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Phospholemman inhibition of the cardiac Na^+/Ca^{2+} exchanger: Role of phosphorylation.

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Abstract

We have previously demonstrated that phospholemman (PLM), a 15 kDa integral sarcolemmal phosphoprotein, inhibits the cardiac Na^+/Ca^{2+} exchanger (NCX1). In addition, p rotein kinase A phosphorylates serine[68] while protein kinase C phosphorylates both serine[63] and serine[68] of PLM. Using HEK293 cells that are devoid of both endogenous PLM and NCX1, we first demonstrated that the exogenous NCX1 current (I_{NaCa}) 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_{NaCa}; (ii) attenuation of the increase in I_{NaCa} by PMA; and (iii) additional reduction in I_{NaCa} in cells treated with forskolin. Mutating serine[63] to alanine (S63A) preserved PLM's sensitivity to forskolin in terms of suppression of I_{NaCa}, whereas mutating serine[68] to alanine (S68A) abolished PLM's inhibitory effect on I_{NaCa}. Mutating serine[68] to glutamic acid (phosphomimetic) resulted in additional suppression of I_{NaCa} as compared to wild-type PLM. These results suggest that PLM phosphorylated at serine[68] inhibited I_{NaCa}. The physiological significance of inhibition of NCX1 by phosphorylated PLM was evaluated in PLM-knockout (KO) mice. When compared to wild-type myocytes, I_{NaCa} was significant larger in PLM-KO myocytes. In addition, PMA-induced increase in I_{NaCa} was significantly higher in PLM-KO myocytes. By contrast, forskolin had no effect on I_{NaCa} in wild-type myocytes. We conclude that PLM, when phosphorylated at serine[68], inhibits Na^+/Ca^{2+} exchange in the heart.

Abbreviations: The abbreviations used are

ANOVA, analysis of variance; 8-Br-cAMP, 8-bromoadenosine 3′, 5′ cyclic monophosphate; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; C_m, whole cell membrane capacitance; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; EGTA, ethylene glycol-bis-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid; E_m, membrane potential; em., emission; ex., excitation; E_{NaCa}, equilibrium potential for Na^+, Ca^{2+} exchange current; FBS, fetal bovine serum; GFP, green fluorescent protein; HEK, human embryonic kidney; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; I_{NaCa}, Na^+, Ca^{2+} exchange current; KO, knock-out; MEM, minimal essential media; NCX1, Na^+, Ca^{2+} exchanger; NIMA, never in mitosis

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A; PKA, protein kinase A; PKC, protein kinase C; PLM, phospholemman; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; SE, standard error; SERCA2, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; SDS-PAGE, sodium dodecyl sulfate- polyacrylamide gel electrophoresis; V_{max}, maximum velocity; WT, wild-type

Introduction

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 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 potentia l phosphorylation sites. In particular, PLM (FXYD1) is the only FXYD family member to have a consensus sequence for phosphorylation by PKA (RRXS), PKC (RXXSXR), and NIMA kinase (FRXS/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 unphosphorylated 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_{max} of sarcolemmal Na^+-K^+-ATPase was 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-knockout (KO) myocytes supports the notion that the inhibitory effects of PLM on Na^+-K^+-ATPase is 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 inhibited Na^+/Ca^{2+} exchange activity (9,10) while downregulation of PLM enhanced NCX1 current (I_{NaCa})(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_{NaCa} when NCX1 was expressed alone in HEK293 cells. Co-expression of PLM with NCX1 resulted in decreased I_{NaCa} in the basal state, additional decrease in I_{NaCa} when stimulated with forskolin, and attenuation of the magnitude of increase in I_{NaCa} by PKC activation. Mutating serine^{68} to glutamic acid (S68E) enhanced while substituting serine^{68} with alanine (S68A) abolished PLM's inhibitory effect on I_{NaCa}. Mutating serine^{63} to alanine (S63A) preserved PLM's sensitivity to forskolin in terms of additional inhibition of I_{NaCa}. Using a fundamentally different model system of murine cardiac myocytes, we first showed that endogenous I_{NaCa} was larger in PLM-KO myocytes when compared to wild-type (WT) myocytes, despite similar NCX1 protein levels. PKC but not PKA activation increased I_{NaCa} in WT myocytes. PLM-KO myocytes exhibited significantly larger increases in I_{NaCa} when stimulated with phorbol 12-myristate 13-acetate (PMA) as compared to WT myocytes. We conclude that PLM, when phosphorylated at serine^{68}, inhibits cardiac Na^+/Ca^{2+} exchanger.

Experimental Procedures

Construction Of PLM Mutants and NCX1 Clones

PLM serine mutants (S63A, S68A and S68E) were constructed with PLM in pAlter-1, using Altered Sites II in vitro 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 since 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 (GFP).

Transfection of HEK293 cells

HEK293 cells (American Type Culture Collection, ATCC; Manassas, VA) were cultured and transfected with various NCX1 and PLM or its mutant clones as previously described (10). Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 containing 10% heat-inactivated fetal bovine serum (FBS) at a density of 1.2 x 10^6 cells per 100 mm dish. After 24 h, medium was changed and cells were transfected with 25 μl Lipofectamine and total of 3μg plasmid DNA per dish: either pAdTrack-CMV alone (3μg), pAdTrack-CMV-NCX1 (1μg) + pAdTrack-CMV (2μg), pAdTrack-CMV-NCX1 (1μg) + pAdTrack-CMV-PLM (1μg) + pAdTrack-CMV (1μg), or pAdTrack-CMV-NCX1 (1μg) + pAdTrack-CMV-PLM serine mutant (1μg) + pAdTrack-CMV (1 μg). The lipid-DNA complex was left on cells for 5 h at 37ºC/5% CO_2. Medium was then replaced with DMEM/Ham's F12 + 10% FBS and cells were cultured for additional 48h before experiments. For patch-clamp applications, 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, while cells co-expressing NCX1 and PLM or its serine mutants are referred as PLM cells or SNNX cells (where NN is either 63 or 68, and X is either A or E).

