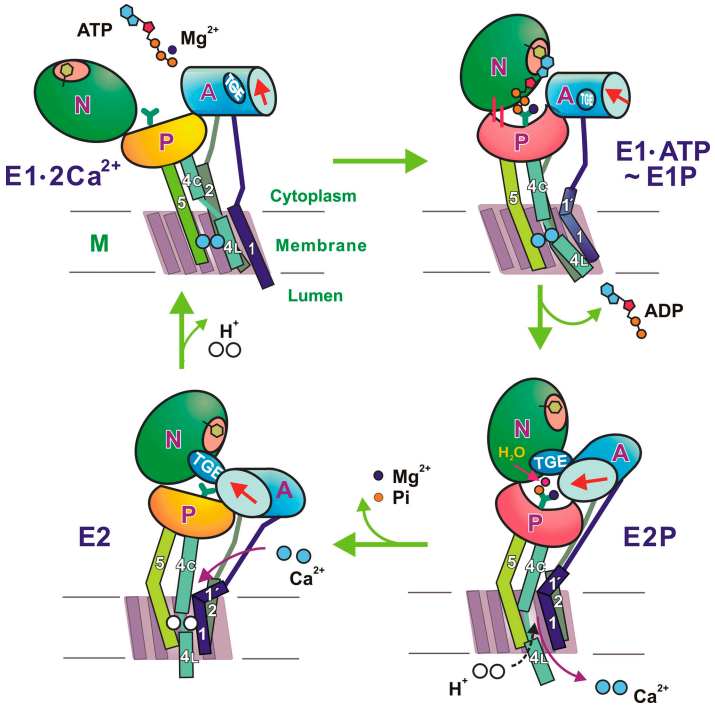
The Journal of General Physiology



ABSTRACTS OF PAPERS AT THE FIFTY-NINTH ANNUAL MEETING OF THE SOCIETY OF GENERAL PHYSIOLOGISTS

Marine Biological Laboratory

Woods Hole, Massachusetts

6–11 September 2005

Organized by

DAVID GADSBY,
JACK KAPLAN,
and
JERRY LINGREL

1. SR Ca^{2+}-ATPase Atomic Structure and Molecular Dynamics Simulations. YUJI SUGITA and CHIKASHI TOYOSHIMA, *Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan* (Sponsor: David Gadsby)

Sarcoplasmic reticulum (SR) Ca^{2+}ATPase (SERCA1a) is an integral membrane protein of 110 kD that establishes the concentration gradient of Ca^{2+} across the SR membrane by transporting two Ca^{2+} per ATP hydrolyzed. Recent X-ray structures of SR Ca^{2+}-ATPase provide atomic models for two high-affinity Ca^{2+}-binding sites in the transmembrane region, which consists of 10 helices. We have studied how the Ca^{2+}-binding sites are stabilized in the transmembrane region by using all-atom molecular dynamics (MD) simulations with explicit solvent and lipids. During the simulations, three cytoplasmic domains as well as the transmembrane domain were very stable. However, the Ca^{2+}-coordination and the H-bonds formed by the protonation of Glu58 and Glu908 are stable in an MD simulation, whereas the H-bonds are disrupted and the Ca^{2+}-coordination geometry is severely altered in another simulation in which these residues are treated as unprotonated. The results clearly indicate that the H-bonds formed by protonation of Glu58 and Glu908 provide extra stability for the Ca^{2+}-binding sites of Ca^{2+}-ATPase (Sugita et al. 2005. *J. Am. Chem. Soc.* In press).

2. Structure and Mechanism of Sarcoplasmic Reticulum Ca^{2+}-ATPase. JESPER V. MØLLER,[1] CLAUS OLESEN,[1] POUL NISSEN,[2] ANNE-MARIE L. JENSEN,[2] RIKKE C. NIELSEN,[2] and THOMAS L.-M. SØRENSEN,[3] [1]*Department of Biophysics and* [2]*Department of Molecular Biology, University of Aarhus, DK-8000 Aarhus C, Denmark*; [3]*Diamond Light Source Ltd., Rutherford Appleton Laboratory, Oxfordshire, UK*

Recent progress in the X-ray analysis of sarcoplasmic reticulum Ca^{2+}-ATPase crystals with bound nucleotides and phosphate analogs (AlF_4^-, MgF_4^{2-}) by our and Toyoshima's groups has led to the structural description, at atomic resolution, of intermediates and transition states related to the Ca^{2+} transport cycle (summarized in Olesen et al. 2004. *Science.* 306:2251–2255). After reaction with ADP and AlF_4 (to mimic the transition state leading to the Ca2E1~P intermediate following reaction with ATP), the cytoplasmic N- and P-domains of Ca^{2+}-ATPase are glued together by AlF_4, forming a transitional complex by an associative SN2 mechanism. This is accompanied by changes in the conformation of the M1/M2 transmembrane segments and a dampened ATPase dynamics that lead to closure of the cytosolic gate for bound Ca^{2+}. After reaction of Ca^{2+}-ATPase with AlF_4 in the E2 state, another complex is formed, which represents the transition state corresponding to E2P dephosphorylation. In this state, the Ca^{2+} liganding residues are occupied by occluded protons, originating from luminal Ca^{2+}/H^+ exchange. In the E2P dephosphorylation transition state, both the A-domain and N-domain are rotated to enable the Thr-181 and Glu-183 in the TGES motif, together with Mg^{2+}, to dephosphorylate Asp-351 by a mechanism that structurally resembles the reaction by which the same Asp-351 is normally phosphorylated from ATP. When dephosphorylation has been accomplished, the TGES motif is disengaged from the phosphorylation site, and the ATPase returns to a conformational state in which it can react with Ca^{2+}. Our data also comprise the structure of an E2 state with bound nucleotide, and a K^+ (Rb^+) site, relevant for understanding the modulatory effect of these ligands on ATPase activity. Overall, our data provide a structural explanation for ATPase function in terms of an orderly sequence of events that leads to effective coupling between the phosphorylation/dephosphorylation and Ca^{2+}/H^+ exchange events. [Supported by the Danish Medical Science Research Council, The Novo Nordisk Foundation, The Lundbeck Foundation, and The Aarhus University Research Foundation.]

3. Purification of Na,K-ATPase expressed in *Pichia pastoris*. Prospects for structural work? STEVEN. J.D. KARLISH, *Department of Biological Chemistry, Weizmann Institute of Science, Rehovot, Israel* (Sponsor: David Gadsby)

Na^+,K^+-ATPase (porcine α/his_{10}-β) expressed in *Pichia Pastoris*, has been dissolved in n-dodecyl-β-maltoside (DDM) and purified in a functional state by metal chelate bead chromatography (Cohen et al. 2005. *J. Biol. Chem.* In press; see abstract for this symposium). The β subunit is expressed as two lightly glycosylated polypeptides and is easily deglycosylated by endoglycosidase-H at 0°C. Added lipid is required to maintain Na,K-ATPase activity, and evidence has been obtained for specific interactions of the protein with the acid phospholipid, dioleoyl phosphatidylserine (DOPS), and probably also the neutral phospholipid, dioleoyl phosphatidylcholine (DOPC), and cholesterol. Recombinant Na^+,K^+-ATPase and pig kidney Na^+,K^+-ATPase, dissolved in DDM, appear to be mainly stable monomers (α/β) as judged by size exclusion HPLC and sedimentation velocity. Their Na^+,K^+-ATPase activities at 37°C are similar but are lower than that of membrane-bound renal Na^+,K^+-ATPase. Both DDM-soluble recombinant and renal Na^+,K^+-ATPase are stabilized in an E_1 conformation, perhaps explaining the lower activities. Human α1 and α2 isoforms have also been expressed with porcine β1 and $\alpha1/his_{10}$-β1 and $\alpha2/his_{10}$-β1 complexes purified, and, in addition, FXYD proteins have been expressed, and α/β/FXYD complexes are being purified (Lifshitz, Garty, and Karlish, abstract for this symposium). The purified complexes of Na,K-ATPase could become important tools for structure–function and biophysical studies, and for studying interactions with lipids and other proteins. Because the recombinant Na,K-ATPase can be produced pure, active, stable, mono-disperse, and deglycosylated, in quantities up to 1 mg, structural work may become feasible. 3D crystallization trials have recently been initiated (with S. Iwata, Imperial College, London).

4. Binding of Na^+ or ATP Control Opening or Closing of the Cytoplasmic Gate to the Na^+ Sites in Na,K-ATPase. PETER L. JORGENSEN, *Institute of Molecular Biology and Physiology, University of Copenhagen, 2100 Copenhagen OE, Denmark*

The initial steps in active Na^+ extrusion are entry of Na^+ through a cytoplasmic gate, followed by binding and occlusion of three Na^+ ions in intramembrane sites to prevent the exchange of ions with those on either side of the membrane, but the details of the structural changes accompanying occlusion have so far not been resolved. High resolution structure analyses of Ca-ATPase of SR (PDB code 1T5S, 1VFP) show that ATP binding closes the gate for access to the intramembrane cation sites I and II primarily by moving the M1 helix. The A-domain tilts by 30° to pull up the M1-M2 helices and to strain the loop to M3. In a homology model of the α-subunit of Na,K-ATPase, the gate for Na^+ entrance on the cytoplasmic side between M2, M4, and M6 can be visualized by Grasp analysis of electrostatic surface potential. Opening and closure of this gate can be monitored by chymotryptic cleavage at Leu-266 in the loop to M3. In a Tris medium, addition of Na^+ ($K_{1/2}$ 1.6 mM) increases the rate constant for chymotryptic cleavage by three- to fourfold, and this effect of Na^+ is reversed by Mg^{2+} and nucleotides. Binding of both Mg^{2+} and the nonhydrolyzable ATP analogs is required to close the gate for entry of Na^+. These structural changes are interpreted to reflect opening of the cytoplasmic gate by Na^+, and closure of the gate upon binding of MgAMP-PCP, accompanied by tilting of the A-domain and changes of the strain on the loop to M3 that can be monitored by ease of chymotryptic cleavage at Leu266.

5. Heavy Metal binding Sites in P_{IB}-type ATPases. JOSÉ M. ARGÜELLO, *Department of Chemistry and Biochemistry, Worcester Polytechnic Institute, Worcester, MA* (Sponsor: Jack H. Kaplan)

P_{IB}-type ATPases transport diverse heavy metals (Cu^+, Cu^{2+}, Zn^{2+}, Co^{2+}, etc.) across membranes. The most recognized members of this subfamily are the two human Cu-ATPases. Their mutations lead to Wilson or Menkes diseases. However, present in archaea, bacteria, and eukaryotes, P_{IB}-ATPases are widely distributed. Previous experimental evidence has shown that individual proteins can transport various metals and that diverse subgroups might present alternative specificities, suggesting a confusing and unpredictable metal selectivity for P_{IB}-ATPases. This can be clarified by identifying and characterizing their various metal binding/transport sites. Many P_{IB}-ATPases, in addition to the transmembrane metal binding site (T-MBS), have NH_2-terminal or COOH-terminal cytoplasmic metal binding domains (MBD). These are apparently regulatory and do not participate in determining metal specificity. By analogy with P_{II}-ATPases, we assumed that P_{IB}-ATPases T-MBS would be constituted by amino acid side chains in transmembrane segments (TMs) flanking the large ATP binding cytoplasmic loop. Bioinformatics analysis of TMs from more than 200 available P_{IB}-ATPase sequences revealed conserved sequences only in TMs H6,

H7, and H8 (equivalent to H4, H5, and H6 of P_{II}-ATPases). These signature sequences allowed the identification of at least five subgroups of P_{IB}-ATPases. Cloning, heterologous expression, and functional characterization of proteins from four of these subgroups revealed unique metal specificities: IB-1 Cu^+/Ag^+; IB-2 $Zn^{2+}/Cd^{2+}/Pb^{2+}$; IB-3 Cu^{2+}; IB-5 Pb^{2+}. Site-directed mutagenesis experiments testing the participation of conserved amino acids in metal coordination during transport indicated that the T-MBS of Cu^+-ATPases (group IB-1) is constituted by two Cys in H6, a Tyr and an Asn in H7, and a Met and a Ser in H8. The essential participation of these residues in heavy metal transport indicates a singular metal coordination in the transmembrane region of Cu-ATPases, distinct from that observed in metalloproteins where the metals play a structural or a catalytic role. [Supported by NSF grant MCM-0235165.]

6. "Next Stop: Binding Sites"—Ion Transport and Binding Sites in P-type ATPases. HANS-JÜRGEN APELL, *Department of Biology, University of Konstanz, 78464 Konstanz, Germany*

Recent biophysical and electrophysiological studies of the three major members of the family of P-type ATPases, i.e., the Na,K-ATPase, the SR Ca-ATPase and the H,K-ATPase, support the concept that a common mechanism of ion transport exists for this family of ion pumps. The basic principle is a central ion-binding moiety deep within the membrane domain of the protein which is connected to the aqueous phases on both sides of the membrane by access channels. A short-circuit pathway is prevented by strict control of two gates which, under physiological conditions, allow only alternating opening to the channels on either side of the binding sites. Although the access channels are (mostly) so narrow that ions migrate through them in an electrogenic manner, the ions move very rapidly, probably controlled by electro-diffusion. Recent kinetic investigations support this concept: only the ion movements in the half channels are electrogenic, while no, or no significant, charge movements could be detected during the phosphorylation/dephosphorylation reactions and the conformation transitions. Subsequent to the approach to the central moiety, binding of the ions in their respective sites is a more complex process which is apparently correlated with conformational relaxations that modify the protein structure adjacent to the bound ion, and thus create or at least optimize the binding sites to allow defined, sequential binding. In addition, complete occupancy of the sites communicates a trigger signal for the subsequent enzymatic action. Such a concept of active ion transport separates rather strictly (passive) ion movements, which are affected by the membrane potential, from the enzymatic activity of the protein, which has to account fully for the energy transduction between chemical energy (stored in ATP) and a "conformational excitation" of the protein that eventually drives the vectorial ion transport. [Supported by DFG grant Ap45/4.]

7. Na,K-ATPase Ion Translocation Pathway. PABLO ARTIGAS, NICOLÁS REYES and DAVID C. GADSBY, *The Rockefeller University, New York, NY*

Palytoxin (PTX) opens an ion channel in the Na/K-ATPase, an effect antagonized by ouabain. We studied PTX-induced channels in outside-out patches, excised from *Xenopus* oocytes heterologously expressing *Xenopus* pumps ($\alpha1\beta3$) mutated to be ouabain resistant, with 125-mM Na solutions containing 100 μM ouabain to inhibit endogenous pumps. Mutation of N131 (corresponding to D129 in rat $\alpha1$) modified single PTX-induced pump-channel conductance (γ_{PTX}) according to introduced charge: γ_{PTX} ~1 pS for positive, γ_{PTX} ~4 pS for neutral, and γ_{PTX} ~6 pS for negative residues. Effects on macroscopic PTX-induced conductance (G_{PTX}) of covalent modification of mutant N131C with charged sulfhydryl reagents resembled those of mutagenesis on γ_{PTX}: cationic $MTSET^+$ reduced, whereas anionic $MTSES^-$ increased, G_{PTX}. This suggests that residue 131 in transmembrane (TM) segment 2, lies near the external entrance to the channel and electrostatically influences its conductance, by controlling the effective local concentration of the conducted ions. $MTSET^+$ and $MTSES^-$ produced corresponding electrostatic effects on G_{PTX} in pumps with single cysteines introduced at several other positions near the extracellular ends of TMs 1, 2, 4, 5, and 6 (including G805C in TM6). However, for the mutant T806C (just intracellular to G805), G_{PTX} was reduced both by $MTSET^+$ (~90%) and by $MTSES^-$ (~20%), suggesting that the channel narrows abruptly at that point, precluding accommodation of $MTSES^-$ without sterically hindering ion conduction. Consistent with these results, homology models based on the SERCA X-ray crystal structures locate T806 at the deepest point of a wide cavity surrounded by TMs 1, 2, 4, 5, and 6. Because many of the residues we mutated in this cavity are reported determinants of ouabain affinity, and were water accessible in the unmodified pump (without PTX), we studied the influence of the presence of the steroid on their modification by $MTSET^+$. The implications of these results for ouabain binding will be discussed. [NIH-HL36783.]

8. Mechanism of SR Ca^{2+}-ATPase by Functional Analysis of Site Mutants. JENS PETER ANDERSEN,[1] JOHANNES D. CLAUSEN,[1] DAVID B. MCINTOSH,[2] ANJA P. EINHOLM,[1] ANNE NYHOLM ANTHONISEN,[1] and BENTE VILSEN,[1] [1]*Institute of Physiology and Biophysics, Department of Physiology, University of Aarhus, Aarhus, Denmark*; [2]*Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa* (Sponsor: David C. Gadsby)

The molecular mechanism of the sarcoplasmic reticulum Ca^{2+}-ATPase can be addressed by combining information from X-ray crystallography with analysis of functional changes in site-specific mutants. We have established a panel of assays for the partial reactions in the Ca^{2+} transport cycle of expressed wild-type and mutant Ca^{2+}-ATPase, including rapid kinetic measurements of the rates of phosphorylation and dephosphorylation, of conformational changes, and of Ca^{2+} binding and dissociation, as well as measurement of the binding affinities for nucleotides, vanadate, fluorides, and other inhibitors. These assays have allowed us to analyze functionally the structural features seen in the atomic models derived from Ca^{2+}-ATPase crystals, such as the movement of the conserved A-domain TGES motif into the catalytic site in E_2 conformations, interaction of the conserved P1-helix residue E340 with transmembrane segment M3 and cytoplasmic loop L6-7, interaction of the bent transmembrane segment M1 with the Ca^{2+}-binding glutamate, E309, of M4, and interaction of conserved N- and P-domain residues with nucleotide and Mg^{2+}. The long known K^+-induced activation of E_2P dephosphorylation by K^+ acting from the cytoplasmic side was disrupted by mutation E732A in domain P, thereby leading to identification of the regulatory K^+ site (Sorensen et al. 2004. *J. Biol. Chem.* 279: 46355–46358). To further understand the regulation of E_2P dephosphorylation, we have analyzed a series of mutants with alterations to the K^+ site and nearby residues of the A-M3 linker that approaches the bound K^+ ion in E_2 conformation. These results will be discussed.

9. Cation Pump Subunit Interactions. KAZUYA TANIGUCHI, *Biological Chemistry, Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo, Japan* (Sponsor: Jack Kaplan)

Na/K-ATPase and other P-type ATPase enzymes utilize the free energy of ATP for ion transport across membranes. Na/K-ATPase and gastric H/K-ATPase retain a catalytic α subunit and a glycosylated β subunit, and kidney Na/K-ATPase also contains a γ subunit. The coupling mechanism of ATP hydrolysis and cation transport in P-type-ATPase can essentially be explained by the Post-Albers mechanism, the sequential appearance of dephosphoenzyme with a high affinity for ATP (E1ATP), an ADP-sensitive phosphoenzyme (E1P), a K-sensitive (and ADP insensitive) phosphoenzyme (E2P), and a dephosphoenzyme with a reduced affinity for ATP (E2), which becomes E1ATP. While protomeric Na/K-ATPase (αβ) is thought to be sufficient for Na/K-ATPase activity, some controversy has arisen concerning whether the functional unit of the enzyme (or the transporter) is a protomer or a much higher molecular weight oligomer, (αβ)n, which would be related to the mechanism of transport, either sequential or simultaneous. Our group, in collaboration with Y. Hayashi (Kyorin University) has successfully demonstrated the simultaneous presence of EP:EATP both in pig kidney Na/K-ATPase and gastric H/K-ATPase, and has been studying enzymatic properties of the oligomer. These data have added new dimensions to the oligomeric properties of the enzyme and are consistent with numerous previous studies by Askari's group (Medical College of Ohio) and W. Schoner's group (Justus-Liebig University). We also succeeded in confirming the presence of tetrameric H/K-ATPase using total internal reflection fluorescence microscopy. Y. Hayashi also was successful in isolating the active teraprotomer of Na/K-ATPase in C12E8 solubilized condition. The presence of subunit interactions in Na/K-ATPase has recently been reported by R.W. Mercer (Washington University), J. Kaplan (Oregon Health Science University), and K. Sweadner (Massachusetts General Hospital). Although the monomeric crystal structure of SR-Ca-ATPase, reported by C. Toyoshima's group (The University of Tokyo) showed an ATP binding site, we showed that each single amino acid mutation in the ATP binding pocket induced different effects on the high and low affinity ATP effects, suggesting a conformational difference in 1 mol of ATP binding/catalytic subunit. Studies of the molecular events occurring in each subunit, and of subunit interactions, during ATP hydrolysis would be the key to a better understanding of the mechanism of energy transduction in P-type ATPases.

10. Characterization of Subunit Interactions and their Role in Na,K-ATPase Delivery to the Plasma Membrane. YIQING CHI, MELISSA LAUGHERY, EDWARD B. MARYON, REBECCA CLIFFORD, and JACK H. KAPLAN, *Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, IL* (Sponsor: Jack H. Kaplan)

The most widespread form of the Na,K-ATPase is the α1β1 heterodimer. Using the baculovirus-infected insect cell system we have shown that the α-subunit ex-

pressed alone is retained in the endoplasmic reticulum, while the β-subunit is able to traffic alone to the plasma membrane. In addition, substitution of the three N-linked glycosylation sites in β1 has little effect on the assembly, activity, or plasma membrane delivery of the Na pump. We have investigated whether this is seen in mammalian cells using polarized monolayers of MDCK cells. In addition, we have investigated similar substitutions in the β2 isoform, which has up to eight potential N-linked glycosylation sites, in both the insect cell and MDCK cell system. Inappropriate expression of the β2 isoform in kidney had been associated with apical mistargeting of Na pump subunits and the development of cysts in polycystic kidney disease. It has been reported that expression of β2 in MDCK cells causes apical delivery of Na pump subunits. We have developed MDCK cell lines that simultaneously express the β1 and β2 isoforms. By using tetracycline-regulated expression and selective siRNA knockdown of one of the isoforms, we have investigated the effects of altered levels and ratios of each β-subunit isoform on Na,K-ATPase targeting and delivery. The effects of these manipulations in β-subunit expression will be discussed as well as the roles of the β-subunit in Na pump delivery. [Supported by NIH grants HL30315 and GM39500.]

11. Na,K-ATPase in Epithelial Cell Polarity and Signaling. AYYAPPAN K. RAJASEKARAN, *Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA*

The Na,K-ATPase is a highly studied molecule best appreciated for its role in intracellular electrolyte homeostasis. Recent studies from our laboratory have established a role for Na,K-ATPase in the formation of tight junctions and establishment of polarity in epithelial cells. In this talk, I will present new evidence that Na,K-ATPase associates with key proteins localized to the apical junctional complex, which contains tight and adherens junctions, in epithelial cells. The significance of these findings in relation to establishment and maintenance of epithelial polarity and its implication in cancer will be discussed.

12. Na,K-ATPase α Subunit Mutations in Neurological Disease. KATHLEEN J. SWEADNER and JOHN T. PENNISTON, *Laboratory of Membrane Biology, Massachusetts General Hospital and Harvard Medical School, Boston, MA* (Sponsor: David Gadsby)

The Na,K-ATPase consumes half of the ATP supply of the brain, and so it would be expected that defects in Na,K-ATPase would result in neurological problems. What is surprising is the very specific nature of the neurological disorders in humans carrying mutations in α3 and α2 Na,K-ATPase subunits. In the nervous system, α3 is found only in neurons. It restores ion gradients drained by electrical activity and makes a small direct hyperpolarizing contribution to membrane potential. Six different mutations of α3 have been found in eight unrelated families with a unique, dominantly inherited disease phenotype, Rapid Onset Dystonia Parkinsonism (RDP). Individuals carrying the gene are symptom free until a metabolically stressful precipitating event, and then they develop symptoms over a period of hours to days: involuntary muscle contractions that result in abnormal movements and postures. We have generated structural models of α3 based on SERCA1a crystal structures and found that all of the mutations lie in places that should be critical for activity. Transfectants showed reduced expression of most of the mutated α3 subunits, suggesting impaired folding or stability. A scheme will be presented that may explain the peculiar characteristics of the disease. In the nervous system, α2 is found in some neurons, but is more abundant in astrocytes. As many as 15 mutations in α2, in 16 families, segregate with familial hemiplegic migraine (FHM2). The disease, like some other migraines and epilepsies, may be due to impairment of astrocyte K^+ clearance. Interestingly, the same disease is produced by mutation of a calcium channel, $Ca_v2.1$, that is expressed exclusively in neurons, and a scheme will be presented that shows a plausible link through the development of cortical spreading depression. Homology maps of FHM2 mutation sites predict, as with the RDP mutations, that most appear to be at structurally critical sites. [Supported by NIH HL036271.]

13. Alterations in the α2 Isoform of Na,K-ATPase Associated with Familial Hemiplegic Migraine Type 2. RHODA BLOSTEIN,[1] LAURA SEGALL,[1] ROSE-MARIE SCANZANO,[1] ALESSANDRA MEZZETTI,[2] ENRICO PURISIMA,[2] and J. JAY GARGUS,[3] [1]*Department of Biochemistry and Department of Medicine, McGill University, Montreal, QC, Canada;* [2]*Biotechnology Research Institute, National Research Council of Canada, Montreal, QC, Canada;* [3]*Department of Physiology and Department of Biophysics and Pediatrics, Section of Human Genetics, University of California, Irvine, CA*

At least nine missense mutations in the α2 subunit of Na,K-ATPase have been identified in familial hemiplegic migraine with aura (FHM2). Whereas two alleles (L764P and W887R) showed loss of function (De Fusco et al. 2003. *Nature Gen.* 33:192–196), we observe that at

least three others, namely T345A (Kaunisto et al. 2004. *Neurogenetics* 5:141–146) as well as R689Q and M731T (Vanmolkot et al. 2003. *Ann. Neurol.* 54: 360–366), are functional but display altered Na,K-ATPase kinetics. Kinetic analyses (apparent cation and ATP affinities, catalytic turnover, and steady-state E_1/E_2 conformational poise) reveal changes effected by T345A, R689Q, and M731T, and suggest that the disease phenotype is the consequence of lowered molecular activity of the α2 pump isoform. The lower activity is due to either decreased K^+ affinity (T345A) or catalytic turnover (R689Q and M731T), thus causing a delay in extracellular K^+ clearance and/or altered localized Ca^{2+} handling/signaling secondary to reduced activity in colocalized Na^+/Ca^{2+} exchange. Information about the mechanistic bases for the kinetic alterations has been obtained from consideration of the structural changes effected by the residue replacements based upon homologous replacements in the known crystal structure of the sarcoplasmic reticulum Ca-ATPase. [Supported by the CIHR grant MT-3876.]

14. Behavioral Abnormalities in Na,K-ATPase Alpha Subunit Haploinsufficient Mice. AMY E. MOSELEY,[1] MICHAEL T. WILLIAMS,[2] TORRI L. SCHAEFER,[2] CHARLES V. VORHEES,[3] and JERRY B LINGREL,[1] [1]*Department of Molecular Genetics, University of Cincinnati, Cincinnati, OH;* [2]*Department of Child Neurology and* [3]*Department of Developmental Biology, Cincinnati Children's Research Foundation, Cincinnati, OH*

Three isoforms of the catalytic α subunit of the Na,K-ATPase are expressed in adult brain. The α1 isoform is expressed in most cell types, while the α2 isoform is mainly expressed in astrocytes and the α3 isoform is expressed in neurons. To explore the role of the Na,K-ATPase in behavior, we performed a battery of tests on adult male mice made heterozygous for the α1, α2, and α3 isoforms. We first tested for anxiety-related behavior. Using the zero maze test, the $\alpha2^{+/-}$ mice spent less time in the open compared with wild type (ANOVA, $F_{3,79} = 4.7$, $P < 0.05$), whereas the $\alpha1^{+/-}$ and $\alpha3^{+/-}$ mice were the same as wild type. All three heterozygous mice performed the same as wild type in the novel object recognition test, spending a similar amount of time with the novel object. General locomotor activity was also assessed. While the $\alpha1^{+/-}$ and $\alpha3^{+/-}$ activity was similar to that of wild-type mice over a period of 1 h, $\alpha2^{+/-}$ mice exhibited reduced locomoter activity throughout this time (ANOVA, $F_{3,84} = 5.5$, $P < 0.05$). The Morris Water maze was used to test for spatial learning deficits. The $\alpha3^{+/-}$ mice displayed longer latency to the platform compared with wild type, using the cued platform version of the Morris Water maze. Using the submerged platform version of the Morris Water maze, the $\alpha2^{+/-}$ and $\alpha3^{+/-}$ mice, but not the $\alpha1^{+/-}$ mice, exhibited longer latency to the platform compared with wild-type animals. While the $\alpha1^{+/-}$ mice displayed behavior similar to wild type, both the $\alpha2^{+/-}$ and $\alpha3^{+/-}$ mice showed memory/learning impairment compared with wild-type mice. The $\alpha2^{+/-}$ mice also showed reduced locomotor activity compared with wild-type, $\alpha1^{+/-}$, or $\alpha3^{+/-}$ mice. In summary, having examined all three Na,K-ATPase α isoform–deficient mice concurrently, we conclude that the α Na,K-ATPase isoforms can differentially modulate behavior. [Supported by NIH grants HL28573, HL66062, DA06733, and DA14269.]

15. Function and Regulation of Human Copper-transporting ATPases, the Menkes Disease and Wilson Disease Proteins. SVETLANA LUTSENKO, NATALIE BARNES, and RUSLAN TSIVKOVSKII, *Department of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, OR*

Human copper-transporting ATPases (Cu-ATPases) play an essential role in cell metabolism. These proteins deliver copper to the secretory pathway that participates in the biosynthesis of secreted copper-dependent enzymes. In addition, Cu-ATPases transport excess copper out of the cells, thereby maintaining the intracellular copper concentration. Mutations in the genes encoding the copper-transporting ATPases ATP7A and ATP7B lead to the severe metabolic disorders Menkes disease and Wilson disease, respectively. Though these Cu-ATPases have distinct tissue-specific distribution, in several tissues, including brain, kidney, and placenta, both ATP7A and ATP7B are present; however, the specific roles of Cu-ATPases in these tissues remain poorly understood. Using high-resolution fluorescent imaging, we demonstrate that in murine cerebellum, ATP7A and ATP7B have distinct cell-specific distribution (Bergmann glia and Purkinje neurons, respectively) and are regulated differently during development. Heterologous expression of ATP7A and ATP7B in Sf9 cells revealed that ATP7A and ATP7B have comparable affinities for either ATP or copper, but they differ substantially in the time courses of their partial reactions, such as catalytic phosphorylation and dephosphorylation. Lastly, we provide experimental evidence that the lack of functional ATP7B in the cerebellum of knock-out mice lacking the Wilson disease gene is compensated by ATP7A, illustrating a tight link between copper homeostasis of Purkinje neurons and of Bergmann glia. [Supported by NIH grant PO1-GM067166.]

16. Na,K-ATPase Regulation by FXYD Proteins. KAETHI GEERING, *Department of Pharmacology and Toxicology, University of Lausanne, CH-1005 Lausanne, Switzerland* (Sponsor: David Gadsby)

Recent studies have provided evidence that four out of seven members of the FXYD family, FXYD1 (phospholemman), FXYD2 (γ subunit), FXYD4 (CHIF), and FXYD7, are auxilary subunits of Na,K-ATPase and regulate its activity in a tissue- and isoform-specific way (for review see Crambert and Geering. 2003. *Sci. STKE.* 2003. 166:RE1). We are interested (1) to identify interaction sites in the transmembrane domain (TM) of the Na,K-ATPase α subunit and of FXYD proteins that mediate the efficient association of the two proteins and/or the functional effect of FXYD proteins, and (2) to elucidate structural and functional properties of FXYD proteins that have so far not been studied. Mutational analysis combined with protein modeling revealed that distinct amino acids in TM9 of the Na,K-ATPase α subunit are involved in either the efficient association or the functional effect of FXYD proteins. Moreover, tryptophan scanning permitted us to identify distinct domains in the TM helix of FXYD7 that are important for the efficient interaction with Na,K-ATPase. Characterization of FXYD3 (Mat-8), a protein that is mainly expressed in stomach and colon and that is up-regulated in certain tumors, revealed that, like other FXYD proteins, FXYD3 also associates with Na,K-ATPase and regulates its transport properties. However, FXYD3 exhibits some unusual characteristics. In contrast to other FXYD proteins, which are type I proteins, FXYD3 may have two TM domains due to lack of cleavage of a signal peptide. Moreover, when expressed in *Xenopus* oocytes, FXYD3 can associate not only with Na,K-ATPase but also with H,K-ATPase. However, in situ (stomach), FXYD3 is associated only with Na,K-ATPase since its expression is restricted to mucous cells in which H,K-ATPase is absent. Finally, we identified a transcript variant of FXYD3 that is expressed in undifferentiated but not in differentiated Caco2 cells. [Supported by the Swiss National Fund grant 31-64793.01.]

17. Structural and Functional Interactions of FXYD Regulatory Protein with Shark Na,K-ATPase. FLEMMING CORNELIUS and YASSER A. MAHMMOUD, *Department of Biophysics, University of Aarhus, Denmark*

In recent studies, we have characterized an FXYD regulatory protein, phospholemman-like protein from shark, PLMS or FXYD10, which is specifically associated with shark Na,K-ATPase (Mahmmoud et al. 2000. *J. Biol. Chem.* 274: 35969–35977; Mahmmoud et al. 2003. *J. Biol. Chem.* 278: 37427–37438). As for phospholemman, the FXYD10 protein contains a COOH-terminal protein kinase multi-phosphorylation domain permitting its interaction with the Na,K-ATPase to be dynamically regulated.

