Additionally, the photon-counting nature of the detector allows spatial and temporal correlations to be obtained very easily. Finally, the detector contains an integrated TCSPC electronics that makes it a perfect tool for fluorescence lifetime imaging. This and other types of detectors currently in development will transform the way single-molecule imaging and spectroscopy experiments are designed and performed.

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3963-Plat
Light Sheet Microscopy Optimized for Depth Penetration to Study Embryogenesis
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Fluorescence light sheet microscopy (FLSM) has gained widespread recognition in recent years, due to its distinct advantages for the 3-dimensional (3D) imaging of living biological samples. FLSM uses a planar sheet of light to illuminate a sample, generating fluorescence over an optical section of the sample that is collected by a wide-field microscope camera oriented orthogonal to the light sheet. The orthogonal geometry between the illumination and detection pathways enables massive parallelization in both illumination and detection; futhermore, it permits optical and physical access to samples (3D cell cultures or whole embryos) in ways that are impossible in the collinear geometry of standard microscopes. Because of these features, FLSM significantly outperforms standard laser-scanning confocal microscopy in imaging speed, phototoxicity, and signal to noise in many imaging applications. An important aspect of any 3D imaging technique is its imaging depth limit (how deep into a sample useful information can be collected). In this respect, standard FLSM fares only slightly better than confocal microscopy. To overcome this hurdle, we have optimized FLSM for imaging of live thick samples by minimizing the degradation of the light sheet due to scattering, while preserving acceptable axial resolution. Using this approach we have imaged whole, live fruit fly embryos and zebrafish embryos. We achieve higher depth penetration than standard FLSM, while maintaining sub-cellular resolution, at imaging speed of about ten times faster than standard confocal microscopy.

Platform BJ: Member-Organized Session: Kinetics, Mechanisms & Regulation of Ion-Transporting ATPases

3964-Plat
Characterization of Partial Reactions in the Catalytic Cycles of Calcium and Copper ATPases
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Calcium and copper P-type ATPases transduce ATP chemical energy to transmembrane osmotic energy, using a catalytic mechanism common to haloacid dehalogenases where phosphoryl transfer from substrate to a conserved aspartate yields a phosphoenzyme intermediate before hydrolytic cleavage of Pi. Favored by its native abundance, characterization of the calcium ATPase is by now quite detailed, including two transmembrane calcium binding sites, a phosphorylation site within the headpiece, a conserved TGES motif for catalytic assistance of hydrolytic cleavage, and domain movements permitting long range linkage of phosphorylation and calcium binding sites. Presently, mutational analysis is still yielding further details on the calcium ATPase. As for bacterial (CopA) and human (ATP7B) copper ATPases, due to low native abundance, heterologous expression in cultured cells is required. In addition to domains present in other P-type ATPases, copper ATPase sequences include an amino terminus extension (NMBD) with one (CopA) or six (ATP7B) copper binding sites. Using recombinant protein, it is possible to demonstrate formation of phosphoenzyme intermediate by utilization of ATP, undergoing rather slow turnover. In analogy to the calcium ATPase, phosphoenzyme intermediate is not formed following mutation of the conserved aspartate or the transmembrane copper site. In addition to the phosphorylated ATPase intermediate, copper dependent phosphorylation of various serine residues occurs in ATP7B. Interference with protein autophosphorylation, both of aspartate and serines, is observed following mutation of a histidine residue in the nucleotide binding domain, a mutation found in Wilson disease of humans. The effects of NMBD copper site mutations and deletions suggest that the NMBD sequence is involved in catalytic regulation as well as protein targeting. (Supported by 5 R01 HL069830-08).

