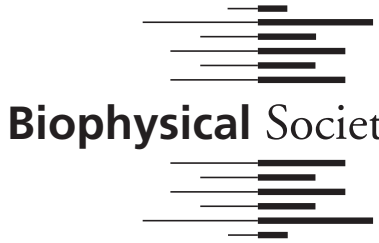


2008 Biophysical Discussions

Calmodulin Modulation of Ion Channels
Asilomar Conference Center, Asilomar, California

October 30 - November 2, 2008

Study Book, Part 2—Poster Abstracts

Co-sponsored by the Biophysical Society and the Office of Rare Diseases (ORD), NIEHS, NIH
With additional support from GlaxoSmithKline

Table of Contents

Poster Board 1

FRET-based mapping of calmodulin bound to ryanodine receptor channels

Bradley Fruen, Florentin Nitu, David Thomas, Razvan Cornea.

University of Minnesota, Minneapolis, MN, USA.

The type 1 ryanodine receptor (RyR1) is regulated by calmodulin (CaM), which activates the channel in nanomolar Ca^{2+} and inhibits the channel in micromolar Ca^{2+}. To investigate the structural basis of RyR1 regulation by CaM, we have used site-directed labeling of channel regulatory proteins and FRET. Sarcoplasmic reticulum membranes were preincubated with a fluorescent FKBP donor (AlexaFluor488-FKBP12.6) and washed to remove unbound donor. FRET, evident as a decrease in donor fluorescence, was then determined following incubations in the presence of fluorescent CaM acceptors (AlexaFluor568-CaMs). Strong FRET between FKBP and CaM was observed when acceptor fluorophore was attached within CaM's N-lobe (E = 0.38 ± 0.2 in 30 nM Ca^{2+}). By comparison, substantially weaker FRET was observed when acceptor fluorophore was attached within CaM's C-lobe (E = 0.08 ± 0.01 in 30 nM Ca^{2+}). Addition of Ca^{2+} evoked little change in FRET to either CaM's N-lobe or C-lobe (E = 0.43 ± 0.2 and 0.13 ± 0.02, respectively, in 30 µM Ca^{2+}). We conclude that CaM binds to the RyR1 in an extended conformation, oriented such that its N-lobe is nearest to FKBP (R = 65-67 Å) and its C-lobe furthest from FKBP (R = 85-93 Å) on a single lateral face of the tetrameric channel. Surprisingly, distance estimates are similar in nanomolar and micromolar Ca^{2+}, suggesting that Ca^{2+} dependent rearrangements of CaM on the RyR1 may operate on a smaller scale than predicted in structural models based on cryo-EM.

Poster Board 2

Calmodulin regulates trafficking of KCNQ channels

Alessandro Alaimo[1], Paloma Aivar[1], Camilo Gómez-Posada[1], Pilar Areso[2], MeriTxell Roura i Ferrer[1], Teresa Zamalloa[1], Alvaro Villarroel[1].

[1]Ion Channels, CSIC, Leioa, Spain, [2]Farmacologia, Universidad Pais Vasco, Leioa, Spain.

We have found that an interaction with Ca^{2+}-calmodulin is required for KCNQ2 channels to exit the endoplasmic reticulum (ER). The association of calmodulin with KCNQ2 is disrupted in some BFNC mutations, preventing the correct trafficking to the plasma membrane. Elevating calmodulin expression partially restored the intracellular distribution of BFNC mutants, whereas over-expression of a Ca^{2+}-binding incompetent calmodulin caused retention at the ER of wild type channels. The EC_{50} for Ca^{2+} binding to calmodulin was in the physiological range and it doubled when calmodulin associated with the KCNQ2 calmodulin-binding domain. It follows that microdomains of intracellular Ca^{2+} could be decoded by calmodulin to permit channel trafficking to discrete regions of the membrane

Poster Board 3

Multiple roles of calmodulin in the functional regulation of TRP channels

Michael X. Zhu, Ph.D., Rui Xiao, M.S., Jisen Tang, M.D.

Neuroscience, The Ohio State University, Columbus, OH, USA.

Transient receptor potential channels (TRP) have emerged as cellular sensors of various internal and external

cues. Generally, the activation of TRP canonical (TRPC) channels is triggered by the stimulation of phospholipase C; however, multiple factors are involved in the regulation of these channels. Among them, Ca^{2+}-mediated feedback channel modulations are often mediated by calmodulin (CaM) and other Ca^{2+}-binding proteins. In vitro binding studies have revealed multiple CaM-binding sites on TRPC proteins. Among them, a common CaM/inositol 1,4,5-trisphosphate receptor-binding site is found at the carboxyl terminus of every TRPC isoform. For some TRPC isoforms, this site also binds to phosphoinositides. Additional non-conserved CaM-binding sites are present at the amino and carboxyl termini of several TRPC proteins. Likewise, multiple CaM-binding sites have been found in TRPV and TRPM proteins. Efforts on elucidating the specific modulatory function of individual CaM-binding sites have begun to reveal the unique and multiple roles of CaM on TRP channel regulation. Here we will present our recent work on CaM binding and CaM-mediated regulation of TRPC and TRPV channels.

Poster Board 4

Calcium-dependent modulation of N-type calcium channels

Lei Zhu, PhD[1], Sarah McDavid, MS[2], Kevin P. Currie, PhD[2].

[1]Anesthesiology, Vanderbilt University, Nashville, TN, USA, [2]Anesthesiology, Pharmacology, & Center for Molecular Neuroscience, Vanderbilt University, Nashville, TN, USA.

N-type voltage-gated calcium channel currents (I_{Ca}) play key roles in many cellular functions including the regulation of neurotransmitter and hormone release. It is now clear that Ca^{2+} itself can modulate these channels by calcium-dependent inactivation (CDI). This is mediated by calmodulin and sensitive to "global" calcium buffering. We have investigated Ca^{2+}-dependent regulation of recombinant N-type I_{Ca} using G1A1 cells (HEK cells stably expressing $Ca_V2.2$ α_{1B}), β_{1b} and $\alpha_2\delta$). Substituting extracellular Ca^{2+} with Ba^{2+} and / or raising the Ca^{2+} buffer concentration in the patch pipette solution (EGTA or BAPTA) enabled estimation of CDI and VDI. Cells were stimulated with an individual step depolarization lasting 1-15s, trains of brief (10ms) step depolarization, or trains of action potential-like waveforms (APW). CDI played a significant role during sustained trains of APW but the onset was relatively slow compared to. Different Ca-channel β subunits can modulate the kinetics of VDI and it has been suggested that in L-type channels CDI is modulated in parallel with VDI. Transient expression of β_{1b} (identical to the "endogenous", stably expressed subunit) did not affect the kinetics of I_{Ca}, but β_{2a} shifted the kinetic profile such that it was dominated by this "exogenous" subunit. Although, VDI was greatly diminished in cells expressing β_{2a} CDI during either a step depolarization or a train of APW was not significantly altered. Recovery of I_{Ca} from inactivation following a 1s or 2s step depolarization was significantly slower in Ca^{2+}. However with long steps or APW trains lasting 15s recovery in Ca^{2+} was significantly faster than in Ba^{2+}. Recovery in Ca^{2+} was significantly slowed by increasing the concentration of either EGTA or BAPTA in the pipette. Our data show that Ca^{2+} modulates both inactivation and recovery from inactivation of N-type I_{Ca}.

Poster Board 5

Ca/CaM-dependent phosphorylation/dephosphorylation regulates chloride channel ClC-3 at the synapse

Lucy Deriy, PhD[1], Xueqing Wang, PhD[1], Danielle Zheng[1], Radmila Sarac, PhD[2], Deborah Nelson, PhD[1].

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

ClC-3, a member of ClC family of chloride channels, is localized to both the postsynaptic membrane and synaptic vesicles of hippocampal neurons and plays important roles in neuronal development and survival. The channel is gated by Ca/CaM kinase II (CaMKII)-dependent phosphorylation of ClC-3 N terminus. In an apparent conundrum, simple elevation of neuronal internal calcium $[Ca^{2+}]_i$ in the presence of the autonomous CamKII, prevents channel opening. We hypothesized that Ca^{2+}-dependent dephosphorylation might be involved in channel closure. Our single channel electrophysiological recordings on inside-out patches from somal membranes of rat hippocampal neurons demonstrated that the Ca^{2+}-dependent decrease in open time probability can be reversed by addition of inhibitors of calmodulin (CaM) or calcineurin (Cn). Identical results were obtained in whole cell recordings from the same preparation. Co-immunoprecipitation demonstrated that Cn associates with ClC-3 in synaptosomal membranes of hippocampal neurons and directly binds to C-terminal GST fusion proteins *in vitro* providing evidence that ClC-3 may serve as a scaffolding protein for channel regulatory elements. In phosphorylation experiments, Cn dephosphorylated ClC-3 N-terminal fusion proteins previously phosphorylated by CaMKII. A combination of site-directed mutagenesis and phosphoimaging we used to pinpoint the exact site(s) of phosphorylation revealed that the ClC-3 N-terminus is phosphorylated at S109 and S114. These results as well as dephosphorylation of S114 by Cn were confirmed by mass spectrometry. Thus, our data provide the first evidence of a single residue involved in protein activation that is controlled by antipodal enzymes sharing the same regulatory elements, namely Ca and CaM. The reciprocal regulation of the same protein by a kinase and a phosphatase, both Ca/CaM-dependent, although without apparent precedent, indicates that CaMKII/Cn can function as a finely coordinated Ca^{2+}/CaM-sensitive switch, sensing spatial and temporal Ca^{2+}/CaM dynamics thereby determining the vector of channel kinetics.

