was maintained with all site II mutants, but was lost with site I mutants, demonstrating that Ca^{2+} binding to site I is sufficient to prevent E2~P formation. On the other hand, N30C-PLB cross-linked strongly to all Ca^{2+} binding site mutants, including those lacking either site I, site II, or both sites, thus demonstrating that PLB binds preferentially to E2, the Ca^{2+}-free state of SERCA2a. 10 μM Ca^{2+} blocked cross-linking of N30C-PLB to site II mutants, yielding K_{Ca} values of 1.25 ± 0.3 μM for E309Q, and 0.32 ± 0.03 μM for N795A, compared to 0.44 ± 0.04 μM for WT-SERCA2a. However, Ca^{2+} had no effect on cross-linking of N30C-PLB to SERCA2a with site I mutants, even at Ca^{2+} concentrations of 100 μM or higher. These results demonstrate that Ca^{2+} binding site I of SERCA2a is the key Ca^{2+}-binding site regulating PLB association and dissociation.

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Concerted but Noncooperative Activation of Nucleotide and Actuator Domains of the Ca-ATPase Upon Calcium Binding

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Calcium-dependent domain movements of the actuator (A) and nucleotide (N) domains of the SERCA2a isoform of the Ca-ATPase were assessed using constructs containing engineered tetracysteine binding motifs, which were expressed in insect High-Five cells and subsequently labeled with the biarsenical fluorophore 4′,5′-bis(1,3,2-dithoarsolan-2-yl)fluorescein (FlAsH-EDT_2). Maximum catalytic function is retained in microsomes isolated from High-Five cells and labeled with FlAsH-EDT_2. Distance measurements using the nucleotide analog TNP-ATP, which acts as a fluorescence resonance energy transfer (FRET) acceptor from FlAsH, identify a 2.4 Å increase in the spatial separation between the N- and A-domains induced by high-affinity calcium binding; this structural change is comparable to that observed in crystal structures. No significant distance changes occur across the N-domain between FlAsH and TNP-ATP, indicating that calcium activation induces rigid body domain movements rather than intradomain conformational changes. Calcium-dependent decreases in the fluorescence of FlAsH bound respectively to either the N- or A-domains indicate coordinated and noncooperative domain movements, where both A- and N-domains display virtually identical calcium dependencies (i.e., $K_d = 4.8 \pm 0.4$ μM). We suggest that occupancy of a single high-affinity calcium binding site induces the rearrangement of the A- and N-domains of the Ca-ATPase to form an intermediate state, which facilitates phosphoenzyme formation from ATP upon occupancy of the second high-affinity calcium site.

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FRET from SERCA to Phospholamban is Decreased by Thapsigargin and Anti-PLB Antibody, but not by Calcium

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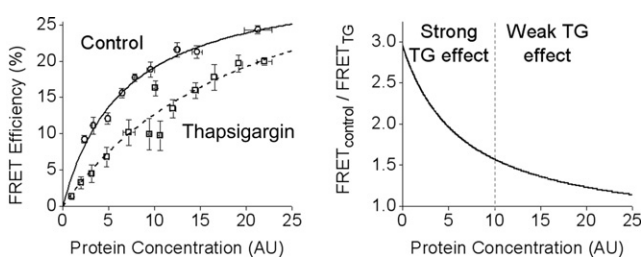
To investigate the regulation of SERCA by phospholamban (PLB) we measured FRET from CFP-SERCA to YFP-PLB. In permeabilized cells, anti-PLB antibody significantly decreased SERCA-PLB FRET consistent with other groups' chemical crosslinking, immunoprecipitation (IP), and functional assays. However, FRET was not abolished by millimolar calcium, suggesting that PLB still interacts with calcium-bound pump. This result contrasts with crosslinking and IP results, but is in harmony with another lab's in vitro FRET studies. In intact cells, SERCA-PLB FRET was decreased by thapsigargin (TG). This observation is compatible with other studies that reported a loss of PLB-SERCA crosslinking with TG but appears inconsistent with reported IP experiments. We measured FRET in a heterogeneous population of cells displaying a wide range of protein concentrations. We observed a decrease in the apparent affinity of PLB for SERCA in the presence of TG. Thus, PLB-SERCA binding was decreased by TG in cells expressing a low protein concentration, but the interaction persisted at high protein concentration. The present results may help reconcile contrasting results reported in the literature and enhance our understanding of the regulation of SERCA by PLB.



739-Pos Board B618

Collapse of TA-Calmodulin (TACaM) upon Binding to Ca^{2+} Pump Peptide C28 Exposes the TA Moiety to Water and Quenches Its Fluorescence

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CaM labeled with a fluorescent triazinylaniline (TA) derivative at Lys-75 shows 2 species upon binding to PMCA or to the CaM-binding peptide from PMCA (C28). The 1st, transient, species is slightly more fluorescent than the free CaM, while the 2nd, stable, species is much less fluorescent. The 1st species can also be emulated in a stable form by binding TA-CaM to a shorter peptide, C20. The fluorescence of TA derivatives is quenched and red-shifted by polarizable solvents such as water. $TANMe_2$ has an emission maximum of 391 nm in a non-polar solvent (toluene), which is red-shifted to 419 nm in ethanol (permittivity = 24.5). The emission maxima of TACaM-C20 and TACaM-C28 are 409 nm and 421 nm respectively. Using the Lippert equation, we find that the effective permittivity that TA sees in TACaM-C20 is about 5 and in TACaM-C28 is about 30. Structures of TACaM-C20 (based on 1CFF) and TACaM-C28 (based on our new NMR data on CaM-C28) were made. The C20 complex has the TA residue surrounded by the extended CaM molecule, in an environment containing relatively little water. In the C28 complex the CaM molecule is collapsed. The surroundings of the TA residue are calculated from these molecular structures of hydrated TA-CaM, and the results are comparable with the experimental fluorescence data. (Supported by grants TW06837 and NS51769 from the NIH)

740-Pos Board B619

Distinct Regulation of pH in the Cytosol and in Acidic Organelles by a Subunit Isoforms of V-ATPase in Human Cancer Cells

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V-ATPases are expressed at the plasma membrane (pmV-ATPases) in highly metastatic cells, in addition to their typical distribution in acidic organelles [endosomes/lysosomes (E/L)]. Distinct a subunit isoforms of V-ATPase target the V-ATPase to different cellular membranes. There are 4 a subunit isoforms (a1, a2, a3, and a4). The a3 and a4 isoforms are found at the plasma membrane in osteoclasts and renal intercalated cells, respectively. We employed isoform-specific siRNA to selectively reduced the mRNA levels of each isoform in highly metastatic human melanoma (C8161) and breast (MB231) cancer cells. Inhibition of V-ATPase with concanamycin decreased in vitro cell invasion. Knockdown of either a3 or a4 also inhibits cell invasion. Simultaneous measurements of pH in the cytosol (pH^{cyt}) and in E/L ($pH^{E/L}$) using pH fluoroprobes targeted to the cytosol or E/L indicated that in C8161 cells, the steady state pH^{cyt} was more acidic in cells transduced with either siRNA-a3 or -a4. Knockdown of a3 in MB231 decreased pH^{cyt}, whereas siRNA-a1, -a2 and -a4 did not affect pH^{cyt}. The $pH^{E/L}$ was more alkaline by knockdown of either a1, a2, or a3 in MB231, whereas in C8161 the $pH^{E/L}$ was increased by siRNA-a1 or -a2. The proton fluxes following an acid load were significantly decreased by knockdown of a1, a2, and a3 in MB231 cells. These data suggest that specific a subunits of V-ATPase control pH in E/L and the cytosol in highly metastatic cells; and that a3 and a4 are significant for pH regulation across the plasma membrane, whereas a1, a2 and a3 are important for $pH^{E/L}$ regulation. These data emphasize the significance of a3 and a4 for the acquisition of an invasive phenotype in metastatic cells.

741-Pos Board B620

Direct Observation Of Rotation Of F1-Atpase From Saccharomyces cerevisiae With mgi Mutations

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Mitochondrial Genome Instability (*mgi*) mutations allow yeast to survive the loss of mitochondrial DNA. A number of these mutations occur in the genes encoding the F_1 portion of the ATP Synthase, and have been shown to uncouple ATP Synthase (Wang et al. 2007). The mutations cluster around the collar region of F_1 where the alpha, beta and gamma subunits interact and are thus likely to affect the kinetics of F_1 rotation.

Single molecule studies of the thermophilic *Bacillus* PS3 F_1-ATPase have revealed kinetic and structural information that cannot be discerned using other