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Calmodulin Modulation of Ion Channels
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Study Book, Part 1—Speaker Materials

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*Please note that not all speakers submitted materials.

Calmodulin-dependent modulation of CaV1.2 channels through reversible phosphorylation

David L. Armstrong

Lab. Neurobiology, NIEHS / NIH, Durham, NC, USA.

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.

Franz Hofmann*, Stefanie Fischer, Sven Moosmang, Andrea Welling.

Institut für Pharmakologie und Toxikologie, TU München, Biedersteiner Str. 29, D-80802 München, Germany

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.

1.   Seisenberger et al. (2000) JBC 275, 39193 –99.
2.   Schulla et al. (2003) EMBO J 22, 3844-54.
3.   Moosmang et. al. (2003) EMBO J 22, 6027-34.
4.   Wegener et al. (2004) FASEB J 18, 1159-61.
5.   Moosmang et al. (2005) J Neurosci 25, 9883-92.
6.   Moosmang et. al. (2006) Circ Res. 98, 105 – 110.
7.   Lee et al. (2006) JBC 281, 25560-25567

Calmodulin control of voltage-dependent relaxation behavior of G protein-gated K⁺ channels

Yoshihisa Kurachi

Department of Pharmacology, Graduate School of Medicine and the Center for Advanced Medical Engineering and Informatics, Osaka University

Acetylcholine activates G protein-gated inwardly rectifying K⁺ (K_G) channels in cardiac myocytes, which underlies vagal deceleration of the heart beat. The Ach-activated KG channel current exhibits a characteristic voltage-dependent property, which is called 'relaxation': The K_G 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 K_G is caused by the apparent voltage-dependent activity of G proteins which control the K_G channel activity as follows: Upon depolarization, Ca^{2+} flows into cell across the cell membrane, which forms Ca^{2+}/Calmodulin (CaM) complex. The Ca/CaM complex removes the inhibition by PIP_3-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 K_G channel activity during depolarizing potentials. Upon hyperpolarization, due to decrease of Ca^{2+} 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 K_G 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

John P. Adelman

Vollum Institute, Oregon Health & Science University, Portland, Oregon, USA

Small conductance Ca^{2+}-activated K+ channels (SK channels) are voltage-independent K⁺ channels that are gated solely by intracellular Ca^{2+} ions. Ca^{2+} gating is accomplished through a unique, constitutive co-assembly of the SK alpha, pore-forming subunits with the ubiquitous Ca^{2+} 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 Ca^{2+} 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 Ca^{2+}-dependent inactivation and facilitation in voltage-dependent Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+}-gating, the mechanisms underlying CK2 and PP2A regulation via CaM, and the consequences of SK-associated CaM modulation for neuronal function.

Apocalmodilin binds to wild type Cav1.4 L-type Ca²⁺ channels and modulates voltage dependent inactivation

C. A. Wahl-Schott, K. Grießmeier, H. Cuny, M. Biel

Munich Center for Integrated Protein Science CIPS^M and Department of Pharmacy—Center for Drug Research, Ludwig-Maximilians-Universität München, Germany

The retinal L-type Ca²⁺ channel Cav1.4 is distinguished from all other members of the high voltage-activated Ca²⁺ channel family by lacking Ca²⁺-dependent inactivation (CDI). In synaptic terminals of photoreceptors and bipolar cells, this feature is essential to translate graded membrane depolarizations into sustained Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ binding.

Session 2 Speaker Materials

Calcium channel regulation and short-term synaptic plasticity

William A. Catterall, Alexandra Few, Nathan Lautermilch, Todd Scheuer, and Sumiko Mochida*

Department of Pharmacology, University of Washington, Seattle, WA 98195-7280 USA
 ***Department of Physiology, Tokyo Medical University, Tokyo, Japan**

P/Q-type calcium currents conducted by $Ca_V2.1$ 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 $Ca_V2.1$, 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 $Ca_V2.1$, which causes inactivation. This biphasic mechanism optimizes calcium channel activity during brief trains of stimuli. The neuronal calcium sensor protein CaBP1 binds to the $Ca_V2.1$ channel, displaces calmodulin, and enhances inactivation without causing facilitation. In contrast, the related neuronal calcium sensor VILIP-2 slows calcium/calmodulin-dependent inactivation and enhances facilitation. Thus, neuro-specific calcium sensor proteins can differentially regulate $Ca_V2.1$ 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 $Ca_V2.1$ 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 $Ca_V2.1$ 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 $Ca_V2.1$ channel. Stimulation of SCGN expressing $Ca_V2.1$ 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 $Ca_V2.1$ 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 $Ca_V2.1$ channels through interaction with calcium sensor proteins is responsible for most of short-term synaptic plasticity.

