pulsed-field-gradient nuclear magnetic resonance spectroscopy has been applied to probe their oligomeric state in solution and conformational changes of specific ligands due to Ca^{2+} binding were investigated using heteronuclear-labeled proteins with different 2D and 3D NMR techniques.

2774-Pos Board B744

CaMKII-Induced Shift in Modal Gating Explains L-type Ca2+ Current Facilitation: A Modeling Study

Yasmin L. Hashambhoy, Raimond L. Winslow, Joseph L. Greenstein.
Johns Hopkins University, Baltimore, MD, USA.

Ca2+/calmodulin-dependent protein kinase II (CaMKII) plays an important role in L-type Ca2+ channel (LCC) facilitation, the Ca2+ -dependent augmentation of Ca2+ current (ICaL) that manifests itself during rapid repeated depolarizing stimuli. Multiple mechanisms may underlie facilitation, including increased LCC rate of recovery from Ca2+−dependent inactivation, and a shift in distribution of LCCs into high activity mode 2 gating, characterized by prolonged channel openings. To understand the mechanisms behind facilitation, a stochastic model was formulated which describes the dynamic interactions among CaMKII, LCCs and protein phosphatases in the cardiac dyad, as a function of subspace Ca2+ and calmodulin levels. This model faithfully reproduces single channel experimental results, and has been incorporated into an integrative computational model of the canine ventricular myocyte. Simulations demonstrate that the phosphorylation dependent shift in LCC modal gating distribution accounts for the hallmarks of ICaL facilitation, namely, ICaL amplitude augmentation, apparent macroscopic increase in rate of recovery from inactivation, and observed slowing of the inactivation rate of ICaL. A shift in LCC gating modes increases the probability of groups of LCCs re-opening during the late phase of the action potential, thus augmenting the risk of early-after depolarizations (EADs). EADs are believed to possibly trigger cardiac arrhythmias, therefore pharmacologic interventions which prevent EADs are likely to have therapeutic value. CaMKII inhibition has been proposed as a therapeutic agent for preventing arrhythmias. However, CaMKII has many phosphorylation targets, including phospholamban and Na+ channels. Our simulation findings suggest that facilitation and risk of EADs can be modulated by reducing mode 2 LCC gating directly. This solution is a favorable alternative to CaMKII inhibition because it will not disrupt the function of other CaMKII targets. This work is supported by National Institute of Health Grant R33HL87345.

2775-Pos Board B745

A Mechanistic, Minimal Model of Ca2+/Calmodulin Dependent Kinase II Signaling in the Cardiac Myocyte

Yasmin L. Hashambhoy, Raimond L. Winslow, Joseph L. Greenstein.
Johns Hopkins University, Baltimore, MD, USA.

Numerous experiments have shown that Ca2+/calmodulin-dependent protein kinase II (CaMKII) expression level and activity are increased in human cardiac myocytes from patients with dilated cardiomyopathy. The role of CaMKII in heart failure remains unclear, in part because of an incomplete understanding of how the kinase helps regulate the normal cardiac environment. CaMKII modulates the behavior of many proteins involved in excitation-contraction coupling, including L-type Ca2+ channels (LCCs), phospholamban (PLB) and Na+ channels. In order to understand the combined effects of CaMKII's modulation of these targets, a stochastic model was built to investigate the molecular mechanisms behind ICaL facilitation. Simulations show that a CaMKII-driven shift in LCC modal gating distribution from mode 1 to mode 2 is sufficient to account for experimentally observed increases in ICaL amplitude, changes in ICaL inactivation kinetics, and alterations in recovery from inactivation. This CaMKII-LCC model was then minimized, reduced to a system of ordinary differential equations, and incorporated into an integrative model of the cardiac myocyte that accounts for graded calcium release. The effects of CaMKII phosphorylation of PLB and Na+ channels were also modeled, based on data from CaMKII-PLB kinase assays and in vitro experiments as well as CaMKII-Na channel patch clamp studies performed in healthy myocytes. Preliminary simulation results show that at high CaMKII levels, hyperphosphorylation of LCCs results in spontaneous early after depolarizations. In addition, increased CaMKII levels result in prolonged action potential duration, primarily because of increased persistent INa. The model therefore serves as an important tool to identify and study the mechanisms by which CaMKII activity modifies action potential shape and duration. This work is supported by National Institute of Health Grant R33HL87345.

