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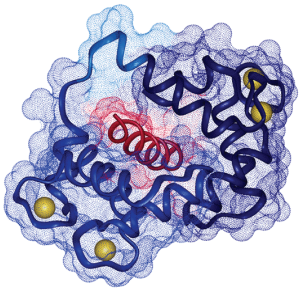
Abstract Book



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SARCOPLASMIC RETICULUM CALCIUM BINDING PROTEINS IN HEALTH AND DISEASE

David H MacLennan

I will describe the background of my entry into the Ca^{2+} binding protein field in 1969 and then discuss contributions from our laboratory aimed at understanding the structure, function, regulation, mechanism of action and involvement in genetic disease of several SR proteins. I will stress how we never associated our work with a specific discipline, but repeatedly learned and utilized new technologies to remain on the advancing edge of the field over many decades. Finally, I will describe recent experiments on animal models of Central Core Disease and Dilated Cardiomyopathy that uncover new ways to think about floppy infant syndrome and terminal events in cardiomyopathy.

THE MOLECULAR MECHANISM OF INTRINSICALLY DISORDERED REGULATORS OF CAM SIGNALING

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Background: Cell signaling pathways must be regulated at multiple levels to control the magnitude and temporal characteristics cellular responses. Calmodulin (CaM) is activated by upstream release of Ca^{2+}, but much less is known about other mechanisms to modulate CaM activity. PEP-19 is small protein with the potential to function as a dedicated Regulator of CaM Signaling, or RCS protein. It is small, with no known activity other than binding to CaM in the presence or absence of Ca^{2+} via an IQ binding motif. Consistent with a role as an RCS protein, gene knockout, and other studies demonstrate that PEP-19 has profound effects on a broad spectrum of cellular processes.

We showed previously that: 1) PEP-19 increases the Ca^{2+} k_{on} and k_{off} rates at the C-domain of CaM 40 to 50 fold without greatly affecting the K_{Ca}; 2) PEP-19 has an acidic sequence located N-terminal to its IQ CaM binding motif is required to modulate Ca^{2+} binding to CaM; and 3) The acidic sequence in PEP-19 was required to sensitize HeLa cells to ATP-induced Ca^{2+} release. Thus synergy between the acidic sequence and IQ motif is necessary to support at least some of the biological activities of PEP-19.

Goal: The goal of the current study was to resolve the NMR solution structure of the PEP-19/apo C-CaM complex, and to determine the structural consequence of mutations in PEP-19 that inhibit its ability to modulate Ca^{2+} binding to CaM. We focused on apo CaM since increasing the Ca^{2+} k_{on} of the C-domain has the potential to increase the rate of association of CaM with other Ca^{2+}-dependent target proteins.

Results: PEP-19 and apo C-CaM form a fuzzy complex in which residues 1-29 in PEP-19 remain disordered and are thus accessible for potential interactions with other proteins. Association with PEP-19 induces apo C-CaM to adopt a semi-open conformation, with helices E and F of Ca^{2+} binding site III showing the greatest change in helical angle relative to free apo C-CaM. However, the semi-open conformation does not account for the effect of PEP-19 on Ca2+ binding to CaM since the IQ peptide from Nav1.5 also causes the C-domain of CaM to adopt semi-open conformation, but does not greatly affect its Ca^{2+} binding properties.

PEP-19 adopts a well-defined alpha helix that extends from Pro37 to Ala58, and binds to a hydrophobic groove in apo C-CaM. This helix includes the entire IQ motif and C-terminal portion of the acidic sequence. In contrast, the N-terminal portion of the acidic sequence (aa 28-36) forms a loop and coil structure that is stabilized by interactions between Ile32 and Met34 in PEP-19 and hydrophobic residues in helices E and F of Ca^{2+} binding site III in C-CaM. The acidic side chains of Asp 31, Asp33, Asp35, Glu38 and Glu40 in PEP-19 extend toward the solvent. Together with residues in helix E of CaM, these acidic side chains in PEP-19 form a negatively charged channel on the surface of the complex that leads to Ca^{2+} binding loop III.

Conversion of Pro37 to Gly greatly decreased the ability of PEP-19 to modulate Ca^{2+} binding to CaM. Backbone amide relaxation and paramagnetic relaxation enhancement show that this mutation increases backbone dynamics in the acidic loop and causes it move away from Ca^{2+} binding loop III. Thus, Pro37 terminates the helical segment of PEP-19 bound to apo-CaM and properly aligns the acidic loop with respect to Ca^{2+} binding site III.

Conclusions: The data support a model in which the IQ motif of PEP-19 facilitates binding to the C-domain of apo CaM. The tethered acidic loop of PEP-19 then associates with helix E and F of CaM to greatly increase the negative surface charge density near Ca^{2+} binding site III relative to free CaM. In contrast, the surface near Ca^{2+} binding loop IV in free CaM is negatively charged and is not greatly changed upon binding PEP-19. This suggests that PEP-19 increases the rate of Ca^{2+} association with the C-domain of CaM by formation of a negatively charged channel that electrostatically steers Ca^{2+} to EF-hand III.

ONE CaM TO BIND THEM: CALMODULIN DISCRIMINATES AMONG NINE HUMAN VOLTAGE-GATED SODIUM CHANNELS

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The human voltage-dependent sodium channel family (Na_V) is responsible for the generation and propagation of the rising phase of action potentials. Na_V channelopathies include epilepsy, "Long QT" syndrome, familial autism and pain insensitivity. Several Na_V channels are known to undergo calcium-dependent regulation mediated by calmodulin (CaM), an essential intracellular calcium sensor. However, the mechanisms of calcium-induced regulation of channel inactivation remain poorly understood, and may vary among Na_V subtypes. Prior structural and thermodynamic studies of the neuronal voltage-gated sodium channel ($Na_V1.2$) demonstrated that the IQ motif [IQRAYRRYLLK] in $Na_V1.2$ binds apo (calcium-free) CaM with high affinity primarily through contacts with the semi-open C-domain of CaM. Calcium binding to CaM slightly reduces affinity for the IQ motif by a factor of 3 (Fig. 1). Despite this small difference, calcium binding is believed to trigger domains of CaM to move to other sites in the channel, possibly including the inactivation gate (DIII-DIV linker) and/or 4-helix bundle upstream of Na_V IQ motifs.

To determine why the $Na_V1.2$ IQ motif binds more favorably to apo CaM than calcium-saturated CaM, and understand contributions of conserved $Na_V1.2$ residues to free energies of interaction, we monitored CaM-induced disruption of FRET in YFP-IQ-CFP biosensors (Fig. 1). All of the tested substitutions affected binding of apo CaM much more severely than calcium-saturated CaM, suggesting the two CaM-IQ interfaces were very different. To understand the molecular mechanism of calcium-triggered conformational change, we determined a solution NMR structure (2M5E.pdb) of $(Ca^{2+})_2$-CaM_C bound to the $Na_V1.2$ IQ motif. It showed the $Na_V1.2$ IQ peptide reversed its orientation relative to the corresponding apo CaM_C–IQ complex (2KXW.pdb; Fig. 1). Calcium had triggered release, with subsequent re-association and reversal.

This pair of solution structures (2KXW and 2M5E) are the first to show apo and calcium-saturated CaM_C bound to the same IQ motif and demonstrate that "the" IQ motif contains nested, anti-parallel sites within one sequence. They offer new insights into the complexity of CaM regulation of $Na_V1.2$ inactivation. However, comparison of CaM binding to the IQ motifs of 9 human sodium channels ($Na_V1.1$ to 1.9; Fig. 2) showed large differences in their calcium-dependent binding of CaM, ranging from ~0.8 kcal/mol preference for apo CaM (NaV1.1) to ~1.5 kcal/mol preference for $(Ca^{2+})_2$-CaM. This raises questions about the generality of CaM-IQ mediated regulation among these channels

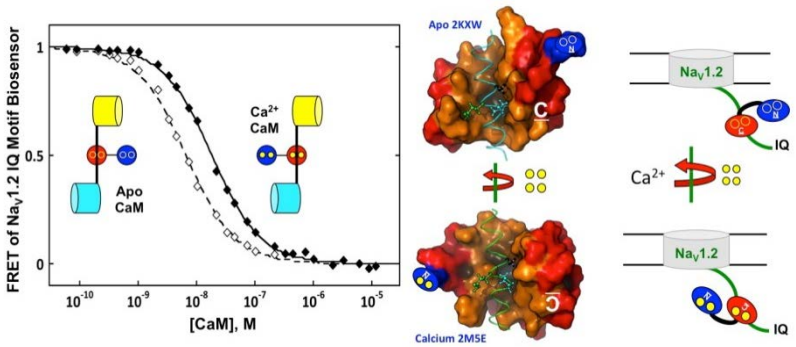

Fig. 1: CaM binding to the IQ Motif of $Na_V1.2$. Biosensors show apo CaM binds more favorably than $(Ca^{2+})_2$-CaM; 2KXW vs. 2M5E; rotation on IQ

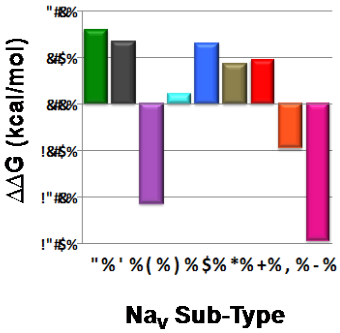

Fig. 2: ΔΔG apo vs. $(Ca^{2+})_2$-CaM $Na_V1.1$, 2, 4, 5, 6, 7 favor apo CaM

CONSERVATION OF CALMODULIN REGULATION ACROSS VOLTAGE GATED CALCIUM AND SODIUM CHANNELS

Manu Ben-Johny

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Voltage-gated calcium and sodium channels comprise two major classes of ion channels that support distinct and vital biological functions: sodium channels sustain brisk spatial propagation of action potentials, while calcium channels link excitation to contraction, secretion and transcription. Accordingly, these channel superfamilies have long been studied as distinct entities. Yet, the carboxyl tails of these channels exhibit remarkable homology, hinting at a purposeful module long-shared amongst these molecules. If this region were to support functions of like correspondence, deep mechanistic insights could be gleaned from joint investigation of these channels, and shared principles obtained for approaching related channelopathic diseases. For numerous voltage-gated calcium channels, dynamic interactions between their carboxyl tail domains and calmodulin (CaM) elaborate robust and recognizably similar forms of feedback regulation. For sodium channels, however, comparatively subtle and variable CaM-dependent effects with conflicting purported mechanisms have been reported, dimming prospects for unified understanding. Here, using Ca^{2+}-photouncaging and through single-channel recordings we find that these dissimilarities in sodium channels are only apparent, and that CaM regulatory function and mechanism are fundamentally conserved. As observed with calcium channels, apocalmodulin binding itself markedly enhances the baseline peak open probability of sodium channels. Furthermore, Ca^{2+} binding to CaM relieves this initial enhancement in peak channel open probability resulting in Ca^{2+}-dependent inhibition of sodium current. These results then help substantiate the persistence of an ancient Ca^{2+}/CaM regulatory design across channel superfamilies.

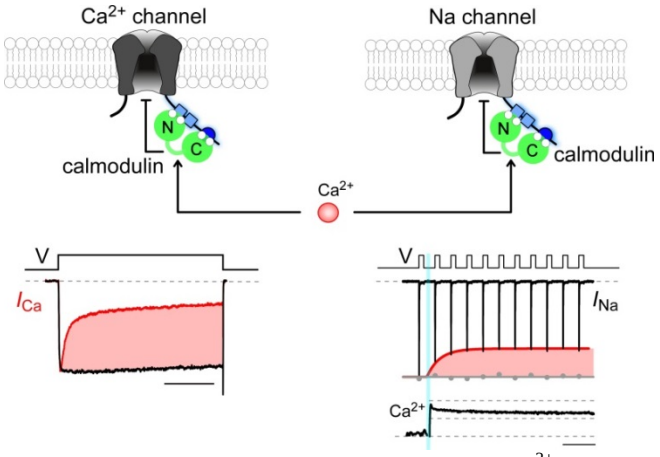

Figure 1. (*Top*) Schemetic shows conserved regulation of both voltage-gated Ca^{2+} and Na channels by Ca^{2+}/calmodulin. (*Bottom left*) Ca^{2+}-dependent inhibition (red shaded area) of Ca^{2+} channels is seen as decrement in Ca^{2+} current (red) compared to Ba^{2+} (black). (*Bottom right*) Rapid Ca^{2+}-photouncaging results in a decrement of peak Na currents (red envelope). In the absence of Ca^{2+}, peak Na currents remain stable (gray dots).

STRUCTURAL BASIS FOR THE LONG RANGE ALLOSTERIC REGULATION AND HIGH ION CONDUCTANCE OF THE RYANODINE RECEPTOR RYR1

Nieng Yan

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Ryanodine receptors (RyRs) mediate rapid release of calcium ions from sarcoplasmic/endoplasmic reticulum and play a pivotal role in the excitation-contraction coupling of skeletal and cardiac muscles. RyRs represent the largest known ion channels and are homotetramers, with each subunit comprising approximately five thousand residues. We determined the structure of the full-length rabbit RyR1 in complex with the modulator FKBP12 at an overall resolution of 3.8 Å, using single particle electron cryomicroscopy. Near-atomic resolution is achieved at the channel domain and its adjoining domains in the cytoplasmic region and resolves 70 percent of the 2.2 million Dalton molecular mass of RyR1. The carboxyl terminal fragment (residues 4545-5037) exhibits the voltage-gated ion channel (VGIC) superfamily fold. An outer site formed by the side chains of Asp4899/Glu4900 and an inner site surrounded by carbonyl oxygen atoms may serve as the Ca^{2+} binding sites along the selectivity filter (SF) vestibule. A highly electronegative hairpin loop connecting S5 and the pore helix is positioned above the entrance to the SF vestibule. The four elongated S6 segments form a right-handed helical bundle that closes the ion-conducting pore at the cytoplasmic border of the membrane. The cytoplasmic vestibule of the pore is enriched with Glu and Asp residues, which likely modulate ion conductance. The S6 segment is followed by a carboxyl-terminal domain (CTD) which contains a previously uncharacterized zinc finger motif. The sequences between S2 and S3 segments fold into a cytoplasmic domain which we name the VSC domain. In addition to the channel domain, structural models were built for three previously uncharacterized major domains: the Central domain, the Handle domain, and the Helical domain. These three domains, all displaying the armadillo repeat fold, together with the amino-terminal domain, constitute a network of superhelical scaffold at the cytoplasmic side. Allosteric regulation of the pore is mediated by the Central domains, which directly interact with the CTD and VSC domains. These structural features explain high ion conductance by RyRs and allosteric regulation of pore conductance through conformational changes in the cytoplasmic domains.

STRUCTURAL INSIGHTS INTO RYANODINE RECEPTOR GATING

Oliver B. Clarke

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Structures of the type 1 ryanodine receptor (RyR1) have revealed the architecture of the closed channel, but the structural basis for RyR1 activation has not previously been described. Here we present cryo-EM reconstructions of RyR1 in multiple defined functional states, including activated states in which the transmembrane pore is dilated.

STRUCTURE AND DYNAMICS OF RYANODINE RECEPTOR

Rouslan Efremov

Ryanodine receptors are the major ion channels responsible for calcium release from endoplasmic or sarcoplasmic reticulum and their function has been extensively characterized in the context of calcium signalling in muscle contraction. RyRs are the 2.2 MDa homotetrameric ion channels that are primarily gated by changes in concentration of calcium ions in the cytoplasm and are regulated by multiple factors including ions, small organic molecules, and interactions with other proteins. Hundreds of mutations in RyRs have been associated with human diseases. The molecular mechanism underlying the complex regulation of RyR has been poorly understood. I will present the architecture of rabbit RyR1 reconstituted in lipid nanodiscs determined at resolution of 6.1 Å by single particle cryo-EM and will show how changes in calcium concentration induce conformational changes in RyR resulting in the channel gating.

MECHANO-CHEMO TRANSDUCTION: X-ROS-DEPENDENT CA^{2+} SIGNALING IN HEART

Jonathan Lederer

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Diastolic filling of the heart is associated with cellular stretching. Recent work has revealed that this physiological cellular stretching underlies the activation of NADPH oxidase type 2 (NOX-2) and the generation of reactive oxygen species (ROS). ROS acts locally to enhance the release of Ca^{2+} from the calcium release units (CRU) of the heart composed of clusters of ryanodine receptors type 2 (RyR2) in the junctional sarcoplasmic reticulum (jSR) and increases the diastolic Ca^{2+} spark rate [1-5]. This process is called "X-ROS signaling" and also enhances the systolic $[Ca^{2+}]_i$ transients under physiological conditions.

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IMAGING AND LOCALIZING IP3-EVOKED CALCIUM SIGNALS AND IP3 RECEPTORS AT THE SINGLE-CHANNEL LEVEL

Ian Parker

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Calcium serves as a universal intracellular messenger, controlling cellular processes as diverse as gene transcription, secretion, and electrical excitability. This versatility arises through the mechanisms by which Ca^{2+} signals are generated and transmitted to act over very different time and distance scales, ranging from waves with periods of minutes to transient domains at nanometer and millisecond scales. We focus on Ca^{2+} signals generated by clusters of inositol trisphosphate (IP_3) receptor/channels (IP_3Rs) that release Ca^{2+} from the endoplasmic reticulum into the cytosol. Channel opening is regulated by Ca^{2+} itself, creating positive and negative feedback loops that result in a hierarchy of signals ranging from openings of single channels ('blips') and concerted openings of channels in a cluster ('puffs') to waves that sweep throughout a cell. The spatiotemporal patterns of cellular Ca^{2+} signals thus depend on the properties of the IP_3Rs, their spatial arrangement in the cell, and their interactions via Ca^{2+} diffusion and possible other mechanisms.

Improvements in fluorescence imaging technology and image analysis now enable recording of Ca^{2+} transients in intact mammalian cells with single-channel resolution (optical 'patch-clamping'). I will describe a spinning laser spot microscope for enhanced 'shadowless' TIRF imaging of fast, dynamic signals, together with novel software for automated detection, localization and analysis of local Ca^{2+} transients. Utilizing these approaches we can dissect the contributions of individual IP_3R channels during puffs, count the numbers of functional channels in a cluster, elucidate mechanisms of puff initiation and termination, and localize puff sites with nanometer precision.

Complementing these studies of IP_3R function and localization via Ca^{2+} imaging, we are further employing single-molecule imaging techniques (STORM and PALM) to localize IP_3R proteins by superresolved imaging of immunostained native receptors, and by tracking of individual IP_3R1 molecules tagged with a photoactivatable fluorescent protein in a knock-in mouse model. Mapping the locations of functional puff sites determined in the same cells reveals a correlation with clusters of immotile IP_3Rs. However, a paradox remains, in that IP_3R proteins appear to be more widely distributed and expressed at much higher numbers than the functional receptors that we observe to generate Ca^{2+} signals.

GENETICALLY-ENCODED TOOLS TO OPTICALLY CONTROL AND IMAGE CA^{2+} DYNAMICS

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In living organism, Ca^{2+} is one of the most versatile second messenger to control biological processes such as muscle contraction, hormonal secretion and apoptosis induction. Its spatial and temporal dynamics has key roles to regulate these physiological phenomena. To reveal such dynamics, variety of Ca^{2+} indicators had been developed. They enabled noninvasive visualization of Ca^{2+} dynamics, provided meaningful information for research in wide range of biological field. However, for deeper understanding of relationship between the spatiotemporal Ca^{2+} dynamics and the following response, development of tools to manipulate intracellular Ca^{2+} level have been desired. In current methods, Ca^{2+} concentration can be controlled by light through Ca^{2+} binding compounds with photocleavable moieties. However, they require irradiation of toxic ultraviolet wavelength light and/or cell loading associated with disruption of the cell membrane. These properties which have possibility to impair cells become big problem especially in the case of in vivo measurement. In addition to this, Ca^{2+} release from such compounds is irreversible. To overcome this, we developed a genetically-encoded photoactivatable Ca^{2+} releaser called PACR (PhotoActivatable Ca^{2+} Releaser). That is composed of Ca^{2+} binding protein and light-sensitive protein. Affinity of PACR for Ca^{2+} was decreased during irradiation of blue light. Thus reversible and repeatable increasing of Ca^{2+} concentration in cell is possible without damage to living specimens. By using PACR, we succeeded nucleus specific temporal Ca^{2+} increase in HeLa cells and excitation of specific neuron in freely moving C. elegans by blue light irradiation. This useful tool is expected to contribute on researches to reveal the role of Ca^{2+} dynamics in complex biological phenomena. In addition to this manipulation tool, I would like to introduce color variants of super-duper luminescent protein that we developed recently, which can be used compatibly with optogenetic actuators.

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STRUCTURAL STUDIES OF RCK-REGULATED CA^{2+} GATING IN TETRAMERIC CATION CHANNELS

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The majority of prokaryotic ligand-gated K^+ channels, as well as eukaryotic Slo channel families (Slo1 or BK, Slo2 and Slo3), contain one or two copies of a conserved C-terminal intracellular ligand-binding RCK domains. RCK domains are also ubiquitously distributed in the bacterial K^+ uptake (Trk or Ktr systems) and efflux machinery (Kef systems) , highlighting their importance in regulating K^+ transport across the cell membrane. In both RCK-regulated K^+ channels and transporters, the basic building blocks of the ligand-gating apparatus are RCK dimers, four of which associate into functional octameric gating rings. Most prokaryotic RCK-containing K^+ channels possess only one covalently-linked RCK domain, which co-assembles with a cytosolic counterpart that is simultaneously expressed via an alternative internal translation start site on the same gene into the functional gating ring. In contrast, Slo K^+ channel families and a subset of prokaryotic K^+ channels contain two tandem RCK domains on each subunit and, therefore, the gating ring assembly no longer requires co-expression of a cytosolic RCK domain. The gating rings of RCK-containing K^+ channels mediate diverse allosteric ligand regulation and one of the major ligands is Ca^{2+}.