Structural interaction of FXYD10 with the Na,K-ATPase α-subunit is investigated by intermolecular cross-linking using homobifunctional thiol cross-linking agents. Cross-linking is identified to take place between the COOH-terminal Cys-74 of FXYD10 and Cys-254 in the A-domain of α, a position optimal for functional regulation. Thus, interaction of FXYD10 with the A-domain inhibits Na,K-ATPase activity by restricting the free rotation of the A-domain toward the N/P-domains, thereby stabilizing the E1P conformation. Such structural interactions are in accord with the kinetic observations of an increased rate of phosphorylation following truncation of FXYD10 to relieve its interaction with the A-domain.

From controlled proteolysis of purified shark Na,K-ATPase, it is proposed that the FXYD10 functional regulation of Na,K-ATPase may be influenced by interactions with the α NH_2 terminus, which control the steady-state E1/E2 conformational poise, and that PKC phosphorylation of the α NH_2 terminus may regulate this interaction. Thus NH_2-terminal truncation of the shark Na,K-ATPase a-subunit abolished FXYD10/α association and the FXYD10 functional interactions were abrogated. [Supported by The Danish Medical Research Council.]

18. Regulation of the Na,K-ATPase by FXYD proteins. HAIM GARTY and STEVEN J.D. KARLISH. *Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel*

The FXYD proteins are a family of single-span transmembrane proteins with unique tissue distributions and cellular regulations. Five members of this group have been shown to interact with the Na,K-ATPase and alter its kinetic properties. Recently, we have demonstrated that a sixth, and the most distinct, family member, FXYD5 (RIC), also interacts with the Na,K-ATPase (Lubarski et al., abstract at this symposium). CHIF and γ are two FXYD proteins that are preferentially expressed in the kidney and have nonoverlapping distributions along the nephron. They have opposite effects on the apparent affinity of the Na,K-ATPase for cytoplasmic Na^+ and thereby provide a convenient means to adjust pumping rates to the unique requirements of different nephron segments. The role of CHIF in aldosterone-dependent electrolyte homeostasis is further established by the phenotypic analysis of CHIF knockout

mice. Other studies have characterized structural and functional interactions between these FXYD proteins and the α1β1 subunits of the pump in transfected HeLa cells and in partly purified renal Na,K-ATPase. Using functional assays, coimmunoprecipitations, and covalent cross-linking we have identified domains and residues participating in the structural and functional interaction of these proteins with the αβ pump complex. These studies have highlighted a central role for a number of specific residues in structural interactions, and demonstrated a similar general disposition of the two FXYD proteins with respect to α and β (Lindzen et al. and Fuzesi et al., abstracts at this symposium). Additional residues have been shown to account for the different functional effects of CHIF and γ. A model of interaction between the transmembrane segments and cytoplasmic sequences of the γ subunit and a homology model of the α1 subunit has been proposed (Fuzesi et al. 2005. *J. Biol. Chem.* In press).

19. Molecular Mechanism of Cardiac Calcium Pump Regulation by Phospholamban, Revealed by Site-directed Spectroscopy. DAVID D. THOMAS, *Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, MN*

We have used an array of site-directed labeling and spectroscopic techniques to probe directly the interactions, structural changes, and dynamics that are crucial to the action of the Ca-ATPase (SERCA) and its regulation by phospholamban (PLB) in the sarcoplasmic reticulum of the heart. Here are some recent highlights:

(1) Spin-labeling and EPR of single-Cys mutants of PLB show that PLB undergoes a large-scale structural change upon SERCA binding, in which the cytoplasmic domain of PLB is lifted high above the membrane surface (Kirby, T., C.B. Karim, and D.D. Thomas. 2004. *Biochemistry.* 42:5842–5852).

(2) Fluorescence resonance energy transfer (FRET) from SERCA to PLB in reconstituted membranes shows that PLB binds extremely tightly to SERCA with a K_d that is much less than the concentrations of SERCA and PLB in the cardiac SR membrane. Activation of SERCA by saturating (10 μM) Ca^{2+} causes a structural change in the SERCA-PLB complex but does not cause significant dissociation of PLB from SERCA under physiological conditions (Mueller, B., C.B. Karim, Negrashov, H. Kutchai, and D.D. Thomas. 2004. *Biochemistry.* 43:8754–8765).

(3) Time-resolved phosphorescence anisotropy (TPA) of SERCA in membranes shows a large-scale change in the structure and dynamics of the P domain of SERCA, with the largest changes observed upon ATP binding, and smaller but opposite changes observed upon Ca binding. ATP causes the P domain to tilt and undergo an order-to-disorder transition (Mueller, B., M. Zhao, I.V. Negrashov, R. Bennett, and D.D. Thomas. 2004. *Biochemistry.* 43:12846–12854). PLB inhibits the ATP-induced dynamic disorder within the P domain.

(4) NMR and EPR of labeled PLB were used to determine the average structure and topology of the PLB monomer in lipid micelles (Zamoon, J., A. Mascioni, D.D. Thomas, and G. Veglia. 2003. *Biophys. J.* 85:2589–2598) and to determine the principal sites on PLB that interact with SERCA (Zamoon, J., F. Nitu, C.B. Karim C., D.D. Thomas, and G. Veglia. 2005. *Proc. Natl. Acad. Sci. USA.* 102:4747–4752).

(5) Solid-phase peptide synthesis was used to label PLB with TOAC, a novel amino acid in which a spin label is rigidly coupled to the α carbon and thus reports directly the dynamics of the peptide backbone. EPR in membranes shows that the transmembrane domain of PLB is a highly ordered helix, but the cytoplasmic domain of PLB is in dynamic equilibrium between helical and dynamically disordered conformations (Karim, C.B., T.L. Kirby, Z. Zhang, Y. Nesmelov, and D.D. Thomas. 2004. *Proc. Natl. Acad. Sci. USA.* 101:14437–14442). Phosphorylation of PLB at Ser 19 increases the population of the disordered conformation (Paterlini, M.G. and D.D. Thomas. 2005. *Biophys. J.* 88:3243–3251). SERCA binding restricts PLB dynamics. Phosphorylation of SERCA-bound PLB does not dissociate PLB from SERCA, but does change the structure of the bound complex.

These results support a model in which PLB binds tightly to SERCA under all physiological conditions. SERCA is inhibited at low Ca^{2+} by transmembrane interactions that prevent an ATP-induced order-to-disorder transition within the P domain. Phosphorylation of PLB induces a disorder-to-order transition within the PLB cytoplasmic domain. It is the dynamically disordered (extended) conformation of the PLB cytoplasmic domain that is poised to bind to the SERCA cytoplasmic domain and actively relieves SERCA inhibition. [Supported by NIH grants GM27906 and GM64742.]

20. Elucidation of the Ouabain-Binding Site in Na-K-ATPase by Chimeric Approaches. JAN JOEP H.H.M. DE PONT, LI YAN QIU, ELMAR KRIEGER, GIJS SCHAFTENAAR, HERMAN G.P. SWARTS, PETER H.G.M. WILLEMS, and JAN B. KOENDERINK, *Department of Biochemistry, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, and Centre for Molecular and Biomolecular Informatics, Radboud*

University Nijmegen, Nijmegen, The Netherlands (Sponsor: Carel van Os)

Although the global structure of gastric H,K-ATPase is similar to that of Na,K-ATPase, it does not bind ouabain. Previously, we showed that a chimera of these enzymes, in which only the M3-M4 and M5-M6 hairpins originated from Na,K-ATPase, bound ouabain with a high affinity (Koenderink et al. 2000. *Proc. Natl. Acad. Sci. USA.* 97:11209–11214). We also demonstrated that only three amino acids (Phe^{783}, Thr^{797}, and Asp^{804}) from the M5-M6 hairpin of Na,K-ATPase were sufficient to confer high-affinity ouabain binding to a chimera that contained only the M3-M4 hairpin of Na,K-ATPase (Qiu et al. 2003. *J. Biol. Chem.* 278:47240–47247). To further pinpoint the ouabain-binding site, we used a loss-of-function strategy and found that M3 was not important, but that four amino acids present in the extracellular half of M4 were crucial for ouabain binding. In a final gain-of-function study, we showed that a gastric H,K-ATPase that contained these seven (=three plus four) amino acids of Na,K-ATPase bound ouabain with a similar affinity to that of the native enzyme. Similar studies were performed with the nongastric H,K-ATPase that has an intrinsic low affinity for ouabain. In the extracellular half of M4, M5, and M6, there are 14 amino acids that differ from those in Na,K-ATPase. Upon introduction of these Na,K-ATPase amino acids into nongastric H,K-ATPase, a high-affinity ouabain-binding site was obtained. By similar approaches to those noted above, we demonstrated that introduction of only five of these amino acids was sufficient to obtain a high-affinity ouabain-binding site in nongastric H,K-ATPase. Based on the E_2P crystal structure of Ca^{2+}-ATPase (Toyoshima et al. 2004. *Nature.* 432:361–368), we constructed a homology model for the ouabain-binding site of Na,K-ATPase in which most of the amino acids we found, as well as several amino acids postulated earlier, play crucial roles.

21. GPCR Signals and Intracellular Traffic of Na^+,K^+-ATPase. ALEJANDRO M. BERTORELLO, *Department of Medicine, Membrane Signaling Networks, Karolinska Institutet, Karolinska University Hospital-Solna, 171 76 Stockholm, Sweden* (Sponsor: David Gadsby)

The establishment of cell polarity in transporting epithelia requires the localization of ion transport proteins (e.g., Na^+,K^+-ATPase) in specific domains of the cell. In response to G protein–coupled receptor signals (such as dopamine, parathyroid hormone), a decrease in Na^+,K^+-ATPase activity from renal epithelial cells is mediated by endocytosis of active units, whereas stimulation of its activity in renal (angiotensin) and lung epithelial cells (isoproterenol, dopamine) is the result of increased recruitment from intracellular organelles (endosomes) to the plasma membrane. It is thus important for the development and maintenance of such polarized structures that during regulation by receptor signals the Na^+,K^+-ATPase units be sorted adequately. Therefore, it is envisioned that movement of Na^+,K^+-ATPase molecules from, or into, the plasma membrane during their regulation by receptor signals would require a highly developed organization and synchronization of spatial and temporal interactions between many signaling networks. An example of such signal compartmentalization is provided by the renal and lung epithelia, where dopamine regulates the same target, the Na^+,K^+-ATPase, by operating different signaling networks that result in opposite consequences for activity of the target.

22. Significance of the Conserved Cardiac Glycoside Binding Site of the α2 Isoform of the Na,K-ATPase. JERRY B. LINGREL,[1] IVA DOSTANIC,[1] JOHN LORENZ,[2] JAMES W. VAN HUYSSE,[3] and JONATHAN NEUMANN,[1] [1]*Department of Molecular Genetics, Biochemistry, and Microbiology and* [2]*Department of Molecular and Cellular Physiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267*; [3]*University of Ottawa Heart Institute, Hypertension Unit, Ottawa, Ontario, Canada*

The Na,K-ATPase contains a highly conserved cardiac glycoside binding site, which occurs in organisms as diverse as *Drosophila*, frogs, chickens, mice, and humans. One exception is the α1 isoform of rodents, which is relatively insensitive to this class of compounds. It is reasonable to hypothesize that this conserved binding site plays a biological role. To test this, we developed animals where the α2 isoform of the Na,K-ATPase of mice is made resistant to ouabain. These animals survive and have normal development, survival rate, and basal cardiovascular hemodynamics. However, when animals are treated with ACTH, which induces hypertension in wild-type animals, no increase in blood pressure occurs in animals with the ouabain-resistant α2 isoform. These findings indicate that the cardiac glycoside binding site plays a physiological role, at least in ACTH-induced hypertension, and further suggests that there is an endogenous ligand interacting with this site. The most logical candidate is the endogenous cardiac glycosides, which have been identified by several laboratories. Our studies indicate that these compounds increase in both the wild-type and targeted mice. The conclusion from our studies is that the cardiac glycoside binding site of the Na,K-ATPase plays an intrinsic physiological role.

23. Gene Targeting Studies of Ca^{2+}-transporting ATPases. GARY E. SHULL,[1] GBOLAHAN W. OKUNADE,[1] MARIAN MILLER,[2] and VIKRAM PRASAD,[1] [1]*Department of Molecular Genetics, Biochemistry, and Microbiology and Department of Environmental Health*,[2] *University of Cincinnati College of Medicine, Cincinnati, OH 45267* (Sponsor: Jack Kaplan)

Ca^{2+} gradients required for Ca^{2+}-signaling and homeostasis are maintained by P-type plasma membrane and intracellular Ca^{2+}-ATPases. Gene-targeting technology is being used to systematically analyze the biological functions of specific Ca^{2+}-ATPase isoforms. The phenotypes of mice carrying null mutations in plasma membrane Ca^{2+}-ATPases PMCA1, PMCA2, and PMCA4 (Kozel et al. 1998. *J. Biol. Chem.* 273:18693–18696; Okunade et al. 2004. *J. Biol. Chem.* 279:33742–33750; Reinhardt et al. 2004. *J. Biol. Chem.* 279:42369–42373; Schuh et al. 2004. *J. Biol. Chem.* 279:28220–28229) indicate that PMCA1 serves essential housekeeping functions, whereas PMCA2 and PMCA4 serve more specialized physiological functions. PMCA1 null mutants die during the early stages of embryogenesis, and loss of a single copy of the PMCA1 gene can exacerbate an apoptosis phenotype observed in vascular smooth muscle of PMCA4 null mice. Although PMCA4 is widely expressed and is the most abundant isoform in many tissues, null mutants appear to be healthy. However, male PMCA4 null mutants are infertile due to a failure of hyperactivated sperm motility resulting from the absence of PMCA4 in the principal piece of the sperm tail, where it serves as the major Ca^{2+} extrusion mechanism controlling sperm Ca^{2+} concentrations. Loss of PMCA2 in sensory hair cells of the inner ear causes profound deafness and balance defects, and loss of PMCA2 in lactating mammary glands causes a severe deficit in the Ca^{2+} concentrations in milk. Homozygous null mutations in sarco(endo)plasmic reticulum Ca^{2+}-ATPase isoform 2 (SERCA2) leads to embryolethality, whereas loss of a single copy of the gene causes impaired cardiac contractility and squamous cell tumors involving keratinized epithelial cells (Prasad et al. 2004. *Biochem. Biophys. Res. Comm.* 322:1192–1203). The latter finding provides the first direct demonstration that a perturbation of Ca^{2+} homeostasis or signaling can be a primary initiating event in cancer. [Supported by NIH grant HL61974.]

Poster Abstracts

24. Purification of Na,K-ATPase Expressed in *Pichia Pastoris*: Specific Interactions with Lipids. EYTAN COHEN,[1] RIVKA GOLDSHLEGER,[1] DANIEL M. TAL,[1] CHRISTINE EBEL,[2] MARC LE MAIRE,[3] and STEVEN J.D. KARLISH,[1] [1]*Department of Biological Chemistry, Weizmann Institute of Science, Rehovot, Israel*; [2]*Institut de Biologie Structurale J.P. Ebel, UMR 5075 CEA-CNRS-UJF, F-38027 Grenoble, Cedex 01, France*; [3]*Unité de Recherche Associée CNRS 2096, Commissariat à l'Energie Atomique Saclay, 91191 Gif-sur-Yvette cedex, France* (Sponsor: David Gadsby)

Na^+,K^+-ATPase (porcine α/his_{10}-β) has been expressed in *Pichia Pastoris*, solubilized in n-dodecyl-β-maltoside and purified by metal chelate bead chromatography combined with size exclusion HPLC. The recombinant protein is inactive if the purification is done without added phospholipids. The neutral phospholipid dioleoyl phosphatidylcholine (DOPC) preserves Na^+,K^+-ATPase activity of protein prepared in a Na^+-containing medium, but activity is lost in a K^+-containing medium. By contrast, the acid phospholipid dioleoyl phosphatidylserine (DOPS) preserves activity in either Na^+- or K^+-containing media. The presence of cholesterol inhibits Na,K-ATPase activity measured at 0°C, while at 37°C, cholesterol stabilizes the protein against thermal inactivation without affecting Na,K-ATPase activity. The stabilizing effect of cholesterol is detectable in the presence of DOPC and not in the presence of DOPS. In optimal conditions, the protein is stable for about 2 wk at 0°C. Both recombinant Na^+,K^+-ATPase and native pig kidney Na^+,K^+-ATPase, dissolved in n-dodecyl-β-maltoside, appear to be mainly stable monomers (α/β), as judged by size exclusion chromatography and sedimentation velocity. Na^+,K^+-ATPase activities at 37°C of the size exclusion HPLC-purified recombinant and renal Na^+,K^+-ATPase are comparable but are lower than that of membrane-bound renal Na^+,K^+-ATPase. The β subunit is expressed in *Pichia Pastoris* as two lightly glycosylated polypeptides and is quantitatively deglycosylated by endoglycosidase-H at 0°C, to a single polypeptide. Deglycosylation inactivates Na^+,K^+-ATPase prepared with dioleoyl phosphatidylcholine, whereas dioleoyl phosphatidylserine protects after deglycosylation, and Na^+,K^+-ATPase activity is preserved. This work demonstrates an essential role of phospholipid interactions with Na^+,K^+-ATPase, including a direct interaction of dioleoyl phosphatidylserine, probably another interaction of either the neutral or acid phospholipids, as well as cholesterol. A role for the β subunit in stabilizing conformations of Na^+,K^+-ATPase (or H^+,K^+-ATPase) with occluded K^+ ions can also be inferred. Purified recombinant Na^+,K^+-ATPase could become an important experimental tool for various purposes including, hopefully, structural work.

25. Thermal Stability of a Thermophilic P-type ATPase. F. LUIS GONZÁLEZ FLECHA,[1] DIEGO I. CATTONI,[1] ATIN K. MANDAL,[2] DIPTI SHARMA,[3] GERMANO S. IANNACCHIONE,[3] and JOSÉ M. ARGÜELLO,[2] [1]*Instituto de Química y Fisicoquímica Biológicas, Universidad de Buenos Aires-CONICET, Argentina*; [2]*Department of Chemistry and Biochemistry and* [3]*Department of Physics, Worcester Polytechnic Institute, Worcester, MA* (Sponsor: Jack H. Kaplan)

Protein stability is the result of a delicate balance between stabilizing and destabilizing interactions. While thermal denaturation of globular proteins is a well-characterized process, little is known about the thermal stability of membrane proteins. In addition, the lack of information on the stability of thermophilic membrane proteins is remarkable. The aim of this work was to initiate the characterization of the thermal denaturation process of CopA, a thermophilic P_{IB}-type Cu-ATPase from *Archaeoglobus fulgidus*. CopA was heterologously expressed in *Escherichia coli*, solubilized in dodecylmaltoside (DDM), and affinity purified. The resulting en-

zyme retained thermophilic characteristics with maximum activity at 75°C and an E^a = 103 kJ/mol. DSC analysis of CopA showed a thermal transition at 81°C, a value significantly higher than that determined for mesophilic P-type ATPases. The presence of ATP-Mg further stabilized the protein, shifting its T_m to 105°C. As expected, CopA denaturation was found to be much slower than that of mesophilic P-type ATPases in similar conditions. The enzyme preparation incubated at 75°C showed an irreversible exponential decrease in enzyme activity and intrinsic fluorescence intensity. This inactivation was not associated with either fragmentation or formation of SDS-stable aggregates of the protein. Moreover, the first-order rate of thermal inactivation suggests a two-state process involving only fully active and inactive molecules. CopA reconstitution in mixed micelles of asolectin and DDM before the inactivation further increased the enzyme stability. These results indicate that thermophilic membrane proteins are more stable than their mesophilic counterparts, that they retain their stability even when heterologously expressed, and therefore, that their stability appears to depend largely on intramolecular interactions. [Supported by NSF grants MCM-0235165 and OISE-0436435 to J.M. Argüello, and DMR-0092786 to G.S. Iannacchione, and ANPCyT grant PICT-11138 to F.L.G. Flecha.]

26. The Modulatory ATP Binding Site of the Calcium Pump. ANNE-MARIE LUND JENSEN,[1] THOMAS LYKKE-MØLLER SØRENSEN,[1] JESPER VUUST MØLLER,[3] and POUL NISSEN,[1] [1]*Department of Molecular Biology and* [2]*Department of Biophysics, University of Aarhus, Aarhus, Denmark*

In skeletal muscles, the dominant P-type ATPase is sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA). SERCA is responsible for the reuptake of cytosolic Ca^{2+} (released during muscle contraction) into the sarcoplasmic reticulum. The active transport by P-type ATPases is fueled by ATP and involves the formation of a covalent aspartyl-phosphoanhydride intermediate. How ATP is involved as the key substrate in formation of the E1~P state has now become well characterized at the biochemical and structural level (Sørensen et al. 2004. *Science.* 304:1672–1675). However, ATP also exhibits a general, stimulatory effect on the functional transitions relating to the E2 states (Stahl et al. 1984. *Biochemistry.* 23:5389–5392), which indicates the existence of a noncatalytic, modulatory site.

We have determined the crystal structure of SERCA in the E2 state with the inhibitor thapsigargin and in presence and absence of the ATP analogue AMPPCP at 3.1 Å and 2.8 Å resolution, respectively. The E2:TG:AMPPCP structure shows important aspects of the modulatory binding site that indeed are different from the catalytic site. Although the N-domain interacts with the adenosine moiety in essentially the same way as in the Ca2E1-AMPPCP state, it is the Glu439 residue of the N-domain, rather than the phosphorylation site centered on Asp351 of the P-domain, that interacts with the phosphate groups of ATP. This is consistent with iron-cleavage data and biochemical studies of an E439A mutant (Patchornik et al. 2002. *Biochemistry.* 41: 11740–11749; Inesi et al. 2004. *J. Biol. Chem.* 279:31629–31637).

We anticipate that the modulatory ATP binding site is fully occupied at typical physiological conditions with millimolar ATP concentrations available. We therefore propose a new route of reactivation of the functional cycle of SERCA, going directly from an E2-ATP state to the Ca2E1-ATP state. [Supported by the Danish Medical Science Research Council, the Novo Nordisk Foundation, and the Lundbeck Foundation.]

27. Crystallization of a Mammalian Membrane Protein Overexpressed in *Saccharomyces cerevisiae.* MARIE JIDENKO,[1] RIKKE C. NIELSEN,[2] THOMAS LYKKE-MØLLER SØRENSEN,[2] JESPER V. MØLLER,[3] MARC LE MAIRE,[1] POUL NISSEN,[2] and CHRISTINE JAXEL,[1] [1]*Unité de Recherche Associée 2096 of the Centre National de la Recherche Scientifique and Service de Biophysique des Fonctions Membranaires, Département de Biologie Joliot Curie, CEA Saclay, 91191 Gif sur Yvette Cedex, and Laboratoire de Recherche Associé 17V and Institut Fédératif de Recherches 46, Université Paris Sud, Paris, France;* [2]*Department of Molecular Biology and* [3]*Department of Biophysics, Institute of Physiology and Biophysics, University of Aarhus, DK-8000 Aarhus C, Denmark* (Sponsor: Philippe Champeil)

The Ca^{2+}-ATPase SERCA1a from rabbit has been overexpressed in *Saccharomyces cerevisiae.* This membrane protein was purified by avidin agarose affinity chromatography based on natural biotinylation in the expression host, followed by HPLC gel filtration. Both the functional and structural properties of the overexpressed protein validate the method. Thus, calcium-dependent ATPase activity and calcium transport are intact after reconstitution in proteoliposomes. Moreover, the recombinant protein crystallizes in a form that is isomorphous to the native SERCA1a protein from rabbit and the diffraction properties are similar. Even if other protein purification methods based on biotin–avidin interaction were used, this is the first example of a successful crystallization of a mammalian membrane protein derived from a heterologous expression system, and it opens the door for the study of mutant forms of

SERCA1a. This procedure is likely to be successful also in the case of other eukaryotic membrane proteins, which are generally difficult to purify and crystallize.

28. Atomic Structure of a Covalently Phosphorylated Intermediate of SERCA1 Ca^{2+}-ATPase: Normal Mode Fits of Electron Densities. EDWARD BEAUMONT,[1] BERTRAND FOURNIER,[2] DAVID STOKES,[3] KONRAD HINSEN,[2] and JEAN-JACQUES LACAPERE,[1] [1]*U683 INSERM 16, F-75870 Paris Cédex 18,* [2]*Laboratoire Léon Brillouin (CEA-CNRS), F-91191 Gif sur Yvette Cedex, Paris, France;* [3]*Skirkball Institute of Biomolecular Medicine, New York University, New York, NY*

Three-dimensional structure of a stabilized phosphoenzyme intermediate of the Ca^{2+}-ATPase has been recently obtained using cryoelectron microscopy of two-dimensional crystals (Stokes et al. 2005. *J. Biol. Chem.* 280: 18063–18072). The electron microscopy (EM) images show tubular crystals formed in the presence of decavanadate where the protein dimers are arranged in rows winding around the tubes. The structure was solved at 8-Å resolution. In a first step, a single protein has been extracted and characterized. We used a recently published method (Hinsen et al. 2005. *Biophys. J.* 88:818–827) for the flexible docking of high-resolution structures into the EM density for this new conformation of the same protein. In brief, the structure fitting consists of an initial step of orientation of the atomic structure relative to the EM density, and an iterative deformation of the structure using a set of low energy normal modes was used to improve the fit of the EM density. We previously showed that normal modes can describe the massive domain changes that the Ca-ATPase undergoes in its catalytic cycle (Reuter et al. 2003. *Biophys. J.* 85:2186–2197). The atomic structures of different reaction intermediates of the Ca-ATPase (Toyoshima et al. 2002. *Nature.* 418:605–611; Sorensen et al. 2004. *Science.* 304:1672–1675; Toyoshima et al. 2004. *Nature.* 430:529–535) were used as starting points leading to different fitted models that were compared. The motions associated with the conformational transition between initial and fitted-final models were further analyzed. In a second step, part of the crystalline tube was used and several Ca-ATPases were fitted into it. Final fitted models obtained after flexible docking were used to characterize the protein–protein interactions within the crystal and the atomic location of the decavanadate complexes.

29. Structural Changes in Sodium Potassium ATPase Induced by Ion Binding and Membrane Cholesterol Depletion. CLAUS HELIX NIELSEN,[1] SALIM ABDALI,[1] JENS AUGUST LUNDBÆK,[2] and FLEMMING CORNELIUS,[3] [1]*Quantum Protein Center, Technical University of Denmark, Lyngby, Denmark;* [2]*Department of Physiology and Biophysics, Weill Medical College, Cornell University, New York, NY;* [3]*Department of Biophysics, University of Aarhus, Aarhus, Denmark*

We have used Raman spectroscopy (30 mW at λ = 532 nm, 5 min acquisition time) as a tool to investigate conformational changes of the unphosphorylated sodium-potassium-adenosine-triphosphatase (Na^+,K^+-ATPase) enzyme from shark rectal gland that occur upon sodium ion binding or membrane cholesterol depletion. Using the well-established effect of buffer composition on the E2–E1 conformational equilibrium, our results show that the protein embedded in native membrane fragments becomes more helical when going from the E2 to the E1 conformation (i.e., upon binding sodium) as evidenced by the changes in the amide I and III bands. Upon cholesterol depletion with methylated-β-cyclodextrin (20 mM for 30 min at 20°C), the $E1_{CholDepl}$ and $E2_{CholDepl}$ spectra are similar and both resemble the E1 spectrum more than the E2 spectrum in the amide I and III bands. For the four conformers (E1, E2, $E1_{CholDepl}$, $E2_{CholDepl}$) the region between 1020 and 1120 cm^{-1} is characterized by at least five overlapping peaks. All conformers share a peak at 1060 cm^{-1}, but E1, $E1_{CholDepl}$, and $E2_{CholDepl}$ all have higher intensities at higher wavenumber bands compared with the E2 spectrum in that region, consistent with the changes observed for the amide I and III bands. In all conformers, the I_{825}/I_{853} tyrosine doublet in the spectra is not significantly changed, indicating no differences in the hydrogen-bonding environment, which poses constraints on models for the E2→E1 transition both in normal and cholesterol-depleted membranes. Taken together, our results show that (part of) the conformational changes in the Na^+,K^+-ATPase upon sodium binding can be mimicked by depletion of membrane cholesterol. This suggests that membrane protein function can be regulated by altering membrane physical properties. [Supported by the Danish National Research Foundation.]

30. Dephosphorylation of the Calcium pump Coupled to Counterion Occlusion. CLAUS OLESEN,[1] THOMAS LYKKE-MØLLER SØRENSEN,[2] RIKKE C. NIELSEN,[2] ANNE-MARIE L. JENSEN,[2] JESPER VUUST MØLLER,[1] and POUL NISSEN,[2] [1]*Department of Physiology and Biophysics and* [2]*Centre for Structural Biology, University of Aarhus, Aarhus, Denmark*

To understand the dephosphorylation mechanism and to reveal the intramolecular coupling between cat-

ion transport and ATP hydrolysis, we crystallized sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA1a) in complex with aluminium fluoride. This represents the transition state of hydrolysis of the counterion-bound (protonated) phosphoenzyme (Olesen et al. 2004. *Science* 306: 2251–2255). The planar aluminium fluoride group is located between the conserved Asp351 side chain and a water molecule, thus representing the transition state of hydrolysis of the phosphoenzyme. The water molecule is positioned, and activated for a nucleophilic attack, by Ser181 and Glu183 of the conserved TGES motif of the A-domain. This arrangement overlaps with the position of ADP:AlF_4^- in phosphoryl transfer in the E1~P structure (Sorensen et al. 2004. *Science* 304:1672–1675). The domain movements associated with the formation of the dephosphorylation site depend on the release of ADP after ATP phosphorylation, and the dephosphorylation reaction cannot proceed before the bound Ca^{2+} ions have been exchanged for protons that become occluded. The helix bundle constituting the proper arrangement of the dephosphorylation site for catalytic activity is stabilized by an integral K^+ site (Sorensen et al. 2004. *J. Biol. Chem.* 279:46355–46358), explaining the stimulatory effect of monovalent cations on dephosphorylation. The new structure provides a rationale for the vectorial transport of Ca^{2+} and couples it with the counterion exchange needed for dephosphorylation.

31. Purification, Kinetic Characterization, and Initial Crystallization of an Archaeal P-type ATPase. BJØRN PANELLA PEDERSEN,[1] THOMAS LYKKE-MØLLER SØRENSEN,[2] and POUL NISSEN,[1] [1]*Department of Molecular Biology, University of Aarhus, Aarhus, Denmark*; [2]*Diamond Light Source Ltd., Rutherford Appleton Laboratory, Oxfordshire, UK*

The sarcoplasmic reticulum Ca^{2+}-ATPase from skeletal muscle (SERCA 1a) is so far the only P-type ATPase cation pump for which atomic structures are available. We therefore aim at structure-based investigations of other P-type ATPases, and we describe the successful cloning and heterologous expression of a number of bacterial and archaeal P-type ATPases.

A large group of P-type ATPases comprising both bacterial and eukaryotic proteins is specific to soft cations such as Cu^{2+} and Zn^{2+} and these cation pumps are essential for many detoxification systems and for maintaining the intracellular metal-ion homeostasis in cells. The best known example being the Wilson and Menkes proteins from humans involved in intracellular copper trafficking.

A P-type ATPase from the archaeal species *Thermoplasma acidophilum* (gene code TA1143) shows sequence homology to the Cu/Ag transporting ATPases and is found to be highly expressed in *Escherichia coli* membranes. Solubilization in DDM followed by affinity chromatography and size-exclusion chromatography yields up to 3–4 mg pure, monodisperse protein per liter *E. coli* culture.

Using a *para*-nitrophenyl phosphate assay, the protein expressed is found to be an active ATPase with a V_{max} of 200 nmol/(mg*min) and a K_m of 5.5 mM. This activity can be competitively inhibited by the addition of ATP or an ATP homologue and it is dependent on Mg^{2+} ions as expected for a P-type ATPase. Identification of the cation specificity is currently in progress.

Crystallization screening using the vapor diffusion technique has produced several hits. The use of the detergent $C_{12}E_8$ proved to be critical for the formation of protein crystals.

32. The Average Conformation at Micromolar [Ca^{2+}] of Ca^{2+}-ATPase with Bound Nucleotide Differs from that Adopted with the Transition State Analogue ADP.AlFx or with AMPPCP Under Crystallization Conditions at Millimolar [Ca^{2+}]. MARTIN PICARD,[1] CHIKASHI TOYOSHIMA,[2] and PHILIPPE CHAMPEIL,[1] [1]*Unité de Recherche Associée 2096 (CNRS) and Service de Biophysique des Fonctions Membranaires (DBJC, CEA), 91191 Gif-sur-Yvette Cedex, France*; [2]*Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan*

Crystalline forms of detergent-solubilized sarcoplasmic reticulum Ca^{2+}-ATPase, obtained in the presence of either a substrate analogue, AMPPCP, or a transition state complex, ADP.fluoroaluminate, were recently described to share the same general architecture, despite the fact that, when studied in a test tube, these forms show different functional properties. Here, we show that the differences in the properties of the E1.AMPPCP and the E1.ADP.AlFx membranous (or solubilized) forms are much less pronounced when these properties are examined in the presence of 10 mM Ca^{2+} (the concentration prevailing in the crystallization media) than when they are examined in the presence of the few μM Ca^{2+} known to be sufficient to saturate the transport sites. This concerns various properties, including ATPase susceptibility to proteolytic cleavage by proteinase K, ATPase reactivity toward SH-directed Ellman's reagent, ATPase intrinsic fluorescence properties (here described for the E1.ADP.AlFx complex for the first time), and also the rates of $^{45}Ca^{2+}$–$^{40}Ca^{2+}$ exchange at site "II." These results solve the above paradox at least partially and suggest that the presence of a previously unrecognized Ca^{2+} ion in the

Ca^{2+}-ATPase.AMPPCP crystals should be reinvestigated. On the other hand, they emphasize the fact that the average conformation of the E1.AMPPCP complex under usual conditions in the test tube differs from that found in the crystalline form. The extended conformation of the nucleotide revealed in the E1.AMPPCP crystalline form might be indicative only of the requirements for further processing of the complex, toward the transition state leading to phosphorylation and Ca^{2+} occlusion. [Supported by HFSPO grant RGP 0060/2001-M.]

