Tuning of Calcium Signaling Pathways by Calmodulin: The Case of \( \text{Ca}_v1.3 \) Channels and Calcineurin

by

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ABSTRACT

The modulation of voltage dependent calcium channels (CaV) by CaM has emerged as an important paradigm for understanding general CaM modulatory processes in biology. The prevailing idea for CaM regulation of CaV channels has been the IQ-centric hypothesis. By combining scanning alanine mutagenesis and FRET 2-hybrid binding assays, the IQ-centric model is challenged. An alternative model whereby CaM departs from the IQ domain is described. By applying the same methodology to naturally occurring RNA edited variants of CaV1.3 channels, a surprising role for apoCaM modulation of CDI is discovered. This apoCaM modulation is then generalized to support a mechanism of tuning of calcium flux into brain cells via ambient fluctuations in CaM. The tuning mechanism is positioned for more general study by developing a novel genetically encoded sensor of calcineurin (CN) activation, CN being the dominant CaM-activated phosphatase throughout biology. By deploying the sensor in neonatal and acutely isolated adult cardiac myocytes, it is observed that CN is capable of being activated in response to single twitch calcium transients in neonatal but not in adult myocytes. The underlying mechanism for the differential CN activation in neonatal and adult myocytes is investigated with a mathematical model capable of fitting the cardiomyocyte sensor data at all experimentally investigated pacing frequencies (0.1Hz-1Hz). Our experimentally-based mathematical model identified free CaM concentration as the main parameter responsible for determining the amplitude and kinetics of CN response in neonatal and adult cardiomyocytes. The tuning conjecture of CaM for the function of both CaV1.3 and CN looms as a potentially central feature of Ca^{2+} signaling in brain and heart.
DEDICATION

To My parents, Vahid and Parisima with infinite gratitude, whose vast and bottomless ocean of love and compassion I may never comprehend.
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Chapter 1

Tuning of CaV 1.3 Calcium Channels by CaM

1.1 Introduction and Background

Calmodulin (CaM) regulation of calcium channels is an intriguing biological phenomenon exuding some of the central paradigms involved in target activation by means of protein-protein interaction. The calcium (Ca) free form of CaM (apoCaM) is normally pre-assocaited with calcium channels (Erickson et al., 2001; Pitt et al., 2001; Erickson et al., 2003a). This preassociation occurs on a conserved IQ domain located on the regulatory c-terminal portion of the channel as depicted in Fig. 1.1 below.

Upon channel opening calcium ions enter the cell and bind the resident calcium sensor CaM. Ca\textsuperscript{2+}/CaM-channel complex then induces conformational changes that enhance channel opening (positive feedback or channel facilitation) in some channel types.

\textsuperscript{1} The results in chapter 1 have been published in Bazzazi H, Ben Johny M, Adams PJ, Soong TW & Yue DT. (2013a). Continuously tunable Ca(2+) regulation of RNA-edited CaV1.3 channels. Cell Rep 5, 367-377. Ben Johny M, Yang PS, Bazzazi H & Yue DT. (2013). Dynamic switching of calmodulin interactions underlies Ca\textsuperscript{2+} regulation of CaV1.3 channels. Nat Commun 4, 1717. The results and portions of the publications are reproduced with permission from the publisher.
Remarkably, binding of Ca\(^{2+}\) ions to individual C- and N-lobes of CaM results in alternate forms of channel regulation (Peterson et al., 1999; DeMaria et al., 2001; Yang et al., 2006). The C lobe of CaM responds to rapidly fluctuating calcium signals near the mouth of the channel (local Ca selectivity) while the N lobe of CaM detects slowly varying global calcium concentration changes (global selectivity) (Lee et al., 2000; DeMaria et al., 2001; Chaudhuri et al., 2007; Dick et al., 2008; Tadross et al., 2008).

Many biological functions including the stability of cardiac action potential are influenced by this CaM dependent calcium feedback regulation and are proposed to be targets for therapeutic treatment and management of cardiac arrhythmias (Alseikhan et al., 2002; Dolmetsch, 2003; Evans & Zamponi, 2006; Dunlap, 2007; Mahajan et al., 2008; Anderson & Mohler, 2009).

The currently accepted conceptual framework for understanding calcium induced conformational changes leading to concrete functional effects is shown in Fig. 1.2. This schematic model summarizes the states traversed from the opening of the channel to the final inactivation or facilitation with a specific reference to L-type Ca\(_{v}1.3\) channels. Channels in state E lack pre-associated CaM. They open normally in response to voltage depolarization pulses from the holding potential of \(-80\) mV (baseline), but fail to undergo Ca\(^{2+}/\)CaM dependent inactivation (CDI) over the typical depolarizing pulse of duration \(\sim 300\) ms (Liu et al., 2010). Pre-association of apoCaM to the channel results in state A. The channels in this state open normally and can also undergo CDI. The bulk cellular apoCaM can not easily access the ‘naked’ channels (channels with no apoCaM), and
switching between states E and A is very slow occurring in 10s of seconds (Chaudhuri et al., 2005). There also exists signal bifurcation between the two lobes of CaM. Binding of Ca\(^{2+}\) ions to the C-lobe results in C-lobe inactivation state $I_C$; while, the binding to the N-lobe induces N-lobe current inactivation and pushes the channels into the state $I_N$.

**Figure 1.2** General scheme for CaM regulation of the representative L-type CaV1.3 channels. (a) Primary configurations of CaM/channel complex with respect to CaM-regulatory phenomena ($E, A, I_C, I_N$, and $I_{CN}$). Inset at far right, cartoon of main channel landmarks involved in CaM regulation, with only the pore-forming $\alpha_1D$ subunit of CaV1.3 diagrammed. Ca\(^{2+}\)-inactivation (CI) region, in the proximal channel C terminus (~160 amino acids (aa)), contains elements potentially involved in CaM regulation. IQ domain (IQ), comprising the C-terminal ~30 aa of the CI segment, long proposed as preeminent for CaM/channel binding. Dual vestigial EF-hand (EF) motifs span the proximal ~100 aa of the CI module; these have been proposed to play a transduction role in channel regulation. Proximal CI (PCI) region constitutes the CI element exclusive of the IQ domain. NSCaTE on channel N terminus of CaV1.2–1.3 channels may be the N-lobe Ca\(^{2+}\)/CaM effector site. (b) Whole-cell CaV1.3 currents expressed in HEK293 cell, demonstrating CDI in the presence of endogenous CaM only. CDI observed here can reflect properties of the entire system diagrammed in (a), as schematized by the stick-figure diagram at the bottom of (b). Here and throughout, the vertical scale bar pertains to 0.2 nA of Ca\(^{2+}\) current (black); and the Ba\(^{2+}\) current (gray) has been scaled ~3-fold downward to aid comparison of decay kinetics, here and throughout. Horizontal scale bar, 100 ms. (c) Currents during overexpression of CaM WT, isolating the behavior of the diamond-shaped subsystem at bottom. (d) Currents during overexpression of CaM$_{12}$, isolating C-lobe form of CDI. (e)
In the presence of wild-type CaM (CaMWT) channels can enter fully inactivated state I_{CN} with strongly reduced opening (Kim et al., 2004; Halling et al., 2006) (current trace in Fig. 1.2b). Moreover, entry into I_{CN} state likely involves cooperative interactions between the C- and N-lobe of CaM modeled by parameter \( \lambda \). Note also that as a result of high level of cooperativity in binding of Ca\(^{2+} \) ions within each lobe of CaM, the model in Fig. 1.2a excludes the binding of single Ca\(^{2+} \) ion to CaM (Linse et al., 1991). Further, only a single CaM is included in the schematic model based on previous observations (Mori et al., 2004; Ben Johny, 2012). The currently held dominant structural basis for the state model of Fig. 1.2a is the IQ centric hypothesis (Halling et al., 2006). According to this view, the IQ domain (Fig. 1.1) serves as not only the apoCaM preassociation site, but also as the effector site for CDI transduced by Ca\(^{2+} / CaM \) (Zuhlke et al., 2000; DeMaria et al., 2001; Pitt et al., 2001). The IQ centric paradigm has motivated several groups to solve the crystal structure of Ca\(^{2+} / CaM \) in complex with IQ domains of Ca\(\text{V} \)1-2 channels (Fallon et al., 2005; Van Petegem et al., 2005; Mori et al., 2008). Several findings; however, fit poorly with this hypothesis. First, crystal structures of Ca\(^{2+} / CaM \) complexed with WT and mutant IQ indicate that the signature isoleucine residue is deeply buried within the C-lobe of CaM and alanine substitutions on this site have negligible perturbations on the overall structure (Fallon et al., 2005). Second, Ca\(^{2+} / CaM \) affinities of WT and mutant IQ are very similar (Zuhlke et al., 2000). It is not clear how mutations on the isoleucin residue communicate with different channel domains to produce the observed drastic CDI deficits. Third, in Ca\(\text{V} \)1.2 and Ca\(\text{V} \)1.3 channels the effector site for the N-lobe of CaM is the N-terminal spatial Ca\(^{2+} \) transforming element (NSCaTe) that as
the acronym suggests is located on the N-terminus of the channel structure (Fig. 1.2a far right) away from the IQ domain. Fourth, analysis of the crystal structure of Cav2.1 hints that the effector site for the C-lobe of CaM is located somewhere outside the classical IQ domain (Mori et al., 2008). These findings pose serious challenge for the IQ centric hypothesis and necessitate new approaches and ideas in identifying the effector sites for CDI.

One major issue with prior IQ-domain analysis was that the experiments were performed with endogenous CaM present. These studies ascribed most of the IQ mutational effects on the weakening of Ca\(^{2+}\)/CaM and IQ binding which was not warranted as the IQ mutations could perturb multiple transition steps of the state model in Fig. 1.2a. In this study we systematically investigate the IQ centric hypothesis by focusing on Cav1.3 channels, a member of L-type calcium channels with robust and separable C- and N-lobe CDI components (Yang et al., 2006; Dick et al., 2008; Tadross et al., 2008). Cav1.3 channels showcase a robust CDI (Fig. 1.2b) when expressed in HEK 293 cells. Compared with the Ba\(^{2+}\) current (grey trace), the calcium current (black trace) shows rapid decline following a fast upstroke. As Ba\(^{2+}\) ions do not bind to CaM (Chao et al., 1984), normalized difference between the two currents after 300 ms conveniently quantifies the extent of steady-state CDI (CDI parameter shown on the side of Fig. 1.2b). Diamond shaped subsystem (Fig. 1.2c below the current trace) is isolated by overexpressing CaM\(_{WT}\) to depopulate state E by mass action. The resulting CDI is indistinguishable from CDI under endogenous CaM levels given the very high apoCaM affinity of WT Cav1.3 channels. The C-lobe CDI component is isolated by overexpressing CaM\(_{12}\), a mutant form of CaM which is incapable of binding to Ca\(^{2+}\) ions.
on its N-lobe. This perturbation pushes the channels out of the state E while preventing the channels from entering states $I_N$ and $I_{CN}$. The current in this case has signature rapid decay as depicted in the bottom of Fig. 1.2d. To isolate the N-lobe CDI component, CaM$_{34}$ (a mutant for of CaM that is incapable of binding Ca$^{2+}$ on its C-lobe) is overexpressed in cells to force the channels by mass action into the state $I_N$. The corresponding N-lobe calcium current showcases slower decline over the duration of the depolarizing pulse (Fig. 1.2e).

Armed with these new conceptual insights, we systematically scan (with alanine mutagenesis) the IQ domain of CaV1.3 channels to investigate whether the effector site for C- and N-lobe of CaM is the IQ domain or not. What we discover is that the IQ domain is in fact not the effector site, and that both lobes of CaM depart from the IQ domain.

### 1.2 IQ Domain is not the Effector Site for Ca$^{2+}$/CaM Mediated CDI

To investigate the correlation between the binding of Ca$^{2+}$/CaM to the IQ domain and N- and C-lobe CDI, we need to develop a theoretical framework for interpreting the CDI vs. affinity plots. In a hypothetical experiment, there are four outcomes for this plot shown in Fig. 1.3.
Figure 1.3 Probing functionally relevant Ca\textsuperscript{2+}/CaM interactions. (a) Unique Langmuir relation (equation 1) that will emerge upon plotting channel CDI (defined Fig. 1.2b, right) as a function of $K_{a,\text{EFF}}$ (association constant measured for isolated channel peptide), if $K_{a,\text{EFF}}$ is proportional to one of the actual association constants in the scheme as in Fig. 1.2a. Black symbols, hypothetical results for various channel/peptide mutations; green symbol, hypothetical WT. (b) Predicted outcome if peptide association constant $K_{a,\text{EFF}}$ has no bearing on association constants within holochannels. (c) Outcome if mutations affect holochannel association constants, but not peptide association constants. (d) Outcome if mutations affect holochannel association constant(s) and peptide association constant, but in ways that are poorly correlated.

One possibility is that the data points (each point represents average CDI value of a hypothetical IQ mutation introduced in Ca\textsubscript{V}1.3 and expressed in HEK 293 cells) lie horizontal to the WT point (Fig 1.3 a-d green circle). This implies that the mutations disrupt the peptide binding but leave CDI unchanged because the mutations do not influence any of the CDI transitions pathways within the holochannel. The second case involves vertical positioning of the points relative to the WT point (Fig 1.3c) implying that the mutations do not perturb binding of Ca\textsuperscript{2+}/CaM with the IQ peptide, but do reduce CDI as they perturb the transitions within the holochannel. It is also possible that mutations disrupt transitions governing CDI within holochannel, but in ways that are uncorrelated with perturbation in Ca\textsuperscript{2+}/CaM binding to a corresponding mutant peptide segment (Fig. 1.3d). The red symbol (Fig. 1.3d) denotes a specific subset of point mutations where the specific mutations disrupt the transitions within the holochannel in a manner as to enhance CDI which is uncorrelated with the diminishing of the Ca\textsuperscript{2+}/CaM binding to peptide segment harboring the same mutation.
To demonstrate that the IQ domain is indeed the effector site for CDI, the data points must follow a binding (or Langmuir) curve described by the Langmuir equation (Fig. 1.3a, Fig. S1.2):

\[ CDI = CDI_{\text{Max}} \frac{K_{a,\text{EFF}}}{K_{a,\text{EFF}} + \Lambda} \]  

(1.1)

\( CDI_{\text{Max}} \) denotes the maximum value of CDI achieved by progressive weakening of the binding. \( \Lambda \) is the affinity when CDI is 50% of its maximum level. If the plot of CDI versus \( K_{a,\text{EFF}} \) for various alanine substitutions follow a Langmuir relation (Eq. 1.1 and Fig. 1.3a), then the domain is the Ca\(^{2+}\)/CaM effector site for CDI. Conceptually, the Langmuir correlation implies that weakening the affinities progressively reduces CDI in a way as to follow a binding curve, and lends strong support to the hypothesis that that the IQ domain is the effector site for Ca\(^{2+}\)/CaM. This concept may be generalized to identify other Ca\(^{2+}\)/CaM effector sites on the holochannel as well. Briefly, if the binding of Ca\(^{2+}\)/CaM to domain X of the holochannel is suspected to be the effector site for CDI, alanine mutagenesis coupled with peptide-Ca\(^{2+}\)/CaM affinity measurements, can in principle prove whether X is the effector site or not. An observed Langmuir correlation curve (Fig. 1.3a and Fig. S1.2) would mean that X is an effector site, while other configurations (Fig. 1.3b-d) would imply otherwise.

We systematically mutated each residue on the IQ domain of Ca\(_V\)1.3 to an alanine (to threonine if alanine occurred naturally in that position) and expressed the mutant channels along with CaM\(_{12}\) or CaM\(_{34}\) to isolate C-lobe or N-lobe CDI respectively. We also performed FRET 2-hybrid assays (Erickson et al., 2001; Erickson et al., 2003a; Dick et al., 2008) to measure binding between the mutated IQ peptides and Ca\(^{2+}\)/CaM in live HEK 293 cells and investigated the correlation between CDI and binding affinities.
according to the theoretical scheme described in Fig. 1.3. Fig. 1.4 summarizes the data. The IQ domain sequence is shown on top of Fig. 1.4a. Accordingly, for all the alanine substitutions the N-lobe CDI is the same as or above the WT level (dashed green line). C-lobe CDI is however drastically attenuated for the signature isoleucine residue (red bar with the exemplar current shown on the right) and the hotspot residues surrounding it (pink bars). For the binding assay, the yellow fluorescent protein (YFP) was fused to specific IQ peptides harboring the mutants. These YFP-fused peptides were then pitted against the cyan fluorescent protein (CFP)–fused CaM. The association constants were then computed as described previously (Erickson et al., 2001; Erickson et al., 2003a) (Fig. 1.4c far left). \( K_{a,\text{EFF}} \) bar graph (Fig. 1.4) illustrates that none of the alanine mutations on the hot spot surrounding the isoleucine residue disrupts \( \text{Ca}^{2+}/\text{CaM} \) binding to any appreciable extent. The only mutation that diminishes the binding to \( \text{Ca}^{2+}/\text{CaM} \) is the \( \text{Y}[+3]D \) mutation (Fig. 1.4c blue circle). Notice that neither C-lobe nor N-lobe CDI is disrupted with \( \text{Y}[+3]D \) mutation (Fig. 1.4a,b blue circle). The bargraphs already demonstrate the incongruency of the IQ domain as the effector site for C- or N-lobe CDI, but to show this concretely, CDI values are plotted against the association constants in Fig. 1.3d and Fig. 1.3e. For N-lobe CDI, three subsets of points are visible. For one subset of cells, the mutations disrupt the binding and increase CDI (the circles to the left of the green WT circle). For the second subset (the points congregating around the WT circle), mutations do not affect the binding or CDI. For the third subset (the two points to the right of the WT circle), the mutations increase both the binding affinity and CDI. In summary, N-lobe CDI and the affinities do not follow a binding curve. This demonstrates that the IQ domain is not the effector site for the N-lobe of CaM, and that the N-lobe
departs from the IQ domain upon its activation by calcium. In fact, the effector site for the N-lobe of CaM has already been determined to be the NSCaTe domain located on the N-terminus of CaV1.3 channels. C-lobe CDI is also uncorrelated with the binding affinities as illustrated by Fig. 1.4e. The structurally severe perturbation Y[+3]D, attenuates binding as described previously, but leaves CDI unchanged (blue point). For four mutations to the left of the WT circle, the binding is diminished while the CDI is left unscathed.

**Figure 1.4** Inconsistencies with IQ domain role as Ca²⁺/CaM effector site. (a) No appreciable deficit in isolated N-lobe CDI upon point alanine substitutions across the IQ domain (sequence at top with bolded isoleucine at ‘0’ position). Left, corresponding subsystem schematic. Middle, bar-graph summary of CDI metric, as defined in Fig.1.2b. Bars, mean±s.e.m. for ~6 cells each. Green dashed line, WT profile; red bar, I[0]A; blue symbol in all panels, Y[3]D. Right, exemplar currents, demonstrating no change in N-
lobe CDI upon I[0]A substitution. Horizontal scale bar, 100 ms; vertical scale bar, 0.2 nA Ca²⁺ current. Red, Ca²⁺ current; gray, Ba²⁺ current. (b) Isolated C-lobe CDI (corresponding subsystem schematized on left) exhibits significant attenuation by mutations surrounding the central isoleucine (coloured bars). Format as in a. I[0]A shows the strongest attenuation (red bar and exemplar currents at right). Bars average ~5 cells±s.e.m. Dashed green line, WT profile. Timebase as in b; vertical scale bar, 0.2 nA Ca²⁺ current. (c) Bar-graph summary of association constants (Kₐ,EFF=1/K₅,EFF) for Ca²⁺/CaM binding to IQ, evaluated for constructs exhibiting significant effects in b (coloured bars, with I[0]A in red), or chosen at random (hashed in b). Error bars, non-linear s.d. estimates. FRET partners schematized on the left, and exemplar binding curves on the right for I[0]A (red) and WT (black). Symbols average ~7 cells. Smooth curve fits, 1:1 binding model. Calibration to efficiency EA=0.1, far right vertical scale bar; Horizontal scale bar corresponds to 100 nM. (d) Plots of N-lobe CDI versus Kₐ,EFF deviate from equation 1, much as in Fig. 1.3b. Green, WT; red, I[0]A; blue, Y[3]D. (e) Plots of C-lobe CDI versus Kₐ,EFF also diverge from Langmuir, as in Fig. 1.3d. This result further argues against the IQ per se acting as an effector site for the C-lobe of Ca²⁺/CaM. Symbols as in d. (d,e) Y[3]D (blue symbol, CDI mean of four cells) yields poor Ca²⁺/CaM binding, but unchanged CDI. Fig.S1.7 and S1.8 for further FRET data.

For four cells including the signature isoleucin (red circle) the affinities are unchanged while the CDI is attenuated. For the two mutations to the right of the WT circle, the binding is increased while CDI is reduced. In short, C-lobe CDI is Langmuir uncorrelated to the binding affinities, implying that the IQ domain is not the effector site for the C-lobe of CaM, and that C-lobe of CaM may depart from the IQ domain and bind elsewhere on the holochannel. The effector site for C-lobe of CaM is determined with similar Langmuir type analysis to be the PCI region (Fig. 1.2a far right) (Ben Johny et al., 2013).