A major project in the lab is designed to reveal the structural basis of Ca^{2+} gating in both prokaryotic and eukaryotic RCK-regulated K^+ channels. Our earlier efforts to address the fundamental question of how Ca^{2+} binding induces gating ring conformational changes were centered on the single-RCK containing MthK channel, a Ca^{2+}-gated K^+ channel from *Methanobacterium thermoautotrophicum* and the human BK channel (hSlo1). Recently, we have also identified a novel plant nuclear membrane channel DMI1 (also named Castor or Pollux) that plays an essential role in the symbiosis between legumes and bacteria (*Rhizobium*) or fungi (*Arbuscular mycorrhizal*) for nitrogen fixation. We have structurally demonstrated that the DMI1 channel functions as a tetramer and its C-terminal ligand-binding domain of each subunit contains two tandem RCK domains, just like other RCK-regulated K^+ channels. Interestingly, this channel is unlikely to be K^+ selective. Our structural studies also reveal multiple Ca^{2+} binding sites on each subunit, suggesting that the channel is also regulated by Ca^{2+}. We are now in the process of performing electrophysiological analysis of the channel to define the selectivity and gating properties of this dual RCK-containing, ligand gated channel.

STRUCTURE AND FUNCTION OF CA^{2+} ACTIVATED TMEM16 CHANNELS AND SCRAMBLASES

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The TMEM16/Anoctamin family of proteins features a remarkable functional diversity. It contains the long sought-after Ca^{2+}-activated chloride channels but also lipid scramblases or cation channels. We have determined the crystal structure of nhTMEM16, a fungal family member that operates as a Ca^{2+}-activated lipid scramblase [1]. Each subunit of the homodimeric protein contains ten transmembrane helices and a hydrophilic membrane-traversing cavity that is exposed to the lipid bilayer as a potential site of catalysis. This cavity harbors a conserved Ca^{2+}-binding site located within the hydrophobic core of the membrane. Mutations of residues involved in Ca^{2+} coordination affect both, lipid scrambling in nhTMEM16 and ion conduction in the Cl^--channel TMEM16A. The nhTMEM16 structure reveals the general architecture of the family and its mode of Ca^{2+}-activation. It also provides insight into potential scrambling mechanisms and serves as a framework to unravel the conduction of ions in certain TMEM16 proteins.

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STRUCTURE AND INSIGHTS INTO THE FUNCTION OF THE BESTROPHIN CALCIUM-ACTIVATED CHLORIDE CHANNEL

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Bestrophin-1 (BEST1) calcium-activated chloride channels regulate the flow of chloride ions across eukaryotic cell membranes in response to intracellular calcium levels. Mutations in BEST1 cause eye disease. This presentation will focus on the insights into the molecular bases of BEST1 function obtained by reconstituting channel function from purified components and determining the channel's atomic structure.

Relevant paper:
1) Dickson, V.K., Pedi, L. and S. B. Long. (2014). *Nature*. **516,** 213-8.
"Structure and insights into the function of a Ca^{2+} -activated Cl^- channel."

INTEGRATED FUNCTIONS FOR ANNEXINS IN THE MAMMALIAN RETINA

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Age-related macular degeneration (AMD) is the leading cause of sight loss in the western world, affecting around 1:10 of the population aged over 65, and with incidence rising in line with population growth and increasing life expectancy. The retinal pigment epithelium (RPE) is a monolayer of cells that abuts both the photoreceptors and the blood vessels of the choriocapillaris, where it effectively forms the blood-retinal barrier. In AMD the RPE has been described as both the instigator and the victim of disease. Understanding the multiple functions of the RPE is key to the eventual development of better AMD therapeutics, and we have used these cells extensively, both in vitro and in vivo, to gain insight into the contributions that annexins make to normal and pathological cell behavior. In my presentation I will discuss three members of the annexin family, AnxA2, AnxA6 and AnxA8, and present work from our group that revealed roles for these proteins in RPE cells in retinal phagocytosis, mitochondrial homeostasis and phenotypic plasticity respectively. AnxA2, which among other activities regulates actin dynamics in a Ca^{2+}-dependent manner, is required for the circadian phagocytosis of shed photoreceptor outer segments, a process that is defective in AMD. Deletion of AnxA6 led to profound changes in mitochondrial morphology in the RPE, and anomalies in intracellular Ca^{2+} handling. Mitochondrial stress is one of the early signs of RPE dysfunction in AMD. The role of AnxA8 in the RPE is proving more elusive to pin down, but we have shown that in the absence of AnxA8, RPE cells in culture trans-differentiate into neuronal-like cells. The mechanism appears to involve suppression of Wnt signaling, and Wnt proteins are known to be important in the embryological development of the RPE from neuroectoderm. Given that RPE cells express several other members of the annexin family, the picture that emerges is one in which individual annexin family members appear to have non-overlapping roles that impinge on some of the most important activities of these multifunctional cells.

CALMODULIN CAPPING OF PSD-95 TRIGGERS ITS POSTSYNAPTIC RELEASE

James B. Ames

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Postsynaptic density protein-95 (PSD-95) is a central element of the postsynaptic architecture of glutamatergic synapses. PSD-95 mediates postsynaptic localization of AMPA receptors and NMDA receptors and plays an important role in synaptic plasticity. PSD-95 is released from postsynaptic membranes in response to Ca^{2+} influx via NMDA receptors. Here, we show that Ca^{2+}/calmodulin (CaM) binds at the N-terminus of PSD-95 (residues 1-60). We also solved the NMR structure of CaM bound to the PSD-95 N-terminal region. Our structure reveals that both lobes of CaM collapse onto a helical structure of PSD-95 formed at its N-terminus (residues 1-16). This N-terminal capping of PSD-95 by CaM blocks palmitoylation of C3 and C5, which is required for postsynaptic PSD-95 targeting and the binding of CDKL5, a kinase important for synapse stability. CaM forms extensive hydrophobic contacts with Y12 of PSD-95. The PSD-95 mutant Y12E strongly impairs binding to CaM and Ca^{2+}-induced release of PSD-95 from the postsynaptic membrane in dendritic spines. We conclude that CaM binding to PSD-95 serves to block palmitoylation of PSD-95, which in turn promotes Ca^{2+}-induced dissociation of PSD-95 from the postsynaptic membrane.

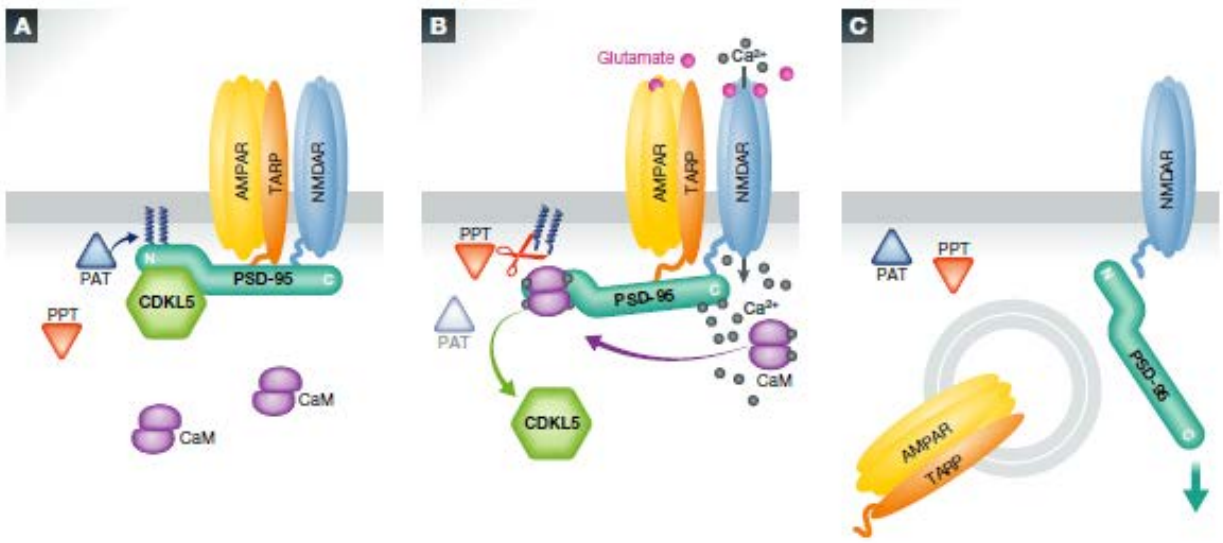

Figure 1. Calcium influx-induced release of PSD-95 and glutamate receptor from the synapse.
At a synapse, cysteine residues of PSD-95 (C3 and C5) are under a continuous cycle of palmitoylation by protein palmitoyl acyltransferase (PAT), and depalmitoylation by palmitoyl protein thioesterase (PPT). A. Palmitoylated PSD-95 associates with the synaptic membrane and CDKL5 and serves as a 'slot' for AMPAR at the synapse through the interaction with TARP/stargazin. B. Upon glutamate stimulation, Ca^{2+} influx through NMDARs induces binding of Ca^{2+}/CaM to PSD-95. Ca^{2+}/CaM blocks the accessibility of PAT, thereby facilitating the depalmitoylation of PSD-95, which subsequently allows PSD-95 to dissociate from the synaptic membrane and CDKL5. C. The dissociation of PSD-95 from the synaptic membrane reduces the number of available 'slots' for AMPAR on the postsynaptic membrane, leading to a reduction of AMPAR-mediated synaptic transmission.

ENGINEERING METAL SENSORS FOR NEURONAL EXOCYTOSIS

Edwin Chapman

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The Ca^{2+} sensor synaptotagmin (syt)-1 regulates neurotransmitter release by interacting with anionic phospholipids. Here we test the idea that the intrinsic kinetics of syt-1•membrane interactions determine, in part, the time course of synaptic transmission. To tune the kinetics of this interaction, we grafted structural elements from the slowest isoform, syt-7, onto the fastest isoform, syt-1, resulting in a chimera with intermediate kinetic properties. Moreover, the chimera coupled a non-physiologically relevant metal, Sr^{2+}, to membrane fusion *in vitro*. In neurons, the chimera slowed the kinetics of synaptic transmission. Neurons expressing the chimera also evinced rapid and efficient Sr^{2+} triggered release, in contrast to the weak response of neurons expressing syt-1. These findings reveal presynaptic sensor-membrane interactions as a major factor regulating the speed of the release machinery, and additionally provide a tool to dissociate synaptic vesicle exocytosis from other Ca^{2+} dependent processes in the synapse.

Syt-1 binds divalent cations via tandem C2-domains that comprise most of the cytoplasmic domain of the protein. In a parallel study, we used a protein engineering approach to constrain and alter the relative orientation of these C2-domains. Molecular dynamics simulation predicted that if the native C2-domain linker is replaced with a nine-residue poly-proline rod, the C2-domains will point in the same direction. Since poly-proline helices have a period of three, large changes in the relative disposition of the C2-domains result from changing the length of the poly-proline linker by a single residue. We varied the linker from six to twelve residues, and these experiments revealed a periodicity of three for the ability of the linker mutants to interact with anionic phospholipids and drive synaptic transmission. Thus, the orientation of the tandem C2-domains of syt-1 is crucial for evoked transmission; linkers of six, nine, and twelve prolines supported syt-1 function, indicating that syt-1 drives release when its tandem C2-domains point in the same direction (i.e. toward the target membrane).

These, and related studies focused on yet another C2-domain-bearing Ca^{2+} sensor for release, Doc2, provide new insights into the nanomechanics of the fusion machines that mediate the release of neurotransmitters from presynaptic boutons, and uncovered a new locus that determines the time course of synaptic transmission.

This work is not yet published, and was supported by NIH and HHMI.

STRUCTURE/FUNCTION AND INHIBITION OF S100B IN MALIGNANT MELANOMA

David J. Weber

S100B and other members of the S100 protein family are elevated in cancers including tumors involving skin, lung, bladder, kidney, cervix, breast, head and neck, larynx, lymph, and mouth to name a few [(1)]. S100s typically function in a cell-specific manner, as signaling proteins, which bind and regulate a number of protein targets in a Ca^{2+}-dependent manner. In addition to being biomarkers, they often contribute to cancer progression when overproduced. One such S100B target is the tumor suppressor protein, p53. In this case, up-regulation of S100B abrogates p53 transcription activation and UV-dependent apoptosis in cancers containing wild-type p53, such as gliomas and malignant melanoma. The focus of this presentation will be on structural and dynamic data (X-ray, NMR) leading to Ca^{2+}-dependent S100B-target formation in malignant melanoma and gliomas (i.e. p53, Rsk1, and others) and how this information can be used to develop novel S100B inhibitors (SBiXs). SBiXs developed over the past several years will be discussed briefly, some of which advanced to *in vivo* studies (mice, canines) including, in one case, a human phase 2 clinical trial; however, it is necessary to engineer next generation SBiXs with increased potency and fewer off-target effects including those involving other S100s (i.e. S100A1). Progress towards this goal will be highlighted and has relied on traditional structure-based drug design approaches including thermodynamic, kinetic, and biophysical considerations. This includes a "Binding and Functional Folding (BFF)" model that considers changes in the dynamic properties of states leading to the formation or inhibition of the Ca^{2+}-S100B-p53 or Ca^{2+}-S100B-Rsk1 complexes [(1)].

REFERENCE

1. Bresnick, A. R., Weber, D. J., and Zimmer, D. B. (2015) S100 proteins in cancer, *Nat Rev Cancer 15*, 96-109.

METASTASIS-INDUCING S100A4 IN THE PRO-INFLAMMATORY PATHWAYS OF NON-COMMUNICABLE DISEASES.

Mariam Grigoriam

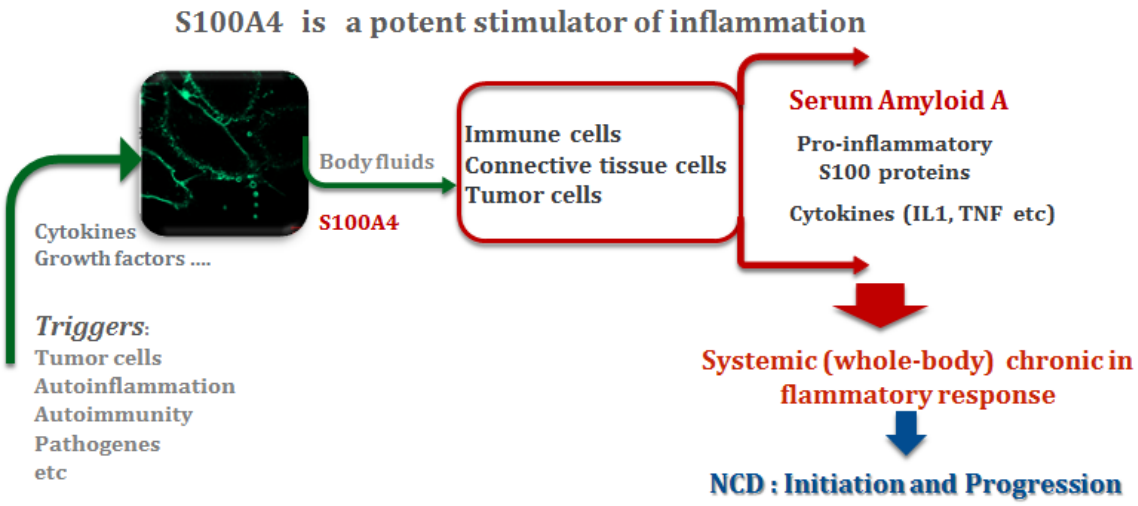
The S100A4 protein is widely acknowledged as a metastasis-inducing protein with proven clinical significance as a poor prognosis indicator.

S100A4 overexpressed by and externalized from various cell types into the extracellular space generates a pro-inflammatory milieu both in the primary tumor and in distant organs. By this it might largely contribute to the formation of pre-metastatic niches and create conditions favorable for homing, growth of tumor cells and formation of lethal metastasis.

The major functional activity of S100A4 is associated with a strong activation of the NF-kB transcription factor and subsequent stimulation of the expression and secretion of MMPs and pro-inflammatory response cytokines, S100A8/A9 and a**cute-phase serum amyloid A proteins (SAA)**. The potent pro-metastatic activity of the downstream target of S100A4, SAA, has been also shown recently by us.
In support to the key role of S100A4 in the induction of metastasis we have shown significant inhibition of tumor spread in response to counteraction to S100A4 in animal models.

In addition to cancer metastasis, the up-regulation of S100A4 also plays a functional role in autoimmune inflammations (e.g. rheumatoid arthritis, psoriasis, systemic sclerosis and others) and disorders of cardio-vascular, nervous and pulmonary systems, suggesting the involvement of common molecular mechanisms and pathways in diverse human Non-Communicable Diseases (NCD).

Conclusion: We consider the metastasis-inducing S100A4 protein as an upstream key instigator of systemic chronic inflammatory processes implicated in the pathogenesis of various NCD including cancer metastasis (Figure).



S100A1 IN CARDIOVASCULAR HEALTH AND DISEASE: FROM MOLECULE TO THERAPY

Julia Ritterhoff

S100A1 is a small EF-hand Ca2+-binding protein with various intracellular and extracellular functions. It is predominantly expressed in cardiac muscle where it plays a crucial role as a modulator of intracellular Ca2+ homeostasis. S100A1 beneficial effects have been attributed to its regulation of sarcoplasmic reticulum function, mitochondrial performance and myofilament cooperativity. Downregulation of S100A1 in cardiomyocytes has been linked to impaired cardiac function and contribution to the development of heart failure. Normalizing the level of S100A1 protein by means of viral gene transfer in animal heart failure models resulted in a disrupted progression towards cardiac failure and enhanced survival. Besides, S100A1 has additional function in endothelial cells and fibroblasts, which adds to its pleiotropic impact on the cardiovascular system and provides a promising therapeutic target for diverse cardiovascular conditions.

DESIGN CALCIUM BINDING PROTEINS FOR MOLECULAR IMAGING

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My lab has been focused on creating novel technologies and reagents for studying calcium signaling, understanding molecular basis of diseases, and diagnostics and therapeutic by analyzing, designing and engineering metal binding sites with high co-ordinations in proteins. I will first discuss our effort in development of computational and bioengineering approaches to visualize the role of calcium in various biological and pathological processes (Calciomics) and intergrate extracellular with intracellular Ca^{2+} signaling & cell-cell communication. I will next discuss our recent progress in designing *de novo* calcium-binding sites into proteins as calcium sensors (CatchER) to probe rapid cellular calcium changes and dynamics in signaling and disease states in high calcium concentration environment) with enhancement in both fluorescence and lifetime. CatchER enables us to monitor SR luminal Ca^{2+} in flexor digitorum brevis (FDB) muscle fibers to determine the mechanism of diminished SR Ca^{2+} release in aging mice for molecular imaging.

COME TOGETHER: DESIGNING S100 PROTEINS TO IDENTIFY NEW BIOLOGICAL COMPLEXES

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The S100 proteins are a large group of homo- and heterodimeric EF-hand calcium-binding proteins involved in cytoskeletal re-organization, membrane repair and enzyme regulation. Most of the members in this family function through calcium binding that triggers a conformational change allowing the S100 protein to interact with a biological target protein. A unique member of the protein family is S100A10 because it does not bind calcium and yet maintains the calcium-like structure observed for other S100 proteins in their calcium-bound form. S100A10 is proposed to play roles in plasma membrane repair through recruitment of the phospholipid-binding proteins annexin A1 and A2, the enlargeosome protein AHNAK and members of the ferlin (dysferlin) family. Several studies have indicated that large S100-mediated protein complexes may orchestrate the repair process and restore the integrity of the membrane.

Although most S100 proteins have the possibility to form homo- and heterodimeric species, most *in vitro* and *in vivo* studies have focused on the homodimeric proteins. On exception is S100A8/S100A9 which exclusively forms a heterodimeric complex. In this work we have examined the unique dimeric properties of some S100 proteins including their abilities to interact with targets and preference for homo- and heterodimer formation. A key approach was the engineering of chimeric S100 proteins using three-dimensional structure information and using these to explore how higher-order S100-protein complexes might be formed. This approach has allowed us to identify some unique and surprising protein-recruiting properties of the S100 proteins.

CA2+ AND βγ-CRYSTALLINS: AN AFFAIR THAT DID NOT LAST

Yogendra Sharma

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A mammalian eye lens contains α-, β- and γ-crystallins. The α-crystallins have been classified within the small heat shock protein family based on similar functions, while the β- and γ-crystallins have been grouped into the βγ-crystallin superfamily on account of their similar topology.

During the last three decades, lens βγ-crystallins have found a huge number of kin from numerous taxonomical sources. Most of these proteins from invertebrates and microbes have been demonstrated or predicted to bind Ca^{2+} at a common binding site involving a distinct double-clamp motif. Based on tentative estimate from genome sequence database, βγ-crystallin superfamily appears to be among the most prevalent groups of Ca^{2+}-binding proteins, though the functional significance of this relationship is yet to be well established.