Na^+/Ca^{2+} Exchange Current (I_{NaCa}) Measurements

Whole cell patch-clamp recordings were performed at 30ºC as described previously (10,11, 18,19). Briefly, fire-polished pipettes (tip-diameter 2–3 μm) were filled with a buffered Ca^{2+} solution containing (in mM): 100 Cs^+ glutamate, 7.25 Na^+ HEPES, 1 $MgCl_2$, 12.75 HEPES, 2.5 Na_2ATP, 10 EGTA, and 6 $CaCl_2$, pH 7.2. Free Ca^{2+} in the pipette solution was 205 nM, measured fluorimetrically with fura 2. Cells were bathed in an external solution containing (in mM): 130 NaCl, 5 CsCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 5 $CaCl_2$, 10 HEPES, 10 Na^+ HEPES, and 10 glucose, pH 7.4. Verapamil (1 μM), ouabain (1mM), and niflumic acid (30 μM) were used to block Ca^{2+}, Na^+-K^+-ATPase, and Cl^- currents, respectively. K^+ currents were minimized by Cs^+ substitution for K^+ in both pipette and external solutions. Only cells that fluoresced green (ex. 380nm, em. 510 nm), indicating successful pAdTrack transfection, were selected for current measurements. Membrane potential (E_m) was held at the calculated reversal potential of I_{NaCa} (−73 mV) for 5 min before stimulation. A descending voltage ramp (from +100 to −120 mV; 500 mV/s) was immediately followed by an ascending voltage ramp (from –120 to +100 mV; 500 mV/s)(Fig. 1A). Membrane currents were measured both before and after addition of 1 mM $CdCl_2$ to the external solution (Fig. 1B). I_{NaCa} was defined as the difference current measured during the descending voltage ramp in the absence and presence of Cd^{2+} (Fig. 1C). To facilitate comparison of NCX1 currents, I_{NaCa} of each cell was divided

by its whole cell capacitance (C_m) to account for variations in cell sizes. Except as otherwise stated, all results were obtained using these standard solutions.

When indicated, PMA (0.1 μM) or forskolin (1 μM)(both dissolved in DMSO) was added to cells after baseline I_{NaCa} was obtained. Repeat I_{NaCa} was measured ~3–5 min. after drug addition.

In a 2nd series of experiments, the effects of PMA on I_{NaCa} were measured under Cl^--free conditions. Pipette solutions consisted of (in mM): 100 Cs^+ glutamate, 7.25 Na^+ HEPES, 1 $MgSO_4$, 12.75 HEPES, 2.5 Na_2ATP, 10 EGTA, and 6 $Ca(OH)_2$, pH 7.2. External solutions contained (in mM): 130 Na^+ aspartate, 5 Cs^+ glutamate, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 5 $Ca(OH)_2$, 10 HEPES, 10 Na^+ HEPES, and 10 glucose, pH 7.4. Verapamil, ouabain and niflumic acid were added to the bath as before. Holding potential was −73 mV. I_{NaCa} was defined as the difference current measured during the descending voltage ramp in the absence and presence of Cd^{2+} (1 mM) or Ni^{2+} (5 mM).

In a 3rd series of experiments, the effects of PMA on I_{NaCa} were measured under high $[Na^+]_i$ conditions. Pipette solutions contained (in mM): 60 Cs^+ glutamate, 40 Na^+ glutamate, 7.25 Na^+ HEPES, 1 $MgCl_2$, 12.75 HEPES, 2.5 Na_2ATP, 10 EGTA, and 6 $CaCl_2$, pH 7.2. External solution consisted of 130 NaCl, 5 CsCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 0.2 $CaCl_2$, 10 HEPES, 10 Na^+ HEPES, and 10 glucose, pH 7.4; and the usual inhibitors. $[Ca^{2+}]_o$ was deliberately lowered to 0.2 mM so that the calculated reversal potential of I_{NaCa} (−103 mV), and thus the holding potential, was closer to the holding potential of −73 mV used in other experiments. Keeping $[Ca^{2+}]_o$ at 5 mM would have resulted in a very negative holding potential of −188 mV. I_{NaCa} was defined as the difference current measured during the descending voltage ramp in the absence and presence of Ni^{2+} (5 mM).

Generation of PLM-KO Mice

A mouse line deficient in PLM was generated by replacing exons 3 to 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 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 PBS, and cut into small pieces. Approximately 60 mg of tissue were suspended in 700 μl of ice-cold lysis buffer containing (in mM): 50 Tris (pH 8.0), 150 NaCl, 1 Na^+ orthovanadate, 1 PMSF, 100 NaF, 1 EGTA, and 0.5% NP40. A Complete Mini protease inhibitor cocktail tablet (Roche, Penzberg, Germany) was also added to 10 ml of lysis buffer. The tissue was homogenized with a glass dounce homogenizer (15–20 strokes), placed on ice for 15 min, before centrifugation at 20,800g for 10 min at 4°C. The supernatant was snap-frozen with dry ice-ethanol and stored at −80°C.

Protein 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 PVDF membranes. Primary antibodies used were 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, Piscataway,

NJ). 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 (Molecular Dynamics; Sunnyvale, CA). 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 Murine Myocytes and Measurement of I_{NaCa}

Cardiac myocytes were isolated from the septum and left ventricular free wall of WT and PLM-KO mice (25–37g) according to the protocol of Zhou et al. (23). Briefly, mice were heparinized (1500 u/kg ip) and anesthetized (pentobarbital sodium, 50 mg/kg ip). The heart was excised, mounted on a steel cannula and retrograde perfused (100 cm H_2O, 37ºC) with Ca^{2+}-free bicarbonate buffer followed by enzymatic digestion (collagenases B and D, protease XIV) as described (23). Isolated myocytes were plated on laminin -coated glass coverslips in a petri dish, and the Ca^{2+} concentration of the buffer was progressively increased from 0.05 to 0.125 to 0.25 to 0.5 mM in 3 steps (10 min interval each). The 0.5 mM Ca^{2+} buffer was then aspirated and replaced with minimal essential medium (MEM, Sigma M1018) containing 1.2 mM Ca^{2+}, 2.5% FBS and antibiotics (1% penicillin/streptomycin). After 1 h (5% CO_2, 37º), media was replaced with FBS-free MEM. 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.

I_{NaCa} was measured in isolated murine myocytes with the same protocol and standard solutions used for transfected HEK293 cells except that pipette tip diameter was increased to 4–6 μm and niflumic acid was decreased to 10 μM.

Statistical Analysis

All results are expressed as means ± _SE. For analysis of a parameter (e.g., I_{NaCa}) as a function of group (e.g., NCX1 vs. PLM) and voltage, two-way ANOVA was used to determine statistical significance. For analysis of C_m, Student's t-test was used. A commercial software package (JMP version 4.0.5, SAS Institute; Cary, NC) was used. In all analyses, $p<0.05$ was taken to be statistically significant.