2776-Pos Board B746

IP_3 Receptor-mediated Ca Release Facilitates RyR-Ca Release To Cause Inotropy And Arrhythmogenicity In Mouse Ventricular Myocytes

Jaime DeSantiago[1], Aleksey V. Zima[2], Timothy L. Domeier[2], Kenneth Ginsburg[1], Jeffery D. Molkentin[3], Lothar A. Blatter[2], Donald M. Bers[1].
[1]University of California, Davis, CA, USA, [2]Rush University, Chicago, IL, USA, [3]University of Cincinnati, Cincinnati, OH, USA.

Endothelin (ET-1)-induced IP_3R-dependent Ca release in atrial myocytes is well established. ET-1-induced positive inotropy and arrhythmogenicity have also been shown in ventricular myocytes from rat and rabbit. Here we used transgenic mice overexpressing IP_3R2 and IP_3 sponge to study ET-1-induced inotropy and arrhythmogenicity in ventricular myocytes. 100 nM ET-1 (14 min) increased ventricular myocyte Ca transients (ΔCa) in IP_3R2OX by 37% (0.5 Hz, fluo4 AM, n=11) vs WT and IP_3 sponge ventricle (-6 ± 10 %). ET-1 increased spontaneous calcium transient (SCT) frequency in IP_3R2OX ventricles compared with WT and IP_3 sponge (10 ± 4 vs 1 ± 1 SCT/min). Similar ΔCa results were obtained using the Ca indicator indo-1 (41 ± 9 vs 19 ± 12 % IP_3R2OX vs WT, n=6). We found no differences between IP_3R2OX (n=17) and WT (n=10) ventricular myocytes in control (0.5 Hz) ΔCa (350 ± 70 vs 330 ± 28 nM Ca_i) or SR Ca content as assessed with 10 mM caffeine (1368 ± 273 vs 1385 ± 78 nM Ca_i). Basal Ca spark frequency in saponin-permeabilized ventricular myocytes did not differ between IP_3R2OX and WT (19 ± 4 vs 16 ± 4 sparks x s^{-1} x (100 μm)$^{-1}$). However, direct application of 10 μM IP_3 produced higher increase in Ca spark frequency on IP_3R2OX (35 %, n=4) than WT (18 %). After 3 min of IP_3 application SR Ca content was depleted to 80 % of control. These data suggest that IP_3R-dependent inotropy is mediated via IP_3R-dependent Ca release that facilitates Ca release through ryanodine receptors. This IP_3-dependent inotropy is associated with spontaneous Ca release activity which may be arrhythmogenic (inducing action potentials). We conclude that IP_3R expression and IP_3-dependent Ca release play an important role in the generation of ventricular arrhythmias.

2777-Pos Board B747

IP_3-induced Ca^{2+} Signals at the Cytoplasm and Nucleus in HL-1 Atrial Cells: Possible Roles of IP_3 Receptor Subtypes

Joon-Chul Kim, Min-Jeong Son, Krishna P. Subedi, Sun-Hee Woo.
Chungnam National University, Daejeon, Republic of Korea.

HL-1 cells are the only adult cardiac cell line available that continuously divides while maintaining an atrial phenotype. We examined the expression and localization of inositol 1,4,5-trisphosphate receptor (IP_3R) subtypes and their functional roles in the local Ca^{2+} signaling of HL-1 cells. RT-PCR and western blot analyses of IP_3R revealed significant expression of type 1 (IP_3R1) and type 2 IP_3R (IP_3R2) in HL-1 and isolated atrial cells. IP_3R1 was more abundant in HL-1 cells than atrial cells, while IP_3R2 protein band was darker in intact atrial cells than HL-1 cells. Immunostaining of the IP_3R subtypes in HL-1 and intact atrial cells demonstrated that IP_3R1 localized to nuclear envelope and that IP_3R2 was distributed at the cytoplasm as a punctate form. Extracellular application of 1 mM ATP, known to generate IP_3, in intact HL-1 cells elicited Ca^{2+} rise with oscillation, while 10 mM caffeine produced a Ca^{2+} transient with no oscillation. Exposure of saponin-permeabilized cells to IP_3 in the presence of tetracaine (1 mM) elicited transient Ca^{2+} increases. The percentage of cells with the IP_3 response, the magnitude of IP_3-induced Ca^{2+} rise, and propensity of Ca^{2+} oscillations were dependent on the concentrations of IP_3. The IP_3-induced Ca^{2+} oscillations were more pronounced in the cytoplasm than the nucleus, such that they developed faster and lasted longer at the cytoplasm, and that their magnitudes were larger. In contrast, IP_3-induced nuclear Ca^{2+} signal showed more prolonged and larger increase in the basal level with less oscillation. The IP_3-induced Ca^{2+} changes were prevented by the blockers of IP_3Rs, heparin and 2-APB. These results suggest that specific subcellular localization of IP_3R subtypes may be responsible for distinct temporal properties of cytoplasmic and nuclear Ca^{2+} signaling.