As functionally diverse the βγ-crystallin domains are, at the culmination of their evolutionary journey, i.e., in lens, the Ca^{2+}-binding ability is reduced considerably. It happens due to the modifications in binding sites, which thus presents a case of complete diversion of the motif distinguishing bacterial proteins from the mammalian ones. Considering the difference in roles played by microbial and lens βγ-crystallins, this change should be a reflection of their functional shift in terms of Ca^{2+}-dependence.

THE MECHANICS OF STIM-ORAI COMMUNICATION

Patrick Hogan

The ER-resident regulatory protein STIM1 triggers store-operated calcium entry by direct interaction with the plasma membrane calcium channel ORAI1. Cell-based research combining the expression of engineered proteins, electrophysiological recording, and advanced light-microscopic techniques has provided considerable insight into STIM-ORAI signalling, but has left tantalizing unanswered questions regarding the gating, modulation, and inactivation of ORAI-family channels. We have taken two complementary paths to investigate these questions. First, in order to dissect the basic mechanisms intrinsic to STIM, ORAI, and the STIM-ORAI complex, we have reconstituted channel gating *in vitro* using the purified recombinant STIM and ORAI proteins. This approach has begun to yield direct insight into ORAI channel gating. Second, in order to identify additional mechanisms that control STIM-ORAI signalling in cells, we have carried out a genome-wide RNAi screen for modulators of store-operated calcium entry. The screen led to the identification of septins and other protein modulators of STIM-ORAI signalling, and to the discovery that there is a striking rearrangement of the plasma membrane microdomain surrounding STIM-ORAI complexes during the onset of store-operated calcium entry. Continued parallel investigations *in vitro* and in cells will be needed for a full understanding of this key calcium entry pathway.

STIM1-MEDIATED CA^{2+} INFLUX CONTROLS IMMUNE REGULATION AND INFLAMMATION IN CHRONIC INFECTION

Stefan Feske

Ludovic Desvignes[1], Carl Weidinger[2], Patrick Shaw[2], Martin Vaeth[2], Theo Ribierre[2], Menghan Liu[2], Lina Kozhaya[2], Lauren McVoy[2], Derya Unutmaz[1,2], Joel Ernst[1,2,3] and Stefan Feske[2]

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Ca^{2+} influx through ORAI1 channels is essential for T cell function and immunity to infection. Patients with inherited mutations in the genes encoding ORAI1 or its activator Stromal interaction molecule (STIM) 1 are immunodeficient and develop chronic infections with a variety of pathogens, including mycobacteria. Chronic infections such as tuberculosis (TB) induce a complex immune response to control pathogen replication but also cause pathology due to sustained inflammation. We find that STIM1 is required not only for acute T cell responses to infection, but especially for T cell-mediated immune regulation during chronic infection. When mice with T cell-specific deletion of *Stim1* were infected with *Mycobacterium tuberculosis (Mtb)*, the pathogen causing TB, they died prematurely during the chronic phase of infection and had increased mycobacterial burdens and severe pulmonary inflammation with increased numbers of myeloid and lymphoid cells. Although T cells lacking STIM1 showed significantly reduced IFNγ production early during *Mtb* infection, this defect did not immediately exacerbate bacterial growth. During chronic infection, however, STIM1-deficient T cells produced large amounts of IFNγ in response to high IL-12 and IL-18 levels. In the absence of STIM1, T cells showed impaired activation-induced cell death upon repeated TCR engagement resulting in pulmonary lymphocytosis and hyper-inflammation. The frequencies of inducible $Foxp3^+$ Treg (iTreg) cells, by contrast, were reduced in chronically *Mtb*-infected STIM1-deficient mice due to a T cell-intrinsic requirement for STIM1 in iTreg differentiation and high pulmonary IFN and IL-12 levels, which suppress iTreg differentiation and maintenance. Taken together, Ca^{2+} influx mediated by STIM1 plays multiple important roles in T cell-mediated immune regulation to limit injurious inflammation during chronic infection.

CALCIUM AND CALCIUM CHANNELS IN INITIATION AND PROGRESSION OF PROSTATE CANCER

Natalia Prevarskaya

Dmitro Gordienko, Charlotte Dubois, Fabien Vanden Abeele, V'yacheslav Lehen'kyi, Maylis Raphael, Pilar Flamenco, Loic Lemonnier, Roman Skryma and Natalia Prevarskaya

Inserm U1003, Equipe labellisée par la Ligue Nationale Contre le Cancer, , Laboratory of Excellence, Ion Channels Science and Therapeutics; Université Lille I Sciences et Technologies, Villeneuve d'Ascq, France.

Normal cell progression to their malignant derivatives is associated with remodeling of the proteins controlling such major cellular functions as apoptosis and proliferation. Here, we show that prostate cancer cells use ORAI and TRP protein redistribution as an oncogenic switch mechanism. In particular, ORAI3 and TRPV6 remodeling results from genomic and microenvironment perturbations that disrupt the equilibrium of channels and favors the formation of novel Ca^{2+} channels activated in a store-independent manner. This remodeling of Ca^{2+} signaling in turn induces cell progression to a more aggressive pro-proliferative phenotype. We also present initial evidence for the major role of calcium signalling in prostate cancer stem cells. Indeed, Orai1 expression is down-regulated in prostate cancer stem cells. The low level of Orai1 is functionally associated with a major decrease in store-operated calcium entry and cytosolic calcium concentration. This functional remodelling of Orai1 proteins in turn down-regulates the Ca^{2+}-calcineurin-NFAT/CBP signalling pathway.

Our study specifically positions these channels at the center of molecular machinery linking deregulated metabolism, calcium homeostasis, and oncogenesis.

LOSS OF MAGNESIUM TRANSPORTER 1 LEADS TO A NOVEL HUMAN IMMUNODEFICIENCY WITH CHRONIC EBV INFECTION AND LYMPHOMA

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Molecular Development Section, Lymphocyte Molecular Genetics Unit, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD

Approximately 90% of people worldwide are latently infected with the B cell-tropic gammaherpesvirus Epstein-Barr Virus (EBV), yet only a subset of immunocompromised hosts develop chronic active EBV infection with a high susceptibility to lymphoma. While the etiology of such immunodeficiency states is sometimes apparent, as in HIV and post-transplant immunosuppression, the underlying immune dysfunction is other cases remains unknown. We have characterized a novel primary immunodeficiency (PID), named X-linked immunodeficiency with magnesium defect, Epstein-Barr virus infection, and neoplasia (XMEN) disease, characterized by loss of function mutations in the X-linked gene for magnesium transporter 1 (MAGT1), chronic EBV infection, and increased susceptibility for developing EBV associated lymphomas. MagT1 is a Mg^{2+}-specific plasma membrane channel highly expressed on hematopoietic cells. We showed that various TCR agonists could induce a transient Mg^{2+} influx in normal T cells but not in MagT1-deficient patient cells. Also, knockdown of MagT1 in normal T cells can causes defects in T cell activation and Mg^{2+} flux defects while expression of MagT1 in patient T cells can restore the fluxes and stimulation. Thus, there is an unexpected requirement for Mg^{2+} flux mediated by MagT1 during T cell activation. In further investigations, we have identified a specific, magnesium-sensitive immune arm of defense against EBV lymphomagenesis that could be amenable to restoration by exogenous magnesium supplementation.

OPPOSING TRANSCRIPTIONAL PROGRAMS DRIVEN BY CALCIUM, CALCINEURIN AND NFAT IN T CELLS

Anjana Rao

Martinez GJ, Äijö T, Pereira RM, Lähdesmäki H, Hogan PG, Rao A.

The calcium/calcineurin-regulated NFAT family of transcription factors plays an established role in T cell activation. We have found that NFAT also controls a cell-intrinsic negative feedback program in which T cell activation is attenuated, both in cultured T cells and in mouse models. NFAT transcriptionally activates this program by regulating the expression of key inhibitory proteins, including signaling proteins such as phosphatases and E3 ligases as well as cell surface inhibitory receptor that dampen TCR signaling.

THE BATTLE FOR METAL BETWEEN BACTERIAL PATHOGENS AND THEIR HOSTS

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Hospital and community-acquired infections caused by bacterial pathogens represent an increasing threat to global public health. This threat is compounded by the fact that bacterial pathogens are rapidly becoming resistant to all existing antimicrobial. Research in my laboratory is focused on identifying nov el targets for therapeutic intervention against bacterial pathogens with a particular emphasis on systems involved in metal trafficking and metabolism. Metals are essential for all life because approximately 40% of all proteins in nature require metals to carry out their biological function. Bacterial pathogens must acquire metals inside their hosts in order to successfully mediate infection. However, vertebrates have evolved strategies to sequester nutrient metal from invading bacteria in a process known as “nutritional immunity”. Based on the strict requirement of bacteria for metal, and the fact that the bacterial and eukaryotic machinery involved in metal acquisition and homeostasis are remarkably different, targeting bacterial machinery involved in metal metabolism has excellent therapeutic potential. This presentation will describe research in my laboratory that is focused on identifying factors and processes involved in the battle for metal between bacterial pathogens and their hosts.

MULTIPLE ROLES OF CALMODULIN IN TUNING CELLULAR FUNCTION, LEARNING AND MEMORY

Richard Tsien

Boxing Li, Michael Tadross, Huan Ma, Samuel Cohen and Richard Tsien

NYU Neuroscience Institute and Center for Neural Science, New York University

The primary language of excitable cells (action potential firing) is converted into the primary language of intracellular activity (biochemical signaling) by voltage-gated Ca^{2+} channels (Ca_Vs). This kind of signaling is vividly exemplified by excitation-contraction (E-C) coupling and excitation-secretion (E-S) coupling. Excitation-transcription (E-T) coupling is arguably a more general event in neurons and other excitable cells but is much less understood. One particular class of Ca^{2+} channels, Ca_V1 (also known as L-type channels) has a privileged role in excitation-transcription coupling to nuclear CREB, a transcription factor critical in learning and memory. However, even the earliest step in this signaling pathway is not fully understood: local Ca^{2+} elevations near Ca_V1 channels are thought to be the main trigger in the signaling cascade, but Ca_V1 channels could also convey a voltage-dependent conformational signal (VCS) to nearby signaling intermediates, analogous to the conformational signal in E-C coupling. We have devised an approach whereby conformational changes required to open the Ca_V pore are experimentally decoupled from Ca^{2+} influx into the channel nanodomain. This dissection uncovered a remarkable requirement for the Ca_V1 VCS in excitation-transcription coupling. Ca_V1 signaling to CREB behaves as a coincidence detector, where both Ca^{2+} and voltage-dependent movements are necessary. The key local signaling intermediates include the $\alpha-$ and βCaMKII isoforms.

Another puzzle is how local signaling at Ca_V1 channels is relayed onward to the nucleus. We have discovered a novel mechanism that mediates long-distance communication within cells: a shuttle that transports Ca^{2+}/calmodulin from the surface membrane to the nucleus. We find that the shuttle protein is yet another CaMKII isoform, γCaMKII. Its phosphorylation at Thr287 by βCaMKII protects the Ca^{2+}/CaM signal, and calcineurin (CaN) triggers its nuclear translocation. Both β CaMKII and CaN act in close proximity to Ca_V1 channels, supporting their dominance, while γCaMKII operates as a carrier, not as a kinase. Upon arrival within the nucleus, Ca^{2+}/CaM activates CaMKK and its substrate CaMKIV, the CREB kinase. This mechanism resolves longstanding puzzles about CaM/CaMK-dependent signaling to the nucleus. The significance of the mechanism is emphasized by dysregulation of Ca_V1, γCaMKII, βCaMKII and CaN in multiple neuropsychiatric disorders including autism and schizophrenia. In all of this, CaM appears to play multiple roles, as an activator of kinase and phosphatase molecules and as a translocating messenger.

CALCINEURIN REGULATION OF NEURONAL CALCIUM CHANNEL ACTIVITY AND SIGNALING TO THE NUCLEUS

Mark L. Dell'Acqua

Jonathan G. Murphy[1,2], Phillip J. Dittmer[1], Jennifer L. Sanderson[1], Jessica A. Gorski[1], John D. Scott[3,4], William A. Catterall[4], William A. Sather[1,2], and Mark L. Dell'Acqua[1,2]

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L-type voltage-gated Ca^{2+} channels (LTCC) couple neuronal excitation to gene transcription that is important for long-lasting forms of synaptic plasticity that underlie learning and memory. LTCC activity is elevated by the cAMP-dependent protein kinase (PKA) and depressed by the Ca^{2+}-dependent protein phosphatase 2B/Calcineurin (CaN), with both enzymes localized to the channel by the postsynaptic scaffold protein A-kinase anchoring protein (AKAP) 79/150. AKAP79/150 anchoring of CaN also promotes LTCC activation of transcription through dephosphorylation of the nuclear factor of activated T-cells (NFAT) to trigger its translocation from the cytoplasm to the nucleus. However, AKAP79/150 anchors CaN through a PxIxIT-like motif that is very similar to the CaN docking sequences found in the NFAT transcription factors themselves, Thus, AKAP79/150 actually competes with NFAT for CaN binding raising the question of how exactly is LTCC-CaN-NFAT signaling promoted by the AKAP? Guided by the crystal structure of the AKAP-CaN complex, mutations in the PxIxIT-like motif designed to either increase or decrease CaN anchoring affinity, were both, surprisingly, found to inhibit NFAT activation. In particular, increasing anchoring affinity immobilized CaN at synapses and prevented NFAT translocation to the nucleus. Thus, AKAP-CaN anchoring is by necessity dynamic and promotes NFAT signaling by balancing strong recruitment of CaN near its upstream activator the LTCC with its efficient release to communicate with its downstream substrate NFAT. Building on this theme of balance in local signaling complexes, we found that disruption of AKAP-PKA anchoring also prevents NFAT signaling; a deficit attributable to profound decreases in basal LTCC phosphorylation, current density, and depolarization-evoked Ca^{2+} influx. Finally, our most recent work indicates that AKAP-PKA enhancement of LTCCs additionally primes these channels for effective coupling to release of Ca^{2+} from ER stores in response to postsynaptic NMDA receptor activation. Interestingly, this ER Ca^{2+} release leads to engagement of the ER Ca^{2+} sensor STIM1 that then interacts with the AKAP-LTCC complex to suppress channel activity and limit the strength of CaN-NFAT signaling to the nucleus. Thus, AKAP79/150 precisely coordinates PKA, CaN, and STIM1 signaling to promote effective LTCC-NFAT excitation-transcription coupling in neurons.

DISORDER IN CALCINEURIN ACTIVATION

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The highly-conserved, heterodimeric Ser/Thr phosphatase calcineurin (CaN) plays vital roles in memory development and retention, cardiac growth, and immune system activation. Alterations in regulation of CaN are believed to contribute to Alzheimer's disease, Down syndrome, autoimmune disorders, and cardiac hypertrophy. CaN possesses a regulatory domain that adopts a disordered state that is an intermediate between two ordered states. This disordered state is essential for activation of the enzyme. At low calcium levels the 95 residue regulatory domain in CaN appears to be folded. As levels rise, the CaN B chain binds calcium and undergoes a conformational change that releases the regulatory domain into a disordered state. The subsequent binding of CaM to CaN results in the regulatory domain folding. Folding of the regulatory domain in turn causes an autoinhibitory domain located C-terminal to the regulatory domain to be ejected from CaN's active site. The disordered state of the regulatory domain is an essential intermediate state in the process of activation.

FKBP12 DETERMINES CALCINEURIN SUBSTRATE SELECTION: IMPLICATIONS TO SYNUCLEINOPATHIES

Gabriela Caraveo Piso

Whitehead Institute for Biomedical Research

Calcineurin is a highly conserved Ca^{2+}-calmodulin-dependent phosphatase critical for transducing the signals generated by Ca^{2+} gradients into cellular responses. We previously showed that the extent of calcineurin activation modulates toxicity resulting from expression of α-synuclein (α-syn), a small lipid binding protein whose misfolding plays a major role in synucleinopathies including Parkinson's disease (PD). Calcineurin activation can be regulated by FK506, which inhibits the phosphatase by forming a ternary complex with the immunophilin FKBP12. While the role of FKBP12 as a cis-trans prolyl isomerase is well understood in protein folding, little is known about its role in FK506-mediated inhibition of calcineurin. Using our yeast model of α-syn toxicity and employing an integrated chemical, genetic and unbiased shotgun proteomic approach, we report that FKBP12 governs calcineurin substrate selection. The phosphorylation of 69 different proteins is affected by the interaction of FKBP12 with calcineurin; among these we identified synaptojanin 1, a synaptic protein that has recently been associated with PD and is critically involved in clathrin-mediated endocytosis. Furthermore, we tested the potential clinical relevance of the inhibition of FKBP12 and calcineurin by FK506 *in vivo* using a rat model for PD. Indeed, FK506 protects against α-syn toxicity. Thus, our data provides new mechanistic insight into calcineurin substrate selection and an endorsement of the use of FK506 at sub-immunosupressive doses in the treatment of synucleinopathies such as PD.

UPREGULATED RYANODINE RECEPTOR ACTIVITY UNDERLIES ALZHEIMER'S DISEASE PATHOLOGY FROM MOLECULAR TO NETWORK LEVELS

Grace Stutzmann

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Abnormalities in ER calcium channels have been widely demonstrated in human AD patients and in animal and cellular models of AD. In particular, the large conductance RyR calcium channel has been implicated, with specific increases in RyR2 expression at early or preclinical disease stages. Her we describe, early in the disease process, evidence of altered RyR expression profiles which likely contributes to the markedly increased RyR-evoked calcium release in AD mouse models. Our evidence indicates that the RyR-mediated calcium dyshomeostasis is driving synaptic structure and plasticity deficits, blunted hippocampal network propagation, increased amyloid and tau pathology, and memory impairments – all of which are central components of AD. Stabilizing RyR activity with both existing and novel compounds has proven highly effective at preventing a wide range of AD features. To this end, we have developed and tested a series of small molecules targeting the RyR2 and assayed these compounds in model cells and AD mice to validate the RyR2 as a therapeutic target. We have found that normalizing RyR function, and the RyR2 in particular, is highly effective in restoring normal intracellular calcium signaling, and subsequently, preserves synaptic structure and plasticity, and reduces histopathology and cognitive deficits. As synaptic loss is the most likely cause of memory loss in AD, targeting RyR2 may be a highly effective and novel therapeutic strategy for preventing cognitive deficits in AD.

NOVEL CALCIUM SIGNALING ROOT OF PARKINSON'S DISEASE

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This presentation will focus on our recent discovery of the previously unknown Ca^{2+} signaling mechanism underlying Parkinson's disease (PD). We will demonstrate that idiopathic, genetic or molecular impairment of the PARK14-dependent store-operated Ca^{2+} entry (SOCE) and depletion of ER Ca^{2+} stores trigger autophagic dysfunction, progressive loss of dopaminergic (DA) neurons in substantia nigra pars compacta (SNc) and age-dependent L-DOPA-sensitive PD, which we could mimic in a new B6.Cg-*Pla2g6*$^{\Delta Ex2\text{-}Vbol}$ (PLA2g6 Ex2^{KO}) mouse model.

IN SEARCH FOR MODELS OF SPORADIC ALZHEIMER'S DISEASE

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AD is a progressive and irreversible neurodegenerative disorder that leads to cognitive, memory and behavioral impairments. Historically, the disease has been defined by the extracellular deposition of diffuse and neuritic plaques composed of amyloid-beta peptide and intracellular hyperphosphorylated neurofibrillary tangles of Tau protein. Vast majority of available animal models of Alzheimer's disease (AD) are based on beta-amyloid/tau hypothesis [1]. These mice overexpress one or more mutated proteins known to be responsible for early onset of familial AD (FAD). The FAD models, which represent less than 5% of all human cases, seem to be of a little value to understand the mechanisms of sporadic AD (SAD). One of the arguments to support such view is the failure of clinical trials aimed to affect the disease by decreasing the level of beta-amyloid in the brain. Altered calcium homeostasis in neurons is proposed to be one of the early events responsible for AD [2]. During human ageing there are changes in the concentration and activity of proteins responsible for Ca^{2+} homeostasis. This leads to slow and discrete increase of basal level of Ca^{2+} in neurons. Disturbances in Ca^{2+} signaling are found in SAD patients before any obvious extracellular Aβ pathology, and Ca^{2+} dysfunction augments Aβ formation and Tau hyperphosphorylation. We detected the enhanced magnitude of Ca^{2+} influx during SOCE in human lymphocytes from SAD patients, and decreased the level of STIM2 protein in human lymphocytes from FAD patients in parallel to an attenuation of SOCE [3, 4]. The decreased level of STIM2 in AD was found in AD models and brains of AD patients [5]. We also showed that the cytoplasmic resting Ca^{2+} level in cultured neurons can be modulated by overexpression of key Store Operated Calcium Entry proteins (STIM1, STIM2 or Orai1) [6, 7]. Based on these observations we generated transgenic mice overexpressing SOCE proteins in brain neurons. We expect that their brain neurons will have elevated basal Ca^{2+} level. If so, this model will be useful to understand the changes in signalosome occurring in the brain due to elevation of basal Ca^{2+} level and may exhibit pathologies characteristic for SAD. The characterization of three transgenic mice lines and preliminary data will be presented.

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CHARACTERIZATION OF STRUCTURE, DYNAMICS AND CALCIUM BINDING OF *PLASMODIUM FALCIPARUM* CDPK3

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Calcium dependent protein kinases (CDPKs) are involved in calcium signaling in plants and several parasites but are absent in mammals. In *Plasmodium falciparum*, the most virulent form of the malaria parasites that causes millions of infections every year with a high rate of mortality, seven different CDPKs have been identified. The proteins comprise a N-terminal kinase domain, an autoinhibitory helix and two C-terminal calcium ligating domains CLD(A) and CLD(B), respectively. Binding of calcium triggers a large conformational change and leads to activation.

P. falciparum CDPK3 is expressed at the sexual stage of the parasitic life cycle and in contrast to most CDPKs, one of the EF-hands in CLD(A) does not bind calcium due to the substitution E⟶Q. Using NMR spectroscopy, we have characterized CLD(A) and CLD(B) biophysically and observed major differences between the two domains.

Both domains fold independently and have the same secondary structure as was observed in the crystal structure of a larger fragment of the protein. Furthermore, the three-dimensional solution structure of calcium-loaded CLD(A) is virtually identical to the crystal structure and we have established that it tumbles as a monomer. The protein is in global exchange with a minor conformation that is populated to 1.1% and the exchange rate is 1600 s^{-1}. Interestingly, also the apo form of CLD(A) is in global exchange. However, here the exchange rate is an order of magnitude faster, which implies that the population of the 'excited' state cannot be determined. The dissociation constant for calcium binding at pH 7.1, 100 mM NaCl is 500 μM, significantly lower than for calmodulin at similar conditions.

The properties of isolated CLD(B) differ significantly from those of CLD(A). First, the protein is not stable in the absence of calcium. Second, the calcium bound form exists as dimers in solution. A third difference is that there are no conformational dynamics.

If our results can be generalized to the intact protein, they imply: 1) CLD(B) is always calcium bound and the structural reorganization is caused by binding of a single Ca^{2+} ion to CLD(A) and 2) CDPK3 is not monomeric but must form homo- or heterodimers.

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SPONTANEOUS CA^{2+} SIGNALING OF INTERSTITIAL CELLS OF CAJAL IN THE DEEP MUSCULAR PLEXUS OF THE MURINE SMALL BOWEL

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Interstitial cells of Cajal located in the deep muscular plexus (ICC-DMP) are closely associated with varicosities of enteric motor neurons and suggested to mediate neuronal responses to smooth muscle. Ca^{2+} handling in ICC-DMP is an important regulator in enteric neurotransmission but their characterization in ICC-DMP has not described in intact GI muscles. In this study we investigate the nature of Ca^{2+} activity in ICC-DMP. Jejunum tissues were taken from c-kit-iCreERT2 mice crossbred with GCaMP3 mice. Ca^{2+} transients in ICC-DMP were recorded *in situ*, using confocal imaging. Ca^{2+} imaging revealed spontaneous calcium transients in ICC-DMP. These Ca^{2+} events had variable firing patterns from discrete localized Ca^{2+} events to propagating events that spread over multi regions in the cell (0.27 to 56.9 µm). The occurrence of these Ca^{2+} events ranged from 12 to 376 events min^{-1} with an average of 134±15 events min^{-1}. We found no correlation between the firing of Ca^{2+} events within a single cell or between multi ICCDMP. Pretreatment of muscles with tetrodotoxin (1µM) increased Ca^{2+} frequency (83±10 to 151±20 min^{-1}) suggesting their involvement in tonic inhibition. Ca^{2+} transients were not inhibited by the absence of Ca^{2+} from extracellular medium indicating that Ca^{2+} influx mechanisms are not the major source of these events. ICC Ca^{2+} transients appeared to rely on intracellular store release as depletion of intracellular calcium stores by thapsigargin and CPA (both; 10 µM) abolished them. ICC calcium transients were also blocked by inhibition of IP_3 receptor antagonists (2-APB and xestospongin C) and ryanodine, indicating that Ca^{2+} release from the ER via both IP_3Rs and RyRs was necessary for their generation. This study reveals spontaneous Ca^{2+} activity in ICC-DMP and describes the nature of the localized-stochastic Ca^{2+} transients. Activation of Ca^{2+} release is likely to be the signaling mechanism in ICC-DMP responsible for transduction of enteric neuronal input in small bowel muscles.

SPHINGOSINE AS A NATURAL MODULATOR OF THE BASAL INTRACELLULAR CALCIUM CONCENTRATION THROUGH INHIBITION OF SERCA ACTIVITY AND ITS RELATIONSHIP WITH APOPTOSIS AND CANCER

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The increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is the key variable for many different and sometimes antagonistic processes, ranging from cell proliferation to apoptosis. The basal $[Ca^{2+}]_i$ usually defines the magnitude and the shape of the cytoplasmic signal-induced increase in the concentration of this cation. However, little is known about the signals involved in the regulation of this important parameter.

The sphingolipid sphingosine (Sph) is involved in the regulation of $[Ca^{2+}]_i$ in may cells. In this work we demonstrated that Sph increases the $[Ca^{2+}]_i$ by totally inhibiting the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) activity, in a similar manner to thapsigargin (Tg), a very specific SERCA inhibitor. We evaluated the effect of Sph on the $[Ca^{2+}]_i$ in different cancer cell lines, as PC-3 (colon), SKBR3, MCF-7 (breast) and Jurkat T cells (human lymphoma). The results showed that addition of sphingosine at a concentration that exerts its effect in many cells (10 µM) produced an increase in the $[Ca^{2+}]_i$ in all cell lines studied, as determined by the use of Fura 2, a Ca^{2+} sensitive fluorophore. This increase could be attributed to the emptying of the endoplasmic reticulum (ER), as assessed by confocal microscopy. As expected, Ca^{2+} release from the ER was followed by an influx of Ca^{2+} from the outside medium through a store-operated Ca^{2+} channel (SOCC), similar to that induced by Tg. Moreover, the addition of Sph after the Tg-mediated increase in $[Ca^{2+}]_i$ induced an additional Ca^{2+} entry, probably through TRP channels, since it is only observed in the presence of extracellular Ca^{2+}.

On the other hand, related to the induction of apoptosis, we also showed that Sph induced DNA fragmentation in MCF-7 cells as evaluated by the TUNEL method using flow cytometry. Moreover, Sph increased the activity of caspase 8 without affecting caspase 3 and 7, and also induced a decrease of the anti- apoptotic protein Bcl-2 in MCF-7, as observed by western blotting. Sph also induced a partial collapse of the mitochondrial electrochemical potential, as determined by loading the cells with rhodamine 123 and following its fluorescence upon addition of the sphingolipid. The results demonstrate that the effect of Sph as an apoptotic inducer is due, at least in part, to an increase in the $[Ca^{2+}]_i$, through the inhibition of the SERCA activity.

This study could be relevant for the understanding of processes related to cancer since it is well known that Tg analogs are under active research as anticancer drugs the onset of. Most importantly, since it is well known that Sph is present in the ER, we postulate that changes in Sph levels could directly control the SERCA activity, and thus the basal $[Ca^{2+}]_I$ in all human cells.

FGF23 EXPRESSION IN CARDIAC FIBROBLASTS IS AUGMENTED BY S100/CALGRANULINS- MEDIATED INFLAMMATION AND ASSOCIATED WITH CARDIAC HYPERTROPHY, BUT NOT IN ANGIOTENSIN II-INDUCED CARDIAC HYPERTROPHY

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Background: Serum S100A12 and fibroblast growth factor (FGF) 23 are biomarkers for cardiovascular mortality in patients with chronic kidney disease (CKD) and are associated with left ventricular hypertrophy (LVH). FGF23 is induced in cultured cardiac fibroblasts in response to cytokines including IL-6, TNF-a, LPS and S100/calgranulins. Moreover, hBAC-S100 transgenic mice with CKD had increased FGF23 in valvular interstitial cells and exhibited LVH. The present study was designed to examine cardiac FGF23 expression in other murine models of LVH in the absence of CKD.

Methods: Hearts from five groups of male mice were studied: (i) C57BL6/J with transgenic expression a bacterial artificial chromosome of the human S100/calgranulins (S1008/9 and S100A12, hBAC-S100), (ii) wild type littermates, (iii) $LDLR^{-/-}$ infused with saline (29 days, 0.9%), (iv) $LDLR^{-/-}$ infused with angiotensin (Ang) II (29 days, 1000 ng/kg/min), and (v) fibroblast specific depletion of angiotensin II type 1a receptor (AT1aR) (S100A4-Cre x $AT1aR^{-/-}$ x $LDLR^{-/-}$) infused with AngII.

Results: hBAC-S100, but not wild type littermate mice, developed significant LVH at 10 months by heart weight/body weight (5.9 ±1.1 mg/g vs. 4.2 ±0.8, $p<0.04$), decreased E/A ratio, and increased LVPW thickness, and associated with increased expression of FGF23 mRNA and protein in cardiac tissue lysates (2-4 fold increase). Similarly, Ang II induced significant LVH compared to saline infused $LDLR^{-/-}$ mice (6.1±1.3 vs. 3.6 ±0.9 mg/g, $p<0.01$), and associated with increased mRNA for hypertrophic genes (ANP, BNP, b-MHC, CTGF and Col1a1). However, there was no significant difference in FGF23 mRNA and protein between Ang II and saline infused mice. Cardiac hypertrophy was attenuated in AngII-infused mice with deficiency of AT1aR (S100A4-$Cre^{+/-}$xAT1aRxLDLR$^{-/-}$). In vitro, Ang II (100nM) did not induce FGF23 in valvular interstitial fibroblasts or myocytes.

Summary: Transgenic expression of S100/calgranulins is sufficient to induce LVH in aged mice with normal renal function, and this is associated with FGF23 expression in cardiac interstitial fibroblasts. Future studies are needed to determine whether cardiac FGF23 promotes LVH in a paracrine manner. However, FGF23 does not play a role in Ang II-induced LVH.

S100A4 PROMOTES SMOOTH MUSCLE CELL PHENOTYPIC TRANSITION. IMPLICATIONS IN ATHEROSCLEROSIS

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Atherosclerosis is the leading cause of cardiovascular diseases worldwide. During atherosclerotic plaque development, smooth muscle cells (SMCs) migrate from the media toward the intima where they proliferate and switch from a contractile to a synthetic phenotype.

From the porcine coronary artery normal media, we isolated, in addition to the classical spindle-shaped SMCs, the rhomboid SMCs. The latter were recovered in higher proportion from experimentally-induced intimal thickening. Rhomboid SMCs exhibited biological features (high proliferative and migratory activities and low level of differentiation), typical of the synthetic phenotype. By means of a proteomic approach, we identified S100A4 as being a marker of the rhomboid SMC population in vitro and of intimal SMCs, both in pig and man. S100A4 is an intracellular calcium-binding protein and exhibits extracellular functions via the receptor for advanced glycation end products (RAGE).

Treatment of spindle-shaped SMCs (devoid of S100A4) with S100A4-rich conditioned medium collected from S100A4-transfected SMCs induced a transition towards a rhomboid phenotype, which was associated with decreased SMC differentiation markers, increased proliferation and migration. It yielded activation of NF-κB in a RAGE-dependent manner. Conversely blockade of extracellular S100A4 in rhomboid SMCs with S100A4 neutralizing antibody induced a transition from rhomboid to spindle-shaped phenotype, decreased proliferative activity and upregulation of SMC differentiation markers. However, silencing of S100A4 mRNA in rhomboid SMCs did not change the level of extracellular S100A4 nor SMC morphology in spite of decreased proliferative activity. Interestingly, compared with native rhomboid SMCs, S100A4-rich conditioned medium treated spindle-shaped SMCs exhibited a proinflammatory signature i.e. increased mRNA expression of matrix metalloproteinases (MMP)-1, -3 and -9 and one of their inhibitors (tissue inhibitors of metalloproteinases-1 [TIMP-1]).

Our results indicate that extracellular S100A4 is essential for the establishment of the rhomboid i.e. synthetic phenotype in porcine coronary artery, shedding light on the mechanisms of SMC accumulation in the intima. It might in addition promotes a deleterious SMC phenotype. S100A4 could be a new target to prevent the evolution of atherosclerotic plaques.

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REGULATION OF MACROPHAGE MOTILITY & INVASION BY S100A4

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Our previous studies have shown that primary bone marrow macrophages (BMMs) derived from S100A4$^{-/-}$ mice form unstable protrusions, due in part to myosin-IIA overassembly, with consequent defects in CSF-1 stimulated chemotactic migration [1]. To evaluate how S100A4 deletion affects the invasive capabilities of macrophages, we examined wild-type and S100A4$^{-/-}$ mature M0, M1-polarized (IFN-γ and LPS-stimulated) and M2-polarized (IL-4-stimulated) BMMs in a Matrigel invasion assay. Under all conditions, S100A4$^{-/-}$ BMMs exhibited a 2-3-fold decrease in CSF-1-stimulated invasion as compared to wild-type BMMs. While the loss of myosin-IIA regulation by S100A4 is likely to contribute to the reduced invasive capabilities of S100A4$^{-/-}$ BMMs, we also considered other mechanisms that might play a role in this defect. Zymography revealed a significant accumulation of the pro form of MMP9 in the conditioned media of M1- and M2-polarized S100A4$^{-/-}$ BMMs, but not M0 S100A4$^{-/-}$ BMMs. Since stabilization of the microtubule network has been linked to MMP9 secretion in macrophages, we compared the levels of α-tubulin acetylation on K40 in wild-type and S100A4$^{-/-}$ BMMs. Tubulin acetylation was elevated in M0, as well as M1- and M2-polarized S100A4$^{-/-}$ BMMs, as compared to wild-type BMMs. Consistent with this finding, immunofluorescence studies revealed a more extensive microtubule network in S100A4$^{-/-}$ BMMs, which was resistant to nocodazole-mediated depolymerization. These studies demonstrate a novel role for S100A4 in the regulation of the microtubule cytoskeleton, and suggest that S100A4 modulates the invasive capacity of macrophages by multiple mechanisms.

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TARGETING PHOTORECEPTOR MEMBRANE GUANYLYL CYCLASE (RetGC) BY CALCIUM-SENSOR PROTEINS: ROLE OF PROTEIN DOMAINS AND THE EFFECTS OF MUTATIONS LINKED TO CONGENITAL BLINDNESS

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Retinal membrane guanylyl cyclase (RetGC1) in complex with its EF-hand Ca2+/Mg2+ sensor proteins, GCAPs, plays a major role in photoreceptor physiology – by supplying second messenger of phototransduction, cGMP, at variable rates in the light *versus* dark, controlled by the negative calcium feedback mediated by GCAPs [1]. The GCAP-regulated activity of RetGC therefore determines the shape and sensitivity of photoresponse [2]. In addition to recently established RetGC-binding interface on GCAP1 [3], we more lately, by using extensive site-directed mutagenesis, functional assays *in vitro* and colocalization tests *in cyto*, established the role of different cyclase domains and made a partial amino acid residues assignment in the cyclase dimerization domain for the GCAP-targeted interface on RetGC1. While in GCAP1 the cyclase-binding interface includes a non-metal binding EF-hand 1 and parts of Ca2+/Mg2+-binding EF- hands 2 and -3, in RetGC1 the interface for different isoforms of GCAPs is encoded in the region of its primary structure comprised of the cyclase kinase-homology and dimerization domains. The RetGC1 dimerization domain providing coiled-coil interaction in RetGC1 homodimer also contributes to the specificity of the GCAP-binding, lacking in hormone receptor cyclase (NPRA). Replacement with homologous sequences from NPRA fails to preserve GCAPs binding in the RetGC1/NPRA chimera protein. Mapping the dimerization domain by point mutations reveals specific residues most important for the binding of the calcium sensor proteins. The residues that impart to the cyclase the ability to bind GCAPs do not form the coiled-coil contact between dimerization domains in the cyclase homodimer, but are specifically required for recognition of GCAPs. When mutated, RetGC and GCAP can cause different forms of congenital blindness via either abnormal gain-of-function or loss-of-function changes in cGMP synthesis in photoreceptors. While some mutations in the cyclase dimerization domain cause congenital rod-cone degeneration by shifting the affinities of the cyclase toward the activator *versus* inhibitor forms of GCAP, a loss-of-function mutation Arg822Pro in dimerization domain and various mutations in cyclase inasehomology domain cause a recessive blindness (Leber's congenital amaurosis, LCA) by disrupting the cyclase ability to bind its calcium-sensor proteins. The cyclase harboring LCA mutations fails to become activated by calcium-free/Mg2+-liganded GCAPs *in vitro* and loses its capability of promoting GCAP association with the membranes in the *in cyto* experiments.

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THE ROLE OF CA2+ INFLUX IN SPONTANEOUS CA2+ WAVE PROPAGATION IN INTERSTITIAL CELLS OF CAJAL FROM THE RABBIT URETHRA

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Interstitial cells of Cajal (ICC) are putative pacemaker cells in the rabbit urethra. Pacemaker activity in ICC results from spontaneous propagating Ca^{2+} waves that are modulated by $[Ca^{2+}]_o$ and whose propagation is inhibited by inositol tri-phosphate receptor (IP_3R) blockers. The purpose of this study was to further examine the role of Ca^{2+} influx and Ca^{2+} release in the propagation of Ca^{2+} waves. Intracellular Ca^{2+} was measured in fluo-4 loaded ICC using a Nipkow spinning disc confocal microscope at fast acquisition rates (50 fps). We identified previously undetected localised Ca^{2+} events originating from ryanodine receptors (RyRs). Inhibiting Ca^{2+} influx by removing $[Ca^{2+}]_o$ or blocking reverse mode sodium/calcium exchange (NCX) with KB-R 7943 or SEA-0400 abolished Ca^{2+} waves, while localized Ca^{2+} events persisted. Stimulating RyRs with 1mM caffeine restored propagation. Propagation was also inhibited when Ca^{2+} release sites were uncoupled by buffering intracellular Ca^{2+} with EGTA-AM. This was reversed when Ca^{2+} influx via NCX was increased by reducing $[Na^+]_o$ to 13 mM. Low $[Na^+]_o$ also increased the frequency of Ca^{2+} waves and this effect was blocked by tetracaine and ryanodine but not 2-aminoethoxydiphenyl borate (2-APB). RT-PCR revealed that isolated ICC expressed both RyR_2 and RyR_3 subtypes. We conclude: i) RyRs are required for the initiation of Ca^{2+} waves, but wave propagation normally depends on activation of IP_3Rs; ii) under resting conditions, propagation by IP_3Rs requires sensitisation by influx of Ca^{2+} via reverse mode NCX; iii) propagation can be maintained by RyRs if they have been sensitised to Ca^{2+}.

CONTRIBUTION OF HUNTINGTIN ASSOCIATED PROTEIN 1 TO REGULATION OF SOCE IN MEDIUM SPINY NEURONS FROM TRANSGENIC YAC128 MICE, A MODEL OF HUNTINGTON'S DISEASE

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Huntington's disease (HD) is a hereditary neurodegenerative disease caused by the expansion of a polyglutamine stretch in the huntingtin (HTT) protein and characterized by deregulated Ca2+ homeostasis [1]. One of the mechanisms that regulate Ca2+ homeostasis is store-operated Ca2+ entry (SOCE) [2], that is enhanced in HD [3]. SOCE is a process in which the depletion of Ca2+ stores in the endoplasmic reticulum (ER) induces Ca2+ influx from the extracellular space. The Ca2+ sensors, stromal interaction molecules 1 and 2 (STIM1 and STIM2), detect changes in Ca2+ concentration in the ER, and aggregate in response to Ca2+ depletion. The aggregates force ER membranes towards the plasma membrane where STIM proteins interact with Orai calcium channels resulting in Ca2+ entry. The mechanism by which mutated HTT affects SOCE is unknown. Therefore, we assessed alterations of the Ca2+ signalosome in the striatum of transgenic YAC128 mice, a model of HD. In medium spiny neurons (MSN) from these mice we detected about 10% increase in basal Ca2+ level. We found that the activity of SOCE was enhanced about 30%, thereby confirming results of Wu et al. [3]. The changes in Ca2+ homeostasis could be explained by increased expression of huntingtin-associated protein 1 (Hap1) mRNA (3-fold) and HAP1 protein (about 2-fold) in the striatum of YAC128 mice, which we detected [4]. The increased level of HAP1 protein may explain changes in SOCE described by Wu et al. [3], if HAP1 influence ER Ca2+ release mediated by IP3R in MSN from YAC128 mice what was showed in other HD models by Tang et al. [5, 6]. In this work we investigated if HAP1 facilitates effect of mutant HTT on IP3R activation, which might explain its involvement in enhanced SOCE in MSN from YAC128 mice. We overexpressed HAP1a or HAP1b using prepared lentiviral constructs and imaged Ca2+ with Fura-2AM. We examine if the increased level of HAP1 activates IP3R and affects SOCE in YAC128 neurons, and in HEK293 cells overexpressing mutant HTT. We also determine changes in the SOC currents in MSN and SK-N-SH cells transduced with HAP1 and mutant HTT using electrophysiology.

