bridges. Therefore the TnTm unit remains open even at moderate Ca^{2+} concentrations. In contrast, flexible Tm chain prevents reattachment of the cross-bridges by partially covering actin sites within a TnTm unit. For the same reason only the flexible chain model predicted the twitch dynamics. In a multiple sarcomere model we tested the hypothesis that the observed heterogeneity of shortening of individual sarcomeres is the principal mechanism causing rapid decrease in overall force upon sudden decrease of Ca^{2+} concentrations. This model somewhat improved the relaxation predicted by rigid TnTm unit models, but only within intermediate range Ca^{2+} concentrations.

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693-Pos Spanning Length And Time Scales Of Muscle Activation With FRET, MD, And Modeling

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Board B537

We approach the dynamics of allosteric change experimentally to resolve which allosteric mechanism—induced-fit and population-shift-stabilization—is employed in the regulatory switch of cardiac muscle. The cardiac Ca-regulatory switch (CRS), the Ca-sensitive binary complex of troponin C (TnC) and troponin I (TnI), is a prototype allosteric signaling network (ASN)—a semi-stable protein assembly that communicates information through coupled intra- and inter-protein domain movements. Most cell biological information processing is performed by ASN. Familiar examples of unbranched ASN include the family of G-protein coupled receptors, ligand gated ion channels, and the ribosome. The relative simplicity of unbranched ASN, compared to branched networks that integrate multiple inputs, make them useful for discovering *emergent properties* in modular hierarchical structure. We show how ASN are useful for discovering *emergent design* issues in modular hierarchical structure.

An engineered intra-molecular FRET pair is used to follow the central structural rearrangement in the CRS. Time resolved FRET measurements indicate that activation of TnC is insensitive to the ordering of Ca / TnI addition. This commutivity is exploited in stopped flow FRET experiments, which are interpreted using a quantitative phenomenology of allostric signaling to reveal that CRS activation proceeds through an induced-fit mechanism. When saturated with Ca, the CRS (15 C) exists as a rapidly inter-converting 3:2 mixture of open (active) and closed (inactive) conformers. Time resolved FRET, transient FRET, and Ca-titration FRET measurements are globally analyzed to resolve the system configurational phase state free-energy landscape (SPEL)—a systems-level partition function—of the regulatory switch. In molecular dynamics (MD) simulations with probes incorporated into structural models derived from NMR and x-ray crystallographic data, we demonstrate consistency between the measured FRET "optical distance" and molecular structure.

Ion Motive ATPases

694-Pos Expression Pattern of Fluorescently Tagged Na pump in *C. elegans.*

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Board B538

The Na,K-ATPase is a transmembrane protein that is responsible for actively transporting $3Na^+$ (out) and $2K^+$ (in) of most eukaryotic cells. The subsequent Na^+ gradient plays a vital role in cell excitability, contractility, and osmotic balance. Na pump physiology is most evident within polarized epithelium where it drives the directional uptake of nutrients from lumen to blood. In order to maintain this unidirectional absorption, Na,K-ATPase expression is restricted to the basal-lateral membrane in epithelial cells. In order to investigate the membrane delivery of Na,K-ATPase further we have constructed fluorescently tagged alpha and beta subunits for expression in *C. elegans.* Two separate plasmids were constructed for injection into *C. elegans;* the α-subunit was constructed with YFP fused to the C-terminus and the β-subunit was constructed with CFP fused to the N-terminus. Both genes are under control of the heat shock promoter, hsp16–41 promoter. The α-YFP plasmid was injected with the cotransformation marker rol-6, whereas the CFP-β plasmid was injected with the lin-15 gene into the syncytial gonad of adults. Transgenic animals were isolated if they displayed the respective contrasformation marker phenotype. In order to view β-subunit expression, CFP-β transgenic worms were heat shocked at 33°C for 2 hours on non pre-warmed plates, then set at 20°C for 4 hours. CFP-β was expressed throughout the intestine of the worm. α-YFP fusion protein has also been viewed within *C. elegans.* α-YFP fluorescence was observed in pharynx, vulva, body-wall muscle cells, nerve cells and throughout the intestine. We are currently characterizing the heat shock time-course and tissue expression intensities of both constructs.

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695-Pos Interaction between Nitric-oxide Synthase Pathways and NAD(P)H Oxidase Pathways Leading to Modulation of the Na^+-K^+ Pump Activity

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Board B539

We have reported that receptor-coupled NADPH oxidase mediates sarcolemmal Na^+-K^+ pump inhibition while receptor-coupled nitric oxide synthase (NOS) mediates stimulation. NOS stimulated the pump via NO-activated guanylyl cyclase (GC), cGMP-activated protein kinase, and okadaic acid-sensitive phosphatase. Interaction between the pathways may fine-tune pump activity. In contrast to the $p47^{phox}$ subunit of NADPH oxidase, interaction at the level of the pump itself is unlikely because its subunits have no readily accessible phosphorylation/dephosphorylation sites. We hypothesised that the NO-activated pathway regulates NADPH oxidase-mediated Na^+-K^+ pump inhibition. We loaded rabbit ventricular myocytes with the O2–sensitive fluorescent dye, dihydroethidium (DHE), and measured fluorescence with confocal microscopy. NADPH oxidase activation with 100nM of the known upstream activator, angiotensinII (AngII), increased DHE fluorescence by 48% compared with controls ($N=4$, $P<0.05$). The increase was abolished with pegylated "cytosolic" superoxide dismutase. Since NO is expected to quench the O2.- signal, we exposed myocytes to 10μM YC-1 to selectively activate GC. YC-1 abolished the Ang II-induced increase in DHE fluorescence ($N=6$, $P<0.05$). However, the AngII-dependent increase in fluorescence was restored in myocytes exposed to both YC-1 and 10nM okadaic acid ($P<0.05$), implicating protein phosphatase 2A. To obtain functional Na^+-K^+ pump correlates we voltage clamped and internally perfused myocytes and measured electrogenic pump current (Ip, normalized for membrane capacitance) as the ouabain-induced (100μM) shift in holding current. Pipette solutions perfusing the intracellular compartment included 10mM Na^+. Mean Ip of 0.50 ± 0.09 pA/pF ($N=7$) was reduced to 0.35± 0.13 pA/pF ($N=10$, $P<0.05$) by 100nM Ang II. YC-1 in the pipette solution restored it to 0.51±0.19 pA/pF ($P<0.05$, $N=12$).