2778-Pos Board B748

Dynamic Measurements of Luminal Ca2+ in the SR of Mammalian Skeletal Muscle

Andrew P. Ziman[1], Christopher Ward[1], George Rodney[1], April Hartford[1], W.J. Lederer[2], Robert J. Bloch[1].
[1]University of Maryland, Baltimore, MD, USA, [2]Medical Biotechnology Center, UMBI, Baltimore, MD, USA.

Excitation-contraction (EC) coupling in skeletal muscle is the process by which an action potential (AP) activates a global increase in [Ca2+]i which then induces contraction. The global increase in [Ca2+]i is mediated by the release of Ca2+ from the lumen of the sarcoplasmic reticulum (SR) through the opening of ryanodine receptors (RyR1). We examined the structure and function of the SR Ca2+ store dynamically, using the low affinity Ca2+ indicator, fluo-5N, which when loaded as an -AM derivative concentrates in the lumen of the SR. Fluo-5N has been used extensively to measure SR Ca2+ and to characterize the Ca2+ stores in cardiac myocytes, with high spatial and temporal resolution (Brochet et al 2005; Wu and Bers 2006). Using dissociated flexor digitorum brevis (FDB) fibers in culture, we studied the dynamics of fluo-5N in

the SR and connecting compartments. Fluo-5N accumulates in transverse structures that align with Z-disks, consistent with the location of a major compartment of the SR in adult skeletal muscle. That these structures are primarily SR in nature is indicated by the fact that fluo-5N fluorescence decreases when fibers are exposed to caffeine. FRAP experiments demonstrated similar recovery constants for SR-trapped fluo-5N to those we have previously recorded in rat cardiac myocytes. We will be using fluo-5N to study the organization of the SR, the changes in Ca2+ dynamics in the SR during EC coupling, and the role of different proteins of the SR in regulating SR [Ca2+].

2779-Pos Board B749

Indo-1 Hybrid Biosensors For Calcium Monitoring In Cellular Organelles

Monika Sztretye[1], Sandrine Pouvreau[2], Michael Bannwarth[3], Ivan R. Corrêa Jr[3], Cindy Fellay[3], Annina Aebischer[3], Leandro Royer[1], Jianxun Yi[1], Jingsong Zhou[1], Kai Johnsson[3], Eduardo Ríos[1].
[1]Rush University Medical Center, Chicago, IL, USA, [2]Université Claude Bernard, Lyon, France, [3]École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland.

The central role of calcium in signal transduction depends on the precise spatial and temporal control of its concentration. The existing possibilities to detect fluctuations in Ca2+ concentration with adequate temporal and spatial resolution and in specific cellular organelles, are limited. We have developed a method to measure Ca2+ concentrations in defined subcellular locations that uses derivatives of the dye Indo-1 covalently bound to fusions of ''SNAP-tag'' (a multiply mutated version of human alkylguanyl DNA alkyl transferase) expressed inside cells. SNAP-Indo-1 conjugates retained the Ca2+−sensing ability of Indo-1 in vitro. One of the derivatives of Indo-1 displayed a four-fold higher fluorescence after coupling to SNAP-tag, which improves specificity of Ca2+ sensing in living cells. In a proof-of-principle experiment, local Ca2+ sensing was demonstrated in muscle cells of mice expressing a SNAP fusion localized to nuclei. [Ca2+] inside nuclei ([Ca2+]n) was evaluated by SEER (shifted excitation and emission ratioing) of confocal microscopic images of fluorescence of the sensor. After permeabilizing the plasma membrane, changes to bathing solutions containing different [Ca2+] induced corresponding changes in [Ca2+]n that were readily detected and used for a preliminary calibration of the technique. Similar hybrid sensors using Indo-1 but targeted to the mitochondrial matrix and the SR were also constructed. In principle, these hybrid sensors should combine the spatial specificity of biosensors with the superior kinetics and dynamic range of small synthetic fluorescent monitors. Factors that tended to limit their performance in initial experiments include targeting specificity of SNAP fusions and unspecific staining by the Indo-1 not reacted with SNAP-tag. Overall, the hybrid biosensor approach is a promising tool for organellar Ca2+ imaging. Support: NIAMS/NIH grants to E.R., MDA to J.Z. and a Marie-Curie Fellowship (EC) to M.B.

