}\) in PLM cells (Fig. 3B).

**Effects of PLM Serine 68 Mutants on \(I_{\text{NaCa}}\) in Transfected HEK293 Cells—**Because serine 68 in PLM is the common phosphorylation target for both PKA and PKC, we next investigated the effects of serine 68 mutants on \(I_{\text{NaCa}}\) in cells co-expressing NCX1 and PLM serine 68 mutants. Mutating serine 68 to alanine (S68A) resulted in abolition of the effect of WT PLM on \(I_{\text{NaCa}}\) (Fig. 4A; \(p < 0.08\)), consistent with our...
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Effects of serine 68 mutants of PLM on \(I_{\text{NaCa}}\) in transfected HEK293 cells. A, HEK293 cells were transfected with either NCX1 alone (open circles, \(n = 14\)) or S68A + NCX1 (open squares, \(n = 7\)). At 48 h post-transfection, \(I_{\text{NaCa}}\) was measured at 5 mM [Ca\(^{2+}\)]\(_o\) and 30 °C as described in Fig. 1. After base-line \(I_{\text{NaCa}}\) was obtained, PMA (0.1 \(\mu\)M) was added to S68A + NCX1 cells (open triangles, \(n = 7\)), and \(I_{\text{NaCa}}\) was again measured. B, HEK293 cells were transfected with NCX1 alone (open circles, \(n = 10\)), PLM + NCX1 (open diamonds, \(n = 8\)), or S68E + NCX1 (open squares, \(n = 6\)). \(I_{\text{NaCa}}\) was measured 48 h post-transfection. In S68E + NCX1 cells, \(I_{\text{NaCa}}\) was measured both before (open squares) and after (open triangles) addition of PMA (0.1 \(\mu\)M). Error bars are not shown if they fall within boundaries of the symbols, pf, picofarad.

Effects of PMA on \(I_{\text{NaCa}}\) in Ventricular Myocytes Isolated from Wild-type and PLM-KO Mice—Results from transfected HEK293 cells strongly suggest that PLM, when phosphorylated at serine 68, inhibits cardiac Na\(^+\)/Ca\(^{2+}\) exchanger. To put the findings in physiological perspective, we examined the effects of PMA on \(I_{\text{NaCa}}\) in cells co-expressing NCX1 and S68A, S68E, or S63A, when considered together, are consistent with the notion that retaining normal serine 68 in PLM is absolutely essential for the stimulatory effect of PMA on \(I_{\text{NaCa}}\) in cells co-expressing PLM and NCX1.

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FIGURE 6. Immunoblots of Na\(^+/Ca^{2+}\) exchanger (NCX1), calsequestrin, and PLM from murine hearts. Left ventricular homogenates were prepared from wild-type and PLM-KO mice of congenic C57BL/6 background as described under "Experimental Procedures." Proteins were separated by gel electrophoresis under non-reducing conditions for NCX1 (50 \(\mu\)g/lane) and calsequestrin (100 \(\mu\)g/lane) and reducing conditions for PLM (5 \(\mu\)g/lane). After transfer to polyvinylidene difluoride membranes, immunoblotting was performed as described under "Experimental Procedures." Numbers on the left refer to apparent molecular mass.

FIGURE 7. Measurement of Na\(^+/Ca^{2+}\) exchange current (I_{NaCa}) in murine cardiac myocytes. I_{NaCa} was measured in ventricular myocytes isolated from adult mouse hearts at 5 \(mM\) [Ca\(^{2+}\)] in and 30 \(^\circ\)C with a descending-ascending voltage ramp protocol (A) as described under "Experimental Procedures." Free Ca\(^{2+}\) in the Ca\(^{2+}\)-buffered pipette solution was 205 \(nM\). Holding potential was at the calculated reversal potential of I_{NaCa} (\(-73 mV\)) under our experimental conditions. Ca\(^{2+}\), Na\(^{+}\)-K\(^{-}\)-ATPase, Cl\(^-\), and K\(^{-}\) currents were blocked by appropriate inhibitors. B, membrane currents recorded in a wild-type myocyte during the descending-ascending voltage ramp from \(+100\) to \(-120\) and back to \(+100 mV\) in the absence and presence of 1 \(mM Ca^{2+}\), derived Ca\(^{2+}\)-sensitive current in the wild-type myocyte shown in B.

