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Regulation of L-type calcium channel by phospholemman in cardiac myocytes

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Abstract

We evaluated whether phospholemman (PLM) regulates L-type Ca^{2+} current (I_{Ca}) in mouse ventricular myocytes. Expression of α_1-subunit of L-type Ca^{2+} channels between wild-type (WT) and PLM knockout (KO) hearts was similar. Compared to WT myocytes, peak I_{Ca} (at −10 mV) from KO myocytes was ~41% larger, the inactivation time constant (τ_{inact}) of I_{Ca} was ~39% longer, but deactivation time constant (τ_{deact}) was similar. In the presence of isoproterenol (1 μM), peak I_{Ca} was ~48% larger and τ_{inact} was ~144% higher in KO myocytes. With Ba^{2+} as the permeant ion, PLM enhanced voltage-dependent inactivation but had no effect on τ_{deact}. To dissect the molecular determinants by which PLM regulated I_{Ca}, we expressed PLM mutants by adenovirus- mediated gene transfer in cultured KO myocytes. After 24 h in culture, KO myocytes expressing green fluorescent protein (GFP) had significantly larger peak I_{Ca} and longer τ_{inact} than KO myocytes expressing WT PLM; thereby independently confirming the observations in freshly isolated myocytes. Compared to KO myocytes expressing GFP, KO myocytes expressing the cytoplasmic domain truncation mutant (TM43), the non-phosphorylable S68A mutant, the phosphomimetic S68E mutant, and the signature PFXYD to alanine (ALL5) mutant all resulted in lower peak I_{Ca}. Expressing PLM mutants did not alter expression of α_1-subunit of L-type Ca^{2+}

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Disclosures

None.

channels in cultured KO myocytes. Our results suggested that both the extracellular PFXYD motif and the transmembrane domain of PLM but not the cytoplasmic tail were necessary for regulation of peak I_{Ca} amplitude. We conclude that PLM limits Ca^{2+} influx in cardiac myocytes by reducing maximal I_{Ca} and accelerating voltage-dependent inactivation.

Keywords

FXYD1; Ca^{2+} channels; phospholemman; arrhythmia

1. Introduction

Phospholemman (PLM), a 72-amino acid phosphoprotein with a single transmembrane (TM) domain [1], is highly expressed in cardiac muscle [2]. PLM co-immunoprecipitates with Na^+-K^+-ATPase [3–5], Na^+/Ca^{2+} exchanger [6–8] and L-type Ca^{2+} channels [8] in the heart. PLM regulates the activities of Na^+-K^+-ATPase [5, 9–11] and Na^+/Ca^{2+} exchanger [6, 12, 13] in cardiac myocytes. In HEK293 cells transfected with α_1-subunit of cardiac L-type Ca^{2+} channel ($Ca_v1.2$) with the auxiliary subunits $\alpha_2\delta$ and β_1b, PLM modulates the gating of L-type Ca^{2+} channels [8]. Specifically, in heterologous expression model systems, PLM slows deactivation and enhances the rate and magnitude of voltage-dependent inactivation (VDI). Ca^{2+}-dependent inactivation (CDI) is not affected by PLM in heterologous expression model systems.

By virtue of its regulatory effects on Na^+-K^+-ATPase and Na^+/Ca^{2+} exchanger, PLM is intimately involved in regulation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and Na^+ concentrations ($[Na^+]_i$) and thus exerts major influences on cardiac excitation-contraction (EC) coupling, both in vitro [10] and in vivo [11, 14]. If PLM also regulates L-type Ca^{2+} channels in cardiac myocytes, the complexity of the relationship between PLM expression and cardiac contractility will escalate greatly. The successful engineering of PLM knockout (KO) mouse [15, 16] permits the hypothesis that PLM regulates L-type Ca^{2+} channels in cardiac myocytes to be rigorously tested. The present study was undertaken to examine whether PLM modulates L-type Ca^{2+} channels in adult cardiac myocytes, whether PLM limits Ca^{2+} influx via L-type Ca^{2+} channels under β-adrenergic stimulation; and to determine the molecular domain of PLM that is involved in regulation of L-type Ca^{2+} channels.

2. Materials and methods

2.1. Generation of PLM-deficient mice and animal care

PLM-KO mice backcrossed to a pure congenic C57BL/6 background were generated as described previously [15, 16]. Homozygous adult littermates ~3 mo old were used. Mice were housed and fed on a 12h:12h light-dark cycle at Temple University Animal Facility and were supervised by veterinary staff members. Standard care was provided to all mice used for experiments. All protocols applied to the mice in this study were approved and supervised by the Institutional Animal Care and Use Committee at Temple University.

2.2. Isolation and culture of adult murine cardiac myocytes

Cardiac myocytes were isolated from the septum and left ventricular (LV) free wall of WT and KO mice according to the protocol of Zhou et al. [17] and as modified by us [15, 18]. Isolated myocytes were plated on laminin-coated coverslips and either used on the same day, or placed in short-term culture [18] for 24h before calcium current measurements.

2.3. L-type Ca^{2+} current (I_{Ca}) measurements

Whole cell patch-clamp recordings were performed at 30°C as previously described [14, 15, 18]. The pipette diameter was 4–6 μm and the pipette resistance was 0.8–1.4 MΩ when filled with standard pipette solution containing (in mM): CsCl 110, TEA.Cl 20, HEPES 10, MgATP 5, and EGTA 10; pH 7.2. Extracellular bathing solution contained (in mM): N-methyl-D-glucamine 137, CsCl 5.4, $CaCl_2$ 2, $MgSO_4$ 1.3, HEPES 20, 4-aminopyridine 4, and glucose 15; pH 7.4. Our solutions were designed to be Na^+- and K^+-free. To ensure steady-state Ca^{2+} loading in the sarcoplasmic reticulum, 6 conditioning pulses (from −70 to 0 mV, 100 ms, 2 Hz) were delivered to the myocyte before the arrival of each test pulse (from −90 to +50 mV, 10 mV increments, 60 ms). In a subset of myocytes, after 6 conditioning pulses, holding potential was changed from −70 to −40 mV before each test pulse (from −40 to + 60 mV, 10 mV increments, 60 ms). Leak-subtracted inward currents were used in analysis for I_{Ca} amplitudes and inactivation and deactivation kinetics. Inward currents obtained under these conditions were blocked by 1 μM verapamil (data not shown). I_{Ca} was normalized to membrane capacitance (C_m) before comparison between WT and KO myocytes. All I_{Ca} amplitudes and τ_{inact} values, when given in Results, were measured at −10 mV. All experiments with Ca^{2+} as ion carrier used these solutions and voltage protocol. To evaluate the effects of holding potential on inhibition of I_{Ca} by PLM, in some experiments holding potential was varied between −90 and −40 mV and peak I_{Ca} was measured at −10 and 0 mV. To eliminate the possibility that at more hyperpolarizing holding potentials, I_{Ca} measured may potentially be contaminated by Ca^{2+} entry via fast Na^+ channels, tetrodotoxin (TTX; 50 μM) was added to the bathing solution before current measurements. TTX at this concentration is known to totally block fast Na^+ current in both rat [19] and mouse [20] cardiac ventricular cells. In another series of experiments, the effects of PLM on VDI were evaluated in freshly isolated WT and KO myocytes by replacing extracellular Ca^{2+} with Ba^{2+}, and lengthening the test pulse to 300 ms.