2780-Pos Board B750

Voltage-Dependent Ca^{2+} Channels Are Clustered But Not Constitutively-Active In Smooth Muscle

John G. McCarron[1], Marnie L. Olson[1], Susan Currie[1], Amanda J. Wright[1], Kurt I. Anderson[2], John M. Girkin[1].
[1]University of Strathclyde, Glasgow, United Kingdom, [2]Glasgow University, Glasgow, United Kingdom.

The organization and distribution of Ca^{2+} signals derived from depolarization-evoked Ca^{2+} entry has been studied in voltage-clamped single vascular and gastrointestinal smooth muscle cells using widefield epi-fluorescence with near simultaneous (2 ms) total internal reflection fluorescence microscopy. Depolarization activated a voltage-dependent Ca^{2+} current (I_{Ca}) and evoked a rise in $[Ca^{2+}]$ in the subplasma membrane space and bulk cytoplasm. The rise which occurred in various regions of the bulk cytoplasm ($[Ca^{2+}]_c$) was approximately uniform; that of the subplasma membrane space ($[Ca^{2+}]_{PM}$) had a wide range of amplitudes and time courses. The $[Ca^{2+}]_{PM}$ variations presumably reflected an uneven distribution of active Ca^{2+} channels (clusters) across the sarcolemma. Constitutive activity in clusters of voltage-dependent Ca^{2+} channels has been proposed to determine bulk average $[Ca^{2+}]_c$. In the present study, channels are not constitutively active. The repetitive localized $[Ca^{2+}]_{PM}$ rises ('Ca^{2+} sparklets') which characterize constitutively-active channels were observed rarely (<1 in 50 cells). Nor did constitutively-active voltage-dependent Ca^{2+} channels regulate the bulk average $[Ca^{2+}]_c$. A dihydropyridine blocker of voltage-dependent Ca^{2+} channels, nimodipine, which blocked I_{Ca} and accompanying $[Ca^{2+}]_c$ rise, reduced neither the resting bulk average $[Ca^{2+}]_c$ (at -70 mV) or the rise in $[Ca^{2+}]_c$ which accompanied an increased electrochemical driving force on the ion by hyperpolarization (-130 mV). Activation of protein kinase C with indolactam-V did not induce constitutive channel activity. Thus while voltage-dependent Ca^{2+} channels appear clustered on the plasma membrane, constitutive activity in the channel is unlikely to play a major role in regulation $[Ca^{2+}]_c$. The voltage-dependent activity of the clustered channels may enable selective activation of various cellular processes by generating a localized rises in subplasma membrane $[Ca^{2+}]$.
Supported by the Wellcome Trust and British Heart Foundation

2781-Pos Board B751

Mitochondria Act Within $InsP_3R$ Clusters To Maintain Ca^{2+} Release In Smooth Muscle

Marnie L. Olson, John G. McCarron.
University of Strathclyde, Glasgow, United Kingdom.

Many smooth muscle activities including contraction, transcription, growth and apoptosis are regulated by transient inositol 1,4,5-trisphosphate ($InsP_3$)-mediated increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). $InsP_3$ binds to receptors ($InsP_3R$) present on the sarcoplasmic reticulum to evoke Ca^{2+} release. $InsP_3R$ exist in clusters and Ca^{2+} released from one receptor may activate nearby $InsP_3R$ within this cluster in a CICR-like process to evoke a 'puff'. Ca^{2+} released may also diffuse to adjacent clusters to trigger further Ca^{2+} release and generate a Ca^{2+} rise throughout the cell. Mitochondrial Ca^{2+} uptake limits a negative feedback process operative on $InsP_3R$ to maintain Ca^{2+} release. Inhibition of mitochondrial Ca^{2+} uptake decreases $InsP_3$-mediated Ca^{2+} waves by $\geq$50%. We addressed whether mitochondria act to maintain release by operating within or between $InsP_3R$ clusters. Ca^{2+} puffs were evoked by localized photolysis of $InsP_3$ in single voltage-clamped colonic smooth muscle cells in which $[Ca^{2+}]_c$ and $\Delta\Psi_M$ were measured simultaneously. EGTA, a slow Ca^{2+} buffer, was used to functionally uncouple puff sites to prevent the formation of Ca^{2+} waves. EGTA was used at a concentration ([300 μM]) which does not affect the magnitude or kinetics of Ca^{2+} puffs. $InsP_3$-evoked Ca^{2+} puffs had amplitudes of 0.5-5.0 F/F_0 and durations of ~200 ms at half-maximum amplitude. Puffs were abolished by the $InsP_3R$ inhibitor 2-APB. The protonophore CCCP and the mitochondrial inhibitor rotenone, each used with oligomycin, depolarized the mitochondrial membrane potential ($\Delta\Psi_M$) and prevented mitochondrial Ca^{2+} uptake. Depolarizing $\Delta\Psi_M$ with CCCP attenuated Ca^{2+} puffs by ~65% while rotenone inhibited them by ~60%. These results indicate mitochondrial Ca^{2+} uptake occurs quickly enough to influence $InsP_3R$ communication at the intra-cluster level. Supported by the Wellcome Trust and British Heart Foundation.

2782-Pos Board B752

Imaging The Individual And Concerted Activity Of IP_3R Ca^{2+} Release Channels In Intact Mammalian Cells

Ian Smith[1], Ian Parker[1,2].
[1]Neurobiology and Behavior, Irvine, CA, USA, [2]Physiology and Biophysics, Irvine, CA, USA.

Cellular signaling mediated by the inositol trisphosphate (IP_3) messenger pathway involves hierarchical Ca^{2+} liberation from the endoplasmic reticulum (ER), whereby local 'elementary' Ca^{2+} transients (Ca^{2+} puffs) serve autonomous signaling functions and as well as constituting the building blocks from which global cellular Ca^{2+} waves are constructed. These channels are inaccessible to single-channel study by patch-clamp in intact cells, and excised organelle and bilayer reconstitution systems disrupt the Ca^{2+} induced Ca^{2+} release (CICR) process that mediates channel-channel coordination. We report here the use of total internal reflection fluorescence (TIRF) microscopy to image single-channel Ca^{2+} flux through individual and clustered IP_3R's in intact mammalian cells. This enables a quantal dissection of calcium puffs involving stochastic recruitment of an average of 6 active IP_3Rs clustered within <400 nm. IP_3R gating kinetics during puffs indicate rapid (~10 ms) recruitment by Ca^{2+}-induced Ca^{2+} release (CICR), followed by a similarly rapid inhibition process that is crucial for terminating puffs by suppressing re-openings of channels in the face of continued high local $[Ca^{2+}]$. Single-channel imaging methodology thus provides nano-scale information of the architecture and dynamic interactions between Ca^{2+} release channels in the native cellular environment; information previously inaccessible by electrophysiological patch-clamp techniques.
Supported by grant NIH GM 40871

2783-Pos Board B753

Analysis of Localized Calcium Alteration During Neural Cell Death

Man-Soo B. Hong.
University of Virginia, Landover Hills, MD, USA.

Fluctuations in intracellular calcium ion ($Ca^{2+}{}_i$) levels are believed to participate in a myriad of physiological and pathological intracellular events. In an attempt to investigate localized alterations in $Ca^{2+}{}_i$ dynamics in a cell-based neurodegeneration model, we used Fura-2/AM dye to monitor $Ca^{2+}{}_i$ ion levels in the human SH-SY5Y neuroblastoma cells induced to undergo apoptosis with 500 nM staurosporine (STS) over a 24 h period. Using rapid illumination frequency at 5 Hz per 340/380 nm excitation wavelength pair, streaming image acquisition and analysis of 12 very small regions of interest (ROI) of ~86.5 μm^2 in either peri-nuclear (PN) or distal (DST) cytoplasmic locations, we captured