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Molecular Assembly of the Mitochondrial Calcium Uniporter Complex

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Mitochondrial Ca^{2+} regulates a wide variety of physiological processes, including ATP production and cell death. Rapid Ca^{2+} uptake into mitochondria is mediated by the mitochondrial calcium uniporter (MCU) complex, which is composed of the pore forming MCU protein, and the regulatory proteins EMRE, and MICU1/MICU2. Currently, the submitochondrial localization and transmembrane orientation of these proteins are either unknown or under debate. We attack these issues using a classical thiol-modification approach. We removed native MCU complex proteins in HEK293 cells using CRISPR/Cas9, and reintroduced mutants containing a single cysteine at defined positions. Treatments of the mitoplast with a bulky, thiol-reactive compound polyethylene glycol (PEG) maleimide would alter the molecular weight of the protein if the engineered Cys is exposed to the intermembrane space. Using this strategy along with other biochemical methods, we demonstrated that (1) MCU adopts an orientation with the signature DIME motif facing intermembrane space (IMS), (2) the conserved C-terminal polyaspartic tail of EMRE is in the IMS, (3) both MICU1 and MICU2 are associated with the outer leaflet of the inner membrane, and (4) although MICU1 is a peripheral membrane protein, MICU2 is an integral component of the inner membrane. Then, applying domain interaction analysis and mutagenesis screening, we identified molecular contacts that govern the Ca^{2+} transport behavior of the MCU complex. In particular, we demonstrate that EMRE interacts with MCU through the transmembrane helices to activate the Ca^{2+} pore, while using the polyaspartic tail to recruit MICU1/2 to gate the pore. This dual functionality of EMRE ensures that all functional MCU complexes respond appropriately to Ca^{2+} stimuli from the cytosol, safeguarding against dangerous Ca^{2+} leakage, which could diminish mitochondrial energy output and potentially trigger apoptotic cell death.

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Mitochondrial Permeability Transition Pore Opening Promotes Calcium Alternans and Waves in Ventricular Myocytes

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The interactions between mitochondria and sarcoplasmic reticulum (SR) calcium (Ca) release form a positive feedback loop, which plays important roles in cardiac excitation-contraction coupling and arrhythmogenesis. We developed a spatially-distributed model for rabbit ventricular myocytes, which consists of a three-dimensional network of coupled mitochondria and Ca release units (CRUs). The L-type Ca channels, the ryanodine receptors, and the mitochondrial permeability transition pores (mPTPs) were simulated using random Markov transitions. We then carried out computer simulations using the detailed ventricular cell model to investigate the effects of mitochondrial depolarization on intracellular Ca cycling dynamics. We show that opening of the mPTPs that cause depolarization of a small portion of the mitochondria promotes Ca alternans and Ca waves due to Ca extrusion from mitochondria elevating SR Ca and enhancing CRU coupling. In experiments of isolated rabbit and mouse ventricular myocytes, we applied FCCP to cause mitochondrial depolarization (measured by TMRM) and mPTP opening (measured by calcein loss from the matrix). For rabbit myocytes at pacing cycle length (PCL) 400 ms, no Ca alternans was observed under control conditions, but Ca alternans appeared 2 minutes after FCCP treatment (50-100 nM) coincident with matrix calcein loss indicating mPTP opening. At the same PCL, spontaneous Ca release was observed 2 minutes after FCCP treatment. For mouse myocytes at PCL 200 ms, no Ca alternans was observed under control condition, but Ca alternans appeared 2 minutes after FCCP treatment. The FCCP-mediated Ca alternans was prevented by the mPTP inhibitor cyclosporine A (CsA). FCCP treatment also sped up Ca oscillations which slowed after CsA. These results show that mitochondrial depolarization and mPTP opening can result in Ca alternans and waves which are known to be arrhythmogenic, mechanistically linking metabolic stress to cardiac arrhythmogenesis.