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Calmodulin regulation of neuronal ion channels

Geoffrey Pitt

Duke University, Ion Channel Research Unit

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

Ludmila Deriy[1], XueQing Wang[1], Guangping Zhang[1], Radmila Sarac[2], and Deborah J. Nelson[1]

[1]Department of Neurobiology, Pharmacology and Physiology, The University of Chicago, Chicago, IL 60637
[2]Department of Biological Sciences, Purdue University Calumet, Hammond, IN

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

From Stobrawa et al. Neuron 29:185-96 (2001)
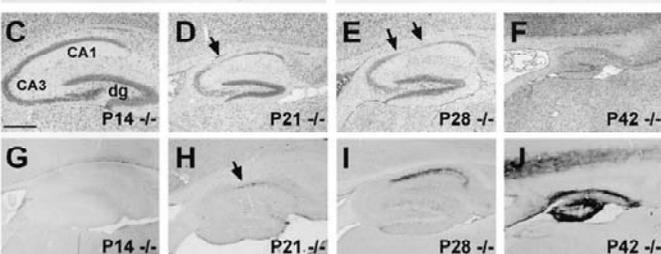
Figure 1. Degeneration of the Hippocampus in Clcn3–/– Mice. (A and B) Frontal sections of an adult (7-month-old) WT and KO mouse, respectively (Nissl stain). The hippocampal formation is nearly totally lost in KO. Nissl-stained sagittal sections (C–F) of KO hippocampi from P14 to P42. (G–J) Adjacent 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 (I). 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).

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 ClC-3 at the hippocampal synapse. Knowing its contribution of synaptic plasticity may yield significant insight into the neurodegeneration observed in the absence of ClC-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 ClC-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 ClC family of Cl- channels, have been identified at the molecular level in selected populations of neurons, i.e. ClC-2 and ClC-3. ClC-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, ClC-2 and not co-transporter activity is postulated to maintain the hyperpolarized ECl. Mutations in the gene encoding ClC-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. Stobrawa and colleagues (2) speculated that defects in endosomal acidification in the ClC-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 ClC-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 ClC-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). ClC-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 GABA$_A$ and NMDA receptors operate in synergy in immature neurons (13), however, ClC-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 GABA$_A$ receptors would generate calcium currents by directly activating voltage-dependent calcium channels (see Fig. 2). The depolarization by GAB$_A$ would not only help to remove Mg^{2+} block of NMDA receptor channels but also participate in the activation of ClC-3; a second anion conductance supporting the same task. Thus, GABAA as well as ClC-3 channels would play the role in neonatal neurons that AMP$_A$ plays in mature neurons i.e. providing initial depolarization and relief of Mg^{2+} block. This is
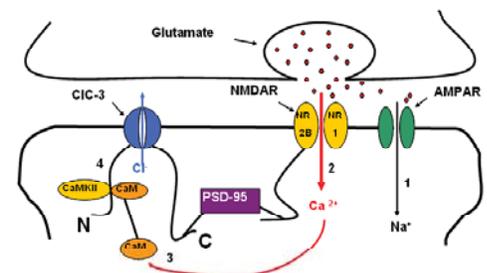
Figure 2. Synergistic actions of ClC-3 and GABA, NMDA and AMPA receptors in developing neurons. Activation of Cl-channels (ClC-3 and GABA-A receptors), excitatory at early developmental times, generates a depolarization which is sufficient to remove the voltage-dependent Mg^{2+} block of NMDA receptors. This may serve as an alternative route for NMDA receptor activation at early developmental times in the absence of AMPA receptor expression. Therefore, in neonatal neurons, a depolarizing Cl- conductance, either GABA-A or ClC-3, serves to remove Mg^{2+} inhibition in NMDA receptors and thereby increase intracellular levels of Ca^{2+}. This mechanism may be active both synaptically as well as extrasynaptically.

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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 ClC-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 Ca^{2+} (15). The developmental consequence of the Ca^{2+} influx during the GDPs has been proposed to provide a significant source of Ca^{2+} involved in activity dependent synaptogenesis and network formation. (15)   Although GDPs would theoretically allow for simultaneous activation of ClC-3, modulation of their activity in the ClC-3 knock-out animal has not been tested. In addition, the impact of ClC-3 expression on the amplitude and duration of the GDPs has not been shown. Accordingly, the contribution of ClC-3 activation to Ca^{2+} handling in the developing nervous system may be, in part, mediated via a potentiated, localized Ca^{2+} influx through NMDA receptors, providing a Hebbian-like modulation of developing synapses; enhancement of release via vesicle acidification, prolonged depolarization, and therefore, Ca^{2+} influx at postsynaptic sites.