We conclude that NOS-coupled pathways attenuates AngII-coupled NADPH oxidase activation and the Na^+-K^+ pump inhibition associated with it, and that such redox-signalling may fine-tune pump activity.

696-Pos The α1 and α2 Isoforms of the Na/K-ATPase are Equally Affected by β-Adrenergic Stimulation in Mouse Ventricular Myocytes

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Board B540

Cardiac Na/K-ATPase (NKA) regulates intracellular Na, which in turn affects intracellular Ca and contractility via Na/Ca exchange. We previously showed (Circ Res. 2005;*97*:252–259) that the NKA-associated small transmembrane protein phospholemman (PLM) regulates NKA activity and PLM phosphorylation mediates the enhancement of NKA function by β-adrenergic stimulation in mouse ventricular myocytes. There are multiple NKA isoforms in cardiac myocytes. NKA-α1 is the dominant, ubiquitous isoform whereas NKA-α2 is present in relatively small amounts and in a species-dependent manner. It has been suggested that NKA-α2 is located mainly in the T-tubules where it could regulate local Na/Ca exchange and thus cardiac myocyte Ca. In this context, it is important to determine whether NKA-α1 and NKA-α2 interact differently with PLM. Here we used myoctes isolated from wild-type mice and mice where NKA isoforms have swapped ouabain affinities (NKA-α1 is ouabain-sensitive while NKA-α2 is ouabain-resistant) to investigate the effect of β-adrenergic stimulation on NKA-α1 and NKA-α2 isoforms. The ouabain-sensitive component was completely blocked with 20 μM ouabain and we measured the Na-dependence of the rate of Na extrusion mediated by the ouabain-insensitive NKA isoform. There was no significant difference between the apparent K1/2 for Na between NKA-α1 and NKA-α 2 (18.1±2.0 mM vs. 16.4±2.5 mM). The maximum Na-extrusion rate (Vmax) was 5.9±0.6 mM/min for NKA-α1 and 1.7±0.7 mM/min for NKA-α2, suggesting that NKA-α2 represents ~22% of total NKA. Isoproterenol (1 μM) significantly increased the affinity for internal Na of both NKA-α1 (K1/2 decreased to 11.5±1.9 mM) and NKA-α2 (K1/2 decreased to 10.4±1.5 mM) with no significant effect on Vmax. In conclusion, β-adrenergic stimulation enhances the activity of both NKA-α1 and NKA-α2 isoforms to a similar extent, suggesting that NKA-α1 and NKA-α2 interact similarly with PLM.

697-Pos Phospholemman (PLM) Regulation of the Na-Pump (NKA): Isoform-Specificity

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Board B541

Phospholemman (FXYD1) is a major target for phosphorylation in the heart. In cardiac myocytes, PLM also associates with NKA and mediates the adrenergic effects on NKA. The paradigm being that PLM-dependent NKA inhibition is relieved upon PLM phosphorylation. We now test the hypotheses that the PLM-NKA interaction and the adrenergic regulation of NKA are isoform-specific. In HEK-293 cells, CFP-NKAα1 (or α2) and PLM-YFP were targeted to the plasmamembrane and their interaction resulted in measurable FRET for both NKA isoforms. PLM-YFP was progressively bleached, resulting in an exponential decline of YFP emission and a concomitant increase in donor fluorescence. The Fdonor increase reached a similar maximum of 23.6±3.4% and 27.5±2.5% for NKAα1 and α2, respectively. Examination of donor *vs.* acceptor fluorescence revealed a linear relationship, indicative of a 1:1 PLM-NKA interaction for both NKA isoforms. PLM-CFP also FRETs with PLM-YFP, but this displayed a nonlinear relationship during progressive bleach, indicating a multimeric complex (3 or more PLM molecules). Activation of PKC (by 100 nM PDBu) decreased FRET so that YFP photobleaching increased Fdonor by only 9.9±1.8% and 6.4±1.9 for NKAα1 and α2. Likewise, PKA activation (10μM forskolin) reduced FRET for both isoforms (4.9±2.3% and 2.4±2.9%). However, submaximal PKA activation with 8-Bromo-cAMP (or lower [forskolin]) produced a smaller FRET reduction for the NKAα2-PLM interaction (13.6±2.6% vs. 5.0±1.7% for NKAα1), suggesting that the local signaling complexes may be different for the two NKA isoforms. Interestingly, application of 10 mM ouabain also completely prevented the observed Fdonor increase for both isoforms and this effect was

mostly phosphorylation independent (based on pretreatment with staurosporine). Our results indicate that NKAα1 and α2 interact similarly with PLM, and both interactions respond similarly to PLM phosphorylation.