DISCUSSION

Phosphorylation of PLM with PKC resulted in increases in \(I_{NaCa}\) in both WT and PLM-KO myocytes (Fig. 8; \(p < 0.0001\)). In another series of experiments, PKC stimulation resulted in increases in \(I_{NaCa}\) in both WT (\(p < 0.0001\)) and PLM-KO (\(p < 0.0001\)) myocytes when compared with their respective unstimulated controls (Fig. 9A). However, the PMA-induced increase in \(I_{NaCa}\) was significantly (\(p < 0.0002\)) higher in PLM-KO (\(-132\%\) increase at \(+100 mV\)) than wild-type myocytes (\(-91\%\) at \(+100 mV\)).

Effects of Forskolin on \(I_{NaCa}\) in Wild-type and PLM-KO Ventricular Myocytes—In a third series of experiments, we measured the effects of forskolin on \(I_{NaCa}\) in murine cardiac myocytes. Baseline \(I_{NaCa}\) was again significantly (\(p < 0.0001\)) higher in PLM-KO than WT myocytes (for clarity, KO data not shown in Fig. 9B). PKA stimulation did not result in appreciable changes in \(I_{NaCa}\) in WT myocytes (Fig. 9B; \(p < 0.11\)). In addition, there were no differences in \(I_{NaCa}\) between WT and PLM-KO myocytes after forskolin treatment (Fig. 9B; \(p < 0.15\)).

We have demonstrated previously in both rat cardiac myocytes (9, 11, 15) and transfected HEK293 cells (10) that PLM, in addition to its well-known modulatory effects on the Na\(^+/\)K\(^{-}\)-pump (4, 6, 8, 24), inhibits cardiac Na\(^+/Ca^{2+}\) exchanger. Specifically, PLM co-localizes and co-immunoprecipitates with NCX1 and functionally decreases \(I_{NaCa}\) and Na\(^+\)-dependent Ca\(^{2+}\) uptake (9,10). Whereas PLM phosphorylation during ischemia (5) or by \(\beta\)-adrenergic stimulation (6) is associated with the inhibition of Na\(^+/K^{+}\)-ATPase, it is not clear whether inhibition of Na\(^+/Ca^{2+}\) exchanger is mediated by phosphorylated or unphosphorylated PLM.

Incorporation of \(^{32}\)P into PLM in intact guinea pig ventricle is enhanced \(-2.6\)-fold with isoproterenol treatment, suggesting WT PLM is partially phosphorylated in the unstimulated state (25). Based on C68PAb and C2AB, which are antibodies specific for phosphorylated (at serine 68) and unphosphorylated PLM, respectively (24, 26), it has been estimated that \(-41\%\) of PLM in adult rat myocytes (24) and \(-25\%\) of PLM in guinea pig myocytes (6) are phosphorylated at serine 68 under the basal state. Using another approach of comparing the effects of WT PLM and its serine 68 and serine 63 mutants on \(I_{NaCa}\) in adult rat myocytes, \(-46\%\) of serine 68 and \(-16\%\) of serine 63 are estimated to be phosphorylated in the resting state (15). The results from these three fundamentally different experimental approaches strongly indicate that PLM is only partially phosphorylated in cardiac myocytes. Overexpression of PLM does not grossly distort the relative level of phosphorylation on serine 68 of PLM in adult rat cardiac myocytes (24). Therefore it is difficult to ascertain which form of PLM (phosphorylated or unphosphorylated) mediates the inhibition of Na\(^+/Ca^{2+}\) exchange in studies using PLM overexpression strategies (9, 15).