Figure 3. Model for the activation of ClC3 as a function of NMDA receptor opening in the postsynaptic membrane. Numbers correspond to the sequence of events leading to ClC3 activation

Our recently published data (1) examining the role of ClC-3 in the modulation of synaptic efficacy in the hippocampus support a model in which Ca^{2+} entry across the postsynaptic membrane via NMDA-receptors promotes CaMKII –dependent phosphorylation and activation of ClC-3 (Fig. 3). In immature neurons, this results in an extended relief of Mg^{2+}-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 Ca^{2+} influx via the opening of voltage-gated Ca^{2+} channels. At late times in development, when ECl is hyperpolarized, the modulatory effect of ClC-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 ClC channels in the phenomenon has not previously been explored. ClC-3 is unique among its family members in that it is gated by Ca^{2+} dependent phosphorylation. Our recent published studies (1) demonstrate that ClC-3 channels colocalize with NMDA receptors at hippocampal synapses. Calcium flowing through NMDA receptors activates ClC-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 ClC-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, Ca^{2+}-dependent ClC-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 ClC-3 channels are ubiquitously expressed in the brain (16), the relationship between ClC-3 channels and NMDA receptors is not likely to be restricted to the hippocampus, however, this remains an open question.

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

Tingting Yang[1], Henry M. Colecraft, Ph.D.[2]

[1]Biomedical Engineering, Johns Hopkins University, Baltimore, MD, USA, [2]Physiology and Cellular Biophysics, Columbia University, New York, NY, USA.

RGK GTPases profoundly inhibit Ca_V1-2 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 Ca_V1-2 channels by either eliminating their association with the plasma membrane or reducing their binding to Ca_Vbeta 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. Constent with this, elevating intracellular Ca^{2+} did not redistribute Rem for the plasma membrae. Further, elevating intracellular Ca^{2+} did not ablate the Rem-CaVbeta interaction assessed by FRET. Finally, elevated intracellular Ca^{2+} did not reverse Rem inhibition of Ca_V channels. We conclude that Ca^{2+}-CaM does not dynamically regulate RGK protein inhibition of CaV channels.

Session 3 Speaker Materials

Ca_V1 and Ca_V2 channels and calmodulin

David T. Yue

Johns Hopkins University School of Medicine, Baltimore, MD

In the context of recombinant Ca^{2+} channels, Ca^{2+}/CaM-dependent feedback regulation at first appeared to represent a unique property of certain Ca_V1 channels. Members of the Ca_V2 clade of these channels seemed devoid of such behavior, and were in fact used as 'inert' partners for chimeric channel analysis of the Ca_V1 channel regulatory process. We now know this initial view to be inaccurate, as nearly all Ca_V1-2 channels exhibit CaM-mediated modulation, according to a common overall mechanism with rich and important variations. Such Ca^{2+} modulation figures crucially in the biological function of Ca^{2+} channels. More broadly, this class of CaM-mediated regulation may be a general model for Ca^{2+} feedback mechanisms that involve local Ca^{2+} sensors affixed to their own Ca^{2+} sources. This pairing of Ca^{2+} sensors and sources—a hallmark of CaM/Ca^{2+} channel regulation—is widespread in many other molecular complexes as well, given the privileged Ca^{2+} signaling afforded by this configuration. Hence, dissecting the mechanisms of CaM/Ca^{2+} channel regulation is a research goal of rather general importance.

The CaM/Ca^{2+} 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 Ca^{2+} regulation, such proximity puts CaM at risk of being 'blinded' by the intense Ca^{2+} entry through the local channel, thus challenging the sensing of far weaker Ca^{2+} 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 Ca^{2+} channels, such functional bipartition of CaM has risen to new heights. Firstly, though a single regulatory CaM persistently associates with channels, Ca^{2+} 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 Ca^{2+}, they somehow favor spatially distinct Ca^{2+} 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 Ca2+ 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 Ca2+ 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$_V$2.2 channels lack *NSCaTE*, and exhibit N-lobe mediated CDI with a global selectivity. Adding *NSCaTE* to Ca$_V$2.2 channels strongly converts their regulation towards a local profile. Residues within *NSCaTE* that are important for this conversion also turn out to be critical for Ca^{2+}/CaM binding. In fact, the extent of global-to-local conversion correlates tightly with the Ca^{2+}/CaM affinity of mutated *NSCaTE* modules. This uncovers a close linkage between the Ca^{2+}/CaM-binding affinity of *NSCaTE* and its ability to transform spatial Ca^{2+} selectivity. Of additional importance, Ca$_V$1.2 and 1.3 channels inherently possess *NSCaTE*, and thereby exhibit N-lobe CDI with local selectivity. Removal of *NSCaTE* converts N-lobe CDI of these channels to a clearly global profile. Thus, *NSCaTE* differentially tunes the spatial Ca^{2+} selectivity of N-lobe CDI across two channel clades (Ca$_V$1 versus Ca$_V$2). 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 Ca2+/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