2.4. Adenoviral infection of adult cardiac myocytes

Recombinant, replication-deficient adenovirus (Adv) expressing either green fluorescent protein (GFP) alone, GFP and WT dog PLM, or GFP and dog PLM mutants (non-phosphorylable S68A, phosphomimetic S68E, and cytoplasmic domain truncation TM43 mutants) were constructed as previously described [21, 22]. A new PLM mutant in which the signature extracellular PFXYD motif was mutated to alanine (ALL5) was generated using Altered Sites II in vitro mutagenesis system (Promega). Two hours after isolation from KO hearts, myocytes were infected with Adv-GFP (5.5×10^8 particles/ml), Adv-GFP-PLM (8.4×10^8 particles/ml) or Adv-GFP-PLM mutants (S68A 5.8×10^8; S68E 1.6×10^9; ALL5 7.5×10^8; TM43 6.4×10^8 particles/ml) in 5 ml of fetal bovine serum-free Eagle minimal essential medium (MEM) containing 0.2 % bovine serum albumin, creatine (5 mM),

carnitine (2 mM), taurine (5 mM), $NaHCO_3$ (4.2 mM), penicillin (30 mg/L), gentamicin (4 mg/L), insulin-transferrin-selenium supplement and 2,3-butanedione monoxime (10 mM) for 3h. An additional 5 ml of MEM (with same supplements) was then added, and myocytes were studied after 24h. Under our culture conditions, adult mouse LV myocytes maintain t-tubule organization and myocyte contractility for up to 48h [18]. For the sake of brevity, KO myocytes infected with Adv-GFP, Adv-GFP-PLM and Adv-GFP-PLM mutants are referred to as KO-GFP, KO-PLM, and the respective designation for the PLM mutant (e.g., KO-ALL5) myocytes, respectively.

2.5. Immunoblotting

Mouse LV homogenates [15, 23, 24] and cardiac myocyte lysates [18, 21] were prepared as previously described. For detection of WT PLM or its mutants (12% SDS-PAGE, reducing conditions with 5% β-mercaptoethanol), either monoclonal B8 which detects the NH_2-terminus of dog but not rodent PLM [22] or polyclonal C2 antibody which detects the COOH-terminus of both rodent and dog PLM [21] was used. For detection of α_1-subunit of L-type Ca^{2+} channel ($Ca_v1.2$)(5% SDS-PAGE, reducing conditions)[25] and calsequestrin (used as loading control)[15, 21], commercially available antibodies were used as previously described.

2.6. Statistics

All results are expressed as means ± SE. For analysis of I_{Ca} and I_{Ba} amplitudes, τ_{inact}, τ_{deact} and protein abundance, 1-way ANOVA was used. A commercially available software package (JMP version 7, SAS Institute, Cary, NC) was used. In all analyses, $p<0.05$ was taken to be statistically significant.

3. Results

3.1. I_{Ca} in WT and KO myocytes

With Ca^{2+} as ion carrier and holding potential of −90 mV before test pulses, I_{Ca} amplitudes were larger in KO compared to WT myocytes (Fig. 1A). Peak I_{Ca} occurred at −10 mV for both WT and KO myocytes (Fig. 1B). At −10 mV, I_{Ca} amplitudes were 8.99 ± 0.49 and 12.67 ± 0.68 pA/pF, respectively, for WT (n=14) and KO (n=12) myocytes with holding potential at −90 mV ($p<0.0002$; Fig. 1C). Time constant of inactivation (τ_{inact}) in KO myocytes (10.65 ± 0.44 ms) was significantly ($p<0.0001$) longer than that in WT myocytes (7.67 ± 0.38 ms)(Fig. 1D). Time constant of deactivation (τ_{deact}) was not different between WT (1.12 ± 0.10 ms) and KO myocytes (1.22 ± 0.18 ms)($p<0.65$). Smaller I_{Ca} amplitude was not due to decreased $Ca_v1.2$ expression in WT (0.92 ± 0.02) compared to KO myocytes (0.94 ± 0.04 arbitrary units)($p<0.8$; Fig.1E). However, when holding potential was stepped to −40 mV (a common practice to inactivate the fast inward Na^+ current) before the arrival of test pulses, I_{Ca} differences between WT and KO myocytes were no longer apparent (Fig. 1F), in agreement with our previous report [15]. In addition, the voltage at which peak I_{Ca} occurred right-shifted from −10 to 0 mV. At 0 mV, I_{Ca} amplitudes were 5.51 ± 0.27 and 5.76 ± 0.50 pA/pF, respectively, for WT (n=6) and KO (n=4) myocytes ($p<0.65$) with holding potential at −40 mV (Fig. 1F). The differences in I_{Ca} amplitudes between WT and KO myocytes progressively decreased at more depolarizing holding potentials (Fig. 1G).

Using TTX (50 μM) to block potential Ca^{2+} entry via activated fast Na^{+} channels, significant ($p<0.0001$) differences in I_{Ca} amplitudes persisted (Fig. 1H). Collectively, these observations suggest that in adult mouse ventricular myocytes held at more physiological membrane potential (−90 vs. −40 mV), PLM decreased I_{Ca} amplitude and enhanced inactivation but had no effect on deactivation.

3.2 Effects of isoproterenol on I_{Ca} in WT and KO myocytes

Addition of isoproterenol (Iso, 1 μM) increased I_{Ca} in both WT and KO myocytes (Fig. 1B). At −10 mV, I_{Ca} amplitude was 20.42 ± 1.98 pA/pF in KO myocytes treated with Iso and was significantly ($p<0.007$) higher than the 13.80 ± 0.91 pA/pF measured in WT myocytes (Fig. 1C). In KO myocytes stimulated with Iso, τ_{inact} (18.43 ± 4.49 ms) was significantly ($p<0.008$) longer than that in WT myocytes exposed to Iso (7.54 ± 0.36 ms)(Fig. 1D).