Armed with the new model of CaM regulation dubbed ‘CaM departure model’, we will attempt to understand the physiologically relevant role of naturally occurring RNA edited variants of Cav1.3 channels. This analysis will in turn lead to a remarkable idea whereby fluctuations in ambient levels of apoCaM may modulate CDI of these channels in the brain.
1.3 CaM Tuning of RNA-edited Ca\textsubscript{V}1.3 Channels

Ca\textsubscript{V}1.3 channels are major portals of calcium entry into pacemaking neurons (Bean, 2007), given the more negative voltages required to open these channels (Xu & Lipscombe, 2001) (Fig.1.5a). It is not therefore surprising that these channels influence many neurobiological functions ranging from circadian rhythms drawn from repetitive spiking in the suprachiasmatic nucleus to movement control modulated by pacemaking in substantia nigra (Chan et al., 2007; Obeso et al., 2008). In fact, Ca\textsubscript{V}1.3 channels contribute to the majority of Ca\textsuperscript{2+} entry in substantia nigra neurons (Cardozo & Bean, 1995; Bean, 2007; Chan et al., 2007; Puopolo et al., 2007; Guzman et al., 2009) whose loss is connected to the pathology of Parkinson’s disease (Surmeier & Sulzer; Bezprozvanny, 2009; Surmeier & Sulzer, 2013). Given the central role of Ca\textsubscript{V}1.3 in pacemaking neurons, it is intriguing that almost half of these channels are RNA edited in the brain. This editing occurs precisely and only on the IQ domain (Fig. 1.5a blue circle) yielding channels whose reduced CDI tunes pacemaking (Huang et al., 2012). The precise mechanisms for the reduction of CDI in RNA-edited channels are unknown; however, given the advances in the previous section, we are well poised to address this problem with single residue effects in terms of down regulation of Ca\textsuperscript{2+}/CaM to the IQ domain. Moreover, based on the crystal structure of the IQ domain of Ca\textsubscript{V}1.2 with only a single glutamate to aspartate difference in complex with Ca\textsuperscript{2+}/CaM, a well constrained homology model of Ca\textsubscript{V}1.3 complex is deduced (Fig. 1.5c). Accordingly, we sought to demonstrate the precise link between CDI deficits in RNA-edited channels and the IQ domain. Contrary to expectation from the IQ-centric hypothesis and consistent with the results of the previous section, these RNA-edited variants fail to reduce the binding to
Ca\textsuperscript{2+}/CaM as do alanine scanning throughout. Surprisingly, these variants reduce CDI by perturbing the binding to apoCaM that we substantiate as being essential for CDI (Bazzazi et al., 2013a; Ben Johny et al., 2013)

Figure 1.5 Functional Effects of RNA Editing of CaV1.3 Channels, Hypothesized to Occur as Perturbation of Ca\textsuperscript{2+}/CaM Complexed Alone with Channel IQ Domain (a) Schematic of main pore-forming \(\alpha\)1D subunit of Ca\textsubscript{v}1.3 channel. Shown are cytoplasmic amino (N) and carboxy (C) termini, containing main elements implicated in CDI. CI, Ca\textsuperscript{2+}-inactivation region spanning proximal channel carboxy tail (~160 aa). CI contains elements involved in CaM regulation. IQ domain (IQ), terminal CI segment (~30 aa) believed preeminent in binding CaM. Dual vestigial EF-hand region (EF) spanning proximal ~100 aa of CI. NSCaTE element on channel N terminus of CaV1.2 and CaV1.3 channels, proposed as N lobe Ca2+/CaM effector site (Dick et al., 2008; Tadross et al., 2008) (b) Popular hypothesis about how CDI arises from CaM interactions with elements described in (a). In this view, Ca\textsuperscript{2+}/CaM binding to the IQ element alone (right) triggers CDI. ApoCaM may prebind to the IQ element in a different way (left), positioning CaM as a “resident” Ca\textsuperscript{2+} sensor (c) Homology model of Ca\textsuperscript{2+}/CaM complexed with CaV1.3 IQ domain (dark blue helix, carboxy-terminal end to right). Ca\textsuperscript{2+} ions, yellow balls (d) Exemplar recombinant CaV1.3 whole-cell currents expressed in HEK293 cells. Leftmost subpanel pertains to prototypic channels with IQDY version of IQ domain. Scale bar (0.2 nA) pertains to Ca\textsuperscript{2+} current (red) throughout. Black Ba\textsuperscript{2+} current scaled down approximately three times to facilitate comparison of decay kinetics, here and throughout. CDI metric, defined at right. Other subpanels pertain to various RNA edited variants, demonstrating a spectrum of reduced CDI strengths. Parentheses contain percentage of corresponding transcripts across mouse brain. (e)
This unexpected outcome suggests that RNA editing changes the affinity of the channels for apoCaM, and that the ambient CaM fluctuations can change the fraction of channels endowed with apoCaM. In this manner, the strength of CDI can be continuously tuned by cellular CaM levels, an effect we substantiate in substantia nigra neurons. This adjustability of CDI by CaM emerges as a key element of Ca²⁺ homeostasis throughout the brain.

1.4 Functional Effect of RNA Editing in the IQ Domain of Caᵥ1.3 Channels

Fig. 1.5d (far left) shows the current trace for the WT IQ domain (IQDY). Such channels comprise ~60% of the transcript in the brain (Huang et al., 2012). With the WT baseline in mind, we can see the resulting CDI for the RNA edited variants shown to the right of the WT trace. The composition of the central IQ domain is shown on top of each trace along with the prelevance of the transcript (Huang et al., 2012). The reduction in CDI is particularly extreme for MQDY and MQDC variants while MRDY and IRDY variants present intermediate levels of CDI attenuation. As with the alanine mutations described in the previous section, we began by investigating whether the CDI deficits correlate with Ca²⁺/CaM binding. To measure the binding we tagged peptides harboring RNA editing
alterations with YFP and pitted them against CFP tagged CaM. We then measured FRET efficiency ($E_a$) in each cell and plotted the values against CFP to obtain binding curves similar to that shown in Fig. 1.5e. The specific curve here corresponds to the signature WT IQDY peptide indicating $K_d=55$ nM. If the IQ domain is the effector site, the CDI versus affinity would produce a Langmuir binding curve (Eq. 1.1) as illustrated in Fig. 1.5f. For the typical IQDY module the association constant $K_{a,\text{EFF}} (=1/K_{d,\text{EFF}}=1/1700)$ would be $5.88 \times 10^{-4}$ reciprocal D$_{\text{free}}$ units, equivalent to an absolute association constant $K_a (=1/K_d=1/55$ nM) approximating 0.018 nM$^{-1}$. For completeness of analysis, we proceed by plotting the CDI levels, not only of RNA editing variant, but also the whole IQ domain under endogenous CaM conditions. Fig. 1.6a summarizes the population effects of CDI in both naturally occurring RNA edited variants (blue bars left) and alanine substitutions (grey and rose bars to the right). The dashed green line denotes CDI level for the WT (IQDY) channel and the IQ domain sequence is aligned atop for ease of visualization. Substitutions at several locations result in strong suppression of CDI (rose bars) with the largest effect occurring with I[0] residue. Exemplar currents for I[0]A, A[-4]T, F[4]A, and V[-9]A are shown. We next obtained binding curves for the substitutions that had the largest CDI deficits (Fig. 1.6c). The green line represents the binding curve for IQDY. Except for the slight decrease in V[-9]A, none of the mutations appretiably perturbed binding, and the curves overlapped with that of the IQDY peptide (Fig. 1.6c).

We repeated the binding experiments for the RNA editing variants as well and observed no decrease in the binding to Ca$^{2+}$/CaM (Fig. 1.6d). Consistent with the previously discussed data in section 1.1, these results support the idea that CDI deficits are not because of perturbations in Ca$^{2+}$/CaM binding to the RNA edited IQ domain.
Figure 1.6 Ca\(^{2+}\)/CaM Effector Role of IQ Domain to Explain Functional Effects of RNA Editing

**a.** Population data for CDI metric of different RNA editing variants (left cluster in blue) and of various point-alanine substitutions (right cluster, rose and gray). Metric for prototypic IQDY species shown in green. Rose bars, strongest CDI reduction by mutations. Dashed gray bars, mutations without appreciable CDI effects that were nonetheless chosen at random for subsequent Ca\(^{2+}\)/CaM binding analysis. Bars show mean ± SEM, derived from four to six cells each for editing, and approximately six cells each for the alanine scan.

**b.** Exemplar current traces corresponding to indicated point-alanine substitutions. Format as Fig. 1.5 in Fig.1.1d.

**c.** FRET two-hybrid interaction curves for Ca\(^{2+}\)/CaM versus IQ domain of point-alanine substitutions. As reference, green curve reproduces fit for prototypic IQDY species (from Fig.1.1e). Black data and fits correspond to whole-cell currents directly above in (b). Each symbol bins data from approximately three, four, seven, and eight cells (left to right).

**d.** FRET two-hybrid interaction curves for Ca\(^{2+}\)/CaM versus IQ domain of RNA editing variants. Format as in (C). Each symbol bins data from approximately seven, six, nine, and six cells (left to right).

**e.** CDI plotted as a function of $K_{a,EFF}$ deviates
To rigorously demonstrate this, we plotted CDI levels versus the association constants and observed no Langmuir correlation (Fig. 1.6e). The blue circles correspond to the RNA edited variants. The plot shows that RNA editing disrupts CDI, but does not perturb the binding to Ca$$^{2+}$/CaM. Indeed, IQ domain does not constitute an effector site for CDI deficits in RNA edited channel variants.

### 1.5 RNA Editing Perturbs apoCaM Binding to the IQ Domain

Despite the fact that the IQ domain is not the effector site for CDI deficits in RNA edited channels, these channels nonetheless show severe reduction in CDI (Fig. 1.5d and Fig. 1.6a). To address this problem we hypothesized that RNA edited variants may influence apoCaM preassociation function of the IQ domain implying that the main reason for reduction in CDI is that RNA editing forces the channels into state E in Fig. 1.2a by mass action. Fig. 1.7a illustrates this in a different way by focusing on the initial transition from channels with no apoCaM present to channels endowed with an apoCaM. The idea is that perturbations on the IQ domain whether by naturally occurring RNA editing or artificial alanine substitutions weaken the affinity of apoCaM to its IQ preassociation site on the channel and thereby move the channels to a state where they open normally but don’t undergo CDI. If the hypothesis of apoCaM binding is correct, CaM overexpression would populate the ‘naked’ channels with apoCaM by mass action and thereby rescue CDI to WT CDI level. Indeed, this is what we observe as illustrated in Fig. 1.7b for both RNA edited channels (left blue bars) and the alanine substitutions (rose bars).
Figure 1.7 ApoCaM Prebinding Role of IQ Domain Explains Effects of RNA Editing (a) Channels require preassociation with apoCaM (configuration $A$) to undergo CDI. Channels without apoCaM (configuration $E$) cannot undergo CDI. RNA editing and alanine scanning might perturb association constant $K_a$ for apoCaM preassociation, thereby reducing CDI by populating configuration $E$. (b) Population data for $CDI$ metric under strong overexpression of CaMWT ($CDI_{CaMhi}$). Different RNA editing variants (left cluster in blue). Various point-alanine substitutions (right cluster, rose and gray). Metric for prototypic IQDY species shown in green. Rose bars, strongest CDI reduction by mutations in Fig.1.6a. Gray bars, mutations without appreciable CDI effects in Fig.1.6a that were nonetheless chosen for CaM overexpression studies. Dashed gray bars, subset of gray-bar constructs chosen at random for additional FRET analysis of apoCaM binding below. Bars show mean ± SEM derived from approximately five cells each. (c) Exemplar current traces for selected constructs during CaM overexpression. Leftmost subpanel, prototypic IQDY species. Other subpanels, RNA-editing variants and point-alanine-substitution constructs, as labeled. Format as in Fig.1.5d. (d) FRET assays of apoCaM binding to entire CaV1.3 CI region (cartoon at left). FRET two-hybrid interaction curves. Green data and fit within MQDY subpanel display properties for IQDY construct (symbols bin approximately ten cells each). Black data, interaction data, and fit corresponding to RNA editing variants and alanine-substituted constructs, as labeled. Symbols
average approximately four, four, six, and five cells each (left to right). (e) Plotting the ratio of $CDI / CDI_{\text{Camhi}}$ (from Fig.1.6a and 1.7b) as function of $K_{a,\text{EFF}}$ decorates Langmuir curve (black line), arguing for apoCaM preassociation function of the IQ domain. Wild-type data in green. See also FigS.1.4 and S1.5. (f) Homology model of C lobe of apoCaM (cyan) complexed with CaV1.3 IQ domain (blue). Left: complex with prototypic IQ domain showing hotspots in red. Right: model with MQDY variant, showing potential steric clash of M[0] with apoCaM.

Remarkably, this simple maneuver restores CDI to the WT level (dashed green line) for all the mutant channels except for the isoleucine where the restoration is 50% of the WT. The exemplar currents illustrate this remarkable CDI rescue in Fig. 1.7c. To show that the binding of apoCaM is affected for altered IQ domains, we fuse the whole C-tail inactivating region (CI) of the channel (harboring the RNA editing or alanine substitutions) with YFP and pit it against CFP-fused CaM. As shown in Fig. 1.7d, RNA editing substitutions disrupt the binding to apoCaM relative to the WT. Plotting $CDI / CDI_{\text{Camhi}}$ (in accordance with Eq. 1.1 and Fig. S1.4) versus the association constants obtained by FRET, yields a Langmuir curve demonstrating that the perturbation in apoCaM preassociation is the main culprit for CDI deficits. Remarkably, the alanine substitutions also fall on this curve demonstrating a unified mechanism for CDI deficits in artificial and naturally occurring substitutions. To lend structural support to this idea, homology models of CaV1.3 IQ domain in complex with the C-lobe of apoCaM were constructed based on the analogous NMR structures for Nav channels (Chagot & Chazin, 2011; Feldkamp et al., 2011). Reassuringly, according to the constructed homology structures shown in Fig. 1.7f, alanine substitutions and RNA edited variants would perturb anchor sidechains deeply embedded within the hydrophobic groove of the C-lobe of apoCaM (Fig. 1.7f structure in the left). The green colored methionine residue in the MQDY RNA editing variant peptide-apoCaM complex (Fig. 1.7f structure in the right).
clearly demonstrates the steric clash of the methionine sidechain with the apoCaM C-lobe.

1.6 Continuously Tunable Ca\(^{2+}\) Regulation in Substantia Nigra Neurons

The newly-found understanding of CDI regulation by RNA editing (Fig. 1.7a) opens the possibility for considerable tunability of Ca\(^{2+}\) regulation of Ca\(_V\)1.3 channels beyond initial expectations. The original concept that editing modulated Ca\(^{2+}\)/CaM interaction with an IQ effector site would limit the range of CDI adjustments to discrete (and even all or none) range much like the discrete settings on a rotary switch. By contrast, the alternate mechanism involving apoCaM preassociation (Fig. 1.7a) predicts a more flexible mechanism as diagramed in Fig. 1.8a. Viewed in this way, the CDI strength of each variant may smoothly be adjusted by fluctuating levels of naturally ambient CaM (\(\Delta\)apoCaM) which could variably redistribute the channels between configurations that lack (configuration E) or manifest CDI (configuration A). In this manner, CDI may be adjusted in the way of a rheostat, with each variant requiring a different level of CaM to achieve half maximal CDI (different values of \(K_{a,\text{EFF}}\)). The biological setting where this connection is likely to be of consequence is the dopamine neurons of the substantia nigra par compacta (SNc), whose loss is linked to Ca\(^{2+}\) dysregulation in Parkinson’s disease (Chan \textit{et al.}, 2007; Guzman \textit{et al.}, 2009). As RNA editing is prominent in these neurons (Huang \textit{et al.}, 2012), we tested for the CaM-mediated upregulation of CDI in isolated murine substantia nigral neurons as exemplified in Fig. 1.8b by GFP expression under the control of a tyrosine hydroxylase promoter. Under control conditions, CDI level was -
Figure 1.8 Tuning of CDI by CaM in Substantia Nigral Neurons. (a) Refocusing the mechanistic scheme to emphasize how changes in ambient apoCaM levels (ΔapoCaM) may continuously tune the strength of CDI. (b) Confocal image of SNC neuron expressing GFP under tyrosine hydroxylase promoter. (c) Whole-cell currents from SNC neurons, averaged from n = 7 (left) and n = 6 (right) cells. Format as in Fig.1.6d. Left: with endogenous levels of CaM. Right: after strongly increasing CaM levels via pipet dialysis of recombinant CaMWT. See also Fig.S1.6. (d) Bar graph summary of data at left. (e) CDI-CaM response curves for various RNA editing species, deduced from FRET two-hybrid data in Fig.1.7d. Black curves, RNA editing species in splice variants lacking a competitive inhibitor ICDI module. Blue curves, parallel behavior of RNA editing species in splice variants containing an ICDI module. (f) Aggregate CDI-CaM response curve (black), averaged over the curves of the entire population of RNA-edited species. Weighting factors specified by transcript and splice prevalence in substantia nigra. Gray and blue zones and curves reproduce response characteristics of individual RNA editing variants. Dashed lines, CDI response and CaM estimates for SNC neurons before and after CaM supplementation, taken from (d). (g) Conceptual
scheme of Ca\textsuperscript{2+} homeostasis, incorporating continuous CaM tuning of CDI strength as projected in (f). Gray outline, generic pacemaking neuron with depolarizing Ca\textsubscript{V}1.3 and hyperpolarizing SK channels. Ca\textsuperscript{2+}-negative feedback gain on Ca\textsubscript{V}1.3 opening is continuously adjustable by CaM (as in f), in the manner of a rheostat-controlled gain element.

modest as illustrated by the current trace (Fig. 1.8c left with endogenous CaM, [CaM]\textsubscript{endo}) averaged for many neurons. Using a FRET-based genetically encoded sensor of CaM (BsCaMIQ), we estimated the free concentration of CaM\textsubscript{endo} to be 3.9 ± 1 \textmu M (n=7) based on the measurements done on the intrinsically nonfluorescent hippocampal neurons (Brody & Yue, 2000; Liu \textit{et al.}, 2010). Dihydropyridine antagonists verified that up to two thirds of the current was carried out by Ca\textsubscript{V}1.3 channels in SNc neurons (Fig. S1.6). Moreover, the CDI strength was half of the value for prototypical IQDY Ca\textsubscript{V}1.3 channels (Fig. 1.8d) measured in HEK 293 cells consistent with significant reduction as a result of RNA editing. Upon perfusion with purified CaM protein (final concentration of \textasciitilde 100 \textmu M), CDI increased by 2-folds approaching the value of the typical IQDY channel (Fig. 1.8d). This outcome is directly consistent with the tunability of CDI by CaM in SNc neurons.

\textbf{1.7 Discussion}

We have demonstrated that RNA editing of Ca\textsubscript{V}1.3 channels negatively modulates their Ca\textsuperscript{2+} regulation by an unexpected mechanism. Rather than attenuate Ca\textsuperscript{2+}/CaM binding to an effector site comprising the channel IQ element alone (Fig. 1.6a), edited variants reduce the affinity of channels for apoCaM (Fig. 1.7d). This effect promotes the occurrence of channels with no resident apoCaM sensor. This mechanism predicts that CDI of edited channels could become a smoothly continuous function of ambient CaM...
levels (Fig. 1.8a), an outcome we corroborate in substantia nigral neurons (Fig. 1.8b-d). These findings demonstrate that naturally occurring RNA editing of Cav1.3 channels acts to modulate CDI in ways that substantiate a recently emerging mechanism where apoCaM begins preassociated with the IQ and other channel elements (Fig. 1.8a, configuration A), but Ca²⁺/CaM effector configuration (configuration I) involves substantial rearrangements (Ben Johny et al., 2013) and differs considerably from that originally proposed in Figs. 1.5b and 1.5c. A surprising outcome here is that even single-residue changes may readily influence configurations outside the Ca²⁺/CaM effector complex. For example, the impression from prior work has been that channels so avidly prebind to apoCaM that they would always possess a resident CaM (Findeisen et al.; Yang et al., 2006; Findeisen et al., 2011), and that mutation of several IQ residues might be required to appreciably affect apoCaM interaction (Erickson et al., 2003a; Liang et al., 2003). This view need not be the norm. More broadly, the mechanism in Fig. 1.8a adds to the growing awareness of direct biological actions by apoCaM, despite the historical focus on the functions of Ca²⁺/CaM (Jurado et al., 1999). Given the strong variation in CaM under various disease states (Chafouleas et al., 1982; Black et al., 2004; Zhang et al., 2005; Lesnick et al., 2007; Yacoubian et al., 2008; Bezprozvanny, 2009; Ikeda et al., 2009), the rheostat-like connection between CaM levels and CDI offers a potentially important dimension of Ca²⁺ homeostasis and dysfunction (Fig. 1.8a). In this regard, it is worth considering the CaM dependence of aggregate CDI exhibited by a channel population comprising prototypic IQDY and editing variants. Fig. 1.8e shows the projected CDI response relations for individual Cav1.3 species, based on our apoCaM binding data (Fig. 1.7d). On top of RNA editing, roughly one-third of Cav1.3 channel
transcripts in substantia nigra exhibit a long splice variant featuring a competitive ICDI inhibitor of apoCaM binding to channels (Liu et al., 2010; Bock et al., 2011). This splicing of the C-terminal portion of the channel results in a parallel set of CDI response relations (Liu et al., 2010; Huang et al., 2012), shown by the set of blue curves in Fig. 1.8e. Each curve represents CDI sensitivity to apoCaM variations over a limited concentration range, as constrained by the 1:1 stoichiometry of apoCaM binding to channels (Ben Johny, 2012). However, when the aggregate response of a population of variants is considered, by averaging the individual curves with weighting factors specified by transcript and splice prevalence in substantia nigra (Bock et al., 2011; Huang et al., 2012), the far more extended relationship shown in Fig. 1.8f results (black curve). Reassuringly, our experimental estimates of CDI responsiveness and estimated CaM (black symbols from Fig. 1.8c and 1.8d) fit well with this projected aggregate response relation (black curve). This agreement between data and prediction should be taken as approximate, because our estimate of free endogenous CaM concentration was obtained using a FRET-based CaM sensor expressed in readily transfectable hippocampal neurons, rather than SNC neurons per se. This outcome then reflects a mechanism to render CDI tunable over a large dynamic range of CaM levels, unachievable by a single Cav1.3 variant. Interestingly, SNC neurons at baseline populate a “set point” right in the middle of this response relationship (Fig.1.8f, dashed line labeled CaMendo), as if to optimally exploit the full dynamics of this system. CDI can thereby adapt smoothly and continuously over a maximal range of CaM levels. Altogether, this system of adjustable interdependence (Fig. 1.8g), based on the role of the IQ domain as an apoCaM
preassociation site, now demands exploration in a vast array of neurophysiological and pathophysiological contexts.