Results

Effects of PMA or Forskolin on I_{NaCa} in HEK293 Cells Expressing NCX1 alone

We have previously shown that HEK293 cells did not express NCX1 or demonstrate measurable I_{NaCa} or Na^+-dependent Ca^{2+} uptake (10). When transfected with rat cardiac NCX1, HEK293 cells exhibited characteristic I_{NaCa} demonstrating both forward (inward current, 3 Na^+ in: 1 Ca^{2+} out) and reverse (outward current, 3 Na^+ out: 1 Ca^{2+} in) Na^+/Ca^{2+} exchange (Fig. 2A). In addition, the reversal potential of I_{NaCa} was between −70 to −60 mV, close to its theoretical equilibrium potential of −73 mV under our experimental conditions (Fig. 2A). There were no significant ($p<0.76$) differences in baseline I_{NaCa} measured with either Cd^{2+} or Ni^{2+} (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 ~120% increase in I_{NaCa}. Control experiments performed in Cl^--free solutions demonstrated that the PMA-induced current increase was not due to increase in Cl^- currents (Fig. 2B). In addition, PMA induced large increases in currents whether Cd^{2+} (~122% at +100 mV)(Fig. 2B) or Ni^{2+} (~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 $[Na^+]_i$ rather than enhancing intrinsic NCX1 activity, experiments were performed in high $[Na^+]_i$ conditions such that I_{NaCa} would not be

so sensitive to small changes in $[Na^+]_i$. Fig. 2C shows that baseline I_{NaCa} was significantly ($p<0.0001$) smaller in high $[Na^+]_i$ and low $[Ca^{2+}]_o$ (0.2 mM) when compared to normal $[Na^+]_i$ and high $[Ca^{2+}]_o$ (5 mM) conditions (Fig, 2A), likely due to the 25-fold reduction of $[Ca^{2+}]_o$. However, addition of PMA increased I_{NaCa} (~84% at +100mV) under high $[Na^+]_i$ conditions, similar to the observations obtained under lower but more physiological $[Na^+]_i$ conditions.

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

Effects of PMA or Forskolin on I_{NaCa} in Cells Expressing both NCX1 and PLM

Co-expression of PLM with NCX1 in HEK293 cells resulted in significant decrease in I_{NaCa} compared to cells expressing NCX1 alone (Figs. 3A & 3B; $p<0.0005$), consistent with our previous observations (10). At +100 mV, PLM inhibited I_{NaCa} by ~26%. PMA treatment of PLM cells resulted in significant increase in I_{NaCa} when compared to unstimulated NCX1 or PLM cells (Fig. 3A; $p<0.0001$). However, the magnitude of I_{NaCa} increase by PMA was much smaller in PLM cells when compared to NCX1 cells (39 vs. 120% at +100mV).

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

Effects of PLM Serine^{68} Mutants on I_{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_{NaCa} in cells co-expressing NCX1 and PLM serine^{68} mutants. Mutating serine^{68} to alanine (S68A) resulted in abolition of WT PLM's effect on I_{NaCa} (Fig. 4A; $p<0.08$), consistent with our previous observations (10). Treating S68A cells with PMA, instead of increasing I_{NaCa} as observed in PLM cells (Fig. 3A), resulted in a modest but significant suppression of in I_{NaCa} when compared to unstimulated NCX1 cells (Fig. 4A; $p<0.0004$).

Mutating serine^{68} to glutamic acid (S68E) resulted in greater suppression of I_{NaCa} when compared to WT PLM (Fig. 4B; $p<0.0001$). Stimulating S68E cells with PMA, in contrast to increases in I_{NaCa} in PLM cells (Fig. 3A), did not result in appreciable changes in I_{NaCa} when compared to unstimulated S68E cells (Fig. 4B; $p<0.70$). The lack of I_{NaCa} stimulation by PMA in both PLM serine^{68} mutants (Figs. 4A & 4B) as compared to WT PLM (Fig. 3A) suggests altered PLM interaction with NCX1 by serine^{68} mutants may somehow interfere with PMA's stimulatory effects on NCX1.

Effects of PLM Serine^{63} Mutant on I_{NaCa} in Transfected HEK293 Cells

Unlike PKA which phosphorylates serine^{68} only, PKC phosphorylates both serine^{63} and serine^{68} in PLM (3). Co-expressing PLM serine^{63} to alanine mutant (S63A) with NCX1 resulted in inhibition of I_{NaCa} compared to cells expressing NCX1 alone (Fig. 5A; $p<0.03$). The magnitude of inhibition by S63A was quite modest (~8% at +100mV) when compared to that by WT PLM (~26% at +100mV; Fig. 3A). Treating S63A cells with forskolin resulted in additional inhibition of I_{NaCa} (~37% at +100mV) when compared to unstimulated S63A cells or NCX1 cells (Fig. 5A; $p<0.0001$).

In another series of experiments, the effects of PMA on I_{NaCa} in S63A cells were evaluated. Unlike cells co-expressing NCX1 and PLM serine^{68} mutants in which I_{NaCa} was not stimulated at all by PMA (Figs. 4A & 4B), S63A cells demonstrated significant PMA-induced

enhancement of I_{NaCa} (Fig. 5B; $p<0.0001$). The effects of PMA on I_{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 PMA's stimulatory effect on I_{NaCa} in cells co-expressing PLM and NCX1.

Effects of PMA on I_{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_{NaCa} in myocytes isolated from WT and PLM-KO mice. Western blots confirmed the absence of PLM in PLM-KO myocytes (Fig. 6). Importantly, NCX1 protein levels (normalized to calsequestrin) were not significantly different ($p<0.52$) between wild-type (99.8 ± 11.7 arbitrary units; n=8) and PLM-KO myocytes (90.7 ± 7.3 arbitrary units; n=8)(Fig. 6). Wild-type and PLM-KO myocytes had similar cell sizes, as evidenced by no differences ($p<0.97$) in whole cell capacitance (a measure of membrane surface area) between WT (184 ± 7 pF, n=13) and PLM-KO myocytes (184 ± 8 pF, n=13). Unlike HEK293 cells expressing exogenous NCX1 (Fig. 1B), in cardiac myocytes a contaminant inward Na^+ current was evident during the ascending voltage ramp (Fig. 7B). For this reason, the Cd^{2+}-sensitive current (I_{NaCa}) was quantitated during the descending portion of the voltage ramp (Fig. 7C). I_{NaCa} was significantly larger in PLM-KO myocytes when compared to WT 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 to their respective unstimulated controls (Fig. 9A). However, PMA-induced increase in I_{NaCa} was significantly ($p<0.002$) higher in PLM-KO (~132% increase at +100mV) than wild-type myocytes (~91% at +100mV).

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, $p<0.15$).