33. Structure of Na.K-ATPase as Analyzed by Cryoelectron Microscopy. PASI PURHONEN,[1] HANS HEBERT,[1] KAREN THOMSEN,[2] and ARVID B. MAUNSBACH,[2] [1]*Department of Biosciences, Karolinska Institutet and School of Technology and Health, Royal Institute of Technology, S-14157 Huddinge, Sweden*; [2]*The Water and Salt Research Center, Department of Cell Biology, Institute of Anatomy, University of Aarhus, DK-8000 Aarhus, Denmark*
(Sponsor: David Gadsby)

We have analyzed the molecular structure of Na,K-ATPase by electron crystallography from frozen-hydrated two-dimensional crystals induced in purified membranes from the outer medulla of pig kidney. Data were collected from 141 small Na,K-ATPase 2-D crystals in E2 conformation, and used for determination of a 3-D structure at <10 Å. The Na,K-ATPase protomer contains all three subunits (α, β, and γ) present in the kidney. We have compared our Na,K-ATPase model from cryoelectron microscopy to the atomic structure of the related Ca-ATPase in different conformations obtained by x-ray crystallography in the laboratories of Toyoshima (Toyoshima et al. 2000. *Nature.* 405:647–655; 2002. 418:605–611; 2004. 430:529–535; 2004. 432: 361–368) and Nissen (Nissen et al. 2004. *Science.* 304: 1672–1675; 2004. 306:2251–2255). The best fit between these two ATPases is observed in the transmembrane region, where the Na,K-ATPase structure shows two groups of distinct densities, being composed of the 10 helices from the catalytic α subunit. Extra density areas in the transmembrane region suggest the positions for the single helices arising from the Na,K-ATPase β and γ subunits, which are absent in Ca-ATPase. Thus, comparisons with Ca-ATPase suggest that the γ subunit of Na,K-ATPase is located in close association with the M2 and M9 transmembrane helices. The overall structure of the α subunit of Na,K-ATPase with respect to the nucleotide binding (N), phosphorylation (P), and actuator (A) domains is similar to the X-ray structure of Ca-ATPase in E2 conformation. The angular difference for the N-domain position of Na,K-ATPase in the observed E2 and in the modeled E1 conformation is ~40°, similar to the difference observed between the E1 and E2 forms of Ca-ATPase. The present observations and comparisons suggest that large conformational changes occur also between the E1 and E2 forms of Na,K-ATPase.

34. Implications of SERCA Structural Models for ATP Binding Events in Na,K-ATPase ("NaKA"). JOHN S. WILLIS and MARK A. MILANICK, *Department of Cellular Biology, University of Georgia, Athens, GA 30601*; *Department of Medical Pharmacology and Physiology, School of Medicine, and Dalton Cardiovascular Research Center, University of Missouri, Columbia, MO 65211*

Based on SERCA structural models, ATP is thought to bind to the N-domain in E1 when N and P domains are separated and there is open access to the nucleotide binding site (PDB 1SU4). Subsequently, the N and P domains converge and the γ-phosphate approaches D351. The close apposition of N and P domains encloses the ATP binding site at this stage and continues to surround it throughout the remainder of the pump cycle. In NaKA, this scenario raises the questions of when ADP can escape and ATP can bind. In NaKA 1 mM ATP binds the enzyme in E2 and promotes the conversion from E2 to E1. In the SERCA model for E2 (1IWO), the ATP binding site is obscured. Similarly, the occurrence of ADP-ATP exchange in NaKA indicates that the binding site is available at E1PADP, even though the SERCA model for this stage is tightly closed (1WPE, F487-D351, 11 Å). However, the subsequent SERCA structure (E2P, 1WPG), although still loosely closed, shows a gap between N and P large enough to allow the passage of nucleotides to the interior. In 1WPG, however, the F487-D351 distance is not only longer than an ATP (27 Å vs. 14 Å), but the TGES region from the A domain also intrudes between N and P. Thus, while the nucleoside head of ATP could slip into the N pocket, the γ-phosphate could not approach D351. With conversion to E1, however, ATP would be well positioned to phosphorylate D351. This scheme offers several explanations for low affinity in E2 (constrained geometry, limited time) and the possibility that the fully open configuration of SERCA (1EUL) might not exist during the NaKA pump cycle at physiological [ATP]. It also offers the prediction that ATP is entrapped during the interval between E2 and E1P.

35. Protonation of Ca^{2+}-ATPase Residues upon Ca^{2+} Release. JULIA ANDERSSON,[1] KARIN HAUSER,[2] and ANDREAS BARTH,[1] [1]*Department of Biochemistry*

and Biophysics, Stockholm University, Stockholm, Sweden; [2]*Institute for Biophysics, Johann Wolfgang Goethe-University, Frankfurt am Main, Germany*

We have studied protonation of the sarcoplasmic reticulum Ca^{2+}-ATPase during Ca^{2+} release and E2P formation ($Ca_2E1 \rightarrow E2P$) using rapid scan Fourier transform infrared spectroscopy. The reaction has been investigated from pH 6.0 to 9.0. Infrared spectra show four signals in the spectral region of protonated carboxyl groups at pH 6.0–7.5 and only two at pH 8.0–9.0. The results show that at least two of the protonated carboxyl groups of E2P have a pK of ~7.7. We have concluded that these carboxyl groups participate in H^+ countertransport. To identify these carboxyl groups and to assign the IR bands, multiconformation continuum electrostatic calculations (MCCE) have been performed to calculate the residues' ionization, at various pH, for the calcium-free and the calcium-occluded structure, respectively. The combination of infrared measurements and MCCE calculations clearly indicates that Asp^{800} is involved in the proton countertransport whereas Glu^{309} is not. The second carboxyl group involved in the countertransport might be Glu^{908}. Our results also indicate a pH-dependent conformational change in a β-sheet or turn structure of E2P.

Additionally, we have tentatively assigned a band to the C=O bond of the phosphorylated Asp 351. Based on infrared data, we concluded that the bond strength is essentially unchanged but is slightly reduced in E2P compared with Ca_2E1P. This reduction is larger when Mg^{2+} is bound to the aspartyl phosphate.

36. Role of the α Subunit Second Extracellular Loop in the Accessibility of K^+ Ions to their Binding Site in Na,K-ATPase. OIHANA CAPENDEGUY and JEAN-DANIEL HORISBERGER, *Department of Pharmacology and Toxicology, University of Lausanne, Lausanne, Switzerland* (Sponsor: J.-D. Horisberger)

By homology with the structure of the SERCA and supported by experimental studies, the fourth, fifth, and sixth transmembrane helices of the α subunit of Na,K-ATPase have been proposed to participate in Na^+ and K^+ binding sites. To improve the understanding of control of access of K^+ ions to their binding sites from the extracellular side, we have focused on the second extracellular loop linking transmembrane segments 3 and 4. To this end we have mutated 12 residues of this loop in the rat α1 subunit, from E314 to G326, into cysteine and studied these mutants by electrophysiological techniques. We measured the functional expression and the accessibility of the cysteines to a membrane-impermeant cationic thiol reagent (MTSET); and, in selected mutants, we studied the voltage-dependent activation by extracellular K^+, and the effect of MTSET binding on activation by K^+ and on the presteady-state ouabain-sensitive current. Several mutations resulted in a large increase of the apparent affinity for extracellular K^+ both in the presence and in the absence of extracellular Na^+. Four mutants (E314C, Y315C, W317C, and I322C) were strongly modified by MTSET, three of these were inhibited and one E314C was stimulated. The effects of the E314 mutation (reduced Vmax, increased apparent affinity for K^+, decreased maximal translocated charges) were all corrected by MTSET treatment. For W317C and I322C, MTSET induced a right shift of the midpoint potential, indicating an increase of the extracellular affinity for Na^+. These results combined with those of a preceding study (Capendeguy and Horisberger. 2005. *J. Physiol.* In press) on the third extracellular loop show that these two loops have a complementary function, controlling the accessibility of K^+ ions to their binding site from the extracellular side by a kind of "gates game" and modulating the E1/E2 equilibrium. [Supported by the Swiss National Fund grant 31-65441.01 to J.-D. Horisberger.]

37. The Cadmium Transport Site of CadA, the Cd^{2+}-ATPase from *Listeria monocytogenes*. CHEN-CHOU WU, ANNE MARTEL, AURELIE GARDARIN, ELISABETH MINTZ, FLORENT GUILLAIN, and PATRICE CATTY, *Laboratoire de Biophysique Moléculaire et Cellulaire, UMR 5090 CEA-CNRS-Université Joseph Fourier, CEA/DRDC/BMC, 38054 Grenoble Cedex 9, France*

The $Zn^{2+}/Cd^{2+}/Pb^{2+}$-ATPases constitute a bacterial subfamily of P1-type ATPases that behave as detoxification pumps, among which is found CadA, the Cd^{2+}-ATPase from *Listeria monocytogenes* (Bal et al. 2001. *FEBS Lett.* 506:249–252; Bal et al. 2003. *Biochem. J.* 369:681–685).

Whereas it is the major determinant of the resistance to Cd^{2+} in *L. monocytogenes*, CadA expressed in the yeast *Saccharomyces cerevisiae* did just the opposite to what was expected, as it strikingly decreased the Cd^{2+} tolerance of these cells. Yeast cells expressing the nonfunctional CadA mutant $Asp^{398}Ala$ could grow on selective medium containing up to 100 μM Cd^{2+}, whereas those expressing the functional protein could not grow in the presence of 1 μM Cd^{2+}. The CadA-GFP fusion protein was localized in the endoplasmic reticulum membrane, suggesting that yeast hypersensitivity was due to Cd^{2+} accumulation in the reticulum lumen (Wu et al. 2004. *Biochem. Biophys. Res. Comm.* 324:1034–1040).

This phenotype was used as a powerful tool to select among 35 mutations in the transmembrane region of

CadA, those that could affect the activity of the protein. Functional studies of the selected mutants produced either in yeast or in Sf9 cells revealed that not only the two cysteines of the canonical CPC motif, but also amino acids located in the third, fourth, and eighth transmembrane helices, were important for CadA activity. This leads us to propose a metal transport site different from those recently proposed for the Cu^+-ATPases, Ccc2p from *S. cerevisiae* (Lowe et al. 2004. *J. Biol. Chem.* 279:25986–25994) and CopA from *Archeoglobus fulgidus* (Mandal et al. 2004. *J. Biol Chem.* 279:54802–54807).

38. Testing the Accuracy of Homology Modeling: Similar Inhibition Patterns by Cytosolically Acting Organic Cations on Na,K- and PM Ca-ATPase. CRAIG GATTO,[1] JEFF B. HELMS,[1] SHENG-YOU HUANG,[2] XIAOQIN ZOU,[2] KRISTA L. ARNETT,[3] and MARK A. MILANICK,[3] [1]*Department of Biological Sciences, Illinois State University, Normal, IL*; [2]*Dalton Cardiovascular Research Center and Department of Biochemistry and* [3]*Department of Medical Pharmacology and Physiology*, University of Missouri, Columbia, MO (Sponsor: Jack H. Kaplan)

We studied the effect of organic cations on the inhibition of the Na,K pump (sheep kidney) and the PM Ca pump (cow erythrocytes). Homology models suggested that the monovalent cations, guanidine and tetramethyl guanidine (TMG), would inhibit both pumps at cytoplasmic accessible binding sites. On the Na pump, inhibition of ATPase activity by guanidine, TMG, and diamine was competitive with Na, and their inhibition of pNPPase, but not of ATPase, was competitive with K, consistent with these organic cations binding predominantly from the inside. The concentration dependence for guanidine and TMG inhibition was consistent with cooperative binding of two inhibitor molecules. These results are in contrast with a known extracellular site inhibitor, tetrapropylammonium (TPA), which does compete with K for ATPase but not with Na (Gatto et al. 2005. *Am. J. Physiol. Cell Physiol.* In press). As also predicted by homology modeling, guanidine and TMG were competitive with calcium for PM Ca-ATPase activity. Again, the concentration dependence of inhibition was consistent with cooperative binding of two inhibitor molecules. Interestingly, the modeling of PMCA predicted that diamine inhibition should differ from guanidine. Subsequent experiments confirmed that diamine inhibited PMCa, but it was not competitive with calcium. Moreover, diamine inhibition was not cooperative and could be accounted for with single molecule binding. In addition, we observed that the extracellular site inhibitor TPA poorly inhibited the PM Ca pump (<10% inhibition at 100 mM TPA). The homology models of both the Na,K and PM Ca pumps provide likely explanations for TPA exclusion from cytoplasmic binding. Even though the models were based on the SERCA structure with occluded calcium (a conformation not expected to bind cytosolic calcium), we were able to predict the distinct cytosolic inhibitor binding characteristics of the Na,K pump and PM Ca pump. Further, these models held up to experimental testing. [Supported by NIH-DK37512 to M.A. Milanick, NIH-GM 061583 and AHA-030161N to C. Gatto, AHA-0315236Z to J.B. Helms, and AHA-0265293Z and NIH-DK61529 to X. Zou.]

39. The Third Na^+ Binding Site of Na,K-ATPase. CIMING LI, OIHANA CAPENDEGUY, KÄTHI GEERING, and JEAN-DANIEL HORISBERGER, *Department of Pharmacology and Toxicology, University of Lausanne, CH-1005 Lausanne, Switzerland* (Sponsor: J.-D. Horisberger)

Na,K-ATPase exports three intracellular sodium ions in exchange for two extracellular potassium ions. In the high resolution structure of the related sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase, two cation binding sites have been identified (Toyoshima et al. 2000. *Nature.* 405:647–655). The two corresponding sites in Na,K-ATPase are thought to be alternatively occupied by sodium and potassium ions, while the location of a third, sodium-specific site has been proposed on the basis of modeling and valence analysis (Ogawa and Toyoshima. 2002. *Proc. Natl. Acad. Sci. USA.* 99: 15977–15982), but has not been demonstrated experimentally. Mutants of residues in the fifth, sixth, and ninth transmembrane segments (TMS) of the α1 subunit of the rat Na, K-ATPase were expressed in *Xenopus* oocytes and studied by two-electrode voltage clamp to evaluate the effects of these mutations on the affinity for intra- and extracellular Na^+ and extracellular K^+. The voltage-dependent translocation of Na^+ to the extracellular medium was also studied by measurement of ouabain-sensitive presteady-state currents upon fast voltage perturbations. Mutation of E961 in TMS-9 did not alter K^+ affinity, but reduced both intracellular and extracellular sodium binding affinity, and altered the voltage-dependent kinetics of Na^+ translocation. Similarly, mutations of G813 and T814 from TMS-6 and of Y778 from TMS-5 altered the voltage-dependent sodium translocation. These results enabled us to define the location of a third sodium-specific binding site in a space between the fifth, sixth, eighth, and ninth transmembrane domains of the α subunit of the Na, K-ATPase at about the same level as the two previously defined cation sites. [Supported by the Swiss National

Fund grant 31-64793.01 to K. Geering and 31-65441.01 to J.-D. Horisberger.]

40. The Third Na^+ Binding Pocket Is Located Near the Amino Acid Residues Thr-774 and Gln-923 in Rat Na,K-ATPase. TOSHIAKI IMAGAWA, TETSUYA YAMAMOTO, SHUNJI KAYA, KAZUYASU SAKAGUCHI, and KAZUYA TANIGUCHI, *Biological Chemistry, Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo, Japan*

Na,K-ATPase, a member of the P-type ATPase family, is an integral membrane protein found in all mammalian cells and forms an acid-stable phosphoenzyme (EP) during the hydrolysis of ATP. Na,K-ATPase is an electrogenic pump that transports three Na^+ ions from the cytosol to the extracellular space and two K^+ ions in the opposite direction. The location of the two Na^+ binding sites were estimated from homology modeling based on the three-dimensional structure of Ca-ATPase, but that of the third site remains obscure. To obtain information concerning the third Na^+ binding site, highly conserved amino acids of rat Na,K-ATPase (Thr-774 in the transmembrane helix M5, Val-920 and Gln-923 in M8, and Glu-953 and Glu-954 in M9) were mutated, and the resulting proteins, T774A, E953A/K, and E954A/K, V920E, and Q923N/E/D/L, were expressed in HeLa cells. Ouabain-resistant cell lines were obtained from T774A, V920E, E953A, and E954A, while Q923N/E/D/L, E953K, and E954K could only be transiently expressed as fusion proteins with an EGFP. The apparent affinities for Na^+, as estimated by Na-dependent EP-formation or Na,K-ATPase activity, were decreased by around two- to eightfold in the case of T774A, V920E, and E954A. The apparent affinities for K^+, as estimated by Na,K-ATPase or K-dependent *p*-nitrophenylphosphatase activity, were only slightly affected by the three mutations, except that V920E showed a 1.7-fold decrease, as estimated by its Na,K-ATPase activity. The E953A mutant showed little significant change in its apparent affinities for Na^+ and K^+. Gln-923 in M8 was crucial for the active transport of Na^+ and/or K^+ across membranes, and the side chain oxygen atom of Thr-774 in M5 was important in the transport of Na^+ but not the transport of K^+. These results suggest that the third Na^+ binding site is located near the Gln-923 and Thr-774 in rat Na,K-ATPase.

41. Salt Induction of a Doubly Lysine-substituted α-subunit Isoform of the Na,K-pump of *Artemia franciscana*. PETER L. JORGENSEN[1] and FRANCISCO AMAT DOMÉNECH,[2] [1]*Institute of Molecular Biology and Physiology, University of Copenhagen, 2100 Copenhagen OE, Denmark*; [2]*Instituto de Acuicultura Torre de la Sal, Castellón, Spain*

Gene sequencing shows that two α-subunit isoforms of Na,K-ATPase are present in the brine shrimp *Artemia franciscana*. The α1-(NN)-subunit is ubiquitous, while the α2-(KK)-subunit, with two substitutions of asparagine by lysine at positions 324 and 776, is expressed exclusively in the salt gland of *Artemia* larvae. In functional studies with lysine substitutions at positions 324 and 776 in the α1-subunit of pig kidney Na,K-ATPase, the stoichiometry of ^{204}Tl binding is reduced from 2 to 1, and Na-dependent phosphorylation from ATP is reduced to 25–30%. To determine the expression of the α2-(KK)-subunit, adult *Artemia franciscana* were cultured for 3 wk with *Tunaliella algae* as fodder in seawater at concentrations increasing stepwise from 3 to 28% salt. Quantitative RT-PCR of transcripts of mRNA from these adult shrimps shows that the abundance of the mRNA of the α2-(KK)-subunit is very low at 3% salt, but it rises steeply in the range of 7–20% salt to levels similar to or higher than those of the mRNA of the α1-(NN)-subunit. Salt induction of the α2-(KK)-subunit of Na,K-ATPase with a reduced stoichiometric ratio of Na^+/ATP may be required for adaptation of the salt gland of *Artemia franciscana*. This can explain why the brine shrimps are the only animal to survive at the extreme salt concentration in tropical lakes.

42. Interaction of Cysteine with CopA, a P_{1B}-type Cu-ATPase from *Archaeoglobus fulgidus*. ATIN K. MANDAL, YING YANG, and JOSÉ M. ARGÜELLO, *Department of Chemistry and Biochemistry, Worcester Polytechnic Institute, Worcester, MA 01609* (Sponsor: Jack H. Kaplan)

Cu-ATPases have an essential role maintaining copper homeostasis inside the cell. These ATPases have a regulatory cytosolic NH_2-terminal metal binding domain (N-MBD) and a metal occlusion/coordinating site constituted by side chains of conserved amino acids located in the sixth, seventh, and eighth transmembrane segments. Previous studies showed that millimolar concentrations of Cys stimulate the ATPase activity of the thermophilic *Archaeoglobus fulgidus* CopA as well as of *Escherichia coli* ZntA and of *Arabidopsis thaliana* HMA2. The high affinity of CopA for metal (2.0–3.5 μM Cu^+) even in the presence of millimolar concentrations of Cys (consider Cu-Cys $K_D < 10^{-10}$ M) suggests a multifaceted interaction of the enzyme with Cys. To elucidate the role of Cys, we studied its effect on the partial reactions of the catalytic cycle of CopA. We observed that 2–50 mM Cys accelerates enzyme turnover with little effect on the Cu^+ affinity of CopA. Cys accel-

erates enzyme phosphorylation, but has no effect on the dephosphorylation rate. Thus, Cys increases steady-state phosphoenzyme levels. Besides, Cys has no significant effect on $E_1 \leftrightarrow E_2$ equilibrium. Similar results were observed in truncated CopA lacking the N-MBD, suggesting that enzyme activation by Cys is independent of the regulatory metal binding sites. Cu^+ uptake into sealed vesicles indicates that Cys acts on the cytoplasmic side of the enzyme. These results and the kinetic analysis of activation curves suggest that through delivery of Cu^+ to the transport site as a Cu-Cys complex, Cys in the mM range stimulates the ATPase, acting as a nonessential activator. [Supported by NSF grant MCM-0235165.]

43. Study on Proton Transfer Mechanisms of the Gastric H^+,K^+-ATPase. MAGOTOSHI MORII,[1] TOMOHIKO ICHIKAWA,[1] HIDEKI SAKAI,[1] NORIAKI TAKEGUCHI,[1] and SHINJI ASANO,[2] [1]*Faculty of Pharmaceutical Sciences, Toyama Medical & Pharmaceutical University, Toyama, Japan*; [2]*College of Information Science and Engineering, Ritsumeikan University, Shiga, Japan*. (Sponsor: Joe Hoffman)

Molecular mechanisms of the proton transfer of the gastric H^+,K^+-ATPase have not been clearly explained because its 3D structures have not been determined. We constructed 3D-structures of the α-subunit of gastric H^+,K^+-ATPase by homology modeling based on the PDB files of the SR Ca^{2+}-ATPase, and refined them by energy minimization and molecular dynamics calculation. In the E_1 form of SR Ca^{2+}-ATPase, a large negatively charged cytosolic open space between M1 and M2 stalks has been recognized to be the entrance for Ca^{2+}. Molecular dynamics calculation and trajectory analysis suggests that this cation entrance is completely closed in the H^+,K^+-ATPase, and that charges (protons) are transferred from the cytosolic basic amino acid residue, Lys-164 (on the M2 stalk), to the acidic amino acid residue in the second cation binding site, Glu-345 (on M4), via the polar (electron donor and acceptor) amino acid residue, Gln-161 (on M2). Then, protons are transferred to the first cation binding site via bound water molecules. In the first cation binding site, Asp-826, Lys-793, Asn-794, Glu-797, and Gln-941 also form a series of charge transfer paths. This charge transfer was interrupted by K793S mutation. We estimated the ATP hydrolysis-dependent water transporting activity in hog GI gastric vesicles by using ^{18}O water, and found that 1.8 mol of water were transported by 1 mol of ATP hydrolysis. The water transport was completely inhibited by SCH 28080. Our results suggest that (1) two cytosolic protons are transferred to the cation binding sites by charge transfer, (2) the protons bind to two water molecules, and (3) two H_3O^+ molecules are transported to the luminal space. [Supported by grants-in-aid from the Ministry of Education, Science, Sports, and Culture in Japan.]

44. Glycerol Changes Calcium-sensing Manner of the Rabbit Sarcoplasmic Reticulum Ca^{2+}-ATPase Molecules. JUN NAKAMURA,[1] GENICHI TAJIMA,[1] AYA KOTAKE,[1] YUSUKE MARUYAMA,[1] and CHIKARA SATO,[2] [1]*Department of Developmental Biology and Neurosciences, Graduate School of Life Sciences, Tohoku University, Sendai, Miyagi, Japan*; [2]*Neuroscience Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan*

The Ca^{2+}-ATPase molecules of the sarcoplasmic reticulum (SR) from rabbit skeletal muscle have been suggested to exist as two conformational variants (A and B) of the chemically equivalent ATPase molecules in the SR membrane at a ratio of 1:1 (Nakamura and Furukohri. 1994. *J. Biol. Chem.* 269:30818–30821); they bind two calcium ions, noncooperatively and positive-cooperatively, respectively, in the absence of a high concentration (≥0.25 mM) of ATP. At a low concentration (10 μM) of ATP and pH 7.4, it was previously shown that Hill plots of the calcium dependence of the total ATPase activity of the ATPase molecules is biphasic with slopes of ∼1.8 and ∼1.0 and an apparent calcium affinity (calcium concentration for the half-maximum activity, $K_{0.5}$) of ∼0.3 μM (Nakamura et al. 2002. *J. Biol. Chem.* 277:24180–24190). This biphasic calcium-dependent profile seems to reflect total calcium binding of the two ATPase forms mentioned above. Here, the Ca^{2+}-ATPase activity was assayed with the method of the two types of coupled enzyme system using the ammonium sulfate–suspended pyruvate kinase and the glycerol-solubilized pyruvate kinase, respectively, at low [ATP]. Glycerol (170 mM; 1.25% vol/vol), not but ammonium sulfate, which originated from the used pyruvate kinase, converted the biphasic calcium-dependent profile of the total ATPase activity to a monophasic profile (a Hill value of ∼1.8 and a $K_{0.5}$ of ∼0.2 μM). The data suggest that glycerol changes the calcium-sensing manner of the ATPase molecules, implying the participation of water molecules in calcium binding of the ATPase molecules. [Supported by grants from NEDO and AIST.]

45. Binding Kinetics of a Quaternary Amine to Extracellular K^+ Site(s) in the Na,K-ATPase. R. DANIEL PELUFFO and JOSHUA R. BERLIN, *Department of Phar-*

macology and Physiology, UMDNJ-New Jersey Medical School, Newark, NJ (Sponsor: Andrew Harris)

Organic quaternary amines competitively inhibit K^+ activation of Na-pump current and Na,K-ATPase activity but are not occluded by the enzyme. Of the quaternary amines tested, benzyltriethylammonium ions and the para-nitro derivative (pNBTEA) inhibit steady-state Na-pump current in a V_M-dependent manner. In light of the data, we predicted that these quaternary amines would produce ouabain-sensitive transient charge movements under conditions supporting extracellular K^+ binding. Cardiac myocytes were whole-cell patch clamped under Na^+-free conditions with electrodes containing high K^+, 0.7 mM ATP, and millimolar concentrations of P_i during superfusion with K^+-free solutions. Ouabain-sensitive transient currents were measured at V_M from −160 to +100 mV with 33–300 μM extracellular pNBTEA. Charge (Q) was measured as the integral of these currents, and fitting Q–V_M relationships with Boltzmann equations showed that total mobile charge (Q_{tot}) was 14 fC/pF for transient currents measured at the beginning (Q_{ON}) and end of voltage pulses (Q_{OFF}) at all pNBTEA concentrations, similar to Q_{tot} for extracellular Tl^+-dependent charge movement. The V_M for half maximal charge movement was shifted by ∼30 mV per e-fold change in amine concentration. The rate constant (k_{tot}) for charge movement was estimated by fitting transient currents with an exponential function. The relationship between k_{tot} and V_M displayed an asymmetric "U" shape with a minimum that shifted to more positive V_M at higher pNBTEA concentrations. The rate constant k_{tot} is also the sum of forward (k_f) and backward rate constants (k_r) modified by V_M, λ (effective charge moved during amine binding), and δ (partitioning of charge between k_f and k_r). λ and δ were calculated to be ∼0.4 and 0.32, respectively. These data suggest that extracellular pNBTEA- and Tl^+-dependent charge movements share similar properties, but amine-dependent charge movements reflect an asymmetric binding reaction independent of other transport steps by the Na,K-ATPase. [Supported by NIH and the American Heart Association.]

46. Temporal Correlations among Extracellular Na Ion Binding/Release Steps in the Na/K-ATPase. MIGUEL HOLMGREN, FRANCISCO BEZANILLA, PAUL DE WEER, ROBERT F. RAKOWSKI, and DAVID C. GADSBY, *The Marine Biological Laboratory, Woods Hole, MA*

In exchanging three intracellular Na ions for two extracellular K ions, the Na/K pump moves net charge through the membrane's electric field. Most of this charge movement occurs as Na ions traverse access channels connecting the extracellular solution to the ion binding sites deep inside the pump. The electrical signals associated with these ion movements can be isolated from normal pump cycle current by constraining the pump to operate in the absence of K ions. Under these conditions, at a given membrane potential and external [Na], the pump population reaches an equilibrium distribution of pumps with zero, one, two, or three Na ions bound. A jump to a new membrane voltage redistributes the pump population, resulting in charge movements evident as transient current that decays as the new equilibrium is established. By fast voltage clamp of the squid giant axon membrane, we identified three components in these current relaxations: fast (which follows the voltage-jump time course), medium speed ($\tau \sim$ 100–200 μs), and slow ($\tau \sim$ 1–5 ms). We proposed that these components reflect distinct, sequential steps of Na ion binding (or release, depending on voltage-jump direction), each associated with an occlusion (or deocclusion) conformational transition. This was suggested by a strong temporal correlation between the decline of the amount of charge that moves as the medium speed component and the relaxation of the slow component, and by the observation that the ability to elicit a fast component was similarly lost after the slow component relaxed. Much improved acquisition capabilities have now clarified the reciprocal temporal correlation between fast and slow components. We find that, at all membrane potentials and external [Na], the time courses of decline, and of recovery, of the fast component closely follow the relaxation time course of the slow component under those conditions, establishing the sequential nature of these charge-moving events. [Supported by NIH Intramural Research Program, GM30376, NS11223, NS22979, and HL36783.]

47. Physical Properties of the Na,K-ATPase Ion Pathway Revealed by Palytoxin. NICOLAS REYES and DAVID C. GADSBY, *Laboratory of Cardiac and Membrane Physiology. The Rockefeller University, New York, NY*

The Na,K pump, like many other ion transport proteins, provides a polar pathway to conduct the ions across the membrane. Access of the transported ions to this pathway is strictly regulated by gating mechanisms within the pump. To study the architecture of the Na,K pump's ion pathway, we took advantage of palytoxin, a marine toxin that disrupts the normally coordinated gating that underlies active Na and K transport; this results in frequent opening of a cation-selective channel through the pump. Residues in transmembrane seg-

ments (TM) 4, 5, and 6 of a ouabain-resistant *Xenopus* Na,K pump were replaced, one at a time, by cysteines, and the effects of modification with external hydrophilic, sulfhydryl-specific, reagents on palytoxin-induced currents were measured. The differential effects of modifications that leave positively or negatively charged adducts suggest that the Na,K pump's ion pathway incorporates a wide extracellular vestibule that reaches deep into the membrane where it becomes constricted, leading to a charge selectivity filter. This selectivity filter in the palytoxin-induced channel is formed, in part, by acidic residues implicated in cation coordination in the untreated Na,K pump (also in Ca- and H,K-ATPases), supporting the identity of ion channel and ion transport pathways. In particular, mutation of the conserved glutamate (E334, in human numbering) in the unwound part of TM 4, to a cysteine, greatly impaired cation selectivity of the palytoxin-induced channel. Furthermore, leaving a positively charged adduct at this cysteine transformed the channel from cation selective to anion selective. These results suggest that the Na,K pump exchanges Na and K ions between ion-coordination site II and the extracellular medium via a funnel-shaped ion pathway partially formed by TM4 and TM6. Strong homology between the catalytic subunits of Ca-, Na,K-, and H,K-ATPases suggests that their cationic transport pathways likely all share the same general architecture. [Supported by NIH grant HL36783.]

48. The K^+-transporting KdpFABC complex from *Escherichia coli*. JOERG-CHRISTIAN GREIE and KARLHEINZ ALTENDORF, *Universitaet Osnabrueck, Arbeitsgruppe Mikrobiologie, D-49069 Osnabrueck, Germany* (Sponsor: David Gadsby)

Based on sequence comparison and biochemical analyses, the membrane-embedded KdpFABC complex belongs to the group of P-type ATPases. In contrast to other well-characterized members of this family (for example the sarcoplasmic reticulum Ca^{2+} ATPase SERCA), KdpFABC consists of four subunits, and, as a unique feature, the sites of substrate transport (KdpA) and ATP hydrolysis (KdpB) are located on two different polypeptides. This, in turn, leads to the need for coupling between the sites of energy conversion and ion translocation.

With the analysis of mutants, this coupling could be attributed to a unique potential dipole within the transmembrane part of KdpB.

KdpB comprises all conserved sequence motifs of P-type ATPases, whereas the sequence of KdpA strongly mimics that of MPM-type K^+ channels (two transmembrane helices with single pore loop). A corresponding K^+ channel activity could be demonstrated by the purification of KdpA and reconstitution into black lipid membranes, resulting in a K^+-selective rectifying phenotype.

Like all other P-type ATPases, the KdpFABC complex undergoes a reaction cycle during catalysis, in which the two main conformations E1 and E2 alternate via phosphorylation of a conserved aspartic acid residue. A K^+-induced E2→E1 transition necessary for ATP binding was demonstrated by CD spectroscopy.