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Mitochondrial SK Channels Attenuate Ca-Dependent Arrhythmia in Hypertrophic Hearts by Regulating Mito-ROS-Dependent Oxidation and Activity of RyR

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Rationale: Small conductance Ca^{2+}-activated K^+ (SK) channels are implicated in ventricular arrhythmias in the context of heart failure and myocardial infarct. In addition to their plasmalemmal localization, SK channels were recently discovered in mitochondria inner membrane (mSK). Activation of these channels is protective against ischemia-reperfusion injury by reducing intracellular levels of reactive oxygen species (ROS). We hypothesized that mSK may also play an important role in regulating mitochondrial function in cardiac disease as well.
Objective: We investigated the role of mSK channels in Ca^{2+}-dependent ventricular arrhythmia using a rat model of hypertrophy induced by banding of the ascending aorta (TAB).
Methods and Results: Dual Ca^{2+} and membrane potential optical mapping of whole hearts from TAB rats revealed that membrane-permeable SK activator improved aberrant Ca^{2+} homeostasis and abolished VT/VF induced by b-adrenergic stimulation. Using whole cell patch-clamp and confocal Ca^{2+} imaging we found that membrane-permeable SK enhancers attenuated frequency of spontaneous Ca^{2+} waves and delayed afterdepolarizations in hypertrophic cardiomyocytes. Furthermore, mSK inhibition enhanced; and activation reduced mitochondrial ROS production in TAB myocytes measured with MitoSOX. Monobromobimane assay demonstrated that increased oxidation of ryanodine receptors (RyRs) in TAB cells was reversed by SK enhancers. Experiments in permeabilized TAB myocytes showed that SK enhancers restored SR Ca^{2+} content, suggestive of substantial improvement in RyR function.
Conclusions: These data suggest that enhancement of mSK channels in hypertrophic rat hearts protects from Ca^{2+}-dependent arrhythmia. This protection is mediated via decreased mitochondrial ROS and subsequent decreased oxidation of reactive cysteines in RyR, which ultimately leads to stabilization of RyR-mediated Ca^{2+} release.

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Diastolic Calcium Leak and the Role of Zinc

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In heart failure, there is a significant accumulation of Zn^{2+} in left ventricular tissue (1). Dysregulation of Zn^{2+}-homeostasis results in altered cardiac contractility and chronically raised cytosolic Ca^{2+} (2). We have recently shown that Zn^{2+} plays a key role in shaping intracellular Ca^{2+}-dynamics through modulation of the cardiac ryanodine receptor (RyR2) (3). In healthy cardiac cells, RyR2 is inhibited by Mg^{2+}, which acts to prevent inappropriate activation of RyR2 during diastole. We have investigated the relationship between cytosolic Ca^{2+}, Mg^{2+} and Zn^{2+} in regulating RyR2 gating. Sheep RyR2 channels were incorporated into artificial membranes under voltage-clamp conditions, as previously described (3). At activating levels of Ca^{2+} (5μM) and in the presence of physiological concentrations of Mg^{2+} (1mM) the addition of 100pM cytosolic Zn^{2+} increased channel open probability (*Po*) from 0.11 ± 0.02 to 0.34 ± 0.12 (SD, n=3, $p<0.05$), suggesting that Zn^{2+} plays a role in enabling RyR2 to operate under conditions of systole. When $[Ca^{2+}]$ was lowered to diastolic levels (100nM), the addition of 1mM Mg^{2+} resulted in complete closure of the channel. Under these conditions, the addition of 100pM Zn^{2+} had no effect on channel *Po*. Elevating Zn^{2+} to pathophysiological levels (1nM) however, caused abnormal channel openings (*Po*=0.12; n=2). At activating levels of Ca^{2+} (5μM), in 38% of our bilayers, we observed currents consistent with those reported for Mitsugumin-23 (MG23) (4), with no contribution from RyR2. Interestingly, the addition of 1nM cytosolic Zn^{2+} increased these currents (mean average current increased from -0.56 ± 0.05 to -0.98 ± 0.1pA (SD, n=4, $p<0.05$)). These data suggest that dysregulated Zn^{2+}-homeostasis leads to irregular gating of both RyR2 and MG23, which is likely to contribute to diastolic Ca^{2+} leak in heart failure.