3965-Plat
The Involvement of Protein-Protein Interactions in the Mechanism of the Na^+,K^+-ATPase
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The Na^+,K^+-ATPase (or sodium pump) was the first ion pump to be discovered (Skou, 1957) and it is one of the most fundametally important enzymes of animal physiology. The electrochemical potential for Na^+, which the enzyme maintains, is used as the driving force for numerous secondary transport systems, e.g. voltage-sensitive Na^+ channels in nerve. ATP provides the energy source to drive ion pumping. However, it also plays a crucial allosteric role, accelerating significantly the enzyme's rate determining E2-E1 transition and the associated release of K^+ ions to the cytoplasm. Based on the results of stopped-flow kinetic experiments and recently published crystal structural data for the related enzyme, the sarcoplasmic reticulum Ca^{2+}-ATPase, it is suggested that the allosteric role of ATP in the mechanism of the Na^+,K^+-ATPase can be explained by an ATP-induced closing of the cytoplasmic domains of the enzyme which relieves steric hindrance arising from interactions between neighbouring pump molecules within the native membrane environment and hence an acceleration of the E2-E1 conformational change (Clarke, 2009). In the presence of millimolar concentrations of ATP, therefore, it is proposed that the enzyme functions as a monomer (alpha-beta protomer), whereas at low ATP concentrations it functions as a dimer ($(alpha\text{-}beta)_2$ diprotomer) or higher aggregate. The physiological advantage of protein-protein interactions is still unclear, but a possibility is that they may lead to an enhancement of the enzyme's ATP affinity and allow it to continue functioning even under hypoxic conditions.

Skou JC. (1957) *Biochimica et Biophysica Acta*, **23**: 394-401.
Clarke RJ (2009) *European Biophysics Journal*, in press.

3966-Plat
The Na/K-ATPase/Src Interaction and the E1/E2 Transition
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In our previous studies, we have shown that Na^+/K^+-ATPase interacts directly with Src to form a signaling receptor complex. This complex is involved in control of basal Src activity and ouabain-induced signal transduction. The aim of this work is to demonstrate that Na^+/K^+-ATPase also regulates Src activity through its conformation-dependent domain movements during pumping cycles. It is known that the Na^+/K^+-ATPase transits from E1 to E2 conformation during an ion pumping cycle. Based on the known crystal structures of Na/K-ATPase, this conformational transition results in a 110 degree turn of the A domain of Na/K-ATPase where Src SH2 domain binds. Computational modeling suggests that the movement of A domain during the pump cycle is likely to release Src from the Na/K-ATPase, resulting in the activation of Src kinase. Indeed, in vitro kinase assays show that while stabilization of Na/K-ATPase by N-ethylmaleimide and AMPPNP in E1P conformation keeps Src in an inactive state, converting the pump into an E2P state by fluoride compounds stimulates Src. Consistently, the Na^+-liganded E1 form of Na^+/K^+-ATPase inhibits Src whereas the K^+-liganded E2 form releases Src from the Na^+/K^+-ATPase and re-activated Src. Finally, Src is completely inactivated by the Na/K-ATPase in the presence of physiological concentrations of Na^+ (150 mM) and K^+ (5 mM). Reduction of K^+ results in an accumulation of E2P Na/K-ATPase and consequently an increase in active Src. Taken together, these new findings suggest that the Na^+/K^+-ATPase/Src complex may function as a pumping receptor, capable of coordinating ion pumping at the plasma membrane and other cellular activities by activating/inhibiting Src kinase.

3967-Plat
Oligomer Structure Detected in Na/K- and H/K-ATPase
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Since Repke's proposal of Flip-Flop model of Na/K-ATPase in 1973, Askari, Schoner, Hayashi, Taniguchi, Froehlich, Clarke and their coworkers have indicated the oligomericity of the enzyme from reactivity to various ligand (2006, J. Biochem. 140, 599 and also see review 2001, J.Biochem.129, 335). One of the most compelling pieces of evidence in favor of the oligomeric nature of Na/K-ATPase and gastric H/K-ATPase is the simultaneous presence of EP:EATP with half site phosphorylation and nearly half site ATP binding (1999, J. Biol. Chem.274,31792, 2002, Biochemistry 41, 2438). Recently

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.

3968-Plat

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.

3969-Plat

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.

3970-Plat

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.

3971-Plat

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.