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Mechanisms of the cyclic nucleotide cross-talk signaling network in cardiac L-type calcium channel regulation

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Abstract

Regulation of L-type Calcium (Ca^{2+}) Channel (LCC) gating is critical to shaping the cardiac action potential (AP) and triggering the initiation of excitation-contraction (EC) coupling in cardiac myocytes. The cyclic nucleotide (cN) cross-talk signaling network, which encompasses the β -adrenergic and the Nitric Oxide (NO)/cGMP/Protein Kinase G (PKG) pathways and their interaction (cross-talk) through distinctively-regulated phosphodiesterase isoenzymes (PDEs), regulates LCC current via Protein Kinase A- (PKA) and PKG-mediated phosphorylation. Due to the tightly-coupled and intertwined biochemical reactions involved, it remains to be clarified how LCC gating is regulated by the signaling network from receptor to end target. In addition, the large number of EC coupling-related phosphorylation targets of PKA and PKG makes it difficult to quantify and isolate changes in L-type Ca^{2+} current (I_{CaL}) responses regulated by the signaling network. We have developed a multi-scale, biophysically-detailed computational model of LCC regulation by the cN signaling network that is supported by experimental data. LCCs are modeled with functionally distinct PKA- and PKG-phosphorylation dependent gating modes. The model exhibits experimentally observed single channel characteristics, as well as whole-cell LCC currents upon activation of the cross-talk signaling network. Simulations show 1) redistribution of LCC gating modes explains changes in whole-cell current under various stimulation scenarios of the cN cross-talk network; 2) NO regulation occurs via potentiation of a gating mode characterized by prolonged closed times; and 3) due to compensatory actions of cross-talk and antagonizing functions of PKA- and PKG-mediated phosphorylation of LCCs, the effects of individual inhibitions of PDEs 2, 3, and 4 on I_{CaL} are most pronounced at low levels of β -adrenergic stimulation. Simulations also delineate the contribution of the following two mechanisms to overall LCC regulation, which have otherwise been challenging to distinguish: 1) regulation of PKA and PKG activation via cN cross-talk (Mechanism 1); and 2) LCC interaction with activated PKA and PKG (Mechanism 2). These results provide insights into how cN signals transduced via the cN cross-talk signaling network are integrated via LCC regulation in the heart.

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Disclosures

None declared.

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Phosphodiesterases; Cyclic nucleotides; Signaling network; L-type calcium channel; Cardiac myocytes; Computational model

1. Introduction

The cardiac voltage-gated L-type calcium (Ca^{2+}) channel (LCC) initiates and coordinates a series of events that give rise to the cardiac myocyte action potential (AP) and mechanical contraction and relaxation within each heartbeat [1,2]. Activated upon membrane depolarization, LCCs allow Ca^{2+} influx across the sarcolemma [3] into nano-structures known as cardiac dyads, defined as regions where the sarcoplasmic reticulum (SR) membrane closely apposes (to within ~ 12 nm) the sarcolemma. Ca^{2+} -binding Ca^{2+} release channels, known as ryanodine receptors (RyRs), are localized to the junctional SR (JSR) membrane of the dyad. LCC openings increase dyad Ca^{2+} concentration ($[\text{Ca}^{2+}]$) and Ca^{2+} binding to RyRs. Upon Ca^{2+} binding, RyRs open to release Ca^{2+} from the JSR Ca^{2+} store in a process known as Ca^{2+} -induced Ca^{2+} release (CICR) [3,4]. The elevated $[\text{Ca}^{2+}]$ promotes Ca^{2+} binding to myofilaments, initiating contraction [5,6]. The process by which electrical excitation leads to mechanical contraction of the myocyte is referred to as the cardiac excitation-contraction (EC) coupling [3,7].

Our previous work constructed a mechanistic model of the cyclic nucleotide (cN) cross-talk signaling network (Fig. 1A) [8,9], composed of the β -adrenergic signaling pathway (red color scheme), the nitric oxide (NO)/cGMP/PKG signaling pathway (blue color scheme), and cross-talk between them (yellow color scheme) as facilitated by five distinct phosphodiesterases (PDEs) (orange boxes) [11–13]. Stimulation of the β -adrenergic and NO/cGMP/PKG pathways exert opposing physiological responses, with the former enhancing cardiac inotropy and lusitropy [3,14] and the latter attenuating cardiac contractility [15–19] and antagonizing β -adrenergic tone [11–13,20–27]. The importance of a delicate balance of cN signaling is reflected by isoform-specific alternations in PDEs in cardiac diseases [23,26,28–30]. As examples, PDE2 upregulation in the failing heart is observed to attenuate β -adrenergic signaling [26], decreased PDE3 activity promotes cardiac myocyte apoptosis [28], and PDE4 downregulation is associated with arrhythmias in cardiac hypertrophy and HF [29]. Drugs that target specific PDE activities have cardio-protective effects [31], such as restoration of PDE3 activity in ischemic and dilated cardiomyopathies [32], restoration of PDE1 and PDE4 activities in cardiac ischemia [33], and inhibition of PDE5 in heart failure, cardiac hypertrophy, and ventricular arrhythmias [34–37].

The cN signaling cross-talk network (Fig. 1A) exerts both stimulatory (green arrow) and inhibitory (red arrow) regulation of LCCs (Fig. 1B). These actions are mediated by the dynamics of the two cyclic nucleotides (cNs), cyclic adenosine-3', 5'-monophosphate (cAMP) and cyclic guanosine-3', 5'-monophosphate (cGMP), as well as the subsequent activation of protein kinase A (PKA) and protein kinase G (PKG) [8,9]. More specifically, PKA isoforms, PKA-I and PKA-II, and PKG isoform, PKG-I, are predominant in cardiac myocytes [8,9]. As shown in Fig. 1B, the random openings and closings of LCCs result from

an interplay between the processes of voltage-dependent activation (left model, horizontal transitions), Ca^{2+} dependent inactivation (CDI; left model, vertical transitions), and voltage-dependent inactivation (VDI; right model) [10]. In addition, Fig. 1B (left model) represents one of a number of possible LCC gating modes (Mode_j) depending upon PKA- and PKG-mediated phosphorylation of the channel, in response to the cN cross-talk signaling network [3,35]. The values of transition rates, f_{mode_j} and g_{mode_j} (colored), depend on the gating mode (Mode_j). Explanation of gating mode transitions will follow in Methods below. In diseases such as cardiac hypertrophy and heart failure (HF) [30,38–41], the imbalances between β -adrenergic and NO/cGMP/PKG signaling and the resultant changes in L-type Ca^{2+} current (I_{CaL}) lead to alterations in EC coupling [42], AP duration (APD) [42], and increased likelihood of after-depolarizations [43]. Despite its physiological significance, mechanisms of LCC regulation by the cN cross-talk signaling network are not yet understood quantitatively.

The large number and inter-dependence of EC coupling-related PKA and PKG phosphorylation targets [3,4,35], as well as the large number of interacting proteins within the cN cross-talk signaling network itself, make understanding of the ways in which this network regulates I_{CaL} challenging [8,9]. Multiple mechanisms may underlie observed changes in I_{CaL} upon activation of the signaling network. Prior studies have modeled the impact of β -adrenergic regulation or PKA activation on LCC gating [44–49]; however, the integration of NO/cGMP/PKG and its cross-talk with the β -adrenergic pathway on LCC gating regulation remains to be carefully studied. In addition, the mechanisms underlying LCC regulation remain elusive, in part because it has been challenging to distinguish the impact on LCC gating from PKA and PKG activation via competitive and compensatory interactions between the cNs and PDEs within the signaling network (Mechanism 1) and from LCC interaction with activated PKA and PKG (Mechanism 2). To address this challenge, we have constructed an integrative mathematical model of the dynamic regulation of stochastic LCC gating as a function of β -adrenergic and NO stimulation (Fig. 1). Three predictions emerge from this model. First, changes in I_{CaL} under varying extents of stimulation of the cN cross-talk network can be explained by redistribution of LCCs among four distinct gating modes. Second, NO suppression of I_{CaL} occurs via potentiation of an LCC gating mode characterized by prolonged closed times [44,50]. Third, individual inhibitions of PDEs 2, 3, and 4 produce no changes in I_{CaL} under basal conditions. Instead, I_{CaL} changes are observed in the presence of β -adrenergic stimulation, with effects being more pronounced at lower, rather than higher levels, of β -adrenergic stimulation.