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Association Between β3-Adrenoceptor Activation and Intracellular Free Zinc Ion Increase Contributes to Hyperglycemia-Induced Cardiac ER-Stress

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The Zn^{2+} release during cardiac cycle results mostly intracellular free Zn^{2+} ($[Zn^{2+}]_i$) increases via triggering production of reactive oxygen/nitrogen

species (ROS/RNS). However, it is not clearly known whether there are relationships between increased ROS/RNS production and $[Zn^{2+}]_i$ changes under hyperglycemic conditions in cardiomyocytes. Thus, herein, we monitored $[Zn^{2+}]_i$ changes and ROS/RNS production by using specific fluorescence dyes, such as FluoZin-3, DAF and DCDFA, respectively, in hyperglycemic cardiomyocytes from streptozotocin-induced diabetic rat heart by using confocal imaging. We have shown that acute increases in either ROS or RNS could induce marked increases in $[Zn^{2+}]_i$ of freshly isolated cardiomyocytes, which could be reversed by a thiol reducing agent DTT. The basal ROS/RNS-induced $[Zn^{2+}]_i$ levels were found to be significantly higher in hyperglycemic cardiomyocytes compared to the controls. Our Western blot analysis showed that hyperglycemia induced marked phosphorylation in some members of macromolecular protein complex of cardiac ryanodine receptors such as PKA, CaMKII and PP2A, which were also hyperphosphorylated with high $[Zn^{2+}]$. Furthermore, other important factors, which have important role in glucose metabolism and are activated with cytosolic $[Zn^{2+}]$, such as a transcription factor NF-κB and GSK-3 and Akt in cardiomyocytes were phophosylated markedly with high glucose incubation. Overall, the present data demonstrated that there are cross-relationships between increased production of ROS/RNS and increased $[Zn^{2+}]_i$ level in hyperglycemic cardiomyocytes, which is mediated with several protein phosphatases and kinases and underlies, in part, diabetes-induced cardiac dysfunction. (Supported by grant of TUBITAK (SBAG-113S466 & SBAG-214S254).

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Shear Stress Enhances Ca^{2+} Spark Occurrence in Rat Ventricular Myocytes Via Mitochondrial NADPH Oxidase-Reactive Oxygen Species Signaling

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It has been reported that shear stress enhances Ca^{2+}-induced Ca^{2+} release during depolarization in ventricular myocytes. To know molecular basis for the increase in global Ca^{2+} releases we assessed the effects of shear stress (~16 dyn/cm^2) on local Ca^{2+} release events (Ca^{2+} sparks) and examined cellular mechanisms for the shear-mediated Ca^{2+} spark regulation using confocal Ca^{2+} imaging in rat ventricular myocytes. The frequency of Ca^{2+} sparks was immediately (within 1 s) increased by shear stress to about 1.5-fold, and further increased to about 2-fold by prolonged (20 s) shear exposure. Inhibition of nitric oxide synthetase and interference of cytoskeletal integrity using L-NAME (1 mM) and colchicine (10 μM), respectively, did not affect the shear-mediated enhancement in spark frequency. Blockers for Na^+-Ca^{2+} exchanger and L-type Ca^{2+} channel, KB-R7943 (5 μM) or Ni^{2+} (5 mM), did not alter the shear effect on spark occurrence. Inhibition of NADPH oxidase (NOX) and mitochondrial uncoupling using diphenyleneiodonium (3 μM) and carbonyl cyanide 3-chlorophenylhydrazone (plus oligomycin, 1 μg/ml), respectively, reduced shear mediated spark enhancements. Pretreatment of reducing agent dithiothreitol (2 mM) also significantly reduced the shear-mediated spark enhancement. Measurement of intracellular reactive oxygen species (ROS) using its specific dye 2′,7′-dichlorofluorescein revealed increase in ROS level by shear stress. Sarcoplasmic reticulum (SR) Ca^{2+} content was not altered immediately after the application of shear stress, but significantly increased after 20-s long shear exposure. Our data suggest that shear stress enhances the frequency of Ca^{2+} sparks partly by producing ROS via mitochondrial NOX, and that prolonged enhancement in spark frequency by shear stress may be also mediated by increased SR Ca^{2+} loading. These mechanisms may partly explain the shear-mediated enhancement in Ca^{2+} transient in ventricular myocytes.

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X-ROS Signaling in Atrial Myocytes Produces Arrhythmogenic Ca^{2+} Waves

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Atrial myocytes undergo stretch during diastole as do ventricular myocytes. We have identified a novel mechanism that links cellular stretch in ventricular myocytes to the tuning of Ca^{2+} release from the sarcoplasmic reticulum (SR)(Prosser et al. Science 2011;333:1440-5). This mechanism, ''X-ROS signaling'', depends on NOX2 (NADPH oxidase) to generate reactive oxygen species (ROS), which appears to oxidize ryanodine receptors (SR Ca^{2+} release channels) and increases their sensitivity to $[Ca^{2+}]$i. Stretch mediates X-ROS signaling through microtubules, which appear to interact with NOX2 to enable it to generate ROS.