Discussion

We have previously demonstrated 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^+ pump (4,6,8,24), inhibited cardiac Na^+/Ca^{2+} exchanger. Specifically, PLM co-localized and co-immunoprecipitated with NCX1, and functionally decreased I_{NaCa} and Na^+-dependent Ca^{2+} uptake (9,10). Whereas PLM phosphorylation during ischemia (5) or by β-adrenergic stimulation (8) was associated with relief of its 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 ventricles was enhanced ~2.6 fold with isoproterenol treatment, suggesting WT PLM was partially phosphorylated in the unstimulated state (25). Based on C68P Ab and C2 Ab 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) were 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] were estimated to be phosphorylated in the resting state

(15). The results from these 3 fundamentally different experimental approaches strongly indicate that PLM was only partially phosphorylated in cardiac myocytes. Overexpression of PLM did 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) mediated the inhibition of Na^+/Ca^{2+} exchange in studies employing 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^+ pump activity by PLM would change the driving force of NCX1 and hence I_{NaCa} magnitude. 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 $[Ca^{2+}]_i$ conditions used in our I_{NaCa} measurements, the $[Na^+]_i$ sensed by NCX1 could be approximated by $[Na^+]_{pip}$. 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 ($[Ca^{2+}]_i$, $[Ca^{2+}]_o$, $[Na^+]_i$, $[Na^+]_o$) 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 previously demonstrated that the protein levels of NCX1 in HEK293 cells were similar in the absence or presence of co-transfected PLM (10). The observed differences in I_{NaCa} 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_{NaCa} 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 α-adrenergic stimulation (27), presumably mediated via PKC (28). Our finding that in HEK293 cells expressing NCX1 alone, PKC activation by PMA resulted in large increase in Na^+/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 Cl^- current since similar current increases were observed under Cl^--free conditions. Another potential concern is that although Ca^{2+} was heavily buffered under our experimental conditions, small changes in $[Na^+]_i$ by PMA may have large effects in I_{NaCa} (proportional to 3rd power of $[Na^+]_i$) with only small effects on E_{NaCa} (proportional to 3rd root of the Na^+ gradient). Under conditions of high $[Na^+]_i$ in which I_{NaCa} would not be expected to be so sensitive to small changes in cytoplasmic Na^+, PKC stimulation still effected a large increase in I_{NaCa}. Our control experiments with Cl^--free solutions and high $[Na^+]_i$ conditions indicate that the observed increase in currents by PMA was due to PKC's enhancement of intrinsic NCX1 activity, rather than an artifactual increase in Cl^- currents or changes in driving force for the exchanger.

PKC activation was 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 which co-expresses both NCX1 and PLM. In HEK293 cells co-expressing both NCX1 and PLM, PMA treatment also resulted in enhancement of Na^+/Ca^{2+} exchange activity, similar to that observed in rat sarcolemmal vesicles (27). The magnitude of I_{NaCa} increase, however, was much smaller in cells co-expressing NCX1 and PLM when compared to cells expressing NCX1 alone. These results suggest that the stimulatory effects of PMA on NCX1 were attenuated by increased PLM phosphorylation. The implication on Na^+/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.

Since 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 Na^+/Ca^{2+} exchanger are quite controversial. For example, PKA activation did not enhance phosphorylation of NCX1 expressed in CCL39 fibroblasts (29) but the catalytic subunit of PKA was quite capable of in vitro phosphorylation of NCX1 immunoprecipitated from xenopus oocytes expressing the Na^+/Ca^{2+} exchanger (30). It is at present equally contentious as to whether the mammalian cardiac Na^+/Ca^{2+} exchange activity is affected by PKA activation. For example, no enhancement of I_{NaCa} by 8-bromoadenosine 3′, 5′ cyclic monophosphate (8-Br-cAMP) was observed in HEK cells expressing dog NCX1 (31). Likewise, 8-Br-cAMP had no effect on Na^+-dependent 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 β-adrenergic stimulation or PKA on Na^+/Ca^{2+} exchange activity was observed (32). In isolated rat sarcolemmal vesicles, isoproterenol had no effect on Na^+/Ca^{2+} exchange activity (27). In intact cardiac myocytes, isoproterenol was reported to increase I_{NaCa} in guinea pig (33) and pig (34) but not in rabbit myocytes (35). Recently, an elegant study 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_{NaCa} by isoproterenol in guinea pig myocytes was due to the activation of a cAMP-dependent and Ni^{2+}-sensitive Cl^- current (36). In rat and mouse ventricular cells in which cAMP did not activate this cAMP-dependent Cl^- current (37), isoproterenol treatment did not increase the amplitude of I_{NaCa} (36). Therefore to-date, the weight of current evidence suggests that β-adrenergic stimulation with subsequent PKA activation had no discernible effects on mammalian cardiac Na^+/Ca^{2+} exchange activity. Our observations that forskolin had no stimulatory effects on I_{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_{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 phosphorylated serine^{68} in mediating PLM's inhibition of I_{NaCa} is supported by the experimental results with serine^{68} mutants. S68A which cannot be phosphorylated resulted in loss of function while S68E which mimicked 100% phosphorylation resulted in additional suppression of I_{NaCa} when compared to 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_{NaCa} are interesting in 3 respects. First, leaving serine^{68} intact but prohibiting phosphorylation at serine^{63} resulted in much more modest inhibition of I_{NaCa} when compared to wild-type PLM. This suggests that phosphorylation at serine^{63} may also contribute to PLM's inhibitory effect on I_{NaCa}. However, the lack of effects on I_{NaCa} by S68A mutant (with or without PMA stimulation) indicates that serine^{68} phosphorylation is of primary importance in PLM's inhibition of NCX1. Second, treating S63A cells with forskolin resulted in a more substantial suppression of I_{NaCa}, again indicating the primacy of serine^{68} phosphorylation in mediating PLM's inhibitory effect on I_{NaCa}. Third and perhaps the most intriguing is that while PMA resulted in large I_{NaCa} increases in cells expressing NCX1 alone or NCX1+PLM, cells which expressed NCX1 and S68A or S68E mutants showed no increases in I_{NaCa} when stimulated with PMA. Cells which expressed NCX1 and S63A mutant (in which serine^{68} is intact), on the other hand, were able to increase I_{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 accessible to PKC action perhaps due to steric hindrance.

The relative lack of effects by S68A and S63A mutants on I_{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 previously demonstrated that both S68A and S63A mutants, similar to WT PLM, were 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_{NaCa} was higher in HEK293 cells expressing NCX1 alone as compared to cells co-expressing NCX1 and PLM, baseline I_{NaCa} was higher in PLM-KO than WT myocytes. PMA treatment resulted in enhancement of I_{NaCa} in both WT and PLM-KO myocytes, although the increase in I_{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_{NaCa} in HEK293 cells and murine myocytes. For example, forskolin treatment resulted in suppression of I_{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_{NaCa}, in agreement with observations by Ginsburg and Bers (35) and Lin et al. (36). The differences between the results obtained in HEK293 cells and murine myocytes with respect to PKA effects on I_{NaCa} are not intuitively obvious but may relate to association of NCX1 with the catalytic subunit of PKA and protein phosphatase 1 (PP1) in rat hearts (30). It is known that NCX1 exhibited significant basal phosphorylation in cardiac myocytes (28). In addition, dephosphorylation of NCX1 by PP1 resulted in reduction of I_{NaCa} (34) whereas increased NCX1 phosphorylation was 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 PP1 such that the net effect would be no measurable changes in I_{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 (BA Ahlers, unpublished observations) or the phosphomimetic S68E mutant would be expected to suppress I_{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 SR 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 fight or flight. Our presented evidence suggests a coordinated paradigm in which PLM, on 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 requires further study.