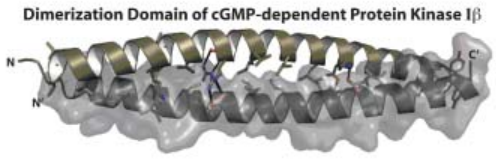
Poster Board 6

The first crystal structure of cyclic GMP-dependent protein kinase Iβ *dimerization domain reveals the molecular features of an extended leucine/isoleucine zipper*

Eric V. Smith-Nguyen, BS[1], Darren E. Casteel, PhD[2], Glen Spraggon, PhD[3], Banumathi Sankaran, PhD[4], Renate B. Pilz, PhD[2], Susan S. Taylor, PhD[1,5], Choel W. Kim, PhD[6].

[1]Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA, USA, [2]School of Medicine, University of California, San Diego, La Jolla, CA, USA, [3]Genomics Institute of the Novartis Research Foundation, San Diego, CA, USA, [4]Howard Hughes Medical Institute, Lawrence Berkeley National Laboratory, Berkeley, CA, USA, [5]Howard Hughes Medical Institute, La jolla, CA, USA, [6]Howard Hughes Medical Institute, University of California, San Diego, La Jolla, CA, USA.

Subcellular targeting of PKG provides an important mechanism for achieving substrate specificity and is mediated by the N-terminal dimerization domain, which binds to defined sets of protein substrates in an isotype dependent manner. Although the N-terminal dimerization domain of PKG Iβ has been shown to provide a docking surface for TFII-I and IRAG, the structural detail was unknown. To understand the targeting mechanism and the molecular detail of the protein docking motif, we solved a crystal structure of the N-terminal domain of PKG Iβ at 2.1 angstrom. The structure reveals two parallel helices warping around each other into a left-handed helix and forming an extended zipper with 10 pairs of leucine/isoleucines packing in a "knobs-into-holes" manner. Most surprisingly, the structure reveals two basic residues at the "d" positions (rather than at the canonical "g" positions) forming interhelical electrostatic interactions with two acidic residues at the "e" positions in a mirror symmetry fashion. These interhelical electrostatic interactions flank a negatively charged surface previously shown to bind TFII-I and IRAG and may aid in stabilizing this important docking surface.



Poster Board 7

Activation of Ca^{2+}-calmodulin dependent protein kinase II (CaMKII) links hypertension to the development of cardiac hypertrophy

Ye Chen-Izu, Ph.D.

Medicine, University of Kentucky, Lexington, KY, USA.

Background—Hypertension is a major risk factor for developing cardiac hypertrophy and heart failure. The molecular link between hypertension and heart disease still remains unclear. We hypothesized that alterations in the Ca^{2+}-calmodulin-CaMKII signaling pathway might be fundamental in the development of hypertensive heart disease (HHD). **Methods and Results—**To test this hypothesis, we examined CaM and CaMKIIδ protein expression and phosphorylation in the left ventricle at distinct stages of HHD: *pre-hypertension*, *hypertension*, *hypertrophy* and *heart failure*, using the spontaneously hypertensive rat (SHR) model. In order to separate HHD-related change from age-related change, we also conducted a longitudinal study using age-matched Wistar-Kyoto (WKY) controls. (1) The onset of hypertension in SHR was associated with a marked increase of CaMKIIδ-Thr287 phosphorylation indicative of increased CaMKII activity; this hyperphosphorylation persisted throughout the development of hypertrophy and heart failure under essential hypertension. (2) The protein expression of δ_B (nuclear) and δ_C (cytosolic) isoforms of CaMKIIδ are similar in SHR and WKY, showing no HHD-related changes, although there are age-related changes in both strains. (3) Using angiotensin converting enzyme (ACE) inhibitor, enalapril, to reduce hypertension in SHR caused reduction of CaMKII-Thr287 phosphorylation and concomitant reversal of cardiac hypertrophy. **Conclusion—**CaMKIIδ signaling is activated by hypertensive stress. Given the known effects of CaMKIIδ on inducing gene transcription and remodeling excitation-contraction coupling, we suggest that CaMKIIδis an important signaling molecule that links hypertension to the development of cardiac hypertrophy.

Poster Board 8

Multiple regions of ryanodine receptor calcium channels determine isoform specificity of calmodulin regulation

Naohiro Yamaguchi, Le Xu, Gerhard Meissner.

Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC, USA.

Numerous endogenous molecules and proteins including calmodulin (CaM) regulate ryanodine receptor (RyR) ion channel activity in cardiac and skeletal muscle. Mice carrying mutations in the highly conserved CaM binding domain of the cardiac RyR isoform show a severe form of cardiac hypertrophy. Prolonged Ca^{2+} transients in neonatal cardiomyocytes indicate that CaM inhibition of RyR2 is required for normal cardiac function. *In vitro* studies show that CaM inhibits the channel activities of the skeletal (RyR1) and cardiac (RyR2) isoforms at micromolar Ca^{2+} concentration, whereas at resting Ca^{2+} CaM activates RyR1 and inhibits RyR2. Corresponding point mutations in the conserved CaM binding site (RyR1 aa 3614-3643; RyR2 aa 3581-3610) result in a different response to CaM. These results suggest that other isoform specific regions have a major role in regulation of RyRs by CaM. To obtain insights into the differential CaM regulation of RyR1 and RyR2, we determined the single channel activities of a series of chimeric RyRs. Inhibition of a chimera carrying the N-terminal portion of RyR1 (RyR1 aa 1-3725; RyR2 aa 3691-4968) by CaM at low and high Ca^{2+} concentrations suggests that the C-terminal region including the channel pore region has a dominant role in CaM regulation of RyR2. A chimera carrying a smaller region of RyR1 (RyR1 aa 2659-3725) is also inhibited by CaM at low Ca^{2+} concentrations. On the oth-

er hand, a chimera containing an even smaller region of RyR1 (RyR1 aa 3581-3725 including CaM binding site) is activated by CaM at low Ca^{2+} concentration, as is wild type RyR1. The results suggest that multiple regions of the RyRs are involved in their regulation by CaM. Additional chimeras are currently constructed.

Poster Board 9

Calmodulin regulation of Cav2 channels and neurotransmitter release

Jamie L. Weiss, Ph.D., Elizabeth P. Seward, Ph.D.

Biomedical Science, The University of Sheffield, Sheffield, United Kingdom.

P/Q-type (Cav2.1) and N-type (Cav2.2) Ca2+ channels are critical to stimulus-secretion coupling in the nervous system and feedback regulation of these channels by Ca2+ can profoundly influence neurotransmission. We have demonstrated differential regulation of Cav2 subtypes by calmodulin (CaM) that impact on the channel's ability to regulate neurotransmitter release in neuroendocrine cells (Wykes, et al J Neurosci. 27: 5236, 2007). We report divergent regulation of Ca2+-dependent inactivation (CDI) of native N- and P/Q-type Ca2+ channels by CaM in chromaffin cells. Robust CDI of N-type channels was observed in response to prolonged step depolarizations, as well as repetitive stimulation with either brief step depolarizations or action potential-like voltage stimuli. Adenoviral expression of Ca2+-insensitive CaM mutants eliminated CDI of N-type channels. The CDI of P/Q-type channels was by comparison modest and insensitive to expression of CaM mutants. Cloning of the 3'-end of Cav2.1 subunits from chromaffin cells revealed multiple splice variants lacking structural motifs required for CaM-dependent CDI. Combining perforated-patch voltage-clamp recordings of pharmacologically isolated Ca2+ currents with membrane capacitance measurements of exocytosis revealed the physiological relevance of CDI on stimulus-coupled exocytosis. Increasing stimulus intensity to invoke CDI resulted in a significant decrease in the exocytotic efficiency of N-type channels compared with P/Q-type channels. Our results reveal unexpected diversity in CaM regulation of native Cav2 channels and suggest that the ability of individual Ca2+ channel subtypes to undergo CDI may be tailored by alternative splicing. We are presently cloning the 3'-end of Cav2.2 channel splice variants in chromaffin cells. In view of the unique advantages offered by chromaffin cells for studying the coupling of voltage-gated calcium channels with the exocytotic machinery, we are currently using these cells to express mutant channels predicted to interfere with neurotransmission.

Poster Board 10

Genomic regulation of INCX by estrogen in rabbit myocytes

Guojun Chen, PhD, Guy Salama, PhD.

Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, PA, USA.

