MODULATION OF CaV1.2 CHANNEL ACTIVITY:
ROLE OF DOMAIN III

A Thesis in
Physiology

by
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ABSTRACT

Cardiac excitation-contraction coupling (ECC) is the fundamental cellular mechanism responsible for the continuous contraction of the heart. PLM, a small membrane protein of the FXYD family, has recently been shown to regulate the activity of both the Na\(^+\)/K\(^+\)-ATPase (NKA) and the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX), two among the numerous molecular components of cardiac ECC. To date, few studies have examined the effect of PLM on the modulation of the activities of other components of cardiac ECC, such as the L-type cardiac Ca\(^{2+}\) channel. Preliminary data obtained by our lab demonstrated that PLM does in fact associate with Ca\(_V\)1.2 and seem to affect Ca\(_V\)1.2 (L-type channel) activation but not Ca\(_V\)2.2 (N-type channel) activation. DIII of Ca\(_V\)1.2 has been shown to play a vital role in Ca\(_V\)1.2 channel regulation by various small organic molecules and toxins, such as the dihydropyridines and calcicludine. Therefore, I hypothesized that PLM’s effect on Ca\(_V\)1.2 is mediated through its interaction with DIII of Ca\(_V\)1.2. Six chimeric Ca\(^{2+}\) channels (NNLL, LLNN, NLLL, LNLL, LLNL, and LLLN) were constructed to identify with which domain of Ca\(_V\)1.2 PLM interacts to regulate channel activity. Channel activation, inactivation, and deactivation were examined via whole-cell patch-clamp electrophysiology in the absence and presence of PLM in Ca\(_V\)1.2, LLNL, and Ca\(_V\)2.2. Despite the importance of domain III in Ca\(_V\)1.2 channel regulation by other small organic molecules and toxins, initial results obtained from this study does not seem to suggest that domain III plays a significant role in PLM modulation of Ca\(_V\)1.2 channel activity.
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Chapter 1

Introduction

Today, cardiovascular disease is the leading cause of death world wide. It accounts for approximately 30% of all deaths, including nearly 40% in high-income countries and about 28% in low- and middle-income countries. In the US alone, 249,000 patients underwent coronary bypass surgeries in 2004. Furthermore, 1.279 million percutaneous coronary intervention procedures were also performed. With a total cost of roughly 20 billion dollars a year, the use of coronary revascularization has been the subject of extensive economic evaluation (1).

1.1 Cardiac Excitation-Contraction Coupling

The average human heart beats approximately 70 times and pumps 5 liters of blood per minute to the rest of the body, delivering oxygen, nutrients, hormones, and other chemicals essential for life. The fundamental cellular mechanism responsible for the continuous contraction of the heart is a process called excitation-contraction coupling (ECC). Cardiac ECC (Fig. 1.1) is initiated when a wave of depolarization from the sinoatrial node propagates through the atrium and the atrio-ventricular node. Upon reaching the ventricle, the wave of depolarization electrically excites ventricular myocytes, opening voltage-dependent Na⁺ channels. The rapid influx of Na⁺ ions from outside the cell further depolarizes the myocyte, activating voltage-dependent Ca²⁺ channels to open.
Ca²⁺ ions enter the myocyte mainly through L-type Ca²⁺ channels. In the cytosol, Ca²⁺ ions bind to and open ryanodine receptors (RyRs) in the membrane of the sarcoplasmic reticulum (SR), inducing additional Ca²⁺ release from the SR. Ca²⁺ entry from outside the cell through voltage-dependent Ca²⁺ channels along with Ca²⁺ released from the SR increases the intracellular concentration of free Ca²⁺ ions ([Ca²⁺]_{free}), which bind to myofilaments within the myocytes initiating cardiac muscle contraction.

Figure 1.1: Ca²⁺ transport in ventricular myocytes. Inset shows the time course of an action potential, Ca²⁺ transient and contraction measured in a rabbit ventricular myocyte at 37 °C. NCX, Na⁺/Ca²⁺ exchange; ATP, ATPase; PLB, phospholamban; SR, sarcoplasmic reticulum (2).

In order for cardiac muscle to relax and be able to contract again, myocytes must return to their original or resting intracellular ionic balance. This means that both Na⁺ and Ca²⁺ need to be extruded from the cell. The Na-K-ATPase (NKA) is the main route of Na⁺ ion efflux from the cell. However, there are four different pathways involved in
the transport of Ca\(^{2+}\) ions out of the cell: SR Ca\(^{2+}\)-ATPase, sarcolemmal Na\(^+/Ca^{2+}\) exchanger (NCX), sarcolemmal Ca\(^{2+}\)-ATPase, and mitochondrial Ca\(^{2+}\) uniporter (3). The extrusion of Ca\(^{2+}\) out of the cell causes the intracellular \([\text{Ca}^{2+}]_{\text{free}}\) to decrease, resulting in dissociation of Ca\(^{2+}\) from and relaxation of myofilaments. This is, of course, a very simplified description of cardiac ECC. In actuality, cardiac ECC requires a delicate balance of different ionic fluxes and the interaction of numerous cellular components. Dysregulation of any one of the cellular components involved in ECC would disrupt the delicate ionic balance and lead to cardiac contractile dysfunction and arrhythmogenesis (2).

1.1.1 Voltage-Dependent Na\(^+\) Channels

When a wave of depolarization reaches a ventricular myocyte, the first cellular component of ECC activated is the voltage-dependent or voltage-gated Na\(^+\) channels that are responsible for the rapid upstroke of the cardiac action potential (Fig. 1.1, inset). Voltage-dependent Na\(^+\) channels are integral membrane proteins that are activated from a closed state to an open state by a change in membrane voltage. They allow the passage of ions, the principle permeating ion being Na\(^+\), across the plasma membrane. Nine members of the voltage-dependent Na\(^+\) channel family (Na\(_{\text{v}}\)1.1 through Na\(_{\text{v}}\)1.9) with similar structure and functional properties from different cell types have so far been characterized, all of which participate in the initiation and propagation of electrical impulses in excitable cells. Voltage-dependent Na\(^+\) channels are also expressed at low levels in nonexcitable cells. Their function in these cells, however, is unclear (4).
The basic structure of the voltage-dependent Na\(^+\) channel (Fig. 1.2) consists of a pore-forming \(\alpha\) subunit of approximately 260 kDa associated with one or more auxiliary \(\beta\) subunits, of which four isoforms (\(\beta_1 - \beta_4\)) have been found in the heart (4). As the major component of voltage-dependent Na\(^+\) channels, the \(\alpha\) subunit is formed by four homologous domains (DI – DIV), each composed of six \(\alpha\)-helical transmembrane segments (S1 – S6). The fourth transmembrane segment (S4) of each domain, believed to be the voltage sensor, contains positively charged amino acid residues at every third position (5). The loop connecting transmembrane segments S5 and S6 in each domain lines the outer, narrow entrance to the channel. The \(\beta\) subunit functions to modify the kinetics and voltage dependence of channel gating. It is also involved in channel localization and channel interaction with cell adhesion molecules, the extracellular matrix, and intracellular cytoskeleton (4).

Figure 1.2: Transmembrane organization of sodium channel subunits. The primary structures of the subunits of the voltage-gated ion channels are illustrated as transmembrane-folding diagrams. Cylinders represent probable \(\alpha\)-helical segments. Bold lines represent the polypeptide chains of each subunit, with lengths approximately proportional to the number of amino acid residues in the brain sodium channel subtypes. The extracellular domains of the \(\beta_1\) and \(\beta_2\) subunits are shown as immunoglobulin-like folds (4).
The principle voltage-dependent Na\(^+\) channel isoform expressed in cardiac myocyte is Na\(_V\)1.5 with a voltage of activation (\(V_a\)) of -47 mV (4), the voltage at which half of the maximum current is elicited through these channels. Because the extracellular Na\(^+\) ion concentration is higher, Na\(^+\) ions flow down its electrochemical gradient into the cell, generating an inward Na\(^+\) current. Na\(^+\) channels inactivate very rapidly at positive potentials (within a few milliseconds), a state in which the channels are impermeable to ions and cannot be activated to open by further depolarizations until they recover, limiting the gain of intracellular Na\(^+\) concentration to just 6 – 15 µmol/L (6) when the resting intracellular Na\(^+\) concentration is around 150 mmol/L (7). Nevertheless, the brief yet profuse influx of Na\(^+\) ions further depolarizes the cell, activating other voltage-dependent channels.

1.1.2 Voltage-Dependent Ca\(^{2+}\) Channels

Responsible for the plateau phase of the cardiac action potential, voltage-dependent Ca\(^{2+}\) channels are members of a gene superfamily of transmembrane ion channel proteins that includes voltage-dependent K\(^+\) and Na\(^+\) channels. Therefore, like them, voltage-dependent Ca\(^{2+}\) channels are integral membrane proteins that are activated by a change in membrane voltage, allowing the passage of ions, principally Ca\(^{2+}\), across the plasma membrane. Ten members of the voltage-dependent Ca\(^{2+}\) channel family (Ca\(_V\)1.1 – Ca\(_V\)1.4, L-type; Ca\(_V\)2.1, P/Q-type; Ca\(_V\)2.2, N-type; Ca\(_V\)2.3, R-type; Ca\(_V\)3.1 – Ca\(_V\)3.3, T-type) from different cell types have been characterized in mammals, serving distinct roles in cellular signal transduction (8).
Voltage-dependent Ca\textsuperscript{2+} channels are multimeric complexes that consist of \(\alpha_1\), \(\beta\), \(\alpha_2/\delta\), and sometimes \(\gamma\) subunits. The largest \(\alpha_1\) subunit contains all of the structural determinants required for ion permeation, voltage-dependent gating, and modulation by drug binding. Like voltage-dependent Na\textsuperscript{+} channels, the pore-forming \(\alpha_1\) subunit of voltage-dependent Ca\textsuperscript{2+} channels is formed by four homologous domains (DI – DIV), each composed of six \(\alpha\)-helical transmembrane segments (S1 – S6). The fourth transmembrane segment (S4) of each domain contains positively charged amino acid residues at every third position, and functions as a voltage sensor. The loop connecting transmembrane segments S5 and S6 in each domain contains a highly conserved negatively charged glutamate residue. Together, these four glutamate residues form a binding site for Ca\textsuperscript{2+} ions called the selectivity filter that enables the channel to preferentially conduct Ca\textsuperscript{2+} ions under physiological conditions where Na\textsuperscript{+} is in excess (9).

Figure 1.3: Subunit structure of Ca\textsubscript{1,1} channels. The subunit composition and structure of calcium channels purified from skeletal muscle are illustrated. The model is updated from the original description of the subunit structure of skeletal muscle calcium channels. This model fits available biochemical and molecular biological results for other Ca\textsubscript{1,1} channels and for Ca\textsubscript{v,2} channels. Predicted \(\alpha\) helices are depicted as cylinders. The lengths of lines correspond approximately to the lengths of the polypeptide segments represented (8).
Cardiac myocytes express two classes of voltage-dependent Ca$^{2+}$ channels, L-type and T-type. Since the Ca$^{2+}$ current ($I_{Ca}$) through T-type channels is negligible in most ventricular myocytes, $I_{Ca}$ generally refers to current through L-type Ca$^{2+}$ channels (2) that have a $V_a$ of -17 mV ($CaV_{1.2}$ channels in 2 mM Ca$^{2+}$) (8). Voltage-dependent Ca$^{2+}$ channels open to allow the flow of Ca$^{2+}$ ions down its electrochemical gradient into the cell, generating an inward $I_{Ca}$. Like Na$^+$ channels, Ca$^{2+}$ channels exhibit voltage-dependent inactivation as well. However, they inactivate at a slower rate, within a few hundred milliseconds at depolarized membrane potentials. Voltage-dependent Ca$^{2+}$ channels are also subject to regulation by Ca$^{2+}$-dependent inactivation. As Ca$^{2+}$ ions enter the cell through Ca$^{2+}$ channels, they interact with the channels to inhibit further Ca$^{2+}$ entry, limiting Ca$^{2+}$ influx via $I_{Ca}$ to $\approx$10 µmol/L (10). Furthermore, studies have shown that calmodulin bound to the carboxy terminus of Ca$^{2+}$ channels to be the mediator of Ca$^{2+}$-dependent inactivation (11, 12). During a typical twitch, $\approx$60 µmol/L of Ca$^{2+}$ entry is required to achieve a contractile force of $\approx$40% of maximum (3). When $I_{Ca}$ increases total intracellular Ca$^{2+}$ concentration by only 10 µmol/L, where does the rest of the required Ca$^{2+}$ come from?

1.1.3 Sarcoplasmic Reticulum Ryanodine Receptors

By far, the most widely accepted mechanism of intracellular Ca$^{2+}$ release is Ca$^{2+}$-induced Ca$^{2+}$ release from the sarcoplasmic reticulum (SR) (2). L-type voltage-dependent Ca$^{2+}$ channels are located primarily at sarcolemmal-SR junctions, where the SR Ca$^{2+}$ release channels, also known as ryanodine receptors (RyRs), localize as well (13). At the sarcolemmal-SR junctions, also called couplons, RyRs are organized into
large Ca\(^{2+}\) release complexes of more than 100 RyRs and up to 200 nm in diameter (14). The opening of one L-type Ca\(^{2+}\) channel (or the binding of 2 – 4 Ca\(^{2+}\) ions to a RyR) is sufficient for complete activation of Ca\(^{2+}\) release at a particular couplon because RyRs in a couplon are activated either by Ca\(^{2+}\) binding or coupled gating between RyRs. However, the presence of 10 to 25 voltage-dependent Ca\(^{2+}\) channels for every 100 RyRs at a couplon guarantees couplon activation, and therefore Ca\(^{2+}\) release at a couplon to be all or none (2). Termination of SR Ca\(^{2+}\) release is achieved either by RyR inactivation (15, 16) or RyR adaptation to high local intracellular Ca\(^{2+}\) concentration (17).

The RyR is both the SR Ca\(^{2+}\) release channel and a scaffolding protein that localizes numerous key regulatory proteins to the couplon, such as calmodulin, FK-506 binding protein, cyclic-AMP-dependent protein kinase (PKA), phosphatases, and sorcin (2). These proteins participate in both intra-SR Ca\(^{2+}\) buffering and the modulation of RyR Ca\(^{2+}\) release. SR Ca\(^{2+}\) release is also regulated by SR Ca\(^{2+}\) load. At moderately low SR Ca\(^{2+}\) content, Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels can fail to induce SR Ca\(^{2+}\) release (18, 19). A high SR Ca\(^{2+}\) load increases not only the amount of Ca\(^{2+}\) available for release, but also the fraction of Ca\(^{2+}\) released from the SR for a given I\(_{\text{Ca}}\) trigger, mediated by a stimulatory effect of high intra-SR [Ca\(^{2+}\)]\(_{\text{free}}\) on RyR open probability (3). SR Ca\(^{2+}\) content can be raised by increasing Ca\(^{2+}\) influx, decreasing Ca\(^{2+}\) efflux, or enhancing Ca\(^{2+}\) uptake into the SR through various pathways such as adrenergic stimulation and an increase in stimulation frequency, action potential duration, I\(_{\text{Ca}}\), or [Na\(^{+}\)]\(_{i}\) (2).
1.1.4 Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase

In order for a contracting ventricular myocyte to relax, intracellular [Ca\textsuperscript{2+}] must decline to allow the dissociation of Ca\textsuperscript{2+} ions from myofilaments. A myocyte accomplishes the task of cytosolic Ca\textsuperscript{2+} ion removal via four Ca\textsuperscript{2+} transporters: 1) SR Ca\textsuperscript{2+}-ATPase, 2) sarcolemmal NCX, 3) sarcolemmal Ca\textsuperscript{2+}-ATPase, and 4) mitochondrial Ca\textsuperscript{2+} uniporter (3). In human ventricular myocytes, SR Ca\textsuperscript{2+}-ATPase (SERCA) removes 70\% of the Ca\textsuperscript{2+} ions that enter during an action potential. NCX removes 28\%, leaving about 1\% each to be removed by the sarcolemmal Ca\textsuperscript{2+}-ATPase and mitochondrial Ca\textsuperscript{2+} uniporter, collectively known as “the slow systems” (20) and will not be described in detail in this thesis.

SERCA is a member of the P-type ATPase protein family characterized by the formation of a phosphorylated enzyme intermediate during catalysis (21). It utilizes the energy from hydrolysis of one ATP molecule to drive the transport of two Ca\textsuperscript{2+} ions from the cytosol into the SR lumen. A single polypeptide of approximately 110 kDa, SERCA folds and matures into a SR membrane protein made up of 10 transmembrane α helices (M1 – M10) and three cytoplasmic domains designated N, P, and A (22). The N domain houses the positively charged binding pocket for ATP and various nucleotide analogs. The P domain contains the crucial aspartate residue phosphorylated during ATP hydrolysis. The A domain, actuator or anchor domain, is believed to assist in the communication between the ATP- and cation-binding sites (23). The two Ca\textsuperscript{2+} binding sites lie side by side in the transmembrane domain near the cytoplasmic surface (22).

In vertebrates, SERCA is encoded by three distinct genes, producing more than 10 isoforms mainly through alternative splicing. The principle isoform expressed in
ventricular myocytes is SERCA2a (22). During ventricular relaxation, it competes with NCX for Ca\textsuperscript{2+} removal from the cytosol and uptake into the SR. The proper functioning and regulation of SERCA dictates SR Ca\textsuperscript{2+} load, and therefore the amount of Ca\textsuperscript{2+} available for release and the fraction of Ca\textsuperscript{2+} released, which ultimately governs cardiac contractility. One of the key regulators of SERCA2a activity in cardiac myocytes is phospholamban (PLB), a small transmembrane protein of 52 amino acids. PLB inhibits SERCA2a activity by lowering its apparent affinity for Ca\textsuperscript{2+} without affecting its enzyme kinetics (24). PLB inhibition of SERCA2a slows the rate of Ca\textsuperscript{2+} removal from the cytosol, affecting muscle twitch relaxation. Furthermore, SERCA2a can no longer compete with NCX as effectively, SR Ca\textsuperscript{2+} content, and therefore, myocyte contractility decreases as well. Phosphorylation of PLB by PKA or calmodulin-dependent protein kinase (CaMKII) relieves this inhibition, allowing faster decline of intracellular [Ca\textsuperscript{2+}] and twitch relaxation, and enhanced SR Ca\textsuperscript{2+} load (2).

1.1.5 Sarcolemmal Na\textsuperscript{+}/Ca\textsuperscript{2+} Exchanger

The sarcolemmal Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) is the second major route of cytosolic Ca\textsuperscript{2+} ion removal during cardiac relaxation. It catalyzes the exchange of Na\textsuperscript{+} and Ca\textsuperscript{2+} ions across the plasma membrane in a stoichiometric ratio of 3 Na\textsuperscript{+} ions for 1 Ca\textsuperscript{2+} ion. This results in an unequal exchange of positive charges across the plasma membrane. NCX is, therefore, electrogenic.

Mammals express three members of the NCX family (NCX1, NCX2, and NCX3), encoded by different genes. The predominant isoform expressed in myocytes is NCX1.1, a splice variant of NCX1. A single polypeptide of 970 amino acids and 110 kDa, NCX’s
inferred and currently accepted architecture consists of a transmembrane domain of nine α helices and a cytoplasmic domain (25). The transmembrane domain contains 2 internal repeat sequences of ≈40 amino acids, designated α-1 and α-2 repeats that are oriented in oppositions within the membrane. They are hypothesized to form part of a vestibule or pathway required for the binding and transport of ions through NCX1 (26). The cytoplasmic domain does not appear to be required for the transport function of NCX, for a mutant lacking most of it retained exchange activity (27). However, the cytoplasmic domain is believed to be involved in the regulation of NCX activity.

A high-capacity but low-affinity Ca²⁺ transporter, NCX competes with SERCA for cytosolic Ca²⁺ during cardiac muscle relaxation. NCX removes nearly all of the Ca²⁺ that SERCA does not pump into the SR (≈28% of Ca²⁺ that enters during an action potential). When measured in purified transporters, the maximum turnover rate and the Kₘ for Ca²⁺ ions for NCX1 is up to 5000 per second and ≈6 μmol/L respectively; compared to those values for SERCA, 100 to 150 per second and ≈0.3 μmol/L respectively (25). The affinity of SERCA for Ca²⁺ is 20-fold greater compared to NCX1, even though its maximum turnover rate is 50-fold less.

Unlike the other ion channels and transporters involved in ECC, NCX is unique in that its direction of Na⁺/Ca²⁺ exchange is reversible depending on the membrane potential and the internal and external Na⁺ and Ca²⁺ ion concentrations. At rest, the membrane potential (Eₘ) is more negative than the reversal potential of NCX (Eₜ Na/Ca), in other words Eₘ < Eₜ Na/Ca. NCX will operate to achieve Eₘ = Eₜ Na/Ca. Therefore, 1 Ca²⁺ ion is extruded in exchange for the entry of 3 Na⁺ ions, resulting in a net movement of positive charges into the cell through NCX as an inward current (Iₜ Na/Ca). In contrast, early in the action
potential, an $E_m$ more positive than $E_{Na/Ca}$ ($E_m > E_{Na/Ca}$) coupled with a high intracellular $[Na^+]$ favors the extrusion of 3 $Na^+$ ions in exchange for 1 $Ca^{2+}$ ion entry, generating a net movement of positive charges out of the cell (outward $I_{Na/Ca}$). While $Ca^{2+}$ influx via NCX alone is much less efficient than L-type $Ca^{2+}$ channels at triggering $Ca^{2+}$ release from the SR, NCX appears to be able to synergistically amplify L-type $Ca^{2+}$ channel induced SR $Ca^{2+}$ release when both are at work (25). Finally, upon repolarization of the action potential, the negative $E_m$ and high intracellular $[Ca^{2+}]$ reverses the direction of NCX activity once again to drive the extrusion of $Ca^{2+}$ from the cytosol.

### 1.1.6 Sarcolemmal Na$^+$/K$^+$-ATPase

In order for a cardiac myocyte to return to its resting state and regain electrical excitability, it must extrude the $Na^+$ that entered the cell at the beginning of the action potential. While there are several pathways through which $Na^+$ ions can enter cells, the Na$^+$/K$^+$-ATPase (NKA) in the sarcolemma is the main pathway for cellular $Na^+$ extrusion (13). NKA plays an important role in maintaining the resting $Na^+$ and $K^+$ ionic gradients across the plasma membrane. It utilizes the energy from ATP hydrolysis to transport three $Na^+$ ions out of and two $K^+$ ions into cells (28), reestablishing cellular $Na^+$ and $K^+$ homeostasis, which in turn ensures the continuous excitability of not only cardiac myocytes, but also neurons and glial cells. Furthermore, NKA is instrumental in a variety of other specialized cellular functions. In the kidney, NKA is expressed in the basolateral membrane of renal epithelial cells. The low intracellular $Na^+$ concentration results in net transepithelial $Na^+$ reabsorption from the renal tubules, thereby regulating extracellular fluid volume and blood pressure. In skeletal and cardiac muscle cells, NKA
activity is also tightly coupled to the activity of NCX, playing a vital role in the control of cardiac contractility (29, 30).

NKA belongs to the protein family of P-type ATPases that includes SERCA, the plasma membrane Ca\(^{2+}\) ATPase, and the H\(^+\), K\(^+\) ATPase (31). As an oligomeric plasma membrane protein, NKA consists of a catalytic \(\alpha\)-subunit with 10 transmembrane segments and a type II \(\beta\)-subunit. The catalytic \(\alpha\)-subunit hydrolyzes ATP, forms the pore through which Na\(^+\) and K\(^+\) ions are transported, and acts as the pharmacological receptor for cardiac glycosides. The glycosylated \(\beta\)-subunit functions as a molecular chaperone. Its proper association with the \(\alpha\)-subunit targets NKA for insertion into the plasma membrane and modulates NKA transport properties (32).

Because NKA affects the functioning of so many cellular processes, a number of mechanisms participate in the regulation of its expression and function. A basic level of control of NKA expression is the stoichiometric synthesis of its \(\alpha\)- and \(\beta\)-subunits. Another level of cellular NKA expression control is the tissue-specific expression of four \(\alpha\)- and three \(\beta\)-isoforms, which potentially permit the formation of 12 different NKA isozymes with distinct functional properties (29, 30). The regulation of NKA function can be either fast-acting, short-term or slow acting, long-term. In terms of acute modulation, NKA activity is constantly regulated by changes in intracellular [Na\(^+\)]. Under resting cellular conditions intracellular Na\(^+\) is limiting for NKA transport activity.
In addition, phosphorylation of the \(\alpha\)-subunit by either PKA or protein kinase C (PKC) in response to peptide hormones and neurotransmitters may affect the transport properties of NKA. Phosphorylation may also influence the distribution of NKA between the plasma membrane and intracellular stores, or both (33, 34). Long-term modulation of NKA is
mediated by aldosterone and thyroid hormones, which alter α- and β-subunit gene transcription and ultimately produce an increased number of NKA units at the cell surface (34). Recently, a novel regulatory mechanism of NKA was discovered that involves the interaction of NKA with phospholemman (PLM), a small membrane protein of the FXYD family.

### 1.1.7 The FXYD Family and Phospholemman

The FXYD family of single membrane spanning proteins was first defined by Sweadner and Rael base on their sequence similarity (35). So far, seven members of the FXYD family (FXYD1 through FXYD7) have been identified in mammals. The FXYD proteins share six conserved amino acids, a Phe-X-Tyr-Asp (FXYD) motif in the extracellular N-terminus and two glycines and one serine residue in the transmembrane domain (36).

FXYD1, also known as phospholemman (PLM), is the first sequenced member of the FXYD family of ion transport regulators (37). A 72 amino acid integral membrane protein, PLM is highly expressed in heart and skeletal muscle, and the liver (21). When first expressed in Xenopus oocytes in 1992, PLM induced chloride-selective currents activated by hyperpolarizing pulses (38). Three years later, Moorman et al. further demonstrated that both the addition of PLM to planar phospholipid bilayers and excised patches of membrane from oocytes expressing PLM not only exhibited currents of various unitary anions, but was most permeant to the zwitterionic amino acid taurine, an osmolyte of animal cells (39). Later experiments showed that in HEK293 cells the overexpression of PLM was responsible for increased taurine efflux and a regulatory
decrease in cell volume in response to cell swelling (40). Furthermore, antisense oligonucleotides against PLM abolished the effect (41). Therefore, it was originally theorized that PLM plays a role in muscle contractility and cell volume regulation (21, 36).

In 1997, the discovery that FXYD2, known to be associated with renal NKA for over 20 years, modulates NKA activity fueled intense research into yet another possible physiological role for PLM (42). Subsequently, all members of the FXYD family except FXYD6 have been shown to co-immunoprecipitate with NKA α subunits and to modulate the function of NKA (13), a key protein in the sarcolemma involved in cardiac ECC. Both Crambert et al. (43) and Despa et al. (44) showed that PLM inhibits NKA mainly by reducing NKA’s affinity for intracellular Na\(^+\) in Xenopus oocytes and myocytes from transgenic mice, respectively. Despa et al. further demonstrated that the inhibition of NKA by PLM is relieved when PLM is phosphorylated during β-adrenergic stimulation, which explains the stimulation of NKA by β-adrenergic agonists observed in many early studies (13).

Concurrently, making the story of PLM even more complex and interesting, Ahlers et al. (45) discovered through co-localization, co-immunoprecipitation, and electrophysiological studies that PLM associates and regulates the function of NCX, yet another key component of cardiac ECC in the sarcolemmal membrane. PLM also inhibits NCX. It is important to note, however, this inhibition is achieved by phosphorylated PLM, specifically at serine 68 (46, 47). By contrast, it is the unphosphorylated form of PLM that actively inhibits NKA (44, 48).
Based on these published studies of NKA and NCX regulation by PLM, our lab theorized that perhaps PLM plays a more universal role in myocyte Ca\(^{2+}\) homeostasis and that PLM may modulate other components of cardiac ECC in the sarcolemma, not restricted to only NKA and NCX.
Chapter 2

Preliminary Data

2.1 PLM Co-Localizes with and Co-Immunoprecipitates CaV1.2

In HEK293 cells that are devoid of endogenous PLM and CaV1.2 and are electrically silent (37), coexpression of exogenous PLM and CaV1.2 results in co-localization of these proteins to the plasma membrane (Fig. 2.1).

Co-immunoprecipitation experiments demonstrate association of PLM with CaV1.2 in transfected HEK293 cells as well (Fig. 2.2). Furthermore, in solubilized adult mouse cardiac myocyte sarcolemma, anti-PLM antibodies immunoprecipitate both PLM and CaV1.2 (Fig. 2.3).
Figure 2.2: Demonstration of association of PLM with Ca\textsubscript{v}1.2 in transfected HEK293 cells by immunoprecipitation. HEK293 cells were transiently transfected with plasmid DNA indicated by "+", "-" means absent. Left, immunoblot of PLM (top) and Ca\textsubscript{v}1.2 (bottom). Immunoprecipitates of Ca\textsubscript{v}1.2 were obtained from 500 \(\mu\)g of whole cell lysate using 5 \(\mu\)g anti-FLAG antibody or control mouse IgG, and then probed with antibodies to either PLM (C2Ab, top) or FLAG for Ca\textsubscript{v}1.2 (bottom). Right, immunoblot of Ca\textsubscript{v}1.2 (top) and PLM (bottom). Immunoprecipitates of PLM were obtained from 500 \(\mu\)g of whole cell lysate using 5 \(\mu\)g anti-PLM antibody (C2Ab) or control mouse IgG, and then probed with antibodies to either FLAG for Ca\textsubscript{v}1.2 (top) or PLM (C2Ab, bottom).
2.2 PLM Differentially Affects Ca\textsubscript{V}1.2 and Ca\textsubscript{V}2.2 Channels

When cotransfected with Ca\textsubscript{V}1.2 in HEK293 cells, PLM appears to slow the activation of Ca\textsubscript{V}1.2 Ca\textsuperscript{2+} channels. This effect is more pronounced at negative potentials as indicated by average raw current traces of Ca\textsubscript{V}1.2 with or without PLM (Fig. 2.4, panel A). PLM also seems to enhance the inactivation of Ca\textsubscript{V}1.2 channels at more depolarized potentials. In contrast, when PLM is cotransfected with Ca\textsubscript{V}2.2 in HEK293 cells, it does not appear to significantly affect channel activation, and seems to enhance channel inactivation at all voltages (Fig. 2.4, panel B).
Figure 2.4: Averaged 150 millisecond (ms) raw current traces from -20 mV to 20 mV step depolarizations of HEK293 cells transfected with CaV1.2 with (red) or without (black) PLM (panel A) and CaV2.2 with (red) or without (black) PLM (panel B).
Chapter 3
Hypothesis and Experimental Approach

3.1 Hypothesis

The differential effect of PLM on CaV1.2 and CaV2.2 channel gating properties creates an ideal paradigm for localizing the domain of CaV1.2 that PLM interacts with to modulate CaV1.2 function. Domain III (DIII) of CaV1.2 has been shown to be important for CaV1.2 channel regulation by various small organic molecules and toxins (9, 49, 50). Therefore, I hypothesized that PLM’s effect on CaV1.2 is mediated through its interaction with DIII of CaV1.2.

3.2 Experimental Approach

The construction of chimeric Ca^{2+} channels that have been exploited previously to determine dihydropyridine binding sites on L-type Ca^{2+} channels (51) was utilized in this study. Chimeric channels were generated by placing either 1 or 2 CaV2.2 (N-type) channel domains into a CaV1.2 (L-type) channel backbone. Accordingly, each domain is identified by a single letter corresponding to the contributing channel type, “L” for CaV1.2 and “N” for CaV2.2. For example, the chimera that has a CaV1.2 backbone but a CaV2.2 DIII is designated LLNL. Six CaV1.2 and CaV2.2 channel chimeras were constructed for this project. However, due to the time constraints of a Masters thesis, electrophysiological data was only gathered from CaV1.2, CaV2.2, and LLNL channels.
4.1 Construction of Chimeric Ca\textsuperscript{2+} Channels

Because the construction of chimeric Ca\textsuperscript{2+} channels was a significant part of this project, it will be described in detail in the Results section of this thesis.

4.2 HEK293 Cell Transfection

cDNAs encoding wild-type Ca\textsubscript{V}1.2 and mutant LLNL Ca\textsuperscript{2+} channel \(\alpha_1\) subunits were cotransfected with \(\alpha_2\delta\), \(\beta_2a\), and TAG (to increase expression efficiency) into HEK293 cells by calcium phosphate precipitation. The calcium phosphate transfection solution contained HEPES buffered saline (HeBS), 50 mM CaCl\(_2\), and cDNA plasmids in the following amounts: 11 \(\mu\)g \(\alpha_1\) subunit (Ca\textsubscript{V}1.2, Ca\textsubscript{V}2.2, or LLNL), 8.5 \(\mu\)g \(\alpha_2\delta\), 5.5 \(\mu\)g \(\beta_2a\), and 2.15 \(\mu\)g TAG. All cDNAs were expressed in the expression plasmid pCDNA3 (Invitrogen, Carlsbad, CA).

HEK293 cells were maintained in DMEM/F-12 medium containing 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin mixture at 37\(^\circ\)C and 5% CO\(_2\). For transfection, 1 mL of the calcium phosphate transfection solution described above was added to HEK293 cells, and incubated for 8 hours, after which the medium was replaced with fresh medium. The transfected cells were then split onto cover slips in 35 mm dishes.
4.3 Patch-Clamp Electrophysiology

Whole-cell currents were recorded at room temperature 2-3 days after transfection. Pipettes were pulled from borosilicate glass (1B150F-3; World Precision Instruments, Inc., Sarasota, FL) using a Sutter P-97 Flaming/Brown micropipette puller (Sutter Instruments Company, Novato, CA) and fire polished on a MF200 microforge (World Precision Instruments, Sarasota, FL). Pipette resistances were typically 2.75-3.25MΩ. External solution for whole-cell recordings contained 130 mM NMG-aspartate, 10 mM HEPES, 10 mM 4-aminopyridine, 10 mM glucose, and 10 mM BaCl₂. The internal solution contained 140 mM NMG-MeSO₃, 10 mM EGTA, 1 mM MgCl₂, 4 mM MgATP, and 10 mM HEPES. The osmolality and pH of the external and internal solutions were adjusted to 300 mmol/kg and 7.4, respectively. Data was acquired using a HEKA Epc9/2 amplifier and PULSE/PULSEFIT software (ALA Scientific Instruments, Inc., Westbury, NY). Currents were sampled at 10 kHz and filtered at 2 kHz. Leaks and capacitive transients were subtracted using a P/4 protocol.

4.4 Data Analysis

Data was analyzed using FitMaster (HEKA Instruments Inc., Bellmore, NY), and graphed using Origin (OriginLab Corporation, Northampton, MA). Statistical significance of the observed differences between cells transfected with and without PLM at each voltage was evaluated using 2 sample independent t-test. All data are mean ± SEM and statistical significance was set at P<0.05. Error bars smaller than symbols do not appear in figures.
5.1 Construction of Chimeric Ca\textsuperscript{2+} Channels

Chimeric Ca\textsuperscript{2+} channels were constructed using cDNAs encoding rabbit Ca\textsubscript{V}1.2 (Genbank No. X15539) and rat Ca\textsubscript{V}2.2 (Genbank No. AF055477) that was generously provided by Dr. Leslie Parent. The sequences of wild-type Ca\textsubscript{V}1.2 and Ca\textsubscript{V}2.2 channels were aligned using Vector NTI (Invitrogen, Carlsbad, CA) and domain boundaries were placed in regions of high amino acid sequence homology between the domains. Ca\textsubscript{V}2.2 channel domains were then amplified using polymerase chain reaction (PCR), subcloned into pCR-Blunt-II-TOPO vectors (Invitrogen, Carlsbad, CA), excised using restriction enzyme digestion, and ligated in frame into an engineered Ca\textsubscript{V}1.2 channel that contained unique AgeI and NotI restriction enzyme sites in the intracellular linker region between the II/III (nucleotide 2751) and III/IV (nucleotide 3680) domains, respectively. These unique restriction enzyme sites were introduced into the cDNA of Ca\textsubscript{V}1.2 via silent mutagenesis using the QuikChange II Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The overall integrity of all cDNA constructs were confirmed by qualitative restriction enzyme digests and DNA sequence analyses. The details of each chimera are described below.
5.1.1 NNLL

CaV1.2 DI and DII (Met-1 to Asn-917) were replaced by DI and DII (Met-1 to Asn-1134) of CaV2.2. CaV2.2 DI and DII were amplified from full-length CaV2.2 and ligated into the HindIII/AgeI sites of the engineered CaV1.2.

5.1.2 LLNN

CaV1.2 DIII and DIV (Leu-895 to Leu2171) were replaced by DIII and DIV (Asp-1112 to Cys-2333) of CaV2.2. This chimera was the first to be constructed for this project and did not utilize the engineered CaV1.2 containing the introduced AgeI and NotI sites. Instead, a single XbaI site at nucleotide 2678 was introduced into CaV1.2, resulting in a missense mutation of a glutamate to arginine at position 894. CaV2.2 DI and DII were amplified from full-length CaV2.2 and ligated into CaV1.2.

5.1.3 NLLL

CaV1.2 DI (Met-1 to Cys-519) was replaced by DI (Met-1 to Pro-447) of CaV2.2. The insert for this chimera was constructed via overlap-extension PCR. CaV2.2 DI and CaV1.2 DII were amplified from full-length CaV2.2 and CaV1.2, respectively. The PCR products of these two domains, containing a small region of overlap, were combined and used as templates in a second round of PCR. The final PCR product was ligated into the HindIII/AgeI sites of the engineered CaV1.2.
5.1.4 LNLL

$\text{CaV}1.2$ DII (Ala-524 to Asn-917) was replaced by DII (Ser-452 to Asn-1134) of $\text{CaV}2.2$. This chimera was constructed using a strategy similar to that described for NLLL above.

5.1.5 LLNL

DIII (Arg-1137 to Ala-1443) of $\text{CaV}2.2$ was amplified from full-length $\text{CaV}2.2$ cDNA and ligated into the AgeI/NotI sites of the engineered $\text{CaV}1.2$, replacing DIII (Arg-920 to Ala-1226) of $\text{CaV}1.2$.

5.1.6 LLLN

DIV (Pro-1445 to Ile-1741) of $\text{CaV}2.2$ was amplified from full-length $\text{CaV}2.2$ cDNA and ligated into the NotI/BstEII sites of the engineered $\text{CaV}1.2$, replacing DIV (Pro-1228 to Ile-1542) of $\text{CaV}1.2$.

5.2 Statistically PLM did not Significantly Alter Gross Gating Properties

Current-voltage relationships from HEK293 cells transfected with $\text{CaV}1.2$, $\text{CaV}2.2$, or LLNL with either empty vector or PLM were generated by depolarizing cells from a holding potential of -100 mV to 300 ms depolarization steps, ranging from -60 to +80 mV. Peak currents were plotted against corresponding voltages to give the current-voltage (I-V) relationship. Data was then fit using the equation, $I = G(V_m - V_{rev})/(1 + \exp((V_a - V_m)/k))$, where $G$ is the maximal slope conductance, $V_{rev}$ is the reversal potential, $V_m$ is the membrane potential, $V_a$ is the half activation potential, and $k$ is the
slope factor. Normalized currents were obtained by dividing the peak current at each voltage by the maximum current elicited from the same cell. I-V relationships indicate that PLM does not significantly alter the gross gating properties of L-type, N-type, or LLNL mutant channels (Fig. 5.1).

Figure 5.1: Current-voltage relationships obtained from HEK293 cells transfected with Ca\textsubscript{v}1.2 (○ n=7, ● n=3), LLNL (○ n=12, ● n=9), and Ca\textsubscript{v}2.2 (○ n=2, ● n=2) with (solid circle, ●) or without (empty circle, ○) PLM.

Although statistically insignificant, PLM shifted the $V_a$ of Ca\textsubscript{v}1.2 slightly to the right (Fig. 5.1, left) from $-15.98 \pm 1.59$ mV for empty vector to $-12.77 \pm 1.23$ mV with PLM, while $V_{rev}$ remained roughly the same $36.63 \pm 0.84$ mV for empty vector and $36.75 \pm 0.74$ for PLM. PLM did not significantly alter $V_a$ for Ca\textsubscript{v}2.2 (Fig. 5.1, right) or the chimeric LLNL channel (Fig. 5.1, middle). $V_a$ for Ca\textsubscript{v}2.2 transfected with the empty vector was $-7.17 \pm 0.20$ mV and $-9.28 \pm 1.29$ mV with PLM, while $V_{rev}$ was $38.14 \pm 0.59$ mV for empty vector and $36.71 \pm 0.89$ mV for PLM. For LLNL, $V_a$ was $-31.79 \pm 1.19$ mV with empty vector and $-29.69 \pm 1.31$ mV with PLM, while $V_{rev}$ was $35.37 \pm 1.00$ mV with empty vector and $36.06 \pm 0.80$ mV with PLM.
5.3 Statistically PLM did not Significantly Alter $T_{peak}$

To assess the effect of PLM on channel activation, the amount of time in milliseconds (ms) taken to reach peak current for each depolarization voltage ($T_{peak}$) was obtained by depolarizing cells from a holding potential of -100 mV to 300 ms depolarization steps, ranging from -60 to +80 mV. Peak current at each voltage was measured by eye from raw data trace, and the time elapsed was recorded. $T_{peak}$ were then plotted against corresponding voltages to give the $T_{peak}$-voltage graphs (Fig. 5.2).

![Graphs showing $T_{peak}$-voltage relationships for different channels with and without PLM](image-url)

The difference in $T_{peak}$ between cells transfected with either empty vector or PLM was not statistically significant ($p > 0.05$) at any voltage for any of the channels studied.

5.4 Statistically PLM did not Significantly Alter $R_{300}$

To examine the effect of PLM on channel inactivation, HEK293 cells transfected with empty vector or PLM were depolarized from a holding potential of -100 mV to either 1000 ms (for $Ca_v1.2$ and LLNL) or 300 ms (for $Ca_v2.2$) depolarization steps, ranging from -60 to +80 mV. The amount of current remaining 300 ms into each
Depolarization step ($I_{300}$) was divided by the peak current ($I_{peak}$) at the same voltage to calculate the residual current ($R_{300}$) as a fraction. $R_{300}$ values were then plotted against corresponding voltages to give the $R_{300}$-voltage graphs (Fig. 5.3).

![Graphs showing $R_{300}$ values for different channels with and without PLM](image)

**Figure 5.3**: Residual current ($R_{300}$) at different depolarization potentials obtained from HEK293 cells transfected with $Ca_{V1.2}$ (○ $n=4$, ● $n=2$), LLNL (○ $n=4$, ● $n=7$), and $Ca_{V2.2}$ (○ $n=3$, ● $n=2$) with (solid circle, ●) or without (empty circle, ○) PLM.

The difference in $R_{300}$ between cells transfected with either empty vector or PLM was not statistically significant at any voltage depolarization for any of the channels studied.

### 5.5 Statistically PLM did not Significantly Alter $\tau_{Deact}$

To examine the effect of PLM on channel deactivation, HEK293 cells transfected with empty vector or PLM were depolarized from a holding potential of -100 mV to a conditioning step of 50 ms at -10 mV, then to a test voltage step of 30 ms, ranging from -100 to -10 mV. The deactivation time constant ($\tau_{Deact}$) was determined by fitting a single exponential function to the current trace elicited at each potential of the test voltage step. $\tau_{Deact}$ values were then plotted against corresponding voltages to give the $\tau_{Deact}$-voltage graphs (Fig. 5.4).
Figure 5.4: Deactivation time constants ($\tau_{\text{Deact.}}$) at different depolarization potentials obtained from HEK293 cells transfected with $\text{Ca}_\gamma\text{1.2}$ ($\bigcirc\ n=4, \bullet\ n=3$) and LLNL ($\bigcirc\ n=4, \bullet\ n=6$) with (solid circle, $\bullet$) or without (empty circle, $\bigcirc$) PLM.
Chapter 6
Discussion

PLM, a small membrane protein of the FXYD family, has recently been shown to regulate the activity of both NKA (43, 44, 48) and NCX (45, 46, 47), two among the numerous molecular components of cardiac ECC. Our lab theorized that, perhaps, PLM plays a more universal role in myocyte Ca\(^{2+}\) homeostasis and that PLM may modulate other components of cardiac ECC in the sarcolemma, not restricted to only NKA and NCX. From our preliminary findings demonstrating that PLM does in fact associate with Ca\(\gamma\)1.2 and seem to affect Ca\(\gamma\)1.2 channel activation but not Ca\(\gamma\)2.2 channel activation (see Chapter 3), I hypothesized that PLM’s effect on Ca\(\gamma\)1.2 channel activation is mediated through its interaction with DIII of Ca\(\gamma\)1.2, since DIII of Ca\(\gamma\)1.2 has been shown to be a pharmacological hot spot (9, 49, 50).

6.1 PLM and Channel Activation

As can be seen from Figure 5.2, both Ca\(\gamma\)2.2 (right panel) and LLNL (middle panel) take less time to reach peak current when compared to Ca\(\gamma\)1.2. However, PLM does not seem to have a significant effect on T\(_{\text{peak}}\) of Ca\(\gamma\)2.2 and LLNL. Unlike Ca\(\gamma\)2.2 and LLNL, T\(_{\text{peak}}\) of Ca\(\gamma\)1.2 (Fig. 5.2, left panel) does not decrease with more depolarizing voltages. Instead, Ca\(\gamma\)1.2 initially takes a longer time to reach peak current until 0 mV,
where $T_{\text{peak}}$ begins to decrease with more depolarizing voltages. Although PLM’s effect on $T_{\text{peak}}$ of Cav1.2 at each voltage is statistically insignificant, a general trend can be observed where PLM appears to initially increase the time taken to reach peak current and then decrease $T_{\text{peak}}$ with more depolarizing voltages. However, this trend may be a result of increased Cav1.2 channel inactivation in the presence of PLM (Fig. 2.4 panel A and Fig. 5.3 left panel), making it appear as if Cav1.2 attains peak current faster with PLM present while in fact less current is passing through the channel due to inactivation. In light of this observation, $T_{\text{peak}}$ might not be an appropriate method of analysis of channel activation. The percentage of current attained at 10 ms or the time required to achieve 75% of peak current may be better analyses of channel activation, and may be able to better demonstrate PLM’s effect on channel activation observed in the preliminary data. Channel activation data should also be repeated and recorded from more cell experiments to correct for the variability apparent in the large error bars (Fig. 5.2).

6.2 PLM and Channel Inactivation

The fraction of peak current remaining 300 ms after depolarization step ($R_{300}$) decreases with more depolarizing voltages for Cav1.2. In other words, Cav1.2 inactivates faster at more depolarizing potentials. Although PLM’s effect on $R_{300}$ of Cav1.2 at each voltage is statistically insignificant, it is apparent that the $R_{300}$ curve for PLM (Fig. 5.3, left panel, solid circles) is steeper compared to empty vector (Fig. 5.3, left panel, empty circles), suggesting that the voltage-dependence of inactivation is stronger, where the same change in voltage results in a bigger change in $R_{300}$, for Cav1.2 when PLM is present.
For the chimeric LLNL (Fig. 5.3, middle panel), $R_{300}$ decreases with more depolarizing voltages as well. However, its $R_{300}$ is significantly less when compared to either CaV1.2 or CaV2.2, indicating that the LLNL channel inactivates much faster. Channel inactivation for CaV2.2 is U-shaped (Fig. 5.3, right panel), consistent with results obtained by Goo et al. (52) and our collaborators (unpublished data). PLM does not have a significant effect on $R_{300}$ of either LLNL or CaV2.2.

6.3 PLM and Channel Deactivation

Due to the time limitations on this project, only the deactivation time constants ($\tau_{\text{Deact}}$) for CaV1.2 and LLNL were obtained. $\tau_{\text{Deact}}$ increases with more depolarizing voltages for both CaV1.2 and LLNL (Fig. 5.4, left and right panels, respectively), suggesting that channel deactivation slows down at more depolarized potentials. Furthermore, PLM does not seem to have a significant effect on ($\tau_{\text{Deact}}$) of CaV1.2 or LLNL.

6.4 Summary and Future Directions

Based on the preliminary data that PLM does in fact associate with CaV1.2 and seem to affect CaV1.2 channel activation but not CaV2.2 channel activation, the goals of this study were to further investigate the effect of PLM on Ca$^{2+}$ channel gating properties and to determine whether PLM’s effects are mediated through its interaction with DIII of CaV1.2. If the molecular determinants on CaV1.2 with which PLM interacts with to bring about its effect were replaced, whatever effect PLM had on CaV1.2 channel gating properties would be lost. Unfortunately, the results of this study did not provide
convincing data in support of my hypothesis that PLM’s effect on CaV1.2 is mediated through its interaction with DIII of CaV1.2, for PLM failed to demonstrate any statistically significant effect on channel activation, inactivation, or deactivation on either CaV1.2, LLNL, or CaV2.2.

However, this study did accomplish the design and construction of six chimeric Ca$^{2+}$ channels (currently utilized by our collaborators to study other means of Ca$^{2+}$ channel modulation), and started the initial characterization of PLM’s effect on LLNL chimeric channel activation, inactivation, and deactivation. If more time had been possible for this study, experiments should be repeated and data recorded from more cells, for data collected from as few as two cells can hardly be representative of any trend, pattern, or effect observed. Secondly, PLM’s effect on the modulation of the other five chimeric Ca$^{2+}$ channels should also be examined. Finally, other methods of data analyses should be explored so that the true effect of PLM on CaV1.2 channel modulation can be clearly represented.
References


52. **Goo YS, Lim W, and Elmslie KS.** Ca\(^{2+}\) enhances U-type inactivation of N-type (Ca\(_{V2.2}\)) calcium current in rat sympathetic neurons. J Neurophysiol 96: 1075-1083, 2006.