2. Methods

The integrated model described in this work (Fig. 1) is referred to as the cN signaling-LCC model and consists of three modules: 1) the cN cross-talk signaling network model; 2) the PKA-PKG-LCC model; and 3) the LCC gating model. To develop this model, we have integrated the previously developed cN cross-talk signaling network model from Zhao et al. [8] with the LCC model of Greenstein and Winslow [10], originally developed by Jafri et al. [51], through PKA- and PKG-mediated regulation of the channel (Fig. 2). Further details of the model are presented in the Supplement: Suppl. Sect. I defines symbols used; Suppl. Sect. II provides all model equations; Suppl. Sect. III defines and gives nominal values for model

parameters; Suppl. Sect. IV lists state variable initial conditions. In addition, Suppl. Sects. IV, V, and VI, provide details on simulation conditions, the bases for model and simulation design, and additional simulation results, respectively. All simulations in this study, including those in the Supplement, were performed with the same set of parameters and state variable initial conditions, unless otherwise stated.

2.1. The cN cross-talk signaling network model

The cN cross-talk signaling network of Fig. 1A describes communication between the PKA and the NO/cGMP/PKG pathways through mechanistic models of PDEs 1, 2, 3, 4, and 5 [8,52]. PDEs 1, 2, and 3 can hydrolyze both cAMP and cGMP, while PDE4 and PDE5 are respectively cAMP- and cGMP-specific [8,52]. The PKA and PKG activities predicted by this model are used to drive the PKA-PKG-LCC model described below.

2.2. The PKA-PKG-LCC model

The PKA-PKG-LCC model is shown in Fig. 2, and consists of two components. The first describes the fraction of LCCs available to gate as a function of PKA-II and PKG-I activations (Fig. 2A). The second (Fig. 2B) describes LCC gating mode as a function of channel phosphorylation state. As shown in Fig. 2A, the distribution of LCCs between the “Unavailable” and “Available” states determine the number of channels available for opening. Increased activation of PKA-II promotes more channels to become available (white) from their unavailable states (grey). This transition is opposed by increased PKG activity. This is supported by experimental evidence that activation of PKA increases [44, 50,53] and activated PKG suppresses channel availability [53,54].

It has been demonstrated that PKA phosphorylation of at least one Serine site on LCC results from β -adrenergic pathway activation [55, 56]. LCC also has PKG phosphorylation sites [57,58]. As reviewed by Benitah, et al. [37], Harvey et al. [55], Keef et al. [59], and van der Heyden et al. [60], PKA and PKG have distinct LCC phosphorylation sites, with the possible exception of Serine 1928. However, the functional role of Serine 1928 in LCC β -adrenergic regulation has been challenged by Ganesan et al. [61]. In the model of Fig. 2B, we therefore assume that PKA and PKG each have distinct LCC phosphorylation sites, and therefore an LCC can be non-phosphorylated (Mode 1), PKA-phosphorylated (Mode A), PKG-phosphorylated (Mode G), or PKA- and PKG-phosphorylated (Mode AG). In Fig. 2B, LCC phosphorylation status is denoted by black (PKA) and grey (PKG) symbols of encircled “P”, with the phosphorylation rates proportional to the activities of the respective kinases (i.e. activated PKA and PKG).

Each phosphorylation state shown in Fig. 2B is associated with a distinct LCC gating mode. Mode 1, in which the channel is not phosphorylated, is the predominant mode under basal non-stimulated conditions, characterized by repeated brief openings [10,50]. PKA-phosphorylated Mode A, which has been referred to as Mode 2 in previous studies [44, 50], is a high-activity gating mode, characterized by prolonged channel openings [44,50]. β -adrenergic stimulation redistributes the relative proportions of channels in each of these modes, such that an increased proportion of LCCs gates in Mode A compared to that under basal conditions [44,50]. Mode G is the PKG-phosphorylated gating mode. Under PKG

activation, histograms of channel closed times show a multi-exponential distribution with widely differing means [54,62,63], indicating the existence of at least one more gating mode other than Mode 1. Compared to Mode 1, Mode G is characterized by a reduction of channel open probability [54,62,64,65] through prolonged channel closed time, with no effects on channel open time or single channel conductance in cardiac myocytes [62,64]. These gating characteristics are also observed in other cell types under PKG activation, such as chromaffin cells [63, 66]. Mode AG is a state of dual PKA and PKG phosphorylation. Single channel recordings show that PKG activation subsequent to pre-stimulation by activated PKA reduces channel open probability and amplitude of I_{CaL} in a manner that renders these properties similar to those observed under basal, non-stimulated conditions [63,64].

In the model of Fig. 2B, protein phosphatase 2 (PP2A) activity controls LCC dephosphorylation. This is supported by the finding that Mode A gating is functionally coupled to a binding site dephosphorylated by PP2A by using inhibitors of differential sensitivity to protein phosphatase 1 (PP1) and PP2A [67]. In addition, the regulatory processes controlling LCC availability (Fig. 2A) and modal gating distribution (Fig. 2B) are assumed to be independent. It is also assumed that LCC phosphorylation (Fig. 2) occurs independently of voltage- and Ca^{2+} -dependent gating (Fig. 1). These assumptions are consistent with previous models describing kinase-mediated phosphorylation of LCC [44,48,68,69].

2.3. The LCC gating model

The LCC model (Fig. 1B) is adopted from Greenstein and Winslow [10], originally developed by Jafri et al. [51]. In this model, LCCs undergo voltage- and Ca^{2+} -dependent gating (Fig. 1B, left) and an independent process of voltage-dependent inactivation (VDI) (Fig. 1B, right). Voltage-dependent transitions to open states (States 6 and 12) occur upon membrane depolarization. When dyadic $[Ca^{2+}]$ is elevated, CDI occurs as a result of downward transitions from Mode Normal (top row) to Mode Ca^{2+} (bottom row), in which transitions into open state (State 12) are rare, thereby effectively inactivating the channel. In addition, as shown in the model on the right in Fig. 1B, VDI promotes (slow) transitions from the active state (State 1) to the inactivated state (State 0) upon membrane depolarization.

Parameters for LCCs gating in Mode 1 are from the original model by Greenstein and Winslow [10]. The LCC model in Mode 1 will henceforth be referred to as the baseline LCC model. Parameters for Mode A gating are obtained by dividing the exit rate from the open state (State 6) by a factor of 10 (i.e. $g_{modeA} = g_{mode1}/10$) while all other parameters remain the same as in the baseline LCC model [44]. Parameters for Mode G gating are obtained by dividing the forward rate into State 6 by a factor of 2 (i.e. $f_{modeG} = f_{mode1}/2$), thereby increasing channel closed times by a factor similar to that measured experimentally [62]. Mode AG gating, corresponding to concurrent LCC phosphorylation by both PKA and PKG, is assumed to incorporate parameter adjustments for both Mode A and Mode G (i.e. $g_{modeAG} = g_{mode1}/10$ and $f_{modeAG} = f_{mode1}/2$). The theoretical basis for adjustments of gating parameters is explained in Suppl. Sect. V-A. Stochastic simulation of LCC gating is performed as described previously [10]. Briefly, for each time step, a uniformly distributed

random variable is generated to determine into which state the Markov processes governing LCC gating (Fig. 1B) transitions, if a transition occurs [10]. In addition, unless otherwise indicated, $[Ca^{2+}]$ is clamped at 4 μM for the entire duration of the simulation, which approximates $[Ca^{2+}]$ in pipette solutions containing Ca^{2+} buffers (e.g. EGTA) typically employed in single channel recordings [70] (Suppl. Sect. VI-C). I_{CaL} currents are smoothed using a moving average with 0.5 ms window.