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ANALYSIS OF DISTINCT ROLES OF CAMKK ISOFORMS USING STO-609-RESISTANT MUTANTS IN LIVING CELLS

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Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) is a member of the CaMK family that is composed of α and β isoforms. CaMKK α was originally identified and cloned from rat brain as an activating protein kinase for CaMKIV, also proving to be a CaMKI activator [1], and another β isoform was later cloned [2]. The kinase can only phosphorylate the activation loop Thr residue of particularly downstream protein kinases, including CaMKI, IV, protein kinase B/Akt (PKB), and 5′AMP-activated protein kinase (AMPK), resulting in induction of their kinase activities [3, 4]. Multiple target protein kinases including CaMKI, IV, PKB, and the AMPK family have been shown to be regulated by CaMKK phosphorylation, suggesting that CaMKK is involved in the regulation of a wide variety of Ca^{2+}-dependent physiological functions through the activation of these downstream protein kinases. Thus, manipulating CaMKK activity in living cells by either gene silencing or pharmacological treatment could yield important information about the signal transduction system mediated by these protein kinase cascades. In 2002, we developed a selective inhibitor (STO-609) of CaMKK that was an ATP-competitive and cell-permeable compound [5]. Cumulative evidences suggest that STO-609 could be a useful tool for investigating the CaMKK-mediated signal transduction pathway. However, Bain *et al.* reported that STO-609 did not show complete specificity toward CaMKK like other protein kinase inhibitors, but could inhibit other protein kinases with similar potency to CaMKK α, suggesting that results obtained by the use of this compound should be interpreted with caution [6]. STO-609 itself is also incapable of distinguishing the signaling pathways mediated by each CaMKK isoform. Of particular interest is the question of whether each CaMKK isoform activates different downstream protein kinase(s) or redundant activators. It is necessary to develop an experimental method to confirm the isoform-dependent effect of the inhibitor, for example by testing whether the effects of STO-609 are no longer observed in cells that express an STO-609-resistant mutant of a CaMKK isoform. Here we describe the development of STO-609 resistant CaMKK isoforms that can be used to analyze the distinct roles of CaMKK isoforms in living cells as well as to confirm the specificity of the inhibitor.

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DEVELOPMENT AND TARGETING OF FLUORESCENT-PROTEIN BASED Ca2+ SENSORS

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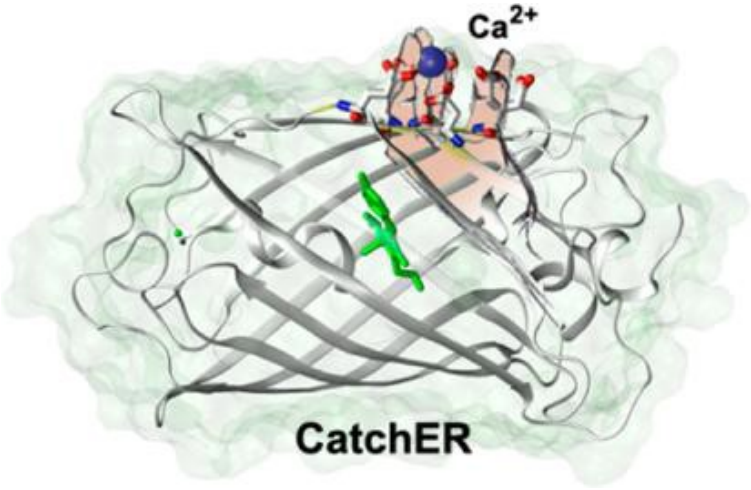

The human body uses many metals in its day to day functions. One such metal is Calcium (Ca^{2+}) commonly known for strengthening bones and teeth. Ca^{2+} plays a role in many biological functions such as gene regulation, the release of neurotransmitters, and muscle contraction. It has also been found to be associated with an assortment of diseases such as cardiovascular disease, dementia, and bone diseases. In order to properly study the intracellular Ca^{2+} dynamics an effective form of measurement must be obtained. While there are many fluorescent chemical dyes and genetically encoded calcium indicators (GECIs) available for intracellular Ca^{2+} measurement they lack targeting capabilities and are sometimes difficult to load into the cell. Our lab has engineered Ca^{2+} sensors using Enhanced Green Fluorescent Protein (EGFP), derived from the wild type Green Fluorescent Protein (GFP), and mCherry, derived from the dsRed protein. These two beta-barrel proteins contain a chromophore located in an alpha-helix running through the center. By substituting amino acid residues on the protein surface for negatively charged ones in the chromophore environment our lab has successfully created a Ca^{2+} binding site on the surface of the proteins. A significant change in the fluorescence is obtained with the binding of Ca^{2+}. Reported here is the latest work on targeting the green sensor CatchER to the membrane of the ER/SR. CatchER has shown strong binding and specificity for Ca^{2+} as well as good optical properties. By adding the ER retention tags it is expressed and retained in the lumen of the ER. The next goal is to anchor the sensor to the membrane of the ER by adding transmembrane domains from known ER membrane proteins. Our lab has also been working to create a pH insensitive red sensor with faster kinetics and greater tissue depth. Work has been made to improve the optical properties of this sensor dubbed RCatchER and the results are shown here. With this work we hope to continue making progress to improve our sensors so that they can be used in further Ca^{2+} studies.

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MOLECULAR MECHANISM OF CONNEXIN26 GAP JUNCTION CHANNEL GATING

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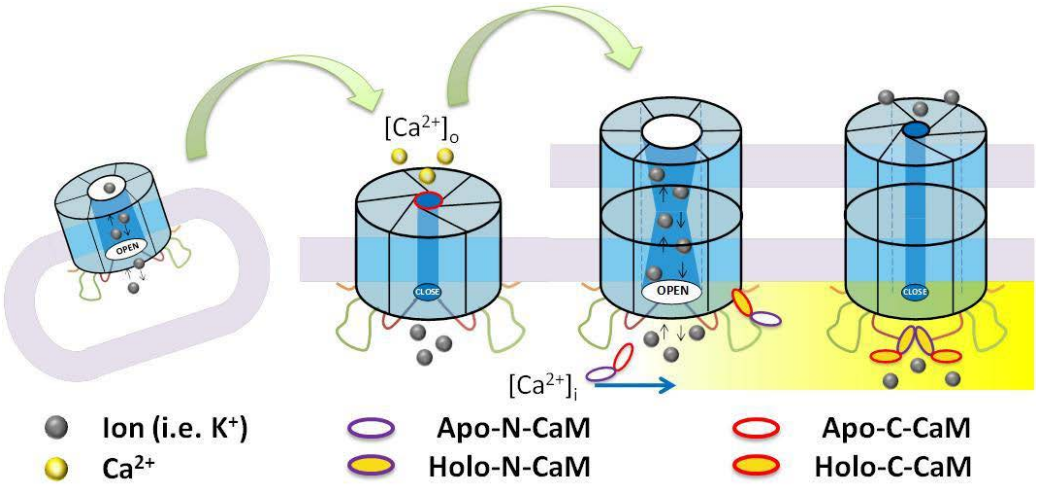

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Gap Junction (GJ) proteins consist of intercellular aqueous channels between adjacent cells that permit exchange of important hydrophilic molecules such as ATP, cAMP, IP3, glutamate, Ca^{2+}, and many more[1]. They play an integral role in maintenance of homeostasis, and vital cellular functions. Connexin26 (Cx26) is the second most ubiquitous GJ proteins, and hundreds of mutations in Cx26 is implicated in many hereditary deafness and dermatological disorders[2]. Understanding the mechanism of GJ gating is crucial to shed light on molecular basis of diseases related to their mutations. However, precise location of the physical gate of these channels is obscure. In our study, we aim to investigate the chemical gating induced by changes in extracellular Ca^{2+} in hemichannels and intracellular Ca^{2+} activated calmodulin protein in GJ channels. We have investigated Ca^{2+} binding affinity, stoichiometry, and Ca^{2+} and CaM induced conformational changes using biophysical methods such as fluorometry, NMR, CD, MS and EM. Furthermore, we have carried out functional studies using electrophysiology and dye transfer assays using mammalian cells to support our in-vitro studies. We have shown Ca^{2+} binding affinity for full length hCx26 to be in mM range, and CaM binding affinity for full length mCx26 and Cx26NTP to be in nM range indicating a direct interaction. Secondary structures of mCx26 was shown to be more stable with the presence of Ca^{2+}, and NMR data supported the conformational change in CaM in the presence of hCx26. By comprehending the structural and molecular basis of gating of hemichannels and GJ channels, we seek to apply it to future therapeutics for GJ related diseases.

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THERMODYNAMIC DISSECTION OF THE INTERFACE BETWEEN SODIUM CHANNEL IQ MOTIFS AND CALMODULIN

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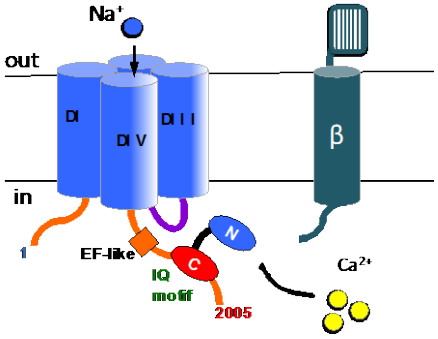
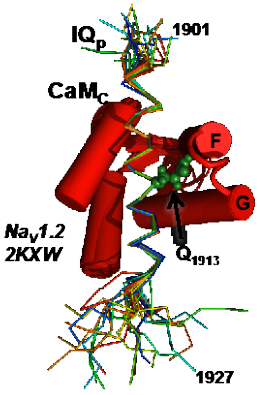
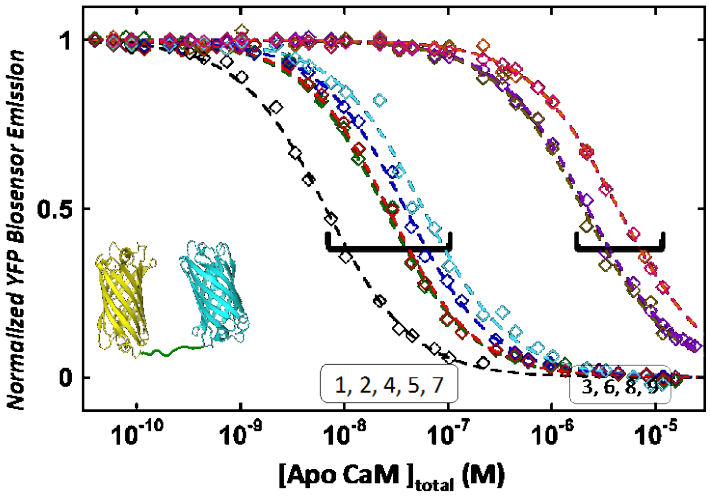
Voltage-gated sodium channels (Na_V) found in excitable cells are responsible for the rising phase of action potentials. These multi-domain transmembrane proteins are regulated by calmodulin (CaM), a highly conserved eukaryotic protein that binds 4 calcium ions to mediate many calcium-triggered signaling events. Fast inactivation of sodium channels, necessary for rapid repolarization of the plasma membrane, depends on Ca^{2+}/CaM-mediated feedback. In the neuronal sodium channel $Na_V1.2$, structures show CaM bound to an intracellular "inactivation" loop between domains DIII and DIV, and an IQ motif [IQRAYRRYLLK] in the cytosolic C-terminal tail. Despite a high degree of sequence identity, the equilibrium constants for CaM binding to nine human Na_V IQ motifs span more than 3 orders of magnitude, with $Na_V1.3$, 1.6,1.8, and 1.9 isoforms only weakly associating with apo CaM. We have investigated the residue-specific thermodynamic determinants of CaM interacting with IQ-motifs by monitoring CaM binding to biosensors containing mutant sequences of sodium channels bracketed by auto-fluorescent proteins YFP and CFP. High-affinity binding was reconstituted by mutating several residues within the $Na_V1.6$ IQ-motif sequence to their $Na_V1.2$ homologues, whereas residues in the post-IQ region appeared to be partly responsible for the weak binding of CaM to $Na_V1.9$. Supported by *NIH R01 GM57001.* **Co-Presenters*







	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
$Na_V1.1$	K	R	K	Q	E	E	V	S	A	V	I	I	Q	R	A	Y	R	R	H	L	L	K	R	T	V	K	Q
$Na_V1.2$	K	R	K	Q	E	E	V	S	A	I	I	I	Q	R	A	Y	R	R	Y	L	L	K	Q	K	V	K	K
$Na_V1.7$	K	R	K	Q	E	D	V	S	A	T	V	I	Q	R	A	Y	R	R	Y	R	L	R	Q	N	V	K	N
$Na_V1.5$	R	R	K	H	E	E	V	S	A	M	V	I	Q	R	A	F	R	R	H	L	L	Q	R	S	L	K	H
$Na_V1.4$	K	R	K	H	E	E	V	C	A	I	K	I	Q	R	A	Y	R	R	H	L	L	Q	R	S	M	K	Q
$Na_V1.3$	K	R	K	Q	E	E	V	S	A	A	I	I	Q	R	N	F	R	C	Y	L	L	K	Q	R	L	K	N
$Na_V1.6$	R	R	K	Q	E	E	V	S	A	V	V	L	Q	R	A	Y	R	G	H	L	A	R	R	G	F	I	C
$Na_V1.8$	R	W	K	Q	E	D	I	S	A	T	V	I	Q	K	A	Y	R	S	Y	V	L	H	R	S	M	A	L
$Na_V1.9$	K	R	K	E	E	E	R	G	A	A	I	I	Q	K	A	F	R	K	Y	M	M	K	V	T	K	G	D

GENETICALLY-ENCODED CHEMILUMINESCENT INDICATOR APPLICABLE IN MILLI-SECOND VOLTAGE PHENOMENA

Shigenori Inagaki[1], Tomoki Matsuda[2], Yoshiyuki Arai[2], Yuka Hinno[3], Hidekazu Tsutsui[3,4], Yashushi Okamura[3], & Takeharu Nagai[1,2]

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Chemiluminescence imaging which doesn't require extrinsic light source has advantages over fluorescence imaging especially in terms of its complete compatibility with optogenetic tools and long-term observation. However, so far limited photon numbers from chemiluminescent proteins restricted use of chemiluminescence imaging to only investigation of slow biological phenomena. In this conference, we introduce Gacky, the world-first chemiluminescent voltage indicator. Since Nanoluc whose brightness is 100 times higher than that of Renilla luciferase is employed as a BRET donor, Gacky enables high-speed (333 frames/s) imaging. Additionally, Gacky successfully visualized voltage change triggered by Channelrhodpsin2 and Halorhodpsin in the single cell, of which genetically-encoded voltage indicators (GEVIs) requiring excitation light are incapable. Morevoer, Gacky is proved to be applicable into drug screening in cardiomyocytes derived from iPSC upon addition of various chemicals for instance causing intracellular Ca^{2+} changes. Voltage imaging done by Gacky completely abolishes excitation light and opens the door to explore detail investigations into fast biological phenomena relating to membrane potential.

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RAGE AS A RECEPTOR FOR EXTRACELLULAR S100 PROTEINS: IS STRUCTURAL PLASTICITY OF THE V-DOMAIN THE KEY TO UNDERSTANDING THE RAGE-S100B INTERACTION?

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The receptor for advanced glycation end products (RAGE) is a multiligand receptor and the main signaling receptor for extracellular S100 calcium binding proteins. The RAGE-S100 signaling axis has been suggested to contribute to the development and progression of several cancers, as well as to brain injury and chronic inflammatory disease states. Recent studies have shown that different RAGE ligands do elicit distinct cellular responses predominantly via binding to the RAGE V-domain.

We hypothesized that structural plasticity of the V-domain is critical for the binding of distinct ligands to RAGE and the formation of structurally distinct complexes capable of ligand-specific signaling.
To test this hypothesis, we have used side directed Trp→Ala mutagenesis of three closely clustered Trp residues within the V-domain and have used biophysical studies to interrogate the role of these Trp residues for V-domain folding stability and binding to S100B.

Our results reveal distinct functions of the three Trp residues for the structural plasticity of the V-domain and subsequently for S100B binding to RAGE. Our X-ray crystallographic data obtained from co-crystals between S100B and RAGE derived peptides suggest at least two distinct binding modes between S100B and RAGE.

Implications of our findings for refined models of RAGE activation by extracellular S100 proteins and discrimination between individual members of the S100-CBP family by RAGE will be discussed.

DENSITY AND TYPES OF CALRETININ- AND PARVALBUMIN-IMMUNOREACTIVE RETINAL GANGLION CELLS IN RABBIT

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Single-cell injection with lipophilic dyes following immunocytochemistry is extremely valuable for revealing the morphology of a cell expressing a protein of interest, and is a more reliable technique for cell type classification than standard morphological techniques. The present study used single-cell injection after immunocytochemistry to describe the density and types of calretinin (CR)- and parvalbumin (PV)-immunoreactive (IR) retinal ganglion cells (RGCs) in rabbit. CR- and PV-IR RGCs were identified by immunocytochemistry and were then iontophoretically injected with a lipophilic dye, DiI. Subsequently, confocal microscopy was used to characterize the morphological classification of the CR- and PV-IR ganglion cells on the basis of their dendritic field size, branching pattern, and stratification within the inner plexiform layer. The results indicated that at least 8 morphologically different types of rabbit RGCs expressd PV, and 10 morphologically different types expressed CR. These RGCs were heterogeneous in their morphology. In addition, CR immunoreactivity was also observed in amacrine cells including AII amacrine cells, and PV immunoreactivity were also found within all cholinergic amacrine cells in the ganglion cell and the inner nuclear layers. The density of CR- and PV-IR cells in the rabbit RGC cell layer was 426 cells/mm$_2$ and 144 cells/mm$_2$, respectively. This approach to integrate the selective expression of a particular protein with spatial patterns of dendritic arborization will lead to a better understanding of RGCs. (NRF-2013R1A1A2059568)

Key words: density, types, retinal ganglion cells, immunocytochemistry, single-cell injection

PARVALBUMIN-IMMUNOREACTIVE CELLS IN THE SUPERIOR COLLICULUS AND VISUAL CORTEX OF DOG: DISTRIBUTION, COLOCALIZATION WITH GAMMA-AMINOBUTYRIC ACID AND COMPARISON WITH CALBINDIN D28K-LABELING

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Although the dog is widely used to analyze the function of the brain, it is not known whether the distribution of calcium-binding proteins (CBP) reflects a specific pattern in the superior colliculus (SC) and visual cortex (VC). We studied the distribution, and morphology of parvalbumin-immunoreactive (PV-IR) cells, and compared PV labeling to that of calbindin D28K (CB) and gamma-aminobutyric acid (GABA) in the SC and VC in dog (*Canis familiaris*). In addition, the effect of monocular enucleation on the PV distribution in the SC was also investigated using immunohistochemistry. PV-IR cells were located throughout the layers with the highest density in layers II/III and IV, while CB-IR cells were predominantly located in layer II/III in the VC. In the SC, PV-IR cells formed three laminar tiers; 1) the upper superficial gray layer (SGL), 2) the lower optic layer (OL) and the upper intermediate gray layer, and 3) the deep layer. The third tier was not very distinct when compared with the other two tiers. The distribution of PV-IR cells is thus complementary to that of CB-IR tiers in the dog SC. Our present data on the distribution of PV-IR cells within the superficial layers are strikingly different from those in previously studied mammals, which show PV-IR cells within the lower SGL and upper OL. PV-IR cells in the dog VC and SC varied dramatically in morphology and size, and included round/oval, vertical fusiform, stellate, horizontal and pyriform cells. Two-color immunofluorescence revealed quantitatively that 11.67% of the PV-IR cells and 11.20% of the CB-IR cells in the SC were colocalized with GABA, while more than 90% of the PV-IR cells and 66.49% of CB-IR cells were colocalized with GABA in the VC of the dog. Monocular enucleation appeared to have no effect on the distribution of PV-IR cells in the contralateral SC. Similar to CB, these data suggest that retinal projection may not control the expression of PV in the dog SC. These data will be informative in appreciating the functional significance of different laminar distributions of CBPs between species. (Dongil Culture and Scholarship Foundation)

Key words: parvalbumin, superior colliculus, localization, enucleation, immunohistochemistry

Ca^{2+} REGULATION OF THE HUMAN CARDIAC SODIUM CHANNEL $Na_V1.5$: CALMODULIN INTERACTING WITH THE FAST INACTIVATION GATE AND EFFECTS OF DISEASE ASSOCIATED MUTATIONS

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Calmodulin (CaM) binding to the human cardiac sodium channel ($Na_V1.5$) Fast Inactivation Gate (FIG) (a linker region between domains 3 and 4) is important for channel inactivation. Previous work from independent laboratories has identified two separate calmodulin binding sites (CBD) contained within the FIG. When studied in isolation, CaM has a modest μM affinity for either CBD site. Using of a FIG construct that contains both CBD sites, CaM binding is significantly increased and a low nM affinity is observed. Importantly, previous results have shown mutations at either of the CBD site are able to modulate $Na_V1.5$ inactivation, suggesting both CBDs are important for proper channel function. Our results indicate CaM interacts with the complete FIG peptide in a manner that is not described by either of the previously published results. Here we present a structural characterization of the molecular details for this novel CaM-FIG interaction. These results are then used to evaluate several heart arrhythmia disease associated mutations that occur in the $Na_V1.5$ FIG and CaM. Preliminary results indicate several of these mutations can disrupt / perturb CaM-FIG complex formation.