Finally, it should be pointed out that the magnitude of inhibition of I_{NaCa} by WT PLM in HEK293 cells was ~26% at +100 mV in our current experiments, 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 μg to 1.0 μg per dish) so that we would better be able to detect additional inhibition of I_{NaCa} when PLM was phosphorylated or when a phosphomimetic PLM mutant was used.

In summary, we have 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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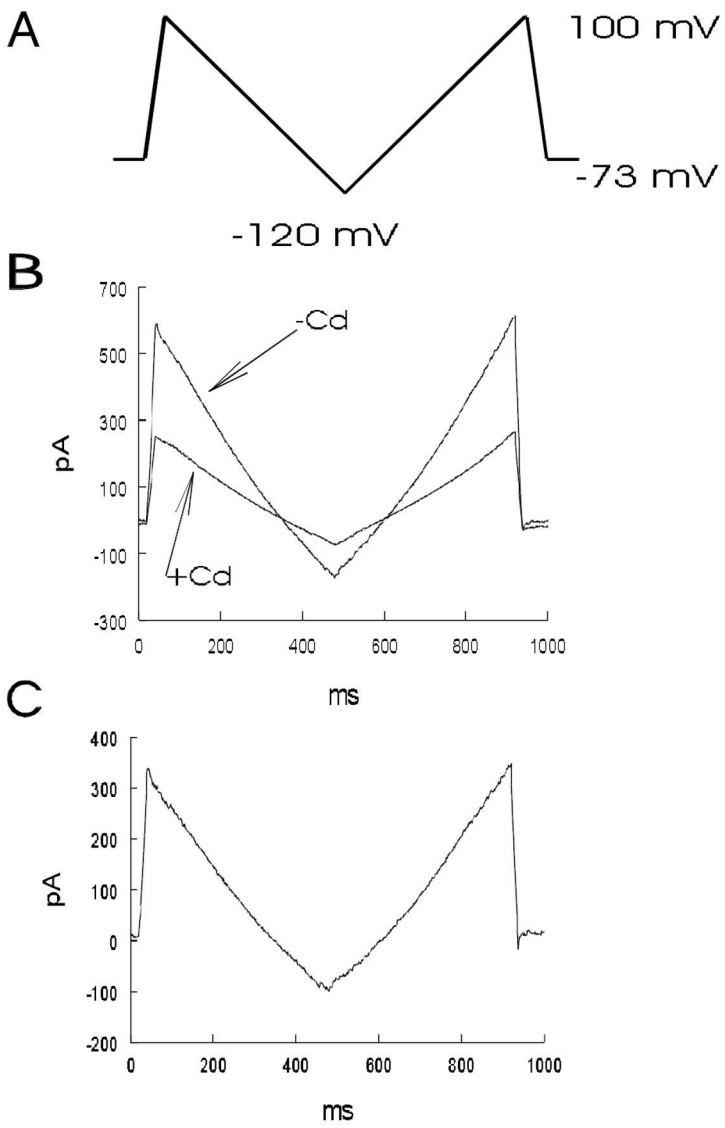

Fig. 1. Measurement of Na^+/Ca^{2+} exchange current (I_{NaCa}) in transfected HEK293 cells

HEK293 cells were transfected with NCX1. At 48h post-transfection, I_{NaCa} was measured at 5 mM $[Ca^{2+}]_o$ and 30°C with a descending-ascending voltage ramp protocol (A) described in 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 transfected cell during the descending-ascending voltage-ramp from +100 to −120 and back to +100 mV, in the absence and presence of 1 mM Cd^{2+}. (C) Derived Cd^{2+}-sensitive current in the transfected cell shown in B.

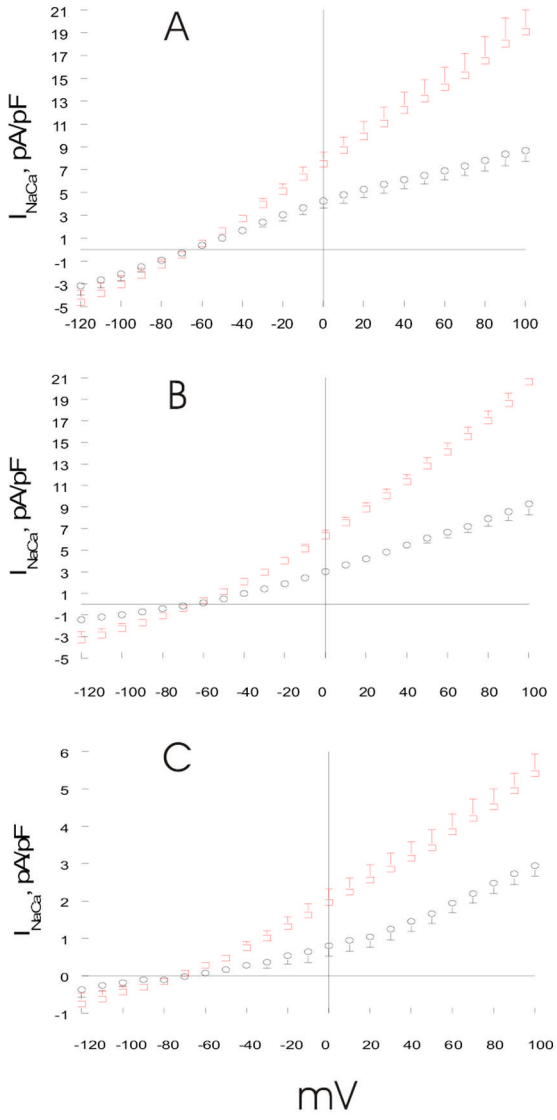

Fig. 2. Effects of PMA on I_{NaCa} in transfected HEK293 cells

(A). HEK293 cells were transfected with NCX1 (open circles, n=14). At 48h post-transfection, I_{NaCa} was measured at 30°C using standard solutions and Cd^{2+} as described in Experimental Procedures and Fig. 1. After baseline I_{NaCa} was obtained, PMA (0.1 μM) was added and I_{NaCa} was measured 3 to 5 min after drug addition (open squares; n=8). (B). I_{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. Cd^{2+} was used to define I_{NaCa}. (C). I_{NaCa} was measured in HEK293 cells transfected with NCX1 under high $[Na^+]_i$ conditions (Experimental Procedures), both before (open circles, n=6) and after (open squares, n=7) addition of PMA. $[Ca^{2+}]_o$ was 0.2 rather than 5.0 mM so that the calculated reversal potential for I_{NaCa} was −103 mV, as compared to the holding potential of −73 mV used in other experiments. Ni^{2+} was used to define I_{NaCa}. Error bars are not shown if they fall within boundaries of the symbols.