1.8 Experimental Methods

Molecular Biology

Our baseline $\alpha_{\text{V}1.3}$ construct ($\alpha 1 \Delta 1 6 2 6$; or $\alpha_{\text{V}1.3}$short in Fig.1.5d) was closely similar to a naturally occurring rat brain variant ($\alpha 1 D$, AF3070009 (Xu & Lipscombe, 2001), encoding 1,643 amino acids) that terminates 18 residues after the IQ domain in the carboxy terminus. To facilitate mutagenesis, $\alpha 1 \Delta 1 6 2 6$ was engineered with a silent and unique KpnI restriction site at a position encoding amino acids G1538T1539, <50 residues upstream of the IQ domain. Additionally, $\alpha 1 \Delta 1 6 2 6$ contains a unique XbaI restriction site followed by a stop codon, both of which reside immediately after the IQ domain ending in V1624G1625. The engineered construct $\alpha 1 \Delta 1 6 2 6$, as cloned within mammalian expression vector pCDNA6 (Invitrogen), thereby permitted rapid substitution of mutated segments of ~260 bp between KpnI and XbaI restriction sites. Actual point mutations were introduced via QuikChange mutagenesis (Agilent Technologies), where the template was a short stretch of $\alpha 1 \Delta 1 6 2 6$ (~1,500 bp) encompassing the KpnI to XbaI segment, all as cloned within an ~3.5 kb pCR-Blunt II-TOPO vector (Invitrogen). After complete sequence verification between KpnI and XbaI restriction sites, mutated segments were cloned into $\alpha 1 \Delta 1 6 2 6$ via these same sites, yielding full-length channel constructs with point mutations. For FRET two-hybrid constructs, fluorophore-tagged CaM constructs were made as described (Erickson et al., 2003a). Other FRET constructs were made by replacing CaM in these constructs with appropriate PCR-amplified and mutated IQ segments, via unique NotI and XbaI sites.
flanking CaM (Erickson et al., 2003a). Throughout, all segments subject to PCR or QuikChange (Agilent) were verified in their entirety by sequencing.

**Transfection into HEK293 Cells**

For electrophysiology experiments, HEK293 cells were cultured in 10 cm plates, and channels were transiently transfected by a calcium phosphate protocol (Brody et al., 1997). We applied 8 μg of cDNA encoding the desired channel α1 subunit, along with 8 μg of rat brain β2a (M80545) and 8 μg of rat brain α2δ(NM012919.2) subunits. β2a minimized voltage inactivation, enhancing resolution of CDI. Additional cDNAs were added as required in cotransfections. All of the above cDNA constructs were driven by a cytomegalovirus promoter. To enhance expression, cDNA for simian virus 40 T antigen (1–2 μg) was cotransfected. For FRET two-hybrid experiments, transfections and experiments were performed as described (Erickson et al., 2003a). Electrophysiology and FRET experiments were performed at room temperature 1–2 days following transfection.

**Neuron Culture**

SNC neurons were isolated from C57BL/6 mice and FVB/N mice expressing GFP under the tyrosine hydroxylase promoter (TH-GFP) (GENSAT (Gong et al., 2003); Rockefeller University). All protocols were approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions. Mouse brains were rapidly removed after decapitation and placed in ice-cold solution containing the following (in mM): NaCl, 59.4; NaHCO3, 25; glucose, 25; sucrose, 75; KCl, 2.5; NaH2PO4, 2.3; CaCl2, 0.9; MgCl2, 14.9. Brains were sectioned into 400 μm coronal slices, and SNC dissected using a 22 gauge needle. SNC pieces were enzymatically digested with 3 mg/ml of proteinase
XXIII (Sigma) for 1 hr at 37°C in dissociation solution containing (in mM): NaSO4, 82; K2SO4, 30; HEPES (pH 7.4), 10; glucose, 10; MgCl2, 5; at 305–310 mOsm adjusted with glucose. All solutions were continuously bubbled with 95% O2, 5% CO2. Following digestion, pieces were washed in Tyrode’s solution containing (in mM): NaCl, 150; KCl, 4; CaCl2, 2; glucose, 10; and HEPES (pH 7.4), 10; at 305–310 mOsm adjusted with glucose. Tyrode’s solution was supplemented with 1 mg/ml of bovine serum albumin and trypsin inhibitor (Invitrogen). After wash, SNC pieces were moved to DMEM/F12 GlutaMAX (GIBCO) supplemented with 3% fetal bovine serum, N2 supplement, B27 supplement, NEAA, and glia-derived neurotrophic factor (GDNF) (25 ng/ml). The pieces were triturated into isolated neurons with a series of glass pipettes and plated on glass coverslips coated with poly-L-lysine and incubated at 37°C, 95% O2, and 5% CO2 until experiments performed between 1 and 5 days later. SNC neurons were identified by characteristic size and morphology, as exemplified by GFP expressing neurons from TH-GFP mice.

**Whole Cell Recording**

For both recombinant channels in HEK cells and endogenous channels in SNC neurons (C57BL/6 mice), whole-cell recordings were obtained at room temperature using Axopatch 200A or 200B amplifiers (Axon Instruments). Electrodes were pulled with borosilicate glass capillaries (World Precision Instruments, MTW 150-F4), resulting in 1–3 MΩ resistances, before series resistance compensation of 80%. The internal solutions contained (in mM) CsMeSO3, 135; CsCl2, 5; MgCl2, 1; MgATP, 4; HEPES (pH 7.3), 5; and BAPTA, 10; at 290 mOsm adjusted with glucose. The bath solution contained (in
mM): TEA-MeSO3, 140; HEPES, 10 (pH 7.3); CaCl2 or BaCl2, 40; 300 mOsm, adjusted with glucose.

**FRET Optimal Imaging**

FRET two-hybrid experiments were carried out in HEK293 cells and analyzed, largely as described (Erickson *et al.*, 2003a). During imaging, the bath solution was a Tyrode’s buffer containing either 2 mM Ca2+ alone for apoCaM interaction experiments or 2 mM Ca2+ and 10 μM ionomycin to elevate intracellular Ca2+ for Ca2+/CaM interaction experiments. In parallel experiments with cells expressing the genetically encoded Ca2+ indicator TN-XL (Mank *et al.*, 2006; Tay *et al.*, 2012), we confirmed that our ionomycin treatment achieved saturating concentrations of Ca2+ with respect to CaM binding. Concentration-dependent spurious FRET was subtracted from raw data prior to binding-curve analysis (Erickson *et al.*, 2003b; Dick *et al.*, 2008). Acceptor-centric measures of FRET were obtained with the 33-FRET algorithm as described (Erickson *et al.*, 2003a). Complementary donor-centric measures of FRET were obtained with the E-FRET method (Chen *et al.*, 2006; Ben Johny *et al.*, 2013). In vitro binding assays were performed as described previously (Erickson *et al.*, 2003a). SD error bounds on Kd,EFF estimates were determined by Jacobian error matrix analysis (Johnson, 1980). BSCaMIQ sensor measurements of free CaM concentration at rest were determined by previously established protocols (Liu *et al.*, 2010) on mouse hippocampal neurons, cultured (Brody & Yue, 2000) and transiently transfected with plasmids encoding the sensor using polyethyleneimine PEI reagent (Polysciences). Sensor
measurements in this setting were favored because of the readily transfectable nature of hippocampal versus SNc neurons.

**Homology Modeling of Ca\(^{2+}\)/CaM Complexed with Ca\(_V\)1.3 IQ Domain**

We used the python-based homology modeling software MODELER (Eswar et al., 2006) to build models of the Ca\(_V\)1.3 IQ domain (comprising positions −12 through +11 in Fig.1.6a) bound to Ca\(^{2+}\)/CaM. Briefly, to model Ca\(^{2+}\)/CaM bound IQ domain, we used Ca\(_V\)1.2 structure (2BE6 (Van Petegem et al., 2005)) as a template. The IQ domains of Ca\(_V\)1.2 and Ca\(_V\)1.3 are different only by a single residue (E\([+2]\) versus D\([+2]\)). Molecular models and atomic structures were visualized and rendered using PyMOL v.1.2r1 (DeLano Scientific).

**Homology Modeling for C Lobe of apoCaM Complexed with IQ Domain**

We used the python based homology modeling software MODELER (Eswar et al., 2006) to build models of Ca\(_V\)1.3 IQ domain bound to the C lobe of apoCaM. Our starting templates were the NMR structures of Na\(_V\)1.5 and Na\(_V\)1.2 IQ domains (Chagot & Chazin, 2011; Feldkamp et al., 2011) with templates aligned as follows:

- Ca\(_V\)1.3 KFYATFLIQDYFRKFRKRV
- Na\(_V\)1.5 EEVSAMVIQRAFRRHLLQR
- Na\(_V\)1.2 EEVSAIVIQRAYRRYLLKQ

Molecular models and atomic structures were visualized and rendered using PyMOL v.1.2r1 (DeLano Scientific).
**Supplementary Figures**

*Figure S1.1* Calibrating FRET Assay for Ca\(^{2+}\)/CaM versus Ca\(_{V1.3}\) IQ Domain, Related to Fig.1.5e. (a) FRET pair of Ca\(_{V1.2}\) IQ domain and Ca\(^{2+}\)/CaM, pertinent to panels b and c. (b) 33-FRET binding curve plots FRET efficiency (\(E_A\), from YFP standpoint) versus free concentration of CFP-CaMWT (\(D_{\text{free}}\)). Each symbol represents the mean ± SEM of ~9 closely similar cell-by-cell measurements. Smooth curve, \(E_A = E_{A,\text{max}} D_{\text{free}} / (K_{d,EFF} + D_{\text{free}})\), where \(E_{A,\text{max}}\) equals plateau value and \(K_{d,EFF}\) shown in (h). Green bar calibrates \(D_{\text{free}}\) units to nM, as given in (h). (c) E-FRET curve plots FRET efficiency (\(E_D\), from CFP standpoint) versus free concentration of YFP-IQ (\(A_{\text{free}}\)). Format analogous to (b). Symbols average ~8 cells each. Smooth curve, \(E_D = E_{D,\text{max}} A_{\text{free}} / (K_{d,EFF} + A_{\text{free}})\), where \(E_{D,\text{max}}\) equals plateau, and \(K_{d,EFF}\) equals that in (b). (d–f) 1:1 binding of Ca\(_{V2.1}\) IQ domain versus Ca\(^{2+}\)/CaM. Symbols average ~10 (e) and 15 (f) cells each. (g) In vitro dansyl-CaM fluorescence assays of Ca\(^{2+}\)/CaM binding to Ca\(_{V1.2}\) (blue) and Ca\(_{V2.1}\) (gray) IQ peptides, from literature (Erickson *et al.*, 2003a). (h) FRET assay calibration. \(K_{d,EFF}\) from live-cell assays on ordinate (a–f), absolute \(K_d\) values from in vitro assays on abscissa. Blue and gray from (g); additional literature \(K_d\) values for Ca\(_{V1.2}\) given by cyan for dansyl-CaM (Zuhlke *et al.*, 2000) and violet for CaM-tryptophan (Black *et al.*, 2005) assays. Linear slope yields green calibrations in (b), (c), (i and j) Corresponding 1:1 binding of Ca\(_{V1.3}\) IQ domain versus Ca\(^{2+}\)/CaM. Symbols average ~19 (j) and 35 cells (k) each. Panel j reproduced from main text Fig.1.5e for ease of comparison. (a–k) To confirm calibration for the technique, Fig.S1.1a and S1.1b show the result for the well-studied Ca\(_{V1.2}\) IQ domain (Zuhlke *et al.*, 1999; Pate *et al.*, 2000; Zuhlke *et al.*, 2000; Erickson *et al.*, 2003a), using our 33-FRET algorithm (Erickson *et al.*, 2001; Erickson *et al.*, 2003a; Erickson *et al.*, 2003b). The relevant FRET pair is cartooned in Fig.S1.1a, and Fig.S1.1b plots the YFP-centric FRET efficiency as a function of the relative concentration of free CFP-tagged Ca\(^{2+}\)/CaM (\(D_{\text{free}}\)). This YFP-centric efficiency is equal to \(E_{Ab}\), where \(E\) is the FRET efficiency if every YFP-tagged IQ were fully bound with CFP-CaM, and \(Ab\) is the fraction of YFP-tagged IQ domains bound (Erickson *et al.*, 2001; Erickson *et al.*, 2003a; Erickson *et al.*, 2003b). Critical to simple interpretation of the relationship in Fig.S1.1b is the confirmation of a 1:1 binding stoichiometry between CaM and the IQ domain. Toward this end, Fig.S1.1 Analyzes this
same FRET pair with an E-FRET algorithm that yields CFP-centric FRET efficiency (Chen et al., 2006) (E. Db, where Db is the fraction of CFP-tagged CaM bound) versus the relative concentration of free CFP-tagged CaM (A_free). If the stoichiometry of binding were 1:1, then the two curves should be identical (Ben Johny, 2012), as confirmed by comparison of Fig.S1.1b and Fig.S1.1c. As another example, the same FRET-based strategy was pursued with the CaV2.1 IQ domain (Fig.S1.1d–Fig.S1.1f), yielding a 1:1 binding stoichiometry with lower affinity interaction. Accordingly, we could compare the half-binding concentrations of live-cell FRET assays with the results of our prior in vitro assays of Ca^{2+}/CaM binding to corresponding IQ peptides (Erickson et al., 2003a) (Fig.S1.2h), yielding a linear fit whose slope furnishes a calibration of ~30. Hence, we could compare the half-binding concentrations of live-cell FRET assays with the \( D_{\text{free}} \) or \( A_{\text{free}} \) units per nanomolar (green bar in Fig.S1.1b, and in main text Fig.1.5e), closely similar to values obtained previously in our laboratory (Erickson et al., 2003a). Multiple in vitro binding assays of Ca^{2+}/CaM and the CaV1.2 IQ domain corroborate our own assays (Zuhlke et al., 1999; Pate et al., 2000; Black et al., 2005) (Fig.S1.2h, rose and cyan symbols). That said, we could turn to the CaV1.3 IQ domain to be studied in this report. Here, Fig.S1.1(panels i-k) confirms a 1:1 stoichiometry, and establishes a \( K_d,\text{EFF} \) for Ca^{2+}/CaM interaction of 1700 \( D_{\text{free}} \) units (\( K_d \sim 55 \) nM). This yields an association constant \( K_a,\text{EFF} \) of \( 5.88 \times 10^{-4} \) reciprocal \( D_{\text{free}} \) units (\( K_a = 1 / K_d \sim 0.018 \) nM\(^{-1}\)).

![Figure S1.2](image_url) Langmuir Equation Analysis of Ca^{2+}/CaM Effector Site, Related to Fig.1.5f. Explicit formulation of Langmuir equation analysis, assuming selective mutational perturbation of Ca^{2+}/CaM binding to effector site (\( \gamma_1 \) step). State 1, preassociated apoCaM bound to channel. State 2, transient apoCaM unbinding in channel alcove. State 3, Ca^{2+} binding to CaM in alcove. State 4, CDI state, with Ca^{2+}/CaM bound to channel effector site (rectangular insets). For approximate representation of the CDI process, we adopt the four-state configuration above in Fig.S1.2. This configuration is closely similar to those previously published to describe CDI (Tadross et al., 2008). Briefly, state 1 represents the channel prebound to apoCaM, at the dual-oval preassociation site cartooned in this complex. Occasionally, apoCaM may flicker off as an unbound apoCaM that is nonetheless ensconced within a channel ‘alcove,’ as illustrated in state 2. Ca^{2+} may bind to the four EF-hand loops within CaM, yielding state 3. As the primary source of Ca^{2+} in our experiments (with a high concentration of the rapid and high-affinity Ca^{2+} chelator 10 mM BAPTA present in the pipet dialyzate) is Ca^{2+} fluxing inward through individual channels, Ca^{2+} driving CDI will mainly be present only when the channels is open (Sherman et al., 1990), at a large and fixed \( C_{\text{spike}} \) value (Sherman et al., 1990) approximating some tens of micromolar (Tay et al., 2012). Thus, the on rate constant relating to transitions from states 2 to 3 is approximated by \( P_O \cdot k_{\text{on}} \cdot C_{\text{spike}} \), where \( P_O \) is the open probability of channels in configuration \( A \) (main text, Fig.1.7a). From this point, Ca^{2+} may unbind from CaM to yield state 2, or Ca^{2+}/CaM may bind the effector site to yield state 4. State 4 corresponds to channels that have undergone CDI, and exhibit a diminished open probability\( P_O/\text{CDI} \). Given this or like representations, we can derive the explicit Langmuir form appropriate to mutations that selectively influence the Ca^{2+}/CaM effector step \( \gamma_1 \). If FRET 2-hybrid measurements yield \( K_a,\text{EFF} = \gamma_1 / s \), where \( s \) is a fixed constant of proportionality, then the following specific Langmuir equation arises
\[ \text{CDI}_{\text{max}} = \frac{K_{a,\text{EFF}}}{\frac{1 + \varepsilon + P_O C_{\text{spike}}^4 k_{\text{on}} / k_{\text{off}}}{\lambda}} \] (S1)

where \( \text{CDI}_{\text{max}} \) is defined as \((P_O - P_{OCDI}) / P_O\), and the steady-state probability of occupying state 4 is given by \(K_{a,\text{EFF}} / (K_{a,\text{EFF}} + \Lambda)\).
**Figure S1.3** Expanded Evidence against the IQ Domain as Effector Site for Ca\textsuperscript{2+}/CaM, Related to Fig.1.6e. (a-d) Ca\textsuperscript{2+}/CaM binding and electrophysiological data for various point substitutions within the Ca\textsubscript{v}1.3 IQ domain (labeled at far left). Left column shows 3\textsuperscript{1}-FRET binding curves in black, with constructs diagrammed at top. For ease of visual comparison, the green trace reports the wild-type interaction relation (derived from main text Fig.1.5e) scaled to a common maximal \(E_{\text{max}}\) amplitude (scale bar at far right). Single-cell FRET measures binned into symbols averaging ~8, 3, 9, and 8 cells for respective panels a-d. Right column, CDI measured with only endogenous CaM present. Red trace using Ca\textsuperscript{2+} as charge carrier, with corresponding 0.2 nA scale bar at right. Black trace with Ba\textsuperscript{2+} as charge carrier, amplitude scaled to facilitate comparison of decay kinetics. Overall, note that T[-10]A (a) did disrupt binding of Ca\textsuperscript{2+}/CaM to the IQ peptide, but alanine substitutions at many other loci did not appreciably alter Ca\textsuperscript{2+}/CaM interaction, as shown in the remaining panels. Interestingly, the decrement in CDI did not seem to correspond with decreased binding, as illustrated in (b) by L[-1]A for which CDI was about halved, while binding remained unchanged.

