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Calmodulin Modulation of Ion Channels
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Dihydropyridine-sensitive, L-type, CaV1.2 calcium channels are regulated by calmodulin through direct and indirect mechanisms. We have studied the opposing effects of the calmodulin-dependent protein kinase, CAMKII, and of the calmodulin-dependent protein phosphatase, calcineurin (PP3), on modal gating of recombinant rabbit cardiac CaV1.2 channels (Erxleben et al., 2006 PNAS 103:3932). Hyperphosphorylation of the channel produced by inhibition of PP3 with the neurotoxic immunosuppressant cyclosporin increases mode 2 gating, which is prevented by prior inhibition of CAMKII with KN-62 or by mutating Ser 1517 to Ala at the cytoplasmic end of helix IVS6. The Timothy disorder mutation, G436R, at the cytoplasmic end of the topologically neighboring IS6 helix increases mode 2 gating without inhibition of PP3, but is also prevented by KN-62 and by mutating Ser 439 to Ala. We have extended this work in two directions by examining the effects of phosphorylation on dihydropyridine sensitivity and by examining the effects of calmodulin mutants that are selectively impaired in their ability to activate CAMKII or PP3.

Analysis of the Cav1.2 channel by conditional mutagenesis.

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Classical inactivation of the L-type Cav1.2 (α1C) calcium channel gene is embryonal lethal before day 14.5 pc (1). We have generated a mouse line in which exon 14 and 15 of Cav1.2 is flanked by two loxP sites. These mice were crossed with various lines expressing tissue-specific Cre recombinase. The off-springs of these mice had severe defects in insulin secretion (2), blood pressure regulation (3), intestinal muscle contraction (4), hippocampal memory acquisition (5). These mice lines showed that the putative T-type calcium channel blocker mibefradil lowers blood pressure by inhibition of the Cav1.2 channel (6). We analysed further the coupling between Cav1.2 and the CaM-Kinase II (7). Facilitation of the Cav1.2 channel by CaM-KII involves the phosphorylation of serine 1512 and 1570 in the C-terminus of Cav1.2. Mutation of S112/1570A results in viable offspring with no obvious phenotype. The electrophysiological consequences will be discussed.

7. Lee et al. (2006) JBC 281, 25560-25567
Calmodulin control of voltage-dependent relaxation behavior of G protein-gated K⁺ channels

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Acetylcholine activates G protein-gated inwardly rectifying K⁺ (Kᵣ) channels in cardiac myocytes, which underlies vagal deceleration of the heart beat. The Ach-activated Kᵣ channel current exhibits a characteristic voltage-dependent property, which is called ‘relaxation’: The Kᵣ current decreases time-dependently during depolarizing potential, while it increases upon hyperpolarization. This property cannot be observed in other types of Kir channels. We showed that the relaxation behavior of Kᵣ is caused by the apparent voltage-dependent activity of G proteins which control the Kᵣ channel activity as follows: Upon depolarization, Ca²⁺ flows into cell across the cell membrane, which forms Ca²⁺/Calmodulin (CaM) complex. The Ca/CaM complex removes the inhibition by PIP₃-binding of Regulator of G Proteins (RGSs). Thus activated RGS proteins can accelerate the hydrolysis of GTP on G proteins, which results in a decrease of Kᵣ channel activity during depolarizing potentials. Upon hyperpolarization, due to decrease of Ca²⁺ influx, the series of reactions will be reversed and results in an increase of the channel activity. With this basic understanding, the CaM/RGS protein-control of voltage-dependent activity of G protein cycle can be modeled, which reasonably explain the temporal behavior of the Ach-activated Kᵣ current. Therefore, Calmodulin-mediated reaction is a key control mechanism of the physiological behavior of G protein-gated K⁺ channels.

Small conductance Ca²⁺-activated K⁺ channels and calmodulin

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Small conductance Ca²⁺-activated K⁺ channels (SK channels) are voltage-independent K⁺ channels that are gated solely by intracellular Ca²⁺ ions. Ca²⁺ gating is accomplished through a unique, constitutive co-assembly of the SK alpha, pore-forming subunits with the ubiquitous Ca²⁺ sensor, calmodulin (CaM). Each SK subunit is bound by a CaM and in this complex, Ca²⁺ binding to the N-lobe E-F hands of CaM initiates conformational changes that ultimately open the gate of the channel, while the C-lobe E-F hands have sacrificed their ability to bind Ca²⁺ ions in favor of anchoring the association between the two protein partners. Our understanding of the molecular mechanisms of SK channel gating were largely facilitated by the development of site directed mutants of CaM that are altered in their ability to bind Ca²⁺ ions. These mutant CaMs have subsequently been applied to the study of a wide range of other ion channels and enzymes, including the opposing processes of Ca²⁺-dependent inactivation and facilitation in voltage-dependent Ca²⁺ channels. More recent results for SK channels reveal that in addition to CaM, the SK channels are constitutively co-assembled with protein kinase CK2 and protein phosphatase 2A (PP2A) that regulate SK channel activity in opposing ways. Surprisingly, CK2 does not phosphorylate the SK channel. Rather CK2 phosphorylates SK-associated CaM, resulting in rapid channel deactivation kinetics and a reduction of the apparent steady state Ca²⁺ sensitivity of the SK channels. The ability of CK2 to phosphorylate SK-associated CaM is itself state-dependent, occurring only in the closed state, lending a second level of Ca²⁺ regulation to SK channel activity. We will discuss the history of ion channel regulation by CaM, the methods and mechanisms that illuminated the details of SK channel Ca²⁺-gating, the mechanisms underlying CK2 and PP2A regulation via CaM, and the consequences of SK-associated CaM modulation for neuronal function.
Apocalmodulin binds to wild type Cav1.4 L-type Ca\textsuperscript{2+} channels and modulates voltage dependent inactivation

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The retinal L-type Ca\textsuperscript{2+} channel Cav1.4 is distinguished from all other members of the high voltage-activated Ca\textsuperscript{2+} channel family by lacking Ca\textsuperscript{2+}-dependent inactivation (CDI). In synaptic terminals of photoreceptors and bipolar cells, this feature is essential to translate graded membrane depolarizations into sustained Ca\textsuperscript{2+} influx and tonic glutamate release. Our previous work demonstrated that CDI of Cav1.4 is prohibited by a specific interaction of a distal C-terminal peptide, termed inhibitor of CDI (ICDI domain), with a region in the proximal C-terminus. Furthermore, the C-terminal components that form the Calmodulin (CaM) binding region are also present and functional in Cav1.4. This finding raises two important questions. Is the Ca\textsuperscript{2+} sensor calmodulin bound to a channel like Cav1.4 that does not display CDI? And if so, is there a functional consequence of CaM binding on channel gating? To clarify these questions we performed coimmunoprecipitation experiments with C-terminal fragments of Cav1.4 and wild-type CaM or a calcium insensitive variant of CaM (apoCaM). The results led us to the conclusion that wild type and apoCaM bind to Cav1.4 channels. To find out the functional consequence of CaM binding for Cav1.4 channel gating we designed mutant Cav1.4 channel variants that lack CaM binding (Cav1.4/5A). By a functional analysis of these channel variants we found out that apoCaM modulates voltage dependent inactivation of Cav1.4 channels. In essence, these data point to a novel action of CaM in the context of ion channels that is independent of Ca\textsuperscript{2+} binding.

Session 2 Speaker Materials

Calcium channel regulation and short-term synaptic plasticity

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P/Q-type calcium currents conducted by Ca\textsubscript{\textgamma}2.1 channels initiate synaptic transmission at many types of synapses. These channels are regulated by binding of calmodulin to a bipartite site in the C-terminal domain composed of an IQ-like motif and a calmodulin binding domain (CBD). Brief, local increases in calcium support high affinity binding of calcium to the C-terminal lobe of calmodulin and interaction with the IQ-like domain of Ca\textsubscript{\textgamma}2.1, which causes facilitation of calcium channel activity. Sustained, global increases in calcium support binding of calcium to the N-terminal lobe of calmodulin and interaction with the CBD of Ca\textsubscript{\textgamma}2.1, which causes inactivation. This biphasic mechanism optimizes calcium channel activity during brief trains of stimuli. The neuronal calcium sensor protein CaBP1 binds to the Ca\textsubscript{\textgamma}2.1 channel, displaces calmodulin, and enhances inactivation without causing facilitation. The neuronal calcium sensor protein VILIP-2 slows calcium/calmodulin-dependent inactivation and enhances facilitation. Thus, neuro-specific calcium sensor proteins can differentially regulate Ca\textsubscript{\textgamma}2.1 channels. Differential regulation by CaBP1 and VILIP-2 requires N-terminal myristoylation of those proteins and depends on the different sequence motifs in the N-terminal domain and the N-terminal pair of EF-hands. To examine the role of this form of calcium channel regulation in short-term synaptic plasticity, we have expressed Ca\textsubscript{\textgamma}2.1 channels in superior cervical ganglion neurons (SCGN) by injection of cDNA encoding wild-type or mu-
tant forms of these channels. Fast cholinergic neurotransmission at synapses between untransfected SCGN in cell culture depends on N-type calcium currents, which are completely blocked by ω-conotoxin GVIA. Synaptic transmission in the presence of conotoxin is partially restored by presynaptic expression of wild-type CaV2.1 channels, and reconstituted neurotransmission is blocked by the specific P/Q-type channel blocker ω-agatoxin IVA. Reconstitution of synaptic transmission is lost if the synaptic protein interaction ('synprint') site in CaV2.1 channels is mutated, due to failure of presynaptic targeting of the CaV2.1 channel. Stimulation of SCGN expressing CaV2.1 channels with paired action potentials or trains of action potentials causes short-term facilitation and depression of synaptic transmission. Mutation of the IQ-like motif of CaV2.1 reduces synaptic facilitation, mutation of the CBD reduces synaptic depression, and all short-term synaptic plasticity is lost in the double mutant. Our results show that, at this model synapse, calcium- and voltage-dependent regulation of CaV2.1 channels through interaction with calcium sensor proteins is responsible for most of short-term synaptic plasticity.


Calmodulin regulation of neuronal ion channels

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Calmodulin (CaM) has been recognized on an increasing basis as a Ca$^{2+}$-sensor for Ca$^{2+}$-dependent gating for various ion channels and, in some cases, has been shown to be a constitutive subunit. Since ion channel subunits assemble early during channel biosynthesis, CaM can also afford Ca$^{2+}$-dependent regulation to various steps of channel biosynthesis, in addition to the well-described roles for the control of gating when the channels are at the cell surface. I present several examples in which calmodulin regulates not only channel gating, but also channel assembly and membrane targeting. These descriptions expand the understanding of Ca$^{2+}$/CaM regulation of ion channel function.

ClC-3: A CaMKII activated channel in hippocampal neurons

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The glutamatergic synapse in hippocampal neurons is the best described interface between electrical activity and memory encoding in the central nervous system. We have found that ClC-3 chloride channels localize to the post-synaptic plasma membranes of hippocampal neurons where they are both spatially and functionally linked to NMDA receptors (1). NMDA dependent-Ca$^{2+}$ entry, activation of CaMKII, and subsequent phosphorylation/gating of ClC-3 by CaMKII links the two channels via a Ca$^{2+}$-mediated positive feedback loop. ClC-3 channels increase neuronal excitability post-synaptically in immature neurons by increasing post-synaptic potentials (EPSPs) at a time when [Cl$^{-}$]$_i$ is high. In stark contrast, ClC-3 channel activation is inhibitory in mature neurons when [Cl$^{-}$]$_i$ is low. Now the opening of ClC-3 channels linked to the Ca$^{2+}$ influx through NMDA channels serves as a charge shunt pathway giving rise to membrane hyperpolarization, reduction in EPSP amplitude, and promotion of the block of NMDRs by Mg$^{2+}$.

ClC-3 knockout mouse phenotype

The ultimate proof of a protein’s identity and, in fact, its important functional role comes from genetically engineered mice in which protein expression is disrupted. ClC-3 was no exception; however, the dominant phenotype was not functional, but anatomical and a stunning surprise. The ClC-3 knockout mouse shows complete postnatal neurodegeneration of the hippocampus (and photoreceptors) beginning in the 2nd postnatal week and continuing through the 4th postnatal week (2-4). Extensive, specific neurodegeneration has been documented in the three ClC-3 knockout mice that have been made (2-4). Although ClC-3 is broadly expressed throughout the brain, neurodegeneration is confined to the hippocampal formation and the retina (see Fig. 1 taken from Stowbrawa and colleagues (2)).

Mice lacking hippocampi survive for one to two years. The spatial-temporal sequence of the neurodegenerative process, however, differs between the mice (3). In one mouse, there appeared to be a lysosomal accumulation of ceroid

![Figure 1. Degeneration of the Hippocampus in Clcn3$^{-/-}$ Mice. (A and B) Frontal sections of an adult (7-month-old) WT and KO mouse, respectively (Nissl stain). The hippocampal formation is nearly totally lost in KO. Nissl-stained sagittal sections (C–F) of KO hippocampi from P14 to P42. (G–J) Adjacent sections labeled with GSA for reactive microglia. At P14 (C and G) the hippocampal formation with the dentate gyrus (dg), CA3, and CA1 region is still well preserved. At P21 (D) the number of pyramidal cells has decreased in the CA1 region (arrow), and microglial cells are slightly labeled (arrow in [H]). At P28 the degeneration has spread over the CA1 region (arrows in [E]) and microglial labeling is stronger (J). At P42 (F) pyramidal cells from the CA1 and the CA3 region have disappeared. Only a few granular neurons are found in the dentate gyrus. The remaining hippocampus is labeled strongly for microglia (J). Figure modified from Stobrawa et al. (2).](image-url)

lipofuscin characteristic of the disease of neuronal ceroid lipofuscinosis (4). Knockout mice, characteristically smaller than WT littermates, show enhanced basal locomotor activity. They are able to learn new motor skills as evidenced in behavioral rotarod tests (2). Electrophysiology of acute hippocampal slice preparations prepared from WT and KO mice between P13 and P15 failed to reveal any significant changes in synaptic function as determined in paired-pulse stimulation of Schaffer collaterals as well as amplitude or frequency of either mEPSPs or mIPSPs (2). There was a measurable decrease in the acidification of endosomes (2) as well as synaptic vesicles (2). Inconsistent with the excitotoxic explanation for the hippocampal and retinal neurodegeneration proposed by Strobrawa and colleagues for the KO mouse (2), the uptake of glutamate into synaptic vesicles isolated from whole brains of adult KO mice was reduced (2). In summary, it is unclear what factor contributes to the selective hippocampal and retinal neurodegeneration that is seen in the clc-3−/− mouse. Our strategy has been to determine the physiological role of CIC-3 at the hippocampal synapse. Knowing its contribution of synaptic plasticity may yield significant insight into the neurodegeneration observed in the absence of CIC-3 expression with respect to both specificity (i.e. hippocampal versus cortical) and mechanism.

**Anion conductances in developing neurons: Potential synergy between GABA activation and CIC-3**

The role of anion conductances in the brain other than GABA (γ-aminobutyric acid) receptor A has been largely overlooked. To date, only two non-ligand dependent Cl− channels, both members of the CIC family of Cl− channels, have been identified at the molecular level in selected populations of neurons, i.e. CIC-2 and CIC-3. CIC-2, an inwardly-rectifying Cl− conductance that activates at membrane potentials more negative than ECl is expressed in classes of neurons that lack inwardly directed Cl− co-transporters (5). In this subset of neurons where GABA is inhibitory, CIC-2 and not co-transporter activity is postulated to maintain the hyperpolarized ECl. Mutations in the gene encoding CIC-2 have been linked to epilepsy (6). The dramatic developmental neurodegenerative phenotype of the clc-3−/− mouse opened the question as to the manner in which other Cl− conductances might control neuronal development and function. Strobrawa and colleagues (2) speculated that defects in endosomal acidification in the CIC-3 knockout “may lead to a mislocalization of membrane proteins (including transporters and receptors), to altered luminal receptor-ligand interactions, or to changes in luminal enzymatic activities.” However, the mechanism whereby this might occur has not been elucidated and the hypothesis remains unproven. We have shown that CIC-3 is also expressed as a plasma membrane CaMKII-activated conductance in the brain as we have shown for non-neuronal cells (7, 8). The question remains as to whether CIC-3 might also be permissive for neuronal development. The literature provides extensive evidence linking early excitatory actions of GABA during development and the construction of cortical networks (9-11). CIC-3 might synergize with GABA receptor activation early in the construction of a functional cortical network and its reinforcement by neuronal activity. The fact that activation of GABAA receptors is initially excitatory and is a direct result of elevated Cl− within developing neurons has been recognized and studied by a number of laboratories (see review (12)). The developmental switch that controls the transition of GABA receptor activation from stimulatory to inhibitory appears to be the transition of expression of the Na-K-2Cl (NKCC1) transporter that accumulates intracellular Cl− to expression of the K-Cl coupled co-transporter (KCC2) that reduces intracellular Cl−. The switch in transporter expression correlates well with changes in the equilibrium potential for Cl− from approximately -40 to -70 mV which occurs over the first 14 days of postnatal development (12). The literature recognizes that GABAγ and NMDA receptors operate in synergy in immature neurons (13), however, CIC-3 expressed at early times in neuronal development may also help to accentuate the role of GABA in generating action potentials and, in general, supporting electrical activity. The depolarization-induced by GABAγ receptors would generate calcium currents by directly activating voltage-dependent calcium channels (see Fig. 2). The depolarization by GABAγ would not only help to remove Mg2+ block of NMDA receptor channels but also participate in the activation of CIC-3; a second anion conductance supporting the same task. Thus, GABAA as well as CIC-3 channels would play the role in neonatal neurons that AMPA plays in mature neurons i.e. providing initial depolarization and relief of Mg2+ block. This is
important in that NMDA-induced currents provide a more substantial component of the overall activation in the developing neuronal network circuitry than in the adult (14). The importance of CIC-3 in this process is underscored in the murine knock-out model in which hippocampal neurodegeneration becomes apparent at approximately P14.

It has been suggested by Leinekugel et al. that spontaneous and network driven Giant Depolarizing Potentials (GDPs) in the neonatal hippocampus are due to the synergy between GABA activation and NMDA receptor activity and provide for spontaneous and synchronous increases in intracellular Ca2+ (15). The developmental consequence of the Ca2+ influx during the GDPs has been proposed to provide a significant source of Ca2+ involved in activity dependent synaptogenesis and network formation. Although GDPs would theoretically allow for simultaneous activation of CIC-3, modulation of their activity in the CIC-3 knock-out animal has not been tested. In addition, the contribution of CIC-3 expression on the amplitude and duration of the GDPs has not been shown. Accordingly, the contribution of CIC-3 activation to Ca2+ handling in the developing nervous system may be, in part, mediated via a potentiated, localized Ca2+ influx through NMDA receptors, providing a Hebbian-like modulation of developing synapses; enhancement of release via vesicle acidification, prolonged depolarization, and therefore, Ca2+ influx at postsynaptic sites.

Our recently published data (1) examining the role of CIC-3 in the modulation of synaptic efficacy in the hippocampus support a model in which Ca2+ entry across the postsynaptic membrane via NMDA-receptors promotes CaMKII dependent phosphorylation and activation of CIC-3 (Fig. 3). In immature neurons, this results in an extended relief of Mg2+-dependent NMDA receptor block, an increase in EPSP amplitude and EPSP time course, all effects leading to an enhancement of synaptic efficacy in generating action potentials and in inducing Ca2+ influx via the opening of voltage-gated Ca2+ channels. At late times in development, when ECl is hyperpolarized, the modulatory effect of CIC-3 activation would depress synaptic potentials.

Chloride channels are a physiologically important, yet under-studied, class of channels in the brain. A role for depolarizing GABA-mediated Cl- driven excitation is now widely accepted as a model for neuronal maturation in cortical circuits; however, a role for CIC channels in the phenomenon has not previously been explored. CIC-3 is unique among its family members in that it is gated by Ca2+ dependent phosphorylation. Our recent published studies (1) demonstrate that CIC-3 channels colocalize with NMDA receptors at hippocampal synapses. Calcium flowing through NMDA receptors activates CIC-3 channels enhancing the excitatory postsynaptic potential (EPSP) in immature neurons and reducing the EPSP in mature neurons. Long-term potentiation (LTP) and its dependence upon NMDA-receptor mediated calcium entry has been widely studied as a mechanism underlying learning and memory. Based upon our data, expression of CIC-3 channels would enhance NMDA-receptor dependent calcium signaling and thereby facilitate the induction of long-term potentiation at early times in development and conversely depress both calcium signaling and induction of LTP in mature neurons. Thus, Ca2+-dependent CIC-3 channels and their unique relationship with NMDA receptor provide a new and important level of regulation in the modulation of synaptic plasticity. In that CIC-3 channels are ubiquitously expressed in the brain (16), the relationship between CIC-3 channels and NMDA receptors is not likely to be restricted to the hippocampus, however, this remains an open question.


Potential role of calmodulin in regulating RGK GTPase inhibition of CaV1-2 channels

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RGK GTPases profoundly inhibit CaV₁-2 channels via auxiliary beta subunits. The C-terminus of RGK GTPases contain a calmodulin (CaM) binding site that overlaps a membrane-targeting domain. This site has been shown to bind CaM in vitro, but not in the context of live cells. Moreover, it has been hypothesized that CaM may relieve RGK protein inhibition of CaV₁-2 channels by either eliminating their association with the plasma membrane or reducing their binding to CaVbeta subunits. To examine this we assessed CaM binding to Rem by FRET in live HEK 293 cells. We detected no interaction between CaM and Rem by FRET suggesting that this association may not be robust in vivo. Consistent with this, elevating intracellular Ca²⁺ did not redistribute Rem to the plasma membrane. Further, elevating intracellular Ca²⁺ did not ablate the Rem-CaVbeta interaction assessed by FRET. Finally, elevated intracellular Ca²⁺ did not reverse Rem inhibition of CaV channels. We conclude that Ca²⁺-CaM does not dynamically regulate RGK protein inhibition of CaV channels.

Session 3 Speaker Materials

CaV₁ and CaV₂ channels and calmodulin

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In the context of recombinant Ca²⁺ channels, Ca²⁺/CaM-dependent feedback regulation at first appeared to represent a unique property of certain CaV₁ channels. Members of the CaV₂ clade of these channels seemed devoid of such behavior, and were in fact used as ‘inert’ partners for chimeric channel analysis of the CaV₁ channel regulatory process. We now know this initial view to be inaccurate, as nearly all CaV₁-2 channels exhibit CaM-mediated modulation, according to a common overall mechanism with rich and important variations. Such Ca²⁺ modulation figures crucially in the biological function of Ca²⁺ channels. More broadly, this class of CaM-mediated regulation may be a general model for Ca²⁺ feedback mechanisms that involve local Ca²⁺ sensors affixed to their own Ca²⁺ sources. This pairing of Ca²⁺ sensors and sources—a hallmark of CaM/Ca²⁺ channel regulation—is widespread in many other molecular complexes as well, given the privileged Ca²⁺ signaling afforded by this configuration. Hence, dissecting the mechanisms of CaM/Ca²⁺ channel regulation is a research goal of rather general importance.

The CaM/Ca²⁺ channel regulatory system has revealed several powerful and unusual Ca2+ sensing capabilities of CaM. While the constitutive association of CaM with channels permits rapid and privileged Ca²⁺ regulation, such proximity puts CaM at risk of being ‘blinded’ by the intense Ca²⁺ entry through the local channel, thus challenging the sensing of far weaker Ca²⁺ signals integrated from distant channels and other Ca²⁺ sources. This latter property is nonetheless crucial for coordinated signaling. CaM solves this challenge in a manner hinted at by Ching Kung, in his pioneering work on CaM regulation within Paramecia. He found that the N- and C-terminal lobes of CaM had separable regulatory functions, where the N-lobe was important for the control of some target molecules, while the C-lobe was important for others. This ‘functional bi-partition’ of the lobes of CaM was unexpected, as the textbook view of time was that both lobes of CaM were required to regulate target molecules. More recently, within the context of CaV1 2 Ca²⁺ channels, such functional bipartition of CaM has risen to new heights. Firstly, though a single regulatory CaM persistently associates with channels, Ca²⁺ binding to its C- and N-lobes can trigger different regulatory processes on the same host channel. Secondly, though the lobes of CaM are less than 60 angstroms apart, and thereby face the same Ca²⁺, they somehow favor spatially distinct Ca²⁺ sources. The C-lobe prefers intense intermittent
Ca\(^{2+}\) influx via the host channel (local selectivity), whereas the N lobe responds selectively to diminutive persistent Ca\(^{2+}\) signals from distant sources (global selectivity). The latter global selectivity thus solves an important challenge for Ca\(^{2+}\) sensors complexed with Ca\(^{2+}\) sources. Nonetheless, completely unknown has been the mechanism underlying the contrasting spatial Ca\(^{2+}\) selectivities of the C- and N-lobes of CaM. In particular, how can the global Ca\(^{2+}\) selectivity of the N-lobe possibly be achieved? These central questions have stymied our laboratory for several years.

A critical clue in this quest for mechanism was the recent discovery that an underappreciated Ca\(^{2+}\)/CaM binding site within the amino terminus of certain CaV channels can utterly transform the spatial Ca\(^{2+}\) selectivity of N-lobe signaling. Thus, we named this site **NSCaTE**, for N-terminal Spatial Ca\(^{2+}\) Transforming Element. Until this finding, the spatial Ca\(^{2+}\) selectivity of CaM regulation appeared an immutable entity. However, as it turns out, native Ca\(_{V2.2}\) channels lack **NSCaTE**, and exhibit N-lobe mediated CDI with a global selectivity. Adding **NSCaTE** to Ca\(_{V2.2}\) channels strongly converts their regulation towards a local profile. Residues within **NSCaTE** that are important for this conversion also turn out to be critical for Ca\(^{2+}\)/CaM binding. In fact, the extent of global-to-local conversion correlates tightly with the Ca\(^{2+}\)/CaM affinity of mutated **NSCaTE** modules. This uncovers a close linkage between the Ca\(^{2+}\)/CaM-binding affinity of **NSCaTE** and its ability to transform spatial Ca\(^{2+}\) selectivity. Of additional importance, Ca\(_{V1.2}\) and 1.3 channels inherently possess **NSCaTE**, and thereby exhibit N-lobe CDI with local selectivity. Removal of **NSCaTE** converts N-lobe CDI of these channels to a clearly global profile. Thus, **NSCaTE** differentially tunes the spatial Ca\(^{2+}\) selectivity of N-lobe CDI across two channel clades (Ca\(_{v1}\) versus Ca\(_{v2}\)). While these findings did not explain the mechanism of spatial Ca\(^{2+}\) selectivity, part of the secret of such selectivity nonetheless had to reside within the context of a compact molecular module.

Along with the discovery of **NSCaTE** function, another crucial advance was being made on the theoretical front. We had long been unable to conceive of any theoretical mechanism whereby global Ca\(^{2+}\) selectivity could be achieved. Finally, by explicitly considering the effects of apoCaM association with channels, and the kinetics of Ca\(^{2+}\) binding and unbinding to C- and N-lobes, we were able to formulate mechanisms with the desired spatial Ca\(^{2+}\) selectivity properties. Local Ca\(^{2+}\) selectivity could be achieved by a ‘slow CaM’ mechanism that responds preferentially to Ca\(^{2+}\) intensity. Global selectivity could be achieved by exploiting an ‘SQS’ mechanism with unprecedented preference for Ca\(^{2+}\) persistence over intensity. This counterintuitive behavior could reflect the interplay of three factors: channel open probability; Ca\(^{2+}\) (un)binding kinetics from CaM; and the balance between channel affinity for Ca\(^{2+}\)/CaM versus apoCaM (parameter r).

Beyond theory, the next key step was the epiphany that the effect of **NSCaTE** might actually be to change the key parameter r in our theoretical SQS mechanism. Accordingly, new methods were devised to enable manipulation of all three factors above, permitting extensive testing and confirmation of our theoretical mechanism. Specifically, open probability was adjusted via a ‘voltage-block’ technique, combined with channels engineered for enhanced opening. Importantly, the ratio of channel affinity for Ca\(^{2+}\)/CaM versus apoCaM (parameter r) is graded via mutagenesis of a novel **NSCaTE** Ca\(^{2+}\)/CaM binding motif. Finally, the theoretical basis of the selectivity mechanisms was confirmed in the stochastic realm, via particle simulations of Ca\(^{2+}\) in the channel nanodomain.

In all, the regulation of Ca\(^{2+}\) channels by CaM has taught us much about the powerful Ca\(^{2+}\) sensing capabilities of CaM, and the extraordinary mechanisms that underlie them. These results, and the approaches taken, may well generalize beyond Ca\(^{2+}\) channels to other spatiotemporally selective Ca\(^{2+}\) decoding systems.
Calcium-dependent inactivation is mediated by multiple determinants

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The voltage-gated inward Ca\textsuperscript{2+} current (IC\textsubscript{a}) is a common mechanism of transient increase in the cytoplasmic free Ca\textsuperscript{2+} concentration that activates a variety of cellular responses. The rapid and complete inactivation of I\textsubscript{Ca} is the critical step terminating Ca\textsuperscript{2+} influx and preventing Ca\textsuperscript{2+} overloading of the cell. In the case of Cav1.2 calcium channels, two different mechanisms are in control of the Ca\textsuperscript{2+} current inactivation. One mechanism is driven by Ca\textsuperscript{2+} ions on the cytoplasmic side of the membrane, whereas the other depends on transmembrane voltage. Replacement of Ca\textsuperscript{2+} for Ba\textsuperscript{2+} as the charge carrier eliminates Ca\textsuperscript{2+}-dependent inactivation (CDI) so that Ba\textsuperscript{2+}-conducting calcium channels inactivate in a voltage-dependent manner by fast and slow mechanisms. Cav\textbeta subunits (e.g., \beta\textsubscript{1a} vs. \beta\textsubscript{2a}) differentially modulate inactivation. CDI was previously co-identifed with two adjacent calmodulin-binding regions (LA/IQ) in the middle part of the \alpha\textsubscript{1C} subunit C-terminal tail. Splice variation of the \alpha1C transcript in this region (e.g., \alpha\textsubscript{1C,86}) accelerates fast inactivation, removes slow inactivation, CDI and differential Cav\beta subunits modulation. However, our findings show that CDI is not mediated solely by LA/IQ. Investigation of other cytoplasmic constituents of the channel complex pointed to the roles of the N-tail, the cytoplasmic pore region, and the \alpha\textsubscript{1C} C-tail folding in inactivation, helping to understand how slow and fast voltage-dependent inactivation and CDI evolve from their interplay.

C-tail folding. Quantitative voltage-dependent FRET microscopy combined with patch clamp in the live cell showed that the \alpha1C subunit C-terminal tail is subject to voltage-gated conformational rearrangements that delivers Ca\textsuperscript{2+} caged in calmodulin, to downstream signaling targets involved in CREB-dependent transcriptional activation. The plasma membrane-anchoring of the \alpha1C C-tail via fusion of the plekstrin homology domain (PH) inhibits slow inactivation, CDI and Ca\textsuperscript{2+} signal transduction. Release of the C-tail fully restores these functions. Thus, a specific functional folding of the \alpha1C C-terminal tail vis-à-vis the pore is crucial for inactivation.

Annual Determinant of Slow inactivation (ADSI). Another determinant relevant to slow inactivation and CDI is composed of four highly conserved amino acids of the transmembrane segment S6, constituting the cytoplasmic end of the pore. Their simultaneous mutation (S405I in IS6, A752T in IIS6, V1165T in IIIS6, and I1475T in IVS6) generates the \alpha\textsubscript{1C,IS-IV} channel. Slow voltage-dependent inactivation of \alpha1C,IS-IV is inhibited, and it does not show CDI and differential Ca\textbeta subunit modulation. N-tail. A very different role was found for the \alpha1C N-terminal tail. It shows limited voltage-dependent mobility in relationship with the plasma membrane conferred by a Ca\textbeta subunit in a manner that facilitates the channel response to voltage gating. Plasma-membrane-anchoring, or deletion, of the \alpha1C subunit N terminus completely inhibited inactivation of the Ba\textsuperscript{2+} (or Ca\textsuperscript{2+}) current in the absence of Ca\textbeta.
Auxiliary $\alpha_2\beta$ and $\alpha_2\delta$ subunits are not crucial for CDI. Co-expression of $\alpha_1C$ with calmodulin in the absence of either (but not both) of these subunits generates fully functional $\text{Ca}^{2+}$ channels supporting CDI. In conclusion, CDI is mediated by determinants of the $\alpha_1C$ C- and N-termini, the ADSI in the cytoplasmic pore region, as well as the folding of the C-tail. Calmodulin integrates these determinants, providing a $\text{Ca}^{2+}$-dependent switch that terminates slow inactivation, releases the $\alpha_1C$ C-tail and shuttles the associated $\text{Ca}^{2+}$/calmodulin acting as an activating stimulus of the $\text{Ca}^{2+}$ signal transduction.

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**Coupling of TRPC4 and TRPC5 channels to rises in cytosolic Ca**

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TRPC4- and TRPC5-channels are members of the TRPC family of Ca-permeable non-selective cation channels which are activated upon stimulation of the phosphoinositide signalling cascade by receptors that stimulate phospholipase C. However, the exact mechanism of channel activation is still open. Like all members of the TRPC subfamily TRPC5 and TRPC4 possess binding sites for calmodulin and IP3 receptors. Three calmodulin binding sites (cambs), cambs1, cambs2 and cambs3, have been identified in the C-terminus of TRPC4 and cambs1 and cambs2 overlap with binding sites for the N- and C-termini of IP3 receptors. In the present study we have explored the specific roles of the three cambs in the modulation of channel activity by selectively disrupting each cambs. Channel activity was induced by perfusing TRPC4-expressing HEK293 cells with GTPgammaS. Channels carrying mutations in cambs2 or cambs3 or in both, cambs2 and cambs3, are still active whereas mutations in cambs1 render the channel inactive. TRPC4 currents require cytosolic Ca, but disruption of cambs2 abolishes this Ca dependence. TRPC5, the closest relative of TRPC4 contains two cambs binding sites within its C-terminus, with cambs1 responsible for overall channel activity and cambs 2 mediating $\text{Ca}^{2+}$/calmodulin dependent channel modulation (1). In contrast to TRPC4, TRPC5 does not require GTPgammaS perfusion but is activated by a rise in cytosolic Ca.

The excess calmodulin recovers activity of Ca\textsubscript{v}1.2 calcium channel in the absence of Ca\textsubscript{v}\beta or α\textsubscript{2}δ subunits

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It is generally accepted that in order to generate calcium currents in response to depolarization, Cav1.2 calcium channels require association of the pore-forming α\textsubscript{1C} subunit with accessory Ca\textsubscript{v}\beta and α\textsubscript{2}δ subunits. A single calmodulin (CaM) molecule is tethered to the C-terminal α\textsubscript{1C}-LA/IQ region and mediates Ca\textsuperscript{2+}-dependent inactivation (CDI) of the channel. Ca\textsubscript{v}\beta subunits are stably associated with the α\textsubscript{1C}-AID site of the cytoplasmic linker between internal repeats I and II and also interact dynamically, in a Ca\textsuperscript{2+}-dependent manner, with the α\textsubscript{1C}-IQ region. Much less is known about the structural aspects of the association between α\textsubscript{1C} and α\textsubscript{2}δ. We found that co-expression of exogenous CaM (CaMex) with α\textsubscript{1C} in COS1 cells in the absence of either Ca\textsubscript{v}\beta or α\textsubscript{2}δ, but not both of these subunits, stimulates the plasma membrane targeting of α\textsubscript{1C}, facilitates calcium channel gating, and supports CDI. Neither real-time PCR with primers complementary to monkey Ca\textsubscript{v}\beta and α\textsubscript{2}δ subunits, nor co-immunoprecipitation analysis with exogenous α\textsubscript{1C} revealed an induction of endogenous Ca\textsubscript{v}\beta and α\textsubscript{2}δ that could be linked to the effect of CaMex. Co-expression of a Ca\textsuperscript{2+}-insensitive CaM mutant CaM1234 also facilitated gating of both the Ca\textsubscript{v}\beta- and α\textsubscript{2}δ-free Ca\textsubscript{v}1.2 channels, but did not support CDI. Our results show that there is a functional matchup between CaMex and Ca\textsubscript{v}\β or α\textsubscript{2}δ that, in the absence of either of these subunits, renders Ca\textsuperscript{2+} channel gating facilitated by CaM molecules other than the one tethered to LA/IQ to support CDI. Thus, co-expression of CaMex creates conditions when the channel gating, voltage- and Ca\textsuperscript{2+}-dependent inactivation, and plasma-membrane targeting occur in the absence of structurally different Ca\textsubscript{v}\β or α\textsubscript{2}δ. We suggest that CaMex affects specific Ca\textsubscript{v}\β/α\textsubscript{2}δ-free conformations of the channel that are not available to endogenous CaM.

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L-channel N-tail: modulation by calmodulin

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The cytoplasmic N-terminus (NT) is an important regulatory site in the α\textsubscript{1C} subunit of the Ca\textsubscript{v} 1.2 channel (L-type). The mammalian α\textsubscript{1C} in different tissues features two major NT isoforms, with a long (46 amino acids) and a short (16 amino acids) initial segment. The two isoforms, which we term “long-NT” and “short NT”, respectively, result from alternative splicing in which either exon 1a (the longer one) or exon 1 (the shorter one) are used (1-3). The long-NT isoforms are abundant in the heart and also present in the brain, whereas the short-NT isoforms are predominant in smooth muscle and brain.