In congenital forms of long QT 2 (LQT2), women are at greater risk of Torsade de Pointes (TdP) than men. In LQT2, TdP is associated with a loss of function of the rapid, delayed rectifying K+ current, IKr resulting in the prolongation of the action potential duration (APD) and QT interval. Sex differences in 'repolarization reserve' have been proposed to account for sex differences in arrhythmia phenotype; yet, in pre-puberty, the arrhythmia phenotype is

reversed with males having a greater risk to TdP than females. We recently showed that the arrhythmia phenotype was due to sex-differences in the expression of the L-type Ca2+ current, ICa,L and now show similar sex-differences in the expression of the Na+/Ca2+ exchanger and its current, INCX, which is a key regulator of intracellular Ca2+. In rabbit myocytes from the base of the left ventricle, INCX was higher in female (12.7 ± 2.1 pA/pF; n=6/3 cell/hearts) than male (5.6 ± 1.5pA/pF; n=5/3 hearts) and higher than myocytes from the apex (2.2 ± 0.8 pA/pF; n=6/4hearts). When cultured in steroid free serum, female INCX decreased in 1-2 days becoming equal to the male INCX then remained stable for 72 hours. Male INCX remained constant over 72 hours. Furthermore, when female myocytes from the base were incubated with 17-beta-estradiol (10 nM), INCX increased and was not down-regulated as in myocytes kept in steroid free-medium. At the apex however, INCX density was not altered by estrogen, indicating spatially heterogeneous effects of estrogen. Thus, estrogen controls the expression of NCX in specific regions of the heart, which may contribute to the arrhythmia phenotype in female hearts with LQT2 and may be relevant to arrhythmia susceptibility in patients with heart failure (where INCX is elevated) or with altered levels of sex steroids.

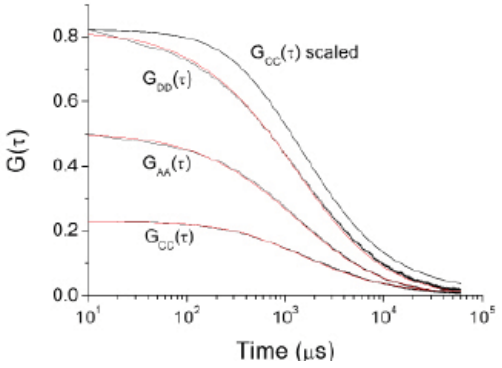
Poster Board 11

Calmodulin dynamics in solution and in cells

Carey Johnson, Ph.D.

Chemistry, University of Kansas, Lawrence, KS, USA.

We have probed calmodulin dynamics in solution by fluorescence correlation spectroscopy and single-molecule FRET experiments. FCS cross-correlation measurements reveal dynamics on the time scale of hundreds of microseconds in holoCaM. The amplitude of dynamics is greatly reduced in apoCaM. We also probed the dynamics of CaM bound to the plasma-membrane Ca^{2+} ATPase. Current work in our lab is aimed at characterizing the conformational states and nature of the dynamics for calmodulin in solution. In collaboration with Prof. Greg Harms, we also detected single molecules of fluorescently labeled calmodulin in HEK and COS cells. Single-molecule tracking revealed a wide range of translational mobilities of calmodulin with diffusion coefficients spanning several orders of magnitude, from roughly 0.01 $\mu m^2\ s^{-1}$ to 10 $\mu m^2\ s^{-1}$, consistent with interaction of calmodulin with a target proteins having a range of mobilities.



FCS of calmodulin labeled with a FRET pair. A comparison of the donor and acceptor autocorrelation functions (GDD and GAA, respectively) with the cross-correlation function, GCC, reveals dynamics on the hundreds of microseconds time scale.

Poster Board 12

Luminescence resonance energy transfer as a sensor for calmodulin conformation

Steen E. Pedersen, PhD[1], Inna Yanez-Orozco, BS[1], D. Brent Halling, PhD[2], Susan L. Hamilton, PhD[1].

[1]Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX, USA, [2]University of Texas, Austin, TX, USA.

Calmodulin (CaM) regulation of calcium channels likely proceeds by calcium-binding inducing CaM to undergo several distinct conformational changes and local shifts in binding sites while CaM is constitutively bound to the channel. To determine the conformational state of CaM, we have developed a lanthanide-based, luminescence resonance energy transfer system to detect the distance between the N- and C-lobes of

calmodulin with high precision. Two sulfhydryl-reactive, sensitized lanthanide chelates were synthesized and shown to provide sensitive, high-precision lifetime measurements. These were reacted along with appropriate acceptor fluorophores to cysteines substituted on CaM (CaM T6C/T118C). Terbium lifetime measurements provided distance measurements between the lobes of CaM when bound to the cardiac calcium channel IQ peptide. Calmodulin was substituted with tryptophans, separately, near each of the four calcium binding sites, to act as a sensitizer for terbium luminescence. This permitted the specific detection of terbium binding at each of the sites separately. Lifetimes at three of the sites were similar at 1.40 ms, with terbium bound at site 2 having a distinctly longer lifetime of 2.26 ms. This indicates stronger shielding from water at site 2. Terbium bound at those sites can act as energy transfer donors to acceptors reacted with a cysteine substitution on the apposing lobe. Tests of luminescence energy transfer between site 1 (T26W substitution)and DABMI reacted to T110C showed a distance of 20.8 Å, which is consistent with the expected value from the calcium-bound CaM-IQ structure. These experiments demonstrate that we can detect the lobe-separation within CaM while it is bound to recognition sequences from the cardiac calcium channel.

Poster Board 13

Androcam: a testis-specific calcium sensor

Kathleen M. Beckingham, Ph.D., Rebecca A. Simonette, M.S., William Deery, Ph.D., Robert Y-S. Lee, M.S.

Biochemistry and Cell Biology, Rice University, Houston, TX, USA.

Males of the genus Drosophila produce sperm of remarkable length, with the 1.8 millimeter long sperm of Drosophila melanogaster being fairly typical. We have identified a Drosophila calmodulin homolog that is testis-specific and which has presumably evolved to mediate the specialized functions associated with spermatogenesis and sperm physiology. Protein interaction data indicate that Androcam may regulate ion channel function in spermatogenesis. Data on the interaction partners of Androcam will be presented.

Poster Board 14

Characterization of CaM binding to the KCNQ channel binding site

Pilar Areso, PhD[1], Alessandro Alaimo[2], Villarroel Alvaro[3].

[1]Farmacologia, Universidad del Pais Vasco, Leioa, Spain, [2]Biofisica, CSIC, Leioa, Spain, [3]Unidad de Biofisica, CSIC, Leioa, Spain.

We have used GST fusion proteins to characterize the calmodulin interaction with the CaM binding site of KCNQ2 channels. The binding site is made up by two discontinuous sequences with a high probability of adopting and alpha helix configuration (helices A+B). In agreement with co-ip experiments with full-length channels, we have found that the interaction is weaker in the presence of calcium. The Kd shifts from 10.4 nM in the absence of Ca^{2+}, to 24.7 nM in the presence of 1500 nM free calcium. As expected, the affinity of CaM for calcium is reduced in the presence of saturating levels of the KCNQ binding site, shifting the IC_{50} from 300 nM in the absence to 590 nM in the presence of the fusion protein. In addition, we have characterized two Bening Familial Neonatal Convulsions causing mutations: R353G and L339R. Both cause a comparable reduction in CaM affinity, ranging from 60 to 80%, but have a differential impact on the CaM binding affinity for calcium. While R353G shifts the IC_{50} to 403 nM, L339R has no effect

on this parameter. This correlates well with the effect on trafficking of KCNQ2 on HEK cells. While over-expression of CaM does not affect the degree of colocalization of L339R with an ER marker, this manipulation reduces the degree of colocalization of R353G towards levels comparable to wt channels. These results lend support to the hypothesis for the existence of a calcium-calmodulin dependent regulation of KCNQ channel traffic.

Poster Board 15

Calcium/calmodulin-dependent protein kinase II regulation of cardiac voltage-gated sodium channels

Nicole M. Ashpole, BS[1], Joseph D. Brogan, BS[1,2], Patrick L. Sheets, PhD[3,1], Theodore R. Cummins, PhD[3,1], Andy Hudmon, PhD[1,2].

[1]Stark Neuroscience Research Institute, Indiana University, Indianapolis, IN, USA, [2]Biochemistry and Molecular Biology, Indiana University, Indianapolis, IN, USA, [3]Pharmacology-Toxicology, Indiana University, Indianapolis, IN, USA.

The predominant voltage-gated sodium channel in heart, Nav1.5, is both a substrate and an anchoring protein for CaMKII. Because voltage-gated sodium channels play an integral role in initiating the action potential and regulating myocyte excitability, understanding VGSC regulation is an important step in identifying potential therapeutic strategies to offset conduction defects associated with heart failure -a condition where calcium signaling effectors, like CaMKII, are upregulated to maximize contractility of the failing heart. We have isolated the region of CaMKII phosphorylation to a particular domain of the Nav1.5 channel and we are currently attempting to define the sites of CaMKII phosphorylation that modulate the biophysical properties of Nav1.5. In addition, we have observed that CaMKII interacts with Nav1.5 only when autophosphorylated, suggesting that the binding site on CaMKII requires a specific state of activation to undergo a stable interaction with Nav1.5. We are in the process of identifying the specific residues that anchor CaMKII to Nav1.5 (on both kinase and channel) in an attempt to study the role that CaMKII targeting plays in regulating Nav1.5 function as well as identifying potential peptide inhibitors that may be used to disrupt this process in cells. Our goal is optimize inhibitors to specifically disrupt Nav1.5 without disrupting CaMKII interaction with other ion channels, like Cav1.2. We are also pursuing the hypothesis that the physical association of CaMKII with Nav1.5 allows calcium and CaM to co-regulate both targets, as calmodulin binds directly to the channel and the kinase. Our working model is that co-regulation of simultaneous CaM targets permits intimate channel-effector coupling in the face of global changes in intracellular calcium levels.

