Phospholemman Modulates the Gating of Cardiac L-Type Calcium Channels

Xianming Wang,‡ Guofeng Gao,§ Kai Guo,‡ Viktor Yarotskyy,‡ Congxin Huang,§ Keith S. Elmslie,‡ and Blaise Z. Peterson*†

†Department of Cellular and Molecular Physiology, and ‡Department of Anesthesiology and Pharmacology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania; and §Renmin Hospital, Cardiovascular Research Institute, Wuhan University, Wuhan, Hubei, People’s Republic of China

ABSTRACT Ca2+ entry through L-type calcium channels (CaV1.2) is critical in shaping the cardiac action potential and initiating cardiac contraction. Modulation of CaV1.2 channel gating directly affects myocyte excitability and cardiac function. We have found that phospholemman (PLM), a member of the FXYD family and regulator of cardiac ion transport, coimmunoprecipitates with CaV1.2 channels from guinea pig myocytes, which suggests PLM is an endogenous modulator. Cotransfection of PLM in HEK293 cells slowed CaV1.2 current activation at voltages near the threshold for activation, slowed deactivation after long and strong depolarizing steps, enhanced the rate and magnitude of voltage-dependent inactivation (VDI), and slowed recovery from inactivation. However, Ca2+-dependent inactivation was not affected. Consistent with slower channel closing, PLM significantly increased Ca2+ influx via CaV1.2 channels during the repolarization phase of a human cardiac action potential waveform. Our results support PLM as an endogenous regulator of CaV1.2 channel gating. The enhanced VDI induced by PLM may help protect the heart under conditions such as ischemia or tachycardia where the channels are depolarized for prolonged periods of time and could induce Ca2+ overload. The time and voltage-dependent slowed deactivation could represent a gating shift that helps maintain Ca2+ influx during the cardiac action potential waveform plateau phase.

INTRODUCTION

Voltage-gated calcium channels regulate important physiological processes such as excitation-contraction coupling, neurotransmitter release, hormone secretion, and gene expression (1). The activity of L-type calcium channels (CaV1.2) is vital for the maintenance of normal excitability and Ca2+ homeostasis in the heart. The dysregulation of CaV1.2 channels can lead to severe cardiac pathologies such as long QT syndrome (2–4). The modulation of CaV1.2 channels by signaling pathways and associated proteins is important for many physiological and pathophysiological processes (5–7). The gating of the α1 subunit of the cardiac CaV1.2 channels is complex and tightly regulated (8). Drugs that target the CaV1.2 α1 subunit are used routinely to treat cardiovascular diseases such as hypertension and angina pectoris (9,10), and α1-associated proteins represent an important mechanism by which CaV1.2 channels are regulated (8,11,12). Thus, drugs that target the pore-forming α1 subunit or its associated regulatory proteins have high clinical relevance.

The FXYD family of ion transport regulators was first defined based on an invariant peptide sequence, known as the FXYD motif (13). The FXYD motif is located on the N-termini of these single membrane spanning proteins and consists of five amino acids (Pro-Phe-X-Tyr-Asp). Seven members of the FXYD family (FXYD1–FXYD7) have been identified in mammals and are distributed widely in tissues that carry out fluid and solute transport (kidney, colon, breast/mammary gland, pancreas, prostate, liver, lung, and placenta), and in electrically excitable tissues (nervous system and muscle) (13,14).

FXYD1, also known as phospholemman (PLM), is localized to the sarcolemma and transverse tubules of cardiac myocytes where it regulates [Ca2+]i through its interactions with Na,K-ATPase (NKA) (15–17) and the Na+/Ca2+ exchanger (NCX) (18–20). As the primary pathway controlling Ca2+ entry into the cardiac myocyte, the observed PLM-dependent changes in [Ca2+]i merit the investigation of L-type calcium channels as a possible target for PLM modulation. We present what we believe to be the first report that currents through cardiac CaV1.2 channels are modulated by PLM. Our detailed kinetic analysis demonstrates that PLM slows the activation, slows deactivation in a time and voltage dependent manner, and enhances voltage-dependent inactivation. Slowed deactivation is predicted to enhance the relative Ca2+ entry during the repolarization phase of the cardiac action potential. These dynamic PLM-dependent changes in CaV1.2 channel gating are expected to directly alter cell excitability, Ca2+ homeostasis and cardiac function.