3. Results

3.1. Model validation of single channel behavior

Model predictions of LCC availability and gating mode distributions in response to the β -adrenergic agonist, isoproterenol (ISO), and the NO donor, S-nitroso-*N*-acetyl-D, L-penicillamine (SNAP), reproduce results from single channel recordings (Fig. 3). Four stimulation scenarios were investigated: “Basal” representing non-stimulated condition, and “ISO”, “SNAP”, and “ISO + SNAP” representing maximal stimulation by the indicated reagent. As shown in Fig. 3A, the fraction of LCCs available under basal conditions is ~25%, validated with results of Greenstein et al. [44]. Consistent with data by Schröder et al. [54], under maximal ISO stimulation, availability increases ~50% from that of basal conditions to ~40%. Availability is suppressed below that of basal condition under maximal NO stimulation, constrained to data by Schröder et al. [54]. Availability under simultaneous NO and ISO stimulation is also below that of basal condition and is validated against data by Schröder et al. [54] and Carabelli et al. [63]. As shown in Fig. 3B, under basal conditions, nearly all channels gate in Mode 1 with the rest gating in Mode A, validated with model results from Greenstein et al. [44]. Upon maximal β -adrenergic stimulation, some channels originally gating in Mode 1 shift to Mode A, so that the fraction of LCCs in gating in Mode A increases to ~20%, validated with model results from Greenstein et al. [44]. Additionally, the model predicts distributions of channel gating modes in response to SNAP and ISO + SNAP measured experimentally (Fig. 3B). Compared to that under basal conditions, more channels gate in Mode G under SNAP stimulation (Fig. 3B). Under simultaneous ISO and SNAP stimulation (Fig. 3B), the fractions of LCCs in Mode A and Mode G respectively are similar to those under ISO and SNAP conditions. In addition, the fraction of LCCs in Mode AG is the highest under ISO + SNAP condition among the four stimulation scenarios.

As shown in Fig. 3C, mean LCC channel open and closed times are calculated from stochastic simulation of five hundred LCCs for each gating mode (bars) and compared to experimental data (dots). The model mean open times of ~0.5 ms for Mode 1 and ~5 ms for Mode A is validated against data of Yue et al. [50] and results of Greenstein et al. [44]. Mean closed time for Mode 1 of ~5 ms is validated by data by Schröder et al. [54]; that of Mode A is similar to Mode 1, validated with data of Yue et al. [50]. In addition, the model is validated with data of Tohse and Sperelakis [62] in which mean channel open time in Mode G is approximately the same as that in Mode 1 at ~0.5 ms, with Mode G mean closed time being approximately twice that of basal conditions at ~10 ms. Finally, the mean closed time of Mode AG is similar to that of Mode G, validated by data by Klein et al. [53]. The model predicts that the mean open time of Mode AG is ~5 ms, similar to that of Mode A.

3.2. Model validation of whole-cell L-type Ca^{2+} current

Fig. 4A and B respectively show the model ensemble current and peak current-voltage (IV) relations of the whole-cell I_{CaL} . In Fig. 4A, I_{CaL} currents are elicited by 150 ms test potentials of 0 mV from a holding potential of -40 mV (top row) under the aforementioned four stimulation scenarios of basal (black), ISO (red), SNAP (blue), and ISO + SNAP (green) stimulation. As shown, β -adrenergic stimulation potentiates the magnitude of I_{CaL} (red) from that under basal conditions (black), consistent with that observed by Katsube et al. [71] and Kameyama et al. [72]. The model also qualitatively reproduces NO suppression of basal I_{CaL} (blue) similar to that measured by Grunshin et al. [73] and Wahler and Dollinger [74]. Finally, under ISO + SNAP condition (green), I_{CaL} is suppressed below that observed under ISO alone, and the peak current is similar to that under basal conditions. The model behavior whereupon SNAP suppresses I_{CaL} pre-stimulated by ISO is consistent with data by Abi-Gerges et al. [75] and Wahler and Dollinger [74].

In Fig. 4B, the model means (filled square dots) and ranges (associated vertical bars) of peak I_{CaL} arising from stochastic LCC gating are calculated from six independent simulations for each test potential of the voltage protocol (top row). The peak IV relations obtained from these simulation results (connected by solid lines) are compared to experimental data (hollow triangular dots). The model reproduces an IV curve under basal conditions (black) consistent with the original modeling results of Greenstein and Winslow [10]. In addition, the degree of IV curve potentiation under ISO (red) qualitatively agrees with data by Katsube et al. [71]. The extent of IV curve suppression due to maximal NO stimulation (blue) agrees with that measured by Sumii and Sperelakis [76]. The IV curve under simultaneous maximal ISO and NO stimulation (green) returns to approximately that observed under basal conditions, a behavior consistent with that observed by Wahler and Dollinger [74]. The model behavior whereupon NO suppresses ISO pre-stimulated I_{CaL} is also consistent with that reported by Abi-Gerges et al. [75].

3.3. Regulation of LCC current by cN signaling network

Results in Fig. 5 show changes in availability and gating mode distribution under various extents of cN cross-talk stimulation. It is to be noted, the simulated LCC gating behavior is a result of two mechanisms: 1) competitive cN binding to PDE in cN network (Mechanism 1) and 2) PKA/PKG effects on LCC (Mechanism 2). As shown in Fig. 5A, the model replicates percent increases in peak I_{CaL} over that under basal condition (grey line) across varying [ISO] as measured by Katsube et al. [71] (black dots), with steep increases in current occurring over the range of ~ 5 nM to 50 nM [ISO]. Model predicts this is due to increased channel availability (Fig. 5B) over the aforementioned range of [ISO]. In addition, with increased β -adrenergic stimulation, there is a shift in channel gating from Mode 1 to Mode A, while the fractions of channels gating in Mode G and Mode AG remain very low across all values of [ISO] (Fig. 5C).

The model was also used to investigate changes in I_{CaL} while varying [NO] as donated from SNAP without (black) and with (grey) simultaneous [ISO] stimulation of 10 μM (Fig. 5D). There is a threshold value of [SNAP] (and therefore [NO]) at which suppression of I_{CaL} occurs. As shown, with or without ISO, low [NO] has little effect on I_{CaL} . Under non-

stimulated conditions (Fig. 5D, black), PKG modulation starts to dominate over basal PKA signaling and suppresses I_{CaL} for [SNAP] above 0.1 μ M. Relative to peak I_{CaL} levels without ISO (black), simultaneous ISO stimulation leads to an increase in I_{CaL} (grey) that is more pronounced at lower than higher [SNAP]. With maximal effective [ISO] (Fig. 5D, grey), PKG signaling begins to diminish LCC β -adrenergic response when [SNAP] surpasses ~50 nM; however, PKA signaling still dominates, until [SNAP] above ~500 nM completely abolishes the ISO-mediated increase in I_{CaL} , such that I_{CaL} returns to that observed under low [SNAP] without ISO (black). On the other hand, the addition of ISO does not shift the range of [NO] over which I_{CaL} exhibits most change. Overall, ISO elicits a greater magnitude of response in LCC than SNAP. In addition, the model predicts that channel availability is suppressed at high [SNAP] either without (grey) or with (black) ISO. Compared to SNAP alone, I_{CaL} is potentiated by addition of ISO across all [SNAP] (Fig. 5E grey vs. black). Furthermore, Fig. 5F shows the proportions of LCCs in each of the four gating modes under varying [SNAP] in the absence of ISO. Increases in [SNAP] caused an increased proportion of channels to gate in Mode G, transitioning from their original Mode 1 gating, while proportions gating in Mode A and Mode AG remain fairly constant across [SNAP] with a very slight increase in Mode AG. Compared to modal distribution under SNAP alone (Fig. 5E), an increase in Mode A gating is the most prominent change caused by addition of ISO, with magnitude of increase slightly higher at lower [SNAP] (Fig. 5G). On the other hand, Mode AG is slightly more pronounced with high [SNAP] under maximal β -adrenergic stimulation. In short, the model predicts that β -adrenergic and NO stimulation primarily arise from potentiation of Mode A and Mode G gating, respectively.