Kinetic data suggest a homodimeric $KdpF_2A_2B_2C_2$ complex. This was confirmed by cross-linking as well as electron microscopic analyses. [Supported by the Deutsche Forschungsgemeinschaft (SFB431/P7).]

49. Influence of pH on the Electrogenic Na^+ Transport by Na^+,K^+-ATPase Studied by Admittance Measurements. V.S. SOKOLOV,[1] A.A. LENZ,[1] K. GRISHANIN,[1] V. HAGEN,[2] and H.-J. APELL,[3] [1]*Frumkin Institute of Physical Chemistry and Electrochemistry RAS, Moscow, Russia*; [2]*Forschungsinstitut für Molekulare Pharmakologie Berlin, 12325 Berlin, Germany*; [3]*Department of Biology, University of Konstanz, 78457 Konstanz, Germany*

The nonsteady-state electrogenic transport of sodium ions through the Na^+,K^+-ATPase was studied by measuring small increments of the admittance of the bilayer lipid membrane with adsorbed Na,K-ATPase-containing membrane fragments. Separate steps of this transport process can be resolved by the frequency dependencies of the admittance increments at different concentrations of sodium ions and pH. The conformational transition E1/E2 of the Na^+,K^+-ATPase, initiated by fast ATP release from caged ATP, activates the electrogenic exchange of sodium ions in the extracellular access channel and depresses their exchange at the cytoplasmic side. The former process can be detected as positive, the latter as negative, changes of membrane capacitance and conductance. A pH increase from 6 to 8 leads to a considerable increase of the magnitude of the negative admittance change. This effect can be explained by a contribution of the transport of two sodium ions on the cytoplasmic side, which is electroneutral at acidic pH due to Na/H exchange, but becomes electrogenic at higher pH. To detect this transport without phosphorylation of the ATPase, a new approach was used, in which a fast increase of the H^+ concentration was initiated by photolysis of so-called "caged protons." The release of H^+ ions from the membrane-bound caged proton molecules leads to a change of the membrane admittance even in the absence of ATP. The effect depends on the sodium ion concentra-

tion in the solution, indicating that at least a part of it can be attributed to the electrogenic transport of sodium ions through the cytoplasmic channel of Na^+,K^+-ATPase (supported by INTAS grant 01-0224).

50. Ca^{2+}-independent Phosphatase Activity of the Plasma Membrane Ca^{2+} Pump. DÈBORA E. RINALDI and HUGO P. ADAMO, *IQUIFIB-Facultad de Farmacia y Bioquímica (UBA-CONICET), Buenos Aires, Argentina* (Sponsor: David Gadsby)

The plasma membrane Ca^{2+} pump (PMCA), like the Na^+-K^+ ATPase and SERCA, is able to hydrolyze p-nitrophenylphosphate (pNPP). However, pNPP is ineffective in supporting the active transport of Ca^{2+} by the PMCA. We have reexamined the role of Ca^{2+}, ATP, and calmodulin and acidic lipids in the phosphatase activity of the PMCA. In the absence of Ca^{2+}, ATP, and calmodulin, the purified PMCA reconstituted in phosphatidylcholine liposomes was able to hydrolyze pNPP at a rate of 0.2 μmol/mg/min. The addition of 1 μM Ca^{2+} and 200 nM calmodulin increased the pNPPase fourfold. Reconstitution in liposomes containing phosphatidylserine eliminated the requirement of Ca^{2+}-calmodulin for maximum phosphatase activity. Indeed Ca^{2+} inhibited this activity with an apparent affinity similar to that of the Ca^{2+} transport site. By homology with the SERCA pump, the Ca^{2+} binding site of the PMCA would be formed by main chain carbonyls of Val418, Ala 419, and Val421 and side chain carbonyls of Asn879, Asp883, and Glu423. A mutant of the human isoform 4xb in which Asn879 was replaced by Asp was expressed in *Saccharomyces cerevisiae*, solubilized by the detergent C12E10 and purified by calmodulin chromatography. The mutant reconstituted in liposomes containing phosphatidylserine had no measurable Ca^{2+}-ATPase activity but it was capable of hydrolyzing pNPP. Furthermore, the pNPPase activity of Asn879Asp was resistant to Ca^{2+} inhibition. Altogether the results suggest that (a) the phosphatase activity of the PMCA does not require Ca^{2+}, and thus the structural arrangement of the cytosolic domains should be similar to that of the E2 conformation; (b) changing Asn879 to Asp disrupts the Ca^{2+} binding site, thereby stabilizing the enzyme in an E2-like conformation; and (c) in E2, maximum phosphatase activity is observed when the pump is not inhibited by the COOH-terminal autoinhibitory domain. [Supported by grants of CONICET and UBA.]

51. New Arguments in Favor of a Second ATP Site in the Sarcoplasmic Reticulum Ca-ATPase of Skeletal Muscle. DEBORA A. GONZALEZ, MARIANO A. OSTUNI, JEAN-JACQUES LACAPÈRE, and GUILLERMO L. ALONSO, *Cátedra de Biofísica, Facultad de Odontología, Universidad de Buenos Aires, Buenos Aires, Argentina; Inserm U683, Faculté Xavier Bichat, Paris, France*

In the absence of ATP, Mn (Mg analogue as ATP cosubstrate) bound with high affinity to the Ca-ATPase, with $K_d \cong 15$ μM. [ATP] up to 30 μM increased (^{54}Mn) Mn binding, but higher [ATP] strongly inhibited it. The complex result suggests the interaction of 2 ATP with K_d around 5 μM and 100 μM.

Although the binding of a second ATP with $K_d \leq 100$ μM has been not described, reinterpretation of old binding measurements of (^{14}C)ATP in the absence of Ca and Mg (Lacapère et al. 1993. *J. Biol. Chem.* 265:348–353) allowed us to consider that two ATPs bind with K_ds 11 μM and 80 μM. In the same way, we provide new insight into ATP-dependence of the intrinsic fluorescence change promoted by addition of ATP to the Ca-ATPase. In the absence of cations, we measured several times the fluorescence variation induced by each [ATP], and clearly obtained a biphasic curve showing two K_ds for ATP (around 6 μM and 60 μM).

We sought a model to explain these Mn and ATP binding results. A simple random binding of one Mn and one ATP largely failed to reproduce the strong inhibitory effect of ATP $>$ 30 μM on Mn binding. A model including the species E.ATP_2, to which Mn binds with low affinity, could satisfactorily simulate the experimental results. According to this model, ATP binds to EMnATP with K_d 300–500 μM, a value compatible with the activating role of ATP on enzyme turnover. Finally, we measured ATPase activity as a function of [Mn] or [Mg], with low (30 μM) and high (3 mM) ATP concentration. With the lowest metal concentrations (up to 0.3 mM Mn and 0.7 mM Mg), the activities were higher with 30 μM ATP than with 3 mM ATP. These results are in accord with the observed inhibitory effect of ATP $>$30 μM on Mn binding, and support the hypothesis of a second ATP locus involved in enzyme activation.

52. Mutations in the Conserved TGES Motif in Domain A of SR Ca^{2+}-ATPase Fall into Two Distinct Functional Classes, Blocking Either E_2P Dephosphorylation or the E_1P–E_2P Conformational Transition. ANNE NYHOLM ANTHONISEN, JOHANNES D. CLAUSEN, and JENS PETER ANDERSEN, *Institute of Physiology and Biophysics, Department of Physiology, University of Aarhus, Aarhus, Denmark* (Sponsor: Anne Nyholm Anthonisen)

Mutation E183A in the conserved TGES motif in domain A of SR Ca^{2+}-ATPase blocks dephosphorylation of E_2P (Clausen et al. 2004. *Proc. Natl. Acad. Sci. USA.* 101: 2276–2281), thus providing the functional correlate of

the crystal structures of Ca^{2+}-ATPase in E_2 form with bound fluoride complex, where the glutamate of TGES is in position to hold the attacking water molecule. In a continuation of this and other previous mutagenesis studies of the TGES motif (Clarke et al. 1990. *J. Biol. Chem.* 265:14088–14092), the present investigation examines mutants S178A, S178L, T181A, T181D, T181R, G182A, G182L, E183D, E183Q, E183L, E183R, E183S, E183H, S184A, S184D, and S184R. Only S178A and S184A displayed significant Ca^{2+} uptake activity. Kinetic studies of the phosphoenzyme showed that two distinct mutant classes could be distinguished. Like E183A, mutants E183Q, E183S, and E183H showed accumulation of the E_2P phosphoenzyme intermediate upon phosphorylation with ATP, indicating that E_2P was formed from E_1P, but was not hydrolyzed. In contrast, the E_1P phosphoenzyme intermediate accumulated in mutants S178L, T181A, T181D, T181R, G182A, G182L, E183D, E183L, E183R, S184D, and S184R, and the E_1P–E_2P transition rate was reduced to <10% that of wild type. E_1P decayed at a significant, although low, rate (5–10% of wild type) in E183D, E183L, and E183R. E183D was able to form E_2P by reaction with P_i in the absence of Ca^{2+}, and catalyzed dephosphorylation of E_2P at a rate approximately one third that of wild type. E183L and E183R, on the other hand, could not form E_2P by phosphorylation with P_i, and for these mutants, the turnover of E_1P phosphoenzyme may reflect an ability of E_1P to dephosphorylate slowly without being first converted into E_2P, i.e., "true" uncoupling of ATP hydrolysis from Ca^{2+} transport.

53. Na/K Pump Slowing by Charge-modifying Mutation at Ouabain-affinity Determinant Position 131 of the α1 Subunit. PABLO ARTIGAS and DAVID GADSBY, *The Rockefeller University, New York, NY*

We studied the function of heterologously expressed Na/K pumps in Na-loaded *Xenopus* oocytes using two-microelectrode voltage-clamp current recording. The continuous presence of 1 μM ouabain silenced endogenous Na/K pumps, allowing selective measurements of signals from exogenous *Xenopus* $\alpha_1\beta_3$ Na/K pumps, made ouabain resistant with α-subunit mutation C113Y. We examined effects on pump function of various substitutions for N131, equivalent to D129 in rat α1, a residue known to influence ouabain binding. The presence of charge at 131 reduced the sensitivity of stationary Na/K-pump current (I_P) to inhibition by ouabain: apparent K_i was tens of micromolar for neutral (N131, N131Q, N131C), hundreds of micromolar for negative (N131D, N131E), and >10 mM for positive (N131K, N131R) substituents. $K_{0.5}$ values for I_P activation by external K, without extracellular Na, were similar in all these mutants, but maximal I_P was greater than or equal to threefold smaller in mutants with a positive charge at position 131. On the other hand, in the absence of external K but presence of external Na, the total quantity (proportional to the number of pumps) of the slowest component of Na-dependent, ouabain-sensitive, transient charge movement (Q_{Na}) elicited by step changes in voltage, was similar for all mutants. However, relaxation of the Na-sensitive transient currents was slowed greater than twofold at all voltages in N131R and N131K mutant pumps. We also modified the charge at position 131 during the course of an experiment by acute exposure of the mutant N131C to sulfhydryl reagents bearing different charges. Though maximal I_P was unchanged by modification with the neutral reagent MTSACE, ouabain sensitivity, maximal I_P, and relaxation rate of Q_{Na} were all reduced by modification with $MTSEA^+$, resembling the above findings with positively charged residues. We conclude that a positive charge at position 131 reduces Na/K pump turnover rate by slowing Na deocclusion/release toward the extracellular surface. [Supported by NIH-HL36783.]

54. Palytoxin-induced Effects on Partial Reactions of the Na,K-ATPase. NADINE HARMEL and HANS-JÜRGEN APELL, *Department of Biology, University of Konstanz, 78464 Konstanz, Germany*

The electrochromic styryl dye RH421 was applied to flat, purified membrane fragments containing Na,K-ATPase in high density to investigate the action of the marine toxin palytoxin (PTX). It was shown that PTX strongly affects the protein only after it has been phosphorylated to reach the state P-E_2. PTX induced a modification of the ion pump, which led to a state with a reduced RH421 fluorescence level that corresponded to an average uptake of about two additional positive elementary charges, such as H^+ or Na^+ (in the absence of K^+), into the membrane domain of the pump protein. The PTX-induced modification of the Na,K-ATPase is reversible and follows an apparent first order reaction with an "on" rate of $1.2 \times 10^5\ s^{-1}M^{-1}$ and "off" rate of $10^{-5}\ s^{-1}$. The stability of the PTX-inhibited ion pump depends on the cations present in the buffer. K^+ ions destabilize the inhibited state significantly more strongly than Na^+. Under saturating K^+ concentrations, an average of ~50% of the pumps are maximally inhibited, and in the presence of Na^+ ions, up to 74% of the enzyme activity can be blocked. When the temperature dependence of the rate constant was determined, a bend in the Arrhenius plot was observed with

an activation energy of 28.5 kJ/mol above 23°C and 71 kJ/mol below. This observation indicates that modification of the Na,K-ATPase occurs by at least a two-step process, e.g., toxin binding and gate opening. To test the accessibility of the ion-binding sites inside the membrane domain, the entrance channels may be blocked by Br_2-TITU from the cytoplasm, and by tetrapropylammonium ions from the extracellular side. The effect of both blockers will be studied to test the current model of PTX action by producing a membrane-spanning ion channel with both external and internal gates opened. [Supported by DFG grant Ap45/4.]

55. P-O bond destabilization accelerates E2P hydrolysis. ANDREAS BARTH[1] and NATALYA BEZLYEPKINA,[2] [1]*Department of Biochemistry and Biophysics, Stockholm University, Stockholm, Sweden*; [2]*Institut für Biophysik, Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany*

Two phosphoenzyme intermediates with different chemical specificity are an essential feature of P-type ATPases. Our aim was to understand the high hydrolysis rate of the ADP-insensitive phosphoenzyme (E2P) of sarcoplasmic reticulum Ca^{2+}-ATPase.

We monitored $^{16}O/^{18}O$ isotope exchange at the E2P phosphate group with infrared spectroscopy. This enabled us to observe three phosphate vibrations against a background of 50,000 protein vibrations.

P-O stretching vibrations of E2P were found at 1194, 1137, and 1115 cm^{-1}. This information was evaluated using the bond valence model and empirical correlations. Compared with the model compound acetyl phosphate, structure and charge distribution of the E2P aspartyl phosphate resemble somewhat the transition state in a dissociative phosphate transfer reaction: the aspartyl phosphate of E2P has 0.02-Å shorter terminal P-O bonds and a 0.09-Å longer bridging P-O bond, the angle between the terminal P-O bonds is wider, and −0.2 formal charges are shifted from the phosphate group to the aspartyl moiety.

The bridging P-O bond of E2P is ∼20% weaker than that of acetyl phosphate in water, which accounts for a 10^{11}- to 10^{15}-fold enhancement of hydrolysis rate. Thus, P-O bond destabilization in E2P facilitates phosphoenzyme hydrolysis. It is caused by a shift of noncovalent interactions from phosphate oxygens to aspartyl oxygens. This shift can be achieved by the relative positioning of Mg^{2+} and Lys 684 between phosphate and aspartyl oxygens with only subtle distance changes being required.

For the Ca^{2+}-ATPase and related phosphoproteins, we suggest that the relative positioning of Mg^{2+} and Lys 684 between phosphate and aspartyl oxygens provides an elegant "handle" for the enzyme to control hydrolysis. [Supported by DFG grant BA 1887/4-1,VR grant 621–2002-5884.]

56. Functional Consequences of Mutation of Asn^{706} at the Catalytic Site of SR Ca^{2+}-ATPase. JOHANNES D. CLAUSEN,[1] DAVID B. MCINTOSH,[2] DAVID G. WOOLLEY,[2] and JENS PETER ANDERSEN,[1] [1]*Institute of Physiology and Biophysics, Department of Physiology, University of Aarhus, Aarhus, Denmark*; [2]*Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa* (Sponsor: Johannes D. Clausen)

Asn^{706} at the catalytic site of SR Ca^{2+}-ATPase was replaced by alanine, cysteine, and serine, and the mutants were analyzed by our panel of functional assays, which now include the binding of the phosphoryl analogs AlF_4^- and MgF_4^{2-} recently used for crystallization, as well as BeF_4^{2-}. All three mutants were devoid of Ca^{2+} transport and ATPase activity. The rate of formation of *E*1P from MgATP was reduced four- and twofold in N706C and N706A, respectively, whereas it was wild type-like in N706S. Binding affinity of Ca_2E1 for AlF_4^- in the presence of ADP ($Ca_2E1{\cdot}P{\cdot}ADP$ transition state analogue) was reduced 15-, 4-, and 1.6-fold in N706C, N706A, and N706S, respectively. The affinity for MgATP was reduced less than twofold. Hence, the reduced rate of phosphorylation from MgATP in N706C and N706A can be ascribed to defective interaction with the phosphoryl transition state. The two major conformational changes in the pump cycle, the Ca^{2+}-binding $E2 \rightarrow Ca_2E1$ transition and the $Ca_2E1P \rightarrow E2P$ transition, were both markedly slower in the mutants compared with wild type, again with N706C exerting the most pronounced effect. All three mutants were unable to form *E*2P by reaction of *E*2 with P_i, and showed >1,000-fold reduction in affinity for vanadate. The affinity of *E*2 for AlF_4^-, MgF_4^{2-}, and BeF_4^{2-} was much less affected in the mutants, particularly the affinity for AlF_4^-, which was wild type-like in N706S and only about threefold reduced in N706C and N706A, indicating that the critical role of N706 in phosphorylation of *E*2 with P_i is related to aspects other than stabilization of the transition state. This contrasts with Glu^{183} in the actuator domain, which is critical to *E*2·P transition state stabilization, as judged from a 16-fold reduction of the affinity of the *E*2 form of mutant E183A for AlF_4^-.

57. Mutation of Gly^{94} in Transmembrane Segment M1 of Na^+,K^+-ATPase Interferes with Na^+ and K^+ Interac-

tions in E_2P Conformation. ANJA P. EINHOLM, MADS TOUSTRUP-JENSEN, JENS PETER ANDERSEN, and BENTE VILSEN, *Department of Physiology, Institute of Physiology and Biophysics, University of Aarhus, DK-8000 Aarhus C, Denmark* (Sponsor: Anja P. Einholm)

The importance of Gly^{93} and Gly^{94} in transmembrane segment M1 of the Na^+,K^+-ATPase for interaction with Na^+ and K^+ was demonstrated by functional analysis of mutants $Gly^{93}Ala$ and $Gly^{94}Ala$. The corresponding residues in the closely related Ca^{2+}-ATPase, Asp^{59} and Leu^{60}, are located just where M1 bends in the Ca^{2+}-ATPase crystal structures. Rapid kinetic measurements of K^+-induced dephosphorylation allowed determination of the affinity of the E_2P phosphoenzyme intermediate for K^+, which normally binds from the extracellular side. In $Gly^{94}Ala$, K^+ affinity was reduced ninefold, relative to wild type, i.e., to the same extent as seen for mutation of the gating residue Glu^{329} at the cation binding sites. Furthermore, in $Gly^{94}Ala$, the E_1P–E_2P equilibrium of the phosphoenzyme showed strongly reduced sensitivity to Na^+, with accumulation of E_2P even at 600 mM Na^+, indicating impaired interaction of E_2P with extracellular Na^+. On the contrary, in $Gly^{93}Ala$, the affinity for K^+ was slightly increased and the E_1P–E_2P equilibrium was displaced in favor of E_1P. In both mutants, the affinity of the cytoplasmically facing sites of E_1 for Na^+ was reduced. Comparison with Ca^{2+}-ATPase mutagenesis data (Einholm et al. 2004. *J. Biol. Chem.* 278:11402–11410) suggests that the role of M1 in binding of the transported ions is universal among P-type ATPases, despite the lack of sequence homology in this region. Structural modeling of Na^+,K^+-ATPase mutant $Gly^{94}Ala$ on the basis of the Ca^{2+}-ATPase crystal structures indicates that the alanine side chain comes close to Ile^{287} of M3, particularly in E_2P, thus resulting in a steric clash that may explain the present observations. [Supported by the Danish Medical Research Council, the Novo Nordisk Foundation, Denmark, the Lundbeck Foundation, Denmark, and the Research Foundation of Aarhus University.]

58. Implications of Dependence on Ionic Strength for the Mechanism of the Conformational Change in Unphosphorylated Sodium Pump. LARRY D. FALLER[1,2] and SHWU-HWA LIN,[2] [1]*Department of Medicine and Department of Physiological Science, University of California Los Angeles, Los Angeles, CA*; [2]*Digestive Diseases Research Center, Veterans Administration Greater Los Angeles Healthcare System, Los Angeles, CA* (Sponsor: Robert A. Farley)

The rate of the Na^+ and K^+ ion–regulated conformational change in unphosphorylated sodium pump covalently labeled with fluorescein depends on ionic strength in both directions (Lin and Faller. 2005. *Biochemistry.* 44:1482–1494). Therefore, electrostatic interactions contribute to the driving force for the change from E_1 to E_2 and to the stability of E_2. The theoretical equation for a bimolecular reaction between charged molecules fits rate data as a function of ionic strength in the $E_1 \rightarrow E_2$ direction with a second-order rate constant (1×10^9 $M^{-1}s^{-1}$) approaching the diffusion-controlled limit. Therefore, a two-step mechanism involving an intermediate encounter complex is required to explain a first-order rate constant (0.002 s^{-1}) in the $E_2 \rightarrow E_1$ direction, far below the diffusion limit. Ionic strength–dependent rates are explained by domain movements in the α subunit of the sodium pump like those imagined in animations based on crystallographic stills of calcium pumps with (E_1) and without (E_2) bound Ca^{2+} (Toyoshima and Nomura. 2002. *Nature.* 418:605–611). Activation parameters for formation of the encounter complex in the sodium pump (Faller, et al. 1991. *Biochemistry.* 30:3503–3510) indicate a crucial role for water in the energetics of the conformational change. The reaction barrier is lowered by an entropy increase attributable to disruption of water structure that accelerates domain association nearly 100,000-fold. Both homology models of the sodium pump and more than two orders of magnitude faster domain separation in proton than sodium pumps predict poorly conserved interdomain interactions. Parameter estimates independent of the fluorescent reporter group or experimental technique support evidence from Na^+ and K^+ titrations for a concerted protein rearrangement (Smirnova et al. 1995. *Biochemistry.* 34: 8657–8667). Ionic strength also varied in titrations with NaCl claimed as evidence for acceleration of domain separation by Na^+ binding to E_2 and for a third conformer induced by binding the third Na^+. [Supported by VA merit review.]

59. Oxygen Exchange Studies of the Phosphoryl Group Transfer Mechanism. LARRY D. FALLER,[1,3] AGNES K. NAGY,[3] UBEN A. DIAZ,[3] and ROBERT A. FARLEY,[2] [1]*School of Medicine, University of California Los Angeles, Los Angeles, CA*; [2]*University of Southern California School of Medicine, Los Angeles, CA*; [3]*Veterans Administration Greater Los Angeles Healthcare System, Los Angeles, CA*

Stable oxygen isotope (^{18}O) exchange has important experimental and theoretical advantages for studying phosphoryl group transfer catalyzed by P-type pumps. The most important experimental advantage is that two parameters, the exchange rate and the probability of forming phosphoenzyme, can be estimated from the

distribution of phosphate isotopomers at a single time point. The most important theoretical advantage is that exchange is catalyzed by a single step in the transport cycle with known formal mechanism. Therefore, intrinsic properties of the protein can be estimated by fitting the rate equation to multivariate arrays of data. Conclusions drawn from studies of purified pumps are that only the metalloenzymes bind P_i and that the sodium pump catalyzes phosphoryl group transfer more efficiently than the calcium pump. Studies of mutations in the signature TGES, TGTKT, DPPR, MVTGD, and TGDGVND sequences of sodium pumps expressed in yeast dramatically illustrate the advantages of ^{18}O exchange measurements. Nearly half of the mutants made so far could be studied as a function of both Mg^{2+} and P_i concentration. Mutations were found that affected only the Mg^{2+} dissociation constant, only the half-maximum concentration for P_i binding to metalloenzyme, or both. Earlier studies of some of the same mutations by other techniques missed the effects on P_i and/or Mg^{2+} binding either because the wrong formal mechanism was assumed, or because offsetting changes in the affinities for Mg^{2+} and P_i masked subtle changes in the half-maximum titrant concentration. Mutations in two of the above signature sequences reduced the probability of the sodium pump forming phosphoenzyme from noncovalently bound P_i by nearly an order of magnitude. The results from oxygen exchange studies of site-directed mutants of the sodium pump generally support the hypothesis that the catalytic machinery of P-type pumps evolved from bacterial haloacid dehalogenases. [Supported by NIH grants DK52802 and GM28673 and a VA merit review.]

60. Nucleotide binding to Na,K-ATPase: pK values of the residues involved in electrostatic interactions with the substrate. NATALYA U. FEDOSOVA and MIKAEL ESMANN, *Institute of Physiology and Biophysics, University of Aarhus, Aarhus, Denmark*

The effect of ionic strength on nucleotide binding to Na,K-ATPase reveals the contribution of electrostatic forces to the interaction process and suggests (according to the Debye-Hückel theory) that the nucleotide binding site and its ligands have effective charges of opposite signs. Quantitative analysis of the dependence of the equilibrium dissociation constant (Kd) on ionic strength provides information on the product of the effective electrostatic charges on the ligand and the binding site (zEzA) and on the equilibrium dissociation constant at zero ionic strength (Kd,o). Investigation of the pH dependencies for both zEzA and Kd,o reveals the pK values for the functional groups of the protein that are critical for nucleotide binding to the enzyme. Comparison of those dependencies allows us to distinguish groups affecting the association rate constant, kon (i.e., involved in long-range coulombic interactions), and those participating in short-range interaction and, therefore, important for the dissociation rate constant, koff. The binding experiments were performed on pig kidney enzyme and its two substrates (ATP and ADP) over the pH range 6.0–9.5 and for ionic strengths 70–540 mM. The pH dependences for zEzA were identical for both substrates and clearly revealed deprotonation of a group with pK $\sim$ 8.5. Deprotonation decreased the positive effective charge of the binding site and, therefore, kon values for both nucleotides by a factor of 2. At the same time Kd,o values increased in the corresponding pH interval. The increase in Kd,o was much more pronounced (10–20 fold) and cannot be explained by the change in kon alone. The dissociation of nucleotides must be faster at basic pH due to the deprotonation of the functional groups with pK $\sim$ 8.5 involved in short-range interactions. The results are consistent with our hypothesis that local interactions are major determinants for affinity by affecting dissociation rate constants.

61. The Influence of Intracellular $[Na^+]$ on the E_1P–E_2P Conformational Change of the Na,K-ATPase Probed by Voltage-clamp Fluorometry. STEFAN GEYS, GIOVANNI ZIFARELLI, ERNST BAMBERG, and THOMAS FRIEDRICH, *Max-Planck-Institute of Biophysics, Frankfurt am Main, Germany*

Site-directed fluorescence labeling and voltage-clamp fluorometry has been used in our laboratory to study conformational dynamics of the Na^+/K^+-ATPase, leading to the identification of enzyme regions (including the M5-M6 loop and the β-subunit), which participate in the major $E_1(P)$–$E_2(P)$ conformational change (Geibel et al. 2003. *PNAS.* 100:964–969; Dempski et al. 2005. *J. Gen. Physiol.* In press). Therefore, Na^+/K^+-ATPase constructs carrying single extracellular cysteines are expressed in *Xenopus* oocytes and sulfhydryl-specific labeling is performed with tetramethylrhodamine-6-maleimide. Voltage steps applied under Na^+/Na^+ exchange conditions evoke characteristic fluorescence changes, which are directly correlated to the voltage dependence of transient charge movement that occurs in conjunction with E_1P–E_2P conformational changes. Here we utilize the Na^+/K^+-ATPase N790C mutant and the L311C mutant (which serves as a sensor for the involvement of the extracellular M3-M4 loop in the E_1P–E_2P reaction). To investigate the influence of $[Na^+]_i$, we coexpressed these constructs with the epithelial Na^+

channel ENaC to effectively control $[Na^+]_i$. When mutant N790C was studied under Na^+/Na^+ exchange conditions, the time courses of the fluorescence responses for a given voltage jump showed a monoexponential behavior at any $[Na^+]_i$. A saturating dependence of amplitudes on $[Na^+]_i$ with a half-maximal concentration of 7.0 (±2.1) mM was found, and also the kinetics at depolarizing potentials were affected. In contrast, fluorescence responses to voltage steps for mutant L311C were biexponential. A slow phase dominated at low $[Na^+]_i$ but successively decreased in favor of a fast phase (half-maximal concentration 8.8 [±0.6] mM), which at saturating $[Na^+]_i$ followed the well-known kinetic behavior of transient charge movements that occur during extracellular Na^+ release/rebinding in conjunction with the E_1P–E_2P conformational change. These results demonstrate that voltage-clamp fluorimetric measurements can be used to study kinetic effects of (weakly or nonelectrogenic) $[Na^+]_i$-dependent steps on electrogenic partial reactions of the Na^+ pump.

62. New Methods for Site-Directed Spectroscopic Labeling of SERCA-type Ca-ATPases. JOSEPH M. AUTRY, DEBORAH L. WINTERS, and DAVID D. THOMAS, *Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, MN 55455* (Sponsor: David D. Thomas)

Sarco/endoplasmic reticulum Ca-ATPases of the SERCA gene family are 110 kD transport proteins that contain around 50 lysine and 25 cysteine residues. Traditional methods of spectroscopic labeling with isothiocyanate, maleimide, and iodoacetamide reagents give heterogeneous labeling of multiple residues in these proteins (Kirley et al. 1985. *Biochem. Biophys. Res. Commun.* 130:732–738; Wawrzynow and Collins. 1993. *Biochim. Biophys. Acta.* 1203:60–70). Here, we report on three alternative strategies for site-directed labeling of SERCA isoforms: tetra-cysteine mutagenesis, domain reconstitution, and transglutaminase transfer. First, site-directed mutagenesis was used to engineer unique tetra-cysteine motifs into the fast-twitch Ca-ATPase (SERCA1) for labeling with fluorescein biarsenical helix/hairpin binder (FLASH), a membrane-permeable probe used for in vitro and in vivo labeling (Tsien. 2003. *Nat. Rev. Mol. Cell Biol.* 4:SS16–SS21). Recombinant SERCA1 was expressed and purified using the baculovirus system, giving a wild-type enzyme of 80% purity with an activity of 6 IU at 25°C. Fluorescence imaging of SDS-PAGE gels revealed that FLASH specifically labels tetra-cys mutants of SERCA1, but not the wild-type enzyme, which contains a native tri-cys site in the phosphorylation domain (^{670}CRRACC), demonstrating the stringent labeling specificity of biarsenical probes. The biarsenical helix motif CCXXCC retained high enzyme activity when introduced at positions 574 and 581 in the nucleotide-binding domain, whereas the biarsenical hairpin motif CCPGCC abolished enzyme activity when introduced at positions 397 and 535 in the same domain. The CCXXCC motif was also scanned around helix 2 in the actuator domain, resulting in a variable labeling efficiency about the helix when labeled in intact Sf21 insect cells. Thus, the tetracys/FLASH system is useful for fluorescent analysis of SERCA, but multiple sites must be examined to optimize activity and accessibility. Second, proteinase K digestion and column chromatography were used to isolate the actuator domain (residues 1–242) from native SERCA1 in fast-twitch sarcoplasmic reticulum vesicles. The actuator domain contains only one cytosolic cysteine residue at position 12, which was labeled with dansyl iodoacetamide (IAEDANS) following reconstitution in lipid bilayers. Third, transglutaminase and fluorescein cadaverine were used to enzymatically label the native Ca-ATPase (SERCA2) in cardiac sarcoplasmic reticulum vesicles. Gel analysis showed that transglutaminase labeled SERCA2 with fluorescein, along with other proteins in cardiac SR. Thus, new methods for site-directed spectroscopic labeling of SERCA-type Ca-ATPases have been developed and are currently being used to examine the functional dynamics of SERCA at previously uncharacterized positions throughout the molecule. Protocols for tetra-cys engineering, domain isolation/reconstitution, and enzymatic labeling will be presented with the hope that these approaches will prove amenable for the biophysical analysis of related members of the ion motive ATPase family. [This work was supported by grants from the Lillehei Heart Institute (J.M. Autry), the American Heart Association (D.L. Winters), and the National Institutes of Health (D.D. Thomas).]

63. Structure of the Nucleotide Binding Domain of the Kdp ATPase in the Free and AMP-PNP Bound State: Implications on the Reaction Cycle. MELINA HAUPT,[1] MURRAY COLES,[1] HORST KESSLER,[1] MARC BRAMKAMP,[2] and KARLHEINZ ALTENDORF,[2] [1]*Institut für Organische Chemie und Biochemie, Technische Universität München, 85747 Garching, Germany*; [2]*Universität Osnabrück, Fachbereich Biologie/Chemie, Abteilung Mikrobiologie, 49069 Osnabrück, Germany* (Sponsor: Jack Kaplan)

P-type ATPases are ubiquitously abundant proteins involved in the active transport of ions and phospholip-

ids across biological membranes (Axelsen and Palmgren. 1998. *J. Mol. Evol.* 46:84–101). The KdpFABC complex of *Escherichia coli*, a unique prokaryotic P-type ATPase, is a high-affinity K^+ uptake system that operates only when the cell experiences osmotic stress or K^+ limitation. The solution structure of the nucleotide binding domain of KdpB (backbone RMSD 0.17Å) was solved and a model of the AMP-PNP binding mode based on intermolecular distance restraints was proposed (Haupt et al. 2004. *J. Mol. Biol.* 342:1547–1558). The calculated nucleotide binding mode shows the purine ring of the nucleotide to be "clipped" into the binding pocket via a π-π-interaction to F377 on one side and a cation-π-interaction to K395 on the other. This binding mechanism seems to be conserved in all P-type ATPases, except the heavy metal transporting ATPases (type IB). The nucleotide binding domain of KdpB is the smallest and simplest one known for P-type ATPases, and represents a minimal version of this functional unit. No evidence of significant conformational changes was observed within the N-domain upon nucleotide binding, thus ruling out a role for ATP-induced conformational changes in the reaction cycle. These findings provide important evidence for a mechanism, in which the reaction cycle is driven by the sequential binding of potassium and ATP, thereby avoiding a futile cycle in the absence of the transported substrate. The recently solved NMR structure (backbone RMSD 0.28 Å) of the AMP-PNP bound form, and nucleotide binding studies on critical mutants, strongly support the proposed binding mode. Based on sequence alignments, it was suggested that the Kdp-ATPase is a progenitor of the heavy metal transporting ATPases. However, based on structural and biochemical data, we conclude that the Kdp-ATPase, which is currently grouped as type IA, has more similarities to type III ATPases. [Supported by the Deutsche Forschungsgemeinschaft (DFG) grant SPP1070.]