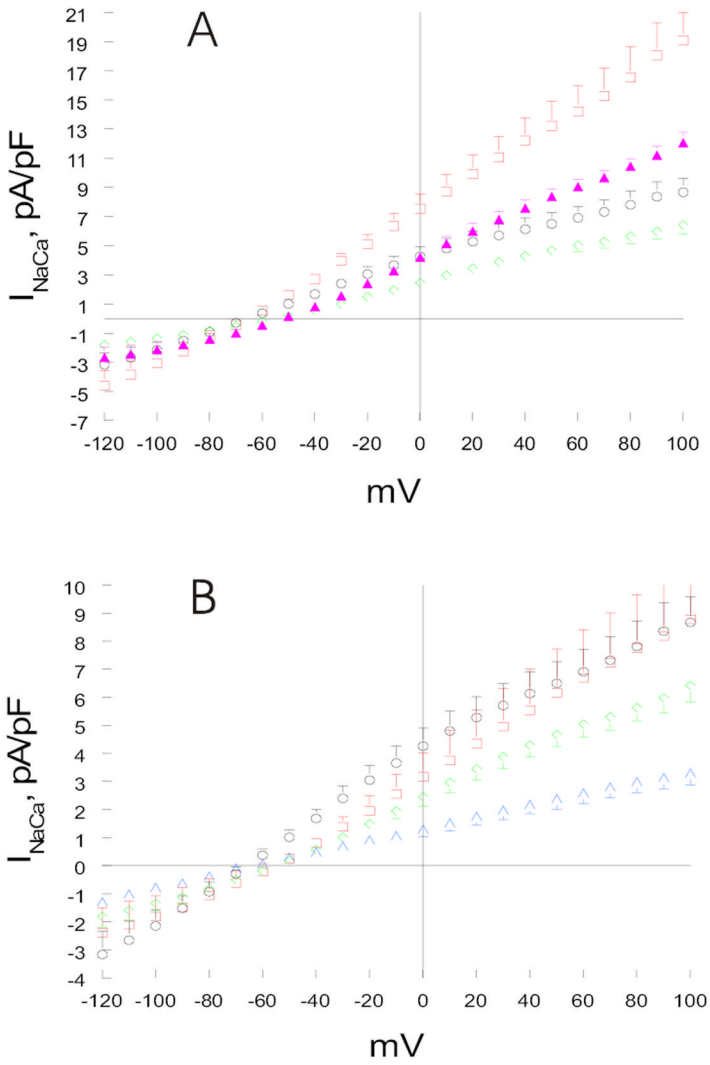

Fig. 3. Effects of PMA and forskolin on I_{NaCa} in transfected HEK293 cells

(A). HEK293 cells were transfected with either NCX1 alone (open circles, n=14) or PLM +NCX1 (open diamonds, n=15). At 48h post-transfection, I_{NaCa} was measured at 5 mM $[Ca^{2+}]_o$ and 30°C as described in Fig. 1. After baseline I_{NaCa} was obtained, PMA (0.1 μM) was added to NCX1 (open squares; n=8) and PLM+NCX1 (filled triangles; n=9) cells. Measurement of I_{NaCa} was repeated ~3 to 5 min after drug addition. (B). HEK293 cells were transfected with either NCX1 alone (open circles, n=14) or PLM+NCX1 (open diamonds, n=15). At 48h post-transfection, baseline I_{NaCa} was obtained. Forskolin (1 μM) was then added to NCX1 (open squares; n=4) and PLM+NCX1 (open triangles; n=7) cells. Measurement of I_{NaCa} was repeated ~3 to 5 min after drug addition. Error bars are not shown if they fall within boundaries of the symbols.

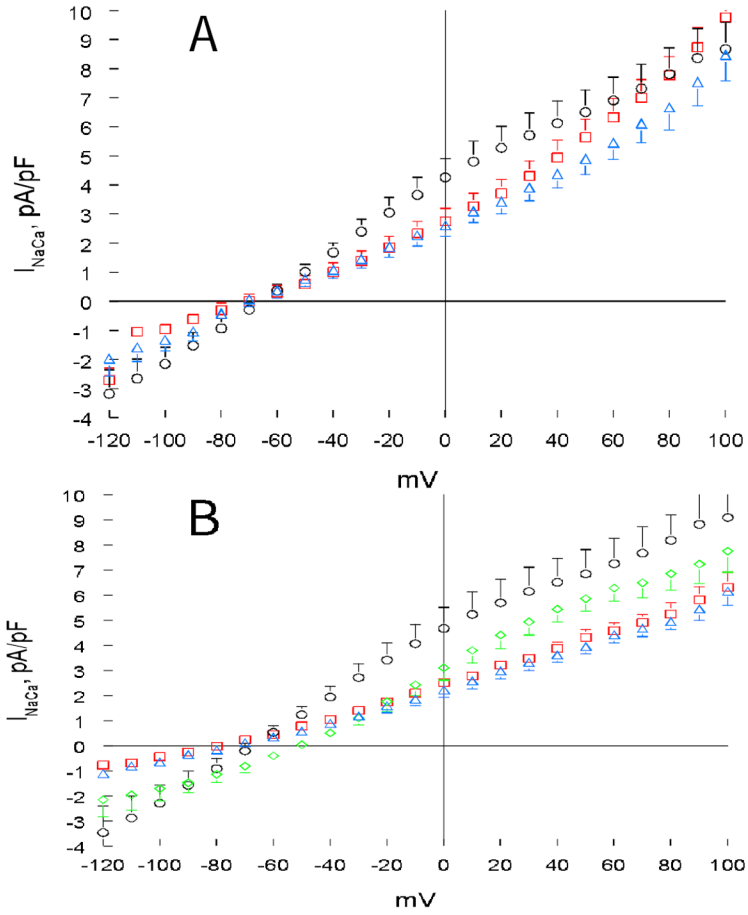

Fig. 4. Effects of serine[68] mutants of PLM on I_{NaCa} in transfected HEK293 cells

(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_{NaCa} was measured at 5 mM $[Ca^{2+}]_o$ and 30°C as described in Fig. 1. After baseline I_{NaCa} was obtained, PMA (0.1 μM) was added to S68A+NCX1 cells (open triangles; n=7) and I_{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_{NaCa} was measured 48h post-transfection. In S68E+NCX1 cells, I_{NaCa} was measured both before (open squares) and after (open triangles) addition of PMA (0.1 μM). Error bars are not shown if they fall within boundaries of the symbols.