Nikolai M. Soldatov

National Institute on Aging, NIH, Baltimore, Maryland, USA

The voltage-gated inward Ca²⁺ current (ICa) is a common mechanism of transient increase in the cytoplasmic free Ca^{2+} concentration that activates a variety of cellular responses. The rapid and complete inactivation of I_{Ca} is the critical step terminating Ca^{2+} influx and preventing Ca^{2+} overloading of the cell. In the case of $Ca_v1.2$ calcium channels, two different mechanisms are in control of the Ca2+ current inactivation. One mechanism is driven by Ca^{2+} ions on the cytoplasmic side of the membrane, whereas the other depends on transmembrane voltage. Replacement of Ca^{2+} for Ba^{2+}

as the charge carrier eliminates Ca^{2+}-dependent inactivation (CDI) so that Ba^{2+}-conducting calcium channels inactivate in a voltage-dependent manner by fast and slow mechanisms. Cavβ subunits (e.g., β_{1a} vs. β_{2a}) differentially modulate inactivation. CDI was previously co-identified with two adjacent calmodulin-binding regions (LA/IQ) in the middle part of the α_{1C} subunit C-terminal tail. Splice variation of the α1C transcript in this region (e.g., $\alpha_{1C,86}$) accelerates fast inactivation, removes slow inactivation, CDI and differential Cavβ 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 α_{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 α1C subunit C-terminal tail is subject to voltage-gated conformational rearrangements that delivers Ca^{2+} caged in calmodulin, to downstream signaling targets involved in CREB-dependent transcriptional activation. The plasma membrane-anchoring of the α1C C-tail via fusion of the plekstrin homology domain (PH) inhibits slow inac-

tivation, CDI and Ca^{2+} signal transduction. Release of the C-tail fully restores these functions. Thus, a specific functional folding of the α1C C-terminal tail vis-à-vis the pore is crucial for inactivation.

Annual Determinan 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 $\alpha_{1C,IS-IV}$ channel. Slow voltage-dependent inactivation of α1C,IS-IV is inhibited, and it does not show CDI and differential $Ca_v\beta$ subunit modulation.
N-tail. A very different role was found for the α1C N-terminal tail. It shows limited voltage-dependent mobility in relationship with the plasma membrane conferred by a $Ca_v\beta$ subunit in a manner that facilitates the channel response to voltage gating. Plasma-membrane-anchoring, or deletion, of the α1C subunit N terminus completely inhibited inactivation of the Ba^{2+} (or Ca^{2+}) current in the absence of $Ca_v\beta$.

PH_N-α_{1C,77}/α_2δ

Ca²⁺

Ba²⁺   200 pA
        200 ms
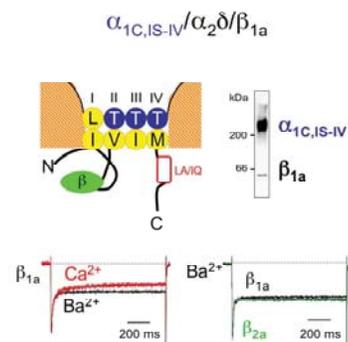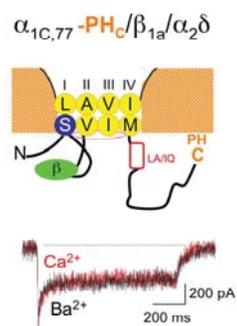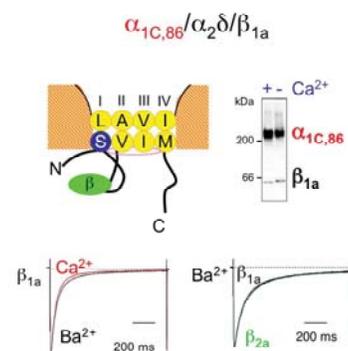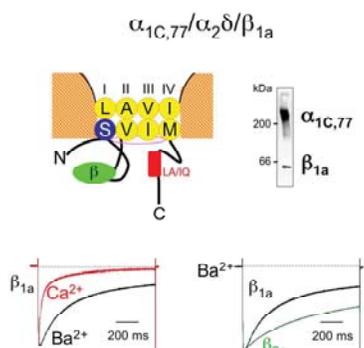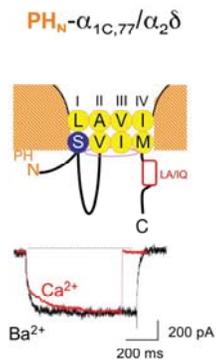
Auxiliary Ca$_\beta$ and α$_2$δ subunit are not crucial for CDI. Co-expression of α1C with calmodulin in the absence of either (but not both) of these subunits generates fully functional Ca²⁺ channels supporting CDI. In conclusion, CDI is mediated by determinants of the α1C C- and N-termini, the ADSI in the cytoplasmic pore region, as well as by the folding of the C-tail. Calmodulin integrates these determinants, providing a Ca²⁺-dependent switch that terminates slow inactivation, releases the α$_{1C}$ C-tail and shuttles the associated Ca²⁺/calmodulin acting as an activating stimulus of the Ca²⁺ signal transduction.

Coupling of TRPC4 and TRPC5 channels to rises in cytosolic Ca

Veit Flockerzi and Adolfo Cavalié

Experimentelle und Klinische Pharmakologie und Toxikologie, Universitaet des Saarlandes, 66421 Homburg, Germany

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 wit 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 Ca-calmodulin dependent channel modulation (1). In contrast to TRPC4, TRPC5 does not require GTPgammaS perfusion but is activated by a rise in cytosolic Ca.

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The excess calmodulin recovers activity of $Ca_v1.2$ calcium channel in the absence of $Ca_v\beta$ or $\alpha_2\delta$ subunits

Qi Zong Lao and Nikolai M. Soldatov

National Institute on Aging, NIH, Baltimore, MD 21224

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 α1C subunit with accessory $Ca_v\beta$ and $\alpha_2\delta$ subunits. A single calmodulin (CaM) molecule is tethered to the C-terminal α1C-LA/IQ region and mediates Ca2+-dependent inactivation (CDI) of the channel. $Ca_v\beta$ subunits are stably associated with the α_{1C}-AID site of the cytoplasmic linker between internal repeats I and II and also interact dynamically, in a Ca^{2+}-dependent manner, with the α_{1C}-IQ region. Much less is known about the structural aspects of the association between α1C and α2δ. We found that co-expression of exogenous CaM (CaM-ex) with α1C in COS1 cells in the absence of either $Ca_v\beta$ or $\alpha_2\delta$, but not both of these subunits, stimulates the plasma membrane targeting of α1C, facilitates calcium channel gating, and supports CDI. Neither real-time PCR with primers complementary to monkey $Ca_v\beta$ and $\alpha_2\delta$ subunits, nor co-immunoprecipitation analysis with exogenous α_{1C} revealed an induction of endogenous $Ca_v\beta$ and $\alpha_2\delta$ that could be linked to the effect of CaMex. Co-expression of a Ca^{2+}-insensitive CaM mutant CaM1234 also facilitated gating of both the $Ca_v\beta$- and $\alpha_2\delta$-free $Ca_v1.2$ channels, but did not support CDI. Our results show that there is a functional matchup between CaMex and $Ca_v\beta$ or α2δ that, in the absence of either of these subunits, renders Ca^{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^{2+}-dependent inactivation, and plasma-membrane targeting occur in the absence of structurally different $Ca_v\beta$ or $\alpha_2\delta$. We suggest that CaMex affects specific $Ca_v\beta/\alpha_2\delta$-free conformations of the channel that are not available to endogenous CaM.

L-channel N-tail: modulation by calmodulin

Adva Ben Mocha[1], Lior Almagor[2], Joel Hirsch[2], Nathan Dascal[1]

[1]Sackler School of Medicine and [2]George Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel. e-mail: dascaln@post.tau.ac.il

