Hayashi and coworkers (2008, Biochemistry, 47, 6039), nicely, isolated soluble Tetraprotomer (T), Diprotomer (D) and Protomer (P) from C12E8-solubilized Na/K-ATPase and showed that Na/K-ATPase activity of T was around 50% of those of D, P and membrane bound enzyme. Each P, D and T bound ATP in the presence of excess EDTA with similar high affinity as the membrane bound enzyme. The binding of ATP and ouabain to T was 50-70% of those of P and D (Hayashi et al., unpublished data). Each retention time for T, D and P accompanying Mg2+-Na+-dependent ATP hydrolysis with or without K+ and that in the absence of ATP was little affected. We measured the amount of EATP and EP accompanying Mg2+-Na+-dependent ATP hydrolysis during the gel filtration in the presence of 12 micro M of [^{32}P]ATP, which concentration nearly saturates EP formation in membrane bound enzyme but not EATP formation. The amount of EATP and EP in T, respectively, was 0.43 ± 0.01 (n=3) and 0.04-0.15 mol/protomer. Those in D and P, which were almost the same as each other, were 0.43 ± 0.04 (n=6) and 0.09-0.21 mol/protomer, respectively. Data suggest some enzymological property may change in the presence of C12E8 or/and by solubilization.

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Structure-Based Design of Phospholamban Mutants for Gene Therapy

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We are using structural biology, molecular biology, physiology, gene delivery and preclinical testing to develop an optimized gene-therapy approach for the treatment of heart failure (HF). A key abnormality in HF is defective function of sarcoplasmic reticulum (SR). Deficient Ca^{2+} uptake during relaxation, associated with a decrease in activity of SR Ca^{2+}-ATPase (SERCA2a), has been identified in failing hearts from both humans and animal models. SERCA activity is regulated by phospholamban (PLB), a small membrane protein that partially inhibits SERCA in the absence of β-adrenergic phosphorylation of PLB. Our approach combining structure-function analysis of PLB and the direct detection of PLB-SERCA interactions relies on (a) fluorescence to show that SERCA inhibition can be relieved without dissociation of PLB, (b) magnetic resonance to define the structural dynamics of PLB's functional interaction with SERCA, and (c) establishing structure-based computational design principles to produce PLB mutants (PLB_M) binding tightly to SERCA but having decreased inhibitory potency. We have obtained evidence that non-inhibitory PLB mutants S16E or L31A are capable of competing with WT-PLB to reverse SERCA inhibition. We are currently testing the physical and functional interaction between SERCA and optimized PLB_M. Based on positive in vitro results, rAAV is used to test PLB_M in rodent and porcine HF models for *in vivo* efficacy.

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Nitroxyl and "Forbidden Disulfides": Phospholamban Cysteines are Targeted to Enhance SERCA2a Activity

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Our enjoyable collaboration with Dr. Jeffrey P. Froehlich focused on the role of phospholamban (PLN) in HNO-induced enhancement of SR Ca^{2+}-ATPase (SERCA2a) activity. We hypothesized that given HNO thiophilic nature it modifies cysteine residues in PLN transmembrane domain, altering its interaction with SERCA2a, thus enhancing pump activity. When HNO, donated by Angeli's salt (AS), was administered to control isolated myocytes, it enhanced both sarcomere shortening and Ca^{2+} transient. However, when AS/HNO was applied to $PLN^{-/-}$ ventriculocytes, HNO inotropy was reduced by ≅ 50% (the remaining likely stemming from enhanced myofilament sensitivity to Ca^{2+}). PLN centrality to HNO cardiotropic action was confirmed incubating SR vesicles from WT and $PLN^{-/-}$ mice with AS/HNO to measure ATP-dependent Ca^{2+} uptake by stopped-flow mixing. In WT, HNO increased Ca^{2+} uptake rate, but it failed to do so in $PLN^{-/-}$ vesicles. The role of cysteines in PLN emerged from studies using ER microsomes from Sf21 insect cells expressing SERCA2a ± PLN (WT or Cys 36-41-46 → Ala mutant) where we assessed SERCA2a dephosphorylation, a measure of E_2P hydrolysis, i.e. a rate-limiting step of SERCA2a activity. AS/HNO augmented SERCA2a dephosphorylation in ER microsomes co-expressing SERCA2a and WT PLN, but this stimulation was absent in microsomes expressing SERCA2a and Cys 36-41-46 → Ala mutant PLN. Thus, Jeff's elegant, creative and passionate approach helped us to show that PLN is essential for HNO-induced faster Ca^{2+} uptake by SERCA2a, suggesting that HNO action occurs, at least partly, via modifications of critical cysteines in PLN transmembrane domain. In Jeff's view, ''forbidden'' disulfide bonds in PLN are involved, and one of his legacies for us is unearthing the cysteine pairs that are involved in HNO-induced formation of an intramolecular bond that could distort the conformation of PLN, thus perturbing its interaction with SERCA2a and relieving the inhibition.