NEW CALCIUM IMAGING METHODS FOR THE VISUALIZATION OF LOCAL ACTIVITIES OF ASTROCYTES IN VIVO

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Astrocytes generate Ca^{2+} signals that are thought to regulate their interactions with neurons and other brain cells. *In vivo* Ca^{2+} imaging data with high spatiotemporal resolution may be instrumental for clarification of astrocytic functions. Here we report a new method for *in vivo* astrocytic Ca^{2+} imaging. An ultrasensitive ratiometric Ca^{2+} indicator, YC-Nano50, was expressed in astrocytes by crossing a conditional knockin mouse line harboring the YC-Nano50 gene with an astrocyte-specific driver mouse line. Using the method, we succeeded in detecting a previously unidentified pattern of spontaneous Ca^{2+} signals (Ca^{2+} twinkles), which occur predominantly in the fine processes but not the cell body. Upon sensory stimulation, astrocytes initially responded with Ca^{2+} signals at the fine processes, and the Ca^{2+} signal subsequently propagated to the cell body. Ca^{2+} twinkles and evoked Ca^{2+} signals were partially and fully dependent on the Ca^{2+} release via the type-2 IP_3 receptor, respectively. These results suggest that astrocytic fine processes function as a highsensitivity detector of neuronal activities, and indicate the importance of intracellular Ca^{2+} stores in the regulation of astrocytic functions [1]. Furthermore, we recently developed a family of genetically encoded intraorganellar Ca^{2+} indicators, named CEPIA, which enable visualization of Ca^{2+} dynamics in the endoplasmic reticulum and mitochondria with high spatiotemporal resolution [2]. A knockin mouse line expressing one of the CEPIA indicators is expected to expand the modality of *in vivo* Ca^{2+} imaging. Thus, these methods provide a useful tool to uncover the functions of astrocyte in intact brain. Moreover, choosing an appropriate driver mouse line, one can use these methods to image Ca^{2+} signals in other cell types *in vivo*.

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IPSC-DERIVED CARDIOMYOCYTES FROM CALMODULINOPATHY PATIENTS DEMONSTRATE DEFICITS IN L-TYPE CHANNEL REGULATION: CUES TO DISEASE MECHANISM

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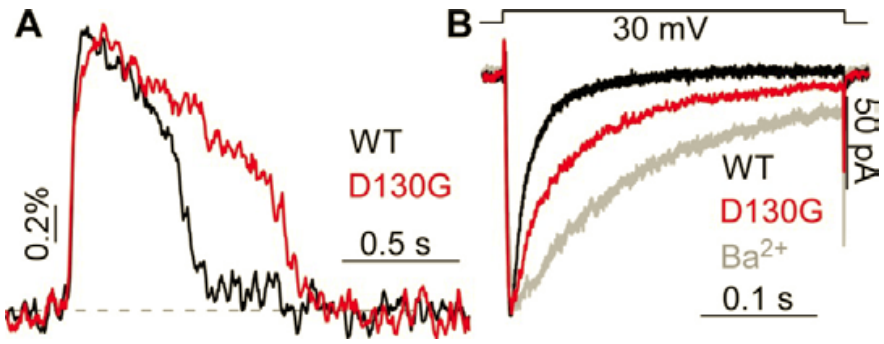
Calmodulinopathies are associated with a small but growing population of patients with severe long QT syndrome (LQTS) and recurrent cardiac arrest. These mutations in calmodulin (CaM) disrupt the Ca^{2+} binding affinity of CaM, implicating a myriad of Ca^{2+}/CaM modulated proteins underlying the disease pathology. As L-type Ca^{2+} channels (LTCCs) play a major role in determining the cardiac action potential duration (APD), disruption of their Ca^{2+} dependent inactivation (CDI) by mutant CaM may be a prominent mechanism leading to LQTS in these patients. Previously, our lab validated the LTCC as a possible contributing factor in mutant CaM related LQTS by demonstrating the ability of overexpressed mutant CaM to disrupt CDI of LTCCs and produce the expected phenotype in guinea pig ventricular myocytes (JMCC **74**:115). However the reliance of these studies on CaM overexpression leaves a major unanswered question: Is a single heterozygous mutation in only one of the three *CALM* genes, as reported in probands, truly sufficient to disrupt CDI of LTCCs and generate the severe LQTS? The preassociation of Ca^{2+}-free CaM with the LTCCs may make it plausible. To test this theory, here we investigate the effect of a heterozygous CaM mutation (D130G) in cardiomyocytes derived from human induced pluripotent stem cells (iPSC-CMs). Taking advantage of ASAP1, a genetically encoded voltage sensor, which robustly reports changes in membrane potential (Nature Neuroscience **17**:884), we are able to follow APD, a cellular correlate of QT interval, as iPSC-CMs mature over time. Indeed, iPSC-CMs harboring the D130G mutation possess significantly prolonged APD compared to wild-type across multiple maturation states, truly recapitulating the phenotype of the proband. Panel **A** shows an example of change of fluorescence intensity of ASAP1 ($-\Delta F/F_0$) in iPSC-CMs (56 days post-differentiation) paced at 0.5 Hz where the APD of iPSC-CMs harboring D130G mutation almost doubles that of the wild-type. Moreover, whole-cell LTCC currents show diminished CDI of the native LTCCs in iPSC-CMs, similar to the observations from overexpressing mutant CaMs in guinea pig myocytes. Panel **B** shows a Ca^{2+} current profile in wild-type 56-day-old iPSC-CMs (black) which decays more rapidly than Ba^{2+} current (gray), indicating the presence of robust CDI in contrast to the sluggish decay of Ca^{2+} current in the mutant counterpart (red). Therefore, a heterozygous D130G mutation in one of the three *CALM* genes is sufficient to suppress the CDI of native LTCCs in patient-derived iPSC-CMs and is likely a key mechanism leading to severe APD prolongation, confirming the prediction drawn from the findings in dissociated cardiomyocytes. Hint



STIM1, STIM2 AND ORAI1 TRANSGENIC MOUSE LINES OVEREXPRESSING KEY STORE OPERATED CALCIUM ENTRY PROTEINS IN NEURONS

Lukasz Majewski, Aleksandra Kurek, Jacek Kuznicki

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Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder. At least two types of AD can be distinguished: sporadic AD (SAD) of unknown etiology, which accounts for most cases, and genetically encoded familial AD (FAD), which affects up to 5% of all patients. Altered calcium homeostasis in neurons is proposed to be one of the early events responsible for AD. Disturbances in Ca2+ signaling are found in SAD patients before any obvious extracellular Aβ pathology, moreover, Ca2+ dysfunction augments Aβ formation and Tau hyperphosphorylation. Our group has shown that the cytoplasmic resting Ca2+ level in cultured neurons can be modulated by overexpression of STIM proteins, ER Ca2+ sensors involved in the Store Operated Calcium Entry (SOCE) [1, 2]. We also detected the enhanced magnitude of Ca2+ influx during SOCE in human lymphocytes from SAD patients [3], and decreased level of STIM2 protein in human lymphocytes from FAD patients in parallel to an attenuation of SOCE [4]. One of the objectives of our present project is to understand how elevated basal Ca2+ level in neurons contributes to neurodegeneration. We have generated three transgenic mouse lines independently overexpressing, specifically in brain neurons, key proteins of SOCE – STIM1, STIM2 and Orai1. The transgene single integration site was confirmed by Southern Blot and FISH technique. As estimated by western blots the level of overexpressed proteins is increased in the brains of transgenic mice. We have started phenotyping these mice using methods of electrophysiology, behavior and Ca2+ imaging.

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PROBING THE STRUCTURAL AND FUNCTIONAL STUDIES OF THE CALCIUM-SENSING RECEPTOR (CaSR)

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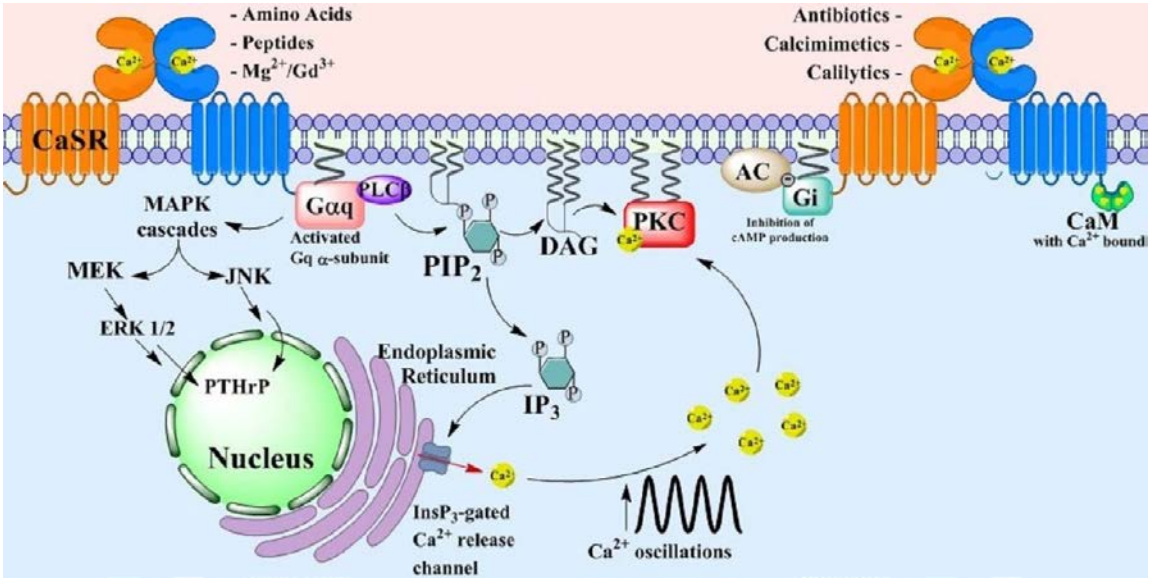
The calcium-sensing receptor (CaSR) is well known for its role in the regulation of the parathyroid hormone (PTH) and maintaining calcium homeostasis. Mutations of CaSR are associated with familial hypocalciuric hypercalcemia (FHH), neonatal severe primary hyperparathyroidism (NSHPT) and autosomal dominant hypocalcemic hypercalciuria (ADHH). However, due to the large size of the extracellular domain (ECD), as well as its high degree of glycosylation, CaSR has neither been visualized using protein crystallography nor NMR. In the present study, we utilized bacterial and mammalian expression of CaSR ECD, where a majority of the ligands interactions occur, to further verify the predicted Ca2+ binding sites. Circular dichroism, fluorescence spectroscopy, and gel electrophoresis have been applied to investigate how calcium modulates the structure of the receptor. In addition, we utilized real-time fluorescence microscopy to examine how calcium induced intracellular calcium signaling could be altered by mutations on Ca2+ binding sites in CaSR transfected HEK 293 cells. The emphasis of this study is to confirm the proposed calcium binding sites within the expressed CaSR and *in vitro*. We have found that Ca2+ binding site mutations lead to a decrease or loss of Ca2+ binding capability as well as a sensitivity reduction *in vitro*.



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INFRARED STUDIES ON THE CA2+-BOUND COORDINATION STRUCTURE OF SYNTHETIC PEPTIDE ANALOGUES OF THE CA2+-BINDING SITE

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Infrared spectroscopy (FTIR) was applied to study the synthetic peptide analogues corresponding to the EFhand motif (helix-loop-helix) of rabbit skeletal muscle troponin C (site III) and *Nicotiana tabacum* calmodulin (NtCaM) isoforms. The regions of the COO– stretches provide information regarding the coordination modes of a COO– group to a metal ion: bidentate, unidentate, and pseudo-bridging [1]. The bands at about 1555-1540 cm-1 for a Ca2+-bound EF-hand motif peptide analogues were assigned to the side chain COO– group of Glu at the 12th position serving as the ligand for Ca2+ in the bidentate coordination mode [2]. As for the site III of rabbit skeletal muscle troponin C, the 17-residue peptide analogue corresponding to loop―helix F seemed to be the minimum Ca2+-binding peptide necessary for the interaction between the side-chain COO– of Glu at the 12th position and Ca2+ in the bidentate coordination mode [3]. First, we examined the 17-residue peptide analogues rabbit skeletal muscle troponin C (site III), where a side chain COO– group of Glu or Asp was labeled by 13C-nuclei. The 13C-labeled COO– group of Glu at the 12th position showed a band at 1526 cm-1 and 1518 cm-1 in the apo and the Ca2+-loaded states, respectively. We confirmed that the band at 1518 cm-1 was due to the 13C-labeled COO– group coordinated to the Ca2+ ion in the bidentate mode. The implications of the 13C-labeled COO– stretches are discussed for an EF hand motif.

Next, we investigated the 17-residue synthetic peptide analogues corresponding to the site I-IV of the three NtCaM isoforms: NtCaM1, NtCaM3 and NtCaM13 [4]. Healthy tobacco leaves mostly contain NtCaM3 and respond to wounding and tobacco mosaic virus (TMV)-triggered hypersensitive reaction by accumulating NtCaM1-type and NtCaM13-type CaMs, respectively [5]. The bands at about 1550 cm-1, which are due to the bidentate coordination mode, were observed for the synthetic peptide analogues for each NtCaM isoform in the Ca2+-bound state, except for the site II of NtCaM13. Some peptide analogues aggregated in the apo and/or Ca2+-loaded states and the Ca2+-bound coordination structure was not formed due to sample aggregation. The FTIR spectra for these peptides are discussed in comparison with those for NtCaM isoforms reported previously [6].

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ARRHYTHMOGENIC CALMODULIN MUTATIONS AFFECT THE ACTIVATION AND TERMINATION OF CARDIAC RYANODINE RECEPTOR MEDIATED CA^{2+} RELEASE

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We recently identified the first two human missense mutations in a calmodulin (CaM) gene (*CALM1*) and linked these to *catecholaminergic polymorphic ventricular tachycardia* (CPVT) and sudden cardiac death in young individuals[1]. More CaM mutations have since been identified in *CALM1* and also in the other two CaM genes (*CALM2 and CALM3)*. All CaM mutations are associated with severe ventricular arrhythmias. CaM regulates several key proteins governing cardiac excitation-contraction coupling (ECC), including the cardiac ryanodine receptor (RyR2) Ca^{2+} release channel. RyR2 mutations also dominantly cause CPVT, where the mutations increase the channel sensitivity to activation and enhance the propensity for pro-arrhythmogenic spontaneous Ca^{2+} release.

Here we investigated the effect of CPVT-linked CaM mutations (N53I and N97S) and two CaM mutations identified in individuals with early onset severe long QT syndrome (LQTS) (D95V and D129G)[2], on spontaneous Ca^{2+} release in HEK293 cells expressing the RyR2 channel. Furthermore, we studied the impact of these mutations on the interactions between CaM and a peptide corresponding to the RyR2 CaM binding domain (CaMBD) residue number 3581-3611, and the Ca^{2+} dependence of this interaction.

In contrast to WT CaM, all four CaM mutations slightly, but significantly, lowered the activation threshold at which spontaneous Ca^{2+} release occurs. In addition, all CaM mutations significantly reduced the threshold at which spontaneous Ca^{2+} release terminates. Taken together, all CaM mutations induced an excessive fractional Ca^{2+} release from internal Ca^{2+} stores.

Interestingly, we demonstrate that the presence of the RyR2 CaMBD decrease the WT CaM C-lobe apparent K_D for Ca^{2+} binding >80 fold to 30 nM. This suggests that Ca^{2+} binding to the C-lobe of CaM would be nearly saturated at diastolic Ca^{2+} concentrations (~100 nM), and that the C-lobe of CaM would constitutively anchor to RyR2 in a Ca^{2+} bound state throughout the ECC cycle. Conversely, the N-lobe of CaM seems poised to be sensing changes in physiological Ca^{2+} with an apparent K_D of ~800 nM in the presence of RyR2 CaMBD.

The D95V, N97S and D129G mutations lowered the affinity of Ca^{2+} binding of the C-lobe of CaM, to apparent K_Ds of ~ 140, 150, and 4000 nM, respectively, consistent with the critical role of these residues in Ca^{2+} binding to the C-lobe. Thus, we suggest that these mutations may shift to an apo-CaM binding state during diastole, leading to dysregulation of RyR2 mediated Ca^{2+} release.

Despite the pronounced impact on RyR2 mediated Ca^{2+} release, the N-lobe N53I mutation only imposed a small lowering of the N-lobe Ca^{2+} affinity (K_D ~1200 nM). Thus, the RyR2 mediated Ca^{2+} release is either highly sensitive to minor changes in CaM N-lobe Ca^{2+} affinity, or the N53I mutation perturbs interactions between CaM and RyR2 outside the 3587-3605 CaM binding domain.

We conclude that dysregulation of RyR2 mediated SR calcium release is likely a major molecular disease determinant of CPVT-linked CaM mutations, and an important component of the clinical manifestation for LQTS-causing CaM mutations.

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OPPOSING ORIENTATIONS OF THE ANTI-PSYCHOTIC DRUG TRIFLUOPERAZINE SELECTED BY ALTERNATE CONFORMATIONS OF M144 IN CALMODULIN

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The anti-psychotic drug trifluoperazine (TFP) is used to treat multiple mental illnesses by inhibition of dopamine receptors and GABA release. TFP is recognized as an antagonist of calmodulin (CaM), an essential eukaryotic calcium-receptor that regulates a multitude of physiological processes including response to inflammation, muscle contraction, intracellular movement, and memory. These essential roles of CaM in health and disease make any pharmacophore-CaM interactions important because they have the potential to disrupt CaM-mediated pathways. TFP has been observed to bind to calcium-saturated calmodulin ($(Ca^{2+})_4$-CaM) at ratios of 1:1 (1CTR), 2:1 (1A29), and 4:1 (1LIN). Each structure contains one TFP bound in the hydrophobic cleft of the C-domain of CaM. However, the orientation of the trifluoromethyl (CF_3) moiety differs among them: it is buried in the C-domain cleft of 1A29 and 1LIN, but protrudes from 1CTR. Because CaM, a small highly acidic protein, is vital to all eukaryotes, it is of interest to understand whether these differences were induced by solvent conditions in crystallization or whether they are evidence of deeper plasticity of the clefts of CaM.

We report a 2.0 Å resolution crystallographic structure (4RJD.pdb) of TFP bound to the (Ca^{2+})-saturated C-domain of CaM (CaM_C) [1]. The asymmetric unit contains two molecules of $(Ca^{2+})_2$-CaM_C. Chain backbones were nearly identical, but the orientation of TFP in the cleft of chain A matched 1A29/1LIN, while TFP bound to chain B matched 1CTR. This was accommodated by a flip of the M144 sidechain and small changes in sidechains of M109 and M145. Docking simulations suggested that the rotamer conformation of M144 determined the orientation of TFP within the cleft of $(Ca^{2+})_2$-CaM_C. Chains A and B show that the open cleft of $(Ca^{2+})_2$-CaM_C is promiscuous in accepting TFP in reversed directions under the same crystallization conditions. Observing multiple orientations of an antagonist bound to a single protein highlights the challenge of designing highly specific pharmaceuticals, and may have importance for QSAR of other CF_3-containing drugs such as fluoxetine (anti-depressant) or efavirenz (reverse transcriptase inhibitor). This study emphasizes that a single structure of a complex represents an energetically accessible state, but does not necessarily show the full range of energetically equivalent states. Supported by NIH R01 GM57001.