3.3 PLM enhanced voltage-dependent inactivation

I_{Ca} inactivation is classically separated into Ca^{2+}-dependent inactivation (CDI) and voltage-dependent inactivation (VDI). In a second series of experiments, Ba^{2+} was used to examine VDI (Fig. 2). Similar to I_{Ca}, peak I_{Ba} occurred at −10 mV (Fig. 2C) and was significantly ($p<0.02$) lower in WT (10.22 ± 0.67 pA/pF; n=5) when compared to KO myocytes (12.76 ± 1.35 pA/pF; n=5). With Ba^{2+} as the permeant ion, τ_{inact} was significantly slower ($p<0.05$) in KO (117.4 ± 15.9 ms) than WT (64.4 ± 15.2 ms) myocytes (Fig. 2B). At −10 mV, compared to WT myocytes, PLM accelerated VDI by 45%, similar to the 28% decrease in τ_{inact} when Ca^{2+} was used as the permeant ion. These observations suggest that the major PLM effect on I_{Ca} inactivation was VDI.

In agreement with observations when Ca^{2+} was the permeant ion, τ_{deact} of I_{Ba} was not different between WT (1.73 ± 0.02 ms) and KO myocytes (1.66 ± 0.11 ms)($p<0.55$).

3.4 Adenovirus-mediated PLM expression decreased I_{Ca} in KO myocytes

In KO myocytes infected with Adv expressing WT PLM, expression of $Ca_v1.2$ was similar to KO myocytes infected with Adv expressing GFP (Fig. 3A). At baseline, peak I_{Ca} amplitudes were significantly ($p<0.03$) lower in KO-PLM (9.34 ± 0.50 pA/pF; n=6) compared to KO-GFP myocytes (10.93 ± 0.41 pA/pF; n=8)(Fig. 3B). At −10 mV, τ_{inact} was significantly ($p<0.04$) longer in KO-GFP (14.04 ± 2.20 ms) compared to KO-PLM myocytes (9.10 ± 0.72 ms)(Fig. 4C). Iso significantly ($p<0.05$) increased I_{Ca} in both KO-GFP (25.32 ± 2.92 pA/pF) and KO-PLM myocytes (16.05 ± 1.35 pA/pF)(Fig. 3B). These results corroborated the observations on I_{Ca} in freshly isolated WT and KO myocytes and indicated that short-term culture and adenovirus infection did not affect the ability of PLM to modulate I_{Ca} amplitudes in adult cardiac myocytes.

3.5 Effects of Ser^{68} phosphorylation on I_{Ca}

Phosphorylation of Ser^{68} of PLM is critical in its regulation of both Na^{+}-K^{+}-ATPase [9] and Na^{+}/Ca^{2+} exchanger [13]. To examine the role of Ser^{68} phosphorylation in regulation of I_{Ca}, we expressed WT PLM or its Ser^{68} mutants (Fig. 4A) in KO myocytes by adenovirus-mediated gene transfer. After 24 h in culture, KO myocytes expressed WT PLM, S68A and S68E mutants as detected by B8 or C2 antibodies (Fig. 3A). Expression of $Ca_v1.2$ was

similar among KO-GFP, KO-PLM, KO-S68A and KO-S68E myocytes (Fig. 3A). At baseline, compared to KO-GFP myocytes (10.93 ± 0.41pA/pF; n=9), both S68A (8.89 ± 0.70 pA/pF, n=6; p<0.02) and S68E mutants (8.19 ± 0.65 pA/pF, n=6; p<0.003) suppressed peak I_{Ca} equally (Fig. 3C). There were no statistically significant differences in I_{Ca} amplitudes between KO-PLM and KO-S68A (p<0.6) and between KO-PLM and KO-S68E (p<0.2) myocytes (Fig. 4B). These results suggest that at baseline, Ser^{68} phosphorylation did not play a major role in regulation of I_{Ca} by PLM.

In the presence of Iso, I_{Ca} amplitudes increased in both KO-S68A (12.27 ± 0.71 pA/pF) and KO-S68E (11.43 ± 1.04 pA/pF) myocytes (Fig. 3C). However, I_{Ca} amplitudes were significantly lower in both KO-S68A (p<0.04) and KO-S68E (p<0.03) when compared to KO-PLM myocytes (16.05 ± 1.35 pA/pF) and less than half (p<0.005) when compared to KO-GFP myocytes (25.32 ± 2.92 pA/pF)(Fig. 4B). These observations suggest that mutating Ser^{68} in PLM may alter the physical interaction between PLM and L-type Ca^{2+} channels in the presence of isoproterenol, resulting in enhanced inhibition of I_{Ca} by the Ser^{68} mutants.

3.6 Role of extracellular PFXYD motif in regulation of I_{Ca}

In heterologous expression systems, the signature PFXYD motif of PLM was found to be important in modulating I_{Ca} [26]. Mutation of the extracellular PFXYD motif did not affect detection by C2 antibody (raised against the C-terminus of PLM) but abolished the B8 (detects the N-terminus of dog PLM) signal as expected (Fig. 3A). Expression of ALL5 mutant in cultured KO myocytes did not affect $Ca_v1.2$ expression when compared to KO-GFP myocytes (Fig. 3A). I_{Ca} amplitudes in KO-ALL5 myocytes (7.21 ± 0.62 pA/pF, n=8; p<0.0006) were lower when compared to KO-GFP myocytes (10.93 ± 0.41 pA/pF; n=9) (Fig. 4B). Compared to KO-PLM myocytes (9.34 ± 0.50 pA/pF), I_{Ca} amplitudes were significantly (p<0.03) lower in KO-ALL5 myocytes (Fig. 4B). These results suggest that mutating the extracellular PFXYD motif to alanine resulted in further suppression of I_{Ca} amplitudes in adult cardiac myocytes.

In the presence of Iso, peak I_{Ca} amplitudes were significantly (p<0.0004) lower in KO-ALL5 (11.82 ± 0.70 pA/pF) when compared to KO-GFP (25.32 ± 2.92 pA/pF) myocytes (Fig. 4B) and τ_{inact} was significantly (p<0.005) shorter (Fig. 4C). Compared to KO-PLM myocytes (16.05 ± 1.35 pA/pF), peak I_{Ca} was significantly (p<0.01) lower in KO-ALL5 myocytes (Fig. 4B).

