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The sarcoplasmic reticulum (SR) achieves selective activation of processes such as gene expression, growth and metabolism, in part, by being able to compartmentalize functions in various regions of the cell. One explanation, for the compartmentalization of function, is the SR comprises a series of structurally-separate Ca^{2+} storage elements each with various arrangements of the release channels and sensitivities to ligands. This study, in single smooth muscle cells, addresses whether or not the SR exists as multiple, separate, Ca^{2+} stores or as a single luminally-continuous entity throughout the cell. From one small site on the cell, the entire SR could be depleted via either ryanodine receptors (RyR) or IP_3 receptors (IP_3R). The entire SR could also be refilled from one small site on the cell. The SR is a single luminally-continuous structure in which Ca^{2+} is in free diffusional equilibrium throughout. Notwithstanding the luminal-continuity, regulation of the opening of RyR and IP_3R, by the $[Ca^{2+}]$ within the SR, may create several receptor arrangements on apparently separate stores. IP_3R and RyR may appear to exist entirely on a single store, and there may seem to be additional SR elements which express either only RyR or only IP_3R. The various SR receptor arrangements and apparently separate Ca^{2+} storage elements exist in a single luminally-continuous SR structure.

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1240-Pos Simultaneous Imaging of Subplasma Membrane and Bulk Cytoplasmic Average Ca^{2+} Concentrations in Single Smooth Muscle Cells

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In smooth muscle, Ca^{2+} controls diverse activities which include cell division, contraction and cell death. Of particular significance in enabling Ca^{2+} to perform these multiple functions is the cell's ability to localize Ca^{2+} signals to certain regions by creating high local concentrations of Ca^{2+} (microdomains) which differ from the cytoplasmic average. Microdomains are acknowledged to occur near the plasma membrane as a result of Ca^{2+} influx, but measuring them has been difficult. Total internal reflection microscopy (TIRF) enables optical sectioning via the use of evanescent wave illumination. In a TIRF system, fluorescence excitation is restricted to within ~200 nm of the coverslip thus it is possible to selectively image the subplasma membrane space. Here subplasma membrane and bulk cytoplasmic average $[Ca^{2+}]$ have been measured simultaneously using TIRF and wide field fluorescent imaging in single voltage clamped smooth muscle cells. A single Ca^{2+} indicator (fluo-3) was used to measure both subplasma membrane $[Ca^{2+}]$ and bulk cytoplasmic average $[Ca^{2+}]$ to simplify analysis of the results since only one set of kinetic parameters and affinity apply in calibrating the Ca^{2+} signals. The results show that, in single voltage clamped smooth muscle cells at rest (–70 mV), the $[Ca^{2+}]$ in the subplasma membrane space was approximately double the bulk cytoplasmic average value. During brief depolarizations to +10 mV the subplasma membrane $[Ca^{2+}]$ was approximately five times greater than the cytoplasmic average value.

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Mitchondrial Channels & Calcium Signaling

1241-Pos Harmonic Generation Spectroscopy of Live Cells: Measurements and Volterra Series Analysis

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We report on measurements and analysis of the fundamental (linear response) and higher harmonics (nonlinear responses) generated by suspensions of live cells, including *S. cerevisiae* (budding yeast) and *S. pombe* (fission yeast), in response to sinusoidal electric fields. Their frequency- and time-dependences exhibit features that correlate with consumption of glucose and oxygen, possibly due to oxidative phosphorylation within the mitochondria. These and other biological systems exhibit nonlinear responses that require a more accurate analysis than that afforded by linearization of the model system. For example, Fourier and Laplace transforms only treat the response of the system at the applied fundamental frequency. By contrast, the higher harmonics and other manifestations of nonlinear response can be treated through the application of Volterra theory. The Volterra series representation thus obtained is not only an explicit nonlinear representation of the system's response to the input signal, but also affords greater insight into the biological system's operation. We discuss the application of Volterra theory to the analysis of systems modeled in terms of certain nonlinear differential equations that include damping. Our main objective here is to describe, and qualitatively understand, the observed behavior of the second harmonic vs. time and frequency. We interpret the behavior in terms of parameter changes within a simple mathematical model, and try to correlate those changes with actual biological processes.

