(1Hz, room temperature) into nucleoplasmic and cytoplasmic [Ca^{2+}]. There was a significant difference in diastolic (121 ± 24nM *vs* 149 ± 35nM; 99 ± 17nM *vs* 121 ± 26nM) and systolic (420 ± 148nM *vs* 364 ± 102nM; 787 ± 172nM *vs* 491 ± 157nM) [Ca^{2+}] between cytoplasmic and nucleoplasmic compartments in mouse and rat cells, respectively (both n=15; P<0.01).
The results reveal that, in cardiac myocytes, the Ca^{2+}-dependent fluorescent properties of Fluo-4 differ between cytoplasm and nucleoplasm and that significant differences between cytoplasmic and nucleoplasmic [Ca^{2+}] exist during diastole as well as systole.

1543-Pos
Control of Ca Release Synchrony by Action Potential Configuration in Murine Cardiomyocytes
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Spatially non-uniform or ''dyssynchronous'' sarcoplasmic reticulum (SR) Ca release has been reported in cardiomyocytes from failing hearts. Using a murine model of congestive heart failure (CHF) following myocardial infarction, we investigated whether altered action potential (AP) configuration promotes release dyssynchrony. We observed that APs (1 Hz) were prolonged in cardiomyocytes isolated from the viable septum of CHF hearts, compared to and sham-operated controls (SHAM). Representative AP recordings were included in a detailed computational model of the Ca dynamics in the dyad. The model predicted reduced driving force for L-type Ca current and more dyssynchronous opening of ryanodine receptors during stimulation with the CHF AP than the SHAM AP. These predictions were confirmed in isolated cardiomyocyte experiments, when cells were alternately stimulated by SHAM and CHF AP voltage-clamp waveforms. However, when a train of like APs was used as the voltage stimulus, the SHAM and CHF AP produced a similar Ca release pattern. In this steady-state condition, both modeling and cell experiments showed that greater integrated Ca entry during the CHF AP lead to increased SR Ca content. We modeled the effect of increased SR Ca content by increasing the Ca sensitivity of the ryanodine receptor, which we observed increased the synchrony of ryanodine receptor activation. Thus, at steady-state, Ca release synchrony was maintained during the CHF AP as greater ryanodine sensitivity offset the de-synchronizing effects of reduced driving force for Ca entry. Our results suggest that dyssynchronous Ca release in failing mouse myocytes results from alterations such as T-tubule re-organization, and not electrical remodeling.

1544-Pos
Imaging of the Ryanodine Receptor Distribution in Rat Cardiac Myocytes with Optical Single Channel Resolution
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We have applied a new optical super-resolution technique based on single molecule localisation to examine the peripheral distribution of a cardiac signalling protein, the ryanodine receptor (RyR), in rat ventricular myocytes. Using high-resolution antibody labeling data we show that the new imaging approach, termed localization microscopy, can give novel insight into the distribution of large proteins, with optical single channel resolution. We present, to our knowledge, the first direct data showing evidence for a two-dimensional array-like arrangement of RyRs in cardiac muscle. Morphological analysis of peripheral RyR clusters in the surface membrane revealed a mean size of ~14 RyRs per cluster, almost an order of magnitude smaller than previously estimated. Clusters were typically not circular (as previously assumed) but elongated with an average aspect ratio of 1.9. Edge-to-edge distances between adjacent RyR clusters were often less than 50 nm suggesting that peripheral RyR clusters may exhibit strong inter-cluster signalling. The cluster size varied widely and followed a near-exponential distribution. We show that this distribution is compatible with a stochastic cluster assembly process and construct simple cluster growth models that generate size distributions very similar to our experimental observations. Based on the placement and morphology of RyR clusters we suggest that calcium sparks may be the result of the concerted activation of several clusters forming a functional 'supercluster' whose gating is controlled by both cytosolic and sarcoplasmic reticulum luminal calcium levels. The new imaging approach can be extended to other cardiac proteins and should yield novel insight into excitation-contraction coupling and the control of cardiac contractility.