3.7 Role of cytoplasmic domain in regulation of I_{Ca}

We next expressed the truncation TM43 mutant (Fig. 4A) in KO myocytes to assess the role of cytoplasmic domain of PLM in regulating I_{Ca}. As expected, C2 antibody did not detect any signal while the B8 signal was present but weak in KO-TM43 myocytes (Fig. 3A). TM43 did not affect $Ca_v1.2$ expression in cultured KO myocytes (Fig. 3A). At baseline, I_{Ca} amplitudes in KO-TM43 myocytes (8.79 ± 0.51 pA/pF, n=9; p<0.005) were lower (Fig. 4B) and τ_{inact} was longer when compared to KO-GFP myocytes (Fig. 4C). I_{Ca} amplitudes (Fig. 4B) and τ_{inact} (Fig. 4C) in KO-PLM myocytes were similar to KO-TM43 myocytes. Collectively, these results suggest that at baseline, the cytoplasmic domain of PLM was not necessary in modulation of I_{Ca}.

In the presence of Iso, peak I_{Ca} amplitudes were significantly ($p<0.0004$) lower in KO-TM43 (13.63 ± 0.56 pA/pF)(Fig. 4B) and τ_{inact} (7.74 ± 1.20 ms)(Fig. 4C) was significantly ($p<0.04$) shorter when compared to KO-GFP myocytes. When compared to KO-PLM myocytes, neither peak I_{Ca} (Fig. 4B) nor τ_{inact} (Fig. 4C) was different in KO-TM43 myocytes.

To summarize, in the presence of Iso, when compared to KO-PLM myocytes, peak I_{Ca} amplitudes were similar in KO-TM43 myocytes ($p<0.08$) but significantly ($p<0.015$) lower in KO-ALL5, KO-S68A and KO-S68E myocytes (Fig. 4B). Our data suggest that it is the transmembrane domain of PLM that was largely responsible for the regulation of I_{Ca} amplitudes and that the extracellular PFXYD motif and cytoplasmic serine^{68} provide additional fine tuning of this regulation.

4 Discussion

It is well established that phospholemman regulates Na^+-K^+-ATPase and Na^+/Ca^{2+} exchanger in the heart [27]. Recent studies utilizing heterologous expression systems suggest that PLM also regulates cardiac L-type Ca^{2+} channels [8, 26]. Enthusiasm for yet another novel regulatory function of PLM, however, must be tempered by the numerous unsuccessful attempts to reconstitute the complete β-adrenergic receptor induced regulation of I_{Ca} using heterologous expression systems [28, 29]. Thus the first major finding is that in freshly isolated adult cardiac myocytes held at the more physiological membrane potential of −90 mV, PLM decreased I_{Ca} amplitude by ~41% (Fig. 1C). It is important to note that both our pipette and bathing solutions were Na^+- and K^+-free, thereby eliminating any effect of PLM on Na^+/Ca^{2+} exchanger and Na^+-K^+-ATPase that might secondarily affect I_{Ca} measurements. In addition, the differences in I_{Ca} amplitudes between WT and KO myocytes were maintained in the presence of TTX used to block potential Ca^{2+} entry via activated Na^+ channels, indicating PLM's effect was exerted on L-type Ca^{2+} rather than on fast Na^+ channels. To evaluate the contribution by different PLM residues and domains on I_{Ca} inhibition, we expressed PLM mutants in KO myocytes cultured for 24 h. Peak I_{Ca} amplitudes in freshly isolated WT myocytes (Fig. 1C) were similar to those in cultured KO myocytes expressing WT PLM (Fig. 4B), consistent with previous report by Zhou et al. (31) that short term culture had no effect on I_{Ca} amplitudes in adult mouse myocytes. In addition, in cultured KO myocytes expressing WT PLM, I_{Ca} amplitudes were lower compared to those measured in KO myocytes expressing GFP (Fig. 4B). The results of the reconstitution experiment independently confirmed the inhibitory effects of PLM on I_{Ca} in freshly isolated myocytes (Fig. 1C).

We have previously reported that peak I_{Ca} amplitudes and inactivation time constants elicited at holding potential of −40 mV were not different between WT and PLM KO myocytes [15]. There were three major differences in experimental conditions that likely account for the differences. The first is that neither pipette nor extracellular solutions were Na^+-free in our previous study, necessitating switching to a less physiological holding potential of −40 mV to inactivate the fast Na^+ current. The second is that the range of test pulses (from −30 to +60 mV; previous study, vs. from −80 to +50 mV; present study) was different. It is known that pulsing from more positive holding potentials would result in

inhibition, as opposed to facilitation, of I_{Ca} [30]. The third is that holding potential was −90 in the present study compared to −40 in the previous study. It is known that in rodent myocytes, I_{Ca} amplitudes elicited at holding potential of −90 mV are larger than those elicited at −40 mV [31]. Indeed, maximal I_{Ca} amplitudes in both WT and KO myocytes were lower when holding potential was at −40 mV (Fig. 1F) when compared to those measured when holding potential was at −90 mV (Fig. 1B). More importantly, differences in I_{Ca} amplitudes between WT and KO myocytes disappeared as holding potential became more positive (Fig. 1G). It is known that inhibition of L-type Ca^{2+} channel by dihydropyridines [32] and the indolizinsulphone SR33557 [33] is voltage-dependent. For example, 10^{-7} M SR33557 induced tonic block of I_{Ca} at holding potential of −50 mV but did not reduce I_{Ca} elicited at holding potential of −80 mV. The proposed mechanism is that both nitrendipine and SR33557 bind with high affinity to the L-type Ca^{2+} channel in the inactivated state (channel closed but not available to be opened). Since we have previously shown that PLM co-immunoprecipitates with $Ca_v1.2$ [8], and our current results with PLM mutants suggest interaction between the TM domains of $Ca_v1.2$ and PLM (Fig. 4), we speculate that PLM interacts with higher affinity to the rested state (channel closed but available to be opened by depolarization) compared to the inactivated state of the Ca^{2+} channel. This will explain the voltage-dependence of PLM inhibition of L-type Ca^{2+} channels. We also add that −90 mV is closer to the resting membrane potential than −40 mV. Thus under physiological conditions, PLM will likely exert its modulatory effect on I_{Ca}.

In the present study, we focused on residues of PLM that have previously been shown to be important in regulation of Na^+-K^+-ATPase and Na^+/Ca^{2+} exchanger in the heart. Ser^{68} is phosphorylated by both protein kinase A and protein kinase C [34]. When phosphorylated at Ser^{68}, inhibition of Na^+-K^+-ATPase is relieved but Na^+/Ca^{2+} exchanger is simultaneously inhibited [27]. Thus a second major finding is that Ser^{68} phosphorylation and indeed the cytoplasmic tail (residues 44 to 72) were not crucial in regulation of I_{Ca} in adult cardiac myocytes under basal conditions (Fig. 4B). Based on the results of the four PLM mutants, the TM domain of PLM appears critical in its regulation of I_{Ca}. The signature PFXYD motif stabilizes interactions between α- and β-subunits of Na^+-K^+-ATPase [35] and modulates L-type Ca^{2+} channel gating in heterologous expression systems [26]. Our data indicate that in addition to the TM domain, the extracellular PFXYD motif contributes to inhibition of I_{Ca}.