In Suppl. Fig. S2 and Fig. 6, the model reveals the behavior of the signaling network driving LCC regulation shown in Fig. 4 and Fig. 5. Despite large increases in activities of all cAMP-hydrolyzing PDEs, PDEs 1–4 (Suppl. Fig. S2C), [cAMP], and subsequent PKA activation (Fig. S2A and B respectively) are substantially increased at elevated [ISO]. Comparably, ISO-induced increases in [cGMP] and PKG activity (Suppl. Fig. S2D and E respectively) as a result of PDE interactions in the cN cross-talk (Suppl. Fig. S2F) [8] are small in magnitude. The large increase in β -adrenergic activation (Suppl. Fig. S2A–C) overcomes cross-talk activation of the NO/cGMP/PKG pathway (Suppl. Fig. S2A–C), leading to promotion of Mode A gating, which dominates over the slight increases in Mode G and Mode AG gating and leads to increased peak I_{CaL} (Fig. 5A–C). In Fig. 6, signal transduction mechanisms under varying [SNAP] stimulation are investigated for the observed LCC responses in Fig. 5D–G. Increasing [SNAP] results in increasing [cAMP] (Fig. 6A) and subsequent PKA activation (Fig. 6B), without (black) or with (grey) simultaneous ISO. As shown in Fig. 6C and D, this behavior is due to cross-talk mechanisms [8]. The activity of PDE3 is suppressed under higher [SNAP], because increased [cGMP] (Fig. 6E) competitively binds to its catalytic domain, diminishing cAMP-occupancy [9]. The rise in PDE2 and PDE4 activities partially compensated for the loss of PDE3 activity. Comparing this with and without high [ISO] (Fig. 6D vs. C), PDE2 compensation for decreased PDE3 activity is more pronounced under high [ISO], because PDE4 is saturated and no longer responsive to changes in the pathway (Fig. 6D).

Because saturation of PDE activities at high [ISO] led to elevated [cAMP] and PKA activation (Fig. 6A and B respectively, grey vs. black), Mode A gating (red) is more favored

under simultaneous SNAP and ISO stimulation (Fig. 5G) versus SNAP alone (Fig. 5F), giving rise to the upward shift of the curve representing peak I_{CaL} upon addition of ISO across [SNAP] (Fig. 5D, grey vs. black). The suppression of peak I_{CaL} curves at high [SNAP] (Fig. 5D, grey and black) is driven by the NO/cGMP/PKG pathway (Fig. 6E–H). In response to increased [NO] with (grey) or without (black) [ISO], activation of PKG (Fig. 6F) substantially increased from the rise in [cGMP] (Fig. 6E). For both cases of with and without [ISO], this is due to saturation of cGMP hydrolysis activities of PDEs 1, 2, 3, and 5 (Fig. 6G and H). Upon addition of ISO (Fig. 6H), despite the decrease in PDE3 rate arising from cAMP competition, PDE2 rate increased sufficiently to compensate. As a result, for both cases, with increasing [SNAP], the substantial increase in PKG activation overcomes the cross-talk response of increased PKA activation. Consequently, with increasing [NO], suppression of LCC availability and Mode A gating and an increase in Mode G gating (Fig. 5E–G) lead to suppression of peak I_{CaL} (Fig. 5D).

3.4. Role of PDE inhibition on LCC regulation under basal conditions

The model predicts that individual inhibition of PDEs 2, 3, and 4 under basal, non-stimulated conditions does not appreciably alter peak I_{CaL} , as indicated by the overlapping peak IV curves in Fig. 7A, which is consistent with the experimental finding of Verde et al. [77]. Our model is able to tease out changes in individual PDE rates during inhibition of each PDE, that is cAMP hydrolysis rate changes of PDEs 1, 2, 3, and 4 (Fig. 7B, black, red, blue, and green bars respectively) and cGMP hydrolysis rate changes in PDEs 1, 2, 3, and 5 (Fig. 7C, black, red, blue, and cyan bars respectively), in addition to the net rate change (grey). For all cases in Fig. 7B and C, the net PDE rate change (grey) is nearly the same as the decrease in rate of the inhibited PDE. As such, the remaining non-inhibited PDEs do not compensate for the loss of the inhibited PDE. This lack of PDE compensation indicates the cross-talk mechanisms are not substantially activated. On the other hand, percent increases in [cAMP] (red) and [cGMP] (blue) under individual inhibition of these PDEs are extremely low (Fig. 7D), so that PKA (red) and PKG (blue) activities are hardly affected (Fig. 7E). As a result, LCC modal distributions remain similar to that under control condition (Fig. 7F). Among these, the only comparatively larger changes are the increases in Modes G (blue) and AG (green) gating under PDE2 inhibition; however, these two modes are lower activity gating modes due to their prolonged closed times (Fig. 3C) and thus do not substantially contribute to I_{CaL} . Additionally, changes in channel availability are negligible (less than 0.1%) for all cases with respect to control. As a result, peak IV for I_{CaL} is not appreciably affected by individual inhibitions of PDEs 2, 3, or 4. Fig. 7B–E reflects that, without β -adrenergic and NO stimulation, individual PDEs are not primary regulators of LCC gating, but rather the direct interaction between the kinases and LCC.

3.5. Role of PDE inhibition on LCC regulation under β -adrenergic stimulation

As shown in Fig. 8A, individual inhibition of PDEs 2, 3, and 4 exerts the most impact on peak I_{CaL} under lower (white and light grey), rather than higher (dark grey) [ISO]. Comparing Fig. 8A and B, the degree to which [cAMP] changes in each case does not necessarily correspond to that of I_{CaL} . Under PDE2 inhibition, higher [ISO] results in larger increases in [cAMP] and PKA activation (Fig. 8B and C respectively); however, the highest [ISO] (1 μ M) resulted in the least change in I_{CaL} (dark grey Fig. 8A). Fig. 8D and E reveal

that, among the three inhibited PDEs, PDE2 inhibition causes the greatest increase in [cGMP] and PKG activation, the extent of which also increased with increasing [ISO]. This is because cAMP gradually replaces cGMP in the PDE2 catalytic domains, leading to decreased PDE2 cGMP hydrolysis, cGMP accumulation, and ultimately increased PKG activation. By decreasing channel availability, promoting Mode G gating, and keeping Mode A gating low (Suppl. Fig. S3), this elevated PKG activation, together with low β -adrenergic tone (Fig. 8B and C), suppresses potential PKA-mediated increases in I_{CaL} and completely annihilates it under high [ISO] (Fig. 8A). Consequently, for PDE2 inhibition under ISO, the interaction between LCC and the two kinases is the principle driver underlying I_{CaL} regulation.

In contrast to PDE2 inhibition, under PDE3 inhibition, higher [ISO] results in smaller increases in [cAMP] and PKA activation (Fig. 8B and C) as well as [cGMP] and PKG activation (Fig. 8D and E). The magnitude of changes in I_{CaL} inversely correlates with [ISO] (Fig. 8A), but positively correlates with changes in [cN] and the extents of PKA and PKG activation of the signaling pathway (Fig. 8B–E). This indicates that the cross-talk mechanisms regulating cAMP and cGMP dynamics are the primary regulators of I_{CaL} under PDE3 inhibition. The responses under PDE4 inhibition are similar to that under PDE3 inhibition in terms of a decrease in I_{CaL} under high [ISO] (Fig. 8A) and that these I_{CaL} changes positively correlate with changes in PKA and PKG activation (Fig. 8C and E respectively). On the other hand, changes in PKA activation (Fig. 8C) inversely correlate with that of [cAMP] (Fig. 8B), which increase with higher [ISO] (Fig. 8B). This is because PKA activation saturated from the steep rise in [cAMP] (Fig. 8B). These observations indicate that regulation of I_{CaL} under PDE4 inhibition operates under a distinct cross-talk mechanism, but similar PKA-PKG-LCC interaction, compared to that under PDE3 inhibition.