The total length of the cytosolic NT of the cardiac (long-NT) α\textsubscript{1C}, up to the beginning of the first transmembrane segment, is 154 amino acid residues. In the past, using a structure-function approach based on deletions and creation of chimeric constructs, we have demonstrated that the initial segment of the cardiac α\textsubscript{1C} is an inhibitory module which specifically reduces the channel’s maximal open probability, P\textsubscript{o,max} (4, 5). The crucial regulatory segment is located at amino acids 1-20 of the long NT, and the partly homologous 16 amino acids of the short-NT isoform cannot perform this function. The removal of the NT-inhibitory module greatly increases the whole-cell current and the P\textsubscript{o,max} without affecting the surface density of the expressed channels (5). This function of the NT
is tightly associated with the effects of the β subunit on the channel: while a prominent effect of the β subunit on the cardiac α1C is to increase the P_o,max (6–8), in the absence of the NT-inhibitory module the coexpression of βsubunit does not increase the P_o,max any more (5). Yet, the NT does not directly interact with the β subunit. Furthermore, the same amino acids, 1-20, are also crucial for the well described enhancing action of protein kinase C (PKC) on the cardiac α1C and their removal abolishes PKC enhancement. However, the phosphorylation site crucial for this PKC effect is not located in the NT (1, 4). Therefore, we envisage that the regulation by the NT-inhibitory module of the actions exerted by the β subunit and by PKC must involve a third “partner” (another part of the α1C itself) with which the NT interacts and via which it affects the gating. This partner has not been identified so far.

The full-length NT of α1C interacts with a number of proteins, among them Gβγ (9), Ca-binding protein 1 (10) and PKC (unpublished results). Importantly, we found that it also directly interacts with calmodulin (CaM) in a Ca2+-dependent manner (9). The removal of most of NT in the α1C CA2-139 mutant rendered the voltage-dependent inactivation (VDI) of the channel faster, whereas the acceleration of the inactivation by Ca2+ was reduced in comparison with the wild-type (wt) channel, suggesting that the NT may play a role in the calcium-dependent inactivation (CDI) (9). Since the C-terminus of α1C harbors the main CaM-binding sites (11-18), and since the distal ends of both NT and CT acted as partially independent but interacting inhibitory gates, we proposed that NT and CT, possibly together with additional cytosolic elements of α1C such as loop I, form a molecular scaffold (NT-CT scaffold) at the cytoplasmic side of the channel, which deters channel opening. We further proposed that the conformation of the NT-CT scaffold changes as a function of the presence Ca2+, which strengthens the inhibitory control exerted by the scaffold in a CaM-dependent manner. We envisaged that “influx of Ca2+ via the open channel is followed by Ca2+ binding to CaM which, in turn, improves the interaction of CaM with its binding sites and further tightens the scaffold, causing more inhibition (which is apparent as Ca2+-dependent inactivation)” (9). Interdependent though distinct roles of NT and CT in CDI have also been supported by a fluorescence energy transfer study (19). A recent work by Yue and collaborators (20) supported and extended this concept, by identifying a crucial CaM-dependent element in the NT between amino acids 80 – 90, which they termed NSCaTE, for “N-terminal spatial Ca2+ transforming element”. They showed that the presence of NSCaTE renders the channel sensitive to Ca2+ ions that enter the channel itself, and noted that “the position of NSCaTE on the channel’s amino terminus indicates that CaM can bridge the amino terminus and carboxy terminus of channels”. It should be noted that the role of N-terminal CaM-binding site in the cardiac α1C (Cav1.2) seems quite modest and the removal of amino acids crucial for NSCaTE function does not greatly change the CDI, but its role in CDI of Cav1.3 is much more prominent, and the addition of NSCaTE to other Cav channels greatly affects their inactivation properties (20).

To explore the hypothesis that CaM forms a “bridge” between NT and CT in the process of CDI and to better understand the role of the N-terminal CaM-binding site, we mapped the interaction sites of NT with calmodulin using pull-down of in vitro synthesized CaM by GST-fused segments of the N-terminus of α1C. We found that CaM binding site in the NT is between amino acids (a.a.) 60-100, in excellent agreement with the findings of Dick et al. (20). The binding of CaM was strictly Ca2+-dependent. We next examined the thermodynamic parameters of the calcium-dependent binding of purified recombinant CaM to the highly purified NT60-120 segment using the Isothermal Titration Calorimetry (ITC). In the presence of 1 or 2 mM Ca2+, but not in the absence of Ca2+, CaM interacted with NT60-120. The equilibrium binding constant (Kb) was about 2*106 M, indicating binding affinity of ~0.5 μM, and the enthalpy change was about -8,000 cal/mole. Mutation in NT60-120 of three amino acids identified by Dick et al. (20) as crucial for the functional role of NSCaTE fully abolished the binding between NT60-120 and CaM. We have next examined the possibility that the N-terminal CaM binding site (NT60-120) directly binds to the C-terminal CaM-binding site, and whether CaM may be involved in such an interaction. To this end, we used purified NT60-120 and an in vitro synthesized C-terminal protein CT1505-1671, which contains both pre-IQ and IQ domains. We did not observe any physical interaction between these two proteins, either in the absence or in the presence of purified recombinant CaM.

While the latter result indicates that CaM does not form a direct “bridge” between the N- and C-terminal CaM binding sites, it should be interpreted with caution. The inhibitory modules in NT and CT are at their distal
ends and do not overlap with CaM binding sites. Yet, functional data suggest that they interact with each other (9), directly or via a third party. Additional binding determinants on both CT and NT may exist. In the context of such interactions among the full-length CT and NT (if they exist), Ca\textsuperscript{2+} -dependent CaM binding to the specific sites identified here and in the past could alter the conformation of the whole NT-CT scaffold to affect the inactivation process.

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Regulation of voltage-dependent inactivation of Cav1.4 Ca\textsuperscript{2+} channels by apocalmodulin

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Voltage-gated calcium channels (Cavs) profoundly contribute to the assembly, function and regulation of neuronal circuits of the retina. There is growing evidence that intracellular channel domains play a pivotal role in the regulation of these proteins. We recently identified a new channel domain in the Cav1.4 L-type calcium channel, termed inhibitor of Ca\textsuperscript{2+}-dependent inactivation (ICDI), that prevents Ca\textsuperscript{2+}-dependent inactivation (CDI). The ICDI is crucially required to confer the permanent Ca\textsuperscript{2+} influx through Cav1.4 needed for tonic glutamate release in photoreceptors and bipolar cells. Interestingly, Cav1.4 contains in its C-terminus a calmodulin (CaM) binding region that in other Cavs confers channel inactivation. By analyzing the functional properties of Cav1.4 mutants we found that apoCaM constitutively binds to Cav1.4 and modulates voltage-dependent inactivation of these channels. In essence, our data point to a novel action of CaM in the context of ion channels that is independent of Ca\textsuperscript{2+}-binding.

Mechanism of Ca\textsuperscript{2+}/calmodulin regulation of TRP channels

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For Ca\textsuperscript{2+}-permeable TRP channels, desensitization mediated by permeating Ca\textsuperscript{2+} ions is a critical mechanism for maintaining Ca\textsuperscript{2+} homeostasis. The two main candidates for transducing the effect of Ca\textsuperscript{2+}, PI(4,5)P\textsubscript{2} (via its degradation by PLC) and CaM have both been proposed to interact with the C-terminal domain of TRP channels. The mechanism for binding of PI(4,5)P\textsubscript{2} to the C-terminal domain may involve nonspecific electrostatic interactions between the anionic PI(4,5)P\textsubscript{2} and clusters of basic amino acids. For many proteins that bind to anionic lipids via such basic clusters, including MARCKS, GAP43, EGFR, and ErbB, Ca\textsuperscript{2+}/CaM can disrupt the protein-lipid interaction. In MARCKS and the EGFR, the effect of Ca\textsuperscript{2+}/CaM has been shown to result from its binding to the same site as the anionic lipids. The Ca\textsuperscript{2+}/CaM complex has a net charge of -16 at neutral pH, making it an effective competitor. The opposing effects of PI(4,5)P\textsubscript{2} and Ca\textsuperscript{2+}/CaM on TRP channel function raise the possibility that the two modulators compete for the same polybasic site in the C-terminal domain of TRP channels.

We propose that Ca\textsuperscript{2+}-dependent desensitization via a PLC-induced reduction in PI(4,5)P\textsubscript{2} levels and via CaM are functionally and structurally coupled. At rest, the positively charged C-terminal domain would binds acidic PI(4,5)P\textsubscript{2} in the plasma membrane, a conformation permissive of activation. Activation then allows Ca\textsuperscript{2+} to enter the cells, and Ca\textsuperscript{2+} produces desensitization by releasing the C-terminal domain from the plasma membrane. The release occurs either when Ca\textsuperscript{2+} activates PLC and PLC degrades PI(4,5)P\textsubscript{2} or when Ca\textsuperscript{2+} binds CaM and Ca\textsuperscript{2+}/CaM displaces PI(4,5)P\textsubscript{2}. TRP channel desensitization could thus occur through either or both mechanisms, depending on the amplitude and spatial distribution of the Ca\textsuperscript{2+} increase, the abundance/activity of PI(4,5)P\textsubscript{2}, PLC and CaM in proximity to the channels, and their relative sensitivity to Ca\textsuperscript{2+}.
Calmodulin mediates the $\text{Ca}^{2+}$-dependent regulation of gap junctions

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Gap junctions mediate cell-to-cell communication of small molecules (< 1 kDa) between neighboring mammalian cells. They are formed by the docking of two hemichannels (termed connexons) in two apposing cells (1). Each connexon is comprised of six connexin (Cx) subunits. To date, at least 20 connexin genes have been identified in the human genome with connexin43 (Cx43) being the most ubiquitous connexin (2). All the Cx proteins share a similar topology comprising four highly-conserved transmembrane regions, a short N-terminal cytoplasmic region, one intracellular and two extracellular loops, with a C-terminal intracellular tail as shown in Fig. 1. Variability in sequence homology across different connexin types is greatest in the intracellular loop and C-terminus. According to sequence similarity, Cx can be further grouped into at least three classes, α, β, and γ (3). A variety of naturally-occurring mutations in the α-Cx class, including the ubiquitous Cx43, and the lens connexins Cx46 (the rodent ortholog of sheep Cx44) and Cx50, are associated with specific defects in the lens (4). In addition, knockout of Cx46 or Cx50 in mice has been shown to result in lens cataract formation or reduction in lens size respectively (5-8).

Gap junctions have been shown to be regulated by the intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) (9). We and others have shown this regulation is likely mediated by CaM interacting directly with the connexin proteins (10-12). We have also shown previously that cell-to-cell communication in lens epithelial cell cultures is inhibited by elevated $[\text{Ca}^{2+}]_i$. Specifically, cell-to-cell transfer of the fluorescent dye AlexaFluor594 was half-maximally inhibited at ~300 nM $[\text{Ca}^{2+}]_i$ in lens cell cultures and this inhibition was prevented by preincubation of these cultures with CaM antagonists (13, 14), consistent with earlier reports that elevated $[\text{Ca}^{2+}]_i$ increased internal electrical resistance in the lens, which was prevented by preincubation with CaM antagonists (13). Indeed, this action of $\text{Ca}^{2+}$ in lens cell cultures is due in part to the inhibition of Cx43, the major connexin in these cell cultures. It has been demonstrated that Cx43-transfected HeLa cells exhibiting a similar $\text{Ca}^{2+}$-dependent inhibition appears to be CaM-mediated (12). The rapid onset of this $\text{Ca}^{2+}$-dependent inhibition of cell-to-cell communication (within seconds) suggests that this is mediated by a direct interaction of CaM with the connexin protein rather than being mediated via the action of a CaM-dependent protein kinase. Indeed, the $\text{Ca}^{2+}$-dependent binding of CaM to rat Cx32 (11, 15), fish Cx35, mouse Cx36 (16) and Cx50 (17) have been reported. Two cytoplasmic CaM binding domains, with one site ($K_d = 27 \text{ nM}$) in the N-terminus and the other site ($K_d = 1.2 \mu \text{M}$) in the C-terminal region have been identified in Cx32 (11), whereas a single CaM binding site ($K : 11-72 \text{ nM}$) was identified in the C-terminus of Cx35 and Cx36 (16). Efforts to map the potential CaM binding sites in the alpha family of connexins have led to conflicting results.

In this study we report our prediction of CaM-binding sites in the intracellular loop that have never been reported before. Peptide fragments encompassing the predicted CaM binding motifs in Cx43 and Cx44 were designed and synthesized. Their abilities to bind CaM were determined using a range of biophysical approaches. Biacore surface plasmon resonance studies demonstrated the binding of this peptide to CaM in the presence, but not the absence, of $\text{Ca}^{2+}$. Far UV circular dichroism studies indicated that, like other CaM binding peptides, the Cx-derived peptides increased their $\alpha$-helical contents on CaM binding. Fluorescence spectroscopy revealed conformational changes of both the peptide and CaM following formation of the CaM:peptide complex. NMR studies demonstrated that both peptides bind to CaM with a 1:1 stoichiometry. The binding of peptides further induce structural changes in both the N- and C-terminal domains, as well as in the linker region of CaM, reflecting a classical wrapping-around mode of interaction. The apparent dissociation constants of the peptides binding to CaM in physiologic $K^+$ is in the range of 0.7-1 $\mu \text{M}$ (for Cx43) and 30-600 nM (for Cx44). On binding of the peptides to CaM, the apparent $K_d$ for CaM decreased, and the Hill Coefficient $n_H$ increased. Isothermal titration studies suggest that the interaction is an exothermic event that is both enthalpically and entropically driven in which electrostatic interactions play an important role. The binding of the Cx44 peptide Cx44129-150 to calmodulin increases the calmodulin intrado-
main cooperativity and further enhances the Ca\textsuperscript{2+}-binding affinities of the C-domain of calmodulin by slowing the rate of Ca\textsuperscript{2+} release from the complex. We further demonstrated that the Ca\textsuperscript{2+}-dependent inhibition of lens epithelial cell-to-cell communication is mediated in part by the direct association of calmodulin with Connexin43 (Cx43). Elevation of intracellular [Ca\textsuperscript{2+}] in HeLa cells transiently transfected with the lens fiber cell gap junction protein Cx44 also resulted in the inhibition of cell-to-cell dye transfer. Our results provide the first direct evidence that CaM binds to a specific region of the ubiquitous gap junction protein Cx43 and Cx44 in a Ca\textsuperscript{2+}-dependent manner. Our data suggest a common mechanism by which the Ca\textsuperscript{2+}-dependent inhibition of the α-class of gap junction proteins is mediated by the direct association of an intracellular loop region of these proteins with Ca\textsuperscript{2+}-calmodulin.

Limiting calmodulin revealed by image and fluorescence correlation spectroscopy

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Many fundamental questions remain regarding cell signaling mediated by calmodulin. Among them are is calmodulin freely available to the hundreds of its potential targets or is it sequestered in cells and if so in what form? What is the range of action of diffusible calmodulin in its apo and Ca\textsuperscript{2+}-bound states? Insights into these basic questions will have a major impact on our understanding of the steps that determine calmodulin's ability to decode alterations in intracellular Ca\textsuperscript{2+}-concentrations and transmit them to downstream targets.

Ca\textsuperscript{2+} signaling is one of the most widely studied second messenger pathways in cells because of its ubiquitous role in modulating cellular processes, ranging from cell division to muscle contraction to neuronal communication among many others. The major pathway for mediating responses to Ca\textsuperscript{2+} flux is through the small ubiquitous protein calmodulin, which cooperatively binds Ca\textsuperscript{2+} and transduces the signal to a family of over 100 calmodulin-binding proteins. It has been estimated that the total concentration of calmodulin-binding proteins is approximately two-fold higher than the total calmodulin concentration in cells. As such, interactions of calmodulin with its target proteins affect its mobility through the cytoplasm, nucleus or near the plasma membrane. The general concept is that calmodulin has been thought of as limiting in its role as a messenger. Thus, the model put forward is that calmodulin is sequestered by its targets in basal conditions and that Ca\textsuperscript{2+} signals activate downstream targets to which calmodulin is already bound or redistribution ensues with calmodulin binding to targets with higher affinity for the Ca\textsuperscript{2+} saturated form.

One of the major targets in cells for Ca\textsuperscript{2+}/calmodulin is Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII), a Ser/Thr directed protein kinase abundant in brain and other tissues. CaMKII is a twelve subunit complex that increases its affinity to calmodulin following autophosphorylation, a phenomenon called calmodulin-trapping. This suggests that CaMKII may have a role in modulating the pool of available calmodulin inside cells. Eventually, activation of CaMKII can lead to physiological changes in the properties of ion channels, receptors and other enzymes which can lead to alterations in synaptic efficiency modulate the force/contraction relationship in cardiac tissues, or many other functions.

Regardless of the amount of information acquired over the years on Ca\textsuperscript{2+} signaling through calmodulin, the question of how calmodulin diffuses throughout the cell to regulate specific pathways is still open. When direct observations of calmodulin mobility and availability have been undertaken some results are contradictory likely due to the lack of spatial resolution, the methodology employed, the cell model used, and the reporter molecule. This presentation will summarize our efforts at determining the intracellular diffusion and interactions of calmodulin using the techniques of fluorescence spectroscopy, raster image correlation spectroscopy (RICS) and number and brightness (N&B) analysis. Together, these techniques provide a methodology to investigate the global diffusive behavior of calmodulin, and determine how calmodulin interacts with binding targets throughout the cell. Using these methodologies, our data show that in HEK293 cells, calmodulin diffuses at a rate slower than expected (10 μm\textsuperscript{2}/s) while eGFP diffuses at ~20 μm\textsuperscript{2}/s. Combining the data from RICS and N&B, we found regions of the cell where only one molecule of calmodulin is interacting with a putative target and regions in which calmodulin is in complexes involving more than one calmodulin molecule. Co-expression of αCaMKII leads to slowed calmodulin diffusion and an increased number of calmodulin molecules in the bound state, thus supporting the hypothesis that CaMKII can modulate calmodulin availability. In total, we conclude that there is little freely available calmodulin at rest or following an increase in Ca\textsuperscript{2+}, supporting the idea that target binding sites must exceed the number of calmodulin
molecules. In addition, these quantitative values for intracellular diffusion bound the issue of the range of action of calmodulin. Time permitting, the presentation will also include recent simulation data using a novel algorithm for calculating diffusion and chemical reactions at the single-molecule level based on these quantitative measurements.

Dynamic Ca-CaM changes in heart and channel regulation by CaMKII

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Ca-CaM regulates many processes in cardiac myocytes, partly via CaMKII and calcineurin (CaN) which have low and high Ca-CaM affinity, respectively. We used FRET-based sensors with different Ca-CaM affinities to measure dynamic beat-to-beat changes in [Ca-CaM] in intact myocytes and assess cumulative signal integration. We also developed computational myocyte models of CaMKII & CaN activation in different cellular domains. CaN in the junctional cleft with SR would always be active in the beating heart, while cytosolic CaN would gradually integrate (e.g. with heart rate changes). CaMKII responds phasically to high local cleft [Ca], but is barely activated in cytosol. We and others have characterized CaMKII effects on cardiac ion channels (e.g. Ca, Na, Ito, RyR), but it is also important to consider how these multiple effects are integrated at the cellular level to influence the cardiac action potential and potentially contribute to arrhythmias. CaMKII-dependent effects on Ca and Na currents tend to prolong AP duration, while Ito changes shorten APD. There are also differences in channel distribution regionally in the heart and also during heart failure. Based on our analysis, the net effect of CaMKII on these channels would be to shorten epicardial APD and prolong endocardial APD, which increases transmural dispersion of repolarization, an effect known to be pro-arrhythmic. CaMKII also activates ryanodine receptors (RyR), Ca sparks and waves, and these can increase the propensity for spontaneous diastolic SR Ca release and triggered arrhythmias. We conclude that CaM is involved with numerous local cellular signaling events, but local channel events (as for other CaM signaling) integrate at the cellular and tissue levels to produce complex integrative effects.

Alternatively spliced calmodulin-2 was found by the yeast-2-hybrid technique as putative partner of Cav2.3, and is expressed in two human cell lines

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Ca2.3 containing (E-/R-type) voltage-activated Ca2+ channels are expressed in excitable cells and trigger neurotransmitter and peptide-hormone release (Kamp et al., 2005;Jing et al., 2005;Zhang et al., 2007). Feedback regulation via incoming Ca2+ ions was detected for the classical Ca2+-dependent inactivation through calmodulin (Liang et al., 2003), and for a cytosolic II-III linker mediated modulation of Ca2+ current (Pereverzev et al., 2002;Leroy et al., 2003;Klöckner et al., 2004).
The expression of R-type Ca\(^{2+}\) channels remote from the fast release sites leads to the accumulation of presynaptic Ca\(^{2+}\) which can both, facilitate and inhibit the influx of Ca\(^{2+}\) ions through Ca\(_{2.3}\). The facilitated Ca\(^{2+}\) influx was recently related to hippocampal postsynaptic facilitation and long term potentiation (Dietrich et al., 2003). To analyze Ca\(^{2+}\) mediated modulation of cellular processes more in detail, protein partners of the carboxy terminal tail of Ca\(_{2.3}\) were identified by yeast-2-hybrid cloning from a human brain library, leading to the detection of a novel, extended and rarely occurring splice variant of calmodulin-2. No classical calmodulin was found as interaction partner. The novel splice variant CaM-2-ext must initiate translation at a separate position in the 5'-untranslated region compared to the classical CaM-2, because the fusion construct from the yeast-2-hybrid screening predicts a novel reading frame. It turns back to the classical reading frame more downstream after the insertion of a 46 nts long insertion (exon 1a) between exon 1 and exon 2. Transcripts, which contain the novel exon 1a, were detected in two human cell lines. Total RNA was isolated from the human embryonic kidney cell line HEK-293 as well as from the human medullary thyroid carcinoma cell line hMTC. RT-PCR analysis from both cell lines yielded cDNA fragments of the expected length for the normal and the extended CaM-2 splice variant, respectively. The transcript level of the normal calmodulin variant (CaM) exceeds by far the level of the extended calmodulin variant (CaM-ext) which is close to the detection limit. To ensure that the minor cDNA-fragment is derived from the predicted calmodulin splice variant CaM2-ext, the amplified cDNA was digested with two restriction enzymes.

To prove our conclusion more strictly that the 308 bp cDNA is indicative for CaM2-ext, a second RT-PCR was performed using as forward primer the oligonucleotide homolog to part of the novel exon 1a. After reverse transcription of total RNA from the same two human cell lines, a single cDNA-fragment of 263 bp was amplified from both cell lines. The amount of cDNA amplified was now clearly beyond detection limit, suggesting that both human cell lines express two splice variants of CaM-2, the classical CaM-2 in high amounts and further the CaM2-ext variant in low concentrations.

The sequencing of the amplified cDNA fragment which should be indicative for CaM2-ext, confirmed its identity as part of an alternate CaM-2 splice variant containing a short part of a retained intron between classical exon 1 and 2, as found in both yeast-2-hybrid clones.

Thus, the finding of an altered calmodulin sequence in two yeast two-hybrid clones and the consecutive amplification of indicative cDNA fragments for CaM2-ext from human cell lines leads to the conclusion that a second splice variant of CaM-2 may exist in vivo, at least in human cell lines. Ongoing analyzes on the protein level may help to determine, if the novel transcript is also translated to a functional protein or if cellular regulation occurs only on the transcript level.


Liang H, DeMaria CD, Erickson MG, Mori MX, Alseikhan BA, Yue DT (2003) Unified Mechanisms of Ca(2+)...


Session 5 Speaker Materials

**Calmodulin regulation of ryanodine receptors**

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Ryanodine receptors (RyRs) are ion channels that release Ca\(^{2+}\) from the sarco/endoplasmic reticulum. The three mammalian isoforms are RyR1 which predominates in skeletal muscle, RyR2 which is present at high levels in cardiac muscle, and RyR3 which was initially identified in brain but is expressed at low levels in many tissues including skeletal muscle. Calmodulin (CaM) inhibits the three mammalian RyR isoforms at micromolar Ca\(^{2+}\) concentrations. At submicromolar Ca\(^{2+}\) concentrations, RyR2 is also inhibited by CaM but RyR1 and RyR3 are activated to different extents by CaM (1-7). \(^{35}\)S\)CaM binding to the homotetrameric receptors shows that each RyR subunit has one high-affinity binding site for the Ca\(^{2+}\)-free (apoCaM) and Ca\(^{2+}\)-bound (CaCaM) forms of CaM (5-9). Cryoelectron microscopy indicates that apoCaM and CaCaM bind to an overlapping region distal to the effector site (ion pore) of RyR1 (10). This suggests that CaM exerts its effects allosterically over relatively long-range interactions within the RyRs.

**Interaction of CaM with skeletal muscle CaM binding domain**

Current models for the interaction of CaM with RyRs are largely based on studies using peptide fragments of RyR1. A gel shift assay showed that apoCaM and CaCaM bind a synthetic peptide (p3614-3643) (11). Deletion of nine C-terminal amino acid residues eliminated apoCaM but not CaCaM binding. Earlier studies showed that alkylation of cysteine 3635 in the deleted part, using N-ethylmaleimide, inhibits apoCaM but not CaCaM binding to RyR1 (12). Binding of Ca\(^{2+}\) to CaM affects its functional interaction with the RyRs (2,13). Whereas mutation of the N-terminal Ca\(^{2+}\) binding sites do not appreciably affect calmodulin modulation of RyR1, elimination of the Ca\(^{2+}\) binding sites in the C-terminal domain switches CaM from an inhibitor to activator at elevated Ca\(^{2+}\) concentrations. Taken together, the results have suggested that the C-terminal half of CaMBD binds apoCaM and Ca\(^{2+}\) binding to CaM shifts binding to the N-terminal site on RyR1, and thereby switches CaM from an activator of RyR1 at low Ca\(^{2+}\) concentrations to an inhibitor at elevated Ca\(^{2+}\) concentrations. Recent fluorescence measurements with engineered CaMs also were interpreted to indicate that the C-terminal and N-terminal domains of CaM sequentially rearrange their positions on the N-terminal and C-terminal domains of p3614-3643, respectively, as the Ca\(^{2+}\) concentration was raised from submicromolar to micromolar levels (14,15).

High resolution structure analysis has provided more direct insights into CaCaM binding to p3614-3643 of RyR1. A 2.0 Å crystal structure analysis of the CaCaM-peptide complex revealed an interaction of the C-terminal and N-terminal domains of CaM with two hydrophobic anchor residues, W3620 and F3636, respectively, at a novel “1-17” spacing (16). Binding brought the two CaCaM domains close toward another, however, without bringing them in direct contact with each other. NMR and FRET measurements indicated that the C-terminal domain of CaCaM tightly binds to the N-
terminal end of the peptide, and furthermore showed that in the complex each domain can interact independently with its own half of the peptide (16). The solution structure of the peptide-apoCaM complex remains to be determined, however.

**Interaction of CaMBD with other regions in the intact RyRs**
The structural consequences of CaM binding to the intact RyRs remain unknown. The sequence of the CaM binding site in RyR2 is identical in RyR3 and differs by two residues in RyR1. Substitution of the two residues in RyR2 with corresponding RyR1 residues did not alter CaM regulation of RyR2, which suggests that they are unlikely to confer isoform specificity (17). Yet the isoforms differ in CaM binding affinity (5,7), thermodynamics of CaM binding (18), and regulation by CaM (5,7,9). Endogenous effector molecules (Mg2+, ATP, reduced vs oxidized glutathione) distinctly alter CaM modulation of the RyRs (5,19). Differences between the three isoforms were also observed in the binding and regulation by apoCaM and CaCaM when the same mutations were introduced in the CaM binding domain (7). These results suggest that RyR domain-domain interactions impose conformational constraints on the CaMBD. Hence, peptides corresponding to CaMBD of RyRs only partially reproduce the binding of CaM to the intact RyRs.

Mutagenesis studies provide further evidence that regions outside of the CaMBD regulate the RyRs. Using a series of RyR1/RyR2 chimeras, Yamaguchi et al.(20) identified five nonconserved amino acid residues that confer CaM RyR isoform specificity. CaMBD peptides modulate RyR1 activity (21,22) and increased the frequency of spontaneous local Ca2+ release events in frog skeletal muscle fibers (23). Use of RyR1 fusion protein suggests that CaMBD peptides may modulate RyR1 activity by interacting with a C-terminal region of RyR1 encompassing residues 4062-4210 (24,25). Zhang et al. (26) report that the N-terminal half of CaM may interact with RyR1 residues 1975-1999 on an adjacent subunit. Substitution of nonidentical residues in RyR1 with corresponding RyR2 residues did not alter CaM regulation of RyR1 (20), which suggests that this region is unlikely to confer isoform specificity.