Poster Board 16

Calmodulin interactions with the neuronal gap junction protein vonnexin35

Xiaofan Li, Ph.D.[1], Cheryl K. Mitchell[1], Ruth Heidelberger, M.D., Ph.D.[2], John O'Brien, Ph.D.[1]

[1]Ophthalmology & Visual Science, University of Texas HSC Houston, Houston, TX, USA, [2]Neurobiology and Anatomy, University of Texas HSC Houston, Houston, TX, USA.

Nearly all gap junctions are uncoupled by high intracellular calcium although the physiological significance of this phenomenon is unclear. Previously, we reported calmodulin (CaM) binding connexin35 (Cx35/36) that could be responsible for Ca^{2+}-dependent uncoupling. Given the widespread occurrence of Cx35/36 in mixed synapses containing Ca^{2+}-permeable NMDA receptors, we hypothesized that this interaction may regulate gap junctional coupling during normal synaptic sig-

naling. We examined the CaM binding site of Cx35 and evaluated whether CaM binding can regulate gap junction coupling. GST fusion proteins containing the C-terminal domain of Cx35 were expressed in bacteria. A series of mutants (KI261EA, I267A, R268A, VQ270AA and I277A) were generated to disrupt elements of CaM binding motifs. Binding interactions were measured by surface plasmon resonance. Cx35 was expressed in stably-transfected HeLa cells, and gap junctional coupling was measured by scrape-loading in the presence or absence of ionomycin to elevate intracellular Ca^{2+}. Changes in Ca^{2+} were measured ratiometrically using Fura-2. CaM binding to the wild type Cx35 C-terminus had an affinity of 1.02 ± 0.16 μM. K261 and I262 were essential for this interaction, since the amount of CaM bound to KI261EA mutant decreased to 7% compared to WT. The binding affinity for I267A and R268A decreased to 6.05 ± 0.62 μM and 2.66 ± 0.25 μM, respectively. The affinity of I277A and VQ270AA mutants were not significantly different than WT, suggesting that the site does not contain an IQ-like motif. Ionomycin treatment caused significant reduction of tracer coupling in Cx35-transfected HeLa cells that was blocked by application of CaM inhibitor W-7. We conclude that CaM plays a role in Ca^{2+}-dependent uncoupling of gap junctions. Experiments are ongoing to determine if the CaM binding site we identified is involved in this regulation.

Poster Board 17

Molecular mechanism of calmodulin-mediated fast desensitization of olfactory CNG-channels

Clemens Waldeck, Nicole Ungerer, Kerstin Vocke, Stephan Frings, Frank Möhrlen.

Department of Moleculare Physiology, Institute of Zoology, University of Heidelberg, Heidelberg, Germany.

Rapid adaptation terminates the receptor current in olfactory sensory neurons (OSNs) after a few seconds, even in the continuous presence of odorants. This phenomenon fundamentally affects OSN function, as it restricts the sensory response to a brief phase of electrical activity. The molecular processes underlying rapid adaptation are not completely understood, but they thought to be related to desensitization of the tetrameric olfactory cyclic nucleotide-gated (CNG)-channel. This channel is composed of 2 principal subunits CNGA2, and the modulatory subunits CNGA4 and CNGB1b. It conducts Ca2+ into the cell and is directly desensitized through Ca2+-camodulin. Both modulatory subunits CNGA4 and CNGB1b have non-conventional calmodulin binding sites of the IQ-type. Previous results (Bradley et al.) lead to the hypothesis that both modulatory subunits (CNGA4 and CNGB1b) have to bind calmodulion to trigger desensitization. Here we show, using a fluorescence spectroscopic binding assay, that only peptides corresponding to the calmodulin-binding site of CNGB1b, but not of CNGA4, bind calmodulin in a Ca2+-dependant way. We confirm this surprising finding functionally using patch-clamp recordings from heterologously expressed channels. The CNGB1b calmodulin-binding site is necessary and sufficent for Ca2+-dependant desensitization of the CNG-channel, while the CNGA4 calmodulin-binding site is not required. The CNGB1b-mediated desensitization by Ca2+-calmodulin increases the K½ for cAMP 8-fold and also increases the voltage dependent rectification of the CNG-channel current. This consistent set of results reveals the pivotal role of the CNGB1b subunit in Ca2+-dependent desensitization of the olfactory CNG-channel.

Poster Board 18

An amino-terminal CaM binding site (NSCaTE) transforms the spatial Ca^{2+} selectivity of CaM-mediated Ca^{2+}-dependent inactivation (CDI) of Ca_V channels

Ivy E. Dick, Michael R. Tadross, Haoya Liang Liang, PhD, Lai Hock Tay, PhD, Wanjun Yang, David T. Yue, MD, PhD.
BME, Johns Hopkins, Baltimore, MD, USA.

Calmodulin-dependent regulation of Ca^{2+} channels impacts numerous realms of biological function. A single calmodulin (CaM) constitutively associates with the channel carboxy tail, the focus of structure-function analysis of CaM regulation. Interestingly, Ca^{2+} binding to the C- and N-terminal lobes of CaM can independently initiate distinct regulatory processes. The C-lobe invariably responds to the ~100 μM Ca^{2+} pulses driven by the associated channel ('local Ca^{2+} selectivity'). Alternatively, the N-lobe somehow ignores these intense pulses and responds preferentially to the Ca^{2+} signals generated by the cumulative effects of Ca^{2+} sources distant from the home channel ('global Ca^{2+} selectivity'). Such global selectivity is essential for coordinated Ca^{2+} regulation across larger cellular expanses. Here, we report that inclusion of a Ca^{2+}/CaM binding site (*NSCaTE: N-terminal Spatial Ca^{2+} Transforming Element)* within the amino terminus of certain Ca_V channels transforms spatial Ca^{2+} selectivity. Firstly, native $Ca_V2.2$ channels lack *NSCaTE*, and exhibit N-lobe mediated CDI with a global selectivity. Adding *NSCaTE* to $Ca_V2.2$ channels strongly converts their regulation towards a local profile. Residues within *NSCaTE* that are important for this conversion also turn out to be critical for Ca^{2+}/CaM binding. In fact, the extent of global-to-local conversion correlates tightly with the Ca^{2+}/CaM affinity of mutated *NSCaTE* modules. This uncovers a close linkage between the Ca^{2+}/CaM-binding affinity of *NSCaTE* and its ability to transform spatial Ca^{2+} selectivity. Secondly, $Ca_V1.2$ and 1.3 channels inherently possess *NSCaTE*, and thereby exhibit N-lobe CDI with local selectivity. Removal of *NSCaTE* converts N-lobe CDI of these channels to a clearly global profile. In sum, *NSCaTE* differentially tunes the spatial Ca^{2+} selectivity of N-lobe CDI across two channel clades (Ca_V1 versus Ca_V2), and this module furnishes a telling clue regarding the mechanism of spatial selectivity.

Poster Board 19

Role of CaM in the interaction with connexins

Yanyi Chen, Jenny Yang.
Chemistry Department, Georgia State University, Atlanta, GA, USA.

The gap junction proteins expressed in fiber cells of human eye lens are formed by the subunit transmembrane proteins, connexins. Mutations of connexins were reputed to result in dominant cataracts in human eyes and heart disease. Calmodulin (CaM) play a role in the regulating connexin's channel activity. We have synthesized several peptide fragments in order to demonstrate the interaction between connexins and CaM. Our results suggested that in the presence of calcium, predicted connexin peptides bind with CaM directly using various spectroscopic method including NMR spectroscopy, CD and fluorescence. We have shown that CaM forms 1:1 complex with connexin peptides with strong affinity using high resolution NMR. A large conformational change of CaM was also observed. Our study further proves that the binding of calcium to CaM is critical for mediating the gap junction in cell to cell communication.

Poster Board 20

Calcium binding to the C- and N-termini of calmodulin: strong and slow vs. weak and fast

Guido C. Faas, PhD, Istvan Mody, PhD.

Neurology, UCLA, Los Angeles, CA, USA.

Calmodulin (CaM) contains two high affinity Ca^{2+}-binding sites on the C-terminus (C-sites) and two lower affinity sites on its N-terminus (N-sites). Activation of some CaM- regulated systems require Ca^{2+} binding either to the N-sites or C-sites or all four sites. The steady-state affinity of these sites for Ca^{2+} has been measured, but in order to gain insight into how Ca^{2+} signals and their temporal patterns are transduced , it is essential to know the precise nature of the Ca^{2+}-binding kinetics. We determined these kinetics *in vitro*, by rapidly uncaging Ca^{2+} in the presence of CaM and measuring the timecourse of [Ca^{2+}] with a fluorescent Ca^{2+} indicator. Fitting the data to a kinetic model incorporating cooperative binding reveal that Ca^{2+} binding to the C-sites is a two step process with k_{on}s of 6.3 and 36 $\mu M^{-1}s^{-1}$ and k_{off}s of 27 and 11 s^{-1} ($K_{d(apparent)}$=1.1 µM). The N-sites had k_{on}s of 39 and 17 $\mu M^{-1}s^{-1}$ and k_{off}s of 2200 and 34 s^{-1} ($K_{d(apparent)}$=11 µM). Despite their lower affinity, the N-sites are faster at binding Ca^{2+} than the C-sites. Accordingly, simulations show that Ca^{2+} binding to the N-sites is capable of linearly following rapid Ca^{2+} signals (50 Hz). Conversely, the C-sites are too slow in releasing Ca^{2+} and accumulate Ca^{2+} thus effectively integrating rapid Ca^{2+} signals. Simulations also show that single Ca^{2+} spikes favor binding to N-sites due to the faster k_{on}s, but during slow changes in [Ca^{2+}], Ca^{2+} preferentially binds to the C-sites because of their higher affinity. The diference between the kinetics of Ca^{2+} binding to the C- and N-sites makes it very likely that in addition to their magnitude, the temporal patterns of Ca^{2+} signals will differentially activate processes regulated by N- and/or C-termini.