To further explain observations of Fig. 8, we examined PDE interactions in response to PDE inhibition (Suppl. Fig. S4). In contrast to non-stimulated condition (Fig. 7), all three [ISO] are sufficient to induce compensatory changes in the non-inhibited PDEs, indicating cross-talk mechanisms are activated by ISO stimulation. Among the three PDE inhibitions, PDE2 inhibition resulted in the smallest changes of PDE cAMP hydrolysis rates (Fig. S4A–C) and the largest net decrease in cGMP hydrolysis rates among PDE inhibitions (Fig. S4D–F), thus promoting PKG-mediated regulation of LCC over that of PKA. For both PDE3 and PDE4 inhibitions, changes in cGMP dynamics are comparatively small (Fig. S4D–F, second and third groups); therefore, the β -adrenergic pathway becomes the principal driver behind the observed changes in I_{CaL} (Fig. 8A). As shown in Fig. S4A–C (second groups), under PDE3 inhibition, PDE4 is a potent compensator in cAMP hydrolysis. In comparison, under PDE4 inhibition, the remaining PDEs are unable to fully compensate for the loss of PDE4, leading to larger [cAMP] rises under PDE4 inhibition (Fig. S4A–C). Consequently, for PDE4 inhibition, it is the saturation of cAMP-mediated PKA activation that prevented further increases in I_{CaL} rather than the compensatory interactions between the PDEs (Fig. 8A).

4. Discussion

4.1. Integrative modeling dissects mechanisms underlying LCC regulation by cN cross-talk signaling network

The cN cross-talk signaling network (Fig. 1A) exerts both stimulatory and inhibitory regulations of LCCs (Fig. 1B), which is essential for the initiation and coordination of cardiac electrical and mechanical properties, such as CICR, AP, and EC coupling [1,2]. On the other hand, the nature of LCC regulation by the cN cross-talk signaling network is poorly understood, in part because it has been challenging to decipher the functional roles of the numerous mechanisms contributing to I_{CaL} regulation. Prior models have studied the impact of β -adrenergic regulation or PKA activation on LCC gating [44–49], but without the integration of NO/cGMP/PKG and its cross-talk with β -adrenergic pathway. Distinct mechanisms of LCC regulation via the interaction between PDEs and cNs (Mechanism 1) and LCC interaction with activated PKA and PKG (Mechanism 2) remain largely unresolved.

In order to explain the functional role of the cN cross-talk signaling network in its regulation of LCCs, we constructed a mechanistic computational model (Fig. 1 and Fig. 2) by functionally integrating our previously validated models of the signaling pathway [8,9] with an existing LCC model [10,51]. The complete model describes I_{CaL} regulation via dynamic interactions among PKA, PKG, and LCCs as a function of extra-cellular stimuli. It reproduces experimentally observed LCC single channel (Fig. 3) and whole-cell current (Figs. 4 and 5A) characteristics as regulated by the signaling network. This model also provides a framework for describing the effects of multiple distinct phosphorylation events, that is those by two kinases, PKA and PKG, on the gating of a single target ion channel. Our model helps tease out the complex interactions underlying existing experimental observations. Model simulations show that PKA- and PKG-mediated regulation of channel open and closed times accounts for changes in whole-cell I_{CaL} under β -adrenergic and NO stimulation (Figs. 3–5). In addition, NO-mediated suppression of basal and β -adrenergic pre-stimulated I_{CaL} occurs via potentiation of LCC Mode G gating. Finally, individual inhibition of PDEs 2, 3, and 4 is compensated through cross-talk mechanisms as well as PKA- and PKG-mediated redistribution of LCC gating modes, so that their effect on I_{CaL} is absent under basal conditions and is most prominent at lower levels of β -adrenergic stimulation (Figs. 7–8 and Suppl. Figs. S3 and S4). Regulation of one signaling pathway by the other is important in maintaining cardiac function, and perturbations within these pathways contribute to the progression of cardiac hypertrophy and heart failure (HF) [30,38–41]. Consequently, delineation of these regulatory mechanisms aids in understanding of how cN signaling imbalances in diseases [30,38–41] modify I_{CaL} and lead to alterations in EC coupling [42], AP duration (APD) [42], and increased likelihood of after-depolarizations [43].

4.2. PKA- and PKG-mediated LCC gating mode redistribution explains regulation of whole-cell I_{CaL}

Quantifying the functional effects of the multiple kinases that regulate LCCs has been challenging because of the many confounding factors in channel regulation [37,78], and the

difficulty of associating channel phosphorylation sites to functional changes in currents [55]. The model presented here explains key aspects of cN regulation of LCCs by relating whole-cell I_{CaL} to the fundamental parameters describing the opening and closing of a single LCC channel. This approach makes it possible to include information on PKA- and PKG-phosphorylated LCC gating properties derived from single-channel experiments [44,50,53,54, 62,63], which would otherwise be impossible if a more phenomenological model were employed. In the model, under basal conditions (Fig. 3A and B, “Basal”; Fig. 3C, Mode 1 in black), PKA phosphorylation increases channel availability (Fig. 3A, “ISO”) and the number of channels gating in Mode A (Fig. 3B, “ISO”), which is characterized by prolonged open time (Fig. 3C). This is consistent with the experimental results of Yue et al. [50], who demonstrated the existence of a distinct gating mode with very long lasting openings as a result of ISO stimulation. Additionally, the model allows for an understanding of how this gating scheme underlies whole-cell I_{CaL} properties – the potentiation of IV curve under maximal ISO stimulation (Fig. 4B) and the full extent of I_{CaL} potentiation under varying [ISO] (Fig. 5A) as observed by Katsube et al. [71]. Consequently, the model elucidated single-channel behavior under a wide range of β -adrenergic stimulation (Figs. 4 and 5A–C).

Furthermore, PKG phosphorylation decreases channel availability (Fig. 3A, “SNAP”) and promotes gating in Mode G (Fig. 3B, “SNAP”), which is characterized by increased channel closed time (Fig. 3C). This is consistent with observations by Tohse and Sperelakis [62] that channel closed time is doubled, while channel open time and single channel conductance remain the same as control when PKG is fully activated by hydrolysis-resistant cGMP analogue (8-bromo-cGMP) [62]. Schröder et al. [54] additionally demonstrated a decrease in channel availability under NO stimulation, with which the model is consistent. The model further relates this gating scheme to the suppression of the peak IV curve for I_{CaL} under maximal PKG activation (Fig. 4B) as observed by Sumii and Sperelakis [76]. Additionally, the model shows that dual phosphorylation of LCC by both PKA and PKG returns channel availability to approximately that of basal conditions (Fig. 3A, “ISO + SNAP”), consistent with the experimental data of Carabelli et al. [63]. Dual phosphorylation also promotes Mode AG gating, a mode characterized by prolonged open and closed times (Fig. 3C). This is consistent with the hypothesis by Carabelli et al. [63] that the effects of PKA and PKG phosphorylation on single channel gating are additive, which is also reflected in the LCC Markov model for Mode AG (Fig. 1B) which incorporates parameter adjustments of both Mode A and Mode G (i.e. $g_{modeAG} = g_{mode1}/10$ and $f_{modeAG} = f_{mode1}/2$). Our model reveals that such arrangement of gating schemes is sufficient to account for NO suppression of pre-stimulated I_{CaL} through β -adrenergic activation (Fig. 4B). This is consistent with experimental finding by Wahler and Dollinger [74] that subsequent NO stimulation restored the I_{CaL} IV curve back to that observed under basal conditions.

4.3. Model elucidates regulation of I_{CaL} via NO/cGMP/PKG signaling axis

The observation that the activation of the NO/cGMP/PKG pathway suppresses pre-stimulated I_{CaL} is consistent across various groups [74–76,79–85]. On the other hand, PKG activation via this pathway has yielded complex results under non-stimulated, basal conditions, including increased [86], decreased [73,87], or unchanged [75,80] I_{CaL} . In

addition, while the hypothesis that PKA-mediated phosphorylation of LCC is required for I_{CaL} potentiation under β -adrenergic stimulation is fairly well established, LCC phosphorylation by PKG has been less studied. We modeled PKG-mediated phosphorylation of LCC (Fig. 2), which is supported by studies demonstrating PKG phosphorylation sites on LCC [57,58]. In addition to the data shown in Fig. 4B by Sumii and Sperelakis [76], NO-mediated suppression of basal I_{CaL} has also been reported by other groups [73,87,88], which supports our model scheme (Figs. 1 and 2) and simulation results (Figs. 3–6). Additionally, Ziolo et al. [87] demonstrated that the observed I_{CaL} suppression is reversed upon application of PKG inhibitor.