698-Pos Role of Phospholemman Phosphorylation Sites in Mediating Kinase-Dependent Regulation of Na/K Pump

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Phospholemman (PLM) is a major target for phosphorylation mediated by both PKA (at Ser68) and PKC (at both Ser63 and Ser68) in the heart. We have previously shown that PLM inhibits Na, K-ATPase (NKA) function and that PLM phosphorylation by PKA or PKC can relieve this inhibitory effect in intact cardiac myocytes. The aim here was to distinguish the role of PLM phosphorylation sites in mediating kinase-induced modulation of NKA function. We measured PKA/PKC effects on NKA-mediated [Na]i decline, using exogenous overexpressed rat alpha1 pump in a stable Hela cell line with adenoviral expression of PLM (WT and mutant), with activation of PKA (20 μM forskolin) and PKC (300 nM PDBu). Expression of WT PLM, S63A mutant PLM or AA PLM (Ser63 and Ser68 to Alanine double mutation) in these cells significantly decreased the apparent intracellular Na affinity of NKA. This decrease in Na affinity was reversed by FSK in cells with WT PLM, but not in cells with AA PLM. FSK did not alter either Vmax or apparent Na affinity of NKA in cells without adenovirus PLM. The Vmax of NKA after PLM expression was similar to that in non-PLM cells and it did not change upon PKA activation. Similarly, PKC activation resulted in an increase in Na affinity (not Vmax) of NKA when WT PLM or S63A-PLM was expressed in those cells, but not without PLM or with AA PLM adenovirus. We conclude that phosphorylation (especially of Ser68) of PLM mediates the PKA and PKC-dependent activation of NKA function, via enhanced Na affinity of rat alpha1 pump expressed in Hela cells.

699-Pos Na/K Pump Isoform and Presence of Phospholemman Alter Extracellular K Dependence of Pump Function in Cardiac Myocytes

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Board B543

Phospholemman (PLM) is a member of the FXYD family of proteins which modulate Na, K-ATPase (NKA) function in a tissue-specific manner. In intact cardiac myocytes we have previously shown that PLM inhibits the intracellular Na affinity of NKA, and that PLM phosphorylation by PKA and PKC reverses this inhibitory effect (thereby stimulating NKA activity). The aim here was to investigate in cardiac myocytes whether the external K affinity of NKA α1 or α2 are different and whether PLM (and its phosphorylation) also modulate Ko affinity. We measured Ko dependence of NKA pump currents with patch-clamp in myocytes from WT and PLM-KO mice and in mice in which the ouabain affinity of NKA-α1 and -α2 was swapped (i.e. where NKA-α1 is the high affinity form). The apparent Ko affinity of NKA was significantly lower in WT myocytes vs. PLM-KO (Km = 2.0 ± 0.2 mM in WT vs. 1.05 ± 0.08 mM in PLM-KO). However, PKA activation by isoproterenol (1 μM) did not alter the Km of NKA for Ko in WT myocytes. On the other hand, selective assessment of NKA-α1 *vs.* -α 2 by 20 μM ouabain that blocks the high affinity form (in WT and ouabain-affinity swapped mice) showed that the NKA-α1 isoform has higher Ko affinity than the –α2 isoform with PLM present (Km= 1.5 ± 0.1 *vs.* 2.9 ± 0.3 mM). We conclude that

1. PLM decreases the apparent Ko affinity of NKA in cardiac myocytes,
2. NKA-α1 has higher Ko-affinity than NKA-α2, and
3. phosphorylation of PLM at cytosolic domain does not alter apparent extracellular K affinity of NKA.

700-Pos Palytoxin-bound Phosphorylation-site Mutant Na/K Pump-channel

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Board B544

To distinguish modulatory from phosphorylation Mg-ATP effects, we mutated the conserved phosphate acceptor Asp (D378 in Xenopus-α1) and examined the mutant pumps in excised patches after transforming them into ion channels with palytoxin. With symmetrical 125 mM Na solutions and cytoplasmic 5 mM Mg-ATP, palytoxin rapidly (τ~8 s) opened D378 (wild-type, WT) pump-channels, but D378N, D378A, or D378E pump-channels opened extremely slowly (τ>500 s) and continued to open after palytoxin washout. This slowing was specific for Mg-ATP (not seen with Mg-AMPPNP or Mg-ADP), and was Na dependent (not seen with symmetrical K solutions). In contrast, Mg-ATP, Mg-AMPPNP, or Mg-ADP all instantly (τ~2 s) increased pump-channel current in inside-out patches with WT or mutant pumps already transformed into channels by palytoxin in the pipette. In outside-out patches with internal Mg-ATP, switching from Na to K external solution decreased pump-channel current extremely slowly (t1/2~600 s) for both WT and mutants with internal Na, but ~10-fold faster with internal K. Without nucleotides, external K-induced current decrease was much faster, t1/2~2 s for WT with internal Na or K, or for the phosphorylation-site mutants with internal K, but 5–10-fold slower for the mutants with internal Na. Thus, with symmetrical K solutions, palytoxin activated only an extremely small current in WT or mutants which, after withdrawal of unbound palytoxin, increased 40–160 fold on exposure to external Na. These results reveal a specific Na-dependent influence of Mg-ATP (but not analogs), in mutant Na/K pumps that cannot be phosphorylated, to stabilize a closed pump-channel conformation after subsequent

binding of palytoxin. They also demonstrate that another modulatory effect specific to Mg-ATP, i.e., marked slowing of external K-induced decrease of pump-channel current, is similarly independent of phosphorylation because it occurs in both WT and mutant pumps. (NIH-HL36783)