**Figure S1.4** Langmuir Equation Analysis of apoCaM Preassociation Site. Langmuir Equation Analysis of apoCaM Preassociation Site, Related to Fig.1.7e Model configuration for understanding exchange of apoCaM with channels. Since, apoCaM preassociation occurs under Ca\textsuperscript{2+}-free conditions, we consider only states 1 and 2 in our 4-state model. Additionally, to account for exchange of apoCaM from cytosol into the alcove of a Ca\textsubscript{v} channel, we introduce a new state 2\textsuperscript{'} where apoCaM resides outside the channel alcove. Here, the rate constants \(k_{\text{in}}\) and \(k_{\text{out}}\) will account for diffusion of apoCaM. \(N_{\text{av}}\), Avogadro’s number. \(V_{\text{alcove}}\), effective volume of channel alcove for apoCaM. The evidence for 1:1 apoCaM interaction with the CI region has been presented elsewhere (Ben Johny et al., 2013). For purposes of deducing the Langmuir form of CDI dependence upon channel affinity for apoCaM, a modified version of the scheme in Fig.S1.2 can be considered. Since apoCaM exchanges slowly with the channel (Chaudhuri et al., 2005)(≥10 s of secs), the majority of the exchange occurs while intracellular Ca\textsuperscript{2+} is very low, during 20-30 s rest periods between brief voltage pulses with 10 mM BAPTA in the whole-cell pipet dialyzate. To deduce the fraction of channels with apoCaM preassociated (= \(F_b\) as defined in the main text), therefore, we only need to consider bulk exchange with states 1 and 2 (relevant to Ca\textsuperscript{2+}-free conditions) of the 4-state model. This yields the configuration shown in Fig.S1.4 above, where state 2\textsuperscript{'} depicts a channel lacking CaM in its alcove; \(\varepsilon\) is apoCaM affinity for the channel; and \(C_{\text{bulk}}\) is the concentration of free apoCaM in bulk cytoplasm. Note that there is no meaning to an interconnection between states 1 and 2\textsuperscript{'} because a channel can only directly release apoCaM into its alcove (Tadross et al., 2008). Based on this configuration, it is straightforward to calculate the equilibrium value for the fraction of channels with onboard CaM, which turns out to equal:
\[ F_b = \Pr\{\text{state1}\} + \Pr\{\text{state2}\} = \]
\[
\frac{\varepsilon}{\varepsilon + 1 + \frac{k_{\text{out}}}{k_{\text{in}}} [C_{\text{bulk}}]} + \frac{1}{\varepsilon + 1 + \frac{k_{\text{out}}}{k_{\text{in}}} [C_{\text{bulk}}]} \quad (S2)
\]

When \( \varepsilon \to 0 \), channels exhibit negligible CDI as observed experimentally in (Liu et al., 2010). Hence, it must be that

\[
0 \sim \lim_{\varepsilon \to 0} F_b = \frac{1}{1 + \frac{k_{\text{out}}}{k_{\text{in}}} [C_{\text{bulk}}]} \Rightarrow \frac{k_{\text{out}}}{k_{\text{in}}} [C_{\text{bulk}}] >> 1 \quad (S3)
\]

With this knowledge, we obtain the simplified Langmuir form

\[
F_b \sim \frac{\varepsilon}{\varepsilon + \frac{k_{\text{out}}}{k_{\text{in}}} [C_{\text{bulk}}]} \quad (S4)
\]

If our FRET 2-hybrid assays experimentally determine \( K_{a,\text{EFF}} = \varepsilon / s \), where \( s \) is a fixed constant of proportionality, then Eq.S4 becomes the Langmuir equation we use to fit actual data in main text Fig.1.7e.

\[
F_b \sim \frac{K_{a,\text{EFF}}}{K_{a,\text{EFF}} + \frac{k_{\text{out}}}{(k_{\text{in}} [C_{\text{bulk}}] s)}} \quad (S5)
\]

In the main text, we can experimentally gauge \( F_b \) for a given construct by measuring the ratio CDI / CDICaMhi as defined for main text Fig.1.7e.
Figure S1.5 Expanded Treatment of Functionally Relevant apoCaM/IQ Preassociation, Related to Fig.1.7e ApoCaM binding and electrophysiological data for various RNA editing variants and mutant point substitutions in CaV1.3 IQ domain (labeled at far left). (a–f) Left column, 3²-FRET assays of apoCaM
interaction with Cl region (cartooned at top). Black shows results for specific variant within IQ domain; light green displays wild-type relation for reference. Format as in Fig.S1.3. Symbols average ~4, 4, 4, 4, 5, and 3 cells for respective panels a-f. Middle column, exemplar traces illustrating CDI measured without CaM supplementation. Right column, exemplar traces illustrating boost of CDI measured after strong CaM supplementation. Format as in Fig.S1.3. The effect of IQ substitution to decrease CDI under endogenous CaM conditions substantially reflects decreased apoCaM preassociation with channels, yielding channels devoid of apoCaM (which thereby lack CDI). Fig.S1.5 (left column) explicitly demonstrates the decreased apoCaM affinity, using 3^3-FRET assays of apoCaM pitted against the carboxy tail of CaV1.3 channels (which includes the IQ domain). That this decreased affinity accounts mechanistically for the decreased CDI observed with only endogenous CaM present (middle column) can be confirmed by the rescue of CDI upon strong overexpression of CaMWT (right column). In particular, there is a clear correlation between the degree of rescue and the extent of decreased apoCaM affinity for the Cl module. For example, the Y[3]A mutation (Fig.S1.5f) spares both binding and CDI under endogenous CaM; moreover, overexpressing CaMWT does not augment CDI. By contrast, mutant A[-4]T (Fig.S1.5e) severely disrupts binding, and there is substantial rescue of CDI upon overexpressing CaMWT. T[-10]A (Fig.S1.5c) and V[-9]A (Fig.S1.5d) yield intermediate weakening of apoCaM binding along with moderate extents of CDI rescue by overexpressing CaMWT.

Figure S1.6 Dihydropyridine Antagonist Sensitivity of Ca^2+ Current in SNc Neurons, Related to Fig.1.8c. Nifedipine blockade of Ca^2+ currents in SNc neurons indicate ~two-thirds of current as L-type channels, largely comprised of the CaV1.3 isoform. Left, exemplar Ca^2+ current traces evoked by steps to 20 mV, before (*) and after (*) application of 10 μM nifedipine. Middle, diary plot of peak Ca^2+ corresponding to exemplar traces at left, as marked. Right, population effects of nifedipine averaged over multiple SNc neurons (number in parentheses), indicating ~2/3 blockade of current. This figure displays the substantial block of Ca^2+ currents in SNc neurons by the L-type channel specific antagonist nifedipine. These results substantiate that a large fraction of Ca^2+ current in these neurons is carried by CaV1.3 channels.
Figure S1.7 Ca\(^{2+}\)/CaM binding and electrophysiological data for various point substitutions within the Ca\(_{v}1.3\) IQ domain (labelled at far left). Leftmost column shows 3\(^{3}\)-FRET binding curves in black, with constructs diagrammed at top. For ease of visual comparison, the gray trace reports the wild-type interaction relation scaled to a common maximal FR amplitude (bar at far right). Single-cell FRET measured binned into symbol averaging ~6 cells for respective panels a–g. Second column from left, shows the Ca\(^{2+}\) and Ba\(^{2+}\) traces for endogenous CaM for each construct. The third column, isolated C-lobe CDI. Red trace with Ca\(^{2+}\) as charge carrier, with corresponding 0.2 nA scale bar at right. Black trace with Ba\(^{2+}\)
Figure S1.8 Ca\(^{2+}\)/CaM binding and electrophysiology data for Y [+3]D substitution in the Ca\textsubscript{v}1.3 IQ domain. Forma as in Fig. S1.8. Symbols in panel a average ~10 cells each.
Chapter 2

Genetically Encoded Sensor of Calcineurin Activation

2.1 Background

The phosphatase calcineurin (PP2B), first isolated by affinity chromatography (Klee & Krinks, 1978), is a central component of many calcium signaling pathways relaying elevated calcium signals from the plasma membrane to the nucleus. It is the only phosphatase that is directly modulated by a second messenger. Structurally, calcineurin (CN) is comprised of two subunits, a regulatory subunit calcineurin-A (CalNA, ~60kD) and a CaM-like calcium binding subunit calcineurin-B (CalNB, ~19kD). CalNA has three isoforms encoded by PPP3CA (α), PPP3CB (β), and PPP3CC (γ) genes. CalNA contains the catalytic phosphatase domain, Ca^{2+}/CaM binding domain, and the autoregulatory domain that under basal calcium levels binds to and blocks the catalytic domain, and prevents the activation of the phosphatase (Fig. 2.1a). The CalNB domain which has two isoforms encoded by PPP3R1 and PPP3R2 is a CaM-like molecule that has four EF-hand domains capable of binding four calcium ions. The two calcium binding sites on the N-terminus have high affinity for these ions and are occupied under basal low calcium conditions (50 nM - 100 nM), while the two sites on the C-terminal domain bind calcium with low affinity (K_d~0.5 µM), and are occupied by calcium ions when the intracellular calcium concentrations rise to hundreds of nM (Klee et al., 1979; Klee et al., 1998; Aramburu et al., 2000; Rusnak & Mertz, 2000; Yang & Klee, 2000). The low-affinity calcium binding sites on the CalNB subunit contribute to ~10% of the overall activity and stabilize the structure of activated calcineurin (Aramburu et al., 2000; Yang & Klee,
Fig. 2.1 (reproduced with permission of the publisher from (Li et al., 2011)) illustrates the domain structure of the two subunits along with the proposed kinetic pathways contributing to CN activation. The most well known target of CN is the Nuclear Factor of Activating T cells (NFAT). NFAT was first discovered as a downstream signaling molecule activated following the engagement of T cell receptor (TCR) with an antigen (Durand et al., 1988; Shaw et al., 1988). It was subsequently discovered that the immunosuppressent compound cyclosporin-A (CsA) specifically blocks the nuclear localization and DNA binding effects of NFAT (Emmel et al., 1989). Shortly thereafter, another landmark paper discovered that the NFAT inhibitory effects of cyclophilin-cyclosporinA and FKBP-FK506 complexes are attributable to the direct and potent inhibition of CN phosphatase activity by these compounds (Liu et al., 1991). The identification of CN as the target of the immunophilin-immunosuppressents suggested that CN is involved in the common step associated with T cell activation. Subsequent investigations overexpressed CN in Jurkat T cells and demonstrated that this action rendered the cells more resistant to the action of CsA and FK506, and augmented NFAT activation (Clipstone & Crabtree, 1992). Moreover, cytoplasmic NFAT was found to be a substrate for CN (Jain et al., 1993).

Although the immune system has been the classical context for discoveries regarding the CN-NFAT pathway, it was soon realized that this pathway is also critical for the development and function of a wide range of cell types and tissues. For example, axonal growth was critically dependent on CN-NFAT. Mice defective in CN signaling show severe defects in axonal growth, and respond poorly to neuronal growth factors such as netrin-1 and neurotrophins. Interestingly, the mice showed no defect in neuronal
differentiation and survival (Graef et al., 2003). CN is also activated by neuronal firing in hippocampal neurons suggesting a role in learning and memory. It has been observed that NFAT is imported to the nucleus in hippocampal interneurons following L-type calcium channel activation (Graef et al., 1999). CN has also been found to be an important regulator of the speed of rapid and slow synaptic cycling in central synapses in mice and rats (Sun et al., 2010), and is also implicated in synaptic plasticity by limiting the synaptic incorporation of AMPA receptors (Sanderson et al., 2012). The bath application or postsynaptic injection of CN inhibitors is shown to prevent NMDA dependent synaptic long term depression\(^2\) (Mulkey et al., 1994; Muller et al., 1995; Kamal et al., 1999; Zhuo et al., 1999; Winder & Sweatt, 2001). Further, LTD was blocked by CN inhibitors in hippocampal slices from adult, but not two-week old rats suggesting a developmentally regulated role for CN in LTD induction (Kamal et al., 1999). It was further shown that induction of LTD in rat hippocampal slices required CN in complex with the scaffolding protein AKAP79/150 (Jurado et al., 2010).

Shifting attention to other tissues, CN signaling is also critical for normal cardiac valve and septum development and morphology (de la Pompa et al., 1998; Ranger et al., 1998), cardiac myocyte differentiation (Kasahara et al., 2013), pancreatic beta cell growth and function (Heit et al., 2006), Schwann cell differentiation (Kao et al., 2009), and normal vascular development (Graef et al., 2001). Disruption of CalNB1 subunit in the heart results in abnormal cardiac growth, disrupted systolic and diastolic function, lower bi-ventricular to body weight ratio, and 100% mortality by the age of 7 months (Schaeffer et

\(^2\) LTD is the weakening of the synaptic transmission in response to low frequency electrical pacing as opposed to long term potentiation (LTP), which refers to the strengthening of the synaptic transmission following high frequency trains of electrical stimulation
CN-NFAT pathway is also critically linked to cardiac metabolic pathways (Schaeffer et al., 2004).

Figure 2.1 Domain Structure of Human Calcineurin (Li et al., 2011). (a) Calcineurin-A subunit (1-521 aa) consists of the catalytic domain that carries out the phosphatase function (71-342 aa), the Calcineurin-B subunit binding site (348-370 aa), CaM binding region (391-414 aa), and the autoinhibitory peptide (457-482 aa) that binds and blocks the activation of the catalytic domain. Human Calcineurin-B subunit (1-170 aa) has 4 EF-hand domains each binding to a calcium ion. The EF1 and EF2 are high affinity binding sites occupied under basal conditions, while the EF3 and EF4 are low affinity. (b) Sequence of kinetic events involved in CN activation. Under basal conditions, the autoinhibitory peptide blocks the catalytic domain of CaLNA (I). Calcium binding to CaNB partially activates Calcineurin (~10%), and stabilizes the
Aside from its function in normal biological processes, there also exists a vast and accumulating body of literature for the role of CN/NFAT in pathophysiological states. To further motivate the results of this chapter, we consider the role of CN/NFAT signaling in relation to three important human diseases: Alzheimer’s disease, cancer metastases, and cardiac hypertrophy.

Alzheimer’s disease (AD) is a neurodegenerative disease characterized by the loss of neurons, the accumulation of extracellular plaques, and intracellular neurofibrillary structures. Recent genetic studies have identified single nucleotide polymorphisms (SNPs) associated with the rate of disease progression. One of these SNPs is located on the regulatory domain of CN (Cruchaga et al., 2010). Optical methods based on fluorescence resonance energy transfer (FRET) have shown that shortly after the treatment of wild type cells with Amyloid beta, derived from a mouse model of AD or human AD brains, CN gets activated within minutes and leads to rapid morphological changes and subsequent loss of synaptic spines. NFAT is also translocated to the nucleus following longer exposures (Wu et al., 2012). In other studies, constitutively active NFAT mimicked neurotoxicity associated with amyloid beta, and inhibition of the NFAT pathway by a short peptide (containing the VIVIT amino acid sequence) alleviated spine loss and morphology changes (Rozkalne et al., 2011; Hudry et al., 2012). In assays of NFAT cytoplasmic and nuclear localization determined in samples from rapidly autopsied postmortem human tissues, the NFAT nuclear localization pattern is differentially modulated depending on different levels of cognitive function and disease progression (Abdul et al., 2009). Overall, a growing body of evidence suggests a crucial
function of the CN/NFAT pathway in regulating different aspects of AD that might provide potential avenues for future therapeutic intervention.

Turning now to cancer metastasis, there is an emerging consensus for the role of CN/NFAT signaling in cancer biology and tumor angiogenesis (Mancini & Toker, 2009). NFAT1 and NFAT5 are expressed in invasive breast ductal carcinomas and promote carcinoma invasion (Jauliac et al., 2002). Sustained CN activation is observed in B- and T-cell lymphomas and in mouse models of lymphoid diseases. Inhibition of CN activity by CsA or FK506 leads to apoptosis of leukemic cells and tumor clearance, and prolongs mouse survival. Moreover, the expression of a constitutively active mutant of CN favors leukemia progression (Medyouf et al., 2007). Application of CsA attenuates endothelial proliferation and pathological angiogenesis in cancer (Nacev & Liu, 2011). Further, targeted deletion of the CN inhibitor gene DSCR1 suppresses angiogenesis and tumor growth (Lee et al., 2014). Indeed, the study of 2814 patients with Down’s syndrome has shown that these patients have a strikingly lower incidence of cancer than the general population (Hasle et al., 2000), which may partly be attributable to the extra copy of DSCR1 gene (Baek et al., 2009). Paradoxically, there is significant increase in cancer incidence in patients on long term immunosuppressent treatment (Dantal & Soulillou, 2005). This neoplastic propensity is probably due to decreased anti-tumor immune function and also re-activation of the quiescent Epstein-Barr virus (EBV)-transformed B lymphoctes. Blocking CN can also prevent dephosphorylation and activation of the targets that negatively affect tumor growth and survival. More selective (more selective than CsA and FK506) peptide inhibitors of NFAT termed VIVIT (as they contain the consensus sequence VIVIT), potently block NFAT dephosphorylation and nuclear
translocation (Aramburu et al., 1998; Aramburu et al., 1999; Mancini & Toker, 2009), prolong survival in fully mismatched islet allografts in mice (Noguchi et al., 2004), and inhibit breast cancer invasion (Jauliac et al., 2002). Overall, there is an emerging consensus favoring a significant role for CN/NFAT signaling in cancer modulation that might provide a potential target for anti-cancer therapy.

Given that CN is the only phosphatase that is directly activated by a second messenger, it is no surprise that CN plays a prominent role in cardiac pathology. In the heart, CN/NFAT pathway has been found to be a central regulator of pathological hypertrophy (characterized by increased myocardial size and the activation of fetal genes) both in vitro and in vivo (Molkentin et al., 1998). NFAT-luciferase activity is dramatically increased following pressure overload or myocardial infarction induced heart failure, but not by exercise induced cardiac enlargement (Lim et al., 2000; Molkentin, 2004; Wilkins et al., 2004). Presence of a constitutively active CN transgene (O'Keefe et al., 1992) in adult mice, results in the activation of the fetal genes, fibrosis, and cardiac growth, all reminiscent of pathological hypertrophy (O'Keefe et al., 1992; Berry et al., 2011). This is also consistent with the finding that cardiac CaM overexpression in mice leads to massive hypertrophy (Gruver et al., 1993). Recent studies using left ventricle samples from human heart failure patients have demonstrated about 4-fold increase in CN activity compared to control subjects (Lim & Molkentin, 1999). It has further been shown that inhibition of CN by CsA prevents pathological hypertrophy in a mouse model of hypertrophic cardiomyopathy (HCM) (Molkentin et al., 1998; Sussman et al., 1998). Considering the failure of traditional therapies for hypertrophy such as angiotensin-converting enzyme (ACE) inhibitors, β-blockers, and Ca²⁺ channel inhibitors, more
selective modulators of CN/NFAT pathway promise an alternative approach to therapy, and reinforce the need for more aggressive research in elucidating the precise role of CN/NFAT dynamics in the onset of cardiac hypertrophy (Olson & Molkentin, 1999; Zhang et al., 2009; Wang et al., 2010; Facundo et al., 2012).

2.2 A FRET-based Tool for Dissecting the Spatial and Temporal Dynamics of CN Activation in Live Cells

Traditional biochemical methods have been very powerful in elucidating the basic biology and structure of CN activation in different cell types (Klee & Krinks, 1978; Klee et al., 1979; Crabtree, 1989; Liu et al., 1991; Jain et al., 1993; Stemmer & Klee, 1994; Klee et al., 1998). However, these population-based tools require fixing of cells and are not suitable for assaying CN dynamics in single live cells. Recent progress in the development of fluorescent-based genetically encoded sensors has opened new windows into studying the complex dynamics of signaling pathways in cells. The advantage of genetically encoded sensors is that they provide a way of targeting the sensors to various organelles and cellular structures in order to study the spatial and temporal variations in protein dynamics. In theory, it should also be straightforward to generate transgenic animals harboring the particular sensor and to investigate protein activation and dynamics in different temporal points and spatial regions of the transgenic animals. These sensors utilize the physical phenomenon of FRET to assay nano-scale conformational dynamics in response to activating signals (Jares-Erijman & Jovin, 2003). The rate of energy transfer by FRET depends sensitively on the distance $r$ ($\sim 1/r^6$) between the fluorophores, orientation of the fluorophores relative to each other (the parameter $\kappa^2$.
below ranges between 0-4), and the refractive index of the media (the parameter \( n^{-4} \), ranges from 1/3-1/5). The equation for the rate of energy transfer by FRET is as follows (Jares-Erijman & Jovin, 2003):

\[
k_f = \frac{k}{\tau_0} \left( \frac{R_0}{r} \right)^6
\]

\[
R_0 = c_0 \kappa^2 J n^{-4} (k_f \tau_0)
\]

\[
E = k_f \tau_0 = \frac{(R_0 / r)^6}{1 + (R_0 / r)^6}
\]

\[
c_0 = 8.8 \times 10^{-28} \text{, } J = 10^{17} \int q_{d,\lambda} \varepsilon_{d,\lambda} d\lambda \text{ in } \text{nm}^6 \text{mol}^{-1}
\]

\(q_{d,\lambda}\) is the normalized donor emission spectrum. The integration is carried out within the overlap region between the donor emission and acceptor absorption. \(E\) is the FRET transfer efficiency. \(\tau_0\) is the unperturbed donor lifetime and \(k_f\) is the rate of radiative transfer. The sensitive dependence of FRET on distance and orientation of the fluorophores provides a powerful method to study protein-protein interactions and conformational dynamics of single proteins in single live cells, and with more sensitive probes in live animals. Impressive array of genetically encoded sensors have already been developed to assay the dynamics of Ca\(^{2+}\) (Miyawaki et al., 1997; Tay et al., 2007; Whitaker, 2010; Tay et al., 2012), PKA (Zhang et al., 2001), PKC (Violin et al., 2003), cGMP (Honda et al., 2001), PI3K/AKT (Gao et al., 2011), cAMP (DiPilato et al., 2004), CaMKII (Takao et al., 2005; Erickson et al., 2011; Erickson et al., 2013), and calcineurin (Newman & Zhang, 2008; Bazzazi et al., 2013b; Mehta & Zhang, 2014).

In this chapter we describe the development of a genetically encoded sensor of CN activation showcasing improved dynamic range and kinetics and demonstrate its function and deployment in HEK293 cells, neonatal rat ventricular myocytes (NRVMS), and
acutely isolated adult guinea pig ventricular myocyte (AGPVMs). To our knowledge, this is the first genetically encoded sensor of CN capable of responding to sub-second calcium fluctuations in primary cells. We also construct a red fluorescent-based probe for NFATc1 (NFAT2) to co-assay NFAT nuclear translocation dynamics in response to CN activation. Moreover, by combining mathematical modeling with data gathered from the sensor, we formulate a remarkable conjecture (consistent with the current understanding of CN in the heart) for tuning of CN activation by CaM in acutely isolated adult cardiac myocytes.

Fig. 2.1 (Li et al., 2011) outlines a 4-state kinetic model for the activation of CN complex in response to calcium elevation. The first step in this process involves the binding of 2 Ca$^{2+}$ ions to CalNB that results in partial activation of CN. Full activation ensues following the binding of Ca$^{2+}$/CaM to the C-terminal CaM binding domain of CalNA, forcing the displacement of the autoinhibitory (AI) domain and large conformational changes relative to the CalNB N-terminus. We hypothesized that this conformational change would be detectable with FRET and proceeded to fuse Cerulean Fluorescent Protein (CFP) to the N terminus of CalNB (CalNB1 isoform) and Venus Fluorescent Protein (VFP) to the C terminus of CalNA subunit (CalNA$\alpha$ isoform) (Mondragon et al., 1997). Our initial approach was to fuse the two domains using glycine linkers (Gly$_n$) of various lengths; however, it was soon apparent that transfecting the constructs in HEK293 cells resulted in severe puncta formation and toxicity. It also seemed that the existence of long glycine linkers introduced instability in the response of the sensors to ionomycin. To alleviate the problem of puncta formation and simultaneously preserve quantitative 1-1 stoichiometry between CalNB1 and CalNA$\alpha$,

3 www.adgene.com, Adgene plasmid 11787 deposited by Dr. Anjana Rao.
viral 2A sequences were employed to fuse the two subunits together as shown below in Fig. 2.2 (Robertson et al., 1985; Trichas et al., 2008).