64. Conformational Changes of the Nucleotide Site of Two Ca^{2+}-ATPases Evaluated by Fluorescence Spectroscopy and Differential Quenching. MIRIAM M. FONSECA,[1] HELENA M. SCOFANO,[1] and JULIO A. MIGNACO,[2] [1]*Laboratório de Membranas Transportadoras,* [2]*Laboratório de Estrutura e Regulação de Proteínas e ATPases, Instituto de Bioquímica Médica, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*

Fluorescence quenching of FITC at the nucleotide site of the plasma membrane Ca^{2+}-ATPase (FITC-PMCA) by the water-soluble quenchers I^- and Cs^+ showed that the E_1Ca+CaM conformer was the least accessible state studied, with smaller suppression constants (*K*q) for both I^- (*K*q ≅ 6.7 M^{-1}) and Cs^+ (*K*q ≅ 0.7 M^{-1}). Accessibility to I^- was equivalent for the E_2VO_4 and E_1Ca states (*K*q ≅ 7.1 and 7.5 M^{-1}, respectively), slightly greater for E_2 (*K*q ≅ 9.1 M^{-1}), and greatest for E_2-P (*K*q ≅ 16.5 M^{-1}) (Fonseca et al. 2002. *Biochemistry.* 41:7483–7489). We now extend these studies to the sarcoplasmic reticulum Ca^{2+}-ATPase (FITC-SERCA), and use TNP-AMP as an additional site probe. With FITC as label, *K*q's are consistently lower for SERCA. At pH 7.4, FITC-SERCA conformational dependence for water accessibility of FITC is equivalent to FITC-PMCA: greater for E_2-P, intermediate for E_2, and lower for other conformations. Lowering pH to 6.0 does not significantly change *K*q's for FITC-PMCA, but with FITC-SERCA, the *K*q's for I^- increase >2x and conformational dependence of suppression disappears. Both FITC-PMCA and FITC-SERCA bind TNP-AMP, leading to a small decrease in fluorescence (FITC-PMCA) or a significantly larger decrease (FITC-SERCA), suggesting for SERCA a closer proximity of the fluorophores. Phosphorylation of the native Ca^{2+}-ATPases by P_i induces superfluorescence of TNP-AMP, much smaller for PMCA than SERCA. The conformational change induced by phosphorylation alters the FITC signal of the complexes with TNP-AMP, and addition of Ca^{2+} reverses the effect. Since PMCA is purified with a $C_{12}E_9$+phosphatidylcholine solution, we added this mixture to SERCA. Although Ca^{2+}-ATPase is fully active, the superfluorescence of TNP-AMP is lost and the *K*q's of phosphorylated FITC-SERCA are greatly altered, suggesting that detergent causes significant alterations in the nucleotide site. Similar hydrophobicity changes occur at the nucleotide site of SERCA and PMCA, but intrinsic site differences give mechanistic distinctiveness to each enzyme. [Supported by CNPq, FAPERJ, FINEP, and PRONEX.]

65. Characterization of the Transition between the Direct and Physiological Routes of Occlusion of K^+ in the Na^+/K^+-ATPase Caused by Addition of Na^+. JOSE LUIS E. MONTI, PABLO G. SCHVARTZ, RODOLFO M. GONZÁLEZ-LEBRERO, SERGIO B. KAUFMAN, PATRICIO J. GARRAHAN, and ROLANDO C. ROSSI, *Instituto de Química y Fisicoquímica Biológicas and Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina*

K^+-occluded intermediates of the Na^+/K^+-ATPase can be obtained either by the direct route, without formation of phosphoenzyme, or by the physiological route, after K^+-stimulated dephosphorylation of the

phosphoenzyme. Since Na^+ is required for phosphorylation by ATP, it is possible to characterize the transition between these two ways of occlusion caused by addition of Na^+. With this aim, we performed experiments at 25°C using a purified preparation of pig kidney Na^+/K^+-ATPase in media containing 2.5 mM ATP, 0.5 mM free Mg^{2+}, imidazole-HCl 25 mM, pH 7.4, and different concentrations of [$^{86}Rb^+$]RbCl (instead of KCl), NaCl, and cholineCl in order to keep constant the ionic strength (the total concentration of the last three salts was always 170 mM). Reaction time was 6–10 s.

Results show that, at fixed [Rb^+] and for increasing [Na^+], occluded Rb^+ first increased along a sigmoidal curve and then decreased along a hyperbola to a nonzero value. When [Rb^+] increases, the K_{05} of both the increasing and decreasing phases increases. In the absence of Na^+, occluded Rb^+ increases with [Rb^+] along a hyperbola with a small but significant value as [Rb^+] tends to zero (~2.5% of the maximal amount of occluded Rb^+), which could represent a high-affinity component.

A model based on the Albers-Post scheme, where three Na^+ must bind to E_1 to prevent occlusion by the direct route and to produce phosphorylation, and where Na^+ and K^+ compete for the cation binding sites in E_1 and in E_2P, approximately predicts the results. However, this model fails to predict that occluded Rb^+ should decrease to a nonzero value as Na^+ concentration increases to infinity. This discrepancy could be partially mimicked by considering that choline can occupy the cation binding sites in E_2P, given the experimental condition that [choline] decreases when [Na^+] increases in order to keep the ionic strength constant. [Supported by CONICET, Agencia Nacional de Promoción Científica y Tecnológica, and Universidad de Buenos Aires.]

66. Substrate Interactions with the Na^+/K^+-ATPase Examined with Fluorescence Resonance Energy Transfer. PROMOD R. PRATAP, *Department of Physics and Astronomy, University of North Carolina at Greensboro, Greensboro, NC 27402* (Sponsor: David Gadsby)

The Na^+/K^+-ATPase uses energy from ATP hydrolysis to move Na^+ out of the cell and K^+ into the cell against their electrochemical gradients. The reaction cycle involves transitions between phosphorylated and unphosphorylated forms of two major conformations of the enzyme (E_1 and E_2). The apparent affinity of the ATPase reaction for ATP is 1–10 μM. In addition, ATP binds to the E_2 conformation with low affinity (apparent $K_d \sim 0.5$ mM). Experiments using FTIR spectroscopy showed that ATP binding to E_1 resulted in conformational changes (Pratap et al. 2003. *Biophys. J.* 85:3707–3717); the apparent K_d of ATP for these changes depended on the ionic conditions in the buffer. These results indicated that there exists a ("reluctant") step following ATP binding that involves a positive change in free energy. We have developed an alternative method for examining ATP binding and subsequent conformational changes using fluorescence resonance energy transfer (FRET) between Cy3- or IAF-labeled ATPase and Alexa-Fluor-647 (AF647)–labeled ATP. When AF647-ATP is added to a suspension of Cy3-ATPase excited at 515 nm, we find an emission increase centered at 686 nm, which could be reversed by the addition of excess ATP. These results indicate that AF647-ATP binds to the ATP binding site on the protein. The magnitude of this fluorescence increase as a function of AF647-ATP concentration could be fitted to a sum of two binding curves with apparent K_d of 49 nM and 8 μM. This implies that ATP binds to the binding site on the ATPase with a K_d of 49 nM, and the binding event is followed by the "reluctant" step. We will discuss these and other results in terms of early events in the enzyme reaction cycle.

67. Testing K^+-like Occlusion of Cations in the Na^+/K^+-ATPase. ROLANDO C. ROSSI, RODOLFO M. GONZÁLEZ-LEBRERO, SERGIO B. KAUFMAN, and PATRICIO J. GARRAHAN, *Instituto de Química y Fisicoquímica Biológicas and Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina*

The K^+-congener Rb^+ can become spontaneously occluded in the Na^+/K^+-ATPase, leading to a state that slowly exchanges cations with the medium. Therefore, the rate of $^{86}Rb^+$ occlusion can be used to test whether any other cation (X^+) can lead the enzyme to a state that slows down $^{86}Rb^+$ occlusion (perhaps with X^+ being occluded). With this aim, we measured the time course of $^{86}Rb^+$ occlusion at 25°C in a purified preparation of Na^+/K^+-ATPase, after addition of $^{86}Rb^+$ (20 μM, final concentration) under two conditions: (1) X^+ was preincubated with the enzyme, or (2) X^+ was added together with $^{86}Rb^+$. Media contained 25 mM imidazole-HCl, pH 7.4. We found that, when the cation was K^+, Rb^+, Cs^+, Tl^+, NH_4^+, or Na^+ + oligomycin, the rate of $^{86}Rb^+$ occlusion for condition 1 was significantly slower than that of condition 2. When Na^+, Li^+, or Mg^{2+} were used, no significant difference between the time courses for the two conditions was observed. To discriminate if those cations that showed differences between conditions 1 and 2 were acting as K^+, we tested the ability of X^+ to block Rb^+ deocclusion (Forbush,

1987. *J. Biol. Chem.* 262:11116–11127). We therefore equilibrated the enzyme with 20 μM $^{86}Rb^+$ and followed the time courses of $^{86}Rb^+$ deocclusion after a 1/20 dilution in media with or without (control) X^+. We found that, when X^+ was K^+, Rb^+, Tl^+, or Cs^+, the rate of $^{86}Rb^+$ deocclusion was slower than that of the control, whereas when Na^+, Li^+, NH_4^+, Mg^{2+}, or oligomycin + Na^+ were used, no significant differences with the control were observed.

These results agree with the rule that those cations that become occluded like K^+ should show differences between the rates of occlusion for conditions 1 and 2, and be good blockers of Rb^+ deocclusion. NH_4^+ seems to be an exception to this rule. [Supported by CONICET, ANPCyT, and UBA.]

68. The Phosphatase Activity of Phosphorylation Site Mutants of Na^+,K^+-ATPase. MICHAELA BARTZ and GEORGIOS SCHEINER-BOBIS, *Institut für Biochemie und Endokrinologie, Fachbereich Veterinärmedizin, Justus-Liebig-Universität Giessen, Giessen, Germany*

The Na^+,K^+-ATPase becomes phosphorylated by the terminal phosphate group of ATP and can only resume its catalytic cycle after the phosphoric ester formed between the phosphate and Asp 369 (sheep α subunit numbering) is hydrolyzed. Besides ATP, the enzyme is capable of hydrolyzing a variety of phosphoric acid esters or anhydrides. This so-called phosphatase activity of the sodium pump has been often controversially discussed.

We investigate here the *p*-nitrophenyl phosphatase activity (*p*NPPase) of Na^+,K^+-ATPase single mutants Asp369Ala and double mutants Asp369Ala/Asp714Glu or Asp369Ala/Asp714Ala.

Asp714 corresponds to the Asp180 of the L-2-haloacid dehalogenase from *Pseudomonas sp.* (L-DEX-ps; EC 3. 8. 1. 2) and was shown to be involved in the dephosphorylation process of Na^+,K^+-ATPase by enhancing the nucleophilicity of a water molecule required for the hydrolysis of the phosphoric ester formed at Asp369 (Su, P., and G. Scheiner-Bobis. 2004. *Biochemistry.* 43: 4731–4740).

The *p*NPPase activity of the Asp369Ala single mutant was at the same level as that observed with the wild-type enzyme, was Mg^{2+} dependent, and was inhibited to the same extent by ATP, acetyl phosphate, or ouabain. With the double mutants, however, *p*NPPase activity was reduced. With the double mutant Asp369Ala/Asp714Glu, where the carboxyl group of Asp714 was replaced by the same of glutamic acid, the *p*NPPase activity was reduced by ~50% and was completely undetectable with the double mutant Asp369Ala/Asp714Ala.

The conclusions of the study are as follows. (1) Phosphorylation of the enzyme by *p*-nitrophenyl phosphate (*p*NPP) is not necessary for hydrolysis. (2) In the mutant enzyme, Asp714 increases the nucleophilicity of the water molecule required for the hydrolysis of *p*NPP and enables it to attack this phosphoric ester in place of the phosphoric ester normally formed at Asp369. (3) Since mutations of Asp714 considerably affect the phosphatase activity of the sodium pump, the hydrolysis of *p*NPP takes place within the Rossmann fold formed around Asp369, the phosphorylation site of the enzyme. [Supported through DFG, Sche 307/5-2.]

69. Kinetic Characterization of Intermediates during Na-ATPase and Na,K-ATPase Activity Using RH421 and Rb^+ Occlusion. PABLO G. SCHVARTZ, JOSE L.E. MONTI, RODOLFO M. GONZÁLEZ-LEBRERO, SERGIO B. KAUFMAN, PATRICIO J. GARRAHAN, and ROLANDO C. ROSSI, *Instituto de Química y Fisicoquímica Biológicas and Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina*

Employment of the fluorescent probe RH421 for studying intermediate states of the sodium pump reveals three main levels of fluorescence, the lowest corresponding to the states having three Na^+ bound, the highest to E_2P, and the intermediate level to the states with two K^+ bound or occluded (Sturmer et al. 1991. *J. Membr. Biol.* 121:141–161). On the basis of this interpretation, quantification of the intermediate states by measurements of fluorescence changes should be possible. This work was aimed to investigate the ability of the fluorescence probe RH421 to kinetically characterize the intermediates E_2P and $E_2(Rb_2)$ as well as the ATPase activity. With this aim, we followed the time courses of the fluorescence change with the enzyme performing Na-ATPase activity, until ATP was completely hydrolyzed. Media contained 150 mM NaCl, 0.5 mM free Mg^{2+}, and ATP from 0.1 to 25.6 μM, at 25°C and 25 mM imidazole-HCl, pH 7.4. Results from these experiments allowed calculation of the Na-ATPase activity, giving a value of K_M for ATP very similar to those found in the literature (Kane et al. 1997. *Biochemistry.* 36:13406–13420). In another series of experiments where different concentrations of Rb^+ were added to the media, we measured in parallel experiments the time courses of fluorescence change and of $^{86}Rb^+$ occlusion. We found that the steady-state levels of both measurements show similar affinities for [Rb^+] and that the increasing parts of the time courses at saturating [Rb^+] have the same rate coefficients. A model based on that of Albers and

Post was fitted to the experimental results. The model allowed simulation of the time courses of both the fluorescence change and of Rb^+ occlusion, after setting a high-fluorescence coefficient value for E_2P and a lower fluorescence coefficient for the Rb^+-bound states. [Supported by CONICET, Agencia Nacional de Promoción Científica y Tecnológica, and Universidad de Buenos Aires.]

70. FTIR Difference Spectroscopy of Na,K-ATPase. MICHAEL STOLZ,[1] ERWIN LEWITZKI,[1] DETLEF THOENGES,[2] WERNER MÄNTELE,[2] ANDREAS BARTH,[3] and ERNST GRELL,[1] [1]*Max-Planck-Institute of Biophysics, Frankfurt am Main, Germany*; [2]*Institute of Biophysics, University of Frankfurt am Main, Frankfurt am Main, Germany*; [3]*Department of Biochemistry and Biophysics, The Arrhenius Laboratories for Natural Sciences, Stockholm University, Stockholm, Sweden* (Sponsor: Andreas Barth)

Changes of protein secondary structure and protein microenvironment associated with partial reactions of Na,K-ATPase have been investigated in detail by reaction-induced FTIR difference spectroscopy. Infrared absorbance changes were recorded in the region from 2000 to 950 cm^{-1} in H_2O and D_2O. Depending on medium composition, three different partial reactions have been induced by the photochemical release of ATP from different precursors (mainly NPE-caged ATP): (1) ATP binding and release, respectively, at the binding site, (2) formation of the ADP-sensitive phosphoenzyme E1P, and (3) formation of the K^+-sensitive phosphoenzyme E2P. All partial reactions lead to distinct changes of the infrared spectrum that are characteristic of the adopted states. The observed band amplitudes in the amide I region of the infrared spectrum indicate that the net change of secondary structure change involves at most ~0.3% of all amino acid residues of Na,K-ATPase. In comparison, the smallest spectral changes are found upon ATP release from the binding site, and the largest for phosphoenzyme conversion (E1P→E2P), indicating larger secondary structure changes. Phosphorylation is accompanied by the appearance of carbonyl bands at 1738 and 1709 cm^{-1}, one of which is tentatively assigned to the phosphorylated Asp^{369} side chain. Spectral changes in the ranges of 1750–1700 and 1610–1400 cm^{-1} found upon phosphoenzyme conversion accompanied with Na^+ release can be attributed to changes in hydrogen bonding, protonation state, and alkali ion coordination of carboxyl groups. The FTIR-difference spectra of Na,K-ATPase and Ca-ATPase (Barth et al. 1996. *J. Biol. Chem.* 271:30637–30646) differ significantly, probably due to different structural features of these two enzymes.

71. Changes in Interactions between Phosphorylation- and Actuator-domains of Sarco(endo)plasmic Reticulum Ca^{2+}-ATPase during Processing of Phosphoenzyme Intermediate. KAZUO YAMASAKI, TAKASHI DAIHO, WANG GUOLI, STEFANIA DANKO, and HIROSHI SUZUKI, *Department of Biochemistry, Asahikawa Medical College, Asahikawa, Japan* (Sponsor: David Gadsby)

Functional roles of the residues located on the interface of the actuator (A) and the phosphorylation (P) domains of sarcoplasmic reticulum Ca^{2+}-ATPase were explored by site-directed substitutions and kinetic analysis. The residues examined were Tyr^{122} and Glu^{123} on the loop linking the A domain and the second transmembrane helix (M2), and Arg^{324} and Arg^{334} on the P domain. The hydrogen-bonding or ionic interactions of Tyr^{122}-Arg^{324} and Glu^{123}-Arg^{334} are found in the crystal structure of Ca^{2+}-bound unphosphorylated Ca^{2+}-ATPase. Results showed the marked inhibition of the isomeric transition of phosphorylated intermediate (*E*P) (loss of ADP-sensitivity) by the substitutions of Arg^{324} and Arg^{334}, and the block of the subsequent hydrolysis of *E*P by those of Tyr^{122} and Glu^{123}. The observed effects of the substitutions indicated that the interactions seen in the Ca^{2+}-bound unphosphorylated structure are not functionally important and further revealed that the positive charge of Arg^{334} and the aromatic ring of Tyr^{122} are critically important for the *E*P transition and *E*2P hydrolysis, respectively. On the basis of the crystal structures, our results strongly suggest that, for the *E*P transition, Arg^{334} moves toward the membrane surface and forms new interaction with M2 (likely Asn^{111}), thus contributing to the inclination of P domain and the gathering of this domain with the largely rotated A domain. Tyr^{122}, on the other hand, most likely functions to produce hydrophobic interactions between the gathered A and P domains and thus to produce the proper configuration of the catalytic site with the phosphatase function. The four residues seem to change their interaction partners and contribute to the coordinated motions of the cytoplasmic domains for processing *E*P to transport Ca^{2+}. [Supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.]

72. Distinct Natures of Be/F-bound, Al/F-bound, and Mg/F-bound Stable Analogs of the ADP-insensitive Phosphoenzyme Intermediate of Sarcoplasmic Reticulum Ca^{2+}-ATPase. STEFANIA DANKO, KAZUO YAMASAKI, TAKASHI DAIHO, and HIROSHI SUZUKI, *Department of Biochemistry, Asahikawa Medical College, Asahikawa, Japan* (Sponsor: David Gadsby)

The structural nature of stable analogs of the ADP-insensitive phosphoenzyme (*E*2P) of Ca^{2+}-ATPase formed in sarcoplasmic reticulum vesicles, i.e., enzymes with bound beryllium/fluoride (Be/F/*E*2), or bound aluminum/fluoride (Al/F/*E*2), or bound magnesium/fluoride (Mg/F/*E*2), was explored and compared with that of actual *E*2P formed from P_i without Ca^{2+} (Danko et al. 2004. *J. Biol. Chem.* 279:14991–14998). Changes in the trinitrophenyl-AMP fluorescence revealed that the catalytic site is strongly hydrophobic in Be/F/*E*2, as in *E*2P, but is hydrophilic in Mg/F/*E*2 and Al/F/*E*2, and yet the three cytoplasmic domains are compactly organized in all these states. Thapsigargin, which fixes the lumenal Ca^{2+} gate in its closed state, reduced tryptophan fluorescence markedly in Be/F/*E*2, as in *E*2P, but only very slightly (hence the lumenal gate is likely closed without thapsigargin) in Mg/F/*E*2 and Al/F/*E*2. Consistent with this, the completely suppressed Ca^{2+}-ATPase activity in Be/F-treated vesicles was rapidly restored in the presence of ionophore A23187, but not in its absence, by incubation with Ca^{2+} at pH 6, and therefore lumenal Ca^{2+} is accessible to reactivate the enzyme. In contrast, no or only very slow restoration was observed with vesicles treated with Mg/F and Al/F even with A23187. Thus Be/F/*E*2 has features very similar to those characteristic of the *E*2P ground state, while Al/F/*E*2 and Mg/F/*E*2 most likely mimic the transition or product state for the *E*2P hydrolysis, during which the hydrophobic environment around the phosphorylation site is lost and the Ca^{2+} gate is closed. The change in hydrophobic nature is probably associated with a change in phosphate geometry from the covalently bound tetrahedral ground state (BeF_3^-) to trigonal bipyramidal transition state (AlF_3 or AlF_4^-) and further to tetrahedral product state (MgF_4^{2-}), and such changes likely rearrange transmembrane helices to prevent access and leakage of lumenal Ca^{2+}. [Supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.]

73. On the Phosphorylation, Dephosphorylation, E_2/E_1 Transition, and the Cation Specificity of Rat and Human Non-gastric H,K-ATPase. HERMAN G.P. SWARTS, PETER H.G.M. WILLEMS, JAN B. KOENDERINK, and JAN JOEP H.H.M. DE PONT, *Department of Biochemistry, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands* (Sponsor: Carel H. van Os)

The catalytic α-subunits of both rat and human non-gastric H,K-ATPase were expressed in Sf9 cells using the baculovirus expression system with several putative β-subunits: $NaK\beta_1$, $NaK\beta_3$, and $HK\beta_1$. In the absence of a β-subunit the enzymes were not active. With the Na,K-ATPase β_1-subunit, the highest ATPase activity was reached and these preparations were used in this study. Both rat and human enzymes could be phosphorylated by ATP, preferentially at lower pH values. The rate of dephosphorylation was relatively fast and could be further stimulated by micromolar concentrations of NH_4^+ or K^+ or millimolar concentrations of Na^+. The rat ATPase activity could be maximally stimulated by NH_4^+; K^+ and Na^+ stimulated the activity to maximally 62 and 18%, respectively. In contrast, the human enzyme was maximally stimulated by either NH_4^+ or Na^+, whereas its maximal K^+-stimulated activity was only 27%. Vanadate inhibited the ATPase activity better with K^+ than with NH_4^+, suggesting that K^+ was a stronger E_2 promotor than NH_4^+. The ATPase activity of both ATPases could also be inhibited by relatively high ouabain concentrations, but hardly by SCH 28080. In addition, oligomycin inhibited the ATPase activity in a cation-independent manner. From the maximal NH_4^+-ATPase activities and phosphorylation levels, turnover numbers of at least 32,000 min^{-1} for the rat and human constructs could be determined, values 20 times higher than that of gastric H,K-ATPase and approximately three times higher than that of Na,K-ATPase. This study shows that nongastric H,K-ATPase is indeed a P-type ATPase. Its mechanism can be described with the Post-Albers reaction scheme, in which it functions more actively with NH_4^+ ions instead of K^+ ions. No hard evidence was obtained that protons, which are the coactivating ions, can be replaced by other cations. [Supported by the Dutch Kidney Foundation.]

74. Importance of Gly^{363} of the Na^+,K^+-ATPase for Catalytic Function and E_2–E_1 Conformational Changes. MADS TOUSTRUP-JENSEN and BENTE VILSEN, *Department of Physiology, Institute of Physiology and Biophysics, University of Aarhus, DK-8000 Aarhus C, Denmark* (Sponsor: Mads Toustrup Jensen)

The highly conserved glycine, Gly^{363}, at the bending point between the P1 helix and the first β-strand of the P-domain of the Na^+,K^+-ATPase was replaced by alanine. The catalytic turnover rate was eightfold reduced in the mutant, and only insignificant phosphorylation from [γ-^{32}P]ATP could be detected in the absence of oligomycin. In the presence of oligomycin, the mutant displayed a fivefold reduction of apparent affinity for Na^+ in activation of phosphorylation, and rapid kinetic measurements at varying ATP concentration showed that the maximal phosphorylation rate was reduced 4.3-fold in the mutant relative to the wild type. It is con-

cluded that Gly^{363}-Ala disturbs the function of the catalytic site in $E_1(Na_3)$ and possibly Na^+ occlusion and/or the communication between the Na^+ binding sites and the catalytic site. The mutant showed 15-fold increase of apparent ATP affinity and 140-fold reduction of apparent vanadate affinity in measurements of ATPase activity, consistent with a shift in the E_1–E_2 conformational equilibrium in favor of E_1. A destabilization of E_2 was further supported by a reduced level of $E_2(K_2)$ and an increased rate of K^+ deocclusion, as well as a large reduction of ouabain affinity. In equilibrium binding experiments, Gly^{363}-Ala displayed a fivefold reduced intrinsic affinity of $E_2(Rb_2)$ for vanadate, indicating a direct effect of the mutation on the catalytic site in E_2, in addition to the effect on the E_1–E_2 equilibrium. The defective catalytic function of the mutant likely results from a change of orientation of the first β-strand of the P-domain that ends with the phosphorylated Asp^{371}, whereas the shift of the conformational equilibrium in favor of E_1 likely results from interference with the interaction between Gly^{363} and Arg^{761} in transmembrane segment M5, which by analogy with the Ca^{2+}-ATPase crystal structures may be important for fixing M5 and the L6-7 loop in correct positions. [Supported by the Danish Medical Research Council, the Novo Nordisk Foundation, Denmark, the Lundbeck Foundation, Denmark, and the Research Foundation of Aarhus University.]

75. Molecular cloning of a putative Na/K-ATPase cDNA from a protist, *Euglena gracilis*. TAKAKO FUKAWA,[1] MARIKO SHONO,[2] MASATO WADA,[3] TAKESHI NARA,[4] TAKASHI AOKI,[4] and YUKICH HARA,[1] [1]*Tokyo Medical and Dental University, Graduate School of Health Sciences, Tokyo, Japan*; [2]*Japan International Research Center for Agricultural Sciences, Ibaraki, Japan*; [3]*National Institute of Fruit Sciences, Ibaraki, Japan*; [4]*Juntendo University School of Medicine, Tokyo, Japan* (Sponsor: David Gadsby)

Most cells other than animal cells had been believed to extrude intracellular sodium ions primarily with a combination of Na^+/H^+-antiporter and H^+-ATPase, rather than with a sodium pump. However, cells of marine alga, *Heterosigma akashiwo*, have Na^+ and K^+-dependent ATPase activity in their plasma membrane, and a novel Na/K-ATPase cDNA was cloned from *H. akashiwo* (Shono et al. 2001. *Biochim. Biophys. Acta.* 1511:193–199). *H. akashiwo* is a unicellular eukaryote and belongs to the protist family. So the Na/K-ATPase gene was expected to be widely distributed in the protist kingdom. Here, we report cloning of a Na/K-ATPase-like cDNA from a protist, *Euglena gracilis*.

A PCR fragment was amplified using degenerate primers corresponding to the highly conserved regions of animal Na/K-ATPase (TM4 and EP site). A full length cDNA was obtained with 5′-RACE and 3′-RACE methods. It was 3,880 bp long and encoded a 1,082-aa protein with a molecular mass of 119 kD. The deduced product exhibited around 40% amino acid identity with animal Na/K-ATPase α-subunits. Hydropathy plot analysis revealed 10 transmembrane domains and its profile was quite similar to those of the α-subunits. The amino acid residues essential for Na^+ or K^+ coordination in Na/K-ATPase were conserved. The 5′ end of the cDNA contained a 26-nucleotide leader sequence (spliced leader sequence), suggesting the occurrence of RNA trans-splicing. Northern blot analysis of *Euglena* RNA revealed a transcript of around 5 kb. It is suggested that the gene codes for a Na/K-ATPase catalytic subunit, and that the Na/K-ATPase gene might be distributed in Euglenoid species.

76. K^+-induced Change in Oligomeric Assembly of $C_{12}E_8$-solubilized Pig Gastric H/K-ATPase Observed by Single-molecule Detection Techniques. KAZUHIRO ABE,[1] SHUNJI KAYA,[2] KAZUYASU SAKAGUCHI,[2] TOSHIAKI IMAGAWA,[2] YUTARO HAYASHI,[3] and KAZUYA TANIGUCHI,[2] [1]*Department of Biophysics, Faculty of Science, Kyoto University, Kyoto, Japan*; [2]*Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo, Japan*; [3]*Department of Biochemistry, Kyorin University School of Medicine, Mitaka, Japan* (Sponsor: David Gadsby)

The pig gastric H/K-ATPase was specifically modified by fluorescein 5′-isothiocyanate (FITC), solubilized by octaethylene glycol dodecyl ether ($C_{12}E_8$), and the composition of oligomeric components was evaluated by single-molecule detection techniques using total internal reflection fluorescence microscopy. The numbers of FITC-H/K-ATPase molecules in oligomeric components were determined from the intensity of FITC fluorescence just after laser illumination (Abe et al. 2003. *Biochemistry.* 42:15132–15138). Solubilization of the enzyme strongly reduced potassium-dependent *p*-nitrophenyl phosphatase (K-pNPPase) activity with the appearance of protomer and diprotomer of FITC-H/K-ATPase. However, solubilization with increasing concentration of K^+ induced significant increases in K-pNPPase activity with an increase in the amount of the tetraprotomer of FITC-H/K-ATPases. The correlation coefficient between the proportion of tetraprotomer and the proportion of the K-pNPPase activity within the same FITC-H/K-ATPase preparation was estimated to be 0.93. Such positive correlations were not obtained between these activities and other oligomeric species.

These data suggest that the catalytic unit of $C_{12}E_8$-solubilized gastric H/K-ATPase is a tetraprotomer and that K^+, a physiological ligand of H/K-ATPase, stabilizes this tetraprotomeric assembly. [Supported by Grant-in-Aid for Scientific Research (17001307 to K. Abe and 13680703 to S. Kaya) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.]

77. Structure-inducing Effects of SDS on NaK-ATPase. MARJORIE E. GABLE, ALEXANDER V. IVANOV, LIJUN LIU, and AMIR ASKARI, *Department of Pharmacology, Medical College of Ohio, Toledo, OH*

Because most structure/function studies of NaK-ATPase have been done on SDS-treated preparations, it is necessary to know how the enzyme's properties are affected by SDS. We reported recently that an SDS-solubilized enzyme form that retains partial structure/function is formed during the purification procedure, and, if not removed, this solubilized and partially unfolded enzyme distorts the properties of the native enzyme (Ivanov et al. 2004. *J. Biol. Chem.* 279:29832–29840). Extending this work, we have studied the effects of nonsolubilizing concentrations of SDS on structure/function. The pig kidney enzyme (purified by the standard SDS-using procedure, but then thoroughly washed) was exposed to low nonsolubilizing SDS levels (one to two orders of magnitude below CMC), and subjected to chemical cross-linking for detection of subunit contacts. Using SH-reactive CuP, SDS greatly stimulated the formation of cross-linked $(\alpha,\alpha)_n$, but not of cross-linked β,β, or α,β. The same low SDS levels did not affect the cross-links induced by amino-reactive BS3 or DSS. The specificities of these SDS effects suggest that binding of SDS monomers to the enzyme increases the clustering of protomers through α,α-contacts at discrete domains. That this clustering is not denaturing was verified by data showing that nonsolubilizing SDS has no effect on Vmax of ATPase activity or on maximal occlusion capacities of Na^+ and Rb^+, but increases ATP and Na^+ affinities. These nondenaturing SDS effects on quaternary structure/function are consistent with known structure-inducing effects of SDS monomers on several partially unfolded proteins, suggesting the presence of partially unfolded domains in the purified enzyme. Whether these domains are native or induced during purification remains to be explored. Having several purified NaK-ATPase preparations that have not seen SDS would be helpful. [Supported by NIH grant HL36573.]