Because PLM is known to regulate Na\(^+/K^{+}\)-ATPase (4–6, 8, 24), it is tempting to explain the effects of PLM on NCX1 as indirect, i.e. changes in \([Na^{+}]_{i}\), due to alterations in Na\(^+/\)K\(^{-}\)-pump activity by PLM would change the driving force of NCX1 and hence \(I_{NaCa}\). The conditions used in our \(I_{NaCa}\) measurements were carefully designed to avoid this ambiguity in that Na\(^+/\)pump activity was eliminated by exclusion of K\(^{+}\) in pipette and bathing solutions as well as by the inclusion of ouabain. In addition, the measured and theoretical equilibrium potentials for \(I_{NaCa}\) (E_{NaCa}) were in reasonable agreement, suggesting that under the heavily buffered [Ca\(^{2+}\)], conditions used in our \(I_{NaCa}\) measurements, the \([Na^{+}]_{i}\), sensed by NCX1 could be approximated by \([Na^{+}]_{pipette}\). Finally, the measured E_{NaCa} between NCX1 and PLM cells were in close agreement, indicating that the \([Na^{+}]_{i}\), sensed by NCX1 were similar in both types of cells. Therefore, the thermodynamic parameters ([Ca\(^{2+}\)], [Ca\(^{2+}\)], \([Na^{+}]_{i}\), and \([Na^{+}]_{i}\)) that determine E_{NaCa} and hence the driving force for \(I_{NaCa}\) (E_{m} - E_{NaCa}), were identical between NCX1 and PLM cells. In
addition, we have demonstrated previously that the protein levels of NCX1 in HEK293 cells are similar in the absence or presence of cotransfected PLM (10). The observed differences in \( I_{\text{Na,Ca}} \) between NCX1 and PLM cells can thus be unambiguously assigned to the direct inhibitory effects of PLM on NCX1. Similar arguments can be advanced that the observed differences in \( I_{\text{Na,Ca}} \) between wild-type and PLM-KO myocytes (with similar NCX1 protein levels) were due to direct inhibition of NCX1 by PLM.

NCX1 is known to be modulated by \( \alpha \)-adrenergic stimulation (27) presumably mediated via PKC (28). Our finding that, in HEK293 cells expressing NCX1 alone, PKC activation by PMA resulted in a large increase in \( \text{Na}^+/\text{Ca}^{2+} \) exchange activity is similar to that observed in CCL39 fibroblasts expressing NCX1 (28). In our experiments on HEK293 cells expressing NCX1, the increase in current by PMA was not due to activation of \( \text{Cl}^- \) current because similar current increases were observed under \( \text{Cl}^- \)-free conditions. Another potential concern is that, although \( \text{Ca}^{2+} \) was heavily buffered under our experimental conditions, small changes in \([\text{Na}^+]_i\) by PMA may have large effects in \( I_{\text{Na,Ca}} \) (proportional to third power of \([\text{Na}^+]_i\)) with only small effects on \( E_{\text{Na,Ca}} \) (proportional to third root of the \([\text{Na}^+]_i\) gradient). Under conditions of high \([\text{Na}^+]_i\), in which \( I_{\text{Na,Ca}} \) would not be expected to be so sensitive to small changes in cytoplasmic \( \text{Na}^+ \), PKC stimulation still effected a large increase in \( I_{\text{Na,Ca}} \). Our control experiments with \( \text{Cl}^- \)-free solutions and high \([\text{Na}^+]_i\) conditions indicate that the observed increase in currents by PMA was due to the enhancement of intrinsic NCX1 activity by PKC rather than an artifactual increase in \( \text{Cl}^- \) currents or changes in driving force for the exchanger.