In agreement with the observations in heterologous expression systems [8], PLM enhanced voltage-dependent inactivation in adult LV myocytes by ~39% (Fig. 2B). In addition, peak I_{Ca} in cardiac myocytes was lower in the presence of PLM (Fig. 1C), consistent with the observations in heterologous expression systems that Ca^{2+} influx was reduced during the depolarization step in the presence of PLM [8]. By contrast, we did not detect any effect of PLM on deactivation, regardless of whether Ca^{2+} or Ba^{2+} was the permeant ion. The major physiological effect of PLM on I_{Ca} in adult cardiac myocytes was modulation of Ca^{2+} influx during the depolarization phase of the action potential, due to both smaller peak I_{Ca} amplitude and speeding of voltage-dependent inactivation. Consistent with this interpretation is the observation that action potential duration (both at 50 and 90% repolarization) was significantly shorter in WT when compared to KO myocytes [15]. In KO myocytes, prolongation of APD_{90} is likely due to enhanced NCX1 activity [15, 36, 37]

whereas prolongation of APD_{50} is consistent with increased I_{Ca} amplitude and slowed inactivation kinetics.

Under resting conditions, ~46% of Ser^{68} of PLM is estimated to be phosphorylated in adult rat myocytes based on inhibition of Na^+/Ca^{2+} exchanger current by S68E mutant [22]. Using phospho-specific anti-PLM antibodies [38, 39], ~30–40% of PLM in adult rat myocytes [38, 40] and ~25% of PLM in guinea pig myocytes [41] are phosphorylated under basal conditions. Since myocyte contractility, $[Ca^{2+}]_i$ transient amplitudes (1.8 mM $[Ca^{2+}]_o$, 1 Hz, 37°C), systolic and diastolic $[Ca^{2+}]_i$ [15] and resting $[Na^+]_i$ [9, 11] are similar between WT and KO myocytes under basal conditions, the regulatory effects of PLM on myocyte $[Ca^{2+}]_i$ dynamics (Na^+-K^+-ATPase, Na^+/Ca^{2+} exchanger and L-type Ca^{2+} channel) are not readily discernible and PLM is functionally quiescent [27, 42]. This conclusion is supported by the observation that in vivo cardiac function under basal conditions is similar between WT and KO mice [11]. Perhaps a subtle hint indicating the presence of PLM in resting myocytes is the shortening of both APD_{50} (accelerated I_{Ca} inactivation) and APD_{90} (partial inhibition of Na^+/Ca^{2+} exchanger) in WT as compared to KO myocytes [15]. Although APD prolongation is associated with increased risks of arrhythmias, the physiological significance of APD prolongation in KO mice is not clear as these mice have normal life-spans compared to WT mice. However, the pathophysiological significance of APD prolongation in KO mice may be unmasked after myocardial infarction (MI), when despite significantly ($p<0.01$) higher ejection fraction (42.4 ± 4.6 vs. 20.8 ± 1.7%), KO-MI mice had reduced survival (50%) compared to WT-MI mice (70%) at 3 weeks post-MI [43], likely as a result of sudden death from increased arrhythmogenesis.

Under stressful conditions when catecholamine levels are high, PLM is phosphorylated at Ser^{68} [34]. Phosphorylated PLM minimizes Na^+ overload (by relieving inhibition of Na^+-K^+-ATPase), thereby increasing the driving force for forward Na^+/Ca^{2+} exchange and promotes Ca^{2+} efflux from cardiac cells under stress. Our present observation that in the presence of isoproterenol, the differences in I_{Ca} (~6.8 pA/pF) and τ_{inact} (~10.9 ms) between KO and WT myocytes was almost 2- and 4-times of those measured under baseline conditions (~3.7 pA/pF and ~3.0 ms, respectively), suggested that another function of PLM is to limit Ca^{2+} entry into cardiac myocytes under stress.

In summary, phospholemman modulated Ca^{2+} entry through L-type Ca^{2+} channels in adult LV myocytes. Phospholemman reduced peak L-type Ca^{2+} current amplitude and enhanced voltage-independent inactivation, but had no effect on deactivation of L-type Ca^{2+} channels. Only the transmembrane and the extracellular (signature PFXYD motif) domains but not the cytoplasmic tail of phospholemman were necessary for regulation of L-type Ca^{2+} channels. We conclude that in concert with its effects on Na^+-K^+-ATPase, Na^+/Ca^{2+} exchanger and L-type Ca^{2+} channel, phospholemman reduces Na^+ and Ca^{2+} overload in cardiac myocytes under high catecholamine stimulation, thereby minimizes risks of arrhythmogenesis and serves as a cardiac “stress” protein.

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Highlights

1. Phospholemman regulates cardiac L-type calcium channels.
2. Inhibition of L-type calcium channels is dependent on holding potential.
3. Phospholemman decreases peak calcium current.
4. Phospholemman enhances voltage-dependent inactivation.
5. Transmembrane domain of phospholemman is critical for inhibition.

