The limiting frequency estimated for the mammalian ear under realistic conditions is up to 1.2 kHz based on CRC model and is up to 2.8 kHz based on TJM model, lower than the mammalian auditory range. If the mechanical characteristics of the MET channel and the adaptation motor are similar, the limiting frequency could be described by the morphological factor in both models, although TJM model is much more sensitive to the operating point. This feature could be used to explain the auditory range of the avian ears and their tonotopic maps by the morphological factor.

2769-Pos Board B739

Frequency Analysis of Complex Waveforms in Sound Stimuli Discriminated by Human Auditory Cortex

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Recognizing objects in the environment from the sounds they produce is one of the primary functions of the auditory system. Recognition is possible, in part, because acoustic features of sounds often represent physical properties of their sources.

This present study is the first to investigate whether a single omitted frequency component in the onset period produces significant differentiation.

Neural activity of 10 human subjects is recorded coincident with the sound stimulus by whole-head Magneto-encephalography (MEG). In the first set of stimuli constructed from a group of carefully chosen anharmoninc frequency, repetitive ('standard') tones with five selected onset frequencies were randomly embedded by rare ('deviant') tones, all tones having randomly varying inter stimulus intervals. In the deviant tones one of the frequency components was omitted during the onset period relative to the standard tones. The frequency of the test partial of this complex tone was intentionally selected to preclude its reinsertion by generation of harmonics or combination tones due to either the nonlinearity of the ear, the electronic equipment or the brain processing.

In the second set of stimuli, time structured as above, repetitive ('standard') tones with five selected sustained partial frequency components were embedded with rare ('deviant') tones for which one of these selected partials was omitted this time in the sustained tone. As before, the carefully frequency selection again precluded their reinsertion by generation of harmonics or combination tones due to the nonlinearity of the ear, the electronic equipment and brain processing.

By comparing the magnetic field responses (mismatch negativity or MMNm) of the two data sets, the presence of significant mismatch negativity is determined to be due to elimination of a single frequency in the onset period of sound stimuli rather than the sustain period.

Local Calcium Signaling

2770-Pos Board B740

Fluorescence Imaging Of Ryanodine Receptor And Caveolin Distribution In Cardiac Myocytes At 30 nm Resolution

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In cardiac ventricular muscle excitation-contraction coupling arises from Ca^{2+} release via clusters of ryanodine receptors (RyRs) in regions of close apposition between the sarcoplasmic reticulum and surface membranes. The local Ca^{2+} signalling in these regions depends on the nanometer organization of RyRs and other protein clusters. Until recently, however, optical imaging was thought to be limited to a resolution of ~250 nm set by the diffraction of light. We have overcome this limitation using a new technique that allows imaging of conventionally labelled fluorescent samples at much higher resolution. Isolated rat cardiac ventricular myocytes were labelled with specific antibodies against cardiac ryanodine receptors and caveolin-3 and secondary antibodies conjugated to the organic fluorochrome Alexa 488. To visualize the distribution of the labelled proteins at the surface sarcolemma cells were imaged on a total internal reflection fluorescence microscope. We observed that Alexa 488 (and several other organic fluorophores) exhibit reversible photobleaching with recovery times >10s. This observation provided the basis for a new imaging technique we term reversible photobleaching microscopy (RPM). RPM relies on intense illumination to reversibly induce a very long-lived dark state from which single fluorochromes slowly return stochastically. These single molecule fluorescence events were detected with a sensitive camera and each event could be localized with an accuracy of better than 30 nm. From several thousand frames we thus reconstructed ultra-high-resolution images of the distribution of RyRs and caveolin at the surface sarcolemma of the stained cells. Both proteins formed dense clusters with diameters that were generally much smaller than the diffraction limit. Our new approach is applicable to measure the nanoscale distribution of many proteins of interest in excitation-contraction coupling or other signalling processes.

2771-Pos Board B741

Simultaneous Determination of Free Zn(II) and Ca(II) with a Single Fluorescent Indicator

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Fluorescent indicators for metal ions such as Ca(II) and Zn(II) are in large measure responsible for our current understanding of the cell biology of these metal ions and others: the success of this approach has fueled the development of scores of fluorescent indicator systems. The inventors of the pioneering ratiometric fluorescent indicators Fura-2 and Indo-1 (Grynkiewicz, et al., 1985) made clear that they had roughly 100-fold better affinity for zinc than calcium, and more recent results have shown that high zinc affinity is frequently the case with other calcium indicators. By comparison, zinc indicator systems have been described with picomolar zinc affinity that are unaffected by 10 mM Ca, 50 mM Mg. Recent evidence now indicates that some phenomena thought to be calcium-dependent are in fact zinc-dependent (Stork and Li, 2006). To help resolve such questions we sought an indicator that would exhibit unique fluorescence signatures when free or bound to Ca or Zn, such that the free metal ions could be determined simultaneously with a single indicator in the same experiment. We found that Calcium Green 5N exhibits significantly different lifetimes in the three states, permitting free Ca and Zn concentrations to be determined simultaneously.

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2772-Pos Board B742

Applications of Genetically Targeted and Optimized Calcium Sensors

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We have recently developed a series of FRET-based genetically encoded sensors for calcium. These sensors (dubbed "cameleons") are made from cyan and yellow fluorescent proteins combined with engineered calcium sensing elements. These optimized cameleon sensors have now been genetically targeted to specific locations within cells including: the ER, mitochondria, Golgi, nucleus, plasma membrane, and cytosol. In the present work we put these sensors to the test in both standard tissue culture cells and in primary hippocampal neurons. We have now used these sensors to define how ER and mitochondrial calcium handling is altered by mutations in the calcium regulatory protein presenilin. Presenilin is an integral membrane protein that localizes to the ER, secretory pathway, and plasma membrane. There is emerging evidence from a number of research groups that presenilin plays a critical role in modulating ER calcium signaling. Using a combination of genetically targeted and small molecule sensors we have identified how mutations in presenilin alter calcium homeostasis in the ER and calcium release through the IP3R. In another line of research aimed at testing the sensitivity and versatility of our sensors, we have identified localized calcium signals generated upon invasion of a host mammalian cell by bacteria. In this work we will discuss the strengths and weaknesses of genetically encoded calcium sensors and potential avenues for further improvement and optimization.

2773-Pos Board B743

Rational Design of Ca^{2+} Biosensor

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Ca^{2+}, the most ubiquitous signaling molecule in human body, regulates numerous biological functions by fluxing between the subcellular compartments. Quantitative and real-time detection of Ca^{2+} concentration fluctuation in specific cellular environments such as the endoplasmic reticulum (ER) is essential to explore the mechanism of Ca^{2+}-dependent cellular signaling. Currently the reported Ca^{2+} sensors based on natural Ca^{2+} binding proteins are limited due to the perturbation of Ca^{2+} signaling. To overcome these limitations, we report the rational design of Ca^{2+} biosensors by engineering a Ca^{2+} binding site into a single enhanced green fluorescent protein (EGFP). These developed Ca^{2+} sensors exhibit a ratiometic fluorescent signal change after binding to Ca^{2+}, with a Kd value optimal for the measurement of Ca^{2+} in the ER. Metal selectivity of the sensors for Ca^{2+} in comparison with Ln^{3+}, and excessive biological metal ions such as Mg^{2+}, K^{+}, Na^{+} has also been examined. In addition, these developed sensors can be targeted to the ER, and exhibit high potential for living cell imaging. Further, their optical and conformational properties have been investigated using various spectroscopic methods. Moreover,

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

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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

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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

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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

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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

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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