PKC activation is associated with increased NCX1 phosphorylation at serine 249, serine 250, and serine 357 (29). In normal cardiac myocytes, however, NCX1 is associated with PLM (10, 11). Therefore the physiologically more relevant model system is one that co-expresses both NCX1 and PLM. In HEK293 cells co-expressing both NCX1 and PLM, PKA treatment also resulted in enhancement of \( \text{Na}^+/\text{Ca}^{2+} \) exchange activity, similar to that observed in rat sarcolemmal vesicles (27). The magnitude of the \( I_{\text{Na,Ca}} \) increase, however, was much smaller in cells co-expressing NCX1 and PLM when compared with cells expressing NCX1 alone. These results suggest that the stimulatory effects of PKA on NCX1 were attenuated by increased PLM phosphorylation. The implication on \( \text{Na}^+/\text{Ca}^{2+} \) exchange in intact myocytes exposed to PKC activators is that the direct stimulatory effects on NCX1 are somewhat opposed by an indirect inhibitory effect by increased phosphorylated PLM.

Because PKC induces phosphorylation at both serine 63 and serine 68 of PLM (3), we next activated PKA to evaluate the effects of PLM phosphorylated only at serine 68 on NCX1. The effects of PKA on the cardiac \( \text{Na}^+/\text{Ca}^{2+} \) exchanger are quite controversial. For example, PKA activation does not enhance phosphorylation of NCX1 expressed in CCL39 fibroblasts (29), but the catalytic subunit of PKA is quite capable of in vitro phosphorylation of NCX1 immunoprecipitated from \textit{Xenopus} oocytes expressing the \( \text{Na}^+/\text{Ca}^{2+} \) exchanger (30). It is at present equally contentious as to whether the mammalian cardiac \( \text{Na}^+/\text{Ca}^{2+} \) exchange activity is affected by PKA activation. For example, no enhancement of \( I_{\text{Na,Ca}} \) by 8-Br-cAMP was observed in HEK cells expressing dog NCX1 (31). Likewise 8-Br-cAMP has no effect on \( \text{Na}^+ \)-dependent \( \text{Ca}^{2+} \) uptake in CCL39 fibroblasts expressing dog heart NCX1 (29). In giant membrane patches excised from blebs of guinea pig ventricular cells, no stimulatory effect of \( \beta \)-adrenergic stimulation or PKA on the \( \text{Na}^+/\text{Ca}^{2+} \) exchange activity is observed (32). In isolated rat sarcolemmal vesicles, isoproterenol has no effect on the \( \text{Na}^+/\text{Ca}^{2+} \) exchange activity (27). In intact cardiac myocytes, isoproterenol has been reported to increase \( I_{\text{Na,Ca}} \) in guinea pig (33) and pig (34) but not in rabbit myocytes (35).
Recently an elegant study has shed light on the confusing literature concerning the effects of PKA activation on mammalian cardiac Na\(^+\)/Ca\(^{2+}\) exchange activity (36). The apparent augmentation of \(I_{\text{NaCa}}\) by isoproterenol in guinea pig myocytes is due to the activation of a cAMP-dependent and Ni\(^{2+}\)-sensitive Cl\(^-\) current (36). In rat and mouse ventricular cells in which cAMP does not activate this cAMP-dependent Cl\(^-\) current (37), isoproterenol treatment does not increase the amplitude of \(I_{\text{NaCa}}\) (36). Therefore to date, the weight of current evidence suggests that β-adrenergic stimulation with subsequent PKA activation has no discernible effects on mammalian cardiac Na\(^+\)/Ca\(^{2+}\) exchange activity. Our observations that forskolin had no stimulatory effects on \(I_{\text{NaCa}}\) in transfected HEK293 cells expressing NCX1 alone and in wild-type mouse myocytes are thus consistent with this view. However, in HEK293 cells expressing both NCX1 and PLM, forskolin resulted in additional suppression of \(I_{\text{NaCa}}\). This observation suggests that PLM, when phosphorylated at serine 68, inhibited cardiac Na\(^+\)/Ca\(^{2+}\) exchange in a heterologous expression system. The importance of phosphorylation of serine 68 in mediating the inhibition of \(I_{\text{NaCa}}\) by PLM is supported by the experimental results with serine 68 mutants. S68A, which cannot be phosphorylated, resulted in loss of function, whereas S68E, which mimicked 100% phosphorylation, resulted in additional suppression of \(I_{\text{NaCa}}\) when compared with WT PLM both in transfected HEK293 cells (current study) and in adult rat cardiac myocytes overexpressing PLM or its serine 68 mutants (15).