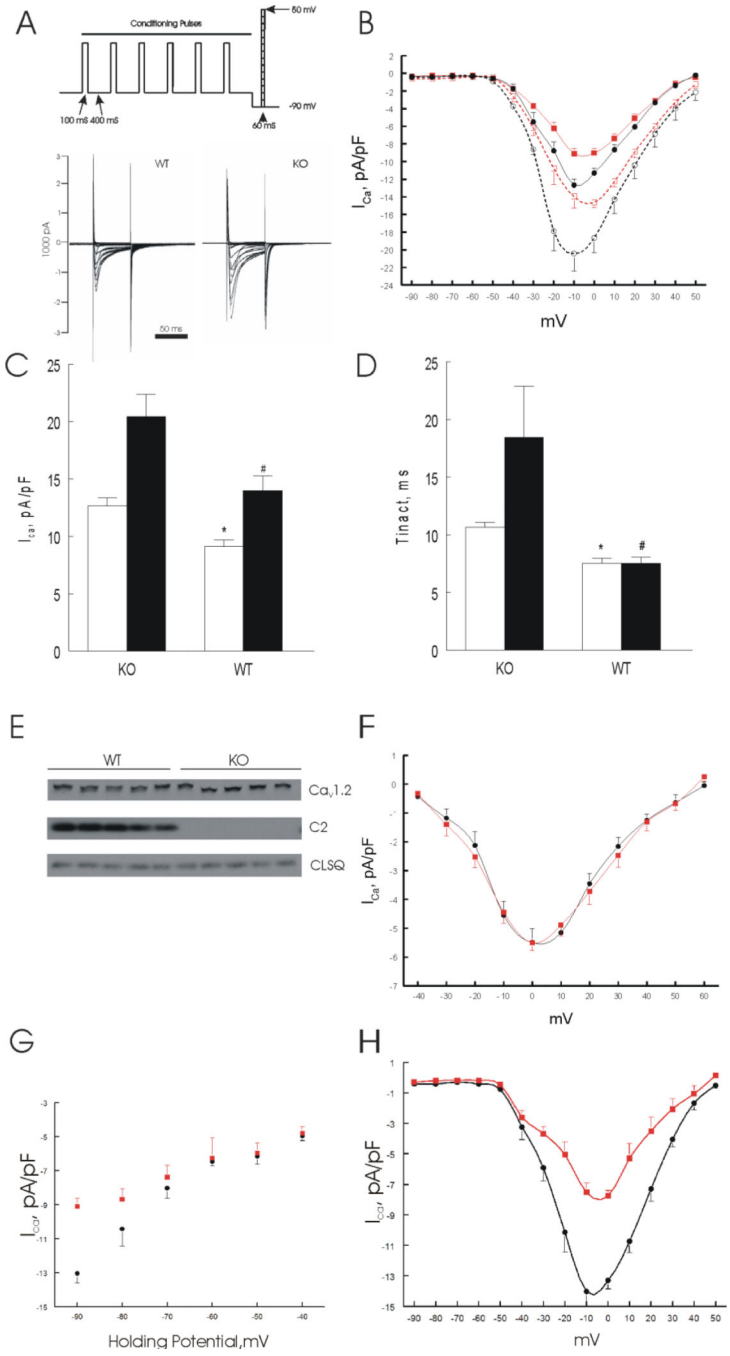

Figure 1.

Phospholemman (PLM) regulates L-type Ca^{2+} current (I_{Ca}) in adult mouse left ventricular (LV) myocytes. Myocytes were isolated from LV of wild-type (WT) and phospholemman knockout (KO) mice and I_{Ca} was measured on the same day. A. Voltage-clamp protocol and representative I_{Ca} traces from WT and KO myocytes. B. Current-voltage (I-V) relationship of I_{Ca} from WT (■; n=14) and KO (●; n=12) myocytes at baseline; and after stimulation with 1 μM isoproterenol (□ WT, n=7; and ○ KO, n=5). Holding potential was −70 mV and stepped to −90 mV before arrival of test pulses. Error bars are not shown if they fall within

the boundaries of the symbols. C & D. Peak I_{Ca} amplitudes and time constants of inactivation (τ_{inact}) (at −10 mV) of KO and WT myocytes, both in the absence (open bars) and presence (solid bars) of 1 μM isoproterenol.*p<0.0002, WT vs. KO; #p<0.008, WT-Iso vs. KO-Iso. E. LV homogenates were prepared from WT and KO mice and probed for α_1-subunit of L-type Ca^{2+} channel ($Ca_v1.2$) and phospholemman (C2); calsequestrin (CLSQ) was used as loading control. There were no differences (p<0.8) in $Ca_v1.2$ expression between WT (0.92 ± 0.02) and KO myocytes (0.94 ± 0.04 arbitrary units)(n=5 each). F. I-V relationship of I_{Ca} from WT (■; n=6) and KO (●; n=4) myocytes at baseline. Holding potential (−70 mV) was stepped to −40 mV before arrival of test pulses. Error bars are not shown if they fall within the boundaries of symbols. Note the difference in ordinate scales between B and F. G. I_{Ca} amplitudes at −10 mV elicited at holding potentials from −90 to −40 mV for WT (■; n=3 to 15) and KO (●; n=3 to 15) myocytes at baseline. Two-way ANOVA indicates significant group (WT vs. KO; p<0.0001), holding potential (p<0.0001) and group × holding potential interaction (p<0.0001) effects, indicating that varying the holding potential significantly affected the inherent differences in I_{Ca} between WT and KO myocytes. H. Baseline current-voltage (I-V) relationship of I_{Ca} ilicited at holding potential of −90 mV from WT (■; n=4) and KO (●; n=4) myocytes in the presence of 50 μM TTX.

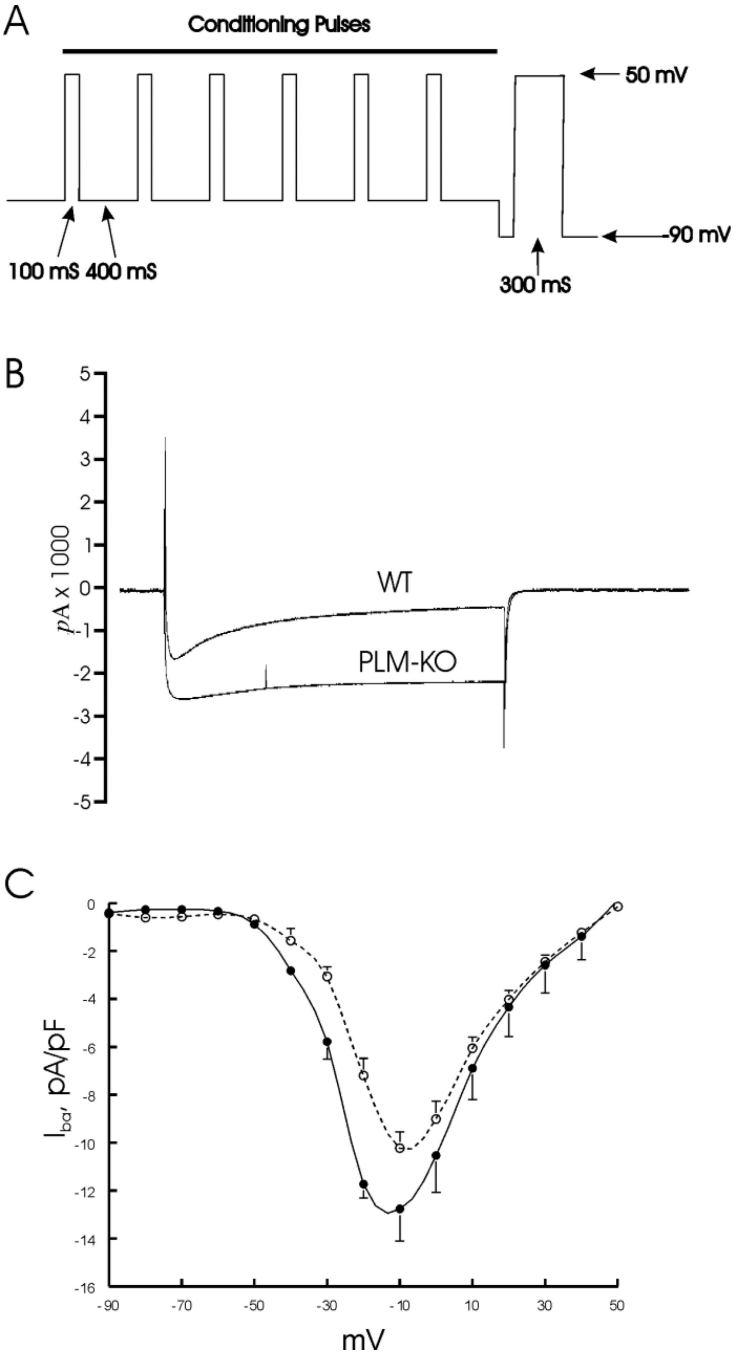

Figure 2.