MATERIALS AND METHODS

Coimmunoprecipitations and Western blot analysis

Antibodies

The mouse monoclonal anti-Flag M2 (Sigma-Aldrich, St. Louis, MO); rabbit polyclonal anti-CaV1.2 (Calbiochem, San Diego, CA); mouse...
monoclonal anti-CaV1.2 (clone L57/46, UC Davis/National Institutes of Health NeuroMab Facility; rabbit polyclonal anti-CaV2.1 (Chemicon, Temecula, CA); rabbit polyclonal anti-PLM (C2) (a generous gift from Dr. J. Y. Cheung, Thomas Jefferson University). Pre-immune mouse IgG, pre-immune rabbit IgG, and protein A/G plus agarose were from Santa Cruz Biotech (Santa Cruz, CA). Isolation of guinea pig ventricular myocytes and subsequent coimmunoprecipitation experiments were carried out as described previously (16). Coimmunoprecipitation experiments carried out on HEK293 cell lysates were carried out as described previously (21).

Whole-cell patch-clamp electrophysiology

Whole-cell patch-clamp experiments were carried out as described previously (22). Briefly, HEK293 cells were transiently transfected with cDNAs encoding CaV1.2 (23) or CaV2.1 (GenBank No. AF055477; generously provided by Dr. Lucie Parent), the auxiliary subunits αdβ2 (24) and β1β2 (25), GFP (Clontech Laboratories, Mountain View, CA) and either PLM/ pAdTrack or empty pAdTrack vector (both generously provided by Dr. Joseph Y. Cheung). The majority of recordings used a bath solution containing (mmol/L): 130 N-methyl-D-glutamine (NMG)-aspartate, 10 HEPES, 10 4-aminopyridine, 10 glucose, 1 MgCl2, and 5 or 10 CaCl2/BaCl2 (Ba2+ was used unless noted otherwise). Internal solutions contained (mmol/L): 140 NMG-MeSO3, 5 EGTA, 1 MgCl2, 4 Mg-ATP, and 10 HEPES. For recording CaV1.2 currents activated by a human cardiac action potential waveform, the bath solution contained (mmol/L): 140 NMG-Cl, 10 NMG-HEPES and 5 CaCl2, and the internal solution contained (mmol/L): 104 NMG-Cl, 14 Creatine-PO4, 6 MgCl2, 10 NMG-HEPES, 10 NMG-EGTA, 5 Tris-ATP, 0.3 Tris-GTP. Data were acquired using either a HEKA EPC-9/2 amplifier and PULSE/PULSEFIT software (ALA Scientific Instruments, Bethesda, MD). Leak and capacitive transients were corrected by compensation and series resistance was compensated at 70%. Tail currents were expressed as the mean ± SD, where p < 0.05 is considered significant.

RESULTS

Association of PLM with native and exogenously expressed CaV1.2 channels

Recent work showing that PLM plays an important role in regulating excitability and Ca2+ homeostasis in myocardium (15–19,26) prompted us to investigate the potential role PLM may play in regulating cardiac CaV1.2 channels. We carried out coimmunoprecipitation (co-IP) experiments to test for the potential association of PLM with CaV1.2 channels using solubilized membranes from guinea pig cardiac myocytes and lysates of transfected HEK293 cells (Fig. 1). As expected, CaV1.2 and PLM were observed when the solubilized membranes were subjected to Western blot analysis (Fig. 1 A, lane 1). Immunoprecipitation (IP) experiments using the anti-CaV1.2 antibodies coprecipitated PLM demonstrating an association between these proteins (Fig. 1 A, lane 4). Both CaV1.2 and PLM were absent in the insoluble and post-IP fractions (Fig. 1 A, lanes 2 and 3) and neither protein was detected in the IgG control lane (Fig. 1 A, lane 5). The plasma membrane Ca2+-ATPase (PMCA) has been shown previously to have no interaction with PLM (16), was used here as a negative control (Fig. 1 B). As shown previously (15,16,19), we found that PLM was able to co-IP with NCX1 (Fig. 1 C) or NKA α1 subunit (not shown). These results indicate that the association between CaV1.2 and PLM is specific.