1545-Pos
Synchronization of Spontaneous Stochastic RyR Activation in Ventricular Myocytes (VM) by Camp or Disengagement of Phospholambam (PLB) From SERCA2
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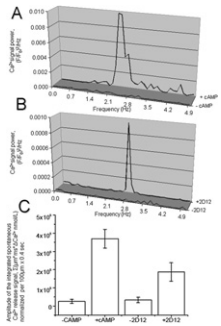
Stochastic RyR activation underlies Ca^{2+} sparks in VM. Here we show that in saponin ''skinned'' VM, bathed in 100 nM Ca^{2+} at 35°C, cAMP converts this stochastic spontaneous RyR activation (Ca^{2+} sparks-confocal linescan imaging) into synchronized, rhythmic RyR activation (Fig. A) about an average dominant frequency 2.2 ± 0.13 Hz (n=3). Of note, cAMP does not alter the SR Ca^{2+} load assessed by the rapid application of caffeine (107 ± 17.3 nM Ca^{2+} n=9 prior and 121 ± 19.3 nM Ca^{2+} n=3 during cAMP exposure). When Ca^{2+} pumping into SR is selectively accelerated by a PLB antibody (2D12, 0.013 mg/ml) that disengages PLB from SERCA2, stochastic RyR Ca^{2+} release becomes rhythmic (Fig. B) about an average dominant frequency of 2.6 + 0.21 Hz (n=5). The amplitude of the integrated spontaneous Ca^{2+} release signal during any given epoch increases when stochastic RyR activation becomes synchronized, i.e. converted to rhythmic activation (Fig. C). This cAMP-generated rhythmicity of spontaneous RyR activation of VM mimics rhythmic spontaneous diastolic Ca^{2+} releases in sinoatrial nodal pacemaker cells which have a basal high level of intrinsic cAMP-dependent signaling.



1546-Pos
Subcellular Mechanisms of Early Impaired Calcium Homeostasis with Chronic Beta$_1$-Adrenergic Stimulation in Mice
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Chronic beta-adrenergic stimulation leads to heart failure (HF). In mice overexpressing beta1-adrenoceptors (TG), increased diastolic Ca load in cardiomyocytes at early age is pivotal for the development of HF. The mechanisms underlying intracellular Ca dysregulation are unclear. We examined cytosolic Ca transients (Fluo4-AM, field stimulation), Na-Ca-exchanger (NCX) function and protein expression, cytosolic Na (SBFI) and T-tubular structures (Di8-ANEPPS) in cardiomyocytes from young (8-16 wks) TG mice and wildtype (WT) littermates.
Results: Systolic [Ca] amplitude was unchanged, time to peak [Ca] (140 ± 5 vs. 127 ± 3 ms) and [Ca] decay (time constant, tau, 223 ± 16 vs. 182 ± 9 ms) were significantly prolonged in TG vs. WT. Diastolic Ca leak from the SR (quantified as tetracaine-sensitive change in diastolic [Ca] or diastolic Ca spark frequency) was unchanged. However, cytosolic Ca removal by NCX during coffein application was significantly slower (tau, 3683 ± 337 in TG vs. 2304 ± 272 ms in WT), indicating reduced forward mode NCX activity. NCX protein expression was unchanged. Preliminary results indicate increased cytosolic [Na] in young TG. Furthermore, confocal line scans revealed delayed (> 15 ms until half-maximal) systolic Ca release in 24.7 ± 2.6 (TG) vs. 4.6 ± 1.4% (WT) of the intracellular regions (n=32 and 31 cells, resp., p<0.01). The extent of dyssynchronous Ca release correlated with time to peak systolic [Ca] (R=0.51, P<0.001) and was associated with a lower density and increased irregularity of T-tubules in TG (22.8 ± 1.6% of cell volume in TG vs. 26.1 ± 2.5% in WT). In summary, in early HF remodeling with chronic beta1-adrenergic stimulation, slowed cytosolic Ca clearance isnot related to increased diastolic SR Ca leak but associated with decreased NCX forward mode activity, which may be related to increased cytosolic [Na]. Reduced T-tubule density with dyssynchronous, slowed systolic Ca release additionally contribute to increased cytosolic Ca load.