78. The Role of N-linked Glycosylation in the Na,K-ATPase β2-subunit. REBECCA CLIFFORD and JACK H. KAPLAN, *Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, IL* (Sponsor: Jack H. Kaplan)

The Na,K-ATPase is a plasma membrane ATPase consisting of αβ heterodimers. The β-subunit allows the α-subunit to traffic to the plasma membrane as a heterodimer. The role of β2-subunit glycosylation in Na,K-ATPase αβ assembly, trafficking, and catalytic activity is not fully understood. The β2-subunit in rat contains eight consensus motifs for N-linked glycosylation and is most homologous to the apically localized H,K-ATPase β-subunit. The H,K-ATPase undergoes reduced apical localization, increased ER retention, and increased degradation when glycosylation of the β-subunit is reduced (Vagin et al. 2004. *J. Biol. Chem.* 279:39026–39034). To determine whether Na,K-ATPase β2 behaves in a similar fashion, we have expressed single and multiple site deglycosylation mutants for Na,K-ATPase β2-subunit with wild-type α1-subunit in High-Five cells. The α1β2 constructs displayed ouabain-sensitive ATPase at the plasma membrane, varying amounts of inactive protein retention in the ER, and cellular degradation. In addition to analyzing how β2 glycosylation mutants affect assembly, transport, and enzymatic activity in insect cells, we investigated their effects on apical/basolateral localization in polarized monolayers of MDCK cells. Our results suggest that the extent of glycosylation of the β2-subunit may play a significant role in Na pump plasma membrane delivery in renal cells. [Supported by NIH grant GM39500.]

79. Interactions Between the α and β Subunits of the Na,K-ATPase. ROBERT E. DEMPSKI, THOMAS FRIEDRICH, and ERNST BAMBERG, *Max-Planck-Institute of Biophysics, Frankfurt am Main, Germany* (Sponsor: Jack H. Kaplan)

The Na,K-ATPase is a multimeric membrane protein that maintains cell homeostasis through the active transport of Na^+ and K^+ against their concentration gradients, using ATP hydrolysis. The protein resides in two primary conformational states in accordance with the Albers-Post scheme: E_1 and E_2. Although there is a wide range of literature that describes ion and inhibitor binding sites as well as the kinetics of charge transport, measurements that follow the conformational dynamics of the ion pump are limited. Toward this goal, our group has previously shown that both the α and β subunits demonstrate fluorescence changes that can be correlated to the conformational state of the protein and to charge transport, using the techniques of cysteine scanning mutagenesis and voltage clamp

fluorometry (Geibel et al. 2003. *PNAS.* 100:964–969; Dempski et al. 2005. *J. Gen. Physiol.* 125:505–520).

Although fluorescence responses at multiple residues can be used to monitor conformational changes of the holoenzyme, more recent studies have focused on understanding the relative conformational dynamics between the α and β subunits. Thus, the purpose of our research is to examine the fluorescence properties of multiple fluorophores and to use fluorescence resonance energy transfer to obtain a qualitative and quantitative analysis of how these subunits interact with each other in order to compare the distances between residues of the α and β subunits. The results from these studies are aimed to provide insight into relevant movement of defined residues between the α and β subunits during conformational changes of the holoenzyme.

80. The Na,K-ATPase β Subunit as a Regulatory Cofactor studied by Voltage Clamp Fluorometry. KATHARINA L. DÜRR, ROBERT E. DEMPSKI, ERNST BAMBERG, and THOMAS FRIEDRICH, *Max-Planck-Institute of Biophysics, Frankfurt am Main, Germany* (Sponsor: Jack H. Kaplan)

The Na^+/K^+-ATPase consists of at least two subunits, a large catalytic α subunit and an ancillary β subunit, which is required for proper trafficking. Although enzymatic functions mediated by the α subunit have been extensively studied, little is known about the role of the β subunit. The participation of the β subunit in Na^+/K^+-ATPase conformational changes has recently been shown by our laboratory using site-directed fluorescence labeling and voltage clamp fluorometry (Dempski et al. 2005. *J. Gen. Physiol.* 125:505–520). Here we utilize the β_1-S62C mutant as a sensor construct to investigate the effects of inserting bulky tryptophan side chains within the β subunit's TM region on voltage dependence and kinetics of the E_1P–E_2P conformational change, which was previously shown to affect Na^+/K^+-ATPase cation affinities (Hasler et al. 2001. *J. Biol. Chem.* 276:16356–16364). Upon expression in *Xenopus* oocytes, sulfhydryl-specific labeling is performed with tetramethylrhodamine-6-maleimide. Voltage steps applied under Na^+/Na^+ exchange conditions evoke characteristic fluorescence changes, which are directly correlated to the voltage dependence of transient charge movement that occurs in conjunction with E_1P–E_2P conformational changes.

Under Na^+/Na^+ exchange conditions, the voltage dependences of both fluorescence amplitudes and charge movements were shifted toward depolarizing potentials when the β_1-S62C construct carrying additional Y39W/Y43W mutations was compared with unmodified β_1-S62C. Due to the voltage dependence of extracellular Na^+ rebinding, this is consistent with an increased Na^+ affinity on the external binding sites and a shift of the E_1P–E_2P conformational equilibrium toward the E1 state, since a lower effective Na^+ concentration is needed to induce the same E_2P–E_1P shift. This was paralleled by a decrease in extracellular K^+ affinity as inferred from the voltage dependences of fluorescence amplitudes in K^+ titration experiments. These observations agree with previous results and underline the role of the Na^+/K^+-ATPase β subunit as a modulating cofactor for enzymatic properties of the holoenzyme. [Supported by DFG SFB 472.]

81. Mutations In β Subunit Transmembrane Domain Residues Reduce Na,K-ATPase Stability. ROBERT A. FARLEY, EVGENIYA DENISOVA, SUSANNE BECKER, and GEORGIOS SCHEINER-BOBIS, *Department of Physiology and Biophysics, University of Southern California, Los Angeles, CA*; *Institut fur Veterinarmedizin, University of Giessen, Giessen, Germany*

Na,K-ATPase and H,K-ATPase each contain two polypeptide subunits, an α subunit of ~1,000 amino acids and a β subunit of ~300 amino acids. Comparison of the amino acid sequences of different β subunits shows only ~30% identity. Within and around the single transmembrane segment of the β subunits, however, there are seven amino acids that are absolutely conserved. These amino acids are all located on one face of a predicted α helix, an arrangement that may have structural or functional significance. Five of the seven conserved residues contain aromatic side chains. To test the significance of this arrangement, alanine and leucine substitutions were made for the conserved aromatic amino acids. Single amino acid substitutions had no effect on ATPase activity, or on the apparent affinity of the pump for either sodium or potassium, and did not affect the stability of the enzyme after heating. Multiple alanine substitutions of these aromatic amino acids also had no effect on apparent ion affinity; however, they markedly reduced the ability of the Na,K-ATPase to bind ouabain after heating. Similar results were obtained after replacement of Phe38 and Phe42 by leucine (Cluster-I), or replacement of Tyr39, Tyr43, or Phe50 by leucine (Cluster-II). Multiple alanine substitutions for amino acids on the opposite face of the anticipated transmembrane α helix, or replacement of Cys45 by phenylalanine to imitate the aromatic acid distribution of the β2 subunit, had no effect on either apparent ion affinity or heat stability. These results can be explained by a model in which the conserved aromatic amino acids of the transmembrane segment of the β subunit interact with at least one transmembrane seg-

ment of the α subunit and stabilize the α/β unit, but do not participate in the binding or recognition of ions by the pump. [Supported by NIH grant GM28673.]

82. An Intermolecular Conformational Coupling Paradigm for ATP-dependent Cation Pump Up-regulation. JEFFREY FROEHLICH, YASSER A. MAHMMOUD, and FLEMMING CORNELIUS, *Department of Biochemistry and Molecular Biology, University of Maryland, Baltimore, MD; Department of Biophysics, Aarhus University, Aarhus, Denmark* (Sponsor: Jack H. Kaplan)

Several of the ATP-dependent cation pumps, including the cardiac muscle sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) and shark rectal gland Na^+/K^+-ATPase, are regulated by intrinsic, low molecular weight proteins (phospholamban/PLB; FXYD10/PLMS). Relief of the inhibition by these proteins leads to an increase in the overall pump activity (≠Vmax) by an unknown mechanism. A recent hypothesis for the regulation of cardiac SERCA2a by PLB (Mahaney et al. 2005. *Biochemistry.* 44:7713–7724) associates the increase in pump activity with SERCA2a oligomerization during catalytic cycling. SERCA2a conformational interactions are prevented in the down-regulated state by the association of PLB with a specific (N domain) site on SERCA2a. Exposure of this site, secondary to PLB phosphorylation, nitroxyl modification, or exclusion from the membrane, enables it to interact with a complementary (A domain) site on an adjacent SERCA2a polypeptide chain, forming a dimer. Staggering of the catalytic reactions and free energy exchange between the protomers enables fast reactions (E1P→E2P) in the cycle to couple to slow (rate-limiting) reactions (E2P→E2 + P_i), decreasing E1P turnover to E2P, and activating E2P hydrolysis. A reciprocal change in the activity of these reactions has also been observed in shark Na^+/K^+-ATPase following PLMS truncation by mild trypsin treatment. The similarities in the kinetic effects that accompany the relief of inhibition by PLB and PLMS suggest that up-regulation of the Ca^{2+}- and Na^+/K^+-ATPases utilizes a common mechanism that involves catalytic subunit oligomerization, intersubunit conformational coupling, and free energy exchange. These molecular events relieve the rate limitation imposed by slow steps in the catalytic cycle, increasing the overall catalytic efficiency (Vmax) of cation pumping. Activation by this mechanism utilizes components that are present in the membrane, and does not involve de novo protein synthesis or trafficking from the Golgi apparatus as required by other models for up-regulation. [Supported by the Danish Medical Research Council and the Aarhus University Research Foundation.]

83. Comparison of Ouabain Binding Amount among Oligomeric Protein Components Constituting Pig Kidney Na^+/K^+-ATPase. Y. HAYASHI, N. SHINJI, Y. TAHARA, E. HAGIWARA, and H. TAKENAKA, *Department of Biochemistry, Kyorin University School of Medicine, Mitaka, Tokyo, Japan* (Sponsor: David Gadsby)

Membrane-bound Na^+/K^+-ATPase purified from porcine renal medulla has been solubilized into $(\alpha\beta)_4$-Tetraprotomer (T), $(\alpha\beta)_2$-Diprotomer (D), and $(\alpha\beta)$-Protomer (P) in $C_{12}E_8$ (Hayashi et al. 2003. *Ann. NY Acad. Sci.* 986:232–234). Under the condition favorable for formation of T, such as the presence of phosphatidylserine under acidic pH, the amount of T exceeded 50% of oligomers, and the isolated T was structurally stable during ATP hydrolysis cycles at 25°C (unpublished data). The amount of ATP bound to T, D, and P were 0.40, 0.70, and 0.71 mol/mol protomer, respectively, in 1 μM ATP (unpublished data). In this report, we estimated the amount of ouabain bound to T, D, and P to identify whether protomers in T were homogeneous with respect to ouabain binding. After incubating the enzyme, before or after solubilization in $C_{12}E_8$, with ATP or Pi for 5 min to form E_2-P via forward or backward directions, 21 μM [^{3}H]-ouabain was added for 2 min to 23 h at 0°C. The resultant enzyme was applied to a TSKgel G3000SWXL column at 0°C, in which A_{280} and radioactivity were simultaneously monitored to estimate the amount of [^{3}H]-ouabain bound to the oligomers. The maximal levels of ouabain bound to T, D, and P were respectively 0.663 ± 0.032, 0.902 ± 0.019, and 0.965 ± 0.053 mol/mol P, which persisted after chasing [^{3}H]-ouabain in the membrane-bound enzyme with 1.3 mM cold ouabain before the solubilization. The amount of [^{3}H]-ouabain bound to T, D, and P in the membrane was thus assumed to be 2.7, 1.8, and 0.94 mol/mol of each oligomer, respectively. These results indicated that protomers in D were homogeneous, while association of four protomers to construct T would conceal the ouabain binding site in one of those protomers.

84. Interaction Region between Catalytic Subunits during ATP Hydrolysis in Oligomeric Na/K-ATPase. SHUNJI KAYA, TOSHIAKI IMAGAWA, KAZUYASU SAKAGUCHI, and KAZUYA TANIGUCHI, *Biological Chemistry, Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo, Japan* (Sponsor: Jack Kaplan)

Nine cysteine residues in the large cytoplasmic region of the rat α 1 Na/K-ATPase were mutated to serine, to give the following substitutions: C452S, C456S & C457S, C511S, C549S, C577S, C599S, C656S,

and C698S. Ouabain-resistant HeLa cells expressing rat α 1 wild type and each of the mutants were selected. All the Cys-substituted enzymes were found to be active. Membranes isolated from the HeLa cells expressing the wild-type and mutant cDNAs all exhibited ouabain-insensitive Na/K-ATPase activity together with intrinsic ouabain-sensitive HeLa Na/K-ATPase activity. The formation of a phosphoenzyme intermediate is known to promote oxidative cross-linking between α subunits catalyzed by Cu^{2+}-phenanthroline (Ganjeizadeh et al. 1995. *J. Biol. Chem.* 270:15707–15710). When wild-type membranes were incubated with Cu^{2+}-phenanthroline under conditions of phosphorylation, formation of α-alpha dimers was observed by SDS-PAGE and Western blotting. Among the mutants, the extent of dimer formation in C456S & C457S was greatly reduced. This suggests that the backside regions of two N-domains come into contact with each other during the formation of E2P in oligomeric Na/K-ATPase. [Supported by grants-in-aid for Scientific Research (13142201 to S. Kaya) from the Ministry of Education, Science, Sports, and Culture of Japan.]

85. Two-hybrid Screening of Mammalian Interactome for Identification of Physiological Partners of βm, a Protein from the Family of X,K-ATPase β-Subunits. TATYANA V. KORNEENKO,[1] NIKOLAY B. PESTOV,[1] ROSSEN RADKOV,[1] MIKHAIL I. SHAKHPARONOV,[2] and NIKOLAI N. MODYANOV,[1] [1]*Medical College of Ohio, Toledo, OH*; [2]*Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia* (Sponsor: Jack Kaplan).

Mammalian βm is the most interesting member of X,K-ATPase β-subunit family. First, it has an unusual NH_2-terminal cytodomain; second, it is dynamically expressed during muscle development; third, it does not associate with X,K-ATPase α-subunits; and, finally, it is not transported to the plasma membrane; instead, it accumulates abundantly in myonuclear membranes. To identify proteins interacting with βm, we have screened a mouse embryo cDNA library in a yeast two-hybrid system against three baits: NH_2-terminal cytodomain, COOH-terminal ectodomain, and full-length protein including its single transmembrane segment. The obtained colonies were tested to exclude obvious false positives using retransformation with bait and control plasmids into fresh yeast cells. Screening against NH_2-terminal cytodomain gave a large number of false positives and one putative true positive identified as borealin, an important component of chromosomal passenger complex. COOH-terminal ectodomain appears to be unsuitable for yeast two-hybrid screening, which yielded diverse peptides containing sequence(s) GR. Most promising results were obtained using full-length βm as bait. For example, βm itself was identified as an interactor. This fact is sufficient to claim that yeast two-hybrid screening using proteins with one transmembrane segment is capable of identifying true interactors. Also, this indicates that βm exists in vivo as a di- or oligomer. The majority of identified proteins are membrane proteins, and an astonishing proportion represents proteins that are active in the nucleus. The candidate true interactors are LAP-1 (a component of inner nuclear membrane that interacts with nuclear lamina), Syne-1 (a large protein enriched in juxtasynaptic nuclei, anchoring inner nuclear membrane to cytoskeleton), and heme oxidases HOX1 and HOX2. The most interesting, however, are transcription factor CREB3 and transcriptional coregulator SKIP. The latter findings suggest that βm may be involved in regulation of transcription, specifically to perinatal skeletal muscle cells. [Supported by NIH grant HL-36573 and RFBR grant 03–04-49046.]

86. Diprotomers are The Smallest Oligomers of Na,K-ATPase in Membranes from Canine Renal Medulla. HOWARD KUTCHAI and DAVID D. THOMAS, *Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, VA*; *Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, MN*

This study provides new information on the quaternary structure of the Na,K-ATPase of canine kidney. Microsomes prepared from canine renal outer medulla were treated with subsolubilizing levels of SDS to produce a purified, membranous Na,K-ATPase preparation. The Na,K-ATPase was labeled with erythrosin iodoacetamide (ErIA) or with erythrosine isothiocyanate (ErITC). Time resolved phosphorescence anisotropy (TPA) measurements were made with the erythrosin-labeled membranes. Fitting theoretical models to the TPA decay curves gave information about the number of distinct rotating oligomeric species present and their rotational diffusion coefficients. EPR spectra of 5-doxylstearate in protein-free liposomes made from lipids extracted from the membranes allowed us to estimate the effective viscosity that opposes protein rotation. The viscosity estimates and the rotational diffusion coefficients obtained from analysis of the TPA data were used in the Saffman-Delbrück equation to estimate the sizes of rotating oligomers. Comparison with our previous data on SERCA1 in skeletal SR suggests that the smallest oligomer of Na,K-ATPase is approximately two times larger in intramembrane volume than the smallest oli-

gomer of SERCA1, which we argue is monomeric SERCA1. This is consistent with the interpretation that the smallest species of Na,K-ATPase present is a diprotomer. Our data are consistent with the presence of two other larger rotating species of Na,K-ATPase, one species being about the size of a tetra-protomer and the other species representing much larger oligomers. [Supported by NIH grants GM27906 and GM50764.]

87. β-Subunit–dependent Polarization of the Na,K-ATPase. MELISSA D. LAUGHERY, YIQING CHI, and JACK H. KAPLAN, *Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, IL* (Sponsor: Jack H. Kaplan)

Abnormal expression of the β2-subunit has been correlated with the mislocalization of the Na,K-ATPase in the disease state of polycystic kidney disease (PKD) (Wilson et al. 2000. *Am. J. of Pathol.* 156:253–268). In normal kidney epithelium cells, the β1-subunit is expressed and the Na,K-ATPase is localized to the basolateral side of the plasma membrane. However, in PKD cells, the β2-subunit and the associated Na,K-ATPase heterodimer have been shown to appear on the apical side of the plasma membrane. To determine if the β2-subunit alone is sufficient to misdirect the Na,K-ATPase to the apical membrane, we have expressed the β2-subunit in a MDCK cell line. Through the methods of (1) confocal microscopy and (2) cell surface–specific labeling, we have documented the polarization of the Na,K-ATPase containing β1 and β2 subunits in the polarized MDCK monolayer. We further these studies to (1) address the potential role of β-subunit glycosylation in cell surface delivery and polarization and (2) determine if polarization of the Na,K-ATPase is consistent in other cell lines that have been derived from kidney epithelium. [Supported by NIH grants HL30315 and GM39500.]

88. βm-Protein, an Enigmatic Member of X,K-ATPase β-Subunit Family. NIKOLAI MODYANOV, NIKOLAY PESTOV, TATIANA KORNEENKO and ROSSEN RADKOV, *Department of Pharmacology, Medical College of Ohio, Toledo, OH* (Sponsor: David C. Gadsby)

From a structural point of view, highly conserved (≥90% identity) mammalian βm-proteins obviously belong to the X,K-ATPase β-subunit family. However, some of their characteristics are unique among the family members. βm-proteins contain two Glu-rich clusters in NH_2-terminal domains. βm-proteins are located in intracellular compartments, being concentrated in the inner nuclear membrane, where no X,K-ATPase α-subunits have been detected. βm does not associate with any X,K-ATPase α-subunit upon heterologous coexpression, thus indicating that mammalian βm does not function as a X,K-ATPase subunit. Expression of mammalian βm-proteins is confined exclusively to skeletal muscle and heart and is strictly developmentally regulated, being the highest in perinatal myocytes. This unique temporal and spatial pattern of βm expression suggests its important role in myogenesis. Some evidence in favor of this hypothesis was obtained using the yeast two-hybrid system, which revealed strong interaction of βm with several nuclear residents, including chromosomal passenger borealin, transcription factor CREB3, and transcriptional coactivator SNW/SKIP.

Analysis of genomic sequences revealed that homologues of human βm gene are located in remarkably conserved chromosomal segments in which the order of multiple genes is the same not only in mammals but also in other vertebrate species. Deduced sequences of chicken, *Xenopus*, and puffer fish *Tetraodon* βm-proteins demonstrate close similarity with mammalian homologues in intramembrane and COOH-terminal domains but not in NH_2-terminal domains whose sequences are highly variable. In contrast to mammals, chicken βm mRNA is present in muscle and brain, whereas message of fish βm exists predominantly in brain. Variability of structure and expression patterns allow us to suggest that "ancient" βm-proteins may differ from mammalian ones with respect to subcellular location and function, and some of them may be actual X,K-ATPase β-subunits. In conclusion, our observations indicate that physiological roles of the X,K-ATPase β-subunit family members are more complex and diverse than currently accepted. [Supported by NIH grant HL36573.]

89. Na,K-ATPase, a Novel Member of the Apical Junctional Complex. SIGRID A. RAJASEKARAN,[1] SONALI P. BARWE,[1] SERGEY RYAZANTSEV,[2] EVELINE E. SCHNEEBERGER,[3] and AYYAPPAN K. RAJASEKARAN,[1] [1]*Department of Pathology and Laboratory Medicine, David Geffen School of Medicine,* [2]*Department of Biological Chemistry, University of California at Los Angeles, Los Angeles, CA*; [3]*Molecular Pathology Unit, Massachusetts General Hospital East, Charlestown*, MA (Sponsor: Ayyappan K. Rajasekaran)

Polarized epithelial cells form a permeability barrier between two biological compartments, the integrity of which is maintained by intercellular junctional complexes. Tight junctions (or zonula occludens) are the most apical component of the junctional complexes and regulate paracellular solute and water flux, and

have been shown to integrate diverse processes such as cell polarity, cell proliferation, and tumor suppression. Tight junctions are multiprotein complexes composed of transmembrane proteins (such as occludin and claudins) that are linked to the actin cytoskeleton through tight junction plaque proteins that also serve as adapters for the recruitment of cytosolic molecules implicated in cell signaling. A functional tight junction is crucial to maintain the barrier function of epithelia. However, mechanisms involved in the formation and maintenance of tight junctions are poorly understood.

In a series of earlier studies, we have shown that Na,K-ATPase function is necessary for the formation and maintenance of tight junctions in polarized epithelial cells. Yet, the molecular mechanism by which Na,K-ATPase regulates tight junctions remained to be deciphered. We now provide evidence that Na,K-ATPase binds to and modulates PP2A activity, a protein serine/threonine phosphatase known to be involved in tight junction regulation. Reduced PP2A activity in Na,K-ATPase–inhibited cells increases occludin phosphorylation and tight junction permeability. Recently we have shown that the β_1-subunit of Na,K-ATPase binds to annexin II, a phospholipid binding protein localized to tight junctions. We found that annexin II binds to occludin in a Na,K-ATPase activity–dependent manner. Together these results suggest that Na,K-ATPase, PP2A, annexin II, and occludin form a novel apical junctional complex involved in the regulation of tight junction permeability in epithelial cells. [Supported by NIH grant DK56216.]

90. Oligomeric Functional Form of the Gastric H,K-ATPase. JAI MOO SHIN and GEORGE SACHS, *Membrane Biology, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA; VA Greater Los Angeles Healthcare System, Los Angeles, CA*

The P_2-type gastric H,K-ATPase is a dimeric heterodimer, $(\alpha\beta)_2$ (Rabon et al. 1986. *J. Biol. Chem.* 261: 1434–1439; Shin, J.M. and G. Sachs. 1996. *J. Biol. Chem.* 271:1904–1908), and undergoes a cycle of conformational changes involving phosphorylation and dephosphorylation during H^+ for K^+ exchange. The functional consequences of this dimeric structure have not been established. To do this, the binding stoichiometry was investigated by phosphorylation with γ ^{32}P-ATP, ^{32}Pi, binding of γ ^{32}P-ATP and INT, a compound that belongs to the class of K^+-competitive inhibitory imidazo[1,2-α]pyridine derivatives. At a low concentration of MgATP ($<$10 μM), the enzyme forms E_1[ATP]•Mg•(H^+):E_2•Mg•(H^+) at a high ATP affinity site, followed by phosphorylation by Mg^{2+} catalysis to form E_1P•Mg•(H^+):E_2•Mg•(H^+), which spontaneously converts to E_2P•Mg•(H^+):E_1•Mg•(H^+). Maximal protein phosphorylation was 2.65 nmol/mg enzyme. At high concentration of MgATP ($>$0.1 mM), the oligomer forms E_1P•Mg•(H^+):E_2[ATP]•Mg•(H^+), which converts to E_2P•Mg•(H^+):E_1[ATP]•Mg•(H^+). The combination of maximal phosphorylation and ATP binding was 5.2 nmol/mg enzyme, similar to the value found for phosphorylation from ^{32}Pi in the presence of Mg^{2+}. INT inhibited the gastric H,K-ATPase K^+ competitively with a K_i of 47 nM and a 35 nM IC_{50} at 10 mM KCl. Maximal binding of INT was 2.64 nmol per mg of the enzyme in the presence of MgATP, Mg^{2+}, Mg-Pi, or Mg-vanadate at 20°C. The rate of binding in the presence of MgATP was much faster than in the presence of Mg only. CDTA pretreatment significantly reduced INT binding to the enzyme, but prior INT binding was not dissociated by CDTA. K^+ ion displaced INT bound in intact vesicles only in the presence of nigericin, showing that INT binding is only to the luminal E_2 form. INT-bound enzyme also formed 2.63 nmol of EP per mg of the enzyme, which is insensitive to K^+. Hence a total of 5.29 nmol binding sites are present per mg of enzyme, with equal binding stoichiometry of INT and phosphoenzyme. INT binding results in the formation of E_2P•Mg•INT_{exo}:E_1•Mg•$(H^+)_{cyto}$ followed by conversion to E_2•Mg•INT_{exo}:E_1•Mg•$(H^+)_{cyto}$ at low concentrations of ATP. However, high ATP concentrations enable formation of a phosphoenzyme, E_2[ATP]•Mg•INT_{exo}:E_1P•Mg•$(H^+)_{cyto}$. Presumably the binding of the inhibitor restricts membrane domain movement, fixing its conformation in the E_2 form, resulting in the inhibition of enzyme activity, and the other half of the oligomer must remain in the E_1.ATP or E_1P form. The combined data suggest that the oligomer is present always in a functional E_1E_2 configuration during enzyme turnover.

91. The Polarized Expression of Na^+,K^+-ATPase Depends on the Association Between β-Subunits Located in Neighboring Cells. LIORA SHOSHANI, RUBEN G. CONTRERAS, MARÍA L. ROLDAN, MARTHA R. HIDALGO, ROSSANA FIORENTINO, AMPARP LÁZARO, and MARCELINO CEREIJIDO, *Department of Physiology, Biophysics, and Neuroscience, Center for Research and Advanced Studies, Mexico City, Mexico* (Sponsored by Jack Kaplan)

The polarized distribution of Na^+,K^+-ATPase plays a paramount physiological role because, either directly or through coupling with co- and counter-transporters, it is responsible for the net movement of ions and solutes across the whole epithelium. MDCK cells do not

express Na^+,K^+-ATPase on the basolateral, but just on the lateral side (Cereijido et al. 2001. *J. Memb. Biol.* 184:299–304). Although the signals and mechanisms achieving this polarization are not yet understood, we have some hints: (1) MDCK cells express the Na^+,K^+-ATPase in the lateral border provided the neighboring cell coexpresses its own Na^+,K^+-ATPase in the contacting border; (2) the β_1-subunit has the typical structure of an adhesion molecule: a short cytoplasmic tail, a single transmembrane segment, and a long and glycosylated extracellular domain; (3) furthermore, Gloor et al. (1990. *J. Cell Biol.* 110:165–174) have shown that the β_2-subunit is in fact an adhesion molecule on glial cells. On these bases, we have suggested that the β-subunit anchors the enzyme at the plasma membrane facing the intercellular space, by virtue of a link between its extracellular domain and a homotypic one present in the neighboring cell. The present results support such a possibility. Although MDCK cells never express Na^+,K^+-ATPase at contacts with CHO cells, they do so when CHO cells are transfected with the β_1-subunit from dog kidney (CHO-β). This may be attributed to the adhesive property of the β_1-subunit, as an aggregation assay using CHO-β cells shows that the expression of dog β_1-subunit in the plasma membrane does increase adhesiveness. This adhesiveness does not involve adherens nor tight junctions. Taken together, our results indicate that MDCK cells express Na^+,K^+-ATPase at a given border provided the contacting cell expresses the dog β_1-subunit. The cell–cell interaction thus established would suffice to account for the polarized expression and positioning of the Na^+,K^+-ATPase in epithelial cells. [Supported by grant of the national research Concil of Mexico.]

92. Differential Targeting of the Na/K-ATPase β-Subunit Isoforms Independent of the α-Subunit in Developing *Drosophila* Eye. YOSUKE ISHIDA, AYA SATO, SHIGE H. YOSHIMURA, PAUL SALVATERA, and KUNIO TAKEYASU, *Laboratory of Plasma Membrane and Nuclear Signaling, Kyoto University Graduate School of Biostudies, Kyoto, Japan; Beckman Institute at City of Hope, Duarte, CA* (Sponsor: David Gadsby)

Drosophila melanogaster genome contains three α-subunit genes and six β-subunit genes for type IIC P-type ATPases (Takeyasu et al. 2001. *Cell. Mol. Biol.* 47:325–333). We report here further studies on the changes in the expression and subcellular localization of these isoforms during development. In situ hybridization using isoform-specific probes demonstrated that only α1-subunit and β1-, β2-, and β3-isoforms were expressed in larval, pupal, and adult eyes of *Drosophila*. For clarifying the subcellular localization of these subunit isoforms, we raised β-isoform–specific antibodies and found the following by immunofluorescence microscopy. (1) The α1-subunit was expressed on all the cell surface membranes including the apical microvilli and the basolateral membranes in the larval and pupal omatidium. However, toward adulthood, the apical distribution was completely abolished and the basolateral localization was sustained, consistent with the previous report (Yasuhara et al. 2000. *Cell Tissue Res.* 300:239–249). (2) The expression of the β1-isoform was restricted to the basolateral and internal endoplasmic reticulum (ER) membranes, and was never localized to the apical membrane throughout the development. (3) The β2-isoform was expressed mainly on the apical membrane and internal ER membranes, but not on the basolateral membranes throughout the eye development. (4) With a striking contrast to the β1 and β2 isoforms, the β3-isoform was expressed intracellularly, i.e., on the ER membranes, in the larval and pupal eyes, but, toward adulthood, this distribution was abolished and exclusively shifted to the basolateral membrane localization. Immunoprecipitation using anti–β3-antibody demonstrated that the β3-isoform did not assemble with the α1-subunit in the larval eye, but did assemble in the adult eye. These results illustrate a complexity of the expression and sorting mechanisms of the type IIC ATPase subunits, and imply the existence of the unique roles of the β-subunit isoforms independent of the α-subunit.

93. High and Low Affinity ATP Effects on Both NaE1 and KE2 of Na/K-ATPase Studied by Substrate Analogues. KAN TANOUE,[1] SHUNJI KAYA,[1] KAZUHIRO ABE,[1] TOSHIAKI IMAGAWA,[1] YUTARO HAYASHI,[2] KAZUYASU SAKAGUCHI,[1] and KAZUYA TANIGUCHI,[1] [1]*Biological Chemistry, Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo, Japan*; [2]*Department of Biochemistry, Kyorin University School of Medicine, Mitaka, Japan* (Sponsor: Jack Kaplan)

A comparison of the fluorescence changes induced by ATP with XTP (CTP, GTP, and UTP) of fully active N-[*p*-(2-benzimidazolyl)phenyl]maleimide probe-labeled Na/K-ATPase at Cys-964 in the α-chain in the presence of Mg^{2+} + Na^+, without or with K^+ (or Rb^+), suggests that XTP also induces the accumulation of phosphoenzyme and occluded K^+, as in the case of *p*-nitrophenyl phosphate (*p*NPP) or acetyl phosphate. The higher activity of Na-dependent XTPase compared with the activity of Na/K-XTPase is due to the spontaneous breakdown of E2P being somewhat more rapid compared with the deocclusion of Rb^+ from (Rb)E2 by

XTP. Stopped-flow measurements of the increase in BIPM fluorescence that accompanies E2P formation, with increasing concentrations of each nucleotide or acetyl phosphate, showed that a plot of rate constant versus concentration gave two intersecting straight lines. A plot of rate constant accompanying the transition, (Rb)E2 to NaE1ATP or NaE1ADP, versus ATP or ADP concentration gave, respectively, two intersecting straight lines. Low concentrations of *p*NPP inhibited ouabain-sensitive Na-ATPase activity by reducing the amount of bound ATP. Higher concentrations of *p*NPP inhibited the activity further by reducing the amount of phosphoenzyme. Chase experiments showed that high concentrations of *p*NPP inhibited the breakdown of phosphoenzyme. These data and others suggest that the Na-bound enzyme showed positive cooperativity for ATP binding, resulting in the formation of phosphoenzyme and EATP complex, and that the phosphorylation of the α-chain of the oligomeric form of the Na-bound enzyme, and the low affinity binding of ATP to that of the K-bound enzyme, both reduced the apparent ATP affinity in each neighboring α-chain, respectively. They also show that the conformation of the adenine binding domain in the Na-bound enzyme is more flexible, in that it can accept nonadenine nucleotide triphosphate, *p*NPP, or acetyl phosphate with positive cooperativity for the phosphorylation compared with the conformation of the K-occluded enzyme, which accepts only an adenine moiety cooperatively.