PLM enhances voltage-dependent inactivation of I_{Ca}. I_{Ca} was measured in freshly isolated myocytes from WT and KO hearts, using Ba^{2+} (2 mM) as permeant ion. A. Voltage-clamp protocol: test pulses from −90 to +50 mV (in 10 mV increments) were extended from 60 to 300 ms duration (for simplicity, only one test pulse is shown). B. Representative I_{Ba} traces at −10 mV from WT and KO myocytes are shown. Note prolonged time course of I_{Ba} in KO compared to WT myocyte. C. I-V curves from WT (○; n=5) and KO myocytes (●; n=5).

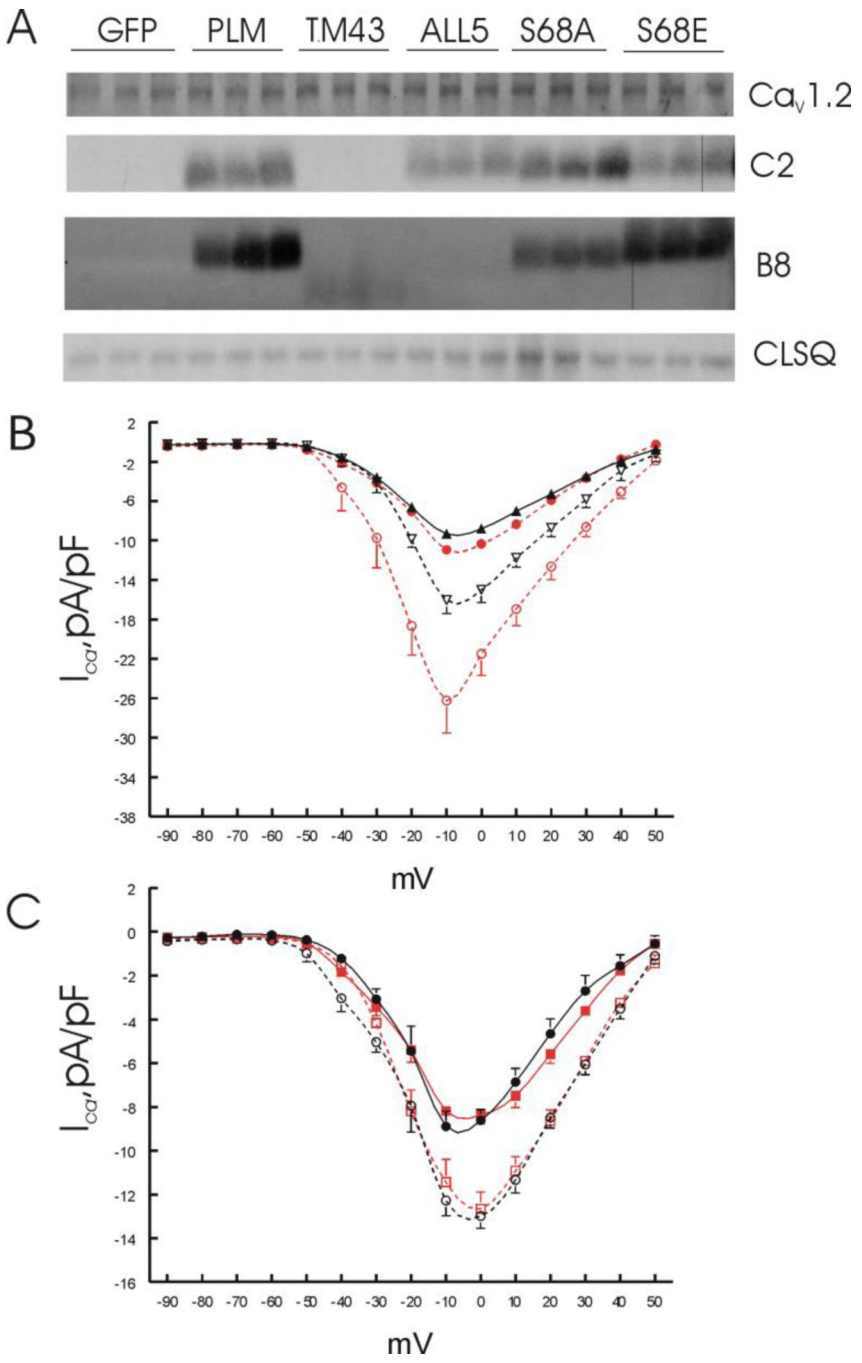

Figure 3.

Effects of PLM mutants on $Ca_v1.2$ expression and I_{Ca}. LV myocytes isolated from KO hearts were infected with adenovirus (Adv) expressing either green fluorescent protein (GFP), WT PLM, the cytoplasmic domain truncation mutant TM43, the signature PFXYD motif changed to Ala mutant (ALL5), the non-phosphorylable Ser^{68} mutant (S68A), or the phosphomimetic Ser^{68} mutant (S68E); and placed in culture for 24 hours prior to I_{Ca} measurements and Western blotting. A. Myocyte lysates were prepared and probed for $Ca_v1.2$, C-terminus (C2) or N-terminus (B8) of PLM and its mutants, with calsequestrin

(CLSQ) serving as the loading control. There were no differences ($p<0.98$) in $Ca_v1.2$ expression among KO myocytes expressing GFP (1.44 ± 0.01), WT PLM (1.42 ±0.01), TM43 (1.45 ± 0.08), ALL5 (1.46 ± 0.07), S68A (1.42 ± 0.00) and S68E (1.46 ± 0.05) mutants (n=3 each; values in arbitrary units). As expected, C2 which detects the cytoplasmic tail of PLM fail to detect any signal in KO-GFP and KO-TM43 myocytes. Likewise, there were no B8 (detects the extracellular NH_2-terminus of dog PLM) signals in KO-GFP and KO-ALL5 myocytes. B8 signals in KO-TM43 myocytes were weak, downward shifted but present. B. I-V curves of I_{Ca} in KO-GFP (●; n=8) and KO-PLM myocytes (▲; n=6) at baseline; and after exposure to 1 μM isoproterenol in KO-GFP (○; n=6) and KO-PLM (▽; n=4) myocytes. C. I-V curves of I_{Ca} in KO-S68A (●; n=6) and KO-S68E (■; n=6) myocytes at baseline, and after addition of 1 μM isoproterenol to KO-S68A (○; n=5) and KO-S68E (□; n=5) myocytes. Note the difference in ordinate scales between B and C.