Poster Board 21

Store-dependent and -independent modes regulating CRAC channel activity of human ORAI1 amd ORAI3

Shenyuan Zhang[1], J. Ashot Kozak[2], Weihua Jiang[1], Andriy V. Yeromin[1], Jing Chen[1], Ying Yu[1], Aubin Penna[1], Wei Shen[1], Victor Chi[1], Michael D. Cahalan[1].

[1]UCI, Irvine, CA, USA, [2]Department of Neuroscience, Cell Biology and Physiology, Wright State University, Dayton, OH, USA.

We evaluated currents induced by expression of human homologs of Orai together with STIM1 in HEK cells. When coexpressed with STIM1, Orai1 induced a large inwardly rectifying Ca^{2+} selective current with Ca^{2+}-induced slow inactivation. Expression of the C-terminal portion of STIM1 with Orai1 was sufficient to generate CRAC current without store depletion. 2-APB activated a large relatively nonselective current in STIM1 and Orai3 co-expressing cells. 2-APB also induced Ca^{2+} influx in Orai3-expressing cells without store depletion or co-expression of STIM1. The Orai3 current induced by 2-APB exhibited outward rectification and an inward component representing a mixed calcium- and monovalent current. A pore mutant of Orai3 inhibited store-operated Ca^{2+} entry and did not carry significant current in response to either store depletion or addition of 2-APB. Analysis of a series of Orai1-3 chimeras revealed the structural determinant responsible for 2-APB-induced current within the sequence from the second to third transmembrane segment of Orai3. The Orai3 current induced by 2-APB may reflect a store-independent mode of CRAC channel activation that opens a relatively nonselective cation pore.

Poster Board 22

Characterization of action potential and inward currents in freshly isolated ventricular myocytes from Zebrafish (Danio rerio)

Fabien Brette, Caroline Cros, Guillermo Luxan, Hayley Dixey, Christopher Wilson, Holly A. Shiels.
Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom.

The Zebrafish is a tropical teleost fish that has been the focus of an increasing number of developmental studies. Physiological interest in this species has been spurred on by the ease of mutants which can be induced by chemomutagenesis. However, our understanding of the basic physiology of Zebrafish, in particular cardiac excitation-contraction coupling is limited. Indeed, there is currently no information about the electrical activity of single myocytes freshly isolated from Zebrafish ventricle; this study addressed this point. Viable ventricular myocytes were obtained by enzymatic digestion. The whole cell configuration of the patch clamp was used to record Na current (INa), Ca current (ICa) and action potential (AP). Results are presented as mean±SE and analyzed with paired t-test. Single ventricular myocytes from Zebrafish are long and thin, as described for other fish species. Cell capacitance was 26.0±1.1 pF (n=69). ICa density (test pulse to 0 mV) was -11.4±3.4 pA/pF and INa density (test pulse to -40 mV) was -104±22 pA/pF (n=6). Resting membrane potential was -66±2 mV (n=19). At 0.1 Hz stimulation frequency, AP duration at 25, 50, and 90% repolarization was 50±17, 105±27, 137±35 ms, respectively (n=12), indicating the presence of a plateau phase. Increasing stimulation frequency to 2 Hz significantly decreased AP duration (n=6, $p<0.05$). To conclude, we have developed a method to obtain viable isolated ventricle myocytes from Zebrafish heart. Ionic currents studied present characteristics similar to other fish species. The presence of a plateau during the AP suggests that this species might be appropriate for ion channels related mutation screening of cardiac alteration.

Poster Board 23

Calmodulin association with connexin32 derived peptides suggests transdomain interaction in chemical gating of gap junction channels

Ryan Dodd[1], Camillo Peracchia, MD[2], Daniel Stolady[1], Katalin Torok, PhD[1].

[1]BMS, SGUL, London, United Kingdom, [2]Pharmacology and Physiology, University of Rochester, Rochester, NY, USA.

Calmodulin plays a key role in the chemical gating of gap junction channels[1]. Two calmodulin-binding regions have previously been identified in connexin32 gap junction protein, one in the N-terminal and another in the C-terminal cytoplasmic tail of the molecule[1,2]. The aim of this study was to better understand how calmodulin interacts with the connexin32 binding domains. Lobe-specific calmodulin interactions were studied by stopped-flow kinetics using Ca^{2+} binding-deficient mutants. Both the N- and C-lobes of calmodulin were found to engage in the interaction with peptides corresponding to the N-terminal tail (residues 1-22) of Cx32; the C-lobe of calmodulin bound with a higher affinity [Ca^{2+} dissociation rate constants $k_{3,4}$, 1.7 ± 0.5 s^{-1}] than the N-lobe ($k_{1,2}$, 10.8 ± 1.3 s^{-1}). In contrast, either the C- or the N-lobe, but only one calmodulin lobe at a time bound to peptides representing the C-terminal tail domain (residues 208-227) of connexin32 ($k_{3,4}$, 2.6 ± 0.1 s^{-1} or k_1, 13.8 ± 0.5 s^{-1} and k_2, 1000 s^{-1}). Calmodulin-binding domains of the N- and C-terminal

tails of connexin32 were best defined as residues 1-21 and 216-227, respectively. Our kinetic and equilibrium binding data obtained by FRET[3], showing separate functions of the N- and C- lobes of calmodulin in the interactions with connexin32, suggest a gap junction gating mechanism that involves trans-domain or trans-subunit bridging by calmodulin.

[1]Peracchia, C. BBA (2004)1662, 61-80.
[2]Torok et al., Biochem J. (1997) 326, 479-483.
[3]Torok et al., Biochemistry (2001) 40, 14878-14890.

This work was supported by the Wellcome Trust.

Poster Board 24

Structural studies of TRPV channels and their regulation by calmodulin

Rachelle Gaudet.

Molecular and Cellular Biology, Harvard University, Cambridge, MA, USA.

TRPV channels play key roles in pain, thermo- and mechanosensation, and calcium homeostasis. The six mammalian TRPV channels partition into two groups: TRPV1-4, involved in sensory signaling; and TRPV5 and TRPV6, expressed in the intestinal tract and kidneys and important for calcium homeostasis. We determined the crystal structures of the N-terminal ankyrin repeat domain of TRPV1, TRPV2 and TRPV6. Superficially, the structures are very similar, as expected from sequence homology. The TRPV ankyrin repeat domains have six ankyrin repeats, with unusually long finger loops. The structures do have notable differences in their details. Notably, the structural differences result in drastically different biochemical properties. The structure of the ankyrin repeats of TRPV1, a channel found in nociceptor neurons and activated by noxious stimuli like heat and capsaicin, shows a bound ATP molecule. Biochemical and electrophysiology data support a role for this ATP-binding site in regulating TRPV1 sensitivity. We showed that intracellular ATP can sensitize the TRPV1 response to capsaicin, whereas calcium-bound calmodulin, acting through the same binding region on the TRPV1 ankyrin repeats, causes desensitization. Mutagenesis studies have refined the determinants for the interaction on both TRPV1 and calmodulin. Furthermore, this binding site for ATP and calmodulin is conserved in TRPV1 from other species, more so than the capsaicin-binding site. However, ATP binding is not conserved in several other TRPV channels, including TRPV2, a close homolog of TRPV1 also involved in sensing hot temperatures, and TRPV5 or TRPV6. Similarly, the TRPV1 ankyrin repeats bind to calmodulin, as do - although weakly - the TRPV5 ankyrin repeats, whereas the TRPV2 and TRPV6 ankyrin repeats do not. These findings contribute to a model for the calcium-dependent regulation of TRPV1 sensitivity. We are pursuing experiments to better understand this mechanism at a molecular level.

Poster Board 25

S100A1 and calmodulin compete for binding the CaM binding domain of RyR1 to modulate skeletal muscle EC coupling

Benjamin L. Prosser, M.S.

Molecular Medicine, University of Maryland School of Medicine, Baltimore, MD, USA.