94. N-linked Glycosylation Provides Apical Sorting Information to the Gastric H,K-ATPase β and to the Na,K-ATPase β_2 Subunit. OLGA VAGIN, SHAHLO TURDIKULOVA, and GEORGE SACHS, *Department of Physiology, School of Medicine, University of California Los Angeles, Los Angeles, CA*; *VA Greater Los Angeles Healthcare System, Los Angeles, CA* (Sponsor: Jack Kaplan)

Two homologous P_2-type pumps, the gastric H,K-ATPase and the Na,K-ATPase, differ markedly in their plasma membrane distribution in polarized cells. The gastric H,K-ATPase resides in intracellular tubulo-vesicles in resting parietal cells and relocates to the canalicular (apical) membrane upon stimulation of acid secretion. When expressed in cultured epithelial cells, the H,K-ATPase resides in the apical domain. Six of seven N-glycosylation sites in the H,K-ATPase β subunit have been found essential for its apical sorting in LLC-PK1 cells. In contrast, the Na,K-ATPase is localized to the basolateral plasma membrane in most native tissues and in cultured polarized cells.

An unusual apical location of the endogenous Na,K-ATPase α_1 subunit was detected in HGT-1 cells (a human gastric adenocarcinoma cell line) using immunohistochemistry and surface-selective biotinylation. An endogenous β_1 subunit was not found in these HGT-1 cells. However, the β_2 subunit was highly expressed in these cells and distributed, along with the α_1 subunit, predominantly to the apical membrane. When the YFP-linked Na,K-ATPase β_1 subunit was expressed in this cell line, it was localized exclusively to the basolateral surface. Of four known isoforms of the Na,K-ATPase β subunits, the β_2 is the most homologous to the H,K-ATPase β subunit and has up to nine glycosylation sites, while the β_1 isoform has only three N-glycosylation sites. Five additional N-glycosylation sites homologous to the ones present in the β_2 subunit were introduced into the β_1 subunit by site-directed mutagenesis. The mutated β_1 subunit was detected predominantly in the apical membrane. Hence, the N-glycans linked to the β subunit isoform of P_2-type ATPases contain apical sorting information. The high abundance of the β_2 subunit isoform is responsible for apical location of the Na,K-ATPase in HGT-1 cells. [Supported in part by NIH grants DK46917, DK58333, 53462, 41301, and USVA.]

95. Functional Consequences of Human Na,K-ATPase α_2 Subunit Mutations Implicated in Inherited Migraine Cases. NESLIHAN TAVRAZ,[1] JAN B. KOENDERINK,[2] GIOVANNI ZIFARELLI,[1] LI YAN QUI,[2] JAN JOEP H.H.M. DE PONT,[2] ERNST BAMBERG,[1] and THOMAS FRIEDRICH,[1] [1]*Max-Planck-Institute of Biophysics, Frankfurt am Main, Germany*, [2]*Radboud University of Nijmegen, Nijmegen, The Netherlands* (Sponsor: Jack H. Kaplan)

The Na,K-ATPase belongs to the family of P-type ATPases, a major class of primary active transport proteins, so called because they become transiently phosphorylated upon ATP hydrolysis. The enzyme utilizes the free energy of ATP hydrolysis to export three Na^+ ions and import two K^+ ions per ATP molecule, thereby maintaining the electrochemical gradients of Na^+ and K^+ across the plasma membrane. The Na,K-ATPase consists of a catalytic α subunit and an accessory β subunit. The human genome encodes four different α subunit and three different β subunit genes. Recently, De Fusco et al. identified two mutations (L764P and W887R) in the Na,K-ATPase α_2 subunit (ATP1A2) associated with familial hemiplegic migraine type 2 (FHM2). This important breakthrough was rapidly followed by four reports in which 11 new FHM2 mutations in the Na,K-ATPase α_2 subunit were identified, which in some cases coincided with additional clinical symptoms. The only functional data available indicates that the L764P and W887R mutants, unlike the wild type, do not support cell survival. It is obvious that the mu-

tants must be functionally impaired, but it is unknown which functions are affected. We measured the functional properties of these mutants upon heterologous expression in *Xenopus* oocytes. The mutated pumps were well expressed at the plasma membrane, but no specific pump currents could be detected. In addition, these mutants did not transport $^{86}Rb^{+}$ or hydrolyze ATP. Although the ATP1A2 mutations do not interfere with α–β subunit interaction, structural alterations are inferred from reduced ouabain binding to membrane preparations containing mutant proteins. It is concluded that the primary disease-causing mechanism is complete loss of function of the Na,K-ATPase α_2 isoform (Koenderink et al. 2005. *Biochim. Biophys. Acta.* 1669:61–68). These methods will be further used to investigate the molecular basis of neurological disorders linked to mutations in the *ATP1A2* gene.

96. Potassium as a Major Player in Cardiovascular Diseases. HENNING BUNDGAARD, *The Heart Centre, National University Hospital, Copenhagen, Denmark*

Heart failure (HF) reduces plasma K, total body K, and myocardial Na,K-pump content. Diuretics and digoxin add to these reductions. These drugs have no proven beneficial effects on mortality, which is in contrast to angiotensin converting enzyme (ACE) inhibitors, β blockers, and aldosterone antagonists, i.e., drugs increasing plasma K and/or total body K. The HF-induced reduction in skeletal muscle K, and likely parallel reduction in myocardial K, may increase intracellular Na and Ca in cardiomyocytes. This may improve systolic but compromise diastolic function. This may be beneficial short term, and diuretics and digoxin probably work through the same mechanisms, but harmful long term. Thus, normalization of the K homeostasis may be a rational approach for prevention and treatment of HF. In keeping with this, a growing body of evidence indicates that part of the positive effects of drugs presently used in HF may be related to their capacity to retain K and increase plasma K. Increasing extracellular K stimulates the Na,K-pump, and this effect may be of crucial importance for the outcome of HF treatment with ACE inhibitors, β blockers, and aldosterone antagonists. Other drugs with positive effects in HF, e.g., NO, statins, and insulin, may also work through pump stimulation. Ongoing clinical studies are expected to provide final evidence for routine use of glucose-insulin-potassium (GIK) infusions in patients with acute myocardial infarction. Heart and muscles release and accumulate considerable amounts of K during activity. This adds to a potential role of K also in arrhythmia disorders, not only in HF and ischemia, since the concentrations of K play a pivotal role in the repolarization phase of the action potential, and also because secondary rises in intracellular Na and Ca may induce after-depolarizations, which are important triggers of arrhythmia. Thus, K and Na,K-pump regulation seem to be of utmost importance in all three major cardiac disease entities. [Supported by the Danish Heart Foundation.]

97. Na,K-ATPase and Sudden Arrhythmic Death. KELD KJELDSEN, *Laboratory for Molecular Cardiology, The Heart Centre, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark*

Nihilism has prevailed regarding potassium (K) in clinical cardiology for the last decades. Moreover, whereas K-depleting diuretics are prescribed to a large fraction of heart failure patients, attention to plasma K values may hitherto have been insufficient in many cases. Recent research, including clinical studies and Na,K-ATPase studies, however, throws new light on K homeostasis. Indications are that plasma K should be maintained high in the normal range in patients with heart disease.

The myocardial Na,K-ATPase is of importance for K handling in the extracellular space (ECV) of the myocardium; moreover myocardial ECV-K is influenced by K delivered by the blood stream. Here skeletal muscle Na,K-ATPase is of importance. Thus, maintaining K in myocardial ECV within safe limits requires optimum regulation of the Na,K-ATPase. Accordingly its dysregulation may cause severe local hyper- or hypokalemia. Moreover, during exercise, K leaks out of muscular cells and is then pumped back by the Na,K-ATPase. Thus, plasma-K may within minutes increase to around 8 mmol/liter during activity and decrease even below resting level during rest. Furthermore, these fluctuations are modified by Na,K-ATPase regulation by, e.g., physical conditioning, disease, and medicine. In diuretic treatment of human subjects, plasma-K is maintained for some time by mobilizing K from intracellular compartments; skeletal muscle Na,K-ATPase and K content is reduced. Similarly muscle magnesium may be reduced. In heart failure patients, myocardial Na,K-ATPase is reduced.

Thus, dysfunction of the K homeostasis may cause inappropriate K shifts at the level of the myocyte membrane. This may, per se, cause arrhythmia. Moreover such dysfunction may disclose conditions that are usually well tolerated but become dangerous when exposed to hyper- or hypokalemia. Thus, in, for example, long QT-syndrome, caused by mutations in genes coding for potassium-channels, exercise and hypokalemia may elicit syncope and sudden death.

Recently, aldosterone antagonist has been added to heart failure treatment, resulting in significant reductions in mortality. A part of this effect may be the outcome of improved extrarenal K homeostasis.

In conclusion, resting plasma-K should be kept high in the normal range (plasma-K 4–5 mmol/liter). This is of special importance in patients with heart disease at risk of arrhythmia. Plasma-K is a dynamic parameter that should be assessed not only during rest but probably also during, e.g., exercise. Major plasma-K shifts and dysfunction of the K homeostasis, per se, as well as combination thereof with preexisting disease may cause arrhythmia, syncope, and sudden death. Nonpotassium sparing diuretics should be reduced to a minimum. In the era of aldosterone-antagonist trials, a trial comparing the protective effect of optimum K supplementation with that of aldosterone antagonism is in demand. Dysfunction of the K homeostasis, Na,K-ATPase and K-channel dysfunction, should be considered in sudden arrhythmic death. Especially in situations with syncope or sudden death during exercise, a thorough evaluation of the K homeostasis seems in demand. The time has come to fend off K nihilism in clinical practice.

98. Essential Role of the Na^+-K^+ Pump in the Control of Cardiac Idioventricular Automaticity. MARIO VASSALLE, *Department of Physiology and Pharmacology, State University of New York, Downstate Medical Center, Brooklyn, NY*

Strong vagal stimulation causes a temporary ventricular standstill by suppressing the discharge of the sinoatrial node (SAN) and thereby unmasking the inhibition that the fast SAN rhythm exerts on Purkinje fiber discharge ("overdrive suppression"). Thus, if during vagal stimulation, ventricles are driven at a rate similar to that of the SAN, cessation of drive is followed by a temporary standstill. If ventricular drive during vagal stimulation is faster or slower than that of SAN, the ensuing pause is longer or shorter, respectively. In complete atrioventricular (A-V) block, vagal stimulation suppresses atrial discharge but not the idioventricular rhythm. Also, the cessation of ventricular drive is followed by a pause, whose duration is a function of rate and duration of drive. In vitro, overdrive of spontaneous Purkinje fibers causes a delayed increase of maximum diastolic potential. Drive cessation is followed by a diastolic depolarization whose slope is less steep than before drive and lasts seconds before the threshold is attained and spontaneous discharge resumes. The pause duration is a function of duration and rate of drive. In the presence of lithium or of metabolic inhibitors, the hyperpolarization and pause are eliminated. During drive, intracellular Na^+ activity (a^i_{Na}) increases as a function of rate and duration of drive, and during the pause, a^i_{Na} may temporarily undershoot the control values. When the Na^+-K^+ pump is inhibited by digitalis, during drive a^i_{Na} increases more and does not return to control values. Also, the pause is abolished and may be substituted by overdrive excitation. Norepinephrine stimulates the Na^+-K^+ pump and shortens the pause. Thus, the inhibitory mechanism of idioventricular pacemakers by the Na^+-K^+ pump is operational when Purkinje discharge is not needed (SAN rhythm) and it is removed when their discharge is needed (e.g., sudden A-V block). [Supported by NIH grants.]

99. Solution Structure of the Nucleotide Binding Domain of the Wilson Disease ATPase. OLEG DMITRIEV,[1] RUSLAN TSIVKOVSKI,[3] FRITS ABILDGAARD,[1] and SVETLANA LUTSENKO,[3] [1]*University of Wisconsin, Madison, WI;* [2]*Oregon Health and Science University, Portland, OR*

The Wilson disease associated protein (WDAP) is a P_1-type ATPase involved in copper metabolism in human tissues. No high-resolution structure of any heavy-metal transporting ATPase has been solved to date. We present a solution structure of the N-domain of the Wilson disease ATPase (WNABD), a 165–amino acid-long protein, which specifically binds ATP and ADP.

The structure of WNABD comprises a twisted β-sheet flanked by two pairs of α-helices. The addition of ATP or ADP to the protein causes major changes in the chemical shifts of many backbone amide groups, a likely indication of significant conformational changes caused by nucleotide binding. We have identified the residues involved in ATP binding by chemical shift perturbation analysis, and we discuss the structural basis for tight ATP binding to WNABD and the effect of the H1069Q mutation, the most frequent cause of the Wilson disease phenotype. [Supported by NIH grants DK55719 and PPG-1P01-GM067166-01.]

100. Lipid Flippase Activity Coupled to a Type IV P-Type ATPase. PARAMASIVAM NATARAJAN, JIYI WANG, and TODD R. GRAHAM, *Department of Biological Sciences, Vanderbilt University, Nashville, TN*

Type IV P-type ATPases are typically the largest subgroup of P-type ATPases in eukaryotes and yet, although defects in one member of this group (FIC1) cause an inherited form of cholestasis in humans, the function of these ATPases is poorly understood. A growing body of evidence suggests these ATPases are

phospholipid translocases (or flippases) responsible for generating phospholipid asymmetry in biological membranes. We have taken a genetic approach to define the physiological and biochemical function of the five yeast members of this group, comprising Drs2p, Neo1p, Dnf1p, Dnf2p, and Dnf3p. These ATPases are required for vesicle-mediated protein transport, with Neo1p acting at the early Golgi, Drs2p at the late Golgi, and Dnf1p and Dnf2p at the plasma membrane and endosomes. Temperature-sensitive mutants were used to show a requirement for Drs2p in budding secretory vesicles from the Golgi complex, and for a flippase activity in purified Golgi membranes. Initial studies suggested that Drs2p specifically pumps phosphatidylserine (PS) from the lumenal to the cytosolic leaflet of the Golgi; however, we could also show that PS is dispensable for Drs2p function in vivo, indicating another substrate for this pump. Recent work indicates that ATP hydrolysis by Drs2p is coupled to bodipy-labeled phosphatidylinositol (PI) translocation to the cytosolic leaflet of the TGN and we are currently testing if endogenous PI is also flipped. The Drs2p ATPase activity, and presumably lipid translocation, is needed to sustain exocytic vesicle biogenesis from the Golgi. We propose that Drs2p facilitates vesicle budding by pumping PS or PI across the membrane to expand the cytosolic leaflet at the expense of the lumenal leaflet. This would induce membrane bending, and perhaps allow coat proteins (such as clathrin) to mold the membrane into a tightly curved vesicle. [Supported by NIH GM62367.]

101. Effects of Sequence Variations on Plasma Membrane Localization of Wilson's Disease Protein Expressed in *Xenopus* Oocytes. ÉVA LÖRINCZI,[1] RULAN TSIVKOVSKII,[2] WINFRIED HAASE,[1] ERNST BAMBERG,[1] SVETLANA LUTSENKO,[2] and THOMAS FRIEDRICH,[1] [1]*Max-Planck-Institute of Biophysics, Frankfurt am Main, Germany*; [2]*Oregon Health and Science University, Portland, OR*

Wilson's disease protein (WNDP) is a copper-transporting ATPase, the high physiological importance of which is underlined by its involvement in Wilson's disease, an inherited disorder with severe hepatic and neurological symptoms caused by accumulation of copper in liver and brain. Despite recent progress in expression and functional characterization of WNDP, many details about the molecular mechanism, the determinants of copper-dependent regulation, and trafficking within cells are still poorly understood, raising the need for new expression systems and independent experimental approaches. Here, epitope insertion, expression in *Xenopus* oocytes, and luminescence measurements have been used to evaluate topological features of WNDP and to quantify its plasma membrane localization. It is shown that the loops between proposed transmembrane segments TMS1 and -2 or TMS5 and -6 of WNDP are extracellularly exposed, while the NH_2 terminus and segment connecting TM4 and -5 are not, consistent with the current topology model. Expression of WNDP at the plasma membrane is additionally verified by electron microscopy of freeze-fractured oocyte preparations. The developed surface detection assay is used to quantify the effects of protein sequence modifications on plasma membrane localization of WNDP. Deletion of the entire amino terminus containing six copper-binding domains or deletion of a carboxy-terminal stretch encompassing a tri-leucine motif leads to increased surface expression. In contrast, the most common Wilson's disease mutation, His-1069-Gln, reduces the amount of protein at the cell surface, consistent with the loss-of-function effect of this mutation. Thus, expression of WNDP in *Xenopus* oocytes opens a novel route to investigate the effects of Wilson's disease sequence variants on subcellular distribution of this important enzyme.

102. Normal Ca^{2+} Signaling in Human Epidermal Keratinocytes Requires Both the ATP2C1 and the ATP2A2. KARIN ABERG, LUCIE FOGGIA, ALAIN HOVNANIAN, and THEODORA MAURO, *Inserm U563, Paul Sabatier University, 31059 Toulouse, Cedex 3, France; Department of Dermatology, University of California, San Francisco, San Francisco, CA; San Francisco Veterans' Hospital, San Francisco, CA*

Intracellular Ca^{2+} stores play a critical role in signaling Ca^{2+}-induced keratinocyte differentiation and cell-to-cell adhesion. Keratinocyte endoplasmic reticulum (ER) Ca^{2+} stores are maintained by the Ca^{2+} ATPase ATP2A2, while a closely related Ca^{2+} ATPase, ATP2C1, localizes to the Golgi and maintains the Ca^{2+} store in this organelle. We find that the ATP2A2 and ATP2C1 act jointly to shape Ca^{2+} release and subsequent Ca^{2+} influx, making keratinocytes the first mammalian cells known to be under dual control of both ER and Golgi Ca^{2+} stores. Inactivating either Ca^{2+} ATPase leads to defects in Ca^{2+}-mediated signaling, cell-to-cell adhesion, or differentiation. Darier disease (DD) and Hailey-Hailey disease (HHD) are inherited skin disorders resulting from dominant mutations in ATP2A2 and ATP2C1, respectively. DD is characterized by loss of adhesion between suprabasal epidermal cells associated with abnormal keratinization while loss of epidermal cell-to-cell adhesion is predominant in HHD. Functional and molecular studies reveal that while ATP2C1

dysfunction is not compensated in HHD keratinocytes, ATP2A2 dysfunction in DD keratinocytes is compensated, in part, by ATP2C1 up-regulation. These compensatory changes allow normal IP_3-mediated Ca^{2+} signaling in DD keratinocytes, and enable DD keratinocytes to remain viable. However, compensatory ATP2C1 up-regulation also leads to abnormally low cytosolic Ca^{2+} concentrations, and may underlie the unique pattern of abnormal differentiation seen in DD keratinocytes. [Supported by the National Institutes of Health grant 2PO1AR39488.]

103. Fast-twitch Muscle-type Sarcoplasmic Reticulum Calcium ATPase (SERCA1) is Expressed in Canine Dilated Cardiomyopathy Myocardium. MARY PETERS, ALI MOBASHERI, NUALA SUMMERFIELD, and IAIN YOUNG. *Musculoskeletal Physiology and Division of Small Animal Studies, Faculty of Veterinary Science, University of Liverpool, Department of Preclinical Veterinary Science, Liverpool, UK*

Dilated cardiomyopathy (DCM) is a degenerative myocardial disease of uncertain etiology resulting in an enlarged and weakened heart. It is seen in many species including humans, but it is particularly common and insidious in large breed dogs. Sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) is responsible for sequestering intracellular Ca^{2+}, allowing the myocardium to relax during diastole. Impaired Ca^{2+} cycling and increased diastolic Ca^{2+} concentration have been suggested as a major causative factor in DCM (e.g., Pieske et al. 1995. *Circulation.* 92:1169–1178). Gupta et al. (1999. *J. Mol. Cell. Cardiol.* 31:1381–1389) show 50% decrease in total SERCA in the left ventricle of dogs with end-stage heart failure but find little change in the right ventricle. This is consistent with the most common clinical manifestation of left-sided heart failure. In our study, we performed an immunohistochemical screen for the expression of SERCA and Na^+/K^+ ATPase isotypes on tissue microarrays of myocardial tissue from unaffected and DCM dogs. Most notably, we found extensive expression of SERCA1 in DCM right ventricle and septum tissue but little or no expression in normal and DCM left ventricle tissue (antibody: fast-twitch skeletal muscle isotype: CaF2-5D2, from the Developmental Studies Hybridoma Bank). This largely agrees with an earlier DNA microarray study that shows expression of SERCA1 in human end-stage cardiac failure hearts (Borlak and Thum, 2003). We propose that the expression of SERCA1 in DCM hearts represents an adaptive response to impaired myocardial function. Indeed, exogenous SERCA1 expression has been shown to increase cardiac contractility in transgenic mice (Loukinaov et al. 1998. *Ann. NY Acad. Sci.* 853:251–259) and has been suggested as a possible direction for gene therapy in the failing heart (Ennis et al. 2002. *J. Clin. Invest.* 109:393–400). [Supported by grants from Petsavers and Petplan, UK.]

104. Enhanced Na,K-ATPase Ouabain Sensitivity and Ouabain-dependent Cell Proliferation in Human Cystic Kidney Cells. ANH-NGUYET NGUYEN, DARREN WALLACE, and GUSTAVO BLANCO, *Department of Molecular and Integrative Physiology and Kidney Institute, University of Kansas Medical Center, Kansas City, KS 66160*

Polycystic kidney diseases (PKD) are characterized by the progressive enlargement of fluid-filled cysts in the kidneys that compromise the function of the organ. Cystogenesis requires that renal epithelial cells proliferate and secrete fluid into the cyst cavity. At present, the relevance of the Na,K-ATPase in the pathophysiology of PKD remains unknown. Besides its role in salt and water transport in the kidney, the Na,K-ATPase also functions as a receptor for ouabain to activate intracellular pathways that lead to cell growth and proliferation. In this work, we show that, when compared with normal human kidney cells (NHK), the Na,K-ATPase of primary cultures of cystic epithelial cells derived from autosomal dominant polycystic kidney patients (ADPKD) have an increased sensitivity to ouabain. Thus, while in ADPKD cells most of the enzyme exhibits a K_i for ouabain similar to NHK cells ($\sim 10^{-7}$ M), they also show a second enzyme population with a K_i for the cardiotonic steroid of $\sim 10^{-9}$ M. This is not due to the aberrant expression of different Na,K-ATPase isoforms, since cystic cells, like NHK cells, express only the α1 and β1 subunits of the enzyme. In addition to the switch in ouabain affinity, nanomolar concentrations of ouabain induce cell proliferation in ADPKD cells that is ~30% higher than that of NHK cells. Concomitantly, ADPKD cells exhibit an increase in ouabain-dependent phosphorylation of the mitogen-activated protein kinases, ERK1/2. Altogether, these results suggest that the high ouabain affinity phenotype of kidney cystic cells may render the cells more prone to the effects of the cardiotonic steroid. The increase in cell growth in ADPKD cells may be important for formation and maintenance of the renal cysts. [Supported by NIH grants HD043044-01 and P20RR017686.]

105. Mutation F785L, Associated With Rapid-onset Dystonia Parkinsonism in the Na^+,K^+-ATPase, Interferes Severely With the Na^+ Binding Properties but Not with the K^+ Binding Properties. BENTE VILSEN, VI-

VIEN RODACKER, and MADS TOUSTRUP-JENSEN, *Department of Physiology, Institute of Physiology and Biophysics, University of Aarhus, DK-8000 Aarhus C, Denmark* (Sponsor: Bente Vilsen, applied for membership 03/29, or David Gadsby)

The Na^+,K^+-ATPase plays key roles in brain function, e.g., neurotransmitter uptake and propagation of the action potential. Recently, six missense mutations in the Na^+,K^+-ATPase were found associated with rapid-onset dystonia parkinsonism (RPD) (de Carvalho Aguiar et al. 2004. *Neuron.* 43:169–175). One of these mutations, E282K, was previously analyzed in our lab and found, among other effects, to interfere with Na^+ binding (Toustrup-Jensen and Vilsen. 2002. *J. Biol. Chem.* 277:38607–38617). To understand the pathophysiological mechanism, it is essential to characterize functionally the disease-causing Na^+,K^+-ATPase mutants and define which partial reaction steps in the enzyme cycle and cation interactions that are affected by the mutations. We ask the question whether there are functional similarities between all the mutants that cause the RPD disorder? Here we report that RPD mutation F785L in transmembrane segment M5, close to the extracellular surface, leads to 13-fold reduction of the apparent affinity for Na^+ determined by activation of phosphorylation. This is an effect on the Na^+-binding E_1 form, and is not caused by displacement of the E_2–E_1 equilibrium away from the E_1 form, toward E_2, because the steady-state concentration of $E_2/E_2(K_2)$ accumulated during ATP hydrolysis was wild type-like, as judged from vanadate and ATP dependencies of Na^+,K^+-ATPase activity. In rapid kinetic measurements, it was revealed that the rate of phosphorylation of the E_1Na_3 form was fourfold reduced. The K^+ binding properties of this mutant were wild type-like, as revealed by the K^+ concentration dependence of Na^+,K^+-ATPase activity and K^+-induced dephosphorylation of E_2P in rapid kinetic measurements. Hence, it is concluded that the RPD mutation F785L interferes only with the Na^+ binding properties and probably, thereby, with Na^+ homeostasis. Other RPD mutations, additional mutations of F785, and mutations causing hemiplegic migraine have also been characterized and will be presented. [Supported by the Danish Medical Research Council, the Novo Nordisk Foundation, Denmark, the Lundbeck Foundation, Denmark, and the Research Foundation of Aarhus University.]

106. Mutations of Vacuolar H^+-ATPase B1 Subunit Affect Proton Pump Assembly and Trafficking. QIONGQIONG YANG,[1] GUANGMU LI,[2] EDWARD A. ALEXANDER,[2] and JOHN H. SCHWARTZ,[2] [1]*Renal Section, First Affiliated Hospital of Zhongshan University, Guangzhou 510080, PR China*; [2]*Boston University Medical Center and Boston University School of Medicine, Boston, MA*

Point mutations in the B1 subunit of vacuolar H^+-ATPase are associated with impaired ability of the distal nephron to secrete acid (dRTA). To test the hypothesis that these mutations interfere with assembly and targeting of the ATPase to the plasma membrane, constructs that mimic eight known point mutations identified in inherited dRTA (M) or wild-type (WT) B1 constructs were transfected into a rat distal nephron (IMCD) cell line to express either GFP-B1WT or GFP-B1M fusion proteins. In coimmunoprecipitation studies, GFP-B1WT formed complexes with other subunits of the H^+-ATPase, whereas the GFP-B1M did not. GFP-B1WT displayed a vesicular distribution by immunohistochemistry while GFP-B1M displayed a diffuse pattern. Proteins immunoprecipitated with anti-GFP antibody from GFP-B1WT cells had ATPase activity, whereas proteins from GFP-B1M samples did not. Proton pump–mediated pHi recovery after an acute acid load was inhibited in all GFP-B1M–transfected cells but not in GFP-B1WT cells. Both GFP-B1WT and GFP-B1M were present in the apical membrane and both were increased with cellular acidification. However, in the GFP-B1WT cells, the amount of GFP-B1, endogenous B1, and the 31KD subunits of the H^+-ATPase all displayed parallel increase in the apical membrane in response to cell acidification. In contrast, in GFP-B1M cells, the endogenous B1 and 31 KD subunits decreased or remained unchanged as the content of GFP-B1M increased in response to acidification. Conclusions: B1 point mutations prevent normal assembly of the H^+-ATPase. The mutants also may act as a dominant negative by competing with WT for the trafficking to the apical plasma membrane.

107. Effect of COX-2 Inhibitor on Expression of the γ Subunit of Na/K-ATPase in Inner Medullary Collecting Duct (IMCD3) Cells. NESTOR E. ALMEIDA, CHRISTOPHER J. RIVARD, TOMAS BERL, and JUAN M. CAPASSO, *Department of Renal Medicine, University of Colorado Health Sciences Center, Denver, CO* (Sponsor: David Gadsby)

The γ subunit of Na/K-ATPase is a member of the FXYD family of small single transmembrane proteins. In IMCD3 cells, the γ subunit is expressed in response to acute hypertonic stress as well as with chronic adaptation to hypertonicity and is thought to act as a modulator of the Na/K-ATPase pump. Expression of the γ subunit has been found to be critical in the adaptation

of IMCD3 cells to hypertonicity (Capasso et al. 2001. *PNAS*. 98:13414–13419). Recently, chronic COX-2 inhibition was found to reduce the medullary expression of Hsp70 and survival of papillary tissues in rats exposed to dehydration (Neuhofer et al. 2004. *Kidney Int.* 65: 431–441). The effect of COX-2 inhibition in IMCD3 cells on γ subunit expression was evaluated both for acute exposure (≤24 h) and chronic exposure (72 h). Analysis of changes in γ subunit protein by Western blot indicated a substantial reduction in protein during chronic COX-2 inhibitor exposure. Analysis of γ subunit mRNA levels by quantitative PCR indicated a time-dependent reduction in message throughout COX-2 inhibitor exposure. Comparison of Hsp70 message with acute and chronic exposure to COX-2 inhibitor reveals an ~45% reduction in message in agreement with the above-mentioned study. These data point to a time-dependent inhibition of γ subunit message, and possibly also of translation, leading to lower γ subunit protein, during chronic COX-2 inhibitor exposure. These effects are similar to those for Hsp70, and taken together they indicate that chronic exposure to COX-2 inhibitor reduces the cell's ability to survive changes in tonicity. [Supported by NIH/NIDDK grant DK66544-01A1S1.]

108. Deletion of Its COOH-terminal Exon Results in Retention of γb in the Endoplasmic Reticulum and Acceleration of Cell Growth. ELENA ARYSTARKHOVA, JENNIFER L. PASCOA, and KATHLEEN J. SWEADNER, *Laboratory of Membrane Biology, Massachusetts General Hospital, Boston, MA* (Sponsor: Jack H. Kaplan)

We have previously demonstrated that plasma membrane expression of FXYD2, the Na^+/K^+ pump γ subunit, in NRK-52E cells either by transfection or induction with hypertonicity resulted in a reduction of the rate of cell proliferation. This correlates with reduced Na^+ affinity and therefore presumably reduced Na^+/K^+ pump activity in vivo. Random mutagenesis of γb followed by generation of stable transfectants was used to identify structural elements of γ influencing cell growth. Western blot analysis of the clones possessing the highest proliferation rate revealed a band with electrophoretic mobility slightly faster than that of γb from rat kidney. The band exhibited strong reactivity with the γb-specific antibody but no reactivity with the antibody against the COOH terminus of γ. Nucleotide sequencing revealed a single point mutation 172C/T in γb, which resulted in creation of a stop codon right at the end of the fourth exon of γ and subsequent generation of a truncated form of γb missing seven amino acids (γb-172C/T). Interestingly, the COOH terminus of γb-172C/T possesses the canonical ER retrieval motif, −KKXX, the sorting signal known to interact with COPI coat proteins in retrograde transport vesicles. Confocal microscopy and subcellular fractionation analysis indicated that the vast majority of γb-172C/T was indeed retained in the ER, a small fraction was found in the Golgi, but none was found at the plasma membrane. Since γb-172C/T was identified in the fastest-growing clones, the data strongly support our hypothesis that γ expression plays an important role in controlling cell proliferation rate. ER retention and rapid growth were replicated with a γb construct with the COOH terminus deleted (γb-CΔ7). We also observed significant intracellular retention of α in γb-CΔ7 cells, suggesting a potential chaperone role for γb-CΔ7 to mediate delivery of α for proteasomal/lysosomal degradation. Whether expression of γb-172C/T occurs in vivo is under current investigation. [Supported by NIH grant HL36271.]

109. Characterization of Spliced Variants of FXYD3. STEPHANIE BIBERT and KAETHI GEERING, *Department of Pharmacology and Toxicology of the University, CH-1005 Lausanne, Switzerland* (Sponsor: David Gadsby)

FXYD3, also known as Mat-8, is able to associate with Na,K-ATPase and to produce a decrease in the apparent affinity both for K^+ and for Na^+ of Na,K-ATPase activity (Crambert et al. 2005. *Mol. Biol. Cell.* 16:2363–2371). In normal tissue, FXYD3 is expressed at high levels in uterus, stomach, and colon but it was found to be up-regulated, e.g., in breast tumors (Morrisson et al. 1995. *J. Biol. Chem.* 270:2176–2182).

It remains to be investigated whether there is a correlation between the regulation of the functional properties of Na,K-ATPase and cell proliferation. Human colon adenocarcinoma cells, Caco2, after reaching confluency, exhibit spontaneously a typical enterocytic differentiation, associated with an up-regulation of FXYD3 (Anderle et al. 2003. *Pharm. Research.* 20:3–15). RT-PCR of Caco2 total RNA revealed the presence of two transcript variants that show variation in the expression during the differentiation process. The full-length transcript appears only in undifferentiated proliferating cells, whereas the second variant, which has an in-frame deletion of 78 nucleotides, is present in both undifferentiated and differentiated cells. RNAs of both variants are also present in three other human colorectal cancer cell lines. These two transcript variants encode two different isoforms of the protein, the long form having a 26–amino acid insertion in the juxtamembrane domain, compared with the short form, which is the common form of FXYD3. After expression

in *Xenopus* oocytes, both variants associate with Na,K-ATPase. Electrophysiological measurements of Na,K-ATPase transport function revealed distinct functional effects of the two variants. The physiological relevance of these effects and their possible role in cell proliferation are under investigation. [Supported by the Swiss National Fund grant 31-64793.01 to K. Geering.]