1242-Pos Two Commercially-Available Antibodies to Kir6.1 Recognize Non-Target Proteins in Bovine Heart Mitochondria

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There is little consensus regarding the identity of the protein subunits that comprise the mitochondrial ATP-sensitive K^+-channel (mitoK_{ATP}). To clarify the issue, we sought to purify any Kir6.x-immunoreactive protein that might co-localize with mitochondria, and identify it by mass spectrometry. Using a Kir6.1 antibody from Santa Cruz Biotechnologies, we observed a 51-kDa immunoreactive band that was enriched in preparations of inner mitochondrial membranes from rat, pig and cow hearts. Localization to the mitochondria was confirmed by immunoelectron microscopy. Immunoreactivity copurified with OXPHOS complex I following two-step purification from solubilized mitochondrial membranes. The 51-kDa immunoreactive band was identified by LC-MS/MS as NADH dehydrogenase flavoprotein 1, the matrix-facing 51-kDa subunit of Complex I. A second Kir6.1 antibody, from Alomone Labs, identified a 48-kDa immunoreactive band in isolated bovine heart mitochondria. Immunofluorescence confirmed that the signal colocalized with ATP synthase *in situ*. Two-step purification from mitochondrial extracts enriched a 48-kDa band, which was subsequently identified as mitochondrial isocitrate dehydrogenase (NADP-binding isoform). Thus, these antibodies gave rise to two false-positive results in heart mitochondria. We would, therefore, be circumspect when analyzing data resulting from their use, particularly as it pertains to the identity of mitoK_{ATP} subunits.

1243-Pos Regulation of Cardiac Mitochondrial Morphology by Intracellular Calcium

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In cardiomyocytes, mitochondria regulate cardiac contractility via ATP production and cellular Ca^{2+} homeostasis regulation. Mitochondria are located closely with the sarcoplasmic reticulum (SR) and plasma membrane to sequester cytosolic Ca^{2+} ($[Ca^{2+}]_c$). $[Ca^{2+}]_m$ uptake increases respiratory chain activity and ATP output. Dysregulation of this process is the cause for many heart diseases. Various diseased cardiac tissues have abnormally small, disorganized mitochondria, suggesting a link between mitochondrial morphology and function. Yet, the role of mitochondrial morphology in regulating mitochondrial energy production, Ca^{2+} homeostasis in the heart remains unknown. To test the hypothesis that cardiac mitochondria fragment by an intracellular Ca^{2+} signal, we used the SR Ca^{2+}-ATPase inhibitor, thapsigargin (TG), which fragments Clone9 mitochondria via an increase in intracellular Ca^{2+} levels and Ca^{2+} influx into mitochondria. Fluorescence microscopy of neonatal rat ventricular myocyte mitochondria showed tubular mitochondria became small and punctate with TG. TG on adult cardiomyocytes caused translocation of mitochondrial fission protein, DLP1/Drp1, from the cytosol to mitochondria, while electron microscopy of mitochondria showed smaller average area and circular morphology. Interestingly, this mitochondrial fragmentation is associated with an increase in reactive oxygen species (ROS) generation. To test if cardiac mitochondria fragment physiologically, we used the β-adrenergic agonist, isoproterenol, on adult rat heart and cardiomyocytes. Isoproterenol increased the heart rate and strength, and caused DLP1 translocation to mitochondria, which was blocked with the β-adrenergic antagonist, propranolol. Currently, we are investigating whether this mitochondrial fission is a key step in altering activity of the electron transport chain and thus dictates mitochondrial ATP and ROS generation during β-adrenergic stimulation. These results suggest that mitochondrial fission proteins are regulated by Ca^{2+} and β-adrenergic signaling during the cardiac excitation-contraction cycles, which could change the function and activity of mitochondria physiologically.