Furthermore, the following experimental results in mammalian cardiac ventricular myocytes support our model by demonstrating the inhibitory regulation of the NO/cGMP/PKG pathway on I_{CaL} and direct interaction of PKG with LCCs, independent of possible confounding factors such as changes in [cAMP] and phosphatase activity via cross-talk mechanisms. First, decreasing cellular [NO], via NOS inhibitor, NO scavengers, or eNOS knockout (eNOS^{-/-}), has a stimulatory effect on basal I_{CaL} [89–91]. Second, basal I_{CaL} suppression by activation of the NO/cGMP/PKG pathway has been demonstrated through methods other than manipulating [NO], such as stimulating soluble guanylate cyclase (sGC) or perfusion of cGMP or cGMP analogue [64,85,87,92,93]. Finally, an increase of basal and pre-stimulated I_{CaL} is observed under inhibition of the NO/cGMP/PKG cascade, such as inhibiting sGC [90,94]. Additionally, the following experiments support the direct interaction between LCC and PKG assumed in our model. First, PKG-mediated suppression of LCC gating is potentiated in PKG-I transgenic mice over-expressing PKG [54]. Second, inhibitory effects of PKG on I_{CaL} has been shown to persist under PDE inhibition and phosphatase inhibition, and this ablates under PKG inactivation [76]. Finally, when I_{CaL} was enhanced by Bay K 8644, which prolongs channel open times without increasing [cAMP] [95]; the enhanced basal I_{CaL} was also reduced by activating PKG [76]. For the experiments that showed no effect [75,80] or slight stimulation [86] of I_{CaL} by NO stimulation, we believe the cause may be variation in experimental protocols. For instance NO, a highly reactive gas, is difficult to manipulate and has many cardiac reaction pathways and targets [18,19,96–101]. In fact, our model simulation shows that there is virtually no effect on I_{CaL} when [NO] is low; suppression only occurs when NO reaches a sufficiently high concentration (above ~50 nM) (Fig. 4, green; Fig. 5D). Consequently, the model predicts a threshold, and the aforementioned experiments may not have produced conditions under which [NO] crossed that threshold.

Because our model allows dynamic transition between gating modes upon activation of the cN signaling pathway (Figs. 1 and 2), we are able to explain the observed whole-cell I_{CaL} in terms of changes in signaling and functional changes in LCC gating. With increased [ISO], substantial increase in [cAMP] and subsequent PKA activation (Fig. S2) overcome the slight increase in ISO-induced PKG activation (Fig. S2) due to cN cross-talk [8], leading to increase in channel availability (Fig. 5B) and shift to Mode A gating (Fig. 5C), finally resulting in steep increases in current over the range of ~5 nM to 50 nM [ISO] (Fig. 5A). We also discovered that NO-mediated suppression of I_{CaL} occurs via potentiation of Mode G gating (Fig. 5D–G). NO limits the physiological β -adrenergic response of LCC via a thresholding effect, below threshold concentration minimal inhibition occurs and above the

threshold suppression of I_{CaL} results. This threshold is $\sim 0.1 \mu\text{M}$ [SNAP] under basal conditions and $\sim 50 \text{ nM}$ [SNAP] under maximal ISO stimulation. Above this threshold, PKG signaling starts to dominate over PKA signaling in its suppression of I_{CaL} . Activation of the NO/cGMP/PKG pathway is sufficiently strong under NO stimulation such that it overcomes β -adrenergic potentiation through cN cross-talk (Fig. 6). In addition, the model shows that PKA and PKG phosphorylation of LCC exerts antagonistic effects on I_{CaL} (Fig. 5). This is supported by the experimental finding that NO ablates the increase in pre-stimulated I_{CaL} through activation of the β -adrenergic pathway [74–76,79–85] and the converse that β -adrenergic activation also stimulates PKG-inhibited I_{CaL} [76], although the latter is not typically assessed experimentally. Our model also shows that the occurrence of Mode AG (Fig. 5C, F, and G) is rare, regardless of the extent of stimulation of the cN cross-talk network. Mode AG therefore contributes the least to I_{CaL} among the four gating modes modeled. This result adheres with intuition that the probability of the simultaneous occurrence of two independent infrequent events (phosphorylation by both PKA and PKG) is low.

In the absence of quantitative experimental data (Suppl. Sect III-B), this study assumed that PKG phosphorylation rate of LCC is equal to that of PKA (i.e. $k_{cat_PKG} = k_{cat_PKA}$). We examined our results on I_{CaL} regulation (Fig. 5, S3, and Fig. 6) through sensitivity analysis (Suppl. Sect. VI-B) by increasing (Suppl. Figs. S5–S7) and decreasing (Suppl. Figs. S8–S10) k_{cat_PKG} ten-fold. Comparing the results from this sensitivity analysis to those of the original model, ten-fold variations in k_{cat_PKG} do not produce significant changes in model outputs on LCC currents, LCC gating, or cN cross-talk signaling (Suppl. Figs. S5–S10). This is because channel dephosphorylation rate (k_{PP2A}) is much larger than the PKA and PKG kinase phosphorylation rates (k_{cat_PKA} and k_{cat_PKG} respectively) and it therefore dominates the behavior of the PKA-PKG-LCC model (Fig. 2). As a result, our key findings are not likely to be affected by the uncertainty in the exact value of the PKG phosphorylation rate of LCC (k_{cat_PKG}).

4.4. Change in I_{CaL} is mitigated by cN cross-talk and redistribution of distinct gating modes

Due to the tightly-coupled and intertwined reaction network (Fig. 1) [8,9], it has been difficult to delineate the contribution of the following two mechanisms to overall LCC regulation in response to β -adrenergic stimulation and/or stimulation of NO: 1) regulation of PKA and PKG activation via cN cross-talk (Mechanism 1); and 2) LCC interaction with activated PKA and PKG (Mechanism 2). Consequently, it has been debated if activation of the NO/cGMP/PKG pathway exerted its effect on LCC through PKG-mediated phosphorylation or its influence on PKA activation [59,102,103]. Analysis through this model is able to distinguish LCC regulation exerted by these two mechanisms. Our model therefore serves as a powerful tool for interpreting experiments on cN signaling and its interaction with I_{CaL} .

The model presented here demonstrates that the effects of PDE inhibition on I_{CaL} are most pronounced under lower levels of β -adrenergic stimulation (Fig. 8A), but are absent under basal conditions (Fig. 7A), consistent with experiments reported by Verde et al. [77]. The

overall trend of peak I_{CaL} current potentiation with 1 nM ISO under PDEs 2, 3, and 4 inhibition are consistent with that reported by Verde et al. [77] (Fig. 8A, white bars). Our model further revealed the mechanisms underlying this phenomenon (Figs. 7–8 and Suppl. Fig. S3). As shown in Fig. 7C and D, the net PDE rate change (grey) is nearly identical to the decrease in rate of the inhibited PDE, indicating that the remaining PDEs do not compensate much for the loss of the inhibited PDE through cross-talk mechanisms. This is because, under basal conditions, [cN] and its change under PDE inhibition are too minute to activate noticeable changes in PDE activities. As a result, PKA (Fig. 7E, red) and PKG (Fig. 7E, blue) interaction with LCC cannot produce sufficient changes in channel availability or modal distribution to induce a change in I_{CaL} . Because PKA-PKG-LCC interaction is preserved similar to that of control and PDE compensation is virtually non-existent under basal conditions, the absence of effects under PDE inhibition is primarily a result of Mechanism 2.

Under β -adrenergic stimulation, the cell activates both Mechanisms 1 and 2 to compensate for PDE inhibition. I_{CaL} increased the least under PDE2 inhibition, because the increase in PDE4 rate prevented large increases in [cAMP] due to strong PDE2-PDE4 coupling [104] (Mechanism 1). In addition, PKG activation under low β -adrenergic tone suppressed PKA-mediated increases in I_{CaL} (Fig. 8) (Mechanism 2). For inhibition of either PDE3 or PDE4, the β -adrenergic pathway becomes the principal driver in I_{CaL} regulation, as changes in PDE cGMP activities are small compared to those under PDE2 inhibition and to changes in PDE cAMP activities (Suppl. Fig. S4 D–F). For PDE3 inhibition, the degree of change in I_{CaL} (Fig. 8A) positively correlates with changes in PKA activation (Fig. 8C) and with that of [cAMP] (Fig. 8B), which is compensated by an increase in PDE2 and PDE4 rates (Suppl. Fig. S4) (Mechanism 1). For PDE4 inhibition, an increase in PDE2 activity partially degrades the excess cAMP, and saturation of PKA activation by cAMP prevents any further increase of LCC Mode A occupancy (Fig. 8 and Suppl. Fig. S4) (Mechanism 1). Understanding of these mechanisms will contribute to the ability to make precise manipulations of I_{CaL} through this pathway.

4.5. Model rationale, limitations, and future work

Our current study focuses on functionally integrating cN cross-talk signaling to LCC channel regulation (Fig. 1). Recent advances have revealed that many signaling components in the model (Fig. 1) form compartments and/or multi-protein signaling complexes (“signalosomes”) [40,105–111]. For instance, in addition to β -adrenergic pathway compartmentalization [112–115], NO-derived cGMP and natriuretic peptide (NP)-derived cGMP have been found to reside in distinct subcellular compartment [116,117] and exert differential regulation of β -adrenergic responses, [11,59,97,118,119], with functional significance in diseased cardiac myocytes [117,119,120]. With advancements in methods for spatiotemporally-resolved recording of cNs [40,105–110, 116,119,120] and in understanding the molecular basis of compartmentalization [109,121–125], extending the model to include these additional mechanisms serves to understand how cN signals are diversified in subcellular micro-domains, how coherent signaling is orchestrated between these compartments, and how down-stream effectors respond to the diversified signals.

Due to lack of experimental data, our model did not study the effect, if any, of LCC CDI on distribution of gating modes or gating characteristics of each gating mode [126,127]. Experimental studies using barium (Ba^{2+}) as a charge carrier, which nearly eliminates CDI [128,129], and with mutations affecting CDI [129] have demonstrated that variations of the CDI process mostly disturb channel gating kinetics in the later phase of the current, but have little effect on peak I_{CaL} . As a result, we limited our analysis to peak I_{CaL} . We further demonstrated that varying $[\text{Ca}^{2+}]$ between 1 nM and 10 μM does not significantly change model peak I_{CaL} (Suppl. Fig. S11). We also did not study the interaction between LCCs and Ca^{2+} dynamics in the micro-domain near the mouth of the LCC or the impact on CICR [78,130–133]. Because of the present uncertainty in phosphorylation sites and their functional roles [55], we modeled whether the LCC is phosphorylated by PKA, PKG, or both, instead of phosphorylation of particular amino acid site(s) (Fig. 2), reflecting a focus on the functional effect of phosphorylation instead of detailed phosphorylation mechanism. This omission of detail in the model may affect the timing of LCC availability changes and modal switching. Additional gating states other than the four gating modes modeled (Fig. 2B) may also exist, characterized by intermediate gating behaviors. On the other hand, because our study focused on steady state behavior of LCC availability and mode switching, and since the model scheme is sufficient to replicate whole-cell LCC currents from single channel behavior, these additional mechanistic details are not likely to dramatically alter model predictions included in this study. With the availability of further experimental data, a more mechanistically detailed LCC phosphorylation model than that presented in Fig. 2 will clarify the transient properties of LCC currents during PKA and PKG phosphorylation events. Furthermore, expansion of the model to include altered cN signaling network [30,38–41] and I_{CaL} [42,43,134] implicated in cardiac hypertrophy and heart failure will also help understand disease mechanisms. Aided by advances in experimental findings, modeling compartmentalization and functional integration of the model into a whole-cell myocyte model in future research will help investigate these unanswered questions in local signaling and cN regulation of Ca^{2+} cycling and AP morphology.

4.6. Summary of findings

A collection of previous studies on the cN cross-talk signaling network and related electrophysiology have allowed us to develop a functionally integrated mechanistic model of cN cross-talk and its regulation of LCCs [8,9]. Most existing approaches, however, cannot readily quantify mechanistic details in the cN signaling network, largely because [NO] is difficult to quantitatively manipulate, NO donors and NO have broad ranges of effects on cardiac myocytes, and LCC phosphorylation sites remain uncertain [96,101,102,135–138]. As a result, the quantitative model presented in this study is needed to interpret the roles of cN cross-talk in LCC regulation revealed by current experimental observations. The major findings of this work are (1) LCC gating mode redistributions underlie changes in whole-cell current under cN cross-talk network regulation; (2) NO regulation occurs via potentiation of a gating mode characterized by prolonged closed times; and (3) the effects of PDE inhibitions on LCCs are mitigated by compensatory actions of cross-talk (Mechanism 1) and antagonizing functions of PKA- and PKG-mediated phosphorylation of LCCs (Mechanism 2). It should be possible to validate findings (1) and (2) via measurements of altered whole-cell I_{CaL} under conditions where regulation of LCC availability and/or LCC gating modes is

pharmacologically disrupted. The relative contribution of Mechanism 1 vs. 2 in finding (3) can be verified if the functional consequences of LCC phosphorylation by PKA and/or PKG can be manipulated. All three findings may be further explored experimentally if the occupancy of the four LCC gating modes could be measured or deduced using pharmacological intervention. In general, we believe experiments that achieve well-controlled NO concentration and dynamics in single channel and whole-cell settings will significantly contribute to understanding of not only the mechanisms identified in this work but also discoveries of new mechanisms in cN signaling. At present, we believe computational modeling may be the most readily accessible approach to dissect PDE interactions, to understand properties of whole-cell currents arising from cN signaling-mediated changes in ion channel gating, and to reveal distinct LCC regulatory mechanisms.

5. Conclusion

We developed a computational model of LCC regulation by the cN cross-talk signaling network (Fig. 1) that functionally integrates signaling and LCC gating to investigate the effect of cN cross-talk on I_{CaL} (Fig. 2). Using the model, we deciphered the underlying mechanisms of three model observations: 1) changes in whole-cell I_{CaL} can be explained by redistribution of LCC gating modes caused by the cN cross-talk network (Figs. 3–5); 2) NO regulation of I_{CaL} occurs via potentiation of Mode G gating (Figs. 5–6); and 3) The effect of inhibition of PDEs 2, 3, and 4 is absent under basal conditions but most pronounced at low levels of β -adrenergic stimulation, because consequent changes in I_{CaL} are mitigated by cN cross-talk and redistribution of LCC gating modes (Figs. 6–8).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.yjmcc.2017.01.013>.

Fig. 1. Model representation of LCC regulation by the cN cross-talk signaling network. (A) The cyclic nucleotide (cN) cross-talk signaling network model from Zhao et al. [8] is composed of the β -adrenergic and NO/cGMP/PKG pathways (red and blue color schemes respectively) as well as the PDEs that regulate cN degradation (yellow color scheme). The PDEs are in turn regulated by the cNs; therefore, they also facilitate cross-talk between the two pathways [8,9]. The entire signal network transduces stimuli (e.g. ISO and NO) to the activation of PKA (isoforms PKA-I and PKA-II) and PKG (isoform PKG-I), which then respectively deliver stimulatory (green arrow) and inhibitory (red arrow) control of LCC (grey color scheme). Model schematics adapted from Zhao et al. [8]. (B) Each individual LCC undergo voltage- and Ca^{2+} -dependent gating (left) and an independent process of voltage-dependent inactivation (VDI) (right), according to a model from Greenstein and Winslow [10]. PKA- and PKG-mediated phosphorylation of LCC promotes LCCs to open and close in distinct gating modes ($Mode_i$), characterized by distinct gating parameters f_{mode_i} (blue) and g_{mode_i} (red). Model schematics adapted from Greenstein and Winslow [10].