110. A Molecular Mechanism for the Dominant Nature of Renal Hypomagnesaemia. EDINIO R. CAIRO, HERMAN G.P. SWARTS, NINE V. KNOERS, LEO A. MONNENS, RENE J.M. BINDELS, JAN JOEP H.H.M. DE PONT, and JAN B. KOENDERINK, *Departments of Biochemistry, Human Genetics, Pediatrics, Physiology, and Pharmacology-Toxicology, Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands* (Sponsored by C.H. van Os)

Dominant renal hypomagnesaemia is a disease characterized by the renal wasting of magnesium. Previous research showed a link between the disease and a G41R mutation in the transmembrane domain of the renally expressed FXYD2 protein (Na,K-ATPase γ-subunit) (Meij et al. 1999. *Nat. Gen.* 26:265–266). We and others have shown that due to this mutation, the protein is absent from the plasma membrane. In addition, the mutated FXYD2 loses its interaction with the Na,K-ATPase. Here we present evidence supporting our hypothesis that the dominant nature of the disease is caused by oligomerization of the wild type and mutant FXYD2 subunits, which might cause misrouting of the wild type. In line with previous studies, our immunoprecipitation experiments of the Na,K-ATPase αβ and FXYD2 show a reduced interaction when the G41R mutation is present. Immunoprecipitation experiments between differentially labeled FXYD2 proteins show that these subunits form oligomers. Addition of the reducing agent DTT, and introduction of mutation C52A (FXYD2 contains only a single cysteine), indicate that the interaction between the transmembrane domains is stabilized by a disulfide bridge. When the G41R mutation is present in both labeled subunits, disruption of the disulfide bridge seems to disturb the oligomerization. We hypothesize that the FXYD2 subunits form dimers, which are stabilized by the disulfide bridge, even when the G41R mutation is present in one or both subunits. In patients, the dimers containing the G41R mutation are probably misrouted, and thus only a minor amount of FXYD2 is able to reach the plasma membrane. This reduced plasma membrane expression might be insufficient to regulate the Na,K-ATPase activity in the renal environment. [Sponsored by ZON-MW and the Dutch Kidney Foundation.]

111. Complex Regulation of the γ Subunit (FXYD2) of Na/K-ATPase: Search for the Promotor. JUAN M. CAPASSO, CHRISTOPHER J. RIVARD, JOSHUA H. CLARK, NESTOR E. ALMEIDA, and TOMAS BERL, *Department of Renal Medicine, University of Colorado Health Sciences Center, Denver, CO* (Sponsor: David Gadsby)

The γ subunit of Na/K-ATPase is a member of the FXYD family of small single transmembrane proteins involved in the modulation of Na/K-ATPase. This protein has been identified as critical to adaptation to hypertonicity in IMCD3 cells. The γ subunit demonstrates complex regulation in transcription and translation (Capasso et al. 2005. *Am. J. Physiol. Renal Physiol.* 288: F76–F81), as well as expression based on cell type. In addition, multiple splicing results in at least three different isoforms (Capasso et al. 2003. *Ann. NY Acad. Sci.* 986:410–415). Recently, we determined two distinctive timing programs for changes in osmotically regulated genes in which either a rapid or delayed transcription onset occurs. The γ subunit follows the second program and demonstrates an increase in mRNA only after a 10-h delay. Hence, the identification of the promoter of the γ subunit is critical to understanding this protein's complex expression.

Multiple amplicons ranging in size from 4 to 0.2 kb were obtained by PCR from mouse DNA using primers that target the FXYD2 gene upstream of the putative γHn-RNA transcription initiation sequence (γ_b exon I), cloned in the vector Blunt II-TOPO, and sequenced. Inserts with the proper sequence and orientation were excised by KpnI/XhoI digestion and spliced into the rapid response reporter vector pGL3(R2.1) down-stream of the poly(A) signal transcriptional pause, and upstream of the firefly luciferase gene.

IMCD3 cells were cotransfected with the above-described plasmid along with the *Renilla sp.* Luciferase reporter vector (as an internal standard).

Firefly luciferase activity was measured under isotonic and hypertonic conditions and corrected for the activity of the *Renilla* luciferase. In this way a DNA fragment extending from −900 to −250 was identified as playing a pivotal role in luciferase activation. Furthermore, this fragment contains the consensus sequence for the tonicity response element YGGAANNNYNY (TonE or MooKwon motif). Since γ regulation is complex, it is likely that this DNA fragment has more than one and probably several regulatory elements. [Supported by NIH/NIDDK grant DK66544.]

112. Endogenous FXYD5 and Transfected FXYD1 Copurify with Na,K-ATPase in HEK293 Cells. CLAUDIA DONNET and KATHLEEN SWEADNER,

Laboratory of Membrane Biology, Harvard Medical School/ Massachusetts General Hospital, Boston, MA (Sponsor: David Gadsby)

It is currently known that phospholemman (FXYD1), γ (FXYD2), MAT-8 (FXYD3), CHIF (FXYD4), FXYD7, and shark phospholemman-like protein interact with the Na,K-ATPase and modulate its activity. HEK293 cells expressed no phospholemman detectable by Western blot. We designed primers to detect all of the other FXYD proteins and performed RT-PCR on the HEK cells, confirming that endogenous phospholemman and most other FXYD mRNAs were absent. mRNA for FXYD5, also known as dysadherin, was present. Dysadherin is known to be 178 amino acids long, including a putative signal sequence and an O-glycosylated extracellular domain. We generated HEK stable transfectants that express human phospholemman at a high level. RT-PCR on these phospholemman-transfected cells showed again the presence of FXYD5 mRNA. Membranes prepared from these HEK cells were submitted to a modified Jorgensen SDS extraction procedure to purify the Na,K-ATPase. Western blot experiments showed that both the transfected phospholemman and the endogenous dysadherin copurified with the Na pump. In our hands, dysadherin appeared in Western blots as several bands. The strongest band at 18 kD correlated well with the molecular weight expected for the unglycosylated form, and other bands were seen as large as 45 kD. Interestingly, the cells expressing phospholemman showed a 2.6-fold increase in the stain intensity of the unglycosylated form of dysadherin, with only a small change in the level of the α subunit. This suggests a mutual regulatory mechanism involving different FXYD proteins.

113. Covalent Cross-links between the γ Subunit (FXYD2) and α and β Subunits of Na,K-ATPase: Modeling the γ–α interaction. MARIA FUZESI, MOSHIT LINDZEN, KAY-EBERHARD GOTTSCHALK, HAIM GARTY, and STEVEN J.D. KARLISH, *Department of Biological Chemistry, Weizmann Institute of Science, Rehovot, Israel* (David Gadsby)

Specific intramolecular covalent cross-links of the γ (FXYD2) to α, or γ to β, subunits of pig kidney Na,K-ATPase, and of rat γ coexpressed with rat α1 in HeLa cells, have been detected and analyzed. Three bifunctional reagents have been used: N-hydroxylsuccinimidyl-4azidosalicylic acid (NHS-ASA), disuccinimidyl tartrate (DST), and 1-ethyl-3-[3dimethylaminopropyl] carbodiimide (EDC). NHS-ASA induces α–γ, DST induces α–γ and β–γ, and EDC induces primarily β–γ cross-links. Using extensive or controlled proteolytic and Fe-catalyzed cleavages, γ–α cross-links have been located to the cytoplasmic surface, upstream of H283 and downstream of V440 of the α subunit. Additional considerations indicate that the DST-induced and NHS-ASA–induced cross-links involve either K347 or K352 in the S4 stalk segment. Mutational analysis of the rat γ subunit expressed in HeLa cells shows that the DST-induced cross-link involves K55 and K56 in the cytpolasmic segment. DST and EDC induce two β–γ cross-links, a major one at the extracellular surface within the segment G143-S302 of the β subunit, and another within G1-R142. We have modeled the interactions of the transmembrane α-helix and an unstructured cytoplasmic segment SKRLRCGGKKHR of γ with a homology model of the pig α1 subunit. According to the model, the transmembrane segment fits in a groove between M2, M6, and M9, and the cytoplasmic segment interacts with loops L6/7 and L8/9 and the S5 stalk segment. Cross-linking of native colonocyte membranes, containing CHIF (FXYD4), with NHS-ASA, DST, and EDC, suggests that the overall disposition of CHIF and γ, relative to α and β subunits, is similar. Thus, functional differences between CHIF and γ may be attributed to local sequence interactions or detailed differences in side chain interactions.

114. Implication of the Transmembrane Domain of FXYD7 in the Structural and Functional Interaction with the Na,K-ATPase. CIMING LI, GILLES CRAMBERT, DELPHINE THULLIARD, and KÄTHI GEERING, *Department of Pharmacology and Toxicology of the University, CH-1005 Lausanne, Switzerland* (Sponsor: David Gadsby)

Members of the FXYD family are tissue-specific regulators of the Na,K-ATPase (Crambert and Geering. 2003. *Sci. STKE.* 2003:RE1). In previous studies (Li et al. 2004. *J. Biol. Chem.* 279:38895–38902), we have shown that residues in the transmembrane (TM) domain 9 of the α subunit of the Na, K-ATPase are implicated in both the structural and functional association with FXYD2, 4, and 7. Here, we have investigated the role of amino acids in the TM domain of FXYD7 in the interaction with the Na,K-ATPase. 20 amino acids of the TM domain were replaced individually by tryptophan, and wild-type and mutant FXYD7 were expressed in *Xenopus* oocytes with or without the Na,K-ATPase. Mutational effects on the stable association with the Na,K-ATPase and on the functional regulation of the Na,K-ATPase were determined by coimmunoprecipitation and two-electrode voltage clamp techniques, respectively. Residues important for the stable association with the Na,K-ATPase are mostly clustered in a re-

gion containing two highly conserved glycine residues, which have been proposed to be important for the structural and functional association with the Na,K-ATPase (Crambert et al. 2004. *J. Biol. Chem.* 279:30888–30895). A few mutants show slight modifications in the functional effect of FXYD7, but no single mutation abolishes the functional effect completely. Residues involved in the functional effect are on the same side of the anticipated TM helix as those important for association. Our results suggest that the regulatory role of FXYD7 is defined by a stretch of amino acids in the TM domain, which influences conformational changes of the α subunit of the Na,K-ATPase during the transport cycle and, consequently, its cation affinities. [Supported by the Swiss National Fund grant 31-64793.01 to K. Geering.]

115. α Isoforms of Na,K-ATPase and FXYD Modulators in the Rat Vascular Muscle Cell Line A7r5. OTTO HANSEN, CHRISTIAN AALKJAER, and HOLGER NILSSON, *Institute of Physiology and Biophysics, Aarhus University, DK-8000 Aarhus C, Denmark*

The cell line A7r5 derived from rat aorta is sensitive to rather low ouabain concentrations (10^{-6} M) and to β-adrenergic and vasopressin stimulation. The presence of ouabain-sensitive α_2 and α_3 isoforms and of FXYD modulators was therefore examined.

Aliquots of A7r5 homogenates were used for Na^+,K^+-ATPase determination or run on SDS gels with purified Na^+,K^+-ATPase preparations in adjacent lanes and blotted to PVDF membranes. The blots were incubated with FXYD-specific or α-isoform–specific antibodies, and radiolabeled secondary antibodies, and the autoradiographic signals were used for identification of FXYD proteins and for semiquantitative determination of α subunits (Hansen. 2004. *Acta Physiol. Scand.* 180: 49–56).

Immunolabeling of Western blots of A7r5 homogenates was compatible with α_1 being almost totally dominating, whereas the presence of α_2 or α_3 was unreliable and probably insignificant. With purified Na^+,K^+-ATPase from rat kidney and or rat brain as references, a density of 14–19 pmol·(mg protein)$^{-1}$ of α_1 and <0.8 and 0.2 pmol·(mg protein)$^{-1}$ of α_2 and α_3, respectively, was determined. Phospholemman (or FXYD1) was identified in A7r5 but not the γ-peptide (or FXYD2). A variable inhibition of the Na^+,K^+-ATPase activity by 10^{-6} M ouabain was seen, but due to the prevailing unspecific ATPase activity of the rough homogenate, no consistent conclusion was reached.

The sensitivity to rather low concentrations of ouabain in A7r5 cell function seems contradictory to the prevailing ouabain-insensitive α_1 isoform. Occupation of a very small part of this isoform by ouabain may suffice or, if present, tiny concentrations of α_2 or α_3 isoforms may have strategic positions in microdomains or favor signal transduction. With the presence of phospholemman, a possible inhibitory factor of Na^+,K^+-ATPase activity is identified. Phospholemman is a target for PKA and PKC activity. Protein kinase activity may release phospholemman inhibition and explain β-adrenergic and vasopressin stimulation. [Supported by grant 2004–6 from Aarhus University Biotech.]

116. Purification of α/β/PLM (FXYD1) Complexes of Na,K-ATPase Expressed in *Pichia pastoris.* YAEL LIFSHITZ, HAIM GARTY, and STEVEN J.D. KARLISH, *Department of Biological Chemistry, Weizmann Institute of Science, Rehovot, Israel* (Sponsor: David Gadsby)

Human phospholemman (PLM, FXYD1) has been stably expressed alone or together with porcine α1 and His-tagged β1 subunits of the Na,K-ATPase (α-His_{10}β) in the methylotrophic yeast *Pichia pastoris.* The α and β subunits were expressed under control of an inducible promoter, and the PLM was expressed constitutively. The yeast membranes were dissolved with n-dodecyl-β-maltoside (DDM), and functional α/β/PLM complexes were purified by metal chelate bead chromatography, as described recently (Cohen et al. 2005. *J. Biol. Chem.* 280:16610–16618). Alternatively, the PLM, expressed without α and β subunits, was reconstituted together with α-His10-β bound to metal chelate beads. In either case, copurification of PLM with α and β subunits indicates the existence of specific interactions, although reconstitution allows better control of the PLM: α/β molar ratio. A comparison of Na,K-ATPase activities of purified α/β and α/β/PLM complexes showed that the presence of PLM was associated with a higher V_{max} and a higher apparent affinity for activation by Na ions. The effect of PLM on the V_{max} is explained by stabilization of the complex and is not due to a higher turnover number. Human α1 and α2 isoforms have been expressed together with pig His_{10}β1, and α1-His_{10}β1 and α2-His_{10}β1 complexes purified. Functional characterization of the α1-His_{10}β1 and α2-His_{10}β1 complexes is being performed. PLM can be reconstituted together with purified human α1-His_{10}β1 and, apparently less strongly, with human α2-His_{10}β1 complexes. PLM raises the apparent affinity of Na ions for human α1-His_{10}β1, as found for porcine α1-His_{10}β1. The observations that PLM raises apparent affinity for Na ions of Na,K-ATPase expressed in the yeast are different from observations in *Xenopus* oocytes in which PLM was found to reduce the apparent affinity for Na ions

(Crambert et al. 2002. *Proc. Natl. Acad. Sci.* 99:11476–11481). These different kinetic effects may indicate the possibility of multiple PLM-α/β interactions, which can be modulated in different experimental systems.

117. Intramembrane Interactions of FXYD Proteins with Na,K-ATPase Studied by Covalent Cross-linking of Proximal Cysteines. MOSHIT LINDZEN, MARIA FUZESI, HAIM GARTY, and STEVEN J.D. KARLISH, *Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel*

Previously, we have established that the transmembrane domains of phospholemman (PLM, FXYD1), the γ subunit of Na,K-ATPase (FXYD2, γ), and CHIF (FXYD4) mediate structural and functional interactions with the αβ subunits of Na,K-ATPase. Chemical cross-linking has been used to gain additional information on the structural interactions and, in particular, identify which residues of the α subunit are involved. Dibromobimane (DB) is a lipid-soluble 6-Å cysteine–cysteine cross-linker. In native membranes, DB cross-links α to CHIF but not to γ or PLM. CHIF has a cysteine residue C49 in the transmembrane segment, whereas the corresponding residue of γ or PLM is phenylalanine. In HeLa cell membranes expressing CHIF, the C49F mutation abolished the α-CHIF cross-link. In agreement, the F to C mutations of γ or PLM (F36C and F48C, respectively) resulted in DB-induced cross-linking of γ or PLM with α. Thus, the three FXYD proteins have a similar proximity to a transmembrane cysteine in the α subunit. The cysteine in the α has been identified by expressing and cross-linking in HeLa cells His-tagged α1 subunit mutated at various transmembrane cysteines. Mutation of C144 located in M2 abolished the above α-CHIF cross-links, while mutation of all other transmembrane α cysteines was without effect. Further information on CHIF C49-α C144 proximity was obtained by inserting cysteine at other positions along the transmembrane segment of CHIF and studying the efficiency of DB-induced cross-linking. These results provide valuable information on the structural interactions between α subunit and FXYD proteins. In essence they are consistent with our recently published model for the α–γ complex (Fuzesi et al. 2005. *J. Biol. Chem.* 280:18291–18301). The role of CHIF C49 and γ F36 in functional effects is being characterized using membranes from HeLa cells expressing wild type and mutated αβ/FXYD complexes.

118. Tissue Distribution and Interaction of RIC (FXYD5) with the Na,K-ATPase. IRINA LUBARSKI,[1] KAARINA PIHAKASKI-MAUNSBACH,[2] STEVEN J.D. KARLISH,[1] ARVID B. MAUNSBACH,[2] and HAIM GARTY,[1] [1]*Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel*; [2]*The Water and Salt Research Center, University of Aarhus, DK-8000 Aarhus, Denmark*

RIC (related to ion channel, FXYD5, dysadherin) is a member of the FXYD family. Four other members of this group have been shown to interact with the Na,K-ATPase and regulate its kinetic properties. However, RIC is unique among FXYD proteins in having a much longer extracellular domain. Moreover, previous studies have suggested a different role of RIC in regulating E-cadherin and promoting metastasis (Ino et al. 2002. *Proc. Natl. Acad. Sci.* USA. 99:365–370). The current study used specific antibodies to determine the cellular and tissue distribution of this protein as well as its possible interaction with the Na,K-ATPase. The antibody labeled a ~22-kD polypeptide in RIC-injected, but not in noninjected, *Xenopus* oocytes. In oocytes injected with HA-tagged RIC, the same polypeptide was identified by the anti-RIC and anti-HA antibodies. Placing the HA tag either before or after the putative signal peptide demonstrated that this peptide is cleaved in the mature protein. RIC is particularly abundant in heart, lung, kidney (cortex > medulla + papilla), and intestine (duodenum > jejunum > ileum ~ colon ~ distal colon). As found for other FXYD proteins, it is effectively immunoprecipitated by antibodies to the α subunit of the Na,K-ATPase and vice versa. Thus, RIC too is likely to be a regulator of the Na,K-ATPase. Currently, RIC's functional effects on the pump kinetics are under investigation. Immunolocalization experiments in kidney revealed that RIC resides in the basolateral membrane of the connecting tubule, the collecting tubule, and the intercalated cells of the collecting duct. This distribution differs from that of the other epithelial-specific FXYD proteins, CHIF and γ. Surprisingly, the anti-RIC antibody labels also the apical membrane in the long thin limb of Henle's loop. This observation may suggest involvement of RIC in the regulation of other transporters.

119. The Structures of Three FXYD Family Proteins in Membrane Environments. CARLA M. FRANZIN, KHANG THAI, JINGHUA YU, and FRANCESCA M. MARASSI, *The Burnham Institute, La Jolla, CA 92037* (Sponsor: David Gadsby)

The FXYD family proteins are tissue-specific and physiological state–specific auxiliary subunits of the Na^+,K^+-ATPase, expressed in tissues that perform fluid and solute transport or that are electrically excit-

able. We have expressed and purified milligram quantities of four FXYD family members: phospholemman (FXYD1), γ (FXYD2), mammary tumor protein (FXYD3), and corticosteroid hormone–induced factor (FXYD4). We are determining their structures, and membrane-associated conformations, by means of solution NMR spectroscopy with the proteins in micelles, and solid-state NMR spectroscopy with the proteins reconstituted in phospholipid bilayers. Results are presented for FXYD1, FXYD3, and FXYD4. [Supported by NIH grant CA082864.]

120. Association of C^{74} of PLMS to C^{254} of the Shark Rectal Na,K-ATPase α-Subunit Revealed by Chemical Cross-linking and a Novel Proteolytic Assay. YASSER A. MAHMMOUD,[1] HENRIK VORUM,[2] and FLEMMING CORNELIUS,[1] [1]*Institute of Physiology and Biophysics and* [2]*Institute of Medical Biochemistry, University of Aarhus, Aarhus, Denmark*

FXYD proteins are tissue-specific sodium pump–interacting proteins. Regulation of Na,K-ATPase activity by interaction with these proteins has been shown to have significant physiological implications. Information about interaction of FXYD proteins with Na,K-ATPase at the transmembrane level is beginning to emerge, but little is known about the cytoplasmic site(s) of interaction of these regulatory proteins on Na,K-ATPase. PLMS (FXYD10) was previously shown to modulate Na,K-ATPase activity in shark rectal glands, and functional studies suggested that PLMS interacts with the A-domain of the shark α-subunit. The present study was undertaken to identify the location of the COOH-terminal domain of PLMS on shark Na,K-ATPase α-subunit, using covalent cross-linking combined with proteolytic cleavage. Treatment of Na,K-ATPase enriched membranes with the homobifunctional cross-linkers 1,6-bismaleimidohexane (BMH) or 1,4-bismaleimidyl-2,3-dihydroxybutane (DMDB) resulted in cross-linking of PLMS to the α-subunit. Cross-linking was not affected by preincubation with sodium or potassium, but was significantly reduced after preincubation with the nonhydrolyzable ATP analogue AMP-PCP. A proteolytic assay was developed, in which control and cross-linked preparations were treated with pepsin at low pH, and the resulting fragments were isolated with immunoprecipitation and resolved by gel electrophoresis. A proteolytic fragment containing PLMS cross-linked to a fragment from the α-subunit was localized on SDS gels. Sequencing of this fragment showed the presence of PLMS as well as a fragment of α comprising 33 amino acids, which included a single Cys residue, Cys254. Thus, regulation by PLMS of Na,K-ATPase occurs, in part, via cytoplasmic interaction of PLMS with the A-domain of the shark α-subunit. [Supported by the Danish Research Academy grant 22–01-0499.]

121. Conformational Changes Involved in the Activation of the Plasma Membrane Ca^{2+} Pump. GERARDO R. CORRADI and HUGO P. ADAMO, *IQUIFIB-Facultad de Farmacia y Bioquímica* (*UBA-CONICET*), *Buenos Aires, Argentina* (Sponsor: David Gadsby)

The autoinhibition of the plasma membrane Ca^{2+} pump is believed to involve the interaction of the COOH-terminal domain with regions of domains A and N (Bredeston and Adamo. 2004. *J. Biol. Chem.* 279: 41619–41625). Calmodulin binds to the inhibitory sequence and promotes an activated state characterized by high affinity for Ca^{2+} and high transport activity. With the aim of studying the inhibition–activation process, we succeeded in creating an active PMCA fused to two fluorescent proteins. The BFP was inserted after Thr2 of the human isoform 4xb while the GFP was located at the COOH-terminal end ∼100 residues downstream of the sequence corresponding to the calmodulin binding peptide C28. The recombinant BFP-PMCA-GFP was obtained by expression in *Saccharomyces cerevisiae* and was purified by affinity chromatography. Excitation of the BFP-PMCA-GFP at 387 nm resulted in a differential GFP emission by energy transfer (FRET) at 509 nm. The efficiency of transfer was estimated by comparing the emission of BFP at 450 nM in the BFP-PMCA-GFP with that of the BFP-PMCA protein. The calculated average distance between chromophores (r) in the BFP-PMCA-GFP was 45 Å. Addition of 10 μM Ca^{2+} did not produce a significant change of r. However the addition of 10 μM Ca^{2+} and 200 nM calmodulin increased the value of r to 50 Å. According to these results, the NH_2 terminus and the COOH terminus of the PMCA would be ∼45 Å from each other, and their relative position would not change significantly during the E2–E1 transition. In contrast, the binding of calmodulin induced a conformational change revealed by a small increase of the distance between the ends of the protein. [Supported by grants of CONICET and UBA.]

122. Phospholemman (PLM) Phosphorylation Alters Its Association with the Na/K Pump (NKA) Assessed by FRET. JULIE BOSSUYT, SANDA DESPA, and DONALD M. BERS, *Department of Physiology, Loyola University Chicago, Maywood, IL* (Sponsor: Donald M. Bers)

Phospholemman, one of the FXYD proteins, is a major target for protein kinase A and C in the heart dur-

ing sympathetic stimulation (β-and α-adrenergic activation). In cardiac myocytes, PLM associates with NKA and mediates adrenergic regulation of NKA (see abstract by Despa et al.). To better understand this regulation, we used fluorescence resonance energy transfer (FRET) between CFP and citrineYFP fusion proteins of Na/K pump α1 subunit and PLM in HEK293 cells to monitor the PLM-Na/K pump interaction. Expression of NKA-CFP and PLM-YFP_c resulted in targeting to the plasma membrane with very high colocalization. Moreover, FRET (measured by conventional and confocal fluorescence microscopy) indicates that PLM and NKA interact at the membrane. Selective photobleaching of YFP_c increased donor fluorescence by 23.8 ± 4.8%. Activation of PKA (by 100 nM forskolin) progressively decreased FRET assessed by $F_{donor}/F_{acceptor}$. After forskolin, YFP photobleaching increased F_{donor} by only 2.2 ± 0.9% ($n = 3$). Activation of PKC (by 100 nM PDBu) similarly reduced FRET. Acceptor photobleaching increased F_{donor} by 3.0 ± 0.9 ($n = 3$) after 15 min of PDBu. These results demonstrate close proximity of PLM and NKA (FRET occurs) and that PLM phosphorylation by either PKA or PKC alters the interaction of NKA-α1-CFP and PLM-YFP_c. Our data are consistent with the hypothesis that PLM regulates NKA activity similar to the way phospholamban regulates SERCA (an inhibitory effect that is relieved upon phosphorylation). [Supported by NIH grant HL-30077, HL64724 (D.M. Bers), and by AHA fellowship (J. Bossuyt).]

123. Phospholemman Phosphorylation Mediates the β-Adrenergic Effects on Na/K Pump in Mouse Ventricular Myocytes. SANDA DESPA,[1] JULIE BOSSUYT,[1] FEI HAN,[1] KENNETH S. GINSBURG,[1] LI-GUO JIA,[2] AMY L. TUCKER,[2] and DONALD M. BERS,[1] [1]*Department of Physiology, Loyola University Chicago, Maywood, IL*; [2]*University of Virginia, Charlottesville, VA* (Sponsor: Donald M. Bers)

Cardiac sympathetic stimulation activates β-adrenergic (β-AR) receptors and leads to protein kinase A (PKA) phosphorylation of several proteins involved in myocyte Ca regulation. The Na/K-ATPase (NKA) is essential in regulating intracellular [Na] ($[Na]_i$), which in turn affects $[Ca]_i$ via Na/Ca exchange. However, the molecular mechanism by which PKA modifies NKA function has been elusive. Phospholemman (PLM), a member of the FXYD family of proteins that interact with and modulate NKA in various tissues, is also a major PKA substrate in the heart. Here we tested the hypothesis that PLM phosphorylation (rather than direct NKA phosphorylation) is responsible for the PKA effects on cardiac NKA function, using wild-type (WT) and PLM knockout mice. We measured NKA-mediated $[Na]_i$ decline and current (I_{Pump}) to assess β-adrenergic effects on NKA function in single isolated myocytes. In WT myocytes, 1 μM isoproterenol (ISO) increased PLM phosphorylation, stimulated NKA activity mainly by increasing its affinity for internal Na (K_m decreased from 18.8 ± 1.4 to 13.6 ± 1.5 mmol/liter), with no significant effect on the maximum pump rate. This leads to a significant decrease in resting $[Na]_i$ (from 12.5 ± 1.8 to 10.5 ± 1.4 mmol/liter). In PLM-KO mice under control conditions, K_m (14.2 ± 1.5 mmol/liter) was lower than in WT, but comparable to that for WT in the presence of ISO. Furthermore, ISO had no significant effect on NKA function in PLM-KO mice. Thus, PLM inhibits NKA activity by decreasing its $[Na]_i$ affinity, and this inhibitory effect is relieved by PKA activation. We conclude that PLM modulates the NKA function in a manner similar to that by which phospholamban affects the related SR Ca-ATPase, i.e., inhibition by reduction of transport substrate affinity, which is relieved by PLM/phospholamban phosphorylation.

124. Relief of Phospholamban Inhibition of the Cardiac Muscle Ca^{2+} Pump (SERCA2a) Raises Vmax by Decreasing the Ca^{2+} Affinity of Luminal Transport Sites. JEFFREY FROEHLICH, GERALD M. WILSON, CARLO G. TOCCHETTI, DAVID KASS, and NAZARENO PAOLOCCI, *Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD; Johns Hopkins Medical Institutions, Baltimore, MD* (Sponsor: Jack H. Kaplan)

Activation of the cardiac muscle sarcoplasmic reticulum (CSR) Ca^{2+} pump (SERCA2a) by β_1-adrenergic agonists, which enhances CSR Ca^{2+} stores, involves PKA-dependent phosphorylation of an intrinsic, low molecular weight protein, phospholamban (PLB). Phosphorylation of PLB increases the apparent Ca^{2+} affinity and Vmax of SERCA2a, reflecting the loss of regulatory control by PLB. Recent studies have shown that the effects of PKA-dependent activation of SERCA2a can be mimicked by incubating CSR vesicles with nitroxyl anion (HNO/NO^-) generated from Angeli's salt (AS; $Na_2N_2O_3$). Exposure of canine CSR vesicles to 0.25 mM AS stimulated the spontaneous rate of E2P hydrolysis ($6\ s^{-1} \rightarrow 77\ s^{-1}$), revealed by chasing with EGTA to block rephosphorylation of SERCA2a. Associated with this change was a threefold increase in the steady-state level of E1P, measured by dephosphorylating SERCA2a with ADP. Stopped-flow studies of ATP-dependent Ca^{2+} uptake by CSR vesicles using arsenazo III as Ca^{2+} indicator showed a biphasic pattern of accumulation that was sensitive to nitroxyl. AS (0.25 mM) increased the veloc-

ity of the slow phase of Ca^{2+} uptake, converting the time course to monophasic behavior without increasing total Ca^{2+} uptake. The slow phase of uptake was abolished by the Ca^{2+} ionophore A23187, demonstrating that it represents luminal Ca^{2+} uptake. We attribute the increase in the velocity of Ca^{2+} uptake to a nitroxyl-induced decrease in the affinity of the luminal transport sites for Ca^{2+}. As Ca^{2+} accumulates, it back-inhibits the pump by binding to these sites, reducing the velocity of Ca^{2+} uptake. Nitroxyl-activated E2P hydrolysis relieves the back-inhibition of the pump by competing with Ca^{2+} binding to the luminal transport sites on E2P. A model accounting for these effects is presented in which covalent modification of SERCA2a and/or PLB by HNO/NO^- causes PLB to dissociate from SERCA2a, relieving its inhibition and raising Vmax. [Supported by a National Center American Heart Association grant to N. Paolocci.]

125. Phospholemman Phosphorylation Mediates the PKC-dependent Modulation of Na/K Pump in Cardiac Myocytes. FEI HAN,[1] SANDA DESPA,[1] LIGUO JIA,[2] AMY L. TUCKER,[2] and DONALD M. BERS,[1] [1]*Department of Physiology, Loyola University Chicago, Maywood, IL*; [2]*University of Virginia, Charlottesville, VA*

Phospholemman (PLM) is a major target for both PKA- (at Ser68) and PKC- (at both Ser63 and Ser68) mediated phosphorylation in the heart. We have shown that PLM modulates the NKA function, and PLM phosphorylation mediates the β-adrenergic effects on NKA in intact cardiac myocytes (Despa et al., poster at this conference). The aim of this study was to investigate how PKC activation affects NKA function in ventricular myocytes and whether PLM is also involved. We measured the effects of the PKC activator PDBu (300 nM) on NKA-mediated $[Na]_i$ decline (fluorescence measurements) and current (I_{Pump}, patch clamp). In WT mouse myocytes, PDBu significantly increased maximal NKA extrusion rate (V_{max}) from 9.2 ± 1.4 to 14.7 ± 2.6 mM/min without any change in the NKA affinity for internal Na (K_m). In PLM-KO mice, PDBu had no effect on either V_{max} or K_m. PDBu also stimulated NKA function in rabbit ventricular myocytes, but, surprisingly, the PDBu effect was mediated by increased NKA affinity for internal Na (K_m decreased from 15.2 ± 1.6 to 8.3 ± 1.1 mM) without altering V_{max}. I_{Pump} measurements in rabbit myocytes supported this conclusion, because PDBu had no effect on I_{Pump} with 100 mM Na in the pipette solution (i.e., V_{max} conditions; 1.16 ± 0.05 vs. 1.09 ± 0.07 pA/pF), but increased I_{Pump} at low, 10 mM, pipette Na (i.e., K_m effect; 0.57 ± 0.04 vs. 0.72 ± 0.03 pA/pF). After pretreatment with isoproterenol (1 μM), PDBu could still increase the Na affinity of NKA (K_m decreased from 13.3 ± 2.1 to 6.9 ± 0.8 mM) without any change in V_{max}. We conclude that (1) PLM mediates the PKC-dependent activation of NKA function in ventricular myocytes, (2) such effects are species dependent (altering V_{max} in mouse, K_m in rabbit), and (3) PKC effects are additive with those of isoproterenol-mediated stimulation of PKA.

126. Coexpression of Phospholamban and Sarcolipin in Cardiac and Skeletal Muscle Differs in Small and Large Mammals. PETER VANGHELUWE,[1] MARLEEN SCHUERMANS,[1] ERNÖ ