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Dynamics of Calcium Uptake and Release by the Mitochondria in the Heart

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Compelling reports suggest that intra-mitochondrial $[Ca^{2+}]_{mito}$ changes dramatically with intracellular $[Ca^{2+}]_i$ transients (1) while other equally compelling investigations suggest there are little to no changes (2). Here we take advantage of the high temporal and spatial resolution of confocal microscopy imaging to examine this question in rat and rabbit ventricular cardiomyocytes. Using a mitochondrially targeted Ca^{2+}-sensitive fluorescent protein "Mitycam" (3), we examined the time course of changes in $[Ca^{2+}]_{mito}$ (matrix mitochondrial $[Ca^{2+}]$) in isolated ventricular myocytes. Examination was performed 48 hours after treatment with adenovirus-dependent Mitycam expression in ventricular myocytes in primary culture.

Mitycam, which co-localizes with mitochondrial-specific markers (e.g. Mito-Tracker red), responds slowly (minutes) to steep changes of $[Ca^{2+}]_i$ in saponin permeabilized myocytes ($[Ca^{2+}]_i << 10$ to 250 nM). Mitycam responds more quickly (~1 s) in intact (i.e. non-permeabilized) myocytes where a large change in $[Ca^{2+}]_i$ (from ~100 nM to ~10 μM) is seen following caffeine (10 mM). However, in these cells, physiologic $[Ca^{2+}]_i$ transients produced no significant increase in $[Ca^{2+}]_{mito}$ in a beat-to-beat manner but can modestly affect the time-averaged $[Ca^{2+}]_{mito}$ following changes in heart rate ($[Ca^{2+}]_{mito}$ responds to changes in $[Ca^{2+}]_i$ like a low-pass-filter). In contrast, isolated Mitycam-containing mitochondria respond to changes in $[Ca^{2+}]$in milliseconds.

We conclude that the cardiac $[Ca^{2+}]_i$ transient does not significantly change $[Ca^{2+}]_{mito}$ in cardiac myocytes in a beat-to-beat manner but slowly influences the time-averaged $[Ca^{2+}]_{mito}$ signal.

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Design and Application of a Class of Sensors to Monitor Ca2+ Dynamics in High Ca2+ Concentration Cellular Compartments

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Quantitative analysis of Ca2+ fluctuations in the endoplasmic/sarcoplasmic reticulum (ER/SR) is essential to defining the mechanisms of Ca2+-dependent signaling under physiological and pathological conditions. Here, we developed a unique class of genetically encoded indicators by designing a Ca2+ binding site in the EGFP. One of them, calcium sensor for detecting high concentration in the ER, exhibits unprecedented Ca2+ release kinetics with an off-rate estimated at around 700 s−1 and appropriate Ca2+ binding affinity, likely attributable to local Ca2+-induced conformational changes around the designed Ca2+ binding site and reduced chemical exchange between two chromophore states. Calcium sensor for detecting high concentration in the ER reported considerable differences in ER Ca2+ dynamics and concentration among human epithelial carcinoma cells (HeLa), human embryonic kidney 293 cells (HEK-293), and mouse myoblast cells (C2C12), enabling us to monitor SR luminal Ca2+ in flexor digitorum brevis muscle fibers to determine the mechanism of diminished SR Ca2+ release in aging mice. This sensor will be invaluable in examining pathogenesis characterized by alterations in Ca2+ homeostasis.

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Rapid Changes in Mitochondrial Ca^{2+}-Concentration in Fast Skeletal Muscle Fibers from Wild Type and Calsequestrin Null Mice

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Mitochondrial Ca^{2+}-uptake is important for the regulation of aerobic ATP production and is involved in apoptosis. Muscle fibers contract in response to transient elevation in intracellular calcium concentration. At rest, the Ca^{2+} stored inside the sarcoplasmic reticulum (SR) is predominantly bound to calsequestrin (CASQ). Since muscle fibers lacking calsequestrin (CASQ) have a reduced SR Ca^{2+} content and present alterations in mitochondrial morphology that may affect function, we investigated mitochondrial Ca^{2+}-handling using a ratiometric FRET-based calcium indicator (mtD3cpv Cameleon) targeted to the mitochondrial matrix. Experiments were conducted in enzymatically dissociated FDB muscle fibers from wild type (WT) and CASQ-null mice, lacking both CASQ isoforms either in the presence (1 mM) or absence of extracellular calcium. Small but significant differences in free mitochondrial Ca^{2+}-concentration ($[Ca^{2+}]_{mito}$) were observed between quiescent WT and CASQ-null fibers. The free $[Ca^{2+}]_{mito}$ during steady state electrical stimulation at 1 Hz showed a rapid increase with a 10% - 90% rise time of 18.4 ± 0.4 ms. The decline in $[Ca^{2+}]_{mito}$ during and after stimulation trains was governed by 3 temporally distinct processes with rate constants of approximately 40 s^{-1}, 1.6 s^{-1} and 0.2 s^{-1} (at 26 °C). During the sustained contractions in WT fibers, frequency-dependent increases in free $[Ca^{2+}]_{mito}$ occurred, which were smaller in the absence than in the presence of external Ca^{2+}. In CASQ-null fibers the increase in free $[Ca^{2+}]_{mito}$ was less pronounced, and in the absence of extracellular Ca^{2+}, the increase in free $[Ca^{2+}]_{mito}$ was virtually absent. These results provide direct evidence for rapid Ca^{2+} uptake by the mitochondria and suggest that mitochondrial Ca^{2+} uptake is sensitive to the amount of Ca^{2+} available inside the sarcoplasmic reticulum as well as in the extracellular spaces.

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Regulation of Voltage-Dependent Anion Channel 2 at Glutamate 73 is Critical for its Role in Cardiac Calcium Handling

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Homozygous embryos of the mutant zebrafish line *tremblor* are deficient for a cardiac specific isoform of the sodium-calcium exchanger (NCX1h) and display only unsynchronized contractions of single cardiomyocytes, comparable to cardiac fibrillation. We have previously shown that pharmacological activation of the voltage-dependent anion channel 2 (VDAC2) can suppress fibrillation and restore rhythmic contractions in *tremblor* hearts by temporal and spatial limitation of Ca^{2+} sparks and suppression of spontaneous propagating Ca^{2+} waves. Consistently, also overexpression of VDAC2 in *tremblor* or NCX1h morpholino knockdown embryos was able to restore rhythmic contractions. Here we show that overexpression of VDAC1 can likewise recapitulate this phenotype, while VDAC3 fails to restore rhythmic contractions, indicating functional differences between the three isoforms. We noticed a prominent distinction between VDAC isoforms at position 73. This position is occupied by a glutamate (E) in VDAC1 and VDAC2, the two isoforms that rescue the *tremblor* phenotype, while a glutamine (Q) occupies position 73 in VDAC3. This unique property of VDAC3 is conserved among different vertebrates. E73 was previously identified as a binding site for Ca^{2+} and the channel inhibitor ruthenium red and to be responsible for regulation of the channel by Ca^{2+} and hexokinase. We therefore mutated E73 to Q in VDAC2 and overexpressed $VDAC2^{E73Q}$ in *tremblor* embryos. Indeed, $VDAC2^{E73Q}$ failed to restore synchronized cardiac contractions in *tremblor*. We further introduced the Q73E conversion into VDAC3 and observed a larger number of embryos displaying synchronized cardiac contractions after injection of $VDAC3^{Q73E}$ mRNA, compared to embryos injected with wild type VDAC3. We therefore conclude that regulation of VDAC at residue E73 is critically involved in the mechanism by which VDAC2 overexpression suppresses cardiac fibrillation in *tremblor*.

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Cardiac Restricted Overexpression of Caveolin-3 Prevents Arrhythmia, Ventricular Hypertrophy and Cardiac Dysfunction in Aging Mice

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Prolongation of the repolarization action potential duration (APD) and altered Ca^{2+} signaling are associated with aging related cardiac hypertrophy and contractile dysfunction. However the mechanism of age dependent alteration in calcium signaling and arrhythmia is not known. Caveolin 3 (Cav3), a muscle-specific scaffolding protein of caveolae, is known to regulate voltage-gated Ca^{2+} channels and Ca^{2+} signaling in cardiomyocytes. To investigate the role of Cav3 in the cardiac aging, we used 4 and 24-month old WT type mice and age matched cardiac restricted Cav3 overexpresser (Cav3OE) mice. The 24-months aged WT mice developed ventricular hypertrophy and fibrosis. A significant reduction in the ejection fraction and fractional shortening confirmed impaired cardiac function in 24-months aged mice. Western blot analysis showed reduced Cav3 expression (50%) in the ventricular myocytes

of 24-months old WT mice as compared to 4-months adult mice. In contrast, cardiac specific Cav3OE mice had normal cardiac function with no detectable fibrosis and cardiac hypertrophy at 24-months, which was similar to 4-months adult WT or Cav3OE mice. Whole-cell patch clamp analysis showed significantly increased APD50 (44.89 ± 8ms) and APD90 (66.5 ± 11ms) (n=6) in the ventricular myocytes of 24-month aged mice compared to 4-months adult mice (APD50: 8.9 ± 2ms; APD90: 17.88 ± 6ms; n=6). A re-expression of $I_{Ca,T}$ (−2.1 ± 1pA/pF) observed in ventricular myocytes from 24-months aged WT mice suggested possible cause for altered Ca^{2+} signaling and induction of ventricular hypertrophy. In contrast, ventricular myocytes from 24-months aged Cav3OE showed a normalized action potential (APD50:11 ± 3ms; APD90:47.5 ± 6ms; n=8) and negligible $I_{Ca,T}$ (−0.3 ± 0.5 pA/pF). We conclude that a reduced Cav3 expression in aged ventricle contributes to arrhythmia, altered Ca^{2+} signaling via re-expressed $I_{Ca,T}$ and cardiac dysfunction. Cardiac specific overexpression of Cav3 prevents aging induced arrhythmia, cardiac dysfunction and hypertrophy.

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Nucleoplasmic [Ca] Transients Alterations and Perinuclear Ca Stores Remodeling after Pressure Overload-Induced Hypertrophy in Adult Cardiac Myocytes

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Nucleoplasmic calcium concentration ([Ca]) in cardiac myocytes (CMs) regulates exitation-transcription coupling and is involved in remodelling processes. Perinuclear Ca stores contribute to the regulation of nucleoplasmic [Ca] transients (CaTs). We thus characterized alterations in perinuclear Ca stores and nucleoplasmic CaTs after pressure overload-induced hypertrophy in adult CMs.
Pressure overload was induced by transverse aortic constriction (TAC) in adult wild-type mice. Sham-operated mice served as controls. Ventricular CMs were isolated 1 and 7 weeks after TAC/Sham. Perinuclear Ca stores were visualized using confocal imaging and staining with Mag-Fluo-4/AM (10μM). CaTs were recorded in electrically-stimulated CMs loaded with Fluo-4/AM (8μM).
In Sham CMs, staining of perinuclear Ca stores revealed a nuclear envelope and tubular structures transversing the nucleus. Rapid application of caffeine (20 mM, n=7) reversibly abolished Mag-Fluo-4 fluorescence. Fluorescence recovery after depletion was identical in nuclear envelope and tubular structures. A significant increase in number of tubules per nucleus was observed during physiological growth (1week: 4.2 ± 0.2; 7weeks: 4.7 ± 0.2 (n=90)). Nuclear dimensions as well as cyto- and nucleoplasmic CaTs remained unaltered. In TAC CMs, the number of tubules progressively decreased (1week; 4.3 ± 0.2; 7weeks: 3.4 ± 0.2 (n=90)), whereas length and width of nuclei increased (1week: 13.3 ± 0.3 and 4.9 ± 0.2μm; 7weeks: 17.8 ± 0.7 and 5.5 ± 0.2μm). One week after TAC changes in the kinetics and amplitude of CaTs were found in the nucleus (TTP: 127 ± 2 vs 194 ± 5ms; RT_{50}: 360 ± 7 vs 380 ± 5ms; amplitude: 491 ± 19 vs 376 ± 28nM; Sham vs TAC (n=15); all $P<0.05$). Seven weeks after TAC similar changes also occurred in the cytoplasm.
Perinuclear Ca stores and nucleoplasmic CaTs undergo significant changes during hypertrophy progression, which appear to precede changes in cytoplasmic Ca regulation. These results raise the possibility that altered nucleoplasmic [Ca] may contribute to the development and/or progression of hypertrophy.

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Histamine-Evoked Signaling in Human Primary Keratinocytes

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Itch, the unpleasant sensation that evokes a desire to scratch, is mediated by a subset of primary afferent C-fibers. Several lines of evidence suggest that keratinocytes also play an active role in itch signaling. First, keratinocytes release nerve growth factor (NGF), a molecule implicated in chronic itch. Second, keratinocytes secrete a number of pruritogens, including histamine, endothelin-1 (ET-1) and leukotriene B_4. Third, keratinocytes are directly activated by a number of pruritogens. To elucidate the role of keratinocytes in the transduction of itch, we are probing the mechanisms underlying itch-evoked calcium signaling in primary human keratinocytes. Using ratiometric Ca^{2+} imaging, we found that keratinocytes respond robustly to three pruritogens: histamine (10 μM), ET-1 (100 nM) and the peptide activator of PAR2, SLIGRL (10 μM). These pruritogens trigger both Ca^{2+} release from stores and Ca^{2+} influx across the plasma membrane. Histamine signaling requires the histamine receptor 1 (H1R), as the H1R-specific antagonist, diphenhydramine (10 μM), completely blocks all calcium signals. Pruritogen-evoked calcium signals also require functional store-operated channels (SOCs), as 2-Aminoethoxydiphenyl borate (2-APB; 50 μM), Gd^{3+} (10 μM) and La^{3+} (50 nM) inhibit pruritogen-evoked calcium influx. Consistent with our pharmacological findings, real time qPCR and microarray gene expression analysis demonstrate that Orai1 is highly expressed in both human and mouse primary keratinocytes. Thus, Orai1 may be a channel onto which multiple itch pathways converge to trigger pruritus.

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Quantitative Characterization of Local Chemical Delivery through Nanopipette

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Scanning Ion Cunductance Microscope (SICM) produces 3D images of live cells with nanometer resolution. In the past, SICM nanopipette probe was used for chemical agent delivery(1) and also to map potassium channels in cardiomyocytes(2). However these applications had little control over the concentration of applied agent that was only estimated from the geometry of the pipette tip. We have used two different methods to quantify and monitor chemical delivery. A highly sensitive electrochemical substrate sensor was fabricated, to quantify the spatiotemporal distribution of delivered electrochemical mediators by SICM nanopipette. The amount of substance released at different pressures and voltages were measured and calibrated by the electrochemical sensor. A double barrel nanopipette with integrated detection sensor was also fabricated to monitor the concentration of mediator near the tip. One barrel was filled with carbon as electrochemical sensor and the other barrel was filled with the mediator. Using this approach and by applying different pressures and voltages to the nanopipette, concentration of chemicals at the tip can be controlled. We were able to trigger localised receptor mediated responses in neurons, by varying local concentration of agonists under the SICM nanopipette tip. This will open the possibility for functional mapping of receptor mediated responses in cells.

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Cytosolic Calcium Dependency of STIM1 De-Oligomerization

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Store operated calcium entry (SOCE) is a universal mechanism that cells use to replenish their intracellular calcium stores. SOCE activation is regulated by STIM1, an endoplasmic reticulum (ER) transmembrane protein with calcium binding EF-hand domains protruding into the ER lumen. Upon depletion of ER calcium stores, the dissociation of calcium ions from STIM1 EF-hands triggers a conformational change that leads to the formation of STIM1 oligomers, which translocate to the plasma membrane to bind and activate calcium channels of the Orai family. Once ER stores are replenished, STIM1 oligomers detach from plasma membrane channels and subsequently de-oligomerize. Whether changes in ER calcium levels regulate STIM1 oligomerization and de-oligomerization in a similar fashion is not known. In this study, we used reversible SERCA inhibitors to deplete and refill ER calcium stores in HeLa cells and measured by FRET the kinetics of oligo- and de-oligomerization between CFP-STIM1 and YFP-STIM1. We found that the refilling of ER calcium stores was not sufficient to de-oligomerize STIM1 molecules. Using BAPTA-AM to increase the cytosolic calcium buffering capacity of cells, we could fully refill ER stores without inducing the de-oligomerization of STIM1. De-oligomerization of STIM1 in BAPTA-loaded cells occurred upon readmission of Ca2+ to the extracellular medium, and was prevented by SOCE channel inhibitors (La3+ and Gd3+). Applying divalent ion Ba2+ in substitution for Ca2+ at the readmission stage induced only a partial and temporal STIM1 de-oligomerization. These data indicate that an elevation of the cytosolic Ca2+ concentration is required for the induction of the de-oligomerization process while ER refilling is not sufficient. Our findings thus indicate that STIM1 oligomerization exclusively depends on ER Ca2+ content, while de-oligomerization depends both on ER and cytosolic Ca2+ concentrations.

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Conformational Rearrangements of STIM1 Cytosolic Portion Sensed by Altered Coiled-Coil Accessibility

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STIM1 and Orai1 are key players of the Ca2+-release activated Ca2+ (CRAC) current that plays an important role in T cell activation as well as mast cell degranulation. Activation of the CRAC channel forming subunit