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Phospholabman Regulation of SERCA2a Kinetics, as Modulated by Nitroxyl

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Activation of cardiac muscle sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) by $_1$-agonists involves cAMP- and PKA-dependent phosphorylation of phospholamban (PLB). Activation increases both the apparent Ca^{2+} affinity and the V_{max} of SERCA2a, requiring a sustained input of free energy during cycling. In collaborative work with Jeffrey Froehlich, MD, and others, we investigated this process by comparing the kinetic and spectroscopic properties of SERCA2a expressed with and without PLB in High Five insect cell microsomes to those of SERCA1 and SERCA2a in native skeletal and cardiac muscle SR. Our data suggests PLB modifies the state of oligomerization of SERCA2a, which in the presence of unphosphorylated, inhibitory PLB is predominantly monomeric. The absence of PLB allows SERCA2a molecules to reorient and interact conformationally, resulting in catalytic behavior characteristic of SERCA1 in skeletal SR, which has no PLB. We propose that intermolecular conformational coupling of SERCA2a units increases the catalytic efficiency of the Ca^{2+} pump (raises V_{max}) and may contribute to activation of the cardiac SR Ca^{2+} pump induced by $_1$-adrenergic agonists. We have recently applied these insights to the mechanism of SERCA2a stimulation by nitroxyl, HNO, which we propose uncouples PLB from SERCA2a by modification of one or more PLB cysteine residues. Dr. Froehlich's kinetics studies indicated the HNO-treated SERCA2a +PLB sample has the same kinetic properties as SERCA2a in the absence of PLB. SERCA2a coexpressed with a null-cysteine PLB construct showed no change in kinetic properties following treatment with HNO. Our ongoing fluorescence and electron paramagnetic resonance spectroscopic experiments agree, showing that HNO-treated SERCA+PLB samples have physical properties similar to SERCA2a without PLB. Our results support the conclusion that HNO-treatment, at least in part, uncouples PLB from SERCA2a, leading to HNO-stimulated SERCA2a activation. Direct effects of HNO on SERCA2a are also under investigation.

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Lost in Translation: Regulation of Na Pump Subunit Abundance

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The Na pump is an αβ heterodimer responsible for maintaining fluid and electrolyte homeostasis in mammalian cells. We engineered MDCK cell lines expressing α1flag-, β1flag-, or β2myc-subunits via a tetracycline (tet)-regulated promoter and a cell line expressing both stable β1myc and tet-regulated β1flag in order to examine regulatory mechanisms of Na pump subunit expression. Overexpression of β1flag increases total β-subunit levels by greater than 200% without changes in α-subunit abundance, however, endogenous β1-subunit (β1E) abundance is decreased. β1E down-regulation does not occur during β2 overexpression. The decrease in β-subunit expression is not accompanied by any change in mRNA levels. In addition, the degradation rate of β-subunits is not altered by β1flag overexpression. Cells stably expressing β1myc, when induced to express β1flag-subunits show reduced β1myc- and β1E-subunit abundance, indicating that these effects occur via the coding sequences of the down-regulated polypeptides. Similarly, MDCK cells over-expressing α1flag-subunits exhibit a reduction of endogenous α1 (α1E) protein with no change in α mRNA levels or β-subunits. The reduction in α1E compensates for α1flag-subunit expression, resulting in unchanged total α-subunit abundance. Thus, regulation of α-subunit expression maintains its native level whereas β-subunit is not as tightly regulated and its abundance can increase substantially over native levels. These effects are also seen in HEK cells. This is the first indication that cellular Na pump subunit abundance is modulated by translational repression. We will discuss the mechanism of this novel, potentially important mode of Na pump regulation.