1547-Pos
Rational Design and Structrual Analysis of Ca^{2+} Biosensor and Application in Skeletal Muscle Cells
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Quantitative and real-time detection of Ca^{2+} signaling in internal Ca^{2+} store sarcoplasmic reticulum (SR) of skeletal muscle cells is essential to explore

the mechanism of various diseases such as malignant hyperthermia, central core diseases, brody diseases and so on highly related with SR calcium abnormal handling. To overcome the limitation of reported genetically encoded Ca^{2+} sensors based on natural Ca^{2+} binding proteins of perturbing Ca^{2+} signaling, we report a novel design of calcium biosensor for the first time by rational de novo engineering a non-natural Ca^{2+} binding site into a single enhanced green fluorescent protein (EGFP), which can successfully quantitatively reveal the subcellular calcium signaling by fluorescence change. These developed Ca^{2+} sensors exhibit K_d values measured inside the mammalian cells in situ optimal for the measurement of Ca^{2+} in the SR. Metal selectivity of the sensors for Ca^{2+} in competition to excessive biological metal ions such as Mg^{2+}, K^+, Na^+ has been examined. In addition, these developed sensors can be targeted to the SR of muscle cells, and detected the Ca^{2+} signaling induced by various agonists and antagonists interacting with SR membrane Ca^{2+} pumps or receptors. Moreover, they exhibit fast response to Ca^{2+}. Further, their optical and conformational properties have been investigated using various spectroscopic methods, including high resonance resolution NMR. Moreover, more than 70% of the amino acids of the EGFP-based designed sensor have been successfully assigned using heteronuclear-labeled proteins. Our studies further reveal the key factors that contribute to the molecular mechanisms of the fluorescence change upon calcium binding and dynamic properties of our designed Ca^{2+} sensors.

1548-Pos

Orai1 Mediates Store-Operated Ca2+ Entry in Normal Skeletal Muscle and Exacerbated Ca2+ Entry in Dystrophic Muscle

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Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy, in which loss of dystrophin expression results in compromised sarcolemmal integrity. Although evidence shows that defects in Ca2+ homeostasis is a causal factor for the progressive cell death observed in DMD, the mechanism of Ca2+ deregulation is still under debate. Several laboratories showed that enhanced Ca2+ entry might serve as a pathological factor in dystrophic muscles. In this study, we explored the role of store operated Ca2+ entry (SOCE) in Ca2+ deregulation of dystrophic muscles. We used real-time PCR and Western blotting to detect known isoforms of Orai and STIM1 and determined that Orai1 was the most abundant in skeletal muscle and was significantly upregulated in muscles from mdx mice, while STIM1 levels remained largely unchanged. Furthermore, Mn2+ quenching of fura-2 fluorescence was applied to measure SOCE activity in flexor digitorum brevis (FDB) fibers and a significant increase in SOCE activity was detected in mdx fibers. Similar levels of resting [Ca]i was identified in wt and mdx groups, while peak response to C/R was significantly higher in mdx fibers than wt. Furthermore, we electroporated shRNA probe against mouse Orai1 into FDB muscle of living mice to produce effective knockdown (KD) of Orai1 expression. Two weeks after Orai1 KD, SOCE activity was eliminated in both wt and mdx muscle fibers and peak response to caffeine and ryanodine in mdx fiber sreturned to a level comparable to wt muscle fiber. Therefore, our study established that Orai1 is an essential component of SOCE machinery in adult skeletal muscle and indicates that Orai1-mediated SOCE could be the major pathway for additional Ca2+ entry into mdx muscle fibers, which would eventually lead to progression of DMD.

1549-Pos

Orai1 and STIM1 Mediate Capacitative Ca^{2+} Entry in Mouse Pulmonary Arterial Smooth Muscle Cells

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Previous studies in mouse pulmonary arterial smooth muscle cells (PASMCs) showed that TRPC1 and STIM1 mediates the sustained component of capacitative Ca^{2+} entry (CCE) but the molecular candidate(s) that mediate the transient component of CCE remains unknown. The aim of the present study was to further examine if Orai1 mediates the transient component of CCE through activation of STIM1 protein in mouse PASMCs.In primary cultured mouse PASMCs loaded with fura-2, cyclopiazonic acid (CPA) caused a transient followed by a sustained rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).The transient but not the sustained rise in $[Ca^{2+}]_i$ was partially inhibited by nifedipine. The nifedipine-insensitive transient rise in $[Ca^{2+}]_i$ and the increase in Mn^{2+} quench of fura-2 fluorescence caused by CPA were both reduced in cells treated with Orai1 siRNA. These responses to CPA were further reduced in cells treated with Orai1 and STIM1 siRNA. Moreover, over-expression of STIM1 enhanced the rise in $[Ca^{2+}]_i$ and the increase in Mn^{2+} quench of fura-2 fluorescence caused by CPA and these responses were reduced in cells treated with Orai1 siRNA. RT-PCR revealed Orai1 and STIM1 mRNAs, and Western blot analysis identified Orai1 and STIM1 proteins in mouse PASMCs. Furthermore, Orai1 was found to co-immunoprecipitate with STIM1 and immunostaining showed co-localization of Orai1 and STIM1 proteins. These data provide direct evidence that the transient component of CCE is mediated by Orai1 channel through activation of STIM1 in mouse PASMCs. [Supported by HL49254, NCRR P20RR15581 (JR Hume) and AHA Scientist Development Grant (LC Ng)]

1550-Pos

In Smooth Muscle, Mitochondrial Movement is Restricted in Native Cells and Unrestricted and Trafficked When Cells are in Culture

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Positioning of mitochondria in the cell is important for the local provision of ATP and for regulation of $[Ca^{2+}]_c$ signals, lipid and reactive oxygen species production, redox control and initiation of cell death signals. In smooth muscle, mitochondrial Ca^{2+} uptake promotes Ca^{2+} release from the sarcoplasmic reticulum via by IP_3R, suggesting a localised removal of the ion from the IP_3R cytosolic face that maintains channel activity[1]. The close physical interaction that this relationship implies is not compatible, however, with the reported free movement of mitochondria throughout the cytosol. Here, image correlation based single particle tracking of mitochondria in freshly-isolated single smooth muscle cells from guinea-pig colon, shows that mitochondria displayed very limited movement. Brownian motion of mitochondria was detected but did not generate any significant displacement of the organelle over time. Neither the actin depolymerising agent latrunculin B (10 μM), nor the microtubule disrupter nocodazole (10 μM) increased mitochondrial movement. In contrast, when freshly-isolated smooth muscle cells were maintained in cell culture conditions for 14 days mitochondrial motility was substantially increased. Mitochondria displayed rapid, directed motion and Brownian movement resulted in displacement of the mitochondria over time. These results suggest that in freshly-isolated smooth muscle cells, mitochondria are either confined or tethered to limit movement; whereas when the same cells divide and proliferate in culture these restraints are lost, mitochondria display random-walk diffusive motion and are accessible to the intracellular trafficking machinery.

1. Chalmers S & McCarron JG (2008) J Cell Sci. 121:75-85.
2. Saunter CD *et al.* (2009) FEBS Lett. 583:1267-73.

*These authors contributed equally to this work. The Wellcome Trust (070854/Z/05/Z), British Heart Foundation (PG/08/066) and Science & Technology Facilities Council (ST/F003722) funded this work.

1551-Pos

Synchronization of Waveform Analysis with Monitoring of Localized [Ca2+] in the Beating Flagellum of Single Sperm

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Much past work indicates that in mammalian sperm Ca^{2+} is the messenger that controls waveform flagellar symmetry, and that the cAMP messenger controls beat frequency. We have now constructed an imaging system that uses dual pulsed-LED sources and records interleaved stop-motion brightfield and fluorescence images of individual loosely-tethered mouse sperm loaded with the Ca^{2+} probe fluo-4. The brightfield images report flagellar beat frequencies of 2-4 hertz for resting sperm, and the fluo-4 images report similar $[Ca^{2+}]$ (~150 nM) in the head, the midpiece, and the cytoplasmic droplet located at the flagellar midpiece/principal-piece junction. Stimulation (10-20s) by local perfusion with alkaline-depolarizing medium K8.6 raises $[Ca^{2+}]$ ~4-fold in each region. The $[Ca^{2+}]$ rises first in the droplet, then the midpiece, and finally in the head. Recovery towards baseline is slow ($t_{1/2}$>30s). Analysis of the waveform shows that increases in flagellar beat asymmetry accompany the increased $[Ca^{2+}]$ but that beat frequency remains unchanged before, during, and after stimulus. The delayed Ca^{2+} responses in midpiece and head are consistent with evoked localized entry through CatSper ion channels in the principal piece with subsequent diffusional redistribution. The accompanying increases in beat asymmetry without increases in frequency suggests that evoked Ca^{2+} entry does not engage cAMP-mediated signaling in sperm.

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