**Figure 2.2** The Design of DuoCalN and UniCalN Sensors of CN Activation. **(a)** Dual CN (thereby called DuoCalN) sensor constructed from fusing CalNB1 to CFP and CalNAα to VFP, and pairing the two together with a p2A-based linker to ensure quantitative 1:1 stoichiometry. **(b)** Unimolecular CN sensor (hereafter called UniCalN) is constructed by fusing the two fluorescent domains together with a mutated (and thus ‘uncleavable’) P2A sequence. The working hypothesis is that raising calcium concentration will result in reduction in FRET ratio relative to the baseline.

2A peptide sequences, first identified by Ryan and colleagues (Ryan et al., 1991), are short amino acid sequences (~20 aa) derived from different members of the picornavirus family. Placing the sequences between the genes of interest results in equal stoichiometric expression at the protein level. This remarkable technology has been used to generate multi-cistronic expression vectors for various applications including correction of multi-gene deficiency in a mouse model lacking a functional T-cell receptor (TCR):CD3 complex (Szymczak et al., 2004), and expressing multi-genes to manipulate brain circuits *in vivo* (Tang et al., 2009). Pertaining to the mechanism of action of the 2A peptide sequences, a ribosome skipping mechanism is proposed to explain the ‘self-
of the gene products and as a result of this idea, the 2A sequences are now referred to as CHYSELs (cis-acting hydrolase elements) (Donnelly et al., 2001a; Donnelly et al., 2001b). Below is a list of the most commonly used 2A sequences in the literature along with the corresponding viral origin (Kim et al., 2011). Note the conservation of the C-terminal NPGP sequence and also the presence of a flanking N-terminal GSG sequence for proper hydrolase activity. Addition of the three amino acid N-terminal sequences was found to be absolutely necessary for proper hydrolase and self-processing function in vivo (Szymczak et al., 2004).

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Sequence</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2A: GSG ATNFSLLKQAGDVEE NPGP</td>
<td>from Porcine Tescho virus</td>
<td></td>
</tr>
<tr>
<td>T2A: GSG EGRGSLLTCGDEVENPGP</td>
<td>from Thosea asigna virus</td>
<td></td>
</tr>
<tr>
<td>E2A: GSG QCTNYALLKLAGDVES NPGP</td>
<td>from Equine rhinitis A virus</td>
<td></td>
</tr>
<tr>
<td>F2A: GSG VQTLNFDLLKLAGDVES NPGP</td>
<td>from Foot-and-mouth disease virus</td>
<td></td>
</tr>
</tbody>
</table>

We selected the corresponding 2A sequence derived from porcine tescho virus (P2A) with variable flanking N-terminal sequence additions and tested the self-processing efficacy of these sequences in vivo with 3FRET (Erickson et al., 2001; Erickson et al., 2003a). We conjectured that adding N-terminal flanking sequences derived from the actual viral sequence may improve the efficiency of the P2A sequence and provide a more rational basis for the P2A design. The following amino acid sequence depicts the portion of the porcine tescho virus containing the P2A sequence (the N-terminal flanking sequences underlined and the P2A sequence highlighted in yellow):

LSATDPIEKAKDSVDNYALPSFTWKPAPVFTCSVPYVSFNSVLPA MYSGFETS
SKIPKRNNQIPQDFGFGLMLVRSSSTAGLAVSVWVKFENVRLFCPRPGIHDPLAY
SRSHPQESREHDEGLSSAMTVMAFQGPGATNFSLLKQAGDVEENPGPSLSKLYQI
LKDPAVDALCEAYDE LKKFKEQATNLLLDSFGDSENPWLNKFIKYLGAYILAWK
SLHDPMTAAAVCFIIGSDVTAFVVSCLAKHLKKFAKTDSPVPPPSPKPRSCHEKCCCGNKHNYPDLENPFSENGFSRFKKGH

The bar graph in Fig. 2.3a shows the ratio of donor (CFP) to acceptor (VFP) molecules computed from 33-FRET measurements (Erickson et al., 2001; Erickson et al., 2003a). The ratio is close to 1 for the P2A constructs. Fig. 2.3b, illustrates the FRET ratio (FR) plotted against the Cerulean (CFP) fluorescence levels. FR values of close to 1 indicate that the fluorescent proteins are completely separated (‘cleaved’ or self-processed).

**Figure 2.3** Analysing the Efficiency of the P2A Peptides with 33-FRET Technology. (a) The ratio of the donor (CFP) to acceptor (VFP) molecules computed by 33-FRET. Variations of the P2A sequences along with the flanking N-terminal addition sequences sandwiched between Cerulean (CFP) and Venus (VFP), P2A (C-P2A-V with no N-terminal flanking sequences), GSGP2A (C-GSG-P2A-V), GPGP2A (C-GPG-P2A-V), QGPGP2A (C-QGPG-P2A-V), FQGPGP2A (C-FQGPG-P2A-V), MAFQGPGP2A (C-MAFQGPG-P2A-V) all result in 1-1 stoichiometry (b) FR vs. S_CER plot showing the FRET ratio vs. Cerulean fluorescence. All N-terminal flanking additions result in FR values of close to 1. P2A without the added N-terminal sequences (C-P2A-V) and the mutant P2A (NPGP→AAAA) have high FR (~5-6) indicating no fluorophore separation.

As expected from the literature, the addition of N-terminal sequences (GSG, GPG, QGPG, FQGPG, and MAFQGPG shown in Fig. 2.3) is essential for proper P2A hydrolysis function (Szymczak et al., 2004; Kim et al., 2011). In the absence of any flanking sequences, the construct C-P2A-V acts as a dimer with high basal FR (Fig. 2.3b). Furthermore, mutating the consensus C-terminal NPGP sequence (to AAAA)
abolishes the self-processing function of P2A. Two types of sensors are constructed by fusing CFP-CalNB1 and CalNAα-VFP. The DuoCalN sensor (Dual CN) is based on using the FQGPGP2A sequence between the fluorescent domains. The UniCalN sensor (Unimolecular CN) is constructed by using the mutant form of P2A (GSGP2Amutant) where the conserved NGPGP sequence was substituted by AAAA (Fig. 2.3b). The plasmid maps (above) and domain structures (below) are shown in Figs. 2.4a and 2.4b. The sensors are then transfected in HEK293 cells to gauge and calibrate the FRET response of the sensors to acute elevations of intracellular calcium concentrations.

Figure 2.4 Plasmid Structures of DuoCalN and UniCalN Sensors. (a) The plasmid map for DuoCalN sensor containing the cleavable P2A sequence FQGPGP2A. The corresponding domain structure and the nucleotide sequence of the linker are shown below the map. (b) Plasmid map of the UniCalN sensor containing the mutated P2A (NGP→AAAA). The corresponding domain structure and the linker nucleotide sequence are shown.
2.3 Calibrating the Calcium Response of the Sensors

HEK 293 cells are transfected with the DuoCalN and UniCalN sensors and loaded with the ratio metric calcium dye Indo-1⁴ (Gryniewicz et al., 1985). The absorption spectrum of Indo-1 lies in the UV region and thus offers the possibility of co-imaging with CFP, VFP, and FRET.

![Graph showing the FRET response of DuoCalN and UniCalN to calcium transients induced by the addition of thapsigargin.](image)

**Figure 2.5** The FRET Response of DuoCalN and UniCalN to Calcium Transients Induced by the Addition of Thapsigargin. (a) Left column, thapsigargin elicits a calcium transient of 500 nM amplitude and duration of ~500 s. (b) Sensor responds by 50% change in FRET efficiency. (c) FRET signal decreases. (d) CFP signal increases consistent with the expected conformational changes in CN complex. Right column, for a cell transfected with UniCalN sensor. (e) Calcium amplitude is about 300 nM with the

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⁴ Indo-1AM, Sigma-Aldrich
duration of ~600 s. (f) The corresponding FRET efficiency change is ~ 25%. (g) FRET signal has a downward transient as expected. (h) CFP signal shows the expected upward transient.

\[
E / E_0 = \frac{FR - 1}{FR_{\text{min}} - 1}
\]

is the normalized FRET efficiency, where \( FR_{\text{min}} \) indicates the basal value of \( FR \).

Slow calcium transients are elicited by applying thapsigargin\(^5\) to cells placed in Tyrode solution with low extracellular calcium concentrations (0-400 \( \mu \)M). As shown in Fig. 2.5, the calcium transient activates the sensor and leads to a decrease in normalized \( FR \) efficiency \((E/E_0 \text{ where } E = FR - 1)\). Furthermore, there is a subsequent return to the initial level following the decay of calcium to its basal level. In HEK 293 cells, the transformation from Indo-1 ratio to calcium (Gryniewicz \textit{et al.}, 1985) is achieved by the following:

\[
R = \frac{F_{405}}{F_{485}}, \quad [Ca](nM) = \beta K_d \frac{R - R_{\text{min}}}{R_{\text{max}} - R}, \quad \beta = 2.14, \quad K_d = 200nM, \quad R_{\text{min}} = 0.44, \quad R_{\text{max}} = 2.92
\]

As a result of the thapsigargin induced slow (~10 min total duration) calcium transients, we can safely assume that the system stays at a quasi-equilibrium state. This assumption can be exploited to calibrate \( FR \) according to the cellular calcium concentration levels.

\( FR \) is normalized according to the form, \( FR_{\text{norm}} = \frac{FR - FR_{\text{min}}}{FR_{\text{max}} - FR_{\text{min}}} = \frac{E - E_0}{E_{\text{min}} - E_0} \), and plotted against \([Ca]\) (nM). This is done for each individual cell at each time point. Fig. 2.6a shows the resulting calibration plots for individual representative cells for DuoCalN sensor. Note that the trajectories of some of the cells in \( FR \) versus \([Ca]\) space show significant hysteresis and departure from the steady-state (this is nonetheless consistent with the quasi-steady state assumption for the system dynamics under slow calcium transients).

\(^5\) Powder form, Sigma-Aldrich
Figure 2.6 Quasi Steady-State Calcium Calibration of DuoCalN Sensor. (a) Individual calibration curves for 14 representative cells elicited by applying 5 µM thapsigargin (Fig. 2.5) to cells transfected with the sensor and loaded with the Indo-1 dye. DuoCalN responds to thapsigargin induced calcium transients. Single Hill curve with n=3.8 and K_D =300nM fits the data. (b) Cumulative calibration plot containing all the data points for 30 cells. The FRET ratio (FR) is normalized by 

\[ FR_{\text{Norm}} = \frac{1}{1 + \left( \frac{[Ca]}{K_d} \right)^{3.8}} \]
Figure 2.7 Quasi Steady-State Calcium Calibration of UniCalN Sensor. (a) Individual calibration curves for 30 cells each elicited by the application of thapsigargin with cells placed in 0-400 µM Ca. DuoCalN robustly and transiently responds to the thapsigargin induced calcium transients. Single Hill curve with $n=3.8$ and $K_d=300nM$ fits the data. (b) Cumulative calibration plot containing the all the data points for 13 cells. FR is normalized by $FR_{Norm} = \frac{1}{1+\left(\frac{[Ca]}{K_d}\right)^{3.8}}$. 

$FR_{Norm} = \frac{FR - FR_{min}}{FR_{max} - FR_{min}}$
The cumulative calibration plot containing the temporal data points from 30 cells is shown in Fig. 2.6b with the fitted Hill calibration equation also shown on the plot. Fig. 2.7a and Fig. 2.7b present parallel set of results for the UniCalN sensor.

According to Fig. 2.1, it would seem that six calcium ions would be necessary for full activation of CN. The binding of two calcium ions partially activates CalNB (~10%) and stabilizes the structure; while, the additional four calcium ions are necessary for activating CaM. According to our calcium calibration data here, a single curve with hill coefficient of n=3.8 fits all the cellular data. This implies that there is high level of cooperativity between the six calcium binding sites. This result is quantitatively consistent with biochemical measurements of the dependence of CN phosphatase activity on calcium concentration carried out by Klee and colleagues (Stemmer & Klee, 1994), who discovered strong cooperativity with a hill coefficient of 3. They (Stemmer & Klee, 1994) conjectured that at least three calcium ions are necessary for full activation of CN. Moreover, they discovered that in the presence of 300 nM of CaM, the half-maximum activation of CN occurred at calcium concentrations of around 1 µM. Our FRET data coupled with simultaneous calcium measurements in the presence of cytoplasmic levels of CaM (~1 µM) suggest that four calcium ions are required, and that half-maximum activation occurs at calcium concentrations of around 300 nM. It is quite satisfying that our FRET-based assay measuring CN conformational activation converges with previous biochemical data measuring actual CN phosphatase activity. This observation hints that the output from our sensors is in fact linearly related to CN activity,
as both activity and FRET activation measurements follow a Hill curve when plotted against calcium concentrations.

### 2.4 Maximum FRET Response of the Sensors

To determine the maximum calcium response of the sensors, they were transfected in HEK 293 cells with saturating amount of CaM achieved by overexpression. Our conjecture was that since CaM binding is a prerequisite for sensor activation, CaM overexpression would saturate the sensors and result in maximal activation. Indeed, after the application of 5 µM ionomycin, the steady-state $FR$ value was 1, implying that there was no FRET in the fully active sensor conformation (~1.19 for DuoCalN and ~1 for UniCalN). Accordingly, the percent $FR$ change (dynamic range) was around 90% for DuoCalN and 100% for UniCalN. We looked at 3 cells transfected with the sensors and CaM<sub>WT</sub> and the response in each cell was similar to the exemplar plots in Fig. 2.8.

The data discussed above demonstrate the utility of the sensors in effectively responding to slow calcium transients (~10 min duration) elicited by thapsigargin. Ionomycin in the presence of elevated CaM levels in HEK 293 cells (Fig. 2.8), resulted in near-complete abolition of steady-state FRET in high calcium conditions in both sensors with 90-100% reduction in $FR$. Near-perfect dynamic range coupled with rapid kinetic response, promise important applications of these sensors in vivo, where large motion artifacts and background fluorescence levels have challenged the utility of the currently existing FRET-based sensors.

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6 Powder form, Sigma-Aldrich
It would be instructive to compare the dynamic range of DuoCalN and UniCalN sensors with the currently available FRET sensors of PKA, CaMKII, and calcineurin. The PKA sensor is designed by linking a phosphobinding domain from 14-3-3τ protein with a PKA consensus peptide substrate, and sandwiching the construct between Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP).

![Figure 2.8 Maximum FRET Response Capacity of DuoCalN and UniCalN under CaMWT Overexpression](image)

<table>
<thead>
<tr>
<th>DuoCalN</th>
<th>UniCalN</th>
</tr>
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<tbody>
<tr>
<td><img src="image" alt="Ionomycin (5 μM)" /></td>
<td><img src="image" alt="Ionomycin (5 μM)" /></td>
</tr>
</tbody>
</table>

**Figure 2.8** Maximum FRET Response Capacity of DuoCalN and UniCalN under CaMWT Overexpression. (a) Maximum conformational change in response to ionomycin with DuoCalN in cells overexpressing CaMWT. Normalized FR level falls rapidly to 1 (90% dynamic change) in less than a minute and stays close to 1 for the duration of the experiment. At the bottom, the simultaneous calcium rise from around 100 nM to 4.5 μM. Calcium rise occurs in two steps: initially increasing to about 1 μM before slowly rising to the final steady-state level. (b) Conformational change in response to ionomycin for UniCalN. The FRET and calcium response parallel that of DuoCalN with 100% FR dynamic change.

The sensor output is the ratio FRET/CFP (Zhang *et al.*, 2001), with maximum attainable ratio change of 25-50%. The authors subsequently deployed this sensor in neonatal
ventricular myocytes, and detected spatiotemporal variations in PKA activity (Saucerman et al., 2006). The genetically encoded sensor of CaMKII activation is constructed similarly to the CN sensors described here, CaMKIIα protein is sandwiched between CFP/YFP or GFP/RFP-based FRET pairs. The sensor is based on the idea that Ca\(^{2+}\)/CaM binding to CaMKII displaces the autoinhibitory domain and induces detectable conformational change (Takao et al., 2005) in the CaMKII dodecamer. Indeed, the authors observed a ~30% change in HeLa cells in response to ionomycin (Fig. 3 of (Takao et al., 2005)). The sensor also responded to calcium spikes in hippocampal neurons. The sensor has also been deployed and demonstrated to respond to calcium transients in acutely isolated adult cardiomyocytes (Erickson et al., 2011). The currently available CN sensor is based on the conformational changes in NFAT protein following dephosphorylation by CN (Newman & Zhang, 2008). The construction of this sensor is based on sandwiching a portion of NFAT protein between CFP/YFP FRET pairs. The changes in FRET/CFP ratio is ~10-15% in response to ionomycin in HeLa cell. The FRET change is abolished by the application of CsA.

DuoCalN and UniCalN sensors (Bazzazi et al., 2013b) showcase a comparable if not improved dynamic range and kinetic response, and expand the genetically encoded sensor toolbox utilized for cellular resolution understanding of signaling pathways in space and time. The next section will describe a Fluorescence-based assay for calibrating the activity of the sensors by coupling the conformational FRET activation with NFATc1 nuclear translocation dynamics in HEK 293 cells.
2.5 Coupling Conformational FRET Activation of the CN Sensors to Activity

Similar to the CaMKII sensor Camuiα (Takao et al., 2005), DuoCalN and UniCalN are sensors of conformational activation state of CN. It may not be apparent how this activation state is related to the actual phosphatase activity of CN, and whether biological conclusions drawn from DuoCalN and UniCalN readouts, would prove directly informative for the role of CN activation in different physiological and pathophysiological contexts. The currently available CN activity sensor (Newman & Zhang, 2008) appears to exhibit slow kinetics and limited dynamic range. Moreover, it is not clear how conformational state of NFAT corresponds to CN enzymatic activity. To guide the use of NFAT in evaluating CN activity, it is crucial to review some of the hallmarks of NFAT biology.

The family of NFAT transcription factors consists of four CN-dependent members, NFATc1 (NFAT2), NFATc2 (NFAT1), NFATc3 (NFAT4), NFATc4 (NFAT3), and one CN-independent member NFAT5. The CN-independent member of this group NFAT5 is activated by osmotic pressure changes (Macian, 2005). The basic domain structure of NFAT is shown in Fig. 2.9a, with the two CN docking sites (Aramburu et al., 1998; Liu et al., 2009; Rodriguez et al., 2009) represented by the consensus motifs PXIXIT and LXVP (where ‘X’ denotes any amino acid), the DNA binding domain (DBD), and the domain that contains the 13 serine residues dephosphorylated by CN for full NFAT activation (Macian, 2005). When calcium concentration is low, NFAT protein is in a hyperphosphorylated state and resides mainly in cytoplasm. The rise in calcium concentration activates CN, which in turn physically docks with and dephosphorylates
NFAT, stripping off phosphates from up to 13 serine residues. Dephosphorylation exposes nuclear localizing signals (NLSs), and yields NFAT translocation into the cell nucleus. Once in the nucleus, NFAT is rephosphorylated by the kinases GSK3 and CK1 that results in its export back to the cytoplasm. Accordingly, the relative ratio of nuclear to cytoplasmic NFAT is a determinant of the relative contribution and competition between CN phosphatase activity and NFAT kinases. It is thus feasible to investigate the coupling between CN sensor activation and activity by hypothesizing the existence of a nonlinear correlation between NFAT nuclear fraction and some measure of CN activation dynamics. Indeed, as will be discussed below, there is a nonlinear relationship between nuclear NFAT fraction and the area under the curve (integral) of the normalized sensor activation profile.

We utilized the human NFATc1 clone (Beals et al., 1997) in our translocation experiments. PCR was used to introduce XhoI and XbaI sites into the NFATc1 gene, allowing subsequent cloning into a pCDNA3 (Invitrogen) plasmid containing the red fluorescent protein mCherry (Shaner et al., 2004). This resulted in mCherry-NFATc1 where, mCherry is fused to the N terminus of NFATc1 as shown in Fig. 2.9b (domain structure below the plasmid structure). The major advantage of mCherry is that it allows for simultaneous imaging with CFP and VFP. mCherry-NFATc1 and sensor constructs (DuoCalN or UniCalN) were transfected into HEK 293 cells and imaged with confocal microscopy. The FRET metric here was the simple ratio $R = \frac{FRET}{CFP}$ where the FRET channel is the emission from VFP under CFP excitation. We initially performed the

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7 www.adgene.com, Addgene plasmid 24219, Dr. Gerald R. Crabtree.
8 Carl Zeiss, LSM780
experiments at room temperature and observed no appreciable NFAT translocation in response to ionomycin or thapsigargin.

After 1-2 hours, less than 10% of the cells treated with 10 μM ionomycin responded with partial NFAT translocation. We then hypothesized that NFATc1 may require physiological temperatures near 37°C for proper function and translocation. In fact, some previous studies did perform all experiments at 37°C when investigating NFAT and NFκB translocation (Dolmetsch & Lewis, 1994; Dolmetsch et al., 1997; Dolmetsch et al., 1998). Per our hypothesis, performing the translocation experiments on the confocal microscope with a mounted temperature-controlled chamber resulted in strong NFAT translocation in 100% of HEK 293 cells treated with 10 μM ionomycin. Moreover, for our particular translocation assay, HEK 293 cells were better models than HeLa or Cos7, as their initial un-stimulated NFATc1 levels (assayed by the intensity of the red fluorescence in the nucleus) were much lower than in HeLa or Cos7 cells. We
observed suboptimal translocations (<10%) for temperatures less than 34°C, which would translate into an ultra steep temperature dependence for NFATc1 nuclear translocation.

By simultaneously observing CFP, FRET, and mCherry channels, we were able to simultaneously evaluate sensor activation and NFATc1 translocation following the application of thapsigargin. Previous experiments with Indo-1 had taught us that varying the external calcium from 0 to 400 μM could produce variable amplitude calcium transients. Therefore for the translocation experiments, the cells were placed in Tyrode solution containing calcium concentrations in the range of 0-400 μM to obtain calcium transients of variable amplitudes, and thus variable sensor activation. The cells were imaged every 30 s, and 1 μM thapsigargin were added to the solution after 10-15 minutes of baseline imaging. This initial delay in imaging was included to obtain a clear fluorescence baseline. NFATc1 nuclear translocation dynamics was calculated using the ratio of nuclear red fluorescence to the whole-cell red fluorescence, \( \frac{N_{\text{Nuclear}}}{N_{\text{WholeCell}}} \) (Figs. 2.10c and 2.10d). The sensor response was quantified by computing the normalized change in \( R = \frac{\text{FRET}}{\text{CFP}} \) \( \left( \frac{-\Delta R}{R_0} = -\frac{R - R_0}{R_0} \right) \). To deduce correlation parameters, we selected data from cells that had low initial nuclear NFAT fraction (<0.15) to retain the full range of NFAT response to CN activation. The change in total nuclear NFAT, \( \Delta N_{\text{Nuclear}} = \left( \frac{N_{\text{Nuclear}}}{N_{\text{WholeCell}}} \right)_x - \left( \frac{N_{\text{Nuclear}}}{N_{\text{WholeCell}}} \right)_0 \) was correlated with the area under the curve of \( \frac{-\Delta R}{R_0} (\int -\frac{\Delta R}{R_0} dt) \) as shown in Fig. 2.10g. A cell with small sensor activation
results in modest NFAT translocation (Fig. 2.10a), while a cell with large and broad amplitude sensor activation, leads to robust and strong NFAT nuclear accumulation (Fig. 2.10b). It is also instructive to note that the initial event in NFAT nuclear enrichment is the appearance of highly fluorescent red puncta in the nucleus that could potentially represent the sites of NFATc1 binding to cellular DNA.

Number of new insights are gained by close inspection of the coupling between CN activation and NFAT translocation dynamics in Fig. 2.10 and Fig. 2.11. In Fig. 2.10c transient sensor activation with duration of 2 minutes is sufficient to result in NFAT dephosphorylation and nuclear import. It is interesting to note that while the sensor activation transient declines to near basal level after 1.5 minutes, the fraction of nuclear NFAT continues to rise. Nuclear NFAT accumulation reaches the steady-state value of 0.3 after ~9 minutes. Similarly in Fig. 2.11c (for UniCalN sensor), NFATc1 nuclear import continues long after the abatement of the CN activation signal. These results are consistent with recent mathematical modeling work positing that high level of cooperativity between the 13 serine sites on NFAT leads to a switch like behavior in NFAT activation dynamics (Salazar & Hofer, 2003, 2009). Our results suggest that once a sufficient fraction of NFAT sites are dephosphorylated, the nuclear import is inevitable even after the fall of calcium transient and CN activation. The NFAT translocation dynamics in HEK 293 cells is also consistent with the data from T lymphocytes and Jurkat T cells. In T cells, TCR engagement with antigen induces calcium transients with a period of about 100s (Dolmetsch & Lewis, 1994) that is sufficient to activate NFAT translocation and subsequent target gene induction. In Jurkat T cells, a single calcium transient with amplitude of 1 μM and duration of 3 minutes leads to near-complete
NFAT translocation that persists long after the decay of the initiating calcium signal (Dolmetsch et al., 1997).

An important aspect of the translocation data pertaining to the kinetics of NFAT translocation is that the export rate of NFAT (biologically determined by nuclear kinases such as GSK3 and CK1) is extremely slow. In fact, in the experiments discussed here, no significant nuclear export was observed even after 1 hour of imaging.

A remarkable nonlinear correlation is discovered for both DuoCalN and UniCalN (Figs. 2.10g and 2.11g) relating the amount of NFAT import, $\Delta NFAT_{\text{nuclear}}$ to the temporal integral of the normalized CN activation, $\int_{\text{t}}\frac{-\Delta R}{R_0}dt : \Delta NFAT_{\text{nuclear}} = 0.42 \times (1 - e^{-\frac{\int_{\text{t}}-\Delta R_{\text{d}}}{R_0}dt}}$ for DuoCalN and $\Delta NFAT_{\text{nuclear}} = 0.45 \times (1 - e^{-\frac{\int_{\text{t}}-\Delta R_{\text{d}}}{R_0}dt}}$ for UniCalN. Note also that the steady-state nuclear NFAT never reaches value of 1. There are two possible reasons for this. First, the calcium transients elicited by thapsigargin might not have the large enough amplitude and long enough duration to fully activate all the available pool of CN. Second, the concentration of expressed CN sensor might not be sufficient to dephosphorylate all the available NFAT pool. In our experiments, the maximum attainable NFAT nuclear fraction was 0.8 achieved by 1 μM ionomycin in the presence of 2 mM external calcium. The use of ionomycin for activation calibration was abandoned as ionomycin had detrimental effects on the cellular morphology and viability. Ionomycin was also found to be inefficient for eliciting calcium transients of variable amplitudes. According to the calibration equations, in order to increase nuclear NFAT by 50% of its maximum value (0.45/2), CN needs to be activated to such an extent as to generate the
area under the curve of \( \int \frac{-\Delta R}{R_0} dt = 6.24s \). Suppose that the shape of the CN activation curve can be approximated as a triangle. The temporal integral can then be computed by the area of the triangle, taking into account the duration and the amplitude of the activation signal:

\[
\int \frac{-\Delta R}{R_0} dt = Amp \cdot (t_{\text{rise}} + t_{\text{fall}}) / 2 = Amp \cdot \frac{t_{\text{duration}}}{2}
\]

\( Amp = \) Amplitude of the signal

The maximum possible normalized sensor activation amplitude in HEK 293 cells under confocal microscopy was determined by the application of 10 \( \mu M \) ionomycin, and the value was about 0.2 (average value observed in \(~10\) cells). This implies that:

\[
\int \frac{-\Delta R}{R_0} dt = 0.2 \times t_{\text{duration}} / 2 = 6.24 \Rightarrow t_{\text{duration}} = 62.4s
\]

This simple calculation illustrates that effective activation of NFAT requires physiological calcium signals with duration of 10s of seconds (Lewis & Cahalan, 1989; Donnadieu et al., 1992; Dolmetsch & Lewis, 1994; Dolmetsch et al., 1997; Dolmetsch et al., 1998). This calculation also predicts that NFAT activation during single calcium transient is negligible in excitable cells such as cardiomyocytes where the duration of the calcium transient is at most 500 ms under physiological conditions.

To summarize: the UV- based calcium dye Indo-1 was utilized to concurrently image calcium concentration changes with FRET measurements. Plotting normalized \( FR, \)
FR_{Norm} = \frac{FR - FR_{min}}{FR_{max} - FR_{min}} \text{ versus calcium concentration } [\text{Ca}](\mu\text{M}) \text{ yielded a Hill equation with the Hill coefficient of } n_{Hill}=3.8, \text{ and } K_d=300 \text{ nM, for both DuoCalN and UniCalN.} 

\left(FR_{Norm} = \frac{FR - FR_{min}}{FR_{max} - FR_{min}} = \frac{1}{1 + \left(\frac{[\text{Ca}]}{300}\right)^{3.8}}\right) \text{ which implied that least 4 calcium ions are necessary for CN activation, and that cellular calcium concentration of 300 nM result in 50\% activation of the sensor. Further, by co- expressing the sensors and WT CaM, the maximum sensor response was found to be limited by CaM_{Total}. These findings are quantitatively consistent with previous in vitro biochemical assays (Stemmer & Klee, 1994) that found a Hill equation with the Hill coefficient of 3 for the dependence of CN activity with [Ca]. In different set of experiments correlating NFATc1 nuclear translocation with sensor activation, the integral of CN activation was found to be correlated with the total NFATc1 nuclear translocation. For both sensor the empirically determined equation was in the form } \Delta F_n = F_{tot} \left(1 - \exp\left(-\frac{Q}{9}\right)\right) \text{ with the maximum recruitable NFAT } F_{tot} \text{ was 0.42 for DuoCalN and 0.45 for UniCalN. This equation is easily seen to be the solution of the following differential equation:}

\frac{dF_n(t)}{dt} = \frac{1}{9} R_{sensor}(t) (F_{tot} - F_n) = k_{in}(t) (F_{tot} - F_0), \text{ where } k_{in}(t) \text{ is the rate constant for NFAT nuclear accumulation as shown in Fig. 2.10 and Fig. 2.11. This means that}

k_{in}(t) = \frac{1}{9} (-\Delta R/R_0). \text{ This remarkable yet simple relation implies that the normalized sensor output is linearly related to the rate constant for the NFAT nuclear important. In other words, this relations strongly hints that the conformational activation is linearly dependent on the activity assayed by the rate of NFAT nuclear import. Thus, the}
normalize sensor output ($-\Delta R/R_0$) is not only a measure conformational activation, but also of CN activity in cells.

In the next section, CN sensors will be deployed in neonatal rat and acutely isolated adult guinea pig cardiomyocytes to evaluate CN activation level in response to calcium transients of varying frequency.
**Figure 2.10** Coupling DuoCalN Sensor Activation to its Activity via NFATc1 Nuclear Translocation.  
(a) Time-lapse imaging of mCherry-NFAT nuclear enrichment in a cell placed in calcium free solution buffered by 1 mM EGTA to ensure the smallest possible thapsigargin induced calcium transient amplitude.
The outline of the nucleus is drawn in dashed white. (b) CFP and FRET channels for DuoCalN distribution. (c) Thapsigargin leads to partial NFAT enrichment ($F_{nuc}=NFAT_{nuc}/NFAT_{total}$) in the nucleus (~0.3, top), and modest activation of the sensor ($R_{sensor}=-\Delta R/R_0$, where $R$=FRET/CFP, bottom) that returns to the baseline after 2 minutes. (d) Time-lapse images of NFAT translocation for a cell placed in 200 µM calcium. (e) CFP and FRET channels are shown. (f) Thapsigargin induces robust NFAT translocation (~0.45, top) and concurrent broad sensor activation profile that lasts for ~5 minutes. (g) Calibration curve correlating the total change in nuclear NFAT ($\Delta F_{nuc}$) for 16 cells vs. the activation integral ($Q$), demonstrating the nonlinear correlation $\Delta F_{nuc}=0.42 \left(1-\exp\left(-Q/9\right)\right)$. This empirical equation is the solution of the ODE $dF_n/dt=k_{in}(t)(F_{tot}-F_n)$ where the rate constant of NFAT nuclear accumulation $k_{in}$ is linearly related to the normalized sensor response $R_{sensor}(t)$ and $F_{tot}$ is the total recruitable NFAT fraction. This implies that the readout of the sensor is linearly related to the activity assayed by NFAT nuclear accumulation rate.
Figure 2.11 Coupling UniCalN Sensor Activation to its Activity via NFATc1 Nuclear Translocation. (a) Time-lapse imaging of mCherry-NFAT nuclear import in a cell placed in calcium free solution buffered by
1 mM EGTA. The outline of the nucleus is drawn. (b) CFP and FRET channels for UniCalN distribution, (c) Thapsigargin leads to partial NFAT enrichment in the nucleus ($F_{\text{nuc}} = NFAT_{\text{nuc}}/NFAT_{\text{total}} \sim 0.4$, top) and modest activation of the sensor ($R_{\text{sensor}} = \text{FRET}/\text{CFP}$, bottom) that returns to the baseline after 2 minutes. (d) Time-lapse images of NFAT translocation for a cell in 200 μM calcium. (e) CFP and FRET channels denoting sensor expression and distribution. (f) Thapsigargin induces robust NFAT translocation ($F_{\text{nuc}} \sim 0.6$, top) with concurrent broad sensor activation profile that lasts for ~5 minutes. (g) Calibration curve correlating the total change in nuclear NFAT vs. the activation integral. The equation describing the calibration is $\Delta F_{\text{nuc}} = 0.45 (1-\exp(-Q/9))$. This empirical equation is the solution of the ODE $dF_{\text{nuc}}/dt = k_{\text{in}}(t)(F_{\text{tot}}-F_{\text{nuc}})$ where the rate constant of NFAT nuclear accumulation $k_{\text{in}}$ is linearly related to the normalized sensor response $R_{\text{sensor}}(t)$ and $F_{\text{tot}}$ is the total recruitable NFAT fraction. This implies that the readout of the sensor is linearly related to the activity assayed by NFAT nuclear accumulation rate.

### 2.6 DuoCalN and UniCalN Deployment in Cardiomyocytes

As discussed earlier in this chapter, CN is the only phosphatase that is activated directly by calcium. It is not only prominent in normal cardiac development and function, but also as a central regulator of the cardiac hypertrophic response. The use of genetically encoded FRET-based kinase sensors have provided novel and remarkable insights into the function and activity of these kinases in live cardiomyocytes (Saucerman et al., 2006; Erickson et al., 2011). In fact the use of the CaMKII sensor, Camui, was instrumental in identifying a previously unknown link between diabetes and cardiac dysfunction (Erickson et al., 2013). Likewise, it is our hope that the use of DuoCalN and UniCalN will aid in better understanding of CN dynamics and function in normal and pathological states of cardiomyocytes.

The first step in transiently expressing the sensor in cardiomyocytes is to use an adenoviral transduction system. The Cre-Lox based adenoviral transduction methodology is well established and detailed elsewhere (Limpitikul et al.; Alseikhan et al., 2002; Colecraft et al., 2002). Briefly, the first step is to clone the constructs into a pADLOX vector (Hardy et al., 1997). VENUS was PCR amplified and subcloned in XbaI/EcoRI sites of the pADLOX vector to generate PADLOX-VEN vector. PCR was then used to
amplify CER-CalNB-FQGPGP2A-CalNA (DuoCalN) or CER-CalNB-GSGP2AM-CalNA (UniCalN) using SalI and XbaI sites and to generate SalI-DuoCalN-EcoRI or SalI- UniCalN- EcoRI respectively as shown in Fig. 2.12. The constructs in pADLOX were then transfected along with the ψ5 shuttle vector to package the sensor into viral particles in HEK 293 cells stably expressing Cre8 recombinase (Cre8 cells). After a few rounds of expansion in Cre8 cells, the viral particles are purified using CsCl column. The density of the viral particles for DuoCalN sensor was $10^{12}$ particles/μl and for the UniCalN sensor was $10^{10}$ particle/μl.

To evaluate the response of the sensor in developing neonatal myocytes, neonatal rat ventricular myocytes were isolated using the standard protocols (Chlopcikova et al., 2001; Tung & Zhang, 2006). The viral particles carrying the sensor constructs were then added to the media 24 hours prior to imaging. On the day of imaging, the cells are washed once with warmed PBS, and fresh media is added. Fig. 2.13 shows the expression of DuoCalN and UniCalN in NRVMs. The sensors are uniformly distributed in cytoplasm and are excluded from the nucleus. On the imaging day, NRVMs were loaded with Indo-1 dye and electrically stimulated with standard grass simulator with stimulating electrodes positioned within 1-2 mm of each other. The voltage amplitude for efficient NRVM field stimulation was in the range of 10 – 20 V with duration of 20-50 ms. Mechanical contraction of cells under the microscope following single stimulation pulse indicated successful field stimulation. Data from each wavelength (FRET, CFP, Indo405, and then Indo485 channels) were gathered in series as shown by the diagram in Fig. 2.14a. A delay of 12 s was inserted to ensure the return to basal state. Indo-1 traces were lowpass filtered with a 20 Hz cutoff frequency, while the same filtering algorithm
was performed on individual CFP and FRET traces with 5 Hz cutoff. It is important to note that this filtering did not alter the FRET response of the sensors, and the transient decrease in FRET and the rise in CFP signals (Fig. 2.14 b) were clearly identifiable from the raw traces alone.

**Figure 2.12** DuoCalN and UniCalN in pADLOX. (a) Plasmid map of the pADLOX plasmid employed in adenoviral vector construction. Molecular cloning site (MCS) is shown along with the Sall, Xbal, and...
The utility of the ‘normal’ protocol for stimulation is shown in Fig. 2.14b, by an exemplar cell (expressing DuoCalN) paced at 0.2 Hz. The left column shows the raw data for the four channels gathered in series (Fig. 2.14b) that highlights the fluorescence bleaching that occurs during the imaging process. On the right hand column of Fig. 2.14b, the bleach corrected versions of the raw traces (obtained by a continuous fit to the baseline decay) are shown. Since the data are gathered in series, occasional ectopic contractions occur in NRVMs that activate the sensor as is apparent in the initial phase of the CFP signal. To clearly visualize and quantify the on and off kinetics of the sensor from the quiescent state, the ‘island’ mode protocol was developed. The island protocol is described by the diagram in Fig. 2.15a. Under this protocol, electrical stimulation (at a specified pacing rate) is performed for a specified duration ($\tau_{\text{island}}$) between the two silent periods with durations denoted by $\tau_1$ and $\tau_2$. The 0.5 Hz paced exemplar cell exhibiting the utility of the island protocol is shown in Fig. 2.15b. Similar bleach correction on the raw traces (left column) was performed to obtain the traces on the right. Also note that the traces in Fig. 2.15b demonstrate the enormous utility and advantage of our ratiometric sensor in automatically correcting for motion artifacts. Individual CFP and FRET signal traces have large amplitude and fast downward spikes overlaid on a slower varying signal. These fast fluctuations are superimposed onto the real FRET-based changes caused by CN conformational activation. Remarkably, the ratio $R=$FRET/CFP (red trace) corrects for the motion induced fluorescence fluctuations, and recovers the real FRET-based signal that shows a much slower dynamic change. This feature of our ratiometric
sensors alleviates the need for applying chemical compounds that block cardiac contraction machinery. Once the stimulation protocols are established, it is crucial to identify and extract parameters from the fluorescence traces that can then be utilized to investigate variations in pacing frequency dependence of different cell types, and cells at specific developmental stages (neonatal vs. adult).

\[ \text{Figure 2.13} \text{ DuoCalN and UniCalN Expression and Distribution in NRVM Cells. (a) DuoCalN expression and distribution in NRVMs with CFP channel (top) and FRET channel (bottom). (b) UniCalN distribution in NRVMs with CFP channel (top) and FRET channel (bottom).} \]
Normal Field Stimulation Protocol for Cardiomyocytes

(a) Computer controlled pacing begins at \( t=0 \) and continues for a specific amount of time with the shutter open to gather data every 1-2 ms. The shutter closes and a delay of 12s or more is introduced before the next wavelength. (b) An NRVM cells expressing DuoCalN is paced at 0.2 Hz. The left four panels show the indo-1 ratio (black), FRET/CFP (red), FRET (green), and CFP (blue) signals respectively from the top. Note the appearance of ectopic CN activation in the CFP (blue) signal as a result of arrhythmogenic NRVM contraction that may occasionally occur without any external stimulation. The right-hand panel depicts the same traces divided by the bleach curve (dashed line). Bleach correction is performed only on FRET and CFP raw traces. The amplitude of the sensor response (red, right column) is determined by a line fit (dashed grey line).
Island Mode Stimulation Protocol for Cardiomyocytes

(a) Stimulation Protocol

Begin pacing

Pacing

End pacing

12sec delay

FRET

τ₁

τIsland

τ₂

CFP

Indo405

Indo485

(b) Raw Data

R_{Indo1}

1.5

1

5s

1

Rsensor

0.9

1

0.9

0.9

FRET

0.9

1

0.9

0.9

CFP

1

1

0.96

0.9

Figure 2.15 Island Mode Stimulation Protocol for NRVMs. (a) There is a delay of duration τ₁ before the pacing begins. The shutter opens to gather data every 1-2 ms. After τ_{Island} seconds, pacing stop for τ₂ seconds. Delay of 12 s is introduced before the beginning of the next series. The shutter is open throughout the island mode for τ₁ + τ₂ + τ_{Island} seconds. (b) The raw data for a cell expressing DuoCalN, and paced at 0.5 Hz. Note the bleaching of CFP (blue) and FRET (green) signals with the the fitted lin (grey dashed). On the right hand side, the same data, but bleach corrected. Large downward motion-induced fluctuations are clearly visible in CFP (blue) and FRET (green) signals, but not in the ratio (red signal).
We have identified two such parameters: one is the normalized amplitude of the sensor FRET ratio ($R_{\text{sensor}}$, Fig. 2.14b and Fig. 2.15b) and the second is the half time to full activation from the quiescent state ($t_{1/2}$) during the island mode stimulation protocol. Fig. 2.16 outlines the general methodology for extracting these parameters from an actual FRET ratio trace. As we will see in the next section, these two parameters have different frequency-dependent profiles in NRVMs and AGPVMs, and quantify the differential activation of CN in neonatal versus adult myocytes.

**2.7 CN Dynamics in Neonatal versus Adult Cardiomyocytes**

The sensors developed here showed great promise in elucidating CN conformational activation in response to calcium elevation in HEK 293 cells. The sensors also expressed well when placed in a viral vector, and added to NRVM cultures (Fig. 2.13). No toxicity was observed for up to 2 days of continuous sensor expression. In this section, we
attempt to understand the pacing frequency dependence of CN activation in cardiomyocytes. This would showcase the first experimental observation of a phosphatase activation dynamics in live excitable cells, and the first observation of differential CN activation in neonatal versus adult myocytes. This may provide a rationale for physiological role of CN during development, and its subsequent pathological role in adult heart. Each sensor is deployed in NRVMs and AGPVMs and amplitudes and time constants are measured for each cell at different pacing frequencies. Fig. 2.17 illustrates the behavior in an exemplar NRVM cell expressing DuoCalN. The data presented here are for cells being stimulated with a single pulse (0 Hz, Fig. 2.17a), 0.2 Hz pacing (Fig. 2.17b), 0.5 Hz pacing (Fig. 2.17c), and 1 Hz pacing (Fig. 2.17d). The continuous line fits (Fig. 2.17a-d second row, solid black lines) to the activation rise from quiescent basal state are also shown. The grey line fits to the decay phase of the sensor activation are also shown (Fig. 2.17 a-d second row). In NRVMs, CN is prone to fast kinetic activation during single twitch calcium transients as demonstrated in Fig. 2.17a. The population data at each frequency are also shown in Fig. 2.17e where normalized amplitudes (Amp) (see Fig. 2.16 for explanation) are plotted against different pacing frequencies. The Amp versus frequency profile supports the idea that CN in NRVMs is readily activatable. A quantitative measure of this statement is given by the zero slope of the Amp versus frequency curve: \( \frac{\Delta Amp}{\Delta f} = 0 \).
Figure 2.17 Frequency Dependence of DuoCalN Dynamics in NRVMs. (a) Single pulse of calcium (~50% amplitude) leads to decrease in FRET ratio of 0.04 (red). FRET signal decreases by 0.02 (green), while the CFP signal increases by about 0.02 (blue signal). (b) 0.2 Hz pacing activates the sensor to about 0.045 with simultaneous decrease in FRET signal (green) and increase in CFP. (c) 0.5 Hz pacing further activates the sensor by about 0.045. Motion induced fluctuations are present in FRET (green) and CFP (blue) signals, while being absent in the ratio (red). (d) 1 Hz pacing leads to rapid activation of the sensor to 0.05 (red) with half time to activation, t1/2, of around 2s with simultaneous decrease in FRET (green) and increase in CFP (blue) signal. (e) Amplitude versus frequency plot of DuoCalN showing a slow increasing nearly flat distribution around 0.05 with \( \frac{\Delta \text{Amp}}{\Delta f} = 0 \text{ s} \). (f) Half time to activation from quiescent state in response to 0.5 Hz and 1 Hz stimulation is around 2s.

Another crucial parameter is t1/2 of the sensor in the island mode protocol (see Fig. 2.15a for explanation of the island protocol). Similar to the amplitude parameter, t1/2 has a sigmoidal shape with the steady-state value achieved at 1 Hz (~2 s). The implications of this plot will become clear when it is compared with the same plot for AGPVMs. We
should again note the utility of the ratiometric character of this sensor in alleviating the motion artifacts produced by often wild myocyte mechanical contractions.

Similar results are obtained for the UniCalN CN sensor as demonstrated by Fig. 2.18. The amplitude versus frequency profile is nearly flat \( \frac{\Delta Amp}{\Delta f} = 0 \), Fig. 2.18e) confirming that both constructs are readily activatable in NRVMs in 0 Hz–1 Hz frequency range. The slope of \( t_{1/2} \) versus frequency for UniCalN seems to be lower than that of DuoCalN (Fig. 2.18f compared with Fig. 2.17f). A possible explanation for this difference is that placing a physical linker between the two CN subunits may put a strain on the structure of the CN complex, and reduce the speed and versatility of the conformational changes. It is in fact remarkable and surprising that the maximum amplitude at 1 Hz is the same (~0.05) for both sensor types. This similarity between DuoCalN and UniCalN implies that the mutant P2A (P2AM) can be quite an effective and efficient linker to be deployed in other FRET-based genetically encoded sensors, where the problem of linker design is often a major obstacle in achieving the full potential of sensors in terms of speed and FRET dynamic range.

To gauge the extent of CN activity in NRVMs in response to electrical pacing, it would also be instructive to estimate the extent of NFAT translocation for a given CN activation transient. This can be done with the calibration equations outlined in Fig. 2.10 and Fig. 2.11. The single calcium transient has duration of about 5 seconds and amplitude of 0.05. The area under the curve is then \(~0.25/2\) assuming a triangular shape for the transient. The amount of NFAT translocation is then estimated to be \( \Delta NFAT = 0.0033 \), which is negligible. By assuming that NFAT nuclear export rate in NRVMS is similar to HEK 293 and Jurkat T cells and hence negligible, this implies that at least 200 pulses are necessary.
to induce 66% NFAT translocation. For 0.2 Hz and 1 Hz pacing rates, the area under the curve can be estimated by assuming a rectangular signal shape as is apparent from Fig. 2.17 and Fig. 2.18. By this assumption we can estimate the duration of 1 Hz train necessary for 50% NFAT translocation. This is easily estimated to be about
\[ T_{\text{pacing}} = \frac{\ln(2)}{0.05} \approx 14 \text{s}, \]
predicting that complete NFAT translocation would occur for the exemplar cells in Fig. 2.17 and Fig. 2.18. The heart rate for 2-3 day old rat pups is around 320 bpm or 5 Hz. For this pacing rate, our data predict that in the absence of endogenous negative regulators of NFAT translocation, NFAT would always be nuclear in beating NRVMs. This is indeed consistent with results of other groups studying NFAT translocation in NRVMs. In fact, one group of investigators has identified CaMKII as a negative regulator of NFAT translocation in NRVMS (MacDonnell et al., 2009). In their experiments, under control quiescent conditions 12% of the cells already had complete GFP tagged nuclear NFATc3, but after transducing the cells with a dominant negative form of CaMKII the fraction of the cells with complete nuclear localized NFATc3 increased to 50% (MacDonnell et al., 2009) under the same culture conditions.

To gain a deeper insight into CN signaling in adult myocytes, the sensors were transduced into acutely isolated guinea pig ventricular myocytes (AGPVMs) and the same protocols were applied to obtain frequency dependence of amplitude and kinetics of sensor activation. The profiles are then compared to that of the NRVMs. Fig. 2.19 illustrates the expression and distribution of DuoCalN and UniCalN sensors in AGPVMs.
Figure 2.18 Frequency Dependence of UniCalN Dynamics in NRVMs. (a) Single pulse of calcium (~80% amplitude) leads to decrease in FRET ratio by 0.085 (red). FRET signal decreases by 0.02 (green), while the CFP signal increases by about 0.1 (blue signal). (b) 0.2 Hz pacing activates the sensor to about 0.1 with simultaneous decrease in FRET signal (green) and increase in CFP. (c) 0.5 Hz pacing rapidly activates the sensor to about 0.05. (d) 1 Hz pacing leads to rapid activation of the sensor to 0.05 (red) with half time to activation of around 2 sec with simultaneous decrease in FRET (green) and increase in CFP (blue) signal. (e) Amplitude versus frequency plot of UniCalN showing a slow increasing nearly flat distribution around 0.05, $\frac{\Delta Amp}{\Delta f} = 0$ sec. (f) Half time to activation from quiescent state is around 1.5 sec.

Note that the sensors are predominantly localized to the z-lines (sarcolemmal regions) where the calcium channels and calcium release machinery (RyR2 receptors) are located in close (~nm) proximity. This is consistent with CN localization to calcium channels by the scaffolding AKAP proteins (Oliveria et al., 2007; Heineke & Ritter, 2012).
frequency dependence of CN activation in AGPVMs has different features from that in NRVMs as illustrated in Fig. 2.20 for DuoCalN and Fig. 2.21 for UniCalN. Single pulses (0 Hz) of calcium do not appreciably activate the sensor which is in stark contrast to NRVMs (Fig. 2.20a compared with Fig. 2.17a). 0.2 Hz pacing also had a similar effect on the sensor, with little activation of the sensor. However, increasing the pacing to 0.5 Hz and 1Hz resulted in cumulative activation of the sensor that closely followed the elevation of the intracellular calcium (Fig. 2.20c and Fig. 2.20d). Fig. 2.20e shows the population distribution of the sensor amplitudes (Amp) versus the pacing frequencies. In the same plot, amp versus frequency profile of AGPVMs is contrasted with that of NRVMs (solid blue line). While in NRVMs the line is flat with zero slope (meaning that CN is activatable in all frequencies), in AGPVMs the activation occurs appreciably only at higher frequencies of 0.5 Hz and 1 Hz with the slope of \( \frac{\Delta Amp}{\Delta f} = 0.05 \). Another important observation is that the time constant \( t_{1/2} \) (defined in Fig. 2.16) is 5-fold smaller in AGPVMs (~5sec at 0.5 Hz and 10sec at 1 Hz). Note that interestingly the maximal amplitude achieved in AGPVMs by the sensor is the same as NRVMs (Amp value at 1Hz). Similar results are obtained for UniCalN sensor expressed in AGPVMs as demonstrated by Fig. 2.21. Likewise, there is no sensor response to single pulse or 0.2 Hz pacing, while the activation peaks at 1 Hz pacing. Accordingly, it is expected that NFAT is fully localized to the nucleus in AGPVMs in response to 0.5 Hz and 1Hz pacing rates. In fact, recent results in the literature show that there is a gradual increase in the fraction of nuclear NFAT from 24% (basal quiescent), to 45% (at 0.5Hz pacing), and eventually to 60% (1Hz pacing) in acutely isolated adult feline ventricular myocytes (AFVMs) (MacDonnell et al., 2009).
**Figure 2.19** CN Sensor Expression and Distribution in AGPVMs. **a.** CFP channel showing rod-shaped cells expressing DuoCalN (40X oil). The expression is predominantly sarcolemmal. Lower panel shows FRET channel for the same cells. **b.** CFP channel for UniCalN with predominant sarcolemmal expression. The bottom panel is the corresponding FRET channel.
Figure 2.20. Frequency Dependence of DuoCalN Dynamics in AGPVMs. (a) single pulse of calcium does not activate the sensor. Normalized CFP (blue), FRET(green), and the ratio(red) are unresponsive. (b) 0.2 Hz train fails to elicit any response in the sensor. (c) 0.5 Hz trains lead to calcium summation that in turn activates the sensor by about 4%. (d) 1Hz pacing for 50s leads to robust increase in sensor activation (red) with subsequent decline to the basal level after the pacing is turned off. The sensor response is similarly around 4%. (e) Amplitude vs. frequency distribution for AGPVMs (red filled circles and solid red line, n=4-10 cells for each point) show a gradual rise in amplitude with $\frac{\Delta Amp}{\Delta f} = 0.05$ s. In contrast, the
This graduate increase in NFAT nuclear fraction parallels the observed increase in amplitude of the sensors in AGPVMs (Fig. 2.20e and Fig. 2.21e). To estimate the maximum activation capacity of the sensors in both NRVMs and AGPVMs and to investigate whether both cells have the same maximum CN activation reservoir, 5 mM caffeine was applied as shown in Fig. 2.21g.

**Figure 2.21** Frequency Dependence of UniCalN Dynamics in AGPVMs. (a) Single pulse of calcium does not activate the sensor. (b) 0.2 Hz calcium train fails to elicit any response in the sensor. (c) 0.5 Hz train leads to negligible sensor activation in this particular cell. (d) 1 Hz pacing for 50s leads to robust increase in sensor activation (red) by 0.05 with subsequent decline to the basal level after the pacing is turned off. (e) Amplitude vs. frequency distribution in AGPVMs (red filled circles and solid red line, n=4-
10 cells for each point) show a gradual rise in amplitude with $\frac{\Delta \text{Amp}}{\Delta t} = 0$ s. In contrast, the corresponding NRVM distribution curve (solid blue) is flat at around 0.05. (f) The half-time to activation from quiescent state in AGPVMs is around 5s in 0.5 Hz and 10s at 1Hz (red filled circles and red line) about 5 times slower than that of NRVMs (solid blue curve). (g) 5 mM caffeine elicits robust sensor response in NRVMs (0.06 for DuoCalN and 0.08 for UniCalN) and AGPVMs (0.07 for DuoCalN and 0.05 for UniCalN). The data are averages from 3-5 cells.

The caffeine response of the sensor amplitudes was quantitatively comparable in both NRVMs and AGPVMs (~0.05-0.09), and was comparable to that achieved by 1 Hz pacing in both cell types (~0.05).

To summarize the findings of this section, both of our CN sensors were deployed in NRVMs and AGPVMs. In NRVMs, CN readily responded to single twitch calcium transients. At 1Hz it required 2 seconds for the sensor to reach half-maximum activation from the basal quiescent state. In contrast, in AGPVMs, CN was unresponsive to single pulse and low frequency pacing of 0.2 Hz; however, the amplitudes began to grow at 0.5 Hz pacing. Pacing at 1 Hz was necessary for efficient activation of CN sensors. The half time to activation or $t_{1/2}$ was around 10 seconds, 5-fold slower than that of NRVMs.

In the next section, we will apply an empirically based mathematical model to understand the underlying dynamics of CN in NRVMs and AGPVMs. Mathematical analysis of the data will aid in formulating concrete ideas and hypothesis regarding differential activation of CN in NRVMs and AGPVMs, and suggest new avenues for further experiments. In fact, we will see that the model predicts that fluctuations in free CaM concentration may tune CN activation level and kinetics in adult cardiac myocytes, analogous to the role of apoCaM in tuning the CDI of CaV1.3 L-type channels in the brain.
2.8 Mathematical Analysis of CN Dynamics in Cardiomyocytes

In order to gain a deeper understanding of the underlying dynamics of biological systems, it is crucial to construct predictive and constrained mathematical models. Without the aid of mathematical models, it would be very difficult to predict the outcome of nonlinear interactions in biological systems. CN/NFAT pathway exudes the nonlinearity inherent in many signaling pathways. Nonlinearities enter the system in different stages of CN activation. Nonlinearity is involved in the binding of Ca\(^{2+}\) ions to C- and N- lobes of CaM, Ca\(^{2+}\) binding to the C- lobe of CalNB subunit, and the subsequent binding of Ca\(^{2+}/CaM\) to CN. It is therefore essential to formally describe these kinetic steps with a mathematical model and constrain the parameters of the model by fitting the sensor data. Indeed, one powerful impetus for developing genetically encoded sensor technology is their utility in obtaining precise temporal and spatial information regarding the activation states and activity of signaling proteins. This new window into the inner workings of signaling pathways coupled with the power of mathematical modeling allows for an unprecedented view of signaling processes that have long eluded traditional biochemical approaches. In the case of CN/ NFAT pathways, researchers have already made inroads in constructing large scale systems models that capture the essential dynamics of the molecular interactions (Shin et al.; Salazar & Hofer, 2003; Arron et al., 2006; Fisher et al., 2006; Saucerman & Bers, 2008; Shin et al., 2011). The application of these models is limited in the absence of suitable genetically encoded sensors. By utilizing these sensors, one can obtain subcellular resolution temporal and spatial information in live cell.
The most comprehensive model of CN activation pathway to our knowledge was constructed by Dr. Jeffery Saucerman and Dr. Donald Bers who surveyed the existing biochemical and qualitative data to model CN activation in different compartments of the adult cardiac myocytes (cytoplasm, sarcolemmal regions, and cytoplasm) (Saucerman & Bers, 2008). In their model they include 3.5 $\mu M$ dyadic and 3 nM cytoplasmic pool of CN. The main biological conclusion from their simulations is that dyadic CN is always active in a beating cardiac myocyte even with 1 Hz pacing. In contrast, cytoplasmic CN gets activated only at pacing rates of 4 Hz and higher, and acts much like a low pass filter. These conclusions are based on the use of very small $k_{\text{off}}$ value of 0.001 s$^{-1}$ for the unbinding of Ca$^{2+}$/CaM from CN. According to our data, CN inactivation roughly follows that of calcium decline and thus the off kinetics of Ca$^{2+}$/CaM departure from CN is expected to be in the order of seconds (in fact $k_{\text{off}}=1$ s$^{-1}$ according to the model fit to the data described below). Even with this faster off kinetics, dyadic CN is still expected to remain fully active from beat-to-beat in a normally beating adult guinea pig heart that beats at a rate of around 200-250 bpm (3-4 Hz). However, according to our sensor data, the active dyadic CN conclusion would change for slower beating rates.

The approach taken in this section is to construct a minimal model that captures the essential known biology and experimentally verified aspects of CN activation. We assume that ~10% of activity is due to calcium binding to CalNB subunit, and that the rest of the contribution to CN activation (~90%) is due to Ca$^{2+}$/CaM binding to CalNA (Stemmer & Klee, 1994; Aramburu et al., 2000; Yang & Klee, 2000; Saucerman & Bers, 2008; Li et al., 2011) (Fig.2.1b). The kinetic pathways describing the steps involved in CN activations are:
\[ 2Ca + CaM \leftrightarrow Ca^2CaM \quad (K_d = 1 \mu M^2) \quad (1) \]
\[ 2Ca + Ca^2CaM \leftrightarrow Ca^4CaM \quad (K_d = 10 \mu M^2) \quad (2) \]
\[ 2Ca + Ca^2CaN \leftrightarrow Ca^4CaN \quad (K_d = 0.5 \mu M^2) \quad (3) \]
\[ Ca^4CaM + Ca^4CaN \leftrightarrow Ca^4CaMCa^4CaN \quad (K_d = 100 pM) \quad (4) \]

Ca\(^2\)CaM = CaM with 2 Ca\(^{2+}\) ions bound.

Ca\(^4\)CaM = CaM with 4 Ca\(^{2+}\) ions bound.

Ca\(^2\)CaN = CN with 2 Ca\(^{2+}\) prebound to the high affinity sites on the N-lobe of CalNB.

Ca\(^4\)CaN = CN with 4 Ca\(^{2+}\) bound to CalNB (fully Ca\(^{2+}\) charged CN).

Ca\(^4\)CaM\(^{4}\)CaN = Activated CN with Ca\(^{2+}\)/CaM bound and fully Ca\(^{2+}\) charged CalNB.

Eqs. (1)-(2) describe the binding of Ca\(^{2+}\) ions to each lobe of CaM. Eq. (3) denotes the binding of 2 Ca\(^{2+}\) ions to low affinity sites on the C-lobe of CalNB, and Eq. (4) describes the binding of Ca\(^{4+}\)/CaM to CN that results in full activation. The activation of the sensor is fitted using the following variable defined by a linear combination of the fraction of calcified CN (Ca\(^4\)CaN in the kinetic scheme) and Ca\(^{2+}\)/CaM-bound (Ca\(^4\)CaMCa\(^4\)CaN) form of CN:

\[
\frac{-\Delta R}{R_0}(t) = P_{sensor} = \alpha_{Ca} \frac{[Ca^4CaN]}{[CaN]_{tot}} + \alpha_{CaM} \frac{[Ca4CaMCa^4CaN]}{[CaN]_{tot}}
\]

\[ (0.1 \leq \alpha_{Ca} \leq 0.15, \quad 0.9 \leq \alpha_{CaM} \leq 1) \]

The first term in Eq. (5) refers to two Ca\(^{2+}\) ions binding to CalNB subunit that constitute \(~10\%) of total activity, while the second term refers to the subsequent binding of Ca\(^4\)CaM to CalNA subunit that accounts for \(~90\%) of activity. The corresponding system of differential equations (ODEs) for the above kinetic scheme are:
\[ rcn02 = k02 \left[ Ca \right]^2 \left[ CaM \right] - k20 \left[ Ca^2 CaM \right] \]  
(7)  
\[ rcn24 = k24 \left[ Ca \right]^2 \left[ Ca^2 CaM \right] - k42 \left[ Ca^4 CaM \right] \]  
(8)  
\[ rcnCa4CaN = kcanCaon \left[ Ca \right]^2 \left[ Ca^2 CaN \right] - kcaCaoff \left[ Ca^4 CaN \right] \]  
(9)  
\[ rcn4CaN = kcanCaM4on \left[ Ca^4 CaM \right] \left[ Ca^4 CaN \right] - kcanCaM4off \left[ Ca^4 CaM Ca^4 CaN \right] \]  
(10)  

\[ \frac{d[Ca^2 CaM]}{dt} = rcn02 - rcn24 \]  
(11)  
\[ \frac{d[Ca^4 CaM]}{dt} = rcn24 - rcn4CaN \]  
(12)  
\[ \frac{d[Ca^4 CaN]}{dt} = rcnCa4CaN - rcn4CaN \]  
(13)  
\[ \frac{d[Ca^4 CaM Ca^4 CaN]}{dt} = rcn4CaN \]  
(14)  

(11)-(14) are solved given the following algebraic constraints:

\[ \left[ CaM \right] = C_1 - \left[ Ca^2 CaM \right] - \left[ Ca^4 CaM \right] - \left[ Ca^4 CaM Ca^4 CaN \right] \]  
(15)  
\[ \left[ Ca^2 CaN \right] = C_2 - \left[ Ca^4 CaN \right] - \left[ Ca^4 CaM Ca^4 CaN \right] - \left[ Ca^4 CaM Ca^2 CaN \right] \]  
(16)  

Where \( C_1 \) and \( C_2 \) are constants of the system (11)-(14) given by:

\[ C_1 = \left[ CaM \right](0) + \left[ Ca^2 CaM \right](0) + \left[ Ca^4 CaM \right](0) + \left[ Ca^4 CaM Ca^4 CaN \right](0) \]  
(17)  
\[ C_2 = \left[ Ca^2 CaN \right](0) + \left[ Ca^4 CaN \right](0) + \left[ Ca^4 CaM Ca^4 CaN \right](0) + \left[ Ca^4 CaM Ca^2 CaN \right](0) \]  
(18)  
\[ C_1 \approx \left[ CaM \right](0) \]  
\[ C_2 \approx \left[ Ca^2 CaN \right](0) \]  

Given that the initial \( (t=0) \) cytoplasmic abundance of Ca-bound forms of CaM and CN are basically negligible, \( C_1 \) and \( C_2 \) denote the initial free CaM \( \left[ CaM \right](0) \) and free CN \( \left[ Ca^2 CN \right](0) \). Note that CN complex is already bound to 2 \( Ca^{2+} \) ions in its CaLB subunit in basal calcium concentrations \((50-100nM)\). The values for the kinetic constants
are based on the values used in Saucerman- Bers model (Saucerman & Bers, 2008). With other parameters modified to best fit our DuoCalN sensor dynamic data\(^9\).