S100A1, a 21-kDa Ca^{2+} binding protein expressed most highly in striated muscle, activates the skeletal muscle Ryanodine Receptor (RyR1)/ Ca^{2+} release channel in vitro. However, a definitive endogenous role of S100A1 in skeletal muscle EC coupling has not been previously demonstrated. Using a transgenic mouse line lacking S100A1, we show that endogenous S100A1 modulates SR Ca^{2+} release in enzymatically dissociated muscle fibers. Utilizing high-speed confocal microscopy we find depressed SR Ca^{2+} release in response to single action potentials in $S100A1^{-/-}$ muscle fibers compared to WT littermates, which can be reversed by adenoviral expression of S100A1 in -/- fibers. Previous studies have shown that apo-Calmodulin (CaM) is a weak activator of RyR1, whereas Ca^{2+}-CaM is an inhibitor of RyR1 in vitro, and we have identified an S100 consensus binding sequence within the previously well characterized CaM binding domain (CaMBD) of RyR1. Through competition assays we now demonstrate that CaM and S100A1 compete for binding this site on the full length RyR1 at similar affinities. Utilizing NMR spectroscopy we have also solved the solution structure of S100A1 bound to a 12 residue peptide from this CaMBD. We propose that endogenous S100A1 and CaM may compete for binding to this site to differentially modulate SR Ca^{2+} release. We present a model where the relative free concentrations of these two proteins available to interact with RyR1 may determine the activation state of the SR Ca^{2+} release channel and therefore significantly affect skeletal muscle EC coupling. We propose to further evaluate the localization and expression of these two proteins, and to explore a potential role of endogenous CaM in EC coupling in intact skeletal muscle fibers.

Poster Board 26

Mechanism of global Ca^{2+} sensing by calmodulin in complex with a Ca^{2+} source

Michael Tadross, Ivy Dick, David Yue.

Johns Hopkins Univ, Baltimore, MD, USA.

Ca^{2+} sensors commonly reside within nanometers of Ca^{2+} sources to enable privileged signaling. Yet, such proximity puts sensors at risk of being 'blinded' by intense Ca^{2+} entry through the local source, thus challenging the sensing of far weaker Ca^{2+} signals integrated from distant sources. This latter property is nonetheless crucial for coordinated signaling. A prototypic solution is found in calmodulin (CaM), as complexed with Ca_V1-2 Ca^{2+} channels. A single CaM persistently associates with channels, and Ca^{2+} binding to its C- and N-lobes trigger different regulatory processes on the host channel. Though the lobes of CaM are ≤6 nm apart, and thereby face the same Ca^{2+}, they somehow favor spatially distinct Ca^{2+} sources: the C-lobe prefers intense intermittent Ca^{2+} influx via the host channel (local selectivity), whereas the N-lobe responds selectively to diminutive persistent Ca^{2+} signals from distant sources (global selectivity). Here, we reveal the mechanisms underlying these contrasting selectivities. Local selectivity is achieved by responding preferentially to Ca^{2+} intensity. Global selectivity exploits a mechanism with unprecedented preference for Ca^{2+} persistence over intensity. This counterintuitive behavior reflects the interplay of three factors: channel open probability; Ca^{2+} (un)binding kinetics from CaM; and the balance between channel affinity for Ca^{2+}/CaM versus apoCaM. New methods enable manipulation of these factors, permitting extensive testing of

mechanism. Specifically, open probability is adjusted via a 'voltage-block' technique, combined with channels engineered for enhanced opening. Importantly, the ratio of channel affinity for Ca^{2+}/CaM versus apoCaM is graded via mutagenesis of a novel *NSCaTE* Ca^{2+}/CaM binding motif, reported elsewhere at this meeting. Finally, the theoretical basis of the selectivity mechanisms is confirmed in the stochastic realm, via particle simulations of Ca^{2+} in the channel nanodomain. These results likely generalize to other spatiotemporally selective Ca^{2+} decoding systems.

Poster Board 27

Functional role of IQ region in voltage geated sodium channel

Subrata Biswas, Ph.D, Deborah Disilvestre, M.S., Yanli Tian, M.D., Victoria L. Halperin, B.S., Gordon F. Tomaselli, M.D.

Cardiology, Johns Hopkins University School of Medicine, Baltimore, MD, USA.

The CaM-IQ motif interaction in the cytoplasmic tail (CT) of voltage gated sodium channels (Na_V) channels influence slow inactivation kinetics, sustained current and current density. In addition, its role in channel trafficking may be critical. In skeletal muscles Na_V ($Na_V1.4$) mutations in the CaM binding motif reduce total and cell surface expression. However truncations of the channel that include the IQ motif does not eliminate channel trafficking but led to loss of Na current. Co-expressing the truncated channel with the remaining portion of the CT-Na_V partially restore the current. Rescued current is sensitive to TTX, indicating the IQ motif distal CT interacts with the IQ motif truncated channel in rescuing the current. The IQ motif supports a role for other regions of the CT of the Na channel in both function and expression. Upstream of CT-Na_V also has a direct Ca^{2+} binding EF-hand like (EFL) motif and, it's role in Ca^{2+} regulation of normal $Na_V1.4$ current is uncertain. The EFL and IQ motifs are at opposite ends of the proximal structured portion of the CT-$Na_V1.4$. In the presence of CaM and Ca^{2+}, it is easy to imagine either competitive or synergistic regulation of the channel by these two motifs. Since both EFL and IQ motifs are associated with regulation of fast inactivation, role of these motifs in CT mutation that affect fast inactivation and, associated with cardiac and skeletal muscle diseases were evaluated.

Poster Board 28

How do the intrinsic (EF-hand domain of hH1) and extrinsic (calmodulin) Ca^{2+} sensors regulate gating of the human cardiac sodium ion channel Nav1.5?

Benjamin A. Chagot, PhD.

Biochemistry, Vanderbilt University, Nashville, TN, USA.

The sodium channel hH1(Nav1.5) has been proven to be a main voltage-gated sodium channel found in human heart. A lot of diseases such as long QT syndromes (abnormal heart rhythm), Brugada syndrome (ventricular arrhythmia leading to sudden death) as well as atrial fibrillation are associated with mutations in hH1 gene. Channel function is mainly involved in action potential initiation and conduction. The activity of the channel could be modulated by a change in the membrane potential but we know that calcium could also modulate the activation of the channel. The voltage-gated sensing takes part in the transmembrane regions of each domain and the calcium sensitivity is given by the C terminal region of the channel. The channel could be inactivated by two mechanisms: a fast- and a slow-inactivation mechanism. But less is known about these inactivation's mechanisms. We are working on the calcium regulation of the channel and think that it could also be related to the fast inactivation, we are trying to understand how the different domains of the protein are interacting together and with calmodulin to modulate the channel activation.

Poster Board 29

Single-channel properties of recombinant retinal L-type Cav1.4 channels

Christian F. Erxleben, PhD[1], Rolf Herrmann, PhD[2], Vadim Arshavsky, PhD[2], David L. Armstrong, PhD[1].

[1]Laboratory of Neurobiology, NIEHS/NIH, Research Triangle Park, NC, USA, [2]Albert Eye Research Institute, Duke University, Durham, NC, USA.

We expressed the retinal Cav1.4 α subunit in a hamster kidney cell line, BHK6, with α2δand β2A accessory subunits and characterized their single-channel properties in cell-attached patches under voltage-clamp. With 90 mM barium as the charge carrier in the patch pipette, depolarization to voltages more positive than -30 mV produced unitary currents with a conductance of 21 pS and a mean open duration of <1 ms. Application of the dihydropyridine agonist enantiomer, (-)BAY K8644, significantly increased the mean open duration and open-time distributions were best fit by an additional component in the ms range. Thus, Cav1.4 have very similar biophysical and pharmacological properties to other L-type calcium channels.

Poster Board 30

Kinetic mechanism of activation of calmodulin-dependent kinase I

Nadja Hellmann[1], Stephen Martin[2], Suleman Bawumia[2], Peter Bayley[2].
[1]Institute for Molecular Biophysics, Mainz, Germany, [2]NIMR, London, United Kingdom.

Calmodulin activates the calmodulin-dependent kinase I (CKI) in a Ca-dependent manner. The activation process involves removal of the autoinhibitory domain of CKI, on which also the calmodulin-binding domain is located. No crystal structure of a complex between calmodulin and a kinase-target is available so far. The calmodulin-binding sequence of CKI has been identified, and the structure of the corresponding peptide with calmodulin was solved. However, there is increasing evidence, that additional interactions apart from the calmodulin-binding domain are existing. This is supported by our data on the fluorescence changes of labels attached to different position in calmodulin. A comparison of the fluorescnce properties of the complex between calmodulin and the kinase and calmodulin and the sequence corresponding to the calmodulin-binding domain indicate that calmodulin interacts also with other parts of the kinase. Furthermore, the analysis of kinetic traces for the binding of calmodulin to the kinases revealed, that calmodulin binds to the closed form of the kinase. Thus, the recognition of the targets by calmodulin is not based on the typical sequence for calmodulin-binding peptides, since these are hidden in the autoinhibited conformation of the kinase.

Poster Board 31

Tuning metal binding affinity and biological function investigation of calmodulin

Jie Jiang, Jenny J. Yang.

Chemistry, Georgia State University, Atlanta, GA, USA.

• Calmodulin (CaM) is a significant mediator by specifically targeting various enzymes, such as phosphatases, ion channels, some metabolic enzymes, and so on. Ca^{2+} cooperatively binds to four EF-hand motifs in both N- and C-domain of CaM, resulting in a Ca^{2+} -induced conformational change. In this study, different mutations located in the

binding sites have been made to determine their effects on the calcium-binding affinities of CaM, including both increase and decrease of the binding affinities. Further, we have examined the conformational properties changes of such mutations which is essential for its activation and inhibition of target molecules by various biophysical methods. In addition, we investigated effects on the biological functions and channel activities due to Ca^{2+} binding affinity changes of these mutants.