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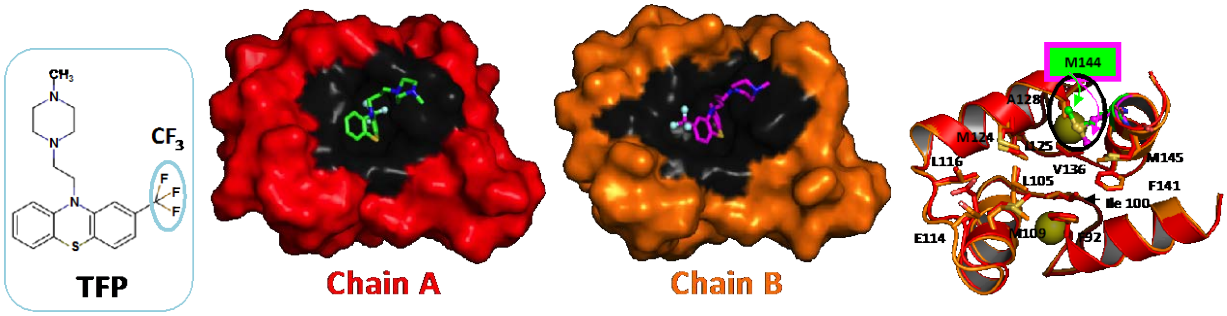

NOVEL CENTRIN-PRP40 COMPLEX: ITS MOLECULAR BIOPHYSICAL CHARACTERIZATION AND CRYSTAL SCREENS

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Centrins are calcium binding proteins belonging to the EF-hand superfamily[1,2,3]. This protein has been found localized to the nucleus along with XPC and RAD23 in the regulation of DNA excision repair[4-6]. More recently, centrin was found to be involved in mRNA export[6]. Prp40 homologue A (Prp40A) is a nuclear protein associated with spliceosome assembly and pre-mRNA splicing. We identified the Prp40A to contain the hydrophobic triad $W_1L_4L_8$ this sequence pattern was found to overlap the Nuclear Export Signal (NES) site[7]. High resolution structure determination has only been ascertained for several domains of this protein. Prp40 is comprised of two tandem WW domains followed by six FF domains, which were found to mediate Prp40-target interactions[7]. Herein our group has provided molecular biophysical evidence through a fragment based drug design approach that validates Prp40A-centrin interaction. Different centrin-Prp40A complexes were studied by Isothermal Titration Calorimetry (ITC), FT-IR and 2D IR correlation spectroscopy. The actual Prp40A peptide studied contained the NES and its sequence was comprised of the following Ac-K_{524}QLRKRNWEALKNILDNMANVTYSTTWSEAQQY$_{556}$-NH_2 referred to as $Prp40Ap_1$ and different centrins. We performed 2D IR correlation spectroscopy of ^{13}C-labelled *Chlamydomonas reinhardtii* centrin (^{13}C-*Crcen*)-$Prp40Ap_1$ at 1:1 mole ratio under varying temperatures. FT-IR spectral data was acquired at 5^oC intervals within the temperature range of 5-95^oC. The use of isotope labelling allowed for the simultaneous monitoring of both centrin and the $Prp40Ap_1$ peptide. 2D IR correlation spectroscopy was used to enhance the spectral resolution and ascertain the molecular events that led to complex formation. Also cross peaks were evident that confirmed the interaction between centrin and Prp40Ap1. In addition, comparative calorimetric study between *Hs*cen2-$Prp40Ap_1$ and *Chlamydomonas reinhardtii* centrin (*Crcen*)-$Prp40Ap_1$ complexes at 25°C were performed. *Cr*cen-$Prp40Ap_1$ complex presented the highest stability and affinity. Also, we explored the interaction between *Cr*cen and $Prp40Ap_1$ as a function of temperature. At a temperature near physiological condition (35°C), the complex exhibited the highest stability (ΔG = -9.49 ± 0.04 kcal/mol) and affinity (K_a = 5.3 ± 0.2 x106 M^{-1}) with a favorable enthalpy (ΔH = -12.1 ± 0.6 kcal/mol) and unfavorable entropy ($-T\Delta S$ = 2.6 ± 0.6 kcal/mol). From the analysis of the binding enthalpies as a function of temperature, we a obtain a change in binding heat capacity (ΔCp) of -0.2402 kcal/mol°C indicating a decrease in exposure of the hydrophobic surface as a result of the interaction between *Cr*cen and $Prp40Ap_1$. Finally, in collaboration with the BioXFEL STC we have performed crystallization screenings of the *Cr*cen-$Prp40Ap_1$ complex. Discussion of the biological implications will be presented.

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DESIGN, OPTIMIZATION, AND ANALYSIS OF GENETICALLY-ENCODED CALCIUM PROBES FOR SUB-CELLULAR APPLICATION

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Calcium is a key effector of many processes necessary for sustaining life. Signaling involving calcium is mediated by many important receptors and binding proteins. The endoplasmic and sarcoplasmic reticulum (ER/SR) are at the center of the calcium signaling cascade with the ability to respond to and release calcium to propagate the stimulus. There is still much to be determined about the rapid, receptor mediated ER/SR calcium dynamics in different cell types and physiological events such as excitation-contraction (E-C) coupling occurring at the junctional face membrane in skeletal muscle cells. We have designed a single fluorophore sensor CatchER to monitor rapid calcium dynamics in the ER/SR of HEK293, C2C12, and FDB muscle fibers. Here, we first report the improvement of the folding and brightness of CatchER in mammalian cells at 37˚C. Compared to CatchER, the new CatchER-T' variant has a 3.5 fold increase in brightness at 37˚C and *in situ* K_D values of 3.2 ± 1.4 mM (HEK293, n = 11) and 3.1 ± 1.4 mM (C2C12, n = 7), which are 3 times the *in situ* K_D of CatchER. We have also created E-CatchER with a 6-fold improvement of calcium binding affinity. We further report the application of developed sensors to monitor both the global and local calcium release dynamics in C2C12 cells with our catchER-JP45 construct. Our sensors will enable the direct monitoring of calcium dynamics in both normal and pathological processes

FAMILIAL ALZHEIMER'S DISEASE MUTATION IN PRESENILIN-1 GENE LEADS TO AN ABNORMAL STORE-OPERATED CALCIUM CHANNELS ACTIVITY INVOLVED IN THE MEMORY LOSS

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Presenilins have been reported to regulate calcium homeostasis in the endoplasmic reticulum, and dysregulation of intracellular calcium has been implicated in the pathogenesis of Alzheimer's disease. Elevated presenilin-1 (PS1) holoprotein levels have been detected in postmortem brains of patients carrying familial Alzheimer's disease (FAD) PS1 mutations. This study deals with the effect of the FAD presenilin mutant that lacks 9 exon (PS1 DE9) and does not undergo an endoproteolysis on store-operated calcium (SOC) entry. Significant enhancement of SOC channels activation has been detected by electrophysiological measurements in hippocampal neurons with PS1 DE9 mutant expression. Moreover, it has been shown that this effect on SOC entry is due to hyperactivity of STIM1 sensor, detected by live cell imaging, and could be attenuated by STIM1 knock-down or 2-APB application. In the same time, the knock-down data have indicated that STIM1 did not regulate SOC channels activity in healthy hippocampal neurons. Transgenic Drosophila fly with expression of PS1 DE9 in cholinergic neuron system has shown a short memory loss, which can be abolished by a 2-APB feeding. All together data suggest that STIM1 sensor could be a possible target for memory loss treatment in FAD caused by PS1 DE9 mutant.

This work was supported by the Russian Scientific Foundation, project no. 14-14-00720, the program of "Molecular and Cellular Biology" RAS, the Russian Basic Research Foundation no. 14-04-31280, the President of Russia Scholarship.

CALMODULIN LOBES FACILITATE DIMERIZATION AND ACTIVATION OF ESTROGEN RECEPTOR-α

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Estrogens exert pleiotropic functions in humans, ranging from neuroprotection and prevention of osteoporosis to cardioprotection and growth of breast tissue. These effects are produced through two members of the nuclear receptor superfamily, estrogen receptor-α (ER-α) and ERβ. In the classic signaling mechanism, the hormone estrogen induces a conformational change in ER-α, which then dimerizes. The estrogen-bound receptor translocates to the nucleus where it binds to DNA at estrogen response elements (EREs) and recruits co-regulatory proteins. These co-activators and co-repressors modulate the transcriptional activation of genes by ER-α. Calcium-dependent regulation of ER-α is critical for activating gene expression and is controlled by calmodulin (CaM). Here we present the NMR structure of Ca^{2+}-bound CaM bound to two molecules of ER-α (residues 287-305). The two lobes of CaM bind to the same site on two separate ER-α molecules (residues 292, 296, 299, 302 and 303), which explains why CaM binds two molecules of ER-α in a 1:2 complex and stabilizes ER-α dimerization. Exposed glutamate residues in CaM (E11, E14, E84, E87) form salt bridges with key lysine residues in ER-α (K299, K302 and K303), which is likely to prevent ubiquitination at these sites and inhibit degradation of ER-α. When expressed in cells, the individual lobes of CaM co-immunoprecipitated with ER-α. Transfection of cells with full-length CaM slightly augmented the ability of estrogen to enhance transcriptional activation by ER-α of endogenous estrogen-responsive genes. By contrast, expression of the separate lobes of CaM abrogated estrogen-stimulated transcription. CaM facilitates dimerization of ER-α in the absence of estrogen, and stimulation of ER-α by either Ca^{2+} and/or estrogen may serve to regulate transcription in a combinatorial fashion. Our data suggest that small molecules that selectively disrupt the interaction of ER-α with CaM may be useful in the therapy of breast carcinoma.

Ca^{2+}/CALMODULIN INTERACTS WITH CONNEXIN43 GAP JUNCTION PROTEIN TO REGULATE THE CHANNEL GATING

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Gap junction is a specialized intercellular connection between a multitude of animal cell-types which directly connects the cytoplasm of two cells and allows electrical and chemical communication and various ions and molecules such as nutrients and metabolite to pass through the channel. Gap junctions are formed by the docking of two gap junction hemichannels (connexins) between neighboring cells, and they have four trasnmemebrane domains, linked by one intercellular and two extracellular loops, with NH_2- and COOHtermini both located in the cytosol. Gap junctions play an important role in maintaining homeostasis and cell and tissue function. Connexin43 is a member of the α family of connexins, the most abundantly expressed human connexin. The low intercellular resistance pathway formed by connexin 43 (Cx43) gap junctions in the working myocardium is critical for propagating electrical signals to allow rapid, synchronized contraction. Loss of function and connexin mutations have been linked to several serious human diseases, including oculodentodigital dysplasia (ODDD) for Cx43. A number of studies indicate that chemical gating of gap junction induced by changes in $[Ca^{2+}]_i$ or $[H^+]_i$ may be mediated by the ubiquitously expressed intracellular Ca^{2+}-binding protein, calmodulin (CaM). Connexin43, which is a CaM target protein, is a membrane protein. Therefore, it is difficult to study the complex due to the challenges associated with the membrane proteins such as; difficulty in expression and purification, low solubility and conformational flexibility. Therefore, the intracellular regions of Cxs are largely "invisible" in the reported X-ray structures. We have reported that CaM interacts with transmembrane region of cytosolic loop (CL) of the major heart connexin, connexin 43, to regulate intracellular channel gating activities, however the role of transmembrane (TM) region adjacent to the loop and the specific residues responsible for the interaction and regulation is still unknown. While several heart disease mutations such as ODDD (occulo-dentodigital dysplasia disease) in these regions were reported, molecular basis for alteration of the cardiac gap junctions via CaM-binding is not yet to be determined due to limitations associated with expression and structural studies of membrane proteins.

Using the unique grafting and peptide/domain approaches developed in our lab, we have obtained preliminary results and they suggest that transmembrane region adjacent to the central loop of connexin 43 is essential for binding to Ca^{2+}/calmodulin. Determination of the mechanism of the interaction between CaM and transmembrane region of cytosolic loop of Cx43 can lead to understand the regulatory properties of cardiac gap junctions formed by Cx43 and molecular basis of heart disease-linked mutations on gap junctions and the prevention of the diseases.

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REGULATION OF THE NEUTROPHIL NADPH OXIDASE AND RELEASE OF S100A8/A9

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S100A8 and S100A9 are members of the S100 sub-family of cytoplasmic EF-hand Ca^{2+}-binding proteins and are abundantly expressed in the cytosol of circulating neutrophils. Formation of Ca^{2+}-dependent heterodimers and heterotetramers [1] is likely a pre-requisite for S100A8/A9 biological activity [2]. At the intracellular level, S100A8 and S100A9 are described, among other, to regulate NADPH oxidase (NOX) activity [3], the major source of reactive oxygen species in neutrophils. These reactive oxygen species are designed to kill invading pathogens but can cause tissue damage and perpetuation of the inflammation when they are excessively produced. In addition to their intracellular roles, S100A8 and S100A9 can be secreted in the extracellular environment and are considered as damage-associated molecular pattern molecules, which amplify the pro-inflammatory response either by activating neutrophils (autocrine mode of action) or other inflammatory cell types (paracrine mode of action) [4]. High concentrations of S100A8/A9 are found at local sites of inflammation or in the serum of patients with inflammatory diseases (e.g. rheumatoid arthritis) [5] and can be used as inflammation biomarkers [6]. Although S100A8/A9 secreted by neutrophils are of importance in the pathophysiology of many inflammatory diseases, the mechanisms by which these proteins are released remain unknown. S100 proteins do not possess the leader sequence typical for transport via the classical endoplasmic reticulum/Golgi pathway and thus are released by an alternative secretory pathway. In this view, it has been suggested that, in monocytes, this non-classical S100A8/A9 secretion is an energy-dependent process, which depends on an intact microtubule network and PKC activation [7].

In this work, we focused both on the intracellular role of S100A8/A9 in the regulation of NOX activity and on the secretion of S100A8/A9.

NOX activation is regulated by the integration of diverse signaling pathways, such as Ca^{2+} signaling and kinase activation. S100A8/A9 could constitute the molecular switch between the Ca^{2+}-dependent signaling cascade and NOX activation. Therefore we characterized S100A8/A9-dependent mechanisms of NOX regulation in neutrophil-like HL-60 cells (dHL60).

The second part of our work intended to study the release of S100A8/A9 from neutrophils. Our results on dHL60 cells and purified human neutrophils show a time-dependent secretion of S100A8/A9 when induced by PMA but not by fMLF or LPS. Further, we observe the formation of neutrophil extracellular traps after 4h PMA but not after fMLF or LPS stimulation both in dHL60 cells and neutrophils. Thus, S100 secretion is only found in conditions where the dHL60 cells/neutrophils produce neutrophil extracellular traps, leading to the postulation that S100A8/A9 could be released by NETosis in human neutrophils.

Clearly, S100A8/A9 are involved in the pathophysiology of many inflammatory diseases by both their intracellular and extracellular functions. Thus, understanding more precisely the mechanisms by which they are secreted as well as their various functions will be a major advance for the development of a selective therapy.

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ENDOGENOUS STORE-OPERATED CALCIUM CHANNELS REGULATED BY STIM2 PROTEINS IN HEK293 CELLS

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Calcium sensors of endoplasmic reticulum - Stim proteins - activate store-operated channels after calcium store depletion. Two isoforms Stim1 and Stim2 differ by their calcium sensitivity.

In cell-attached mode there are three types of endogenous calcium channels in HEK293 cells: I_{max}, I_{NS} and I_{min}. In our single-channel patch clamp experiments on HEK293 cells, selective activation of native Stim2 proteins or Stim2 overexpression results in store-operated activation of I_{min} channels, while Stim1 activation blocks this process. Changes in the ratio between active Stim2 and Stim1 proteins can switch I_{min} channels regulation between store-operated and store-independent modes. The results of this study show that Stim1 and Stim2 differ in the ability to activate endogenous store-operated channels: I_{min} channels are regulated by Stim2, TRPC3-containing I_{NS} channels are induced by Stim1, and TRPC1-composed I_{max} channels are activated by both Stim1 and Stim2. These new data about cross-talk between Stim1 and Stim2 and their different roles in store-operated channel activation are indicative of an additional level in the regulation of store-operated calcium entry pathways.

In further experiments we study effects of store-operated channel modulator 2-APB and overexpression of dominant negative Orai1 E106Q or Orai3 E81Q on activity of endogenous I_{min} channels.

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CALCIUM DYNAMICS AS A POTENTIAL READOUT OF MECHANOTRANSDUCTION AT EPITHELIAL WOUNDS

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When epithelial tissues are wounded, the earliest observed response is a calcium wave. The calcium wave occurs immediately upon wounding, originating at the wound site and spreading through gap junctions multiple cell lengths away from the wound. We hypothesize that this calcium wave is triggered by changes in the mechanical properties of a tissue at wound sites and then drives downstream wound response programs. Although it is known that this calcium wave is important for wound healing, less is known about what induces this earliest wound response.

We have characterized this conserved calcium wave using *in vivo* live imaging of the *Drosophila* pupae notum. Preliminary results reveal three distinct stages of the calcium wave in the pupae notum: an initial release, a controlled spread, and a stochastic wave. The initial release appears multiple cell lengths away from the wound immediately upon wounding. We predict that this calcium is released via mechanically gated channels or via channels that bind a diffusible factor originating from the site of the wound. The second stage is a controlled spread of calcium away from the initial release occurring seconds after wounding. Minutes after wounding, in the third stage, we observe a stochastic wave characterized by random flashes of calcium that flare around the wound.

I will correlate the changes in force distribution across the tissue at wounds with the calcium wave using CellFIT analysis (a tool developed by the Hutson lab). I will then use novel genetic tools to manipulate tissue tensions prior to wounding to rigorously test their role in the wound response. Understanding the environmental cues that trigger a wound response will help us understand normal wound healing and disease states such as cancer.

IDENTIFICATION OF SCGN AS A CYTOSOLIC INSULIN BINDING PROTEIN

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Secretagogin (SCGN) is a sparsely explored hexa EF-hand Ca2+ binding protein abundantly expressed in β-pancreatic cells. It is implicated in insulin and corticotropin-releasing hormone (CRH) release and is shown to favour cell survival. In our in-silico analysis (and in previous reports), we observed a striking subsistence of secretagogin at all possible locations of insulin existence. This observation prompted us to explore the functionality of the coexistence of two critical proteins. We report here an extremely important feature of secretagogin, that is, it binds insulin. Our data evoke a soft role of Ca2+ on the complex formation. Weaker affinity (albeit strong enough to make a stable complex in the presence of Ca2+) may also suggest an insulin transporter like function of secretagogin, which binds/releases insulin at specific Ca2+ signals. We elucidate that secretagogin protects insulin from reducing agent induced aggregation in a Ca2+ independent manner which suggests a chaperone like function of secretagogin for insulin. This observation suggests that secretagogin might be assisting insulin to fold correctly in-vivo. In addition, treatment of insulin responsive C2C12 cells with (SCGN + Insulin) complex shows less response than equimolar dose of free Insulin possibly due to much slower dissociation rate in the cell culture media allowing a sustained release of Insulin pointing towards a medicinally interesting property of SCGN. Since decline in insulin level/effectiveness (i.e., diabetes), with the ever increasing victims, is among the most rampant disease world-wide, our study implies that secretagogin might become a novel target for developing future therapeutics for diabetes and other insulin-related pathologies.

CALCIUM SIGNALING DYNAMICALLY CONTROL CHAPERONE SYSTEM IN MITOCHONDRIA TO REGULATE LEAF SENESCENCE

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Calcium signaling plays an essential role in plant cell physiology, and chaperone-mediated protein folding directly regulates plant programmed cell death. However, how these two processes crosstalk in plants remains unknown. The *Arabidopsis thaliana* protein AtBAG5 (Bcl-2-associated athanogene 5) is unique in that it contains both a BAG domain capable of binding Hsp70 and a characteristic IQ motif that is specific for Ca2+-free CaM binding and hence acts as a hub linking calcium signaling and the chaperone system. Here, we demonstrate that AtBAG5 localizes to mitochondria and that its overexpression leads to leaf senescence symptoms including decreased chlorophyll retention and massive ROS production in dark-induced plants. Structural and biochemical studies revealed that Ca2+-free CaM and Hsp70 bind AtBAG5 independently, whereas Ca2+-saturated CaM and Hsp70 bind AtBAG5 with negative cooperativity. Additional *in vivo* studies confirmed that extracellular calcium can suppress premature senescence in dark-induced leaves of transgenic plants that overexpress AtBAG5. Collectively, we propose a molecular mechanism in which CaM senses Ca2+ to dynamically control the binding of AtBAG5 to Hsp70 in mitochondria, leading to an alteration in the ROS level and senescence-associated gene expression, thus regulating plant senescence.

SUPER-RESOLUTION TRACKING OF NATIVE IP3R1 IN A PA-MCHERRY-IP3R1 KNOCKIN MOUSE MODEL

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Puffs are local Ca2+ signals that arise by Ca2+ liberation from the endoplasmic reticulum (ER) through tightly clustered inositol trisphosphate receptors (IP3Rs). Local Ca2+ signals arise at just a few, fixed locations within a cell, suggesting clusters are stable entities; and Ca2+ blips generated by 'lone' IP3Rs are also immotile. In contrast, GFP-tagged IP3Rs show a dense distribution throughout a cell with the majority of GFP-tagged IP3Rs able to diffuse freely within the ER membrane.

These paradoxical observations may be explained because Ca2+ imaging studies detect only functional IP3Rs, whereas imaging studies utilizing GFP-tagged IP3Rs report on the entire population of IP3R proteins. Using super-resolution imaging of photoactivatable mCherry (PAmCherry) tagged IP3R1 over-expressed in COS-7 cells, we have previously shown that the majority (>70%) of IP3Rs were freely motile whereas the remaining molecules were immotile; and that a fraction of these immotile molecules were clustered with dimensions (a few hundred nanometers across) comparable to those previously estimated for IP3R clusters underlying functional puff sites. This led us to speculate that these immotile clustered IP3R molecules underlie local Ca2+ puffs [1].

In common with all such overexpression systems, there are concerns as to whether the over-expressed fluorescently tagged IP3R protein truly reflects the functioning and localization of the native protein. We have thus generated a transgenic mouse where PAmCherry has been knocked into the ITPR1 locus. In this way all native IP3R's express the photoactivatable protein PAmCherry tagged onto their N-terminus.

Our preliminary tracking of tagged native receptors in fibroblasts, astrocytes and cortical neurons from the transgenic mouse show that in contrast to our over-expression studies, the majority of IP3Rs are immotile, with a smaller population freely motile. Furthermore, we find that a proportion of these immotile molecules are clustered and are preferentially localized near to sites of local Ca2+ release.

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KNOCKING DOWN MITOCHONDRIAL CALCIUM UNIPORTER AMELIORATES DOPAMINERGIC NEURONAL LOSS IN PINK1 MUTANT ZEBRAFISH

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Parkinson's disease (PD) is a progressive neurodegenerative disease that commonly affects the elderly and remains incurable. PTEN-induced putative kinase 1 (PINK1) is a mitochondrial serine/threonine-protein kinase and loss of function mutation in *PINK1* leads to familial PD. One of the attributed mechanisms behind dopaminergic (DA) neuronal loss in PD is mitochondrial dysfunction. In our study we are trying to understand the role of calcium homeostasis in mitochondrial dysfunction during pink1 deficient condition. We used a *pink1* mutant zebrafish line with a premature stop mutation (Y431*) in the *Pink1* kinase domain as the modal organism1. Our approach was to alter multiple mitochondrial Ca2+ influx mechanisms in *pink1-/-* zebrafish and to evaluate the resulting molecular and cellular changes in respect to PD pathology. We used morpholino based knockdown strategy to silence mitochondrial calcium uniporter (*mcu)* and voltage dependent anion channel (*vdac1),* which are responsible for Ca2+ influx into mitochondria. We analyzed dopaminergic neuronal loss by *in situ* hybridization using tyrosine hydroxylase riboprobe. We also studied mitochondrial complex I-IV activity to evaluate the effect of mcu knockdown on mitochondrial respiration. Our results shows that alteration of Ca2+ sequestration through knockdown of *mcu*, but not *vdac1* leads to restored mitochondrial respiration and thereby contributing to viable DA neurons in *pink1 -/-* zebrafish.