**Regulation of in vivo SR Ca2+ release by CaM**
To investigate the regulation of skeletal muscle excitation-contraction by CaM, RyR1-L3624D deficient in apoCaM and CaCaM binding and RyR1-W3620A deficient in only CaCaM binding were expressed in RyR1 deficient (dyspedic) myotubes (27). Neither RyR1-L3624D nor RyR1-W3620A eliminated voltage-gated SR Ca2+ release. In permeabilized adult mouse skeletal myofibers, CaM decreased Ca2+ spark frequency and size, whereas a Ca2+-insensitive mutant of CaM increased both spark frequency and amplitude (28). However, dissociation of CaM from the myofibers using a CaM binding peptide did not alter Ca2+ spark frequency. The results provide some evidence that CaM binding to RyR1 is not essential for skeletal muscle excitation-contraction coupling.

To determine the physiological importance of CaM regulation of RyR2 in cardiac muscle, Yamaguchi et al. (29) generated a mutant mouse deficient in the regulation of RyR2 by CaM at diastolic and systolic Ca2+ concentrations. Mice that express only the mutant form of RyR2 show signs of cardiac hypertrophy as early as 1 day after birth and die around 2 weeks of birth. Sustained Ca2+ transients were observed in homozygous cardiomyocytes isolated from 1-day old mice. The results suggest that a direct interaction with RyR2 facilitates the termination of SR Ca2+ release.

**Conclusion**
In summary, CaM regulates the RyRs by a direct interaction. Both the Ca2+-free and Ca2+ bound forms of CaM bind to a highly conserved CaM binding domain corresponding to residues 3614-3643 in RyR1. The mechanism of a differential regulation of RyR isoforms by CaM appears to be outside and distal to the CaM binding domain. Understanding of the conformational changes associated with CaM binding will ultimately require that the solution structures of the RyRs and RyR-CaM complexes become available. Some progress has been made in understanding the physiological importance of a direct interaction between CaM and RyR2 in cardiac muscle. The functional significance of a direct interaction of CaM with RyR1 is less clear. A possible explanation is that the two isoforms are regulated by distinct mechanisms in skeletal and cardiac muscle. In cardiac muscle, control of SR Ca2+ release by Ca2+ evidently requires that RyR2 is regulated by CaM. A direct interaction with the L-type Ca2+ channel tightly controls RyR1 activity, which may minimize the effects of CaM in skeletal muscle.

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Voltage-gated calcium channels convert electrical activity into long-term changes in the structure and function of neurons and are thus critically important for the plasticity of the nervous system. Our lab studies how calcium channels control gene expression, how cells control the number of calcium channels on the cell membrane and how mutations in calcium channels lead to disease. We recently identified a transcription factor derived from the C-terminus of the CaV1.2 voltage gated calcium channel called CCAT. We found that CCAT is produced in developing inhibitory neurons by transcriptional initiation from a 5′ start site that is distinct from the site used to generate the mRNA that encodes the channel. This 5′ start site is controlled by a promoter and enhancer that are different from those that control the generation of full-length CaV1.2 channels. Alternative start sites in the CACNA1C gene also generate at least two proteins, one that contains a voltage sensor and a transcription factor domain and one that encodes CCAT. Our results suggest that alternative initiation of CACNA1C and of other voltage gated calcium channels, generates a variety of proteins that share amino acid sequences but have dramatically different functions. A second project in the lab is focused on understanding how neurons regulate the number of calcium channels on the cell membrane. We found that activation of glutamate receptors leads to internalization and degradation of dendritic calcium channels including L-type channels and AMPA receptors. This process is mediated by lysosomes and is regulated by the lipid kinase PIKfyve. PIKfyve is the sole kinases that generates PI(3,5)P2 in cells, it binds directly to CaV1.2 channels, and regulates the motility of late endosomes and lysosomes. PIKfyve is necessary for neuronal survival during intense stimulation with glutamate as might occur during epilepsy or stroke. Mutations in the complex of proteins that regulate PIKfyve lead to neuronal death and epilepsy in mice. Finally, we have studied how a mutation that is associated with autism in patients with Timothy Syndrome (TS) alters channel signaling in neurons. We found that mutant calcium channels from TS patients trigger profound dendritic retraction and alter neuronal migration in the developing central nervous system. This effect of TS channels is largely mediated by a conformational change in the channels that leads to ectopic activation of the RhoA signaling cascade. These studies help elucidate the mechanisms by which electrical activity regulates neuronal function and also provide insight into the molecular underpinnings of autism and other neurological diseases.
tant CaM<sub>1234</sub> OR mutation of CaM binding site in the α1C C-terminal-tail both terminated transcriptional signal transduction, but did not inhibit Ca<sup>2+</sup> currents through the channels. Two-dimensional continuous wavelet transform analysis of FRET signals recorded in COS1 cells revealed that CREB-dependent transcriptional activation occurs in discrete nuclear signaling microdomains. Dependence of the appearance of these domains on alternative stimuli has been investigated. In conclusion, Ca<sup>2+</sup>-induced inactivation mechanism that terminates channel opening plays a crucial role in transcriptional activation. Ca<sup>2+</sup> ions for CREB-dependent transcriptional activation are caged in calmodulin, transferred to downstream target(s) by the voltage-dependent conformational rearrangements of the channel C-terminal tail, and occur in discrete nuclear microdomains.

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**Multifaceted regulation of Cav2.1 Ca<sup>2+</sup> channels by Ca<sup>2+</sup> binding proteins**

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Voltage-gated Cav<sub>2.1</sub> channels conduct P/Q-type Ca<sup>2+</sup> currents that regulate neurotransmitter release and neuronal excitability. These channels undergo a dual feedback regulation by incoming Ca<sup>2+</sup> ions, Ca<sup>2+</sup>-dependent facilitation and inactivation, which is mediated by calmodulin binding to the Cav2.1 α1-subunit. We showed previously that the Ca<sup>2+</sup> binding proteins, parvalbumin (PV) and calbindin (CB), may buffer global Ca<sup>2+</sup> levels that support inactivation, but not local Ca<sup>2+</sup> signals that cause facilitation of Cav2.1. To determine if PV and CB similarly influence neuronal Cav2.1 channels, we turned to cerebellar Purkinje neurons, which express high levels of PV, CB, and Cav 2.1. Patch-clamp recordings of dissociated Purkinje neurons from wild-type (WT) mice and those lacking PV and CB (PV/CB<sup>−/−</sup>) revealed a broader role of these Ca<sup>2+</sup> binding proteins in regulating Cav2.1. First, inactivation of Ca<sup>2+</sup> currents, but also Ba<sup>2+</sup> currents, was significantly greater in PV/CB<sup>−/−</sup> neurons than in WT neurons. Second, Ca<sup>2+</sup>-dependent facilitation was significantly less in PV/CB<sup>−/−</sup> than in WT neurons during trains of action potential waveforms. Of the multiple Cavβ subunits detected in Purkinje neurons, Cavβ<sub>2a</sub> confers Cav2.1 channels with the least inactivation and greatest Ca2+-dependent facilitation in transfected HEK293T cells. Therefore, downregulation of Cavβ<sub>2a</sub> could account for increased inactivation and decreased Ca<sup>2+</sup> dependent facilitation of Cav 2.1 in PV/CB<sup>−/−</sup> neurons. Consistent with this possibility, Cavβ<sub>2a</sub> was detected in a significantly smaller fraction of PV/CB<sup>−/−</sup> neurons than WT neurons by single-cell RT-PCR. Due to the decreased Ca<sup>2+</sup> buffering capacity of PV/CB<sup>−/−</sup> neurons, downregulation of Cavβ<sub>2a</sub> may serve as a homeostatic response to restrict activity-dependent Ca2+ influx through Cav 2.1 channels. Our findings highlight the significance of Cavβ<sub>2a</sub> as a key determinant for Ca<sup>2+</sup>-dependent facilitation and inactivation of Cav 2.1 in neurons and suggest a mechanism that may contribute to abnormal Purkinje cell rhythmicity and ataxia shown previously in PV/CB<sup>−</sup> mice.
Structural studies of CaM interaction with cardiac L-type voltage-gated calcium channels and ryanodine receptors

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The physiological events leading to cardiac muscle contraction and relaxation can be attributed to distinct protein-calcium binding events. An electrical signal (action potential) activates the opening of the L-type voltage gated calcium channels (LTCCs) leading to a small influx of calcium into the cell that triggers opening of the sarcoplasmic reticulum (SR) cardiac ryanodine receptors (RyR2). Calcium from the SR stores is released into the cytosol via the open RyR2 channels leading to a transient rise in intracellular [Ca²⁺]. The binding of calcium to troponin C produces structural changes that enable myosin interaction with actin initiating muscle contraction. In order for the heart to relax [Ca²⁺]i is rapidly removed through the concerted action of the Na⁺/Ca²⁺ exchanger (NCX1), the plasma membrane Ca²⁺ pump (PMCA) and the SR Ca²⁺ pump (SERCA2). These processes are governed by the action potential, calcium and a complex network of protein-protein interactions. Of particular interest to this group is the elucidation of the molecular mechanisms, and structural basis, underlying the regulation of LTCC and RyR2 function through protein binding. Studies are directed at how calmodulin (CaM), by a direct association with each of these channels, regulates both inactivation and activation of the channels; though there remains some controversy as to the functional effects of CaM upon RyR2. One of our aims is to understand how CaM discriminates between its binding partners in the cardiac calcium cycling process in response to physiological changes to [Ca²⁺]. We have employed an array of techniques including transmission electron microscopy and single particle analysis, fluorescence spectroscopy, NMR and isothermal titration calorimetry to investigate the association of CaM, in different calcium bound states, with the cardiac LTCC and RyR2. Studies currently involve: (i) LTCC-CaM interaction at the channel and subunit level (ii) elucidation of the molecular basis for both calcium-loaded and calcium-free CaM binding to RyR2 and (iii) determining the role of the C-terminal domain flanking the RyR2-CaM binding region for regulating receptor function.