Poster Board 32

Calcium-dependent facilitation of $Ca_v2.1$ (P/Q-type) calcium channels is attenuated by familial hemiplegic migraine type 1 mutations and in a splice-dependent manner

Paul Adams, Esperanza Garcia, PhD, Sian Spacey, MD, Terrance P. Snutch, PhD.

University of British Columbia, Vancouver, BC, Canada.

The calmodulin mediated calcium-dependent facilitation (CDF) of $Ca_v2.1$ channels is gaining acceptance as a relevant channel property capable of affecting synaptic plasticity. It has been shown that disruption of calcium sensor binding to the $Ca_v2.1$ subunit can prevent CDF and result in a marked reduction in synaptic plasticity in a model system. We hypothesized that naturally occurring P/Q-type channel mutations might alter CDF and could likewise affect synaptic plasticity and thereby compromise neuronal function. In the present study we investigated for the first time how mutations implicated in Familial Hemiplegic Migraine type 1 (FHM-1) affect CDF of $Ca_v2.1$ channels. Our results show that all three FHM-1 mutations examined can result in a significant reduction in the ability of P/Q-type channels to undergo CDF. However, the effects were highly alternative splice-dependent with some FHM-1 mutations nearly completely abolishing CDF in certain $Ca_v2.1$ splice variants, while in other $Ca_v2.1$ splice variants the mutations had little effect on CDF. It is likely that the observed reduction in prepulse-dependent facilitation is due to channels being in a constitutively facilitated state. We hypothesize that the selective gain-of-function effects of FHM-1 mutations on certain $Ca_v2.1$ splice variants may underlie the increased glutamate release at central synapses relevant to the disease pathophysiology. Overall, our results provide new insights into the disease mechanisms related to FHM-1 and also provide the first evidence for P/Q-type channel CDF as being potentially altered in a human disease state.

Poster Board 33

Intracellular calcium potentiates and inactivates the sensory ion channel TRPA1 as revealed by a pore mutant

Yuanyuan Wang, Rui Chang, Hang Waters, Emily Liman, Ph.D.

University of Southern California, Los Angeles, CA, USA.

The transient receptor potential A1 (TRPA1) channel is the molecular target for environmental irritants and pungent chemicals, many of which bind covalently to the channel and cause long-lasting activation. Extracellular Ca2+ is a key regulator, both potentiating and subsequently inactivating TRPA1 channels through mechanisms that are not well understood. To determine if Ca2+ entry is required for either or both processes, we generated a pore mutant of TRPA1 in which Ca2+ permeability was greatly reduced. Currents through mutant channels were neither potentiated nor inactivated by extracellular Ca2+ and both were restored when intracellular Ca2+ buffering was reduced, indicating that elevation of intracellular Ca2+ is essential for both potentiation and inactivation. To gain insight into the structural basis for potentiation and inactivation of TRPA1, we characterized the divalent cation sensitivity and specificity of

both processes. Both inactivation and potentiation could be induced by extracellular Ca2+, Ba2+, and Sr2+, whereas only inactivation could be induced by Mg2+. We also tested whether potentiation or inactivation could be attributed to a putative EF hand in the N terminus of the protein, to PI(4,5)P2 hydrolysis or to calmodulin. Our data doe not support any of these possibilities, leaving open the question of how Ca2+ promotes potentiation and inactivation of TRPA1. Our identification of a pore mutant of TRPA1 provides an important tool for further investigating the contribution of Ca2+ influx through TRPA1 to cellular physiology.

Poster Board 34

Calcium- and voltage-dependent regulatory mechanisms of cardiac L-type Ca^{2+} channels

Satomi Adachi-Akahane, Ph.D.

Pharmacology, Toho Univ. Fac. Med. Sch. Med., Tokyo, Japan.

Cardiac L-type Ca^{2+} channels play critical roles in pace-maker activity and excitation-contraction coupling through shaping action potential waveform, triggering local Ca^{2+} release from the SR, and fine-tunng the SR Ca^{2+} concent *via* reciprocal regulation between L-type Ca^{2+} channels and RyRs. The activation and inactivation kinetics of L-type Ca^{2+} channels are dynamically regulated by voltage and $[Ca^{2+}]i$. The elevation of local Ca^{2+} around the L-type Ca^{2+} channel turns on the Ca^{2+}-dependent inactivation and facilitation, where calmodulin (CaM)-binding 'IQ' and CaMKII are both implicated. In atria, two-types of L-type Ca^{2+} channel alpha subunits, $Ca_V1.2$ and $Ca_V1.3$, are expressed, while $Ca_V1.2$ is dominantly expressed in ventricle. To elucidate Ca^{2+}- and voltage-dependent regulatory mechanisms of cardiac L-type Ca^{2+} channels, we compared the voltage- and Ca^{2+}-dependent inactivation and facilitation of $Ca_V1.2$ and $Ca_V1.3$. We have been further exploring the gating kinetics of atrial and ventricular L-type Ca^{2+} channels and their relationships with modulation by protein kinases such as PKA and CaMKII. The molecular determinants of the gating regulation of $Ca_V1.3$, in comparison with those of $Ca_V1.2$, are under investigation.

Poster Board 35

Structural basis for calmodulin-mediated regulation of the ryanodine receptor

Adina Maximciuc Kilpatrick, Ph.D.

Biochemistry and Cell Biology, Rice University, Houston, TX, USA.

We investigate the structural basis of calmodulin (CaM)-mediated regulation of the skeletal muscle ryanodine receptor (RyR1) by pursuing NMR and X-ray crystallographic studies of CaM/RyR1 peptide complexes at low and high calcium concentrations. We determined the 2.0 Å crystal structure of Ca^{2+}CaM in complex with a 30-residue RyR1 peptide (P3614-3643). The structure reveals that hydrophobic anchor residues in the target arranged in a novel '1-17' spacing allow each calmodulin lobe to interact with the peptide independently. NMR ^{15}N relaxation measurements and residual dipolar couplings confirm the structure of each calmodulin lobe and show that the complex undergoes segmental domain motion. Fluorescence measurements indicate that CaM binds with both domains to the 3614-3643 peptide, whereas if the second anchor is unavailable, CaM can bind without collapsing on the target. The independence of the two lobes of calmodulin offers a structural explanation for how other domains may compete for binding to this region to regulate the channel. NMR studies of these interaction partners indicate that the RyR1 target binds to only the C-lobe of CaM at low

calcium concentrations, similar to other ion channels whose activity is modulated by CaM. The conformation of the C-lobe in the calcium-free complex closely resembles the one seen in the crystal structure of the calcium-loaded complex, suggesting that binding of the RyR1 peptide locks the C-domain of CaM in a conformation similar to that of the calcium-loaded protein. Comparison of the CaM/peptide complexes at low and high calcium concentrations provides a model for how CaM interacts with this region of RyR1: the C-lobe is constitutively tethered to the 3614-3643 target and is calcium-loaded even at low, resting calcium levels, whereas high calcium induces the N-lobe to bind to this region.

Poster Board 36

Protein flexibility and function: theoretical study of conformational transitions of calmodulin domains

John J. Portman, Ph.D.

Physics, Kent State University, Kent, OH, USA.

The key to understand a protein's function often lies in its conformational dynamics. We develop a coarse-grained variational model to investigate the interplay between structural transitions, conformational flexibility, and function of the N-terminal, C-terminal, and even-odd fragment of calmodulin. The mechanisms are investigated in part in terms of cracking (local unfolding).

Poster Board 37

Differential effects of phospholamban and Ca^{2+}/calmodulin-dependent kinase II on Ca^{2+} uptake and release in isolated cardiac myocytes

Andreas A. Werdich[1], Eduardo A. Lima, PhD[1], Dzhura Igor, PhD[1], Madhu V. Singh, PhD[2], Jingdong Li, PhD[2], Mark E. Anderson, PhD[2], Franz Baudenbacher, PhD[1].

[1]BME, Vanderbilt University, Nashville, TN, USA, [2]Medicine, Carver College of Medicine, Iowa City, IA, USA.

In cardiac myocytes, the activity of the multifunctional Ca^{2+} and calmodulin-dependent protein kinase II (CaMKII) is hypothesized to regulate Ca^{2+} release from and Ca^{2+} uptake into the sarcoplasmic reticulum (SR) via phosphorylation of the ryanodine receptor 2 (RYR2) and phospholamban (PLN), respectively. We tested the role of CaMKII and PLN on the frequency adaptation of $[Ca^{2+}]_i$-transients in nearly 500 isolated ventricular cardiomyocytes from transgenic mice chronically expressing a specific CaMKII inhibitor, interbred into wild-type (WT) or PLN null ($PLN^{-/-}$) backgrounds under physiologically relevant pacing conditions (i.e. frequencies ranging from 0.2 to 10 Hz and at 37 ^{o}C). Our results suggest that in isolated mouse ventricular cardiac myocytes (1) combined chronic CaMKII inhibition and PLN ablation lead to a slowing of Ca^{2+} release at physiological frequencies, (2) frequency-dependent decay of the amplitude and shortening of the $[Ca^{2+}]_i$-transient occurs independent of chronic CaMKII inhibition and in models with PLN ablation, (3) PLN-mediated regulation of Ca^{2+} uptake is diminished at higher stimulation frequencies well within the physiological range.