701-Pos Rapid Na^+ deocclusion from an Antarctic octopus Na^+/K^+-ATPase

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Board B553

In order to operate in different thermal environments, enzymes like the Na^+/K^+-ATPase must have evolved structural changes to regulate their kinetics. To understand specific changes that underlie cold temperature adaptation, we have compared transport properties of a Na^+/K^+-ATPase cloned from an Antarctic *(Pareledone sp.* (PS); −1.8°C) and a temperate *(Octopus bimaculata* (OB); 12–20°C) octopus species. As a first step, we tested the temperature sensitivity of forward pump currents expressed in *Xenopus oocytes.* Between 22°C and 12°C the Q10 values were strikingly different (PS = 3.69; OB = 10.58). At room temperature (22°C) the pumps' overall turnover rate did not differ significantly (PS = 26 ions*s^{-1}; OB = 22 ions*s^{-1}). To begin identifying precise reaction steps that differ between species, the process of Na^+ deocclusion was studied in isolation. Transient currents under conditions that favor Na^+/Na^+-exchange were recorded in response to voltage steps over the range of −200 to +40mV. Single-exponential fits to the slow component revealed that the relaxation rate for Na^+ deocclusion was ~1.5 times faster at 22ºC (PS = 235.1 s^{-1}; OB = 152.7 s^{-1}) and ~2.2 times faster at 12ºC (PS = 30.2 s^{-1}; OB = 13.7 s^{-1}), for PS than for OB. The Q10 values of Na^+ deocclusion (PS = 7.78; OB = 11.15) are quite steep, suggesting that this transition (E1-to-E2) could be rate-limiting at low temperatures. The amino acid sequences of the two pumps are very similar (96% identity). Based on their location and conservation among phyla, three residues were selected as likely candidates to explain the kinetic differences. Two of these are located within transmembrane spans (M2 and M9) and the other within the phosphorylation domain. The effect of these residues on Na^+ deocclusion is presently being tested.

702-Pos Thermodynamics Of ATP Binding To The Na^+,K^+-ATPase

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Board B546

The Na^+,K^+-ATPase is a P-type ATPase, found in all animal cells. The enzyme uses the free energy from hydrolysis of ATP for transporting Na^+ and K^+ ions across the cell membrane. Previous work using stopped-flow fluorimetry suggests that the enzyme exists as a dimer with two gears of pumping depending on the number of ATP molecules bound (Clarke & Kane , in press). To test this hypothesis isothermal titration calorimetric (ITC) experiments have been performed to see if evidence for two ATP binding equilibria could be detected. Initial experiments showed heat signals due to a very slow ATP hydrolysis in addition to ATP binding. These signals disappeared, however, on adding the specific Na^+,K^+-ATPase inhibitor, ouabain, which blocks the enzyme in a phosphorylated state. The slow ATP hydrolysis must, therefore, be due to Na^+,K^+-ATPase activity stimulated by small amounts of divalent metal ions, which act as ATP cofactors for hydrolysis. The slow ATP hydrolysis could also be inhibited by the inclusion in the buffer of 10 mM CDTA, a strong Mg^{2+} chelator. Under these conditions any heat signals measured could be confidently attributed to ATP binding alone. ATP binding was also investigated by measuring the fluorescence anisotropy of the fluorescent ATP derivative, BODIPY FL ATP (Molecular Probes), over a range of concentrations at constant protein concentration. When the probe binds to Na^+,K^+-ATPase a significant increase in its fluorescence anisotropy occurs due to the reduced rotational mobility of the fluorophore. Analysis of both the ITC and fluorescence anisotropy data enabled ATP dissociation constants to be determined and a critical discussion of the dimer Na^+,K^+-ATPase hypothesis.

References

Clarke RJ & Kane DJ (2007) *Biophys. J*, in press.

703-Pos Analysis of Relative Binding Energies of Cardiac Glycosides to the Na, K-ATPase

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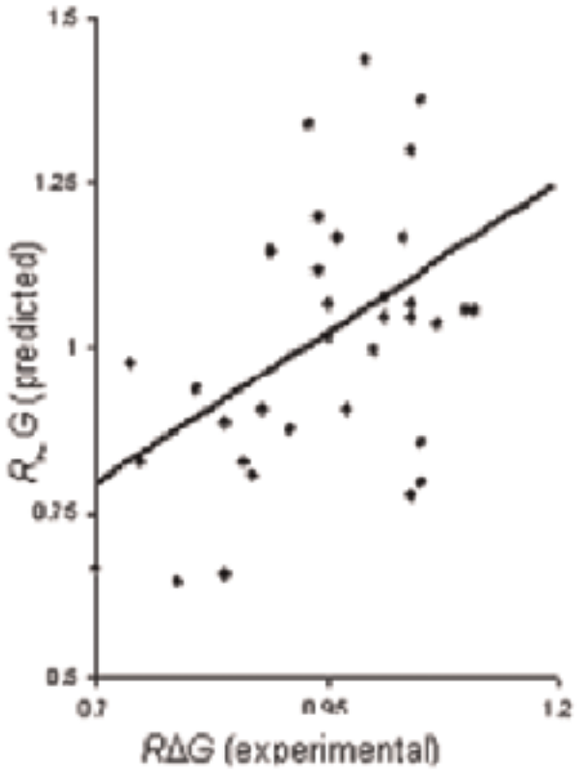
Cardiac glycosides are believed to inhibit the Na,K-ATPase by binding at extracellular sites on the enzyme; however, the protein structure and amino acids involved in molecular recognition of glycosides by the Na,K-ATPase are unclear. In this study, the crystal structure (1WPG) of a rabbit Ca-ATPase *E*2P-like conformation was used as a template for homology modeling of the sheep Na,K-ATPase alpha1 subunit with MODELER. Ouabain was manually docked and the complex was refined using AMBER force field. The binding mode was consistent with the results obtained from GOLD docking and other published models. Binding energies of 35 cardiac glycosides were also calculated with a similar approach and then compared to free energies of binding derived from the equilibrium binding study of Paula *et al. (Biochemistry* ***44***: 498–510, 2005). Predicted and experimentally-derived binding energies of these glycosides were normalized (RΔG) to those values for ouabain. The normalized values of predicted RΔG were well correlated *(r^2* = 0.45) with experimentally-derived values (see Figure). Further refinement of glycoside binding calculations is required, but this procedure provides a reasonable approach to predicting the binding modes of unknown cardiac glycosides to the Na,K-ATPase



704-Pos Fluorescence Measurements of ATP Binding to Na/K-ATPase Labeled with Fluorescent Probes

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Board B548

The Na^+/K^+-ATPase, a membrane-associated ion-motive ATPase, uses energy from the hydrolysis of ATP to move Na^+ out of and K^+ into cells. We have labeled ATPase isolated from duck supraorbital salt glands with one of Cy3-maleimide, and Alexa Fluor 546 carboxylic acid, succinimidyl ester. The fluorescence at the emission peak of these labeled proteins decreased upon addition of unlabeled nucleotide (ATP or ADP) or of ATP labeled with Alexa Fluor 647. We observe that:

(a) the fluorescence change of all three labeled enzyme as a function of nucleotide concentration could be fitted with a Hill equation, with a Hill coefficient of 0.3 – 0.5 and K0.5 values ranging from 0.75 to 20 μM for ATP and ADP in Na^+ buffer and 50 μM in K^+ buffer.

(b) Addition of 1 mM ATP to enzyme labeled with Alexa Fluor 546 carboxylic acid, succinimidyl ester gave a fluorescence decrease at 582 nm of 1.6%; addition of 997 μM ATP + 3.3 μM Alexa Fluor 647 ATP resulted in decrease in fluorescence at 582 nm of 16.6%. The difference between the fluorescence changes in the presence and absence of Alexa Fluor ATP is attributable to FRET between labeled enzyme and labeled ATP. This difference also indicates that Alexa Fluor 647 ATP binds with higher affinity than ATP.

(c) Activity of unlabeled enzyme as a function of ATP concentration could be fitted to a Hill equation with a Hill coefficient of 0.6 for both Na^+/K^+-ATPase and Na^+-ATPase activity.

These results indicate that both ATP binding to the enzyme and ATPase activity demonstrates significant cooperativity. This cooperativity may be due to enzyme aggregation or may be an intrinsic property of the enzyme.

705-Pos Dimers Are Forever: New Developments In The Mechanism Of The Na^+,K^+-ATPase

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Board B549

The Na^+,K^+-ATPase (sodium pump) was the first ion pump to be discovered (Skou, 1957) and is one of the most fundamentally important enzymes of animal physiology. The electrochemical potential gradient for Na^+, which it maintains, is used as the driving force for numerous secondary transport systems, e.g. voltage-sensitive Na^+ channels. The mechanism of the Na^+,K^+_ ATPase is universally described in biology textbooks by the Albers-Post cycle, which represents the catalytic subunit as a monomer undergoing a cyclical sequence of conformational changes, ion binding and release steps and ATP phosphorylation/dephosphorylation reactions. Although this mechanism is consistent with the vast majority of experimental data, for many years research groups around the world have discovered reproducible results which are inconsistent with this mechanism:

1. multiple ATP binding affinities;
2. multiple rate constants for the same reaction from the same source;
3. phosphorylation of half of the ATP binding sites;
4. simultaneous presence of two intermediate states of the cycle; and
5. two-step release of K^+ ions.

These inconsistencies indicate that the widely accepted Albers-Post model cannot be the full truth. The results of stopped-flow kinetic experiments and theoretical simulations (Clarke & Kane, in press) indicate that the enzyme exists as a functional dimer within the membrane. To explain these results and previous inconsistencies with the Albers-Post model, we propose a new mechanistic model in which the enzyme cycles at a low rate with ATP hydrolysis by one catalytic subunit or at a high rate with ATP hydrolysis by two catalytic subunits simultaneously within a dimer, depending on the concentration of available ATP. Thus, we propose a bicyclic model with two gears to replace the classical Albers-Post model.

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706-Pos Low Cytoplasmic Na^+ Concentration $[Na^+]i$ is maintained by the 2nd Na^+ Pump in Insect Cells

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Board B550

The control of $[Na^+]i$ has been attributed to the action of the Na^+/K^+ ATPase. However, a 2nd Na^+ pump has been described in mammalian kidney, colon and malpighian tubules of the insect Rhodnius prolixus [MT] [Biochim. Biophys. Acta ***394***:281–292,1975; Comp. Biochem. Physiol B ***119***:807–811,1998]. This ATPase does not require K^+, is Ouabain insensitive and Furosemide inhibited. The biochemical entity of this pump has been identified and characterized in enterocyte membranes solubilized with detergent C12E9 [*J. Gen. Physiol.****126***:77a, 2005], which preserves both ATPase activities allowing their *in vitro* determination by inorganic phosphate (Pi) liberation. We measured Ouabain (Na^+/K^+) and Furosemide (Na^+) sensitive ATPase activities in MT and in C6/36 insect culture cells homogenates. In both preparations, the specific activity of the 2nd Na^+ pump [in nmol Pi/mg protein.min] was much higher than that of the Na^+/K^+ pump, 49±11 vs 14±4 in MT (n=7) and 58±12 vs 20±7 in C6/36 (n=14), respectively. In order to investigate the physiological relevance of each of the pumps, we measured $[Na^+]i$ with a microfluorometric method. Either MT or C6/36 cells were loaded with SBFI and the fluorescence ratio (340/380) was determined with an Ionoptic system. Application of Ouabain (1 mM) has no effect on $[Na^+]i$ in either MT or C6/36 cells; while 1 mM Furosemide produced a sustained and reversible $[Na^+]i$ elevation. Thus in the present experiments, resting low $[Na^+]i$, is maintained by the 2nd Na^+ pump in insect cells These new findings have to be incorporated to a secretion model in *Rhodnius* MT [J. Membrane Biol. ***202***:105–114,2004].

Dr. Jesús Castillo reinitiated us in these experiments which were partly supported by Fonacit and Fundación Polar.

707-Pos Nitroxyl (HNO) Activates the Cardiac Sarcoplasmic Reticulum Calcium Pump in a Phospholamban-dependent Manner by Interacting with Cysteine Residues in the Transmembrane Domain

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Nitroxyl (HNO) donated by Angeli's salt (AS) is a positive ionotropic/lusitropic agent *in vivo* and *in vitro*. In isolated cardiomyocytes, HNO enhances intracellular Ca^{2+} cycling by activating Ca^{2+} release from ryanodine (RyR2) receptors and Ca^{2+} re-uptake by the sarcoplasmic reticulum Ca^{2+} pump (SERCA2a). Activated Ca^{2+} release is reversed by the reducing agent dithiothreitol (DTT), implicating critical thiols in RyR2 (Tocchetti et al. Circ. Res. *100*:96–104, 2007). Preliminary data obtained with canine cardiac SR vesicles shows that activation of Ca^{2+} uptake by AS/HNO is accompanied by enhanced dephosphorylation of SERCA2a, but whether this is a direct effect on SERCA2a or HNO-induced modulation of its "brake" phospholamban (PLB) is unclear. To characterize the protein sites modified by nitroxyl, we studied AS/HNO-induced activation of SERCA2a dephosphorylation using SERCA2a and PLB expressed in Sf21 cell ER microsomes. Brief exposure of microsomes expressing SERCA2a and PLB to 0.25 mM AS produced a 10-fold stimulation (0.5 to 5 s^{-1}) of the initial phase of phosphoenzyme decay following an EGTA chase. Conversely, no stimulation was detected in microsomes expressing SERCA2a alone. Expression of SERCA2a with mutant PLB in which all 3 Cys in the TM domain were replaced by Ala showed no stimulation of dephosphorylation by AS/HNO. Regulation of SERCA2a Ca^{2+} uptake activity by Cys→Ala PLB remained intact as evidenced by the decrease in Km for Ca^{2+} following exposure to anti-PLB antibody. Exposure to dithiothreitol (2 mM) reversed the activation of dephosphorylation by AS/HNO. We conclude that activation of the cardiac SR Ca^{2+} pump by AS/HNO results from covalent modification of the Cys residues in the TM domain of phospholamban and that this induces a conformational change in PLB that relieves the inhibition of SERCA2a.

708-Pos Phospholamban Oligomerization, Quaternary Structure, and SERCA-Binding Measured by FRET in Living Cells

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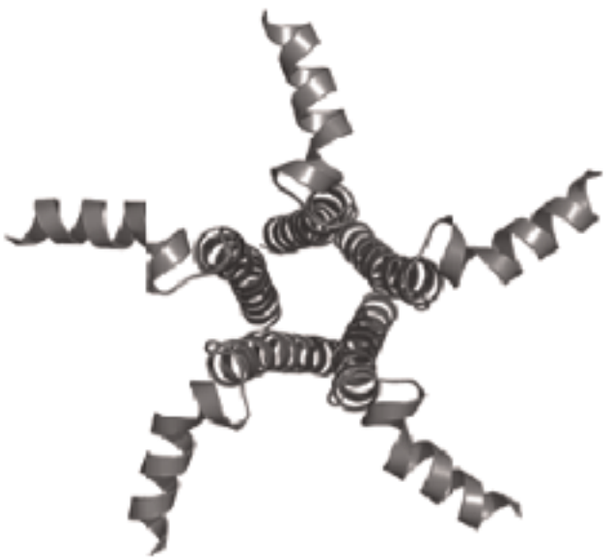
The interactions of phospholamban (PLB) with itself and its regulatory target SERCA were investigated by fluorescence resonance energy transfer (FRET) in AAV-293 cells. A computational model of pentamer FRET indicated the average probe separation distance in the pentameric PLB structure was 58.7 ± 0.5 angstroms. This is consistent with a "pinwheel" quaternary conformation, in which cytoplasmic domains fan out from the central bundle of transmembrane helices (Fig. 1), as opposed to a more compact "bellflower" conformation. I40A mutation of PLB did not alter pentamer conformation, but increased the concentration of half-maximal FRET (KD) by $>$ 4 fold. PLB association with SERCA, measured by FRET between CFP-SERCA and YFP-PLB, was increased by I40A mutation. Consistent with a decreased pentamer:monomer ratio, YFP-I40A-PLB showed faster photobleaching (indicating decreased homotransfer FRET), and an increased rate of fluorescence recovery after photobleaching (FRAP). In addition, a naturally-occurring PLB mutation, L39Stop, greatly reduced PLB oligomerization and SERCA-binding. L39Stop-PLB was also mislocalized to

the cytoplasm and nucleus. Saponin permeabilization of cells expressing YFP-L39Stop-PLB caused total loss of fluorescence from the cells, indicating that the truncation of the transmembrane domain abolished membrane anchoring of PLB.



709-Pos Golgi Ca^{2+}-ATPases Regulate A Novel Ca^{2+} Signaling Pathway

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Board B553

The Secretory Pathway Calcium ATPases (SPCA) comprise a newly identified family of Ca^{2+} transporters. There are two human isoforms: hSPCA1 is ubiquitously expressed at variable levels in different human tissues, whereas hSPCA2 has a more restricted distribution and is found in brain and gastrointestinal system. These transporters supply the Golgi lumen with Ca^{2+} and Mn^{2+} ions that are essential for protein sorting, processing and glycosylation. Based on their distribution and properties, the SPCA are implicated in diverse physiological functions, including regulation of desmosomal contacts in keratinocytes, calcium absorption in intestine, and milk secretion in mammary gland. We show that the pumps are localized to the Golgi and to vesicular compartments that are close to the plasma membrane. Isoform-specific trafficking to the plasma membrane correlates with induction of Ca^{2+} influx and activation of the calcineurin signaling pathway. We use knockdown and overexpression strategies in conjunction with Ca^{2+} imaging and electrophysiological approaches to document the contribution of these pumps. Both N- and C-termini of the SPCA appear to be involved in the regulation of trafficking and Ca^{2+} signaling. We speculate that a novel Ca^{2+} signaling pathway may be important for regulation of Golgi and vesicular stores, analogous to the refilling of endoplasmic reticulum stores.