To obtain the temporal profiles of the state variables, first the Indo-1 measured calcium transient is used as an input to the system and then the set of ODEs (11-14) is numerically solved using the stiff ODE solver ode15s in MATLAB\(^{10}\). To demonstrate the methodology used in fitting the data, Fig. 2.22a shows the sensor data for an NRVM cell along with the fit (red). The predicted state variables are also shown (Fig. 2.22b). A free CaM concentration of 45nM best fits the data in Fig. 2.22a, and the off kinetics of \(\text{Ca}^{2+}/\text{CaM} \) unbinding from CN complex is \(k_{\text{canCaMoff}}=1 \text{ s}^{-1}\), which is three orders of magnitude faster than the value used in Saucerman- Bers model (Saucerman & Bers, 2008). Table 2.1 lists the relevant model parameters along with their values used in simulations. The initial impetus for developing the mathematical model was to understand the extent by which free cytoplasmic CaM levels ([CaM\(_{\text{free}}\)]) modulate amplitude and kinetics of CN activation in both NRVMs and AGPMVs. It was thus essential to be able to compare sensor activation in both cell types and normalize for the differences in sensor expression levels. To achieve this, we calculated the initial CFP fluorescence before pacing and took the mean value as representing the sensor expression for each cell type. As shown in Fig. 2.23j, the average value of CFP in NRVM cells was 2.16 times larger than the same value in AGPVMs. This means that the \(C_2\) parameter (representing total CN level) was multiplied by 0.46 in AGPVMs for all subsequent simulations. Table 2.1 also reflects this scaling factor in the parameter \(C_2\).

\(^9\) We used DuoCalN data for fitting because we think that it best represents the CN complex in its physiological form without any physical linkers between the two subunits.

\(^{10}\) Version R2010b, MathWorks Inc., 7.11.0.584, Licence: 703789
our satisfaction the simple kinetic scheme was capable of fitting the DuoCalN data at different pacing rates as demonstrated in Fig. 2.23 (a-d) for NRVMs and Fig. 2.23 (e-h) for AGPVMs. The predicted mean free CaM concentrationss are shown in Fig. 2.23j bargraph. The means value of \([\text{CaM}_{\text{free}}]\) in NRVMs is computed to be around 250 nM, 2.5 times larger than the value in AGPVMs \((\text{[CaM}_{\text{free}}]=100 \text{ nM})\). These values are consistent with the accepted literature range \([\text{CaM}_{\text{free}}]\) (50 nM-100 nM) in adult cardiac myocytes (Bossuyt & Bers; Persechini & Stemmer, 2002; Maier et al., 2006; Wu & Bers, 2007; Bossuyt & Bers, 2013). Our computer simulations predict that overexpressing CaM in AGPVMs would convert them into NRVM- like cells in terms of their CN activation properties. We find experimental evidence for this prediction in literature. Maier et al. (Maier et al., 2006) expressed a FRET sensor of Ca-CaM (BsCaMIQ with Kd=45 nM) in adult rabbit ventricular myocytes (ARVMs) and observed that wild type CaM overexpression was necessary for obtaining detectable FRET response. The authors attributed this result to low level of free CaM that they estimated to be between 50-100 nM. Another important idea emerging from the computational modeling is that ambient free CaM levels can modulate and tune CN activation and kinetics in cardiomyocytes. This is illustrated in Fig. 2.24a for an AGPVM cell paced at 1 Hz. By computationally varying free CaM from 30 nM to 100 nM, different CN activation curves are obtained with varying kinetics and amplitudes. A dramatic example of CaM rescue and modulation of sensor activation is achieved in Fig. 2.24b. The particular AGPVM cell shown, is paced at 0.5 Hz, and experimentally does not show any detectable sensor activation; however, it is computationally predicted that 2.5-fold increase (to ~70 nM) in free CaM concentration would restore CN response in this particular cell (solid blue line).
What this suggests is that free CaM is the limiting factor in CN regulation, consistent with evidence from other investigators (Persechini & Stemmer, 2002; Maier et al., 2006).

Figure 2.22 Fitting the sensor data with the mathematical model. (a) DuoCalN is expressed in an NRVM cell paced at 0.1 Hz. Indo-1 signal (black) is converted to calcium and interpolated and fed into the ODE model. The solution of the ODE model was fit (solid red) to the normalized ratio of the sensor (noisy black) by equation (5). The fitted free CaM level is $[\text{CaM}_{\text{free}}]=45 \text{nM}$. (b) The computed values for the state variables along with the input $[\text{Ca}]$ signal. The change in $[\text{Ca}]$ is 400 nM, resulting in 3 nM change in $\text{Ca}^2\text{CaM}$, 20 pM change in $\text{Ca}^4\text{CaM}$, 100 nM increase in $\text{Ca}^4\text{CaN}$ and 20 nM increase in $\text{Ca}^2\text{CaM}\text{Ca}^4\text{CaN}$. 
Combining mathematical modeling with the dynamic CN sensor data has provided intriguing biological insights into dynamics of CN in neonatal versus adult cardiomyocytes. Because a single kinetic model fits all the cellular data paced at varying frequencies, the underlying biology of CN activation is the same in neonatal versus adult myocytes. The simplest hypothesis that emerges from the modeling exercise is the remarkable idea that the main difference in CN response in NRVMs and AGPVMs is the concentration of free CaM. This is consistent with the biology of developing cells such as NRVMs. These cells are at a developmental stage, where the constitutive physiological -
Figure 2.23 Fitting the Computational Model to DuoCalN Dynamics in NRVMs and AGPVMs Paced at Different Frequencies. The calcium transients (not shown) are fed into the ODE model and the numerical solutions (solid red, see Eq. 5) are fit to the experimental activation data of DuoCalN. For NRVMs (a-d) (a) Model fits to a single pulse of CN activation with 30 nM free CaM. (b) 0.2 Hz pacing leads to a strong response. The computed CaM level is [CaM]$_{free}$=40 nM. (c) 0.5 Hz summation response in CN sensor is fit with the model with CaM=120 nM. (d) 1 Hz pacing results in strong CN activation which is fit with the model in both on and off phases of the activation curve. The free CaM=70 nM. For AGPVMs (e-h) (e) Single pulse fails to elicit any response and is captured by the model with 30 nM free CaM (f) A non responsive cell paced at 0.2 Hz is also fit with the model (g) A cell paced at 0.5 Hz is fit with the model. The activation kinetics (6s) and the final amplitude (.04) are captured by the computational fit. (h) A cell paced at 1 Hz for 30s. The model correctly follows the on and off phases of the sensor, (i) Sensor expression is evaluated by the initial pre-pacing CFP fluorescence level. For NRVMs, mean CFP$_0$ level is 4.79×10$^3$ (n=10), which is 2.16 times higher than its value in AGPVMs (2.22×10$^3$, n=10), (j) Computed CaM levels in NRVMs (mean value 250 nM, n=20) compared to AGPVMs (mean value 100 nM, n=8).
Figure 2.24 CaM Tuning of CN Dynamics in AGPVMs. (a) AGPVM cell expressing DuoCalN. The cell is paced at 1 Hz in island mode for 15 s. The models with free CaM=30 nM. CaM is computationally varied from 5 to 95 nM. The curves shown correspond to CaM levels of 5, 15, 30, 75, and 95 nM from bottom to the top. The initial activation kinetics increases as CaM is elevated. The steady-state sensor amplitude rises as well. 2.5-fold increase in the fitted CaM level (30 nM for the third red line from the bottom to the blue line corresponding to 75 nM of free CaM) nearly doubles the amplitude and the kinetics of the rise phase (b) Another AGPVM cell paced at 0.5 Hz. Sensor is non-responsive. CaM is varied from 10 nM (lowest curve) to 100 nM (highest curve). The curves shown correspond to 10, 20, 30, 50, 70, and
activation of CN/NFAT is necessary for efficient development and physiological cardiac growth. In AGPVMs the activation of CN/NFAT is hypertrophic and these cells need to tightly control limit the activation of pathological hypertrophic genes and one way that this is achieved is by limiting the pool of free CaM (Fig. 2.23j and Fig. 2.24b).

2.9 Discussion

To summarize the results obtained in this section, both DuoCalN and UniCalN express in NRVMs and AGPVMs without detectable toxicity for up to 36 hours. Expression in NRVMs is cytoplasmic and uniform while in AGPVMs the expression is sarcolemmally enriched consistent with CN biology in acute cardiomyocytes (Fig. 2.14 and 2.19). Both DuoCalN and UniCalN show FRET change in response to twitch calcium transients in NRVMs with activation summation when paced at 0.5Hz and 1Hz. In contrast to NRVMs, the sensors do not respond in AGPVMs at pacing rates of 0.2Hz or lower. The activation begins to emerge when adult cells are paced at 0.5Hz and 1Hz. The initial kinetics of activation from the quiescent state is much slower in AGPVMs when compared to that in NRVMs (5-fold slower, Fig.2.20 and Fig.2.21). Moreover, the slope of the Amplitude versus frequency is drastically different in each cell type.

In NRVMs, the distribution is flat implying that \( \frac{\Delta Amp}{\Delta f} = 0 \), while in AGPVMs \( \frac{\Delta Amp}{\Delta f} = 0.05 \). Moreover, the application of caffeine results in comparable sensor activation (amplitudes of about 0.06-0.08) in both NRVMs and AGPVMs (Fig. 2.21e).
Fig. 2.25 summarizes the differences in parameters of CN activation between NRVM and AGPVMs.

The mathematical model constructed in this section was able to fit the sensor data in both NRVM and AGPVMs in wide range of pacing frequencies (Fig. 2.23a-h). The computed mean free CaM concentration in NRVMs was ~250 nM while the corresponding mean concentration in AGPVMs was ~100 nM. Further, Computational modeling predicted that CaM overexpression would transform AGPVMs into NRVM- like cells in terms of their CN activation properties. It also proposed a mechanism by which fluctuations in cellular free CaM can modulate and tune CN activation amplitude and kinetics (Fig. 2.24a and Fig. 2.24b) much like the apoCaM tuning of CDI in CaV1.3 discussed in chapter 1 (Fig. 1.8f). The slow activation of CN in AGPVMs compared to NRVMs as quantified by t1/2 and the combined computational/experimental methodology that proposed low CaM_free levels as the culprit for the alternate response in AGPVMs, hint at a potential mechanism by which cardiomyocytes can modulate CN/NFAT dependent physiological and pathophysiological hypertrophic response (Fig. 2.26). This is consistent with the incident of massive hypertrophy in a mouse model overexpressing CaM in the heart (Gruver et al., 1993), and the responsiveness of the genetically encoded sensor of CaM_free, (BsCaMIQ) only in the presence of overexpressed CaM_WT in adult rabbit ventricular myocytes (Maier et al., 2006). Accordingly, any pathological perturbations in ambient CaM levels is predicted and expected to influence CN dynamics.
Figure 2.25 CN Activation Parameters in NRVM versus AGPVMs. (a) the mean value of t_{1/2} (time to half-maximal activation amplitude) in NRVM and AGPVMs paced at 1 Hz. The data for DuoCalN (Duo) and UniCalN (Uni) is plotted. The value in NRVMs is about 2s, 5-fold faster than that of AGPVMs (~10s). (b) The mean value for the slope of the normalized sensor amplitude versus pacing frequency (Fig. 2.17, Fig. 2.18 and Fig. 2.20, Fig. 2.21) illustrating stark difference between AGPVMs (value of ~0.05) and NRVMs (~0). (c) Computational estimate of mean [CaM]_{free} values (~10 cells) (Eq.17, parameter C_1 in the mathematical model) in NRVMs (~250 nM) versus AGPVMs (~100 nM) based on the fitting of the mathematical model to the pacing induced DuoCalN FRET response (Fig. 2.22 and Fig. 2.23).

In recent experiments by Yang et al. (Yang et al., 2014), FRET measurements were employed to demonstrate that 90% of the z-line CaM in adult myocyte was RyR2-bound, and, that the binding affinity was decreased by 3-fold in a post-myocardial rat infarction heart failure model (Yang et al., 2014). Combining with the empirical and mathematical insights obtained by the sensors developed in this section, this would imply that failing
myocytes have higher free CaM levels, and accordingly are more prone to CN activation. This 'free CaM' conjecture developed here can be experimentally tested by co-expressing CaM$_{WT}$ in AGPVMs alongside the sensor and mCherry-NFAT to investigate concurrent activation of CN and NFAT in response to elevated CaM levels.

The tools and protocols developed in this chapter, substantially expand the toolbox for precise and live-cell investigation of CN/NFAT dynamics, not only in cultured cardiomyocytes, but also in live animals. The sensors can also be deployed in other cell types such as neurons and lymphocytes where CN/NFAT activation is crucial in both basic biology and pathophysiology. The genetically encoded sensor can also be used to identify more potent compounds for inhibiting conformational activation of CN. The currently available compounds CsA and FK506 intercholate (Liu et al., 1991; Huai et al., 2002; Jin & Harrison, 2002) within the catalytic domain of CN and do not (neither expected to) block the initial conformational activation assayed with our FRET-based sensors. High throughput drug screening with the current sensors would be an essential step in identifying compounds with lower liver and neurological toxicity associated with the used of CsA and FK506 (Palmer & Toto, 1991; Burdmann et al., 2003).
Figure 2.26 Maturation and Adult Hypertrophy and CN Dynamics. Ca flux from calcium channels activates CaM that in turn bind and activate CN complex. In neonatal heart kinetics of CN activation is fast and free CaM level is high so that CN is prone to activation and hypertrophic genes are on. In adult heart, CN kinetic is slow and free CaM level is low. CN is less prone to activation. CN activation in adult heart leads to pathological hypertrophy.

Conclusion

In chapter 1, we carried out scanning alanine mutagenesis of the whole IQ domain of CaV1.3 channels, and challenged the prevailing IQ centric model of CDI induction in calcium channels. An alternative model of CaM regulation ‘CaM departure model’ emerged from our whole-cell patch clamp and quantitative live cell FRET-binding assays (Fig. 1.4). By applying these insights to naturally RNA-editing variants of CaV1.3 channels, we discovered a surprising role for apoCaM preassociation in modulating the CDI of these channels. Moreover, apoCaM modulation of the CDI offered a mechanism
by which the ambient CaM fluctuations could tune CDI in the brain much like switches on a rheostat.

The second chapter, described the development of novel genetically encoded sensors for CN activation, and discovered equations for coupling conformational activation to activity via modulation of NFAT nuclear translocation. The sensors were then deployed in neonatal and adult cardiomyocytes, and obtained the frequency dependent activation of CN in NRVMs and AGPVMs. In NRVMs, CN is activated in response to single twitch calcium transients, while in AGPVMs a pacing rate of at least 0.5 Hz is required for sensor activation. Moreover, by constructing a mathematical model of CN activation, we were able to fit the sensor data in both NRVMs and AGPVMs that revealed that the same underlying biological processes are responsible for CN activation in both cell types. The model also identified free CaM as a central regulator and the limiting factor in modulating the amplitude and kinetics of CN in both cell types corroborating the prevailing idea of the field that free CaM is a limiting factor in CaM target regulation (Persechini & Stemmer, 2002). Indeed, in NRVMs, a mean free CaM concentration of 250 nM was required to fit the data, 2.5 times higher than that of AGPVMs (mean free CaM concentration of 100 nM). The computed free CaM concentration of 100 nM in AGPVM cells is consistent with the accepted value in literature which is in the range of 50 nM-100 nM (Wu & Bers, 2007; Bossuyt & Bers, 2013). The tuning of CDI of CaV1.3 channels in the brain and the conjectured tuning of CN activation in cardiomyocytes (and potentially other cell types), unify the results of chapters 1 and 2, bringing together the two seemingly disparate fields. The general tuning concepts discovered and defended in this thesis, imply that a simple parameter such as protein concentration can achieve
widely different biological outcomes in different cell types. The specific conceptual breakthrough here is to realize the versatility of CaM regulatory function in different cell types. It is likely that similar phenomena also occur in other cell types such as cancer cells and the immune lymphocytes at different developmental stages. Targeting CaM in both apo- and Ca\(^{2+}\)-bound form may be a major focal point in the journey towards better understanding of calcium-dependent signaling cascades in both health and disease.
Bibliography


characteristics of the heart. *Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia* 145, 49-55.


Stemmer PM & Klee CB. (1994). Dual calcium ion regulation of calcineurin by calmodulin and calcineurin B. *Biochemistry* 33, 6859-6866.


Chlopčíková S, Psotová J & Miketová P. (2001). Neonatal rat cardiomyocytes--a model for the study of morphological, biochemical and electrophysiological characteristics of the heart. Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia 145, 49-55.


DeMaria CD, Soong TW, Alseikhan BA, Alvania RS & Yue DT. (2001). Calmodulin bifurcates the local Ca2+ signal that modulates P/Q-type Ca2+ channels. Nature 411, 484-489.


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EDUCATION

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Thesis: Synchronization in heterogeneous networks of hippocampal inter- neurons.  
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2003 BSc in Applied Mathematics with minors in Physics and Pure Mathematics  
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RESEARCH INTERESTS


RESEARCH POSITIONS

2007-2014 JOHNS HOPKINS UNIVERSITY, DEPARTMENT OF BIOMEDICAL ENGINEERING  
(Advisor: Dr. David T. Yue)  
FRET-based genetically encoded sensor design, structure-function analysis of calcium channels, alanine-scanning mutagenesis

2005-2007 JOHNS HOPKINS UNIVERSITY, DEPARTMENT OF BIOMEDICAL ENGINEERING  
(Lab rotations and coursework)  
Stochastic simulation of calcium diffusion and capture in cardiac dyad, FRET-based genetically encoded sensor of PDK1 and CaMKII, flow-cytometry based assay for L-type calcium channel trafficking.
2005  UNIVERSITY OF WATERLOO, DEPARTMENT OF APPLIED MATHEMATICS  
(Advisors: Dr. Sue A. Campbell and Dr. Brian Ingalls)  
*Bifurcation analysis and phase reduction of the differential equations modeling the electrical activity of a network of interconnected hippocampal inter-neurons*

2000-2004  UNIVERSITY OF CALGARY, DEPARTMENT OF PHYSIOLOGY AND BIOPHYSICS  
(Advisors: Dr. Wayne Giles, Dr. Gary Kargacin, Dr. Don Welsh)  
*Mathematical modeling of calcium diffusion and regulation in smooth muscle cells, calcium dynamics in cardiomyocytes, membrane potential propagation in arteries, and the role of coupling*

1999-2000  UNIVERSITY OF CALGARY  
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*Pharmacokinetic modeling of Cisplatinum and Carboplatinum, Mathematical modeling of tumor growth, reaction kinetic analysis of Cisplatinum binding to Mesna.*

TEACHING EXPERIENCE

JOHNS HOPKINS UNIVERSITY, DEPARTMENT OF BIOMEDICAL ENGINEERING  
2013  Instructor: Intersession course entitled ‘Random Walks in Biology’ targeted to freshman and sophomore students. Nine students successfully completed the class. The topics included: History of Brownian motion, history of atomism, basic mathematics of random walks including ordinary differential equations describing diffusion to capture, applications in immunology, gene therapy, cancer biology, calcium diffusion in restricted biological structures, and insect pheromones  
2010  Teaching assistant: Ion channels of excitable membranes  
2009  Teaching assistant: Thermodynamics and statistical mechanics

UNIVERSITY OF WATERLOO, DEPARTMENT OF APPLIED MATHEMATICS  
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JOURNAL PUBLICATIONS

1.  Bazzazi H, Yang W, Yue DT, FRET- based genetically encoded sensor of Calcineurin reveals stimulus-dependent differential activation of calcineurin in cardiomyocytes (in preparation)


**CONFERENCE PRESENTATIONS**


2. Sang LJ, **Bazzazi H**, Johnny MB, Yue DT. Resolving the Grip of the Distal Carboxy Tail on the Proximal Calmodulatory Region of CaV Channels *Biophysical Journal*, 102(3): 126a Poster Presentation at the 56th annual Biophysical Society meeting in San Diego, CA.


5. **Bazzazi H**, Greenstein JL, Tanskanen AJ, Winslow RL, Stochastic dynamics of calcium ion motion and channel gating in the cardiac dyad during excitation-
contraction coupling, Presented as a poster at the workshop on the application of methods of stochastic systems and statistical physics in biology, held at the University of Notre Dame, Indiana, October 28-30, 2005.


7. **Bazzazi H**, Clark RB, Giles WR, Mathematical simulations of the effects of altered AMP-kinase activity on I\(_{Na}\) in mammalian ventricle, presented as a poster at the international conference on mechanisms of maintaining sodium and calcium homeostasis in the mammalian heart: implications for ischemia and left ventricular dysfunction, University of California, San Diego, March 29-31, 2005.