a heterodimeric bb'from a cyanobacterial ATP synthase exist as traditional left-handed coiled coils for most of their lengths. Upon binding of the soluble E. coli b2 to soluble F1, the tether region of the b-dimer disengages to form a more loosely packed arrangement that then seems to repack closely before entering the membrane-phase.
Using the completely assembled F1Fo-ATPase, we recently observed changes in the packing interactions of b2 during the formation of ATP-hydrolysis transition states. These transitions were observed through the release of spin label catalyzed by non-modified cysteine on the adjacent b-subunit. Only those spin-labels were released that were predicted by us to be close to or at the left-handed coiled coil b-dimer interface, while no release of label from positions that were predicted to be at the outer surface of the helices was observed. The results suggest that while modest conformational changes of the b2-dimer occur during catalysis, the dynamics of these changes do not appear large enough to support a left-handed to right-handed coiled coil conversion that has previously been suggested.

3818-Pos
Partial Reactions of the ATP Synthesis Reaction in the E. Coli βD380C Mutant F1Fo-ATP Synthase
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The initial stage of ATP synthesis by the *E. coli* wild type (WT) and mutant βD380C F_1F_o-ATPase was compared at saturating substrate concentrations and a proton-motive force (*pmf*) of ~315 mV. The enzymes catalyze both ATP hydrolysis (Baylis Scanlon et al., 2008, *J. Biol. Chem.* 283, 26228-26240) and ATP synthesis with similar steady-state parameters. ATP synthesis by the WT enzyme proceeded with neither burst nor lag-phase while ATP synthesis by βD380C (+DTT) showed a burst phase with a stoichiometry of ATP/F_1F_o equal to 1. The burst was simulated using a kinetic model in which the βD380C mutant has a less than 10-fold increase in Pi binding rate in combination with a less than 10-fold decrease in ATP release rate compared to WT. Resolution of the burst allows us to distinguish the partial reactions of the ATP synthesis pathway.
Comparison of the ATP release rates in ATP hydrolysis (Baylis Scanlon et al., 2007, *Biochemistry* 46, 8785-8797) and ATP synthesis suggest differences in the cooperative behavior in the two directions of the reaction. In ATP hydrolysis, positive cooperativity in catalysis is induced by nucleotide (ATP) only, while in ATP synthesis it is induced by both nucleotide (ADP) and the *pmf*. Fast steady-state ATP hydrolysis proceeds through a trisite mechanism, while ATP synthesis uses a bisite mechanism.
We further analyzed the effects of the mutation by forming a stator-rotor disulfide cross-link, βD380C-γC87 induced by DTNB. The cross-linked enzyme catalyzed absolutely no ATP synthesis in the millisecond time domain after inducing a *pmf*. This result suggests that γ subunit mobility is required for the ATP synthetic reaction to occur and is consistent with the model that high affinity Pi binding cannot be achieved without $\Delta\mu_H^+$-dependent γ-subunit rotation.

3819-Pos
Electric Field Driven Torque in ATP Synthase
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Dysfunction of mitochondrial enzymes, including ATP synthase, has been implicated in type-2 diabetes, cancer, heart disease, and neurodegenerative diseases. Our electric field driven torque (EFT) model of ATP synthase predicts a scaling law relating torque to the number of proton binding sites in the rotor (*c*-ring) and the proton motive force (pmf) across the mitochondrial inner membrane. When the F_O complex of ATP synthase is coupled to F_1, the model predicts a critical pmf to drive ATP production. In order to fully understand how the electric field resulting from the pmf drives the *c*-ring to rotate, it is also important to examine the charge distribution on the protonated *c*-ring and in the *a*-subunit, which contains the proton half-channels and acts as a stator. A self-consistent field approach is used in our calculations, based on a refinement of reported ac_{12} structural data. The calculations reveal changes in pKa for key residues on the *a*-subunit and *c*-ring, as well as titration curves and protonation state energy diagrams. Implications for the EFT model will be discussed. Support was provided by R21CA133153 from NHLBI and NCI at NIH and from NSF, and by grant E-1221 from the R. A. Welch Foundation. Additional support was provided by the State of Texas through the Texas Center for Superconductivity and the Norman Hackerman Advanced Research Program.

3820-Pos
Cardiac Pacemaker Cells Uniquely Match ATP Supply to Demand
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Sinoatrial node cells (SANC) within the heart's pacemaker govern the heart rhythm and contraction due to constitutively active, Ca^{2+}-activated adenylyl-cyclases (AC) that generate a high level of cAMP/protein kinase A (PKA) dependent, localized, submembrane-compartmentalized Ca^{2+}- and AC-cycling. This rhythmic Ca^{2+} cycling generates rhythmic action potentials (APs), which initiate Ca^{2+} release and SANC contraction. To drive the heart beat SANC must tightly regulate ATP production to supply sufficient ATP for cAMP production, Ca^{2+}-cycling and contraction. To explore how ATP supply-demand is tightly balanced in isolated SANC we measured ATP levels: in control; during PKA-inhibition by specific inhibitor peptide PKI (15μM) or by H-89 (6μM); during intracellular Ca^{2+}-buffering by BAPTA (25μM); during muscarinic-receptors (MR) activation by carbacol 1μM ± MR-inhibition by atropine (10μM). PKA-inhibition by PKI or H-89, which blocked the major ATP-consumption processes, depleted ATP by 45 ± 6% and 44 ± 5%, respectively. Thus, complete inhibition of these major ATP consumption processes in SANC significantly reduces ATP levels **even though** ATP consumption is reduced. Buffering intracellular Ca^{2+} depleted ATP by 54 ± 8%. Hence, Ca^{2+} not only has direct effects on the surface membrane potential to ignite APs, and to activate myofilament-displacement/force production, but also regulates ATP production. In contrast to SANC, in either stimulated (3Hz) or quiescent rabbit ventricular cells the same experimental perturbations had only minor (<6%) effects on ATP levels. These control mechanisms identified by pharmacological-perturbations are apparently utilized in nature, because MR-stimulation of SANC also depleted the ATP by 45 ± 10% (by blocking the ATP-consumption processes). Notably, atropine, a MR-antagonist, substantially reversed the MR-stimulation effect, resulting in only 18 ± 3% ATP depletion. These data suggest that in contrast to ventricular cells, the same signals that drive, and are derived from, the utilization of ATP in SANC also tightly couple the production of ATP to match energy demand.

3821-Pos
Analysis of Intracellular ADP Compartmentation Reveals Functional Coupling between Pyruvate Kinase and ATPases in Rat Cardiomyocytes
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Cardiomyocytes have intracellular diffusion restrictions, which spatially compartmentalize ADP and ATP. According to mathematical models, diffusion restrictions are localized in certain areas of the cell. However, the models so far have used data sets generated on rat heart permeabilized fibers, where diffusion distances may be heterogeneous. This is avoided when using isolated, permeabilized cardiomyocytes. The aim of this work was to analyze the intracellular diffusion of ATP and ADP in rat permeabilized cardiomyocytes. Intracellular energetic communication between mitochondria and ATPases was approached from several angles, where respiration rate, ATPase rate and ADP concentration in surrounding solution were determined under several conditions. The data was analyzed by mathematical models reflecting different levels of cell compartmentation. In agreement with previous studies, we found significant diffusion restriction by the outer mitochondrial membrane and confirmed a functional coupling between mitochondria and a fraction of ATPases in the cell. In addition, our experimental data shows that a considerable activity of endogenous pyruvate kinase (PK) remains in the cardiomyocytes after permeabilization. Intriguingly, a fraction of ATPases was inactive without ATP-feedback by this endogenous PK. When analyzing the data, we were able to reproduce the measurements only with the mathematical models that include a tight coupling between fraction of endogenous PK and ATPases. To our knowledge, this is the first time such a strong coupling of PK to ATPases has been demonstrated in permeabilized cardiomyocytes indicating the importance of glycolysis in energy production for cardiac function.

3822-Pos
Diffusion Restrictions in Cardiomyocytes from Low-Performance Heart
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In adult mammalian cardiomyocytes, intracellular diffusion restrictions affect metabolic regulation. Despite extensive studies on rat cardiomyocytes, their cause and role in vivo is still unclear. Intracellular membrane structures may play a role. Previous studies suggest that rainbow trout permeabilized

cardiac fibers also have diffusion restrictions. This is surprising because rainbow trout cardiomyocytes are thinner and have fewer intracellular membrane structures than adult rat cardiomyocytes. However, results from fibers may be affected by incomplete separation of the cells. The aim of this study was to verify the existence of diffusion restrictions in trout cardiomyocytes by comparing ADP-kinetics of mitochondrial respiration in permeabilized fibers, permeabilized isolated cardiomyocytes and isolated mitochondria from rainbow trout heart. We developed a new solution specific for trout cardiomyocytes, where they retained their shape and showed stable steady state respiration rates. The apparent ADP-affinity of permeabilized cardiomyocytes was different from that of fibers. It was higher, independent of temperature and not increased by creatine. However, it was still about ten times lower than in isolated mitochondria. This suggests that intracellular diffusion of ADP is indeed restricted in trout cardiomyocytes. The difference between fibers and cardiomyocytes suggest that results from trout cardiac fibers were affected by incomplete separation of the cells. The lack of a creatine effect indicates that trout heart lacks mitochondrial creatine kinase tightly coupled to respiration. These results from rainbow trout cardiomyocytes are similar to those from neonatal mammalian cardiomyocytes. Thus, it seems that metabolic regulation is related to cardiac performance. It is likely that rainbow trout can be used as a model animal for further studies of the localization and role of diffusion restrictions in low-performance hearts. Next step will be to identify the contribution of mitochondrial outer membrane and cytosolic factors in intracellular diffusion restriction.

3823-Pos

Novel Method for Investigation of Interactions between Mitochondrial Creatine Kinase and Adenine Nucleotide Translocase

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The aim of this study was to elaborate fluorescent labeling of mitochondrial creatine kinase (MtCK) and adenine nucleotide translocase (ANT) to investigate the mechanism of their functional coupling with Förster resonance energy transfer (FRET) technique. New alternative fluorescent labeling technique - Fluorescein Arsenical Hairpin (Flash/tetracystein) binder technology was exploited to fluorescently label MtCK. Implementation of fluorescent proteins such as GFP for MtCK fluorescent tagging was excluded because of the functional importance of MtCK C- and N-terminal part and insertion of large fluorescent protein inside the MtCK protein imposes potential risk to interfere the structure, localization and function of the fused protein. Tetracysteine motifs were introduced into five different positions in MtCK by mutagenesis. Sequentially the recombinant MtCK constructs were expressed in different eukaryotic cells lines and activity of the constructs were determined. The cells were stained with Flash labeling reagent and the expression of tetracysteine tagged MtCK mutants were visualized *ab inito* with epifluorescent and confocal microscopy. Improved variant of cyan fluroescent protein Cerulean as an appropriate FRET partner for Flash was chosen to fluorescently label ANT. Both N- and C-terminally fused ANT-Cerulean constructs were generated. ANT fusion proteins were expressed in different eukaryotic cell lines and their expression was visualized with epifluorescent and confocal microscopy. Functional constructs of MtCK and ANT-Cerulean were selected for studies of their interaction in cardiomyocytes by applying FRET technique.

3824-Pos

VDAC Phosphorylation Regulates Interaction with Tubulin

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Mitochondria and mitochondrial bioenergetics are believed to be involved in glycogen synthase kinase (GSK3β)-related cardioprotection. Recently it was suggested that cardioprotection could be achieved through the preservation of mitochondrial binding of hexokinase II (HXKII), or/and through GSK3β phosphorylation of voltage dependent anion channel (VDAC) (Pastorino et al., *Cancer Res.,* 2005; Das et al., *Circ. Res*., 2008). VDAC, the most abundant channel in the mitochondria outer membrane (MOM), is known to be responsible for most of the metabolite and ATP/ADP fluxes across MOM. Recently we have found that dimeric αβ-tubulin regulates mitochondrial respiration by directly blocking VDAC and hence, permeability of MOM for ATP/ADP (Rostovtseva et al., *PNAS*, 2008). Here, using mammalian VDAC reconstituted into planar lipid membrane, we show that tubulin-VDAC interaction appears to be very sensitive to the state of VDAC phosphorylation. When VDAC is phosphorylated *in vitro* by either GSK3β or protein kinase A (PKA), the on-rate of tubulin binding increases up to 100 times compared with untreated VDAC. Importantly, the basic properties of VDAC, such as single-channel conductance, selectivity, and voltage gating, remain almost unaltered after phosphorylation. Nonspecific alkaline phosphatase and tyrosine kinase inhibitor PP2A dephosphorylate VDAC, which results in decreased tubulin binding. Gel analysis and subsequent phospho-staining confirm that VDAC contains motifs recognized by both GSK3β and PKA. Phosphorylation causes a pronounced asymmetry of tubulin binding to VDAC. These findings allow us to point to the tentative GSK3β and PKA serine/threonine phosphorylation sites positioned on the cytosolic loops of VDAC. The results show that VDAC phosphorylation enhances tubulin-induced VDAC closure and thus could reduce MOM permeability and mitochondria respiration. We suggest that GSK3β cardioprotective effect is more complex that was initially thought because along with HXKII it involves tubulin as a potent regulator of VDAC and hence, cellular respiration.

3825-Pos

Free Tubulin and cAMP-Dependent Phosphorylation Modulate Mitochondrial Membrane Potential in Hepg2 Cells: Possible Role of VDAC

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BACKGROUND: Conductance of the voltage-dependent anion channel (VDAC) in the mitochondrial outer membrane has been proposed to limit mitochondrial metabolism in cancer cells and contribute to the Warburg effect. Since tubulin binding and phosphorylation promote VDAC closure, we hypothesized that free tubulin and cAMP-dependent phosphorylation by protein kinase A (PKA) modulate $\Delta\Psi$ in cancer cells by regulating VDAC-dependent flux of substrates into mitochondria. Our **AIM** was to modulate VDAC closure and opening in intact cells by increasing and decreasing endogenous free tubulin and by promoting and blocking PKA activation. **METHODS**: HepG2 human hepatoma cells were incubated in Hank's solution with 5% CO_2/air, and $\Delta\Psi$ was assessed by confocal microscopy of TMRM. Free and polymerized tubulin was determined using a commercial kit. **RESULTS**: Myxothiazol (10 μM), a respiratory inhibitor, caused only a slight decrease of (TMRM fluorescence), but subsequent addition of oligomycin (10 μg/ml), a F_1-F_0-ATPase inhibitor, collapsed $\Delta\Psi$ nearly completely, showing that inhibition of both respiration and ATPase are required to collapse $\Delta\Psi$. Stabilization of microtubules by paclitaxel (10 μM) increased $\Delta\Psi$ by 60%, whereas disruption by colchicine (10 μM) or nocodazol (10 μM) decreased $\Delta\Psi$ by 60-70%. Paclitaxel pretreatment prevented the depolarizing effect of colchicine and nocodazol. Dibutyryl cAMP (1 mM) decreased $\Delta\Psi$ by 45% whereas H89 (1 μM), a specific inhibitor of PKA, increased $\Delta\Psi$ by 94% and blocked the effect of dibutyryl cAMP. **CONCLUSION**: Free tubulin and cAMP/PKA-dependent phosphorylation modulate mitochondrial $\Delta\Psi$ in HepG2 cells, most likely by regulating VDAC conductance. Up and down regulation of $\Delta\Psi$ by tubulin polymerization/depolarization and PKA dependent phosphorylation/dephosphorylation is consistent with the hypothesis that VDAC is rate-limiting for mitochondrial metabolism in cancer cells and responsible, at least in part, for the Warburg effect.

3826-Pos

Hypothermic Cardioprotection Attenuates Mitochondrial Permeability Transition Pore Opening and Calcium Loading in Isolated Cardiac Mitochondria

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Ischemia-reperfusion injury (IRI) is associated with mitochondrial permeability transition pore (mPTP) opening and impaired mitochondrial respiration. Hypothermia attenuates IRI. We examined mitochondrial function in mitochondria obtained from isolated hearts subjected to warm or cold ischemia. Guinea pig isolated hearts were perfused at constant pressure with Krebs-Ringer's solution at 37°C and subjected to 30 min global ischemia at 37°C or 17°C. After 5 min of reperfusion mitochondria were isolated. Mitochondrial $[Ca^{2+}]_m$, membrane potential ($\Delta\Psi_m$), and NADH were measured by spectrophotometry at appropriate wavelengths with indo-1, BCECF, rhodamine 123 fluorescent dyes, and autofluorescence, respectively. After energizing with pyruvic acid, 0-100 μM $CaCl_2$ (0.03-60 μM free $[Ca^{2+}]_e$) was added followed by 250 μM ADP. Ca^{2+} -induced mPTP opening was assessed by collapse of $\Delta\Psi_m$. 10 μM $[Ca^{2+}]_e$ resulted in mPTP opening after 37°C IRI, but only at 35 μM $[Ca^{2+}]_e$ after 17°C IRI. ADP decreased $\Delta\Psi_m$ and NADH and increased $[Ca^{2+}]_m$ in all mitochondria, but the fall in $\Delta\Psi_m$ was greater and the responses to ADP with Ca^{2+} overloading were worse after 37°C IRI vs. 17°C IRI. The incidence of no state 4 respiration was 25% with no added