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CHARACTERIZATION OF A NOVEL ECHINODERM PUTATIVE CALCIUM BINDING PROTEIN FROM THE SEA CUCUMBER *HOLOTHURIA GLABERRIMA*

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Holothuria glaberrima exhibits the capacity to regenerate body parts after injury or loss. This ability is mediated by an initial blastemal formation which requires proliferation and reorganization of precursor cells. Accordingly, various molecules play key roles in different aspects of the regeneration of a body part. The use of microarrays and high-throughput sequencing lead our laboratory to identify a series of new molecules associated to different processes of regeneration. Such is the case for the protein Orpin.

Based on an EST database, we found that Orpin gene is over expressed at early stages of intestinal regeneration. Its expression occurs concomitantly with remodeling of the extracellular matrix. Also, another isoform was identified and both sequences were present in libraries from regenerating nerve and intestinal tissues. Sequence analyses revealed both sequences encode two EF-hand motifs. Unlike other known calcium binding proteins, they have a signal peptide. Orpin antibodies display labeling mainly of the mesentery epithelium attached to the intestine of non-regenerating sea cucumbers. Furthermore, these antibodies are being used to determine the tissue and temporal specificity of Orpin isoforms. In order to evaluate the effect of Orpin during regeneration, recombinant fusion proteins are expressed to be injected into regenerating animals. Thus, determining the role of Orpin isoforms during a regenerative process would help to elucidate the molecular mechanisms involved in different aspects of nerve and intestinal regeneration.

IN VITRO ANALYSIS OF HUMAN S100 PROTEIN HETERODIMER COMPLEX FORMATION

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The S100 proteins are dimeric Ca^{2+}-binding sensor proteins that are comprised of two EF-hand motifs per protomer. There are 27 members of the human S100 protein family with each being preferentially expressed in different tissues or cell types throughout the body [1,2]. The S100 proteins are involved in numerous intracellular regulatory pathways including protein phosphorylation, enzyme activity, cytoskeletal reorganization and gene transcription. Most S100 proteins that have been isolated from tissues and biophysically characterized are noncovalent homodimers comprised of two identical polypeptide chains. However, heterodimeric S100 proteins with two different polypeptide sequences have also been observed. Calprotectin (S100A8-S100A9) is a prime example because of its strong preference to form heterodimers [3,4]. S100A8 and S100A9 proteins are almost always co-expressed and function together in mediating macrophage recruitment to sites of vascular injury [5] and exacerbating reperfusion following an ischemic event [6]. Other S100 heterodimer complexes including S100B with S100A1, S100A6 and S100A11 have also been characterized by using yeast-two hybrid experiments [7,8]. However, the identification and analysis of homo- and heterodimer populations of S100 proteins has proven quite difficult and the preference for the formation these different species is poorly understood. In this work, the first steps towards determining the relative strengths of homo- and heterodimer S100 protein interactions and the relative populations of each complex were examined. We have designed and developed a high-yield *Escherichia coli*-based human S100 protein co-expression system using the pETDuet-1 expression plasmid (Novagen) for the purpose of creating homo- and heterodimeric S100 protein complexes *in vitro*. Using ESI-MS, 2D NMR spectroscopy, and a split-GFP fusion protein system to observe and biophysically characterize these S100 protein complexes, our work aims to provide better insight and context for the formation of homo- versus heterodimeric S100 protein complexes.

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S100A4: KEY PLAYER IN CANCER METASTASIS AND SIGNALING-BASED INTERVENTIONS FOR METASTASIS RESTRICTION

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We identified the metastasis gene S100A4 as transcriptional ß-catenin target [1]. S100A4 led to strongly increased migration and invasion in cell culture, and to the development of liver and lung metastases in xenografted mice. We demonstrated in patient samples of primary CRC that high expression of S100A4 predicts metastases formation and reduced patient survival. RAGE mediates S100A4-induced effects and is a prognostic biomarker for human metastasis by itself [2,3]. Our results link pathways important for tumor progression and metastasis: the Wnt signaling pathway and S100A4, which regulates motility, invasiveness and inflammation [1,4]. Very recently, we discovered the reciprocal interplay of S100A4 with Wnt antagonists, with S100A4 as the predominant actor [submitted].

S100A4 suppression has potential for therapeutic intervention [5]. We established in vivo intervention strategies targeting S100A4 for metastasis inhibition. As proof of principle, we applied shRNA acting on S100A4 systemically and prevented metastasis in mice [6]. Furthermore, we identified small molecule inhibitors from high-throughput screens (HTS) of pharmacologically active compounds employing an S100A4 promoter-driven reporter [7-9]. Best hits act via intervening in the Wnt pathway. S100A4 inhibitor treatment reduced cell motility and, importantly, restricted metastasis in mouse models.

We are now translating our findings on restricting S100A4-driven metastasis into clinical practice. Our assay for circulating S100A4 transcripts in patient blood is used to monitor treatment success [10]. These repositioned FDA-approved inhibitory compounds, which we identified via HTS, are currently being tested in two prospective phase II clinical trials for treatment of CRC patients.

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COMPREHENSIVE IDENTIFICATION OF HUMAN CALMODULIN-TARGETS

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Calmodulin (CaM) is an intracellular Ca^{2+}-signal transducer that can interact and regulate the biochemical functions of numerous proteins, including enzymes, receptors and cytoskeletal proteins in a Ca^{2+}-dependent manner. Recent studies using a proteomic approach have successfully identified CaM-binding proteins including novel CaM-targets such as Wolframin and PRG-1 (plasticity related gene 1) [1,2]. The proteomic approach for identification of CaM-target proteins is initiated by purification of CaM-targets from tissue or cell extracts by using CaM-coupled affinity matrix, followed by identification of purified proteins by LC-MS/MS (liquid chromatography tandem MS). However, partially purified proteins by the affinity chromatography are not necessarily to be *bona fide* CaM-target proteins since many proteins interacted with CaM-targets, which are often co-purified by this method. Therefore, in order to overcome such disadvantage of the proteomic approach, O'Connell *et al.* reported a protein array screening of 10,000 unique human neural proteins on PVDF membrane with fluorophore-labeled CaM and identified 72 novel CaM-targets [3]. Here, we have used protein arrays (Protein Active Array®) carrying human full-length cDNAs derived GST-fusion proteins synthesized by the method of wheat germ protein expression system, which are captured at the bottom of each well of 1536 well formatted array by magnetic glutathione-coupled beads to perform CaM-binding screening with biotinylated CaM. When we tested this high throughput CaM-binding screening with known CaM-kinases including CaMKI, CaMKII, CaMKIV, CaMKK and non-enzyme CaM-target, Wolframin [1], all of the known CaM-targets captured in 1536 well formatted array was specifically and readily detected. When we performed CaM-binding screening with Protein Active Array® carrying 19,676 GST-fused human proteins, we detected >100 CaM-binding proteins including known and unknown CaM-targets. In this report, we identified and characterized a striated muscle activator of Rho signaling (STARS) [4] as a novel CaM-target. *In vitro* CaM-binding experiments including CaM-Sepharose chromatography, CaM-overlay and zero-length crosslinking confirmed a Ca^{2+}-dependent and stoichiometrical CaM-binding of STARS. Immunoprecipitation revealed the Ca^{2+}-dependent interaction of CaM with STARS in transfected COS-7 cells. Based on our results, Protein Active Array® is useful to screen and identify binding partners for the signaling molecule such as CaM. Identification of STARS as a novel target for Ca^{2+}/CaM complex may suggest that intracellular Ca^{2+}, which simultaneously mediates contraction, regulates the physiological function of STARS in muscles.

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ELUCIDATING KEY INTERACTIONS BETWEEN CALMODULIN AND PSD-95 THAT CONTROL SYNAPTIC PLASTICITY

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The Ca^{2+} sensor protein, calmodulin (CaM) regulates neuronal synaptic plasticity by controlling the density of glutamate receptors positioned at the postsynaptic membrane of hippocampal neurons. Here, we present an NMR structural model of CaM bound to the postsynaptic density protein-95 (PSD-95) in order to understand the mechanism by which CaM and PSD-95 both control AMPA receptor localization at the postsynaptic membrane. Previously, our lab published an NMR structure of Ca^{2+}-CaM bound to the first 13 residues of PSD-95, revealing that CaM binding blocks the palmitoylation of PSD-95 at N-terminal residues, Cys3 and Cys5 [1]. Thus CaM binding to depalmitoylated PSD-95 caps the N-terminus and helps promote the Ca^{2+} -induced dissociation of PSD-95 from the postsynaptic membrane. Recent data have shown that beyond these N-terminal interactions, phorphorylation of PSD-95 at threonine-19 is necessary for PSD-95 mobilization and long-term depression (LTD). Using NMR-guided computational docking, we report a structural model of Ca^{2+}-CaM bound to PSD-95 peptide (residues 1-19) that is phosphorylated at threonine-19 (backbone RMSD of 1.0 Å for 200 structures). Along with Thr19, PSD-95 residues Gln15 and Glu17 were also identified to make contact with CaM and these contacts were verified by mutagenesis. Our structural model of CaM bound to PSD-95 with phosphorylation at Thr19 now shows a complete picture of calmodulin's role in binding PSD-95 to regulate its anchoring of AMPAR at the post synapse.

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NA+/CA2+ EXCHANGE CONTRIBUTES TO THE REGULATION OF THE PACEMAKER ACTIVITY OF INTERSTITIAL CELLS OF CAJAL WITH INTERACTION WITH ANO1

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Interstitial cells of Cajal (ICC) are the pacemaker cells that generate electrical slow waves in gastrointestinal (GI) smooth muscles that organize the motor patterns in many regions of the GI tract into phasic contractions. Slow waves result from activation of Ca^{2+}-activated Cl^- channels (CaCC) encoded by *Ano1* and depolarize ICC to the Cl^- equilibrium potential (E_{Cl}). How activation of CaCC is sustained through the duration of slow waves is unknown. We tested the role of the Na^+/Ca^{2+} exchanger (NCX) in regulating pacemaker activity in ICC. ICC of small intestine and colon expresses NCX isoforms. NCX3 co-immunoprecipitated with ANO1 and these proteins were co-localized in ICC using a proximity ligation assay. Inhibitors of NCX increased CaCC current in ICC, suggesting that NCX, acting in Ca^{2+} exit mode, helps to regulate basal $[Ca^{2+}]_i$ in these cells. Shifting NCX into Ca^{2+} entry mode by replacing extracellular Na^+ with Li^+ increased spontaneous transient inward currents (STICs) recorded under voltage clamp. The STICs were due to activation of CaCC. Stepping ICC from -80 to -40 mV activated slow wave currents. These events were reduced in amplitude and duration by KB-R7943 and SN-6 and enhanced in amplitude and duration by increasing the driving force on NCX. Our results suggest that NCX regulates the activation of ANO1 in ICC. Dynamic changes in ionic gradients during slow waves may flip the directionality of the exchanger, allowing it to remove Ca^{2+} during the inter-slow wave period and to sustain activation of ANO1 during the slow wave plateau phase.

SYNAPTOTAGMIN 1 IN ALZHEIMER'S DISEASE – GUARD OR PARTNER IN CRIME?

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Synaptic loss is the strongest correlate of memory deterioration in Alzheimer's disease (AD). It is primarily caused by local accumulation of amyloid β (Aβ), and in particular neurotoxic Aβ42. Aβ is a proteolytic product of a subsequent processing of the amyloid precursor protein (APP) by two enzymes - β-secretase and presenilin 1 (PS1)/γ-secretase. Continuous, default or experimentally induced neuronal activity causes an increase in Aβ production, which is strongly related to the intracellular calcium flux and synaptic vesicle exocytosis. However, the molecular mechanisms of the Ca^{2+}-dependent regulation of the Aβ production remain unclear.

To get thorough understanding of the mechanisms underlying evoked Aβ production upon increased Ca^{2+} concentration, we performed a mass spectrometry screen for Ca^{2+}-dependent PS1 interacting proteins, and found synaptotagmin 1 (Syt1) as a novel PS1 interactor that binds directly to PS1 N-terminal loop fragment in high Ca^{2+} condition. We verified that Ca^{2+} binding to Syt1 is a pre-requisite for the interaction to occur. Since Syt1 is a Ca^{2+} sensor in neurotransmitter release, involved in trafficking of synaptic vesicles at the active zone of the synapse, and interacts not only with PS1 but also with APP, we hypothesized that it may modulate trafficking and/or maturation of APP and its processing enzymes at the synapse. Therefore, we applied subcellular fractionation, cycloheximide pulse chase assay, enzyme-linked immunosorbent assay (ELISA), and fluorescence lifetime imaging (FLIM) to investigate the role of Syt1 in Aβ40 and Aβ42 production, and in the stability and trafficking of APP, β- and γ-secretases in cell lines with Syt1 knock-down or overexpression.

Our experiments demonstrate that Syt1 directly interacts with PS1 in a Ca^{2+}-dependent manner, and can modulate PS1 conformation and A β production/secretion by affecting trafficking and maturation of APP processing enzymes. Interestingly, in the absence of Syt1, PS1 adopts closed, "pathogenic" conformation, which correlates with higher Aβ42/40 ratio, suggesting possible protective role for Syt1. On the other hand, Syt1 overexpression and knock-down in PC12 cell line resulted in increased and decreased levels of secreted total Aβ, respectively.

To conclude, our novel discoveries link together important players in the AD pathogenesis: synapse, Ca^{2+}, PS1 and Aβ production, and open new avenues for therapeutic interventions focusing at the synapse.

DIRECT VISUALIZATION OF INTERACTION BETWEEN CALMODULIN AND CONNEXIN45

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Gap junction are channels formed between adjacent cells which act in many vertebrate mature and developing cells to allow signal molecules and ions freely through. Connexin45 (Cx45) a member of the γ connexin family, is expressed in embryo, heart and neurons. Cx45 gap junction contributes to heart development and its regulation. Like all other gap junctions, their alterations in regulation result in various human diseases. Despite extensive efforts and studies devoted since 1980's, the direct interaction between CaM and connexins including Cx45 remains obscure to date. Here we report the first interaction of CaM in Cx45 in living cell using a luminescence resonance energy transfer method. Such interaction is strongly dependent on intracellular calcium concentration and can be blocked by CaM inhibitor W7. Furthermore, we dissect the molecular basis of this interaction using a peptide model. Peptide at intracellular loop of Cx45 directly binds to Ca^{2+}/CaM with an affinity of 5 nM using fluorescence-labeled CaM and surface plasma resonance. The addition of this Cx45 cytosolic peptide results in a global chemical shift changes of ^{15}N labeled CaM in the presence Ca^{2+} and which implies that both N- and C-domain of CaM are participating in the association with Cx45. Cx45 peptide binding to CaM also enhance the calcium sensitivity of both the N- and C-domain. Together, our findings reveal that Ca^{2+}/CaM directly interacts with the cytoslolic loop of CX45 with an extended conformation that differs from that of alpha family Connexins.

ABERRANT CALCIUM SIGNALING BY TRANSGLUTAMINASE-MEDIATED POSTTRANSLATIONAL MODIFICATION OF IP_3 RECEPTORS

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Reversible and repetitive structural changes are essential for ligand-gated ion channels to mediate biological signaling. Here we show a new mode of posttranslational modification that robustly controls the structural changes in the ligand-gated ion channels. The inositol 1,4,5-trisphosphate receptor (IP_3R) in the endoplasmic reticulum assembles ligand-gated ion channels that mediate calcium signaling. IP_3Rs are allosteric proteins comprising four subunits that form an ion channel activated by binding of IP_3 at a distance. Defective allostery in IP_3R is considered crucial to cellular dysfunction, but the specific mechanism remains unknown. We demonstrate that a pleiotropic enzyme transglutaminase type 2 (TG2) targets the allosteric coupling domain of IP_3R type 1 (IP_3R1) and negatively regulates IP_3R1-mediated calcium signaling and autophagy by locking the subunit configurations. The control point of this regulation is the covalent posttranslational modification of Gln2746 residue which TG2 tethers to the adjacent subunit. Modification of Gln2746 and IP_3R1 function was observed in Huntington's disease models, suggesting a pathological role of this modification in the neurodegenerative disease. Our study reveals that cellular signaling is regulated by a new mode of posttranslational modification that chronically and enzymatically blocks allosteric changes in the ligand-gated channels which relate to disease states. This is the first demonstration of transglutaminase-catalyzed posttranslational modification in ligand-gated ion channel allostery and provides a new framework for enzymatic regulation of ligand-gated ion channels.

[1] K. Hamada, *et al.*, PNAS 111(38), E3966-75 (2014)

IRBIT SUPPRESSES CAMKII-ALPHA ACTIVITY AND CONTRIBUTES TO CATECHOLAMINE HOMEOSTASIS

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Inositol 1,4,5-trisphosphate receptor (IP_3R) binding protein released with IP_3 (IRBIT) contributes to various physiological events (electrolyte transport, mRNA polyadenylation, and the maintenance of genomic integrity) through its interaction with multiple targets. However, little is known about the physiological role of IRBIT in the brain. In this report, we identified calcium calmodulin-dependent kinase II alpha (CaMKIIα) as an IRBIT-interacting molecule in the central nervous system. IRBIT binds to and suppresses CaMKIIα kinase activity by inhibiting the binding of calmodulin to CaMKIIα. In addition, we show that IRBIT knockout mice show elevated catecholamine levels, increased locomotor activity, and social abnormalities. The level of tyrosine hydroxylase (TH) phosphorylation by CaMKIIα, which affects TH activity, was significantly increased in the ventral tegmental area of IRBIT deficient mice. We concluded that IRBIT suppresses CaMKIIα activity and contributes to catecholamine homeostasis through TH phosphorylation.

[1] Katsuhiro Kawaai, Akihiro Mizutani, Hirotaka Shoji, Naoko Ogawa, Etsuko Ebisui, Yukiko Kuroda, Shigeharu Wakana, Tsuyoshi Miyakawa, Chihiro Hisatsune, Katsuhiko Mikoshiba, *PNAS*, in press doi:10.1073/pnas.1503310112

ERP44 EXERTS REDOX-DEPENDENT CONTROL OF BLOOD PRESSURE AT THE ENDOPLASMIC RETICULUM

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Blood pressure maintenance is vital for systemic homeostasis, and angiotensin II is a critical regulator. The upstream mechanisms that regulate angiotensin II are not completely understood. Here, we show that ERp44, a factor involved in disulfide bond formation in the endoplasmic reticulum (ER), regulates angiotensin II. In mice, genetic loss of ERp44 destabilizes angiotensin II and causes hypotension. We show that ERp44 forms a mixed disulfide bond with ERAP1, an aminopeptidase that cleaves angiotensin II. ERp44 controls release of ERAP1 in a redox-dependent manner to control blood pressure. Additionally, we found that systemic inflammation triggers ERAP1 retention in the ER to inhibit hypotension. These findings suggest that ER redox state calibrates serum angiotensin II levels via regulation of the ERp44-ERAP1 complex. Our results reveal a link between ER function and normotension and implicate ER redox state as a potential risk factor in the development of cardiovascular disease.

[1] C. Hisatsune et al., Molecular Cell, 2015, in press

Board #49

FUNCTIONAL ANALYSIS OF THE ATAXIA RELATED MUTATION OF THE PLASMA MEMBRANE CALCIUM ATPASE ISOFORM 3

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Raffaele Lopreiato, Tito Cali', Martina Frizzarin, Ilenia Bertipaglia, Nunzio Damiano, Maria Ruzzene, Oriano Marin, Giuseppe Zanotti, Marisa Brini, Ernesto Carafoli

Board #50

A NOVEL MUTATION IN ISOFORM 3 OF THE PLASMA MEMBRANE CALCIUM PUMP IMPAIRS CELLULAR CALCIUM HOMEOSTASIS IN A PATIENT WITH CEREBELLAR ATAXIA AND LAMININ SUBUNIT 1 ALPHA MUTATIONS.

Tito Cali', Raffaele Lopreiato, Joshua Simony,Marisa Vineyard, Martina Frizzarin, Ginevra Zanni, Giuseppe Zanotti, Marisa Brini, Marwan Shinawi, Ernesto Carafoli.

Board #51

AN IN SILICO STUDY OF THE MOLECULAR MECHANISM OF THE PMCA PUMP AUTO-INHIBITION.

Francesco Zonta, Tito Cali', Giuseppe Zanotti, Raffaele Lopreiato, Marisa Brini, Ernesto Carafoli.

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A NEW LOOK AT THE PLASMA MEMBRANE CALCIUM PUMP.

Raffaele Lopreiato, Tito Cali', Marta Giacomello, Marisa Brini, Ernesto Carafoli.