The results of S63A mutant on \(I_{\text{NaCa}}\) are interesting in three respects. First, leaving serine 68 intact but prohibiting phosphorylation at serine 63 resulted in a much more modest inhibition of \(I_{\text{NaCa}}\) when compared with wild-type PLM. This suggests that phosphorylation at serine 63 may also contribute to the inhibitory effect of PLM on \(I_{\text{NaCa}}\). However, the lack of effects on \(I_{\text{NaCa}}\) by S68A mutant (with or without PMA stimulation) indicates that serine 68 phosphorylation is of primary importance in the inhibition of NCX1 by PLM. Second, treating S63A cells with forskolin resulted in a more substantial suppression of \(I_{\text{NaCa}}\) again indicating the primacy of serine 68 phosphorylation in mediating the inhibitory effect of PLM on \(I_{\text{NaCa}}\). Third and perhaps the most intriguing is that although PMA resulted in large \(I_{\text{NaCa}}\) increases in cells expressing NCX1 alone or NCX1 + PLM, cells that expressed NCX1 and S68A or S68E mutants showed no increases in \(I_{\text{NaCa}}\) when stimulated with PMA. Cells that expressed NCX1 and S63A mutant (in which serine 68 is intact), on the other hand, were able to increase \(I_{\text{NaCa}}\) with PKC activation, similar to cells expressing both NCX1 and wild-type PLM. Our results on the serine 63 and serine 68 mutants suggest that changes in conformation in PLM by mutating serine 68 may alter its interaction with NCX1, resulting in NCX1 not being accessible to PKC action perhaps due to steric hindrance.

The relative lack of effects by S68A and S63A mutants on \(I_{\text{NaCa}}\) in transfected HEK293 cells may be due to loss of interaction between these PLM mutants and NCX1. This is unlikely, however, as we have demonstrated previously that both S68A and S63A mutants, similar to WT PLM, are able to co-immunoprecipitate NCX1 in HEK293 cells co-expressing NCX1 and PLM or its serine mutants (15).

The physiological relevance of serine 68 phosphorylation in PLM on NCX1 function was examined in WT and PLM-KO myocytes. There are many similarities between the results obtained in transfected HEK293 cells and murine myocytes. For example, similar to the observation that \(I_{\text{NaCa}}\) was higher in HEK293 cells expressing NCX1 alone as compared with cells co-expressing NCX1 and PLM, base-line \(I_{\text{NaCa}}\) was higher in PLM-KO than WT myocytes. PMA treatment resulted in enhancement of \(I_{\text{NaCa}}\) in both WT and PLM-KO myocytes, although the increase in \(I_{\text{NaCa}}\) was much higher in PLM-KO myocytes. This is also similar to our findings in the heterologous expression system. On the other hand, there are some differences between the effects of PKA on \(I_{\text{NaCa}}\) in HEK293 cells and murine myocytes. For example, forskolin treatment resulted in suppression of \(I_{\text{NaCa}}\) in HEK293 cells co-expressing NCX1 and PLM. By contrast, PKA stimulation in WT myocytes did not result in any detectable changes in \(I_{\text{NaCa}}\) in agreement with observations by Ginsburg and Bers (33) and Lin et al. (36). The differences between the results obtained in HEK293 cells and murine myocytes with respect to PKA effects on \(I_{\text{NaCa}}\) are not intuitively obvious but may relate to association of NCX1 with the catalytic subunit of PKA and protein phosphatase 1 in rat hearts (30). It is known that NCX1 exhibits significant basal phosphorylation in cardiac myocytes (28). In addition, dephosphorylation of NCX1 by protein phosphatase 1 results in reduction of \(I_{\text{NaCa}}\) (34), whereas increased NCX1 phosphorylation is associated with enhancement of Na\(^+\)/Ca\(^{2+}\) exchange activity (28). PKA stimulation of intact cardiac myocytes would be expected to simultaneously increase phosphorylation in both NCX1 (stimulatory) (30) and PLM (inhibitory) plus or minus other unknown effects on protein phosphatase 1 such that the net effect would be no measurable changes in \(I_{\text{NaCa}}\). In NCX1 expressed heterologously in HEK293 cells, there may not be such close association of PKA with NCX1 in an assembled "macromolecular complex" (38) so that PKA can exert its effects on NCX1. On the other hand, in our simplified heterologous expression system, phosphorylation of PLM by ubiquitous PKA present in these cells or the phosphomimetic S68E mutant would be expected to suppress \(I_{\text{NaCa}}\).

In the intact heart, β-adrenergic stimulation increases Na\(^+\) influx into the myocytes because of the chronotropic effect (more frequent depolarizations). In addition, L-type Ca\(^{2+}\) current and SERCA2 activity are also increased in response to β-adrenergic stimulation, resulting in increased Ca\(^{2+}\) entry and Ca\(^{2+}\) loading of the sarcoplasmic reticulum. Increased sarcoplasmic reticulum Ca\(^{2+}\) available for release largely accounts for the increased inotropy of β-adrenergic agonists. To maintain steady-state Ca\(^{2+}\) balance, the increased myocyte Ca\(^{2+}\) entry must necessitate increased Ca\(^{2+}\) efflux mediated by forward Na\(^+\)/Ca\(^{2+}\) exchange, thereby bringing more Na\(^+\) into the cell. Therefore, enhanced Na\(^-\)K\(^+\)−ATPase activity (by PLM phosphorylation) during β-adrenergic stimulation is necessary to prevent cellular Na\(^+\) overload. On the other hand, unchecked stimulation of Na\(^-\)K\(^+\)−ATPase would decrease intracellular Na\(^+\) concentration, thereby increasing the thermodynamic driving force of forward Na\(^+\)/Ca\(^{2+}\) exchange, resulting in Ca\(^{2+}\) depletion. The ensuing decreased inotropy is clearly not desirable under the circumstances of flight or flight. Our presented evidence suggests a coordinated paradigm in which PLM, upon phosphorylation at serine 68, enhances Na\(^-\)K\(^+\)−ATPase (5, 8) but inhibits Na\(^+\)/Ca\(^{2+}\) exchange activities in cardiac myocytes. The consequences of Na\(^-\)K\(^+\)−ATPase stimulation on the one hand and Na\(^+\)/Ca\(^{2+}\) exchange inhibition on the other on cellular Ca\(^{2+}\) homeostasis and contractility are complex and difficult to predict or model and clearly require further study.

Finally it should be pointed out that the magnitude of inhibition of \(I_{\text{NaCa}}\) by WT PLM in HEK293 cells was ~26% at +100 mV in our current experiments; this is much more modest than our previous results of ~80% inhibition at +100 mV (10). This is because we deliberately decreased the amount of plasmid DNA encoding PLM used in the transfection (from 1.5 to 1.0 µg/dish) so that we would better be able to detect additional inhibition of \(I_{\text{NaCa}}\) when PLM was phosphorylated or when a phosphomimetic PLM mutant was used. In summary, we demonstrated that phospholemman phosphorylated
at serine 68 inhibited Na\(^+\)/Ca\(^{2+}\) exchange in both transfected HEK293 cells and mouse myocytes. We conclude that, in intact cardiac myocytes, phosphorylation of phospholemman results in relief of inhibition of Na\(^+\)/K\(^{-}\)-ATPase and inhibition of Na\(^+\)/Ca\(^{2+}\) exchange.

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