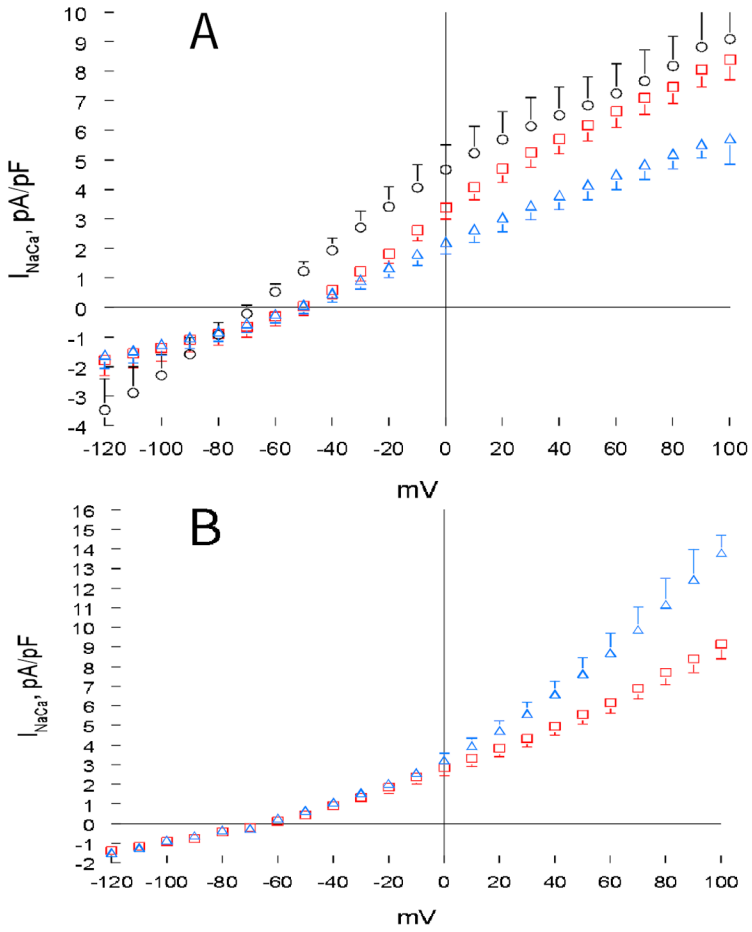

Fig. 5. Effects of serine^{63} mutants of PLM on I_{NaCa} in transfected HEK293 cells

(A). HEK293 cells were transfected with either NCX1 alone (open circles, n=10) or S63A +NCX1 (open squares, n=5). At 48 h post-transfection, I_{NaCa} was measured at 5 mM $[Ca^{2+}]_o$ and 30°C as described in Fig. 1. After baseline I_{NaCa} was obtained, forskolin (1 μM) was added to S63A+NCX1 cells (open triangles; n=5) and I_{NaCa} was again measured. (B). HEK293 cells were transfected with S63A+NCX1 (open squares, n=6). I_{NaCa} was measured 48h post-transfection, both before (open squares) and after (open triangles) addition of PMA (0.1 μM). Error bars are not shown if they fall within boundaries of the symbols.

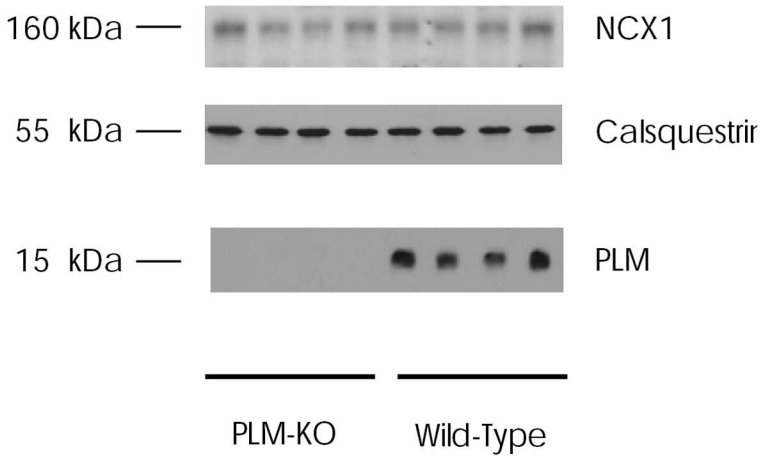

Fig. 6. Immunoblots of Na^+/Ca^{2+} exchanger (NCX1), calsequestrin and phospholemman (PLM) from murine hearts

Left ventricular homogenates were prepared from wild-type and PLM-KO mice of congenic C57BL/6 background, as described in Experimental Procedures. Proteins were separated by gel electrophoresis under non-reducing conditions for NCX1 (50 μg/lane) and calsequestrin (100 μg/lane), and reducing conditions for PLM (5 μg/lane). After transfer to PVDF membranes, immunoblotting were performed as described in Experimental Procedures. Numbers on the left refer to apparent molecular mass.

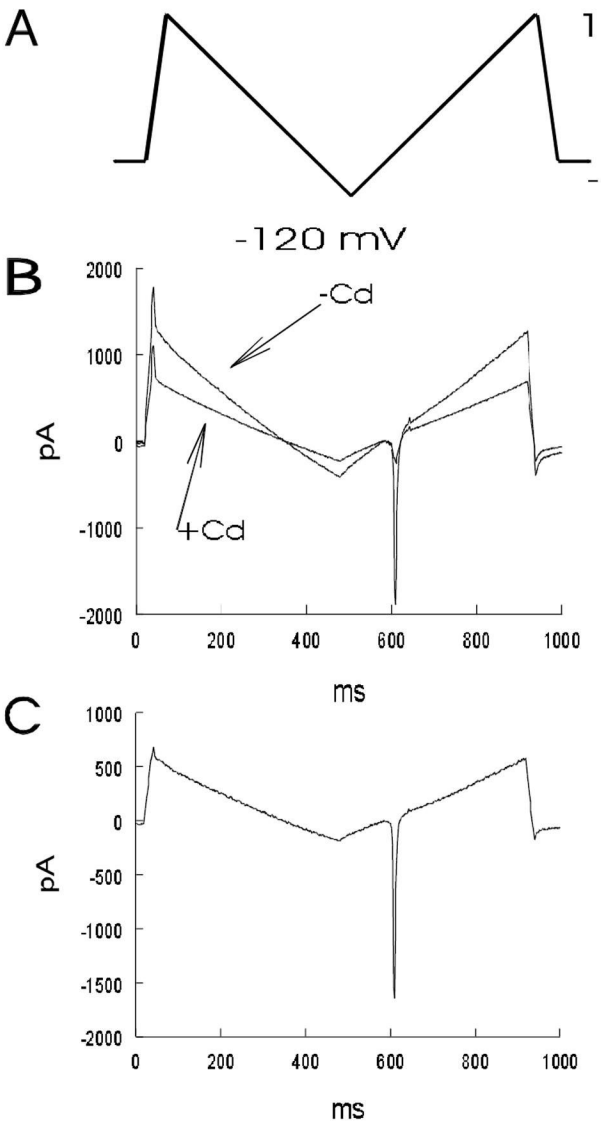

Fig. 7. Measurement of Na^+/Ca^{2+} exchange current (I_{NaCa}) in murine cardiac myocytes