710-Pos Hinge-Bending Mechanisms in F1-ATPase: Vertical Excitation and the Role of the Load

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Board B554

ATP binding to a beta subunit in its open state induces the hinge to undergo a transition to a closed state. One mechanism for this open-to-closed transition has been proposed based on the "switching Go model" (Koga & Takada, PNAS *103*, 2006) and a "two-basin" (−ATP and +ATP) energy landscape. Here, the hinge transition is initiated via a fast "vertical" excitation from its initial equilibrium open state to an unstable open state on the +ATP-surface. This is followed by a slower relaxation down the +ATP-surface into its new closed equilibrium final state.

Another mechanism for this transition has been proposed (Oster & Wang, BBA 1458, 2000) using a different kind of two-basin energy landscape: a -ATP "ground-state" energy surface, and a +ATP "excited-state" surface. Here the vertical hinge transition is initiated from an initial equilibrium open state on the –ATP-ground surface to an unstable open state on the +ATP-excited surface. This is followed by a relaxation to the closed hinge state along the +ATP-excited surface to a final non-equilibrium closed state on this surface.

We suggest that the vertical excitation process is similar in principle to the "optical" Franck-Condon transition in small-molecule photochemistry. This kind of rapid excitation may also represent the local injection of external energy into the beta subunit upon binding ATP. Secondly, we propose that the final non-equilibrium closed state in the Oster-Wang mechanism represents a type of mechano-chemical force-balanced state. In this state, the chemical forces (ATP binding energy) are balanced by opposing mechanical forces (elastic strain in an internal load). Thus, the role of the load is to permits storage of ATP binding energy - and to preclude attainment of a true equilibrium final state.

711-Pos Brownian Dynamics Simulation of the F1-ATPase Molecular Motor

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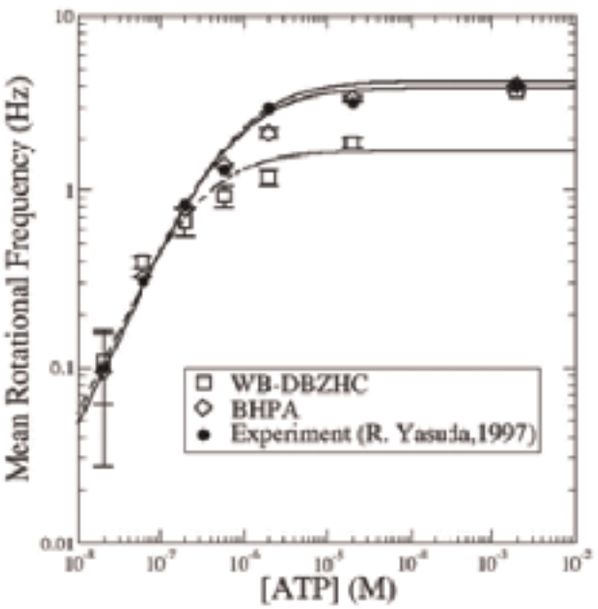
Board B555

In a series of prodigious experiments, Noji (Nature *386*: 299–302, 1997), Yasuda (Cell *93*:1117–1124, 1998) and coworkers shown that the F1-ATPase is able to rotate huge actin filaments, two orders of magnitude larger than the motor itself. Hereby we simulate these experiments by using Brownian dynamics and a reduced set of kinetic equations, with all but one rate constants from experimental reports. Two reaction pathways for the F1-ATPase were chosen: a first one by the groups of Boyer (Biochim. Biophys. Acta *140*:215–250, 1993; Annu. Rev. Biochem. *66*:717–74, 1997) and Walker (Nature *370*:621–628, 1994), and modified by Cross and co-workers (PNAS *92*:10964–10968, 1995), -BW-DBZHC- and another one by

M. Amzel and coworkers (PNAS *95*:11065–11070, 1998; J. Bioener. Biomemb. *32*:517–521, 2000) -BHPA-, including a fourth conformational state for the *Beta* subunit, "*Closed*", with an ATP (ADP) intermediate affinity of Kd=30μM (35μM) (Senior, Biochim. Biophys. Acta 1553:188–211, 2002). Our simulations with BHPA reproduces extraordinary well the measured frequencies by Yasuda et. al. for all ATP concentrations (20nM to 2mM) and all filament lengths (0.5×10^{-6}m to 4.0×10^{-6}m). In contrast, the BW-DBZHC pathway gives frequencies that are systematically lower than observed.



712-Pos Renumbered to 724.01-Pos Board B568.01

Board B556

No Abstract

713-Pos Renumbered to 724.02-Pos Board B568.02

Board B557

No Abstract

714-Pos Light Modulated Rhodamine Efflux in the Intestinal Parasite *Giardia duodenalis*

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Board B558

A light-modulated efflux pathway has been identified in trophozoites of the human intestinal parasite *Giardia duodenalis*. It is manifested by the net increase of the intracellular concentration of the cationic fluorophore Rhodamine 123 from the background solution in response to near UV or blue light illumination. The light-modulation is shown to be reversible, with the cells accumulating dye in the presence of illumination and effluxing the dye otherwise. The overall process of the dye uptake is governed by two competing processes: Charge dependent uptake between the cationic dye and the negative membrane potential, and ATP dependent efflux of the dye that can be modulated by illumination. The optical activation spectrum of the process has been determined and features two peaks, at approx. 380 nm and 500 nm. The light-modulated efflux of a homologous series of Rhodamine 110 and Rhodamine B esters of varying lipophilicity is also investigated in drug sensitive and drug resistant *G. duodenalis* strains. An assay of the uptake of these dyes showed differences in the membrane permeability of the different strains. Esters of Rhodamine B were not accumulated in response to light but were strongly localised within the cells even in the absence of illumination, independent of the lipophilicity. The presence of two ethyl groups attached to the amines in Rhodamine B appeared to alter the affinity of the efflux transporter for the dye such that Rhodamine B and its esters were not effluxed.