Poster Board 38

Phosphorylation determines the calmodulin-mediated calcium response and water permeability of AQP0

Katalin Kalman, Ph.D., Karin Nemeth-Cahalan, M.S, Alexandrine Froger, Ph.D., James E. Hall, Ph.D.

Physiology, UCI, Irvine, CA, USA.

In Xenopus oocytes the water permeability of AQP0 (P_f) increases with removal of external calcium, an effect that is mediated by cytoplasmic calmodulin (CaM) bound to the C-terminus of AQP0. To investigate the effects of serine-phosphorylation on CaM-mediated Ca^{2+} regulation of P_f, we tested the effects of kinase activation, CaM inhibition, and a series of mutations in the C-terminus CaM binding site. Calcium regulation of AQP0 P_f manifests four distinct phenotypes: Group 1: with high P_f upon removal of external Ca^{2+} (wt, S229N, R233A S235A, S235K, K238A, R241E); Group 2: with high P_f in elevated (5 mM) external Ca^{2+} (S235D, R241A); Group 3: with high P_f and no Ca^{2+} regulation (S229D, S231N, S231D, S235N, S235N/I236S); Group 4: with low P_f and no Ca^{2+} regulation (PKA and PKC activators, S229D/S235D, S235N/I236S). Within each group, we tested if CaM binding mediates the phenotype, as shown previously for wt AQP0. In the presence of CDZ, a CaM inhibitor, S235D showed high P_f and no Ca^{2+} regulation, suggesting that S235D still binds CaM. Contrarily, S229D showed a decrease in recruitment of CaM, suggesting that S229D is unable to bind CaM. Taken together, our results suggest a model in which CaM acts as an inhibitor of AQP0 P_f. CaM binding is associated with a low P_f state, and a lack of CaM binding is associated with a high P_f state. Pathological conditions of inappropriate phosphorylation or calcium/CaM regulation could induce P_f changes contributing to the development of a cataract.

Poster Board 39

Gating modulation of L-type calcium channels by intra- and intermolecular protein-protein interactions

Alexandra Koschak[1], Anamika Singh[1], Mathias Gebhart[1], Gabriella Juhasz-Vedres[1], Reinhard Fritsch[2], Martina J. Sinnegger-Brauns[1], Jutta Engel[3], Christoph Romanin[2], Joerg Striessnig[1].

[1]Pharmacology and Toxicology, Institute of Pharmacy, University Innsbruck, Austria, [2]Institute of Biophysics, University Linz, Austria, [3]Institute of Physiology II and Tübingen Hearing Research Centre, University Tübingen, Germany.

$Ca_V1.3$ L-type channels control excitability in sensory and central neurons and sinoatrial node (SAN) function by means of their low-voltage activation and inactivation properties. For example, $Ca_V1.3$ inward calcium current (I_{Ca}) inactivates rapidly in SAN cells whereas in contrast, almost no inactivation occurs in cochlear inner hair cells (IHC). We have recently reported that $Ca_V1.3$ channel gating is modulated by an intramolecular C-terminal mechanism. This mechanism was elicited during analysis of human C-terminal splice variants and modulates the channel's negative activation range and slows calcium-dependent inactivation (CDI) (Singh et al., JBC 2008). Another candidate suggested to slow $Ca_V1.3$ channel inactivation (via interaction with $Ca_V\beta$) is the presynaptic Ribbon-synapse protein RIM. We reproducibly detected RIM2α and RIM2β isoforms in the total organ of Corti before onset of hearing and RIM2α transcripts also in IHC preparations (P4-6). None of the RIM transcripts was detected at the later develop-

mental stage of P20. We expressed $Ca_V1.3$ channel complexes ($\alpha1,\beta3,\alpha2\delta1$) in tsA-201 cells together with either GFP or GFP-hRIM1α_{1503}, hRIM2β_{932} or GFP-rRIM2α_{1168} for whole-cell patch-clamp recordings. All constructs shifted the voltage-dependence of I_{Ca} activation and inactivation significantly to more depolarized potentials (shift $V_{0.5,\ act}$:7-12 mV,$V_{0.5,inact}$:10mV) and also affected I_{Ca} inactivation (2.5-3.5-fold decrease of % inactivation during 5s-depolarization to V_{max}P<0.001, Mann Whitney test vs. $Ca_V1.3$+GFP). Coexpression of GFP-rRIM2α_{1168} also significantly affected the inactivation of $Ca_V1.3$ barium currents and maximal r_{50} values as quantitative measure of CDI were not significantly changed. In an early developmental stage, RIM might therefore partly account for the slow voltage-dependent inactivation of $Ca_V1.3$ currents. Together these data show that cells have developed different ways to fine tune $Ca_V1.3$ channel gating according to their specific physiological needs which can be achieved in various ways reaching from alternative splicing to protein-protein interactions.

Poster Board 40

CaMKII phosphorylation of the CaV1.2 β2a subunit is required for afterdepolarizations in adult ventricular myocytes

Olha Koval, PhD, Xiaoqun Guan, PhD, Jinying Yang, PhD, Mark Anderson, MD, PhD, Professor of Medicine and Physiology.

Internal Medicine, Iowa University, Iowa City, IA, USA.

Introduction: The voltage-gated L-type Ca^{2+} channel ($Ca_v1.2$) is the main entry point for Ca^{2+} into contracting heart muscle cells. This Ca^{2+} entry is critical for initiating excitation-contraction coupling, but when disordered contributes to arrhythmia-inducing afterdepolarizations, activation of cell death pathways and initiation of hypertrophic gene programs. The main auxiliary regulatory protein for $Ca_v1.2$ is the β-subunit. The importance of the β-subunit as a point of regulation for protein kinase modulation of Ca^{2+} entry in heart, both for physiology and disease, has become increasingly clear over the past several years. Over the last two years, exciting new data have emerged showing that the β-subunit is a target for the multifunctional Ca^{2+} calmodulin-dependent protein kinase II (CaMKII) at Thr498. CaMKII phosphorylation of the β-isoform causes increased L-type Ca^{2+} current that our laboratory has linked to early after depolarizations, arrhythmias, apoptosis, and hypertrophy. **The goal of this study is to determine the proarrhythmic interaction between CaMKII and Beta 2a subunit in an adult ventricular myocyte system.** **Methods:** We infected cultured adult rabbit ventricular myocytes with recombinant adenoviruses permitting dominant-negative over-expression of mutant $Ca_V1.2$ β-subunits (β2a) lacking the CaMKII phosphorylation site (Thr498Ala) or WT β2a-controls. We confirmed β2a-subunit expression was physiologically targeted to T-tubules by immunodetection of a FLAG-epitope. **Results:** Here we show that cells overexpressing the β2a-(WT) subunit demonstrated afterdepolarizations, dramatic action potential prolongation, increased I_{Ca} amplitude, and enhanced dynamic increases (facilitation) that are a signature of CaMKII signaling to $Ca_V1.2$. In contrast, the CaMKII phosphorylation defective β2a-mutant infection caused similar increases in action potential prolongation, but not ICa facilitation or afterderpolarizations. **Conclusions:** A single mutation of β2a-(Thr498Ala) abrogates proarrhythmic CaMKII signaling effects on I_{Ca} in cardiomyocytes.

Poster Board 41

Disrupting inter-domain interactions in IP_3 receptors

Yi Sun, Colin Taylor, PhD.

Department of Pharmacology, University of Cambridge, Cambridge, United Kingdom.

Calmodulin (CaM) regulates inositol 1,4,5-trisphosphate receptors (IP_3R), but neither the mechanisms nor the role of CaM in mediating Ca^{2+} regulation of IP_3R are resolved. A recent report suggested that CaM tightly tethered to IP_3R was essential for IP_3R activation (Nadif Kasri, N. *et al.*, (2006) *J. Biol. Chem*. 281, 8332-8338). We confirm that a CaM-binding peptide derived from myosin light chain kinase (MLCK) inhibits IP_3-evoked Ca^{2+} release via all three IP_3R subtypes (~90% inhibition of maximal release evoked by IP_3 for IP_3R1, no effect on EC_{50}). However, inhibition by MLCK peptide is not mimicked by other CaM antagonists that effectively block regulation of IP_3R by CaM. Inhibition by MLCK peptide is rapid (<8s), fully reversible, and occurs under conditions where there is no CaM associated with IP_3R. MLCK peptide stimulates IP_3 binding to IP_3R1 and to its bacterially expressed N-terminal, but not after removal of the suppressor domain (residues 1-224) (>2-fold). We suggest that MLCK peptide binds directly to a sequence within the suppressor domain that is similar to a 1-8-14 CaM-binding motif. The peptide thereby unzips an interdomain interaction that is essential for IP_3R activation. We conclude that CaM is not essential for IP_3R activation, and that MLCK peptide is a selective non-competitive antagonist of IP_3R that binds directly to the N-terminal and thereby uncouples IP_3 binding from channel gating.

Nadif Kasri, N., Torok, K., Galione, A., Garnham, C., Callewaert, G., Missaen, L., Parys, J.B. and De Smedt, H. (2006) Endogenously bound calmodulin is essetial for the function of the inositol 1,4,5-trisphosphate receptor., *J. Biol. Chem.* **281**, 8332-8338

Supported by the EPSRC and Wellcome Trust.