I_{NaCa} was measured in ventricular myocytes isolated from adult mouse hearts at 5 mM $[Ca^{2+}]_o$ and 30°C with a descending-ascending voltage ramp protocol (A) as described in 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 Cd^{2+}. (C) Derived Cd^{2+}-sensitive current in the wild-type myocyte shown in B.

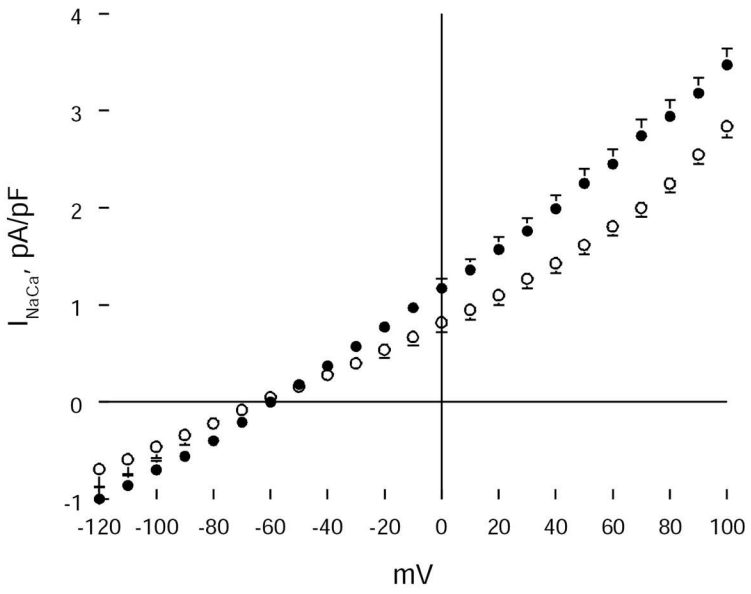

Fig. 8. I_{NaCa} is larger in PLM-KO when compared to wild-type cardiac myocytes

I_{NaCa} was measured in ventricular myocytes isolated from wild-type and PLM-KO mouse hearts at 5 mM $[Ca^{2+}]_o$ and 30°C as described in Fig. 7. Shown are current density-voltage relationships of I_{NaCa} (means ± SE) from wild-type (open circles; n=20) and PLM-KO (filled circles; n=23) myocytes. Error bars are not shown if they fall within the boundaries of symbols.

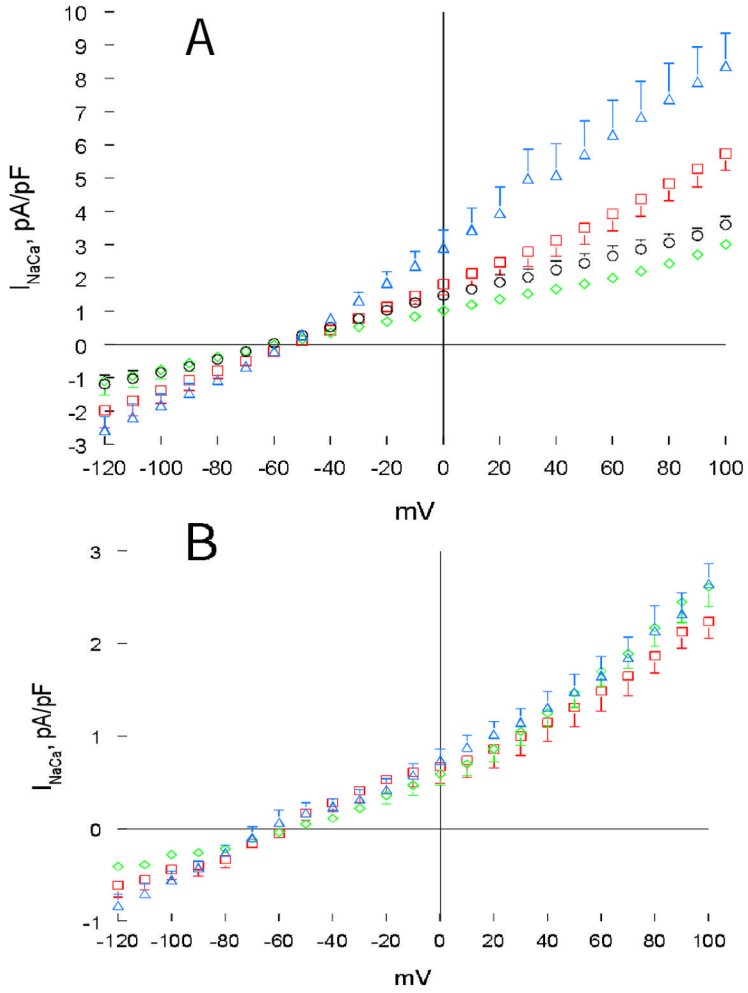

Fig. 9. Effects of PMA and forskolin on I_{NaCa} in wild-type and PLM-KO cardiac myocytes

(A). I_{NaCa} was measured in a second group of ventricular myocytes isolated from wild-type (diamonds, n=7) and PLM-KO (circles, n=7) mouse hearts at 5 mM $[Ca^{2+}]_o$ and 30°C as described in Fig. 6. After baseline I_{NaCa} was obtained, PMA (1 μM) was added to both wild-type (squares, n=7) and PLM-KO (triangles, n=7) and I_{NaCa} measurement was repeated. (B). I_{NaCa} was measured in a third group of wild-type myocytes, both before (diamonds, n=6) and after (squares, n=6) addition of forskolin (1 μM). Similarly, I_{NaCa} was measured in PLM-KO myocytes. For clarity of presentation, only data from PLM-KO myocytes treated with forskolin (triangles, n=6) are shown. Error bars are not shown if they fall within boundaries of the symbols.