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1244-Pos Metabolic Control Of Mitochondrial Fusion-fission Dynamics

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Mutual dependence between mitochondrial function and morphology is becoming evident and is likely to be important for cell survival under both physiological and pathophysiological conditions. Based on studies of mitochondrial uncoupler-treated cells, loss of $\Delta\Theta_m$ is thought to halt mitochondrial fusion, through cleavage of Opa1, the inner mitochondrial membrane fusion protein. However, the uncoupler evokes multiple changes in mitochondrial chemistry: $\Delta\Theta_m$, pH and $[Ca^{2+}]$, which changes may also affect mitochondrial dynamics through Opa1-dependent and -independent mechanisms. To isolate each component we tested the effect of selected ionophores and mitochondrial inhibitors on $\Delta\Theta_m$, pH and $[Ca^{2+}]$ by multiparameter single cell imaging and on mitochondrial morphology and fusion by confocal imaging of mitochondria-targeted fluorescent highlighters. The protonophore, carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) caused dissipation of $\Delta\Theta_m$, concomitant matrix pH decrease and a cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) rise that could be suppressed by incubation of the cells in a Ca^{2+} free medium. These changes were followed by proteolytic processing of Opa1 and inhibition of mitochondrial fusion that could be partially rescued by Opa1 overexpression. Unexpectedly, the mitochondrial morphology in the FCCP-treated cells also showed donut formation resulted from end-to-end or end-to-side fusion, which was not affected by Opa1 or Drp1K38A overexpression. The K^+/H^+ ionophore, nigericin caused mitochondrial hyperpolarization, decrease in the matrix pH and a rise in $[Ca^{2+}]_c$. Nigericin caused no Opa1 cleavage but mitochondrial fusion became inhibited and fragmentation of the mitochondria occurred. Thus, mitochondrial depolarization activates the Opa1 cleavage pathway to suppress fusion in conjunction with stimulation of distinct donut-forming mechanisms. Furthermore, matrix acidification and the $[Ca^{2+}]_c$ rise fail to evoke Opa1 cleavage but still prevent fusion while $\Delta\Theta_m$ is sustained.

1245-Pos Distribution, Responses To Ca^{2+} Transients And Calibration Of A Mitochondria-targeted Cameleon Biosensor Expressed In Muscle Of Live Mice

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D3 and D3cpV are calmodulin-based two-fluorophore biosensors (cameleons) redesigned to reduce interferences (Palmer et al. 2006), which have an affinity (K_D ~1 μM) suitable for monitoring [Ca^{2+}] in mitochondria. We studied the distribution and characterized the response of these sensors in muscle fibers of mice transfected as described (DiFranco et al. 2006). 4mtD3cpV (where 4mt indicates four repeats of the signal sequence of cytochrome c oxidase) expressed abundantly and was located in mitochondria as assessed in dual staining images. Cells were patch-clamped with pipettes containing X-rhod (for monitoring cytosolic Ca^{2+}) and line-scanned confocally to derive the ratio ***R*** of cameleon fluorescence at 530 and 480 nm (excited at 458 nm). Under pulse depolarization ***R*** increased, peaked at 2.5-fold 80 ms later than the simultaneously measured cytosolic Ca^{2+} transient and then decayed to a ~70% lower level, which was maintained for seconds. D3cpV without the mt signal sequence was also used. It distributed as in cells expressing GFP, presumably in the cytosol. Its response, however, was similar to that of the mitochondria-localized form. A fit to the response with a two Ca^{2+}-site binding model required two vastly different reaction times (~10 ms and 20 s) but still could not reproduce some features. The peculiar kinetics suggests that the sensor adopts more than three states with different spectra. The initial component of the response is sufficiently fast to be useful for monitoring rapid transients, while the slow component is highly sensitive. A better understanding of the reaction mechanism or at least a better reaction model is necessary for a quantitative use of this cameleon. We are grateful to Amy Palmer (U of Colorado) for providing the plasmids.