715-Pos Role Of The F0F1-ATPase In Bacterial Effects Of Electromagnetic Irradiation With Extremely High Freguency And Low Intensity

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Board B559

Electromagnetic irradiation (EMI) of extremely high frequency has being studied due to two aspects at least, the first of which is in that bacteria and yeast cells may communicate with each other by EMI of millimeter or sub-millimeter range and the second one is regarding with using in therapy and development of telecommunication technology. Inhibitory effects on Escherichia coli and other bacteria have been shown with "noisy" and coherent EMI of extremely high frequency.

The marked changes in membrane H^+-conductance and H^+ and K^+ fluxes through the membrane, including H^+ fluxes via the F0F1-ATPase and through the potassium uptake Trk system, correspondingly, were determined with the coherent EMI of extremely high frequency of 51.8 and 53 GHz with low intensity (the flux capacity of 0.6 mW/sm^2) and irradiation duration of 30 or 60 min. The total and N,N'-dicyclohexylcarbodiimide (DCCD)- and azide-inhibited ATPase activity of membrane vesicles after irradiation of bacteria noticeably decreased. Bacterial cell sensitivity to DCCD, inhibitor for F0F1 under fermentation, was lowered; production of H2 by whole cells became less. However, membrane potential was kept on the same level. These results indicate input of membranous changes in bacterial action of coherent EMI of extremely high frequency, when the F0F1-ATPase is probably playing a key role. Resonant interaction of EMI of this frequency with F0F1 is possible.

716-Pos Effects Of Thiol Reagents And Cu^{2+} On *Escherichia Coli* F0F1-ATPase

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Board B560

Thiol (SH-) groups have been shown can modulate ion transport and ATPase activity of *Escherichia coli* proton-F0F1-ATPase and be significant for an energy transfer by interchange of 2 SH-⋄-S-S- + 2H between F0F1 and the other proteins when the change in the number of SH-groups were determined. A modification of SH-groups in membrane proteins of fermenting *E. coli* (pH 7.5) by 5.5-dithiol-bis-(2-nitrobenzoic) acid or Ellman's reagent and succinimidyl-6-(β-maleimidopropionamido) hexanoate decreased H^+-K^+-exchange, disturbed the K^+-dependent and *N,N'*-dicyclohexylcarbodiimide- and azide-inhibited ATPase activity of F0F1 and the production of H2. But these reagents did not affect these processes upon cysteine in b subunit of F0 substituted by alanine. Moreover, a drop in redox potential caused by H2 produced upon fermentation of glucose or utilization of formate was less in the presence of these reagents. Such effects were not observed with the other thiol reagent - *N*-ethylmaleimide (NEM). Besides, Cu^{2+}, breaking the disulfides of membrane proteins, increased the number of SH-groups independently on K^+ and suppressed the increased level of SH-groups by ATP in the presence of K^+. The increase in the number of SH-groups and the inhibition in ATP-dependent increasing SH-groups number by Cu^{2+} lacked when treated with NEM. Such effects were not observed with Zn^{2+}, Co^{2+} or Cu^+.

The results indicate a role of SH-groups and cysteine in the b subunit of F0 for H^+-K^+-exchange and the production of H2 by *E. coli* those are participating in a dithiol-disulfide interchange within the F0F1-ATPase and in K^+ uptake system and hydrogenases 4 or 3. A difference in properties of thiol reagents causing different effects in *E. coli* and a discrimination between divalent ions are discussed.

Membrane Transport

717-Pos How Do Arginine Rich Peptides Cross The Lipid Membrane? - A Molecular Dynamics Study

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Board B561

The recombinant HIV-1 Tat protein contains a small region which is capable of translocating cargoes of different molecular sizes, such as proteins, DNA, RNA, or drugs, across the cell membrane in an apparently energy independent manner. The pathway that these peptides follow to enter the cell has been the subject of strong controversy for the last decade. This peptide is highly basic and hydrophilic (47YGRKKRRQRR57R). Therefore, a central question that any candidate mechanism has to answer is how can this highly hidrophilic peptide be able to cross the hydrophobic barrier imposed by the cell membrane. We will present a mechanism for the spontaneous translocation of the Tat peptide across a lipid membrane revealed by extensive molecular dynamics simulations. This mechanism explains how key ingredients such as the cooperativity among the peptides, the large positive charge, and the arginine amino acids contribute to the uptake. These results provide a general mechanism that describes how cell penetrating peptides rich in arginine amino acids are able to translocate across the membrane in an energy-independent manner.

718-Pos Do Porins Pass CAPs?

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Board B562

The cationic antimicrobial peptide (CAP) protamine is known to inhibit bacterial survival (Pink et al., *Langmuir **19***, 8852 (2003), and references therein), but the mechanism of attack is as yet undetermined. For Gram-negative bacteria, two pathways have been proposed:

(a) self-promoted uptake, and
(b) passage through porins.

Here, we study the latter possibility, and model part of the outer membrane of a Gram-negative bacterium in an aqueous solution containing multivalent ions and CAPs. The intent is to determine whether CAPs could pass through porins and, if so, what aspects of external (e.g., ionic concentration) and internal (e.g., porin and O-sidechain characteristics) parameters affect their passage. This study is accomplished via Monte Carlo computer simulations of a "minimal model" of the outer membrane of a Gram-negative bacterium with an embedded porin.

719-Pos Drug permeation through the cell membrane and OmpF investigated by Molecular Dynamics simulations

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Gram negative bacteria represent a serious threat to human health. In particular, their mechanism of drug resistance is invalidating the currently used antibiotic therapies and new chemical entities are urgently required to fight this ever increasing problem. One promising line of research includes drugs that may act as inhibitors of the Beta-Lactamase enzyme, thus interfering with the metabolic pathway of drug resistance. The crucial problem for these drugs is their translocation through the outer membrane of the bacteria. Here, we

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