We evaluated X-ROS signaling in murine and rabbit atrial myocytes. Ca^{2+} sparks and Ca^{2+} waves were recorded before and after stretch (10-20% of cell length). The frequency of Ca^{2+} release events (Ca^{2+} sparks and Ca^{2+} waves) increased in proportion to the amount of cell stretch (up to 20% of cell length). Higher single cell stretch (15-20% of cell length) produced arrhythmogenic Ca^{2+} waves, while lesser stretch leads to an increase in frequency of Ca^{2+} sparks. Stretch-induced Ca^{2+} waves were prevented by the NOX inhibitor DPI (Diphenyleneiodonium chloride). Interestingly, microtubule density was higher and protein expression levels were increased in murine atria compared to ventricles. Similarly, protein expression levels of the catalytic NOX2 subunit gp91phox were higher in atria compared to ventricles. Stretch-induced Ca^{2+} waves did not occur in rabbit cells treated with the NOX inhibitor DPI. Thus, X-ROS signaling is operative in atrial myocytes and can modulate Ca^{2+} signaling.

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Novel Insights into Sinoatrial Nodal Cell Local Calcium Releases (LCRs) from Automated Computer Analysis in Spontaneously Beating Cells

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LCRs are crucial events of cardiac pacemaker cell function. However, specific algorithms for their automatic detection and analysis have not been developed in 2D$^+$time for live spontaneously beating pacemaker cells. Here we measured calcium signals by a high-speed 2D-camera in isolated rabbit SA-node cells. Our new algorithm tracks points along the midline of the moving/contracting cell. It uses these points as a coordinate system for affine transform, producing a transformed image series where the cell does not move/contract. AP-induced calcium transients and LCRs were isolated from noise by applying a series of spatial filters. We detected LCR birth/death events by a differential (frame-to-frame) sensitivity algorithm applied to each pixel (cell location). An LCR is detected when its signal changes sufficiently quickly within a sufficiently large area. The LCR dies when its amplitude decays substantially or when it merges into a rising transient.

The algorithm is capable of rapidly analyzing long recordings, e.g. we detected and analyzed as many as 1746 individual LCR in 81 consecutive cycles from one cell. On average, there were 21.6 LCR events per diastolic period (SD=8.0), ranging between 8 and 48, with mean LCR duration being 28.4 ms. Ultimately our algorithm finds not only known key LCR parameters such as period, signal mass, and duration, but also novel parameters crucial for further biophysical insights such as LCR birth and death statistics, propagation path and ''hot-spots'' (areas with higher LCR activity). As the LCRs travel in live cells, the algorithm identified their splits and merges, validating predictions of our recent 3D-numerical model of SA-node cell and indicating importance of locally propagating calcium-induced-calcium release for the LCR fate and power critical for the diastolic depolarization acceleration via electrogenic sodium/calcium exchanger.

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Synchronization of Local Calcium Releases (LCRs) in Guinea Pig Single, Isolated SA Node Cells Contributes to Generation of Rhythmic Action Potential-Induced Ca^{2+} Transients

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Spontaneous, rhythmic LCRs are an important factor of pacemaker function of SA node cells (SANC). LCRs operate within the context of a coupled-clock system involving interactions of plasma membrane and sarcoplasmic reticulum (SR)-based calcium clocks. To date, the functional importance of this coupled-clock system has been demonstrated in rabbit SANC that beat spontaneously and rhythmically. The contribution of LCRs to action potential firing in guinea pig SANC, however, is controversial.We quantified the entire ensemble of LCRs in healthy appearing, single guinea pig SANC prior to and in the presence of beta-adrenergic stimulation (isoproterenol, 1μM). LCRs were measured by a high-speed 2D-camera and analyzed by a novel computer program capable of identifying LCRs in video-recordings.Although nine of the 22 SANC examined did not exhibit Ca^{2+} transients, LCRs were present in all cells. LCRs were smaller and more intense in cells without Ca^{2+} transients than in those with Ca^{2+} transients (rhythmic or dysrhythmic). Isoproterenol increased LCR size and induced rhythmic Ca^{2+} transients in majority of cells (6/9) without Ca^{2+} transients prior to isoproterenol. SANC that exhibited Ca^{2+} transients prior to isoproterenol generated approximately 80 LCRs/s. Cells in which Ca^{2+}