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1246-Pos Vdac2 Dependence Of tbid-induced Apoptosis

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Board B222

Bid, a pro-apoptotic Bcl-2 family protein induces apoptosis by mediating the release ofcytochrome c (cyto c) and other intermembrane space proteins from mitochondria to the cytosol. Bid is activated by proteolytic cleavage to form truncated (tBid) that binds to the outer mitochondrial membrane (OMM) and engages Bak/Bax-dependent breakdown of the OMM barrier. The voltage-dependent anion channel (VDAC) is the major permeability pathway for metabolites and other molecules in the OMM but its role in the tBid-induced OMM permeabilization remains controversial. Here, we have studied the dependence of tBid-induced apoptosis on each VDAC isoform. Fluorometric measurements of mitochondrial membrane potential ($\Delta\Theta m$) were conducted in suspensions of permeabilized mouse embryonic fibroblasts (MEFs) lacking each VDAC isoform. tBid (3.7-37nM) induced complete loss of $\Delta\Theta m$ in wild type (WT), VDAC1-knockout (KO), VDAC3-KO and VDAC1/3-double KO (DKO) MEFs but it had no effect on VDAC2-KO MEFs. Immunoblot analysis of cytosol and membrane fractions showed complete release of cyto c from tBid-treated WT, VDAC1-KO, VDAC3-KO and VDAC1/3-DKO MEFs but no release from VDAC2-KO MEFs. It has been reported that VDAC2 is required for proper targeting of Bak in OMM. Immunoblot analysis showed that the membrane fraction of VDAC2-KO MEFs lacks BAK, whereas substantial amount of Bak is present in WT and in other VDAC-KO MEFs. Fluorometric measurements of $\Delta\Theta m$ and cyto c immunoblotting were conducted in suspensions of permeabilized WT, Bak-KO and Bax-KO cells. Bak-KO MEFs were less sensitive to tBid than WT or Bax-KO MEFs but responded to at least maximal tBid (37nM). Thus, complete insensitivity of VDAC2-KO against tBid is partly due to the lack of mitochondrial Bak but also depends on another factor (Bax recruitment?). Taken together, our data suggest that VDAC2 is essential for the ordered execution of tBid-induced apoptosis.

1247-Pos In Yeast, Ca2+ and Octylguanidine Interact with Porin (VDAC) Preventing the Mitochondrial Permeability Transition

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The mitochondrial permeability transition is defined as the sudden increase in mitochondrial conductance due to the opening of an unspecific pore. The yeast permeability transition pore, also termed yeast mitochondrial unspecific channel (YMUC), is active in situ and is inhibited when ATP is depleted or phosphate accumulates. The composition of this pore is unknown since the pore still opens when the genes encoding either the adenine nucleotide carrier (ANC) from the inner mitochondrial membrane or the voltage-dependent anion channel (VDAC) from the outer mitochondrial membrane are deleted. This pore is closed by the addition of different effectors such as Ca2+, octylguanidine (OG) and poly-anions. In order to define the yeast mitochondrial unspecific channel interaction site for these effectors, we decided to test the effect of these compounds on two laboratory strains, a wild type and a VDAC deletion mutant. In addition, the VDAC modulator decavanadate

(dVO4) was used as a control. We measured mitochondrial oxygen consumption, transmembrane potential and mitochondrial swelling in two conditions: Open YMUC using 0.4 mM phosphate in the reaction mixture and closed YMUC using 4 mM phosphate. Our results indicate that in open YMUC conditions and in the absence of VDAC, the YMUC is desensitized to OG, dVO4 and to Ca2+ but not to inorganic phosphate. Thus, it is suggested that VDAC is dispensable for YMUC opening, but necessary for Ca2+, OG and dVO4-mediated closure of the channel.

1248-Pos Exposure to Aqueous Extract of Cigarette Smoke Increases Susceptibility of Mouse Ventricular Myocytes to the Mitochondrial Permeability Transition (MPT)

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Board B224

Exposure of animals to cigarette smoke (CS) increases myocardial infarct size, but it is not clear whether this is due to CS effects on myocytes.

Smoke from 2 cigarettes was passed through 100 ml Hepes solution, and adult mouse ventricular myocytes (M) were exposed to Hepes solution containing 0.1% of this extract (CSE). We measured changes in the susceptibility to MPT (argon laser illumination-induced TMRM fluorescence and hypercontracture, confocal microscopy); and effects on mitochondrial Ca^{2+} uptake in digitonin-permeabilized myocytes (5 min exposure to 1000 nM Ca^{2+}, Rhod 2A fluorescence, confocal microscopy).

CSE 0.1% increased mitochondrial Ca^{2+} uptake (control Rhod2A F/F_0 2.76±0.43 vs 0.1% CSE 4.45±0.42, means±SEM, n=8, $p<0.05$). The superoxide scavenger Tiron 10 mM abolished the effect of 0.1% CSE on Ca^{2+} uptake (F/F_0 2.36±0.37). Tiron alone had no effect (F/F_0 2.25±0.52). CSE 0.1% decreased the time to an abrupt increase in TMRM fluorescence (control 312±28 sec vs 192±16 sec) and the time to hypercontracture (control 392±32 sec vs 286±23 sec, n=19–35, $p<0.05$). In Tiron 10 mM, CSE did not significantly shorten the time to MPT as detected by TMRM fluorescence or hypercontracture, and Tiron alone did not influence MPT susceptibility. The nicotine concentration in 0.1% CSE measured by HPLC was 15.6 ng/ml, in the range of arterial plasma nicotine concentrations in humans smoking a single cigarette.

These findings indicate that oxidative radicals in CSE increase Ca^{2+} uptake and increase susceptibility to MPT. Development of the MPT is a major factor in irreversible myocardial injury caused by ischemia/reperfusion. Therefore these direct effects of CSE on myocyte mitochondrial sensitivity to MPT could account for increased infarct size produced in vivo in intact animals by exposure to CS.

1249-Pos Huntingtin Modulates Cytoplasmic And Mitochondrial Calcium Signaling By Attenuation Of Ip_3 Receptor Sensitivity Of ER

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The polyglutamine expansion (polyQ) in huntingtin protein (Htt) is a cause of Huntington's disease (HD). Deletion of the Htt gene homolog (*Hdh*) is embryonic lethal in mice establishing it as an essential gene. Previous studies have implicated impaired calcium signaling in the pathogenesis of HD and have shown that Htt binds to the IP_3 receptor Ca^{2+} release channels at the ER. Furthermore, expression of polyQ-Htt has been shown to affect both the IP_3 receptor activity and mitochondrial bioenergetics. To systematically search for Htt function in calcium signaling, three mouse embryonic fibroblast (MEF) cells lines were used which contain a single copy of the *Hdh* gene (*Hdh*-HET) and three MEF lines in which the *Hdh* gene was deleted (*Hdh*-KO) utilizing *Hdh* +/– and Hdh-floxed mice. $[Ca^{2+}]$ was monitored in both cytoplasm and mitochondria using fluorescence imaging and fluorometry. We found that the cytoplasmic Ca^{2+} spikes resulting from the activation of IP_3 receptor and the ensuing mitochondrial Ca^{2+} signals were suppressed in the *Hdh*-KO cells when compared to *Hdh*-HET cells. In experiments using permeabilized cells, a suboptimal dose of IP_3 caused lesser ER Ca^{2+} mobilization in *Hdh*-KO cells; the IP_3 sensitive fraction of the ER Ca^{2+} store was approximately 75% in both control and *Hdh*-HET cells. Neither passive Ca^{2+} buffering nor the mitochondrial Ca^{2+} uptake was altered in the *Hdh*-KO cells. These results suggest that the IP_3 sensitivity of the IP_3 receptor is attenuated in the *Hdh*-deficient cells, providing a mechanism for attenuation of the IP_3-linked $[Ca^{2+}]$ signaling in the *Hdh*-KO cells. Thus, Htt plays an important role in modulating IP_3 receptor-mediated cytoplasmic and mitochondrial calcium signaling.

1250-Pos Mitochondrial Free [ca] During Sr Ca Release In Permeabilized Rat Ventricular Myocytes

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Board B226

Cardiac mitochondria can take up Ca, competing with other Ca transports (e.g. SR Ca-ATPase & Na/Ca exchange). Some have reported rapid mitochondrial Ca transients, relatively synchronized with normal cytosolic $[Ca]_i$ transients, invoking physical proximity between SR Ca release sites and mitochondria to explain rapidly

rising in intra-mitochondrial free [Ca] ($[Ca]_m$). However, $[Ca]_m$ is generally not calibrated, and the Ca indicator signal may not be exclusively mitochondrial. Here we measured calibrated $[Ca]_m$ in single rat myocytes using ratiometric Ca indicator fura-2 AM and plasma membrane permeabilization by saponin (to eliminate cytosolic fura-2). First we measured the steady state $[Ca]_i$ vs. $[Ca]_m$ relationship at high [EGTA] (5 mM; pH 7.2, 23°C). $[Ca]_m$ had a sigmoid dependence on $[Ca]_i$ and was lower than $[Ca]_i$ for $[Ca]_i$ below 600 nM. Mitochondrial Ca uptake was Ru360- and FCCP-sensitive. Additional studies used low [EGTA] (50 μM) and 75–150 μM $[Ca]_i$, where cyclical spontaneous SR Ca release occurred (5–10/min), in the absence of Na (to limit mitochondrial Ca extrusion). Transient increases in fluorescence were seen at both 340 and 380 nm wavelengths, but the ratio signal (and $[Ca]_m$) hardly changed during an individual beat, such that $[Ca]_m$ gradually rose at ~2–8 nM/beat. Notably, massive SR Ca release by 10 mM caffeine abruptly stopped the progressive rise in $[Ca]_m$ and the spontaneous Ca transients (confirming that SR Ca releases caused the $[Ca]_m$ rise). A 5 nM $[Ca]_m$ rise per beat is a total uptake of ~0.5 μmol/l cytosol (assuming 100:1 Ca buffering in mitochondria) consistent with prior estimates from $[Ca]_i$ measurements (*J Physiol* 1994; *476*:279–293) and is $< 1\%$ of the SR Ca uptake in rat myocytes. Thus, while $[Ca]_m$ rises during Ca transients, the total amount is small, but can be cumulative over many beats.

1251-Pos VDAC Closure Sensitizes Rat Liver Mitochondria toward Ca^{2+}-induced Permeability Transition

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BACKGROUND Recently we demonstrated that the acute ethanol exposure closes voltage-dependent anion channels (VDAC) of the outer mitochondrial membrane. Here, we study the effect of VDAC closure on the Ca^{2+}-induced mitochondrial permeability transition (MPT) in isolated liver mitochondria. Our **AIM** was to evaluate the effect of G3139, a VDAC inhibitor (Biophys J, ***93***:1184), on the Ca^{2+}-induced MPT and reactive oxygen species (ROS) production in isolated mitochondria.

METHODS: Rat liver mitochondria were isolated by differential centrifugation. G3139 was obtained from Genta (USA). Mitochondria (1 mg/ml) were incubated in buffer containing (mM): sucrose 200, KH_2PO_4 1, EGTA 0.02, succinate 5, rotenone 0.002, oligomycin (1 μg/mg protein), Tris/HEPES 20, pH 7.4. After treatment with G3139 (0–5 μM), 50–300 μM of $CaCl_2$ was added, and swelling was monitored by absorbance at 540 nm. ROS formation was measured by dihydroethidine (1 μM) fluorescence.

RESULTS: The efficiency of Ca^{2+} to induce the MPT was expressed as the time required for 50% swelling to occur. This time decreased with increasing Ca^{2+}. G3139 treatment dose-dependently led to more rapid onset of the MPT at all Ca^{2+} doses, demonstrating increased potency of Ca^{2+} in mitochondria with closed VDAC. Dihydroethidine fluorescence also revealed increased ROS production after VDAC closure. The antioxidant, butylated hydroxytoluene (10 μM) reversed the effect of G3139, postponing MPT onset.

