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Detection of Sarcolipin Dimerization and SERCA Binding using FRET Microscopy

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We have monitored molecular interactions of sarcolipin (SLN) and the sarcoplasmic reticulum Ca-ATPase (SERCA) by measuring Förster resonance energy transfer (FRET) between fusion proteins labeled with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). SLN regulates contractility in cardiac and skeletal muscle by regulating SERCA calcium uptake and perhaps by acting as an ion channel. Here, live cell FRET microscopy provided novel evidence for self-association of SLN and for regulatory complex formation between SERCA and SLN. Fluorescence photobleaching revealed that SLN self-assembles into dimers but not higher oligomers, and that SERCA and SLN monomers bind together in a 1:1 binary complex. FRET between CFP-SLN:YFP-SLN and CFP-SERCA:YFP-SLN exhibited a hyperbolic dependence on protein concentration, with maximum efficiency of 62 ± 1% and 52 ± 1%, respectively. The concentration of half-maximal FRET was 7.6 ± 0.4 AU for SLN:SLN and 17.0 ± 1.0 AU for SERCA:SLN, demonstrating that the binding affinity of SLN:SLN homo-dimers is 2.2-fold greater than SERCA:SLN hetero-dimers. Mutating SLN residue Ile-17 to Ala (I17A) decreased maximum FRET for SERCA:SLN by 14 ± 4% with no change in binding affinity, indicating that the I17A-SLN mutation causes a structural rearrangement with distance increase of 5 ± 1 Å within the binary regulatory complex. Addition of wild-type SERCA (cardiac and fast-twitch isoforms) decreased the binding affinity of the SLN:SLN interaction by 24 ± 6% with no change in maximum FRET, indicating that SLN monomers are in competition between dimerization and SERCA binding. We propose that SLN exists as monomers, homo-dimers, and hetero-dimers in sarcoplasmic reticulum membranes, and that Ile-17 of SLN acts as an allosteric switch in the regulatory complex with SERCA.

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Impairment of PMCA Activity by Amyloid β-Peptide in Membranes from Alzheimer's Disease-Affected Brain and from Other Model Systems

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High-affinity Ca^{2+}-transport ATPases are key components in the machinery of cytosolic Ca^{2+} regulation. We have found that the Ca^{2+}-dependence of plasma membrane Ca^{2+}-ATPase (PMCA) activity is constant between pCa values of 6.5 and 4.5 in membranes from Alzheimer's disease (AD) affected-brain whereas in normal brain the activity increases from pCa 6.5 to 5.5 and then decreases from 5.5 to 4.5. However, the Ca^{2+}-dependencies of sarco(endo)plasmic- (SERCA) and secretory pathway- (SPCA) Ca^{2+}-ATPases activity are similar in AD and normal brains. Addition of amyloid β-peptide (Aβ) to normal brain decreases the activity of PMCA at pCa 5.5, resulting in the same Ca^{2+}-dependence as that seen in AD brain, whereas addition of Aβ to AD membranes has no effect on PMCA activity. Aβ also decreases the activity of PMCA purified from pig cerebrum, the effect being isoform specific, with greatest inhibition for PMCA4, smaller inhibition for PMCA3 and no inhibition for PMCA2. The effect of peptide on PMCA was also seen in primary culture of mouse hippocampal neurons, and the lack of effect on the other pumps was seen on SERCA and SPCA from cultured neurons and on SERCA1 from rabbit skeletal muscle and on SERCA2a and SERCA2b overexpressed in COS cells. Besides, cholesterol blocked the inhibitory effect of Aβ on PMCA in different model systems and in the raft fraction of pig synaptosomal membranes. From these data we can conclude that impairment of PMCA functioning with respect to Ca^{2+} is closely linked to the presence of amyloid-β peptide in AD brain.