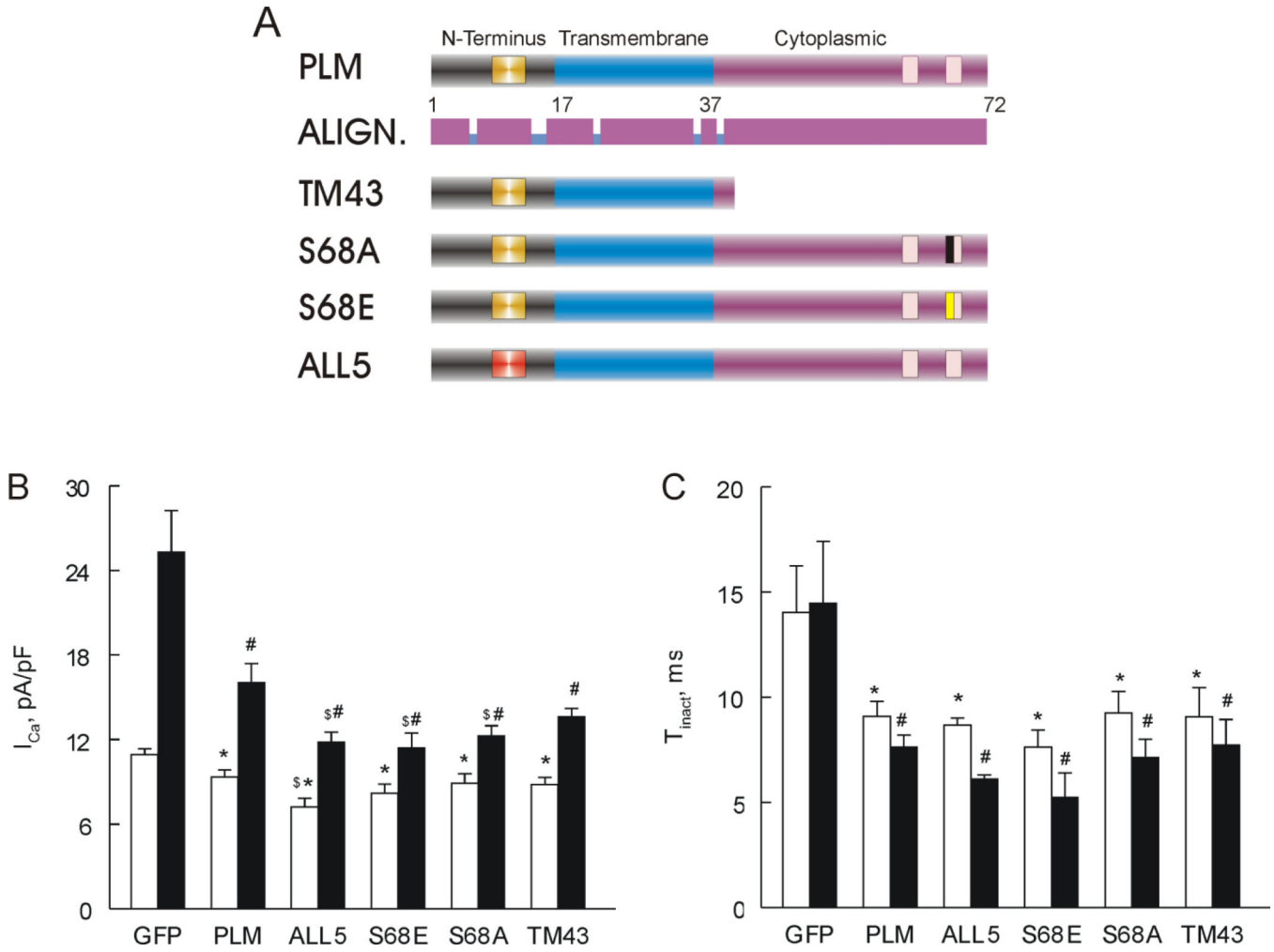

Figure 4.

Effects of PLM and its mutants on peak I_{Ca} in cultured KO myocytes. A. Schematic representation of WT PLM (both dog and rat) and dog PLM mutants. The structure of dog PLM (PLM) is shown to consist of an extracellular NH_2-terminal domain (black), a single-span transmembrane domain (blue), and cytoplasmic COOH-terminal domain (purple). The signature PFXYD sequence is shown in gold at the NH_2-terminus, and the 4 potential phosphorylation sites (Ser^{62}, Ser^{63}, Ser^{68} and Thr^{69}) are represented by pink boxes at the COOH-terminus. The 6 amino acid differences between dog PLM and rat PLM (ALIGN) are shown. Mouse PLM is identical to rat PLM except that Thr^{69} is replaced by Ser^{69} (not shown). All PLM mutants were constructed on the dog PLM backbone. Shown are representations of COOH-terminal deletion mutant (TM43), Ser^{68} substitution mutants (S68A and S68E), and ALL5 mutant in which the signature PFXYD motif is changed to Ala (shown as gold to red). Ser to Ala mutation is shown as pink to black, whereas Ser to Glu mutation is shown as pink to yellow. B. Summary of peak I_{Ca} (at −10 mV) measured in adenovirus-infected KO myocytes followed by culture for 24 hours, both in the absence (open bars) and presence (filled bars) of 1 μM isoproterenol. *p<0.03, KO-GFP vs. KO-PLM or KO-PLM mutants at baseline; #p<0.05, KO-GFP vs. KO-PLM or KO-PLM mutants

in the presence of 1 μM isoproterenol; $p<0.04, KO-PLM vs. KO-ALL5 at baseline, and KO-PLM vs. KO-ALL5 or KO-S68E or KO-S68A in the presence of 1 μM isoproterenol. C. Summary of τ_{inact} (at −10 mV) measured in adenovirus-infected KO myocytes followed by culture for 24 hours, both in the absence (open bars) and presence (filled bars) of 1 μM isoproterenol. *p<0.035, KO-GFP vs. KO-PLM or KO-PLM mutants at baseline; #p<0.0045, KO-GFP vs. KO-PLM or KO-PLM mutants in the presence of 1 μM isoproterenol.