A. Availability **B. Gating Mode Switching** **Fig. 2.**

State diagrams for the PKA-PKG-LCC Model. The PKA-PKG-LCC Model regulates LCC channel availability (A) and distribution of LCCs among four distinct gating modes (B). (A) The proportions of channels in the available (white) and unavailable (grey shaded) states are determined by the extent of PKA-II and PKG-I activation. (B) Increased activations of PKA-II and PKG-I promote the channels to be phosphorylated by the respective kinases, with dephosphorylation regulated by protein phosphatase, PP2A. Phosphorylation by PKA-II and PKG-I are respectively denoted by black and grey symbols with encircled “P”. Clockwise from upper-left corner, the proportions of channels in each of the non-, PKA-, PKA-and-PKG-, and PKG-phosphorylated states (grey-shaded boxes on top) correspond to distinct gating modes of Mode 1, Mode A, Mode AG, and Mode G (white boxes at the bottom).

Fig. 3.

LCC single channel properties under cN cross-talk signaling regulation. The model reproduces experimental data and prior modeling results. Dotted symbols are data from prior studies, with black and grey indicating experimental and modeling studies respectively; bars represent simulation results of the current study. (A) and (B) Simulations are performed under four stimulation conditions, “Basal” (no stimuli), “ISO” ([ISO] = 10 μ M), “SNAP” ([SNAP] = 100 μ M), and “ISO + SNAP” ([ISO] = 10 μ M and [SNAP] = 100 μ M, applied simultaneously). (B)–(C) Gating modes Mode 1, Mode A, Mode G, and Mode AG are plotted and labeled in order. (A) Percent of LCCs available under basal, ISO, SNAP, ISO + SNAP conditions versus results of Greenstein et al. (modeling) [44], Schröder et al. [54], and Carabelli et al. [63] respectively. (B) Distribution of gating modes under four stimulation conditions, versus modeling results for Basal and ISO conditions from Greenstein et al. [44]. (C) Mean open (first group) and closed (second group) times for channels gating in each mode. Mean channel open times for Mode 1 and Mode A versus data by Yue et al. [50], that for Mode G versus data by Tohse and Sperelakis [62]. Mean closed times for Mode 1, Mode A, Mode G, and Mode AG respectively versus results by Schröder et al. [54], Greenstein et al. (modeling) [44] and Yue et al. [50], Tohse and Sperelakis [62], and Klein et al. [53]. Currents are elicited by 150 ms pulses to 0 mV from a holding potential of -80 mV. The mean open and closed times of each gating mode are calculated from independent simulation of 500 sweeps, with distribution shown in Suppl. Fig. S1.

Fig. 4.

Regulation of whole-cell I_{CaL} by the cN cross-talk signaling network. Simulation are performed under four stimulation conditions, “Basal” (no stimuli, black), “ISO” ([ISO] = 10 μ M, red), “SNAP” ([SNAP] = 100 μ M, blue), and “ISO + SNAP” ([ISO] = 10 μ M and [SNAP] = 100 μ M applied simultaneously, green). (A) Whole-cell LCC ensemble currents (bottom row) elicited by 150 ms test potentials to 0 mV from a holding potential of -40 mV (top row). (B) In the bottom row, data are shown in hollow triangular symbols with vertical bars indicating ranges; simulations are shown in filled square symbols and are connected with solid lines. The peak IV curve under basal conditions (black) versus results from Greenstein and Winslow (modeling) [10]. The extents to which the IV curve changes under ISO (red), SNAP (blue), and ISO + SNAP (green) are similar to those observed by Katsube et al. [71], Sumii and Sperelakis [76], and Wahler and Dollinger [74] respectively. Model means (dots) and ranges (vertical bars) from stochastic LCC gating are shown for six independent simulations. Currents are elicited by 150 ms duration test potentials over the range of -40 mV to $+60$ mV in 10 mV steps from a holding potential of -40 mV (top row).

Fig. 5.

Redistribution of LCC gating modes by the cN cross-talk signaling network. LCC gating behavior under varying [ISO] (no SNAP) (A–C) and under varying [SNAP] with 10 μM [ISO] and without ISO (D–G). (A) and (D) The mean (dot) and range of Peak I_{CaL} (vertical bar) of six independent stochastic simulations are shown. (A) Percent increases in Peak I_{CaL} under varying [ISO] over that under basal conditions (no ISO or SNAP) (grey line) versus data by Katsube et al. [71] (black dots). (D) Peak I_{CaL} under varying [SNAP] without ISO (black) and with 10 μM [ISO] (grey). (B) and (E) Percent of LCCs available. Percentages of LCCs gating in Mode 1, Mode A, Mode G, Mode AG are shown for varying [ISO] (C), varying [SNAP] (F), and varying [SNAP] under simultaneous ISO stimulation (10 μM) (G).

Fig. 6.

Role of cN cross-talk in LCC regulation. (A) and (B) respectively, [cAMP] and percent active PKA-II under varying [SNAP] without (black) and with $10\mu\text{M}$ [ISO]. (C) cAMP hydrolysis rates for PDEs 1, 2, 3 and 4 for simulation without ISO. (D) Similar to C, for simulation with ISO. (E) and (F) respectively, [cGMP] and percent active PKG under varying [SNAP] without (black) and with $10\mu\text{M}$ [ISO]. (G) and (H) The cGMP hydrolysis rates for PDEs 1, 2, 3, and 5 for simulation without and with ISO respectively.

Fig. 7.

Results of individual PDE inhibition on I_{CaL} under basal condition. All simulations are performed under basal, non-stimulated condition, without ISO or NO. Results are compared to responses prior to the indicated PDE inhibition. (A) Peak IV curves for I_{CaL} under control condition without PDE inhibition (black) and with 90% inhibition of PDE2 (red), PDE3 (blue), and PDE4 (green). Currents are elicited from a holding potential of -50 mV to test potentials of 300 ms duration from -40 mV to $+50$ mV in 10 mV increments. The means (dots) and ranges (vertical bar; very minute) of three runs are shown for each IV curve. Under PDE 2, 3, and 4 inhibitions, increases in PDE cAMP hydrolysis rates are shown in (B), increases in PDE cGMP hydrolysis rates in (C), percent increases in [cAMP] (red) and [cGMP] (blue) in (D), percent increases in PKA (red) and PKG (blue) activation in (E), and percent increases in gating modes in (F). (C) and (D) The increases in hydrolysis rates for PDEs 1, 2, 3, 4, and 5 are shown in black, red, blue, green, and cyan respectively, with the

net rate shown in grey. (F) Percent increases in gating modes, Modes 1, A, G, and AG are shown in black, red, blue, and green outlined bars respectively.

Fig. 8.

Result of PDE inhibition on I_{CaL} under β -adrenergic stimulation. Individual inhibitions of PDEs 2, 3, and 4 (abscissa) under 1 nM (white), 10 nM (grey), and 1 μ M (dark grey) [ISO]. Percent increases in model responses are shown with respect to responses prior to the indicated PDE inhibition. For PDEs 2, 3, and 4 inhibition respectively, the application of 10 μ M EHNA, 1 μ M Cilo, and 0.3 μ M Rol have been simulated in accordance with the experiment by Verde et al. [77]. (A) Increases of peak I_{CaL} over control under low, median, and high levels of [ISO] with PDEs 2, 3, and 4 inhibition (first, second, and last bar groups respectively). The means (dots) and ranges (vertical bars) of peak I_{CaL} from three runs are shown for each bar. (B)–(E) respectively, percent increases in [cAMP], PKA-II activation, [cGMP], and PKG activation under the indicated PDE inhibition over control for each [ISO].