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HNO Uncouples PLN from SERCA2a Enhancing Pump Activity

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The sarco(endo)plasmic Ca-ATPase (SERCA2a) sequesters Ca^{2+} ions from the cytosol into the cardiac sarcoplasmic reticulum (CSR) to promote cardiac relaxation. Phospholamban (PLN) is an integral membrane protein that regulates SERCA2a in CSR. Phosphorylation of PLN in response to β-adrenergic stimulation enhances cardiac inotropy by increasing CSR Ca^{2+} uptake. Nitroxyl (HNO), a new candidate drug therapy for congestive heart failure (CHF), improves overall cardiovascular function by increasing Ca^{2+} release and Ca^{2+} re-uptake in CSR through direct effects on RyR2 and SERCA2a, respectively. We propose that HNO, a thiol oxidant, modifies one or more PLN sulfhydryls and thereby decreases the regulatory interaction of PLN with SERCA2a, contributing to SERCA2a activation. To test this model, we have used fluorescence and EPR spectroscopy to determine the effects of HNO on the physical regulation of SERCA2a by PLN. We used expressed SERCA2a ± coexpressed PLN in High Five insect cells microsomes and Angeli's Salt (AS) as our HNO donor. Steady-state fluorescence intensity studies of FITC-labeled SERCA2a coexpressed with PLN showed that AS/HNO increased the amplitude of the Ca^{2+}-dependent E2 to $E1Ca_2$ conformational change by nearly two fold, similar to the effect observed when PLN is uncoupled from SERCA2a by anti-PLN antibody treatment. This matches our previous finding that AS/HNO stimulates SERCA2a activity nearly two fold. Saturation-transfer EPR studies of maleimide spin-labeled SERCA2a coexpressed with PLN showed that AS/HNO decreased the rotational mobility of SERCA2a, but not significantly more than that of control SERCA2a without PLN. Taken together, our results suggest HNO physically uncouples PLN from SERCA2a increasing SERCA2a conformational flexibility and SERCA2a activity. We are investigating the role of individual PLN cysteines in this mechanism by repeating these experiments using SERCA2a coexpressed with PLN cysteine mutants, and direct effects of HNO on SERCA2a are also being investigated.

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Oligomeric Interactions in Cardiac Calcium Regulation

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We used time-resolved fluorescence resonance energy transfer (TR-FRET) to determine the oligomeric state of phospholamban (PLB) and its inhibited target, sarcoplasmic reticulum Ca-ATPase (SERCA). Previous work on our lab has shown that PLB is primarily pentameric but SERCA binds preferentially to the monomeric form in lipid vesicles. Recent EM studies suggest that the PLB pentamer might also bind to SERCA. We tested this hypothesis by labeling SERCA at C674 with a fluorescent donor (TMRIA) and labeling PLB at K3 with a non-fluorescent acceptor (MGITC), then reconstituting the proteins into lipid vesicles and performing TR-FRET as a function of the fraction of acceptor-labeled PLB (x_A), keeping the total PLB/SERCA molar ratio constant at 10. Simulations showed that if a PLB monomer binds to SERCA, the dependence of FRET on x_A should be linear, but the binding of a PLB oligomer should produce distinct curvature in the plot. The observed plot was quite linear, and was indistinguishable from control experiments using a monomeric mutant of PLB. We conclude that PLB binds to SERCA only as a monomer. These results have important implications for the design of PLB mutants to be used in gene therapy for heart failure.

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Probing the Mechanism of SERCA-PLB Regulation by Time-Resolved Fret

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We are using solid-phase peptide synthesis, membrane reconstitution, an enzyme-coupled Ca-ATPase activity assay, and time-resolved fluorescence resonance energy transfer (TR-FRET) to investigate the molecular mechanism by which the cardiac Ca-ATPase (SERCA) is regulated by phospholamban (PLB). In human heart failure, SERCA activity is inadequate, and current therapeutic research focuses on the goal of increasing SERCA activity by reducing PLB inhibition of SERCA. PLB inhibition is relieved by $[Ca^{2+}] > \mu M$ or by phosphorylation of S16 by PKA. It has been proposed that relief of this inhibition requires dissociation of the SERCA-PLB complex. To test this hypothesis, we have designed and synthesized monomeric PLB variants with a FRET acceptor (DABCYL), with and without phosphorylation at S16, and then reconstituted them with SERCA labeled with a FRET donor (IAEDANS). After reconstitution, the interactions of these PLB variants with SERCA were characterized both functionally (Ca-ATPase activity) and physically (TR-FRET), as affected by Ca^{2+} and PLB phosphorylation. We found that Ca^{2+} completely relieves SERCA inhibition, while phosphorylation partially relieves SERCA inhibition. We also found that Ca^{2+} and phosphorylation have slight but opposite effects on FRET. Time resolution provided independent measurements of protein association and structure. We conclude that inhibition of SERCA is relieved by structural rearrangement within the SERCA-PLB complex, without dissociation of PLB from SERCA, with distinct mechanisms for relief by Ca^{2+} and PLB phosphorylation.