The cytoplasmic N-terminus (NT) is an important regulatory site in the α1C subunit of the $Ca_v1.2$ channel (L-type). The mammalian α_{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_{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 α1C is an inhibitory module which specifically reduces the channel's maximal open probability, $P_{o,max}$ (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 Po,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 α_{1C} 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 Po,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 α_{1C}, 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 Ca^{2+} - dependent manner (9). The removal of most of NT in the $\alpha_{1C\Delta2-139}$ mutant rendered the voltage-dependent inactivation (VDI) of the channel faster, whereas the acceleration of the inactivation by Ca^{2+} 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 α_{1C} 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 α_{1C} 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 Ca^{2+}, which strengthens the inhibitory control exerted by the scaffold in a CaM-dependent manner. We envisaged that "influx of Ca^{2+} via the open channel is followed by Ca^{2+} 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 Ca^{2+} transforming element". They showed that the presence of *NSCaTE* renders the channel sensitive to local Ca^{2+} (the Ca 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 α_{1C} ($Ca_v1.2$) 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 $Ca_v1.3$ 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 α_{1C}. 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 Ca^{2+}-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 Ca^{2+}, but not in the absence of Ca^{2+}, CaM interacted with NT_{60-120}. 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 NT_{60-120} of three amino acids identified by Dick et al. (20) as crucial for the functional role of *NSCaTE* fully abolished the binding between NT_{60-120} and CaM. We have next examined the possibility that the N-terminal CaM binding site (NT_{60-120}) 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 $CT_{1505-1671}$, 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^{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 Ca²⁺ channels by apocalmodulin

Martin Biel

Munich Center for Integrated Protein Science CIPSM and Department of Pharmacy – Center for Drug Research, Ludwig-Maximilians-Universität München, Butenandtstr. 5–13, 81377 Germany.

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 Ca²⁺- dependent inactivation (ICDI), that prevents Ca²⁺-dependent inactivation (CDI). The ICDI is crucially required to confer the permanent Ca²⁺ 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 Ca²⁺-binding.

Mechanism of Ca²⁺/calmodulin regulation of TRP channels

Sharona E. Gordon, PhD, William N. Zagotta, PhD.

Physiology and Biophysics, University of Washington, Seattle, WA, USA.

For Ca²⁺-permeable TRP channels, desensitization mediated by permeating Ca²⁺ ions is a critical mechanism for maintaining Ca²⁺ homeostasis. The two main candidates for transducing the effect of Ca²⁺, PI(4,5)P2 (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)P2 to the C-terminal domain may involve nonspecific electrostatic interactions between the anionic PI(4,5)P2 and clusters of basic amino acids. For many proteins that bind to anionic lipids via such basic clusters, including MARCKS, GAP43, EGFR, and ErbB, Ca²⁺/CaM can disrupt the protein-lipid interaction. In MARCKS and the EGFR, the effect of Ca²⁺/CaM has been shown to result from its binding to the same site as the anionic lipids. The Ca²⁺/CaM complex has a net charge of -16 at neutral pH, making it an effective competitor. The opposing effects of PI(4,5)P2 and Ca²⁺/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 Ca²⁺-dependent desensitization via a PLC-induced reduction in PI(4,5)P2 levels and via CaM are functionally and structurally coupled.. At rest, the positively charged C-terminal domain would binds acidic PI(4,5)P2 in the plasma membrane, a conformation permissive of activation. Activation then allows Ca²⁺ to enter the cells, and Ca²⁺ produces desensitization by releasing the C-terminal domain from the plasma membrane. The release occurs either when Ca²⁺ activates PLC and PLC degrades PI(4,5)P2 or when Ca²⁺ binds CaM and Ca²⁺/CaM displaces PI(4,5)P2. TRP channel desensitization could thus occur through either or both mechanisms, depending on the amplitude and spatial distribution of the Ca2+ increase, the abundance/activity of PI(4,5)P2, PLC and CaM in proximity to the channels, and their relative sensitivity to Ca²⁺.

Calmodulin mediates the Ca²⁺-dependent regulation of gap junctions

Yubin Zhou,* Yanyi Chen *, Wei Yang, * Hing Wong*, Monica M. Lurtz,† *Jie Jiang, * Yun Huang, Charles F. Louis,† and Jenny J. Yang*

*Department of Chemistry, Georgia State University, Atlanta, GA 30303 USA; and the †Department of Cell Biology and Neuroscience, University of California, 900 University Avenue, Riverside, CA 92521, USA

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 Ca²⁺ concentration ($[Ca^{2+}]_i$) (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 $[Ca^{2+}]_i$. Specifically, cell-to-cell transfer of the fluorescent dye AlexaFluor594 was half-maximally inhibited at ~300 nM $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ increased internal electrical resistance in the lens, which was prevented by preincubation with CaM antagonists (13). Indeed, this action of Ca²⁺ 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 Ca²⁺ -dependent inhibition appears to be CaM-mediated (12). The rapid onset of this Ca²⁺-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 Ca²⁺-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 (Kd = 27 nM) in the N-terminus and the other site (K_d = 1.2 μM) in the C-terminal region have been identified in Cx32 (11), whereas a single CaM binding site (K: 11-72 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 Ca²⁺. Far UV circular dichroism studies indicated that, like other CaM binding peptides, the Cx-derived peptides increased their α-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 μM (for Cx43) and 30-600 nM (for Cx44). On binding of the peptides to CaM, the apparent K_d of Ca²⁺ for CaM decreased, and the Hill Coefficient nHill 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 Ca²⁺-binding affinities of the C-domain of calmodulin by slowing the rate of Ca²⁺ release from the complex. We further demonstrated that the Ca²⁺-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 $[Ca^{2+}]_i$ 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 Ca²⁺-dependent manner. Our data suggest a common mechanism by which the Ca²⁺- 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 Ca²⁺-calmodulin.

Figure 1 Membrane topology of Cxs and the predicted calmodulin binding regions.

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Session 4 Speaker Materials

Limiting calmodulin revealed by image and fluorescence correlation spectroscopy

M. Neal Waxham

U.T. Med. School at Houston, Neurobiology

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^{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^{2+}-concentrations and transmit them to downstream targets.

Ca^{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^{2+} flux is through the small ubiquitous protein calmodulin, which cooperatively binds Ca^{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^{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^{2+} saturated form.

One of the major targets in cells for Ca^{2+}/calmodulin is Ca^{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^{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 µm2/s) while eGFP diffuses at ~ 20 µm2/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^{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

Donald M. Bers, PhD.

Pharmacology, University of California, Davis, Davis, CA, USA.

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, I_{to}, 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

Marcel A. Kamp, Dr. med.[1,2], Margit Henry[2], Kayalvizhi Radhakrishnan[2,3], Marco Weiergräber, Dr. med., Dr. nat. med.[2,3], Toni Schneider, Prof. Dr.[2,3]

[1]Department of Neurosurgery, University Hospital of Heinrich-Heine University, Duesseldorf, D-40225 Duesseldorf, Germany, [2]Institute for Neurophysiology, University of Cologne,, D-50931 Cologne, Germany, [3]Center for Molecular Medicine Cologne (CMMC), D-50931 Cologne, Germany.

$Ca_v2.3$ containing (E-/R-type) voltage-activated Ca^{2+} 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 Ca^{2+} ions was detected for the classical Ca^{2+}-dependent inactivation through calmodulin (Liang et al., 2003), and for a cytosolic II-III linker mediated modulation of Ca^{2+} 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 $Ca_v2.3$. 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 $Ca_v2.3$ 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 an 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.

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Session 5 Speaker Materials

Calmodulin regulation of ryanodine receptors

Gerhard Meissner and Naohiro Yamaguchi

University of North Carolina, Chapel Hill, NC 27599-7260

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 Ca2+ 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 (Mg^{2+}, 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 Ca^{2+} spark frequency and size, whereas a Ca^{2+}-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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} release.

Conclusion

In summary, CaM regulates the RyRs by a direct interaction. Both the Ca^{2+}-free and Ca^{2+} 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 Ca^{2+} release by Ca^{2+} evidently requires that RyR2 is regulated by CaM. A direct interaction with the L-type Ca^{2+} channel tightly controls RyR1 activity, which may minimize the effects of CaM in skeletal muscle.

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The mysterious lives of calcium channels

Ricardo Dolmetsch

Assistant Professor, Dept. of Neurobiology, Stanford University, 299 Campus Drive D227 Stanford CA 94305

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.

Calmodulin/L-type calcium channel-mediated activation of CREB-dependent transcription in nuclear microdomains

Evgeny Kobrinsky and Nikolai M. Soldatov

National Institute on Aging, NIH, Baltimore, Maryland, USA

Voltage-gated $Ca_v1.2$ calcium channels couple membrane depolarization to intracellular Ca^{2+} signaling that is crucial for many processes including transcriptional activation. The channels respond to membrane depolarization by a transient increase in Ca^{2+} conductance that causes brief increase in intracellular free Ca^{2+} concentration, and is terminated by a negative feed-back mechanism called Ca^{2+}-induced inactivation (CDI). A key element mediating CDI is calmodulin (CaM) tethered to the C-tail of the pore-forming α1C subunit of the channel. Using FRET microscopy combined with patch clamp we have found that activation-inactivation cycles of the channel are accompanied by reversible rearrangements of the α1C C- terminal tail. Anchoring of C-tails to the plasma membrane blocked these rearrangements and inhibited activation of CREB-dependent transcription in spite of large inward Ca^{2+} current due to inhibited CDI. Release of the tails restored transcriptional activation. Substitution of CaM for Ca^{2+}-insensitive mu-

tant CaM_{1234} OR mutation of CaM binding site in the α1C C-terminal-tail both terminated transcriptional signal transduction, but did not inhibit Ca^{2+} 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^{2+}-induced inactivation mechanism that terminates channel opening plays a crucial role in transcriptional activation. Ca^{2+} 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.
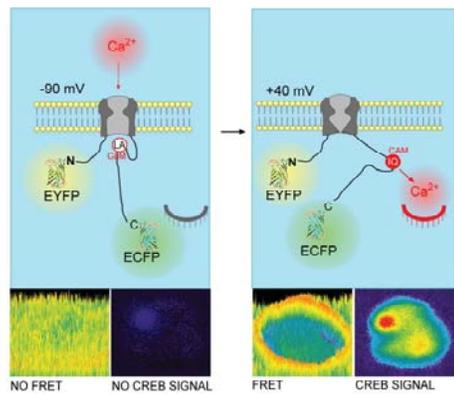
Multifaceted regulation of $Ca_v2.1$ Ca2+ channels by Ca^{2+} binding proteins

Lisa Kreiner[1], Beat Schwaller[2] and Amy Lee[1]
[1]Dept. of Pharmacology and Center for Neurodegenerative Disease, Emory University, Atlanta, GA, USA and [2]Institute of Histology and General Embryology, University of Fribourg, Fribourg, Switzerland

Voltage-gated $Ca_v2.1$ channels conduct P/Q-type Ca^{2+} currents that regulate neurotransmitter release and neuronal excitability. These channels undergo a dual feedback regulation by incoming Ca^{2+} ions, Ca^{2+}-dependent facilitation and inactivation, which is mediated by calmodulin binding to the Cav2.1 α1-subunit. We showed previously that the Ca^{2+} binding proteins, parvalbumin (PV) and calbindin (CB), may buffer global Ca2+ levels that support inactivation, but not local Ca^{2+} 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 $Ca_v2.1$. Patch-clamp recordings of dissociated Purkinje neurons from wild-type (WT) mice and those lacking PV and CB ($PV/CB^{-/-}$) revealed a broader role of these Ca^{2+} binding proteins in regulating $Ca_v2.1$. First, inactivation of Ca^{2+} currents, but also Ba2+ currents, was significantly greater in $PV/CB^{-/-}$ neurons than in WT neurons. Second, Ca^{2+}-dependent facilitation was significantly less in PV/CB-/- than in WT neurons during trains of action potential waveforms. Of the multiple Cavβ subunits detected in Purkinje neurons, $Ca_v\beta_{2a}$ confers Cav2.1 channels with the least inactivation and greatest Ca2+-dependent facilitation in transfected HEK293T cells. Therefore, downregulation of $Ca_v\beta_{2a}$ could account for increased inactivation and decreased Ca2+-dependent facilitation of $Ca_v2.1$ in PV/CB-/- neurons. Consistent with this possibility, $Ca_v\beta_{2a}$ was detected in a significantly smaller fraction of PV/CB-/- neurons than WT neurons by single-cell RT-PCR. Due to the decreased Ca^{2+} buffering capacity of $PV/CB^{-/-}$ neurons, downregulation of $Ca_v\beta^{2a}$ may serve as a homeostatic response to restrict activity-dependent Ca2+ influx through $Ca_v2.1$ channels. Our findings highlight the significance of $Ca_v\beta_{2a}$ as a key determinant for Ca^{2+}-dependent facilitation and inactivation of $Ca_v2.1$ in neurons and suggest a mechanism that may contribute to abnormal Purkinje cell rhythmicity and ataxia shown previously in $PV/CB^{-/-}$ mice.

Structural studies of CaM interaction with cardiac L-type voltage-gated calcium channels and ryanodine receptors

Sarah L. Bent[1], Conor P. Walsh[1], Lu-yun Lian[2], Annette C. Dolphin[3] and Ashraf Kitmitto[1]*

[1]Cardiovascular Medicine, School of Clinical & Laboratory Sciences, Faculty of Medical & Human Health, University of Manchester, M13 9NT, UK
[2] The University of Liverpool, School of Biological Sciences, Liverpool L69 7ZB UK
[3] Department of Pharmacology, University College London, London WC1E 6BT, UK

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 recticulum (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 intracelluar $[Ca^{2+}]_i$. 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 $[Ca^{2+}]i$ is rapidly removed through the concerted action of the Na^+/Ca^{2+} exchanger (NCX1), the plasma membrane Ca^{2+} pump (PMCA) and the SR Ca^{2+} 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 $[Ca^{2+}]_i$. 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.