We were interested determining whether PLM was capable of associating with other members of the CaV superfamily. Therefore, we carried out co-IP experiments using HEK293 cells transiently expressing either Flag-tagged CaV1.2 (Fig. 1 D), or CaV2.1 (Fig. 1 E) channels plus PLM. Consistent with our findings using guinea pig membranes, we found that co-IP was obtained for PLM with an anti-Flag Ab only in cells expressing both Flag-tagged CaV1.2 channels and PLM (Fig. 1D), whereas no PLM co-IP was observed when using the CaV2.1 Ab (Fig. 1 E). The reverse experiment of using the PLM Ab showed a co-IP of CaV1.2, but not CaV2.1 (not shown). Thus, PLM seems to specifically interact with CaV1.2 channels.

PLM specifically alters CaV1.2 channel gating

The association between PLM and CaV1.2 channels suggests the potential for gating modulation, which was examined using the HEK293 cell heterologous expression system. Whole-cell recordings from HEK293 cells expressing CaV1.2 (Fig. 2, A–C) or CaV2.1 (Fig. 2, D–F) channels in the absence (empty vector) and presence of PLM were conducted. We found that PLM slows CaV1.2 channel activation and left-shifts the activation versus voltage relationship (Fig. 2, A and C). These currents were generated by 25 ms depolarizing pulses to minimize the effect of voltage-dependent inactivation in our experiments (see below), and Ba2+ was used as the charge carrier to eliminate Ca2+-dependent inactivation (12,27–29). The sample traces in Fig. 2 A show normalized currents (at −10 mV) from different cells (± PLM) to highlight the effect of PLM to slow activation. The activation versus voltage relationship measured from tail currents was left-shifted ≥20 mV so that the half activation voltage (V1/2) changed from 25.0 to 4.1 mV with PLM and the relationship exhibited a much steeper slope factor (e-fold/23.3 vs. 12.9 mV) (Fig. 2 C). Consequently, at +10 mV, <40% of the channels were activated in the absence of PLM, whereas nearly 70% of the channels are activated in the presence of PLM. In contrast to CaV1.2, PLM failed to alter the activation kinetics (Fig. 2 D) or the voltage-dependence of activation of neuronal CaV2.1 (Fig. 2, E and F) or CaV2.2 (not shown) channels. Thus, PLM selectively associates with and modulates CaV1.2 but not CaV2.1 and CaV2.2 channels.

The voltage-dependence of PLM-induced slowed CaV1.2 channel activation was determined using currents generated during the I/V protocol (Fig. 3). The speed of activation was quantified by measuring the time required for the current
to increase from 10 to 90% of the peak current ($T_{10-90}$). Activation was significantly slowed at hyperpolarized voltages close to the threshold for channel activation, but no significant effect was observed at voltages $\geq$ 0 mV (Fig. 3 B). It is possible that the effect of PLM on activation kinetics is voltage dependent. However, inactivation has been shown to impact the measurement of activation kinetics (30), so this interpretation is confounded by our observation that PLM expression also enhances voltage dependent inactivation at more depolarized voltages (see below).

**PLM slows deactivation of CaV1.2 channels**

Activation and deactivation share the same gating machinery operating in opposite directions. Thus, the PLM effect on activation may also be reflected in altered deactivation kinetics. Fig. 4 A shows superimposed tail currents from CaV1.2 channels $\pm$ PLM to highlight the effect of PLM on deactivation. Tail current deactivation was fit using a single exponential equation to determine the deactivation time constant ($\tau_{\text{Deact}}$). Using the I/V protocol (Fig. 2). $\tau_{\text{Deact}}$ was determined following a range of depolarizing voltages and showed an increase with voltage that was enhanced by PLM (Fig. 4 B). $\tau_{\text{Deact}}$ was significantly larger at all voltages for (+)PLM compared with (−)PLM. These findings suggest that the interaction of PLM with CaV1.2 channels slows channel closing.

It is well established that deactivation is slowed when CaV1.2 channels switch from mode 1 (low $P_o$) into mode 2 (high $P_o$) gating (31,32) and this gating switch is both
time- and voltage-dependent (32). Thus, if PLM enhances mode switching, the effect should depend on both step duration and voltage. We tested this prediction by measuring the time course (0–250 ms step duration) for slowed deactivation at $-100$ mV (Fig. 4 C), $+30$ mV (Fig. 4 D), and $+80$ mV (Fig. 4 E). As expected, $\text{CaV}_{1.2}$ currents in the absence of PLM exhibited a moderate ($+80$ mV) time- and voltage-dependent slowing of deactivation (increased $\tau_{\text{Deact}}$), suggesting that some channels are transitioning from mode 1 to mode 2 gating (Fig. 4, C–E). In the presence of PLM, $\tau_{\text{Deact}}$ was significantly larger than ($-\text{PLM}$) at $+80$ mV for pulse durations $\geq 80$ ms, and for $+30$ mV at 250 ms. It is noteworthy that changes in $\tau_{\text{Deact}}$ were not correlated with current amplitude, indicating that the PLM-induced changes do not result from voltage-clamp errors. Thus, PLM directly alters $\text{CaV}_{1.2}$ gating by enhancing the voltage- and time-dependent slowing of deactivation, consistent with PLM promoting mode 2 gating. Additional single channel experiments are required to confirm this intriguing hypothesis.

**PLM specifically enhances voltage-dependent inactivation of $\text{CaV}_{1.2}$ channels**

Examination of the records in Fig. 3 A shows an apparent enhancement of voltage-dependent inactivation (VDI) at the most depolarized voltage (20 mV) by PLM. This was further investigated using longer voltage steps of 300 ms (Fig. 5 A) and 1000 ms (Fig. 5 C) where VDI is larger and more easily measured. $\text{CaV}_{1.2}$ channels exhibit little inactivation at the end of a 300 ms step pulse from $-100$ to $+100$ mV, and the data were fitted with a Boltzmann equation (smooth lines) to generate the half activation voltage ($V_h$) and slope factor ($k$). For $\text{CaV}_{1.2}$: $V_h = 25.0 \pm 2.2$ mV and $k = 23.3 \pm 1.57$ for ($-\text{PLM}$) and $V_h = 4.1 \pm 0.95$ mV and $k = 12.9 \pm 0.81$ for ($+\text{PLM}$). $V_h$ and $k$ for $\text{CaV}_{1.2}$ (but not $\text{CaV}_{2.1}$) currents are significantly different when measured in the absence and presence of PLM ($p < 0.05, n = 9$).

**FIGURE 2** PLM alters the kinetics and voltage-dependence of $\text{CaV}_{1.2}$ channel activation. $\text{Ba}^{2+}$ currents evoked by 25 ms depolarizing steps to $-10$ mV in the absence ($-\text{PLM}$; black) and presence ($+\text{PLM}$; gray) for (A) $\text{CaV}_{1.2}$ and (D) $\text{CaV}_{2.1}$ channels were normalized to current amplitudes measured at the end of each step. Current-voltage relationships were generated by a series of 25 ms step pulses ranging from $-60$ to $+50$ mV from a holding potential of $-100$ mV for (B) $\text{CaV}_{1.2}$ and (E) $\text{CaV}_{2.1}$. Steady-state activation curves measured from tail currents are left-shifted in the presence of PLM for (C) $\text{CaV}_{1.2}$, but not (F) $\text{CaV}_{2.1}$ channels. Tail currents were measured at $-50$ mV after a series of 25 ms step pulse 5 from $-100$ to $+100$ mV, and the data were fitted with a Boltzmann equation (smooth lines) to generate the half activation voltage ($V_h$) and slope factor ($k$). For $\text{CaV}_{1.2}$: $V_h = 25.0 \pm 2.2$ mV and $k = 23.3 \pm 1.57$ for ($-\text{PLM}$) and $V_h = 4.1 \pm 0.95$ mV and $k = 12.9 \pm 0.81$ for ($+\text{PLM}$). $V_h$ and $k$ for $\text{CaV}_{1.2}$ (but not $\text{CaV}_{2.1}$) currents are significantly different when measured in the absence and presence of PLM ($p < 0.05, n = 9$).
suggesting that PLM may enhance the transition rate from $O \rightarrow I$ (see below). $\text{Ca}^{2+}$ influx via $\text{CaV}_{1.2}$ channels induces $\text{Ca}^{2+}$-dependent inactivation (CDI), but we found that PLM does not alter CDI (Fig. S1 in the Supporting Material). Therefore, it seems PLM specifically enhances the speed of VDI.

In addition to a dynamic role in shaping the cardiac action potential, inactivation is critical for establishing the number of available channels for activation and, as a result, the amount of $\text{Ca}^{2+}$ entering the cell during each cardiac cycle (9,10). We used a standard 3-pulse protocol to determine the voltage-dependence of channel availability in the absence and presence of PLM. Peak currents were measured at 0 mV before and after 30-s steps to voltages ranging from −110 to 0 mV. The postpulse/prepulse ratio ($I/I_{\text{max}}$) plotted against voltage (i.e., the $h_{30}$ curve) is depicted in Fig. 5 F. PLM steepens the voltage-dependence (Boltzmann slope factor) and induces a ~10 mV depolarizing shift in the $h_{30}$ curve. Similar experiments carried out on cells expressing neuronal $\text{CaV}_{2.1}$ channels in the absence and presence of PLM exhibited no change in either the half inactivation voltage or the slope factor (not shown). Thus, PLM seems to have the capacity to selectively increase the number of available $\text{CaV}_{1.2}$ channels at depolarized resting membrane potentials.

**PLM promotes a deep inactivated state from which recovery is slow**

To better understand how PLM affects inactivation of $\text{CaV}_{1.2}$ channels, we investigated the recovery from inactivation in the absence and presence of PLM. Inactivation was induced by 500 ms steps to +10 mV and quantified as the ratio of peak postpulse to prepulse currents evoked by steps to 0 mV (Fig. 6 A). The recovery from inactivation time course was fit by a single exponential function to determine the recovery $\tau$ (Fig. 6 B). $\text{CaV}_{1.2}$ channels inactivated by 58% in (−)PLM and 64% in (+)PLM over the 500 ms step, and the recovery $\tau$ was nearly identical for $\text{CaV}_{1.2}$ channels ± PLM (Fig. 6 B). However, the magnitude of recovery was significantly reduced in the presence of PLM, suggesting that PLM places the $\text{CaV}_{1.2}$ channels into a deeper inactivated state from which recovery is slow. This hypothesis was tested using longer (20 s) voltage steps to place more channels into the deeper inactivated state (Fig. 6 C). The fraction of inactivating current resulting from 20 s depolarizing steps to +10 mV was significantly larger in the presence of PLM (98%) than was observed in the absence of PLM (87%) (Fig. 6 D). Interestingly, 36% of the inactivated $\text{CaV}_{1.2}$ channels (−)PLM had recovered from inactivation by the first measurement point (30 ms), whereas only 12% of the channels (+)PLM had recovered by this time. This fast recovery is consistent with the recovery $\tau$ (−20 ms) measured after the 500 ms inactivating steps (Fig. 6 B), and further supports our hypothesis that PLM enhances the transition of channels into a deep inactivated state. $\text{CaV}_{1.2}$ channels ± PLM recovered substantially more slowly after the 20-s step compared to the 500-ms step (10 s vs. 20 ms), but the recovery rates from the deep inactivated state were indistinguishable in the absence and presence of PLM (Fig. 6 D). Therefore, PLM increases the fraction of channels that enter the deep...
inactivated state, but does not alter the rate at which the channels exit from that state.

PLM promotes dynamic changes in Ca\(^{2+}\) entry

PLM-induced slowing of activation and enhancement of inactivation are expected to combine to reduce Ca\(^{2+}\) entry, yet the slowing of deactivation is expected to increase Ca\(^{2+}\) entry during the cardiac action potential (4). Because PLM-induced changes in activation, inactivation, and deactivation all appear to be voltage- and time-dependent, it is likely that PLM temporally changes Ca\(^{2+}\) entry during membrane depolarization and repolarization. The effect of PLM-induced gating changes on Ca\(^{2+}\) flux can be demonstrated by comparing difference currents (Fig. 7, A–E) recorded in external solution with 10 mM Ca\(^{2+}\) as charge carrier. The integral of difference current provides a quantitative measure of the changes in the number of Ca\(^{2+}\) ions permeating the channels ± PLM at various time points during the depolarizing step (Fig. 7 F). The integral is negative at potentials <10 mV, indicating that Ca\(^{2+}\) influx is reduced during the depolarizing step in the presence of PLM, which results from slowed activation. The difference current integral becomes positive at potentials >10 mV because slowed deactivation greatly increases Ca\(^{2+}\) influx after the membrane is repolarized.

PLM increases Ca\(_{\text{V}1.2}\) currents during the repolarization phase of the cardiac action potential

It is important to note that the changes in ionic flux elucidated by the difference currents (Fig. 7) clearly highlight important temporal aspects of PLM-induced changes in Ca\(_{\text{V}1.2}\) channel gating. For example, slowed activation seems to play a dominant role at reducing influx during early depolarization, but slowed deactivation greatly increases flux upon repolarization. Given the time and voltage-dependent slowing of deactivation, we postulated that PLM would increase calcium entry during the repolarization phase of the cardiac action potential (cAP) after the ~200 ms plateau phase. To test this, we recorded Ca\(_{\text{V}1.2}\) currents ± PLM generated by a human cAP waveform in 5 mM Ca\(^{2+}\) (4,33). The cAP-generated currents ± PLM exhibited a stable plateau phase and monotonic decline during repolarization. The effect of PLM on Ca\(^{2+}\) flux during the repolarization phase was assessed by normalizing currents ± PLM during the plateau phase, which showed that the duration of cAP induced-currents is increased when Ca\(_{\text{V}1.2}\) channels are expressed with PLM (Fig. 8 A). This enhancement was verified by integrating the normalized current over the last 200 ms of the cAP (225–425 ms), which showed a significant increase (+)PLM. Thus, the interaction of PLM with Ca\(_{\text{V}1.2}\) channels significantly increases Ca\(^{2+}\) influx during the repolarization phase of the cAP, which suggests that PLM plays a role in setting cAP duration and the QT interval.

DISCUSSION

We believe our findings are the first to show that PLM associates with and modulates the gating kinetics of Ca\(_{\text{V}1.2}\) (L-type), but not Ca\(_{\text{V}2.1}\) (P/Q-type) or Ca\(_{\text{V}2.2}\) (N-type; not shown) channels. PLM was found to alter four important gating processes of Ca\(_{\text{V}1.2}\) channels: 1) activation kinetics were slowed at voltages near the threshold for channel
activation; 2), deactivation kinetics were slowed following voltage steps comparable in magnitude and duration to that of the human cardiac action potential (cAP); 3), VDI was enhanced at potentials corresponding to the plateau phase of the cAP; and 4), an increased number of channels enter a deep inactivated state from which recovery is slow. Below, we discuss these PLM-induced changes in CaV1.2 gating in greater detail and how these PLM-dependent changes are likely to affect the cardiac action potential and contractility.

**PLM-induced changes in inactivation**

Recent publications have presented evidence highlighting the importance of calcium channel inactivation to cardiac function (2–4,34–39), which motivated our investigation of a potential PLM-dependent modulation of VDI. Over step depolarizations similar to the plateau phase of the cAP, PLM accelerates the rate of and increases the magnitude of VDI. The accelerated VDI at depolarized voltages (i.e., +20 mV) suggests the PLM increases the rate constant governing transition from the open (O) to inactivated (I) state, whereas the increase in VDI magnitude suggests that PLM does little to the I → O rate. This latter point is substantiated by the observation that PLM enhances the fraction of inactivating channels, which seems to be correlated with increased occupancy of a deep inactivation state from which recovery is slow (τ > 10 s). We also found that the co-expression of PLM affects steady-state inactivation by shifting the half inactivation voltage −10 mV depolarized and increasing voltage-dependence inactivation (smaller Boltzmann slope factor). The combination of right-shifted V1/2 and steeper voltage dependence by PLM increases the number of available channels at voltages near resting membrane potential (−80 to −50 mV). Thus, the association of PLM with CaV1.2 channels may play an important role in regulating the number of available channels under physiological and pathological conditions.

**PLM-induced slowing of activation and deactivation**

We observed a PLM-induced slowing of activation, but this slowing is strongly voltage-dependent and most easily
observed at potentials <0 mV. The voltage-dependence of slowed activation may result from the concurrent PLM-induced enhancement of inactivation that is more prevalent at depolarized voltages. However, during preliminary mutagenesis experiments we have identified PLM mutants that fail to enhance VDI, but still selectively slow activation at voltages <0 mV (K. Guo, X. Wang, G. Gao, C. Huang, K. S. Elmslie, B. Z. Peterson, unpublished data). This supports the idea that the PLM effect on activation is strongly voltage-dependent. Given that the \( C \rightarrow O \) transition is voltage independent (4,36), the mutation findings suggest that the effect of PLM is to decrease the voltage-dependent \( C \rightarrow C \) transitions, which predicts that \( \text{CaV}_{1.2} \) channel On-gating currents will be slowed by PLM. Those experiments are ongoing currently.

\( \text{CaV}_{1.2} \) channels have been shown to exhibit modal gating behavior (31,40), where the channels exist in one of three gating modes: mode 1 gating is characterized by short

![Figure 6](https://example.com/figure6.png)

**FIGURE 6** PLM promotes a deep inactivated state from which recovery is slow. (A) Recovery from a short inactivating pulse. Ba\(^{2+}\) currents were evoked using a three-pulse protocol with 100 ms pre- and postpulses to 0 mV bracketing a 500-ms inactivating pulse to +10 mV. The interval between the inactivation pulse and postpulse (recovery time) was increased to determine the recovery time for inactivation. The interval between sweeps was 30 s. \( \text{CaV}_{1.2} \) currents (±PLM were scaled to peak pre-pulse current and superimposed for easier comparison. (B) The ratio of Ba\(^{2+}\) currents measured before and after 500 ms inactivating steps (\( I_{\text{post}}/I_{\text{pre}} \) ratio) is plotted versus recovery time. Smooth lines are single exponential fits; \( \tau = 30.6 \pm 5.1 \) and 27.3 ± 4.3 ms for (–)PLM and (+)-PLM respectively (n = 5, not significantly different). (C) Recovery from a long conditioning pulse. Currents generated as described for A except inactivation was generated by a single 20-s step to +10 mV and the pre- and post-pulse voltages were −10 mV. Recovery from inactivation was determined by a train of postpulses (shown). The 20-s inactivating pulse is not shown, but its position is indicated by a dashed line after the prepulse in pulse protocol. (D) The \( I_{\text{post}}/I_{\text{pre}} \) is plotted versus recovery time for 20-s inactivating data. Smooth lines are single exponential fits: \( \tau = 10.1 \pm 1.9 \) s and 12.5 ± 1.8 s for (–)PLM and (+)-PLM, respectively (n = 7, not significantly different). Asterisks above data points indicate significant difference in the fraction of recovered current between (–) and (+) PLM at each time point.

![Figure 7](https://example.com/figure7.png)

**FIGURE 7** Dynamic effects of PLM on ionic flux. (A–E) Difference Ca\(^{2+}\) currents (10 mM Ca\(^{2+}\) as charge carrier) were calculated from averaged currents \( (n = 10–12) \) from cells recorded in the absence and presence of PLM for 25 ms at potentials from −10 to +30 mV. (F) Integrated difference currents (gray shading) versus step voltage were used to quantify voltage-dependent changes in Ca\(^{2+}\) flux induced by PLM.
open times, low $P_O$ and represents the predominant mode of gating; mode 2 gating occurs much less frequently and is characterized by very long open times and high $P_O$; and mode 0 gating is observed when the channel fails to open during a depolarizing step. Occupancy of mode 2 gating greatly slows CaV1.2 channel deactivation as measured by whole-cell tail currents (32,41,42)(5,43). Long depolarizations that mimic the cardiac action potential can induce CaV1.2 channels to switch from mode 1 into mode 2 gating (32), but these depolarizations have only modest effects to slow deactivation in HEK293 cells expressing CaV1.2 channels (32). Although single channel experiments are needed to confirm that PLM-enhancement of slowed deactivation results from mode switching, it seems likely that the interaction of PLM with CaV1.2 channels in cardiac myocytes regulates the voltage- and time-dependent mode switching to enhance Ca$^{2+}$ influx.

Role of PLM in regulating excitability and contractility

The precise physiological role of PLM is not yet resolved partly because the relationships between PLM and NKA (6,15,44–47), NCX (7,48,49) and, as we report here, CaV1.2 channels, are complex, and partly because there exists some variability in the literature regarding the effect PLM expression has on contractility (7,44,45,48,50). Results from isolated myocytes suggest PLM induces a decrease in contractile strength in low Ca$^{2+}$ and has no effect on contractility in physiological Ca$^{2+}$ (7,48,50). However, results from whole heart suggest that PLM increases contractility in physiological Ca$^{2+}$ (45).

Our finding that PLM modulates the gating of cardiac CaV1.2 channels further increases the complexity of the relationship between PLM expression and contractility. Changes in calcium channel gating, such as impaired VDI and slowed deactivation, profoundly affect cardiac function (2–4,34–38,51). PLM-dependent slowed deactivation seems to increase Ca$^{2+}$ entry during the repolarization phase of the cAP and may account for the observed decrease in left ventricular pressure measured from PLM knockout mice (45). In addition, by increasing the fraction of channels occupying the deep inactivated state, PLM may help protect the heart under conditions such as ischemia or tachycardia where the channels undergo prolonged depolarization and the myocytes are vulnerable to Ca$^{2+}$ overload.

SUPPORTING MATERIAL

A figure is available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)01799-8.

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