CONCLUSIONS: VDAC closure enhances the susceptibility of mitochondria to the Ca^{2+}-induced MPT. An accompanying increase of ROS production and reversal of the effect of G3139 on the MPT by antioxidant suggest that sensitization of mitochondria to Ca^{2+} by G3139 could be due to increased oxidative stress following VDAC closure.

1252-Pos Redox Regulation of Mitochondrial Calcium Signaling

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Board B228

Mitochondria are crucial for proper cellular Ca^{2+} signaling and energy production and are a significant source of cellular reactive oxygen species (ROS). Just as Ca^{2+} plays a role in the production of ROS, cellular redox state can modulate Ca^{2+} signaling. Redox regulation of sarcoplasmic reticulum and plasma membrane Ca^{2+} channels including RyR, IP_3 receptors, and L-type Ca^{2+}channels is well documented, but data demonstrating redox control of mitochondrial Ca^{2+} channels is sparse. We provide evidence for redox regulation of mitochondrial Ca^{2+} uptake using isolated rat heart mitochondria in the presence of reducing and oxidizing compounds. In isolated rat heart mitochondria, redox active compounds were found to influence mitochondrial Ca^{2+} uptake. Mitochondria loaded with fluorescent Ca^{2+} indicators were subjected to various extramitochondrial Ca^{2+} concentrations ($[Ca^{2+}]_o$) in the presence of reducing agents and oxidants. Results indicate that redox active compounds alter mitochondrial Ca^{2+} uptake by decreasing mitochondrial free Ca^{2+} ($[Ca^{2+}]_m$) ~20% in the presence of 2 mM DTT (reducing conditions) and increasing $[Ca^{2+}]_m$ by 67% in 2 mM GSSG (oxidizing conditions). We also studied redox modulation of $[^3H]$ryanodine binding to isolated rat heart mitochondria. GSSG increased $[^3H]$ryanodine binding to the mitochondrial ryanodine receptor (mRyR) by 42% while DTT decreased $[^3H]$ryanodine binding to the mRyR by 80%. Moreover, the redox-dependent changes of $[Ca^{2+}]_m$ in isolated mitochondria are blocked by ruthenium red (RuR) suggesting that the dominant redox effects are on mitochondrial Ca^{2+} influx mechanisms (i.e. mitochondrial Ca^{2+} uniporter (MCU), mRyR, and non-selective cation channels). This redox regulation may serve as a mechanism to optimize channel function within the microdomains formed between cardiac mitochondria and the sarcoplasmic reticulum. Reciprocal interaction between redox modulated Ca^{2+} signaling and Ca^{2+} modulated ROS production underlies the concept of ROS and Ca^{2+} crosstalk.

1253-Pos Physical Coupling Supports Local Ca^{2+} Transfer Between SR Subdomains And The Mitochondria In Heart Muscle

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Mitochondrial Ca^{2+} signals associated with ryanodine receptor (RyR) dependent SR Ca^{2+} release are commonly supported locally by high [Ca2+] microdomains at close contacts between the organelles. Here we studied if the SR-mitochondrial interface underlying the local Ca^{2+} communication was secured via inter-organellar physical links.

Rat heart homogenates were tested for mitochondria-associated SR fragments capable for RyR-dependent Ca^{2+} mobilization and mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) signal generation. Ample presence of SR markers (calsequestrin, SERCA2a, phospholamban) was detected by western blotting of crude mitochondria, most of which disappeared after percoll purification. 'Immunomitochemistry' revealed numerous SR particles in the crude fraction co-localized with mitochondria, while failed to show SR markers in the percoll-purified fraction. Sub-fractionation of crude mitochondria on a linear sucrose density gradient following osmotic lysis and sonication resulted in fractions of outer mitochondrial membrane (OMM) devoid of inner mitochondrial membrane (IMM) and also lacking SR markers. Fractions containing both OMM and SR markers also contained IMM. Fluorescence Ca^{2+}-imaging in rhod2-loaded adherent particles revealed $[Ca^{2+}]_m$ responses to caffeine stimulation, which were prevented by Ca^{2+}-predepletion of the SR, or by inhibitors of mitochondrial Ca^{2+} uptake. Also, no concomitant rise of $[Ca^{2+}]$ occurred in the gross volume of the incubation medium, indicating local Ca^{2+} transfer between the particles. Surprisingly, even the percoll-purified 'heavy' mitochondrial fraction displayed Tg-sensitive Caffeine-induced $[Ca^{2+}]_m$ rise despite the hardly detectable SR markers.

Our data suggest that physical links support the SR-mitochondrial associations where the local Ca^{2+} transfer occurs. Mitochondria bind a relatively small fraction of the total SR and utilize domains where the OMM also binds the IMM (contact points) for the SR anchorage.

1254-Pos Isoflurane enhances buffer Ca -induced mitochondrial Ca ion flux: Role of mitochondrial Ca ion uniporter?

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Background: There is ample data suggesting that cardiac preconditioning against ischemia/reperfusion (I/R) injury is initiated within mitochondria, possibly on proteins involved in electron transport and or cation channels and exchangers. We tested if the anesthetic isoflurane directly modulates mitochondrial matrix $[Ca^{2+}]$ via the mitochondrial Ca^{2+} uniporter (CaU), which along with ROS, may participate in the triggering phase that leads to the cytosolic signaling cascade necessary to protect the mitochondrion from injury.

Methods: We examined effects of isoflurane on mitochondrial respiration, mCa^{2+} dynamics (by indo 1 AM), redox state (NADH), and membrane potential ($\Delta\Theta m$, by rhodamine 123/HRP) using spectrofluorometry in guinea pig heart isolated mitochondria energized with complex I substrate, Na-pyruvate, or complex II substrate Na-succinate in Ca^{2+} free buffer. The direct effect of isoflurane on mCa^{2+} handling was assessed by challenging mitochondria ± isoflurane (0.5–2 mM) with 0.5 to 3.0 mM $CaCl_2$ in 2.5 mM EGTA buffer (ionized $[Ca^{2+}]$ about 25–500 nM). Ruthenium red (RuR) was given to block CaU.

Results: Adding $CaCl_2$ caused step-wise increases in matrix $[Ca^{2+}]$ of 25–500 nM; this effect was blocked completely by RuR indicating Ca^{2+} uptake was through the CaU rather than a cation exchanger. Isoflurane promoted added matrix Ca^{2+} uptake (by 50–100 nM) in a dose-independent manner, while it decreased states 3/4 respiration (control index), and $\Delta\Theta m$ with no change in redox state.

Discussion: Isoflurane-induced increases in matrix Ca^{2+} uptake (cation leak) down its large electrical and chemical gradient are associated with a small decrease in $\Delta\Theta m$ and slowed respiration. Isoflurane may increase matrix Ca^{2+} influx indirectly via changes in electrochemical potential or by a direct effect on CaU proteins to enhance conductance. Increased cell Ca^{2+} is known to initiate a cascade of protective kinases leading to cardioprotection.

Endoplasmic Reticulum & Protein Trafficking

1255-Pos Characterization Of Endosomal Insulin Receptor Complexes By Mass Spectrometry

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Insulin binding to its cognate receptor at the cell surface triggers autophosphorylation of the β-subunit on regulatory tyrosine residues and rapid internalization of the complexes into the endosomal apparatus. Using highly purified hepatic endosomes, we characterized here, by mass spectrometry, IR immunocomplexes prepared at the peak time of internalization (2 min post-insulin injection). Mono-phosphorylated tyrosines residues located within the catalytic loop (Y1146,Y1150,Y1151), and the C-terminal domain (Y1316, Y1322) of the β-subunit were identified. Among the co-immunoprecipitated proteins, we identified unambiguously ATIC (5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase), an enzyme involved in the two last steps of de novo purine biosynthesis as well as the molecular adaptors grb7/grb14, coatomer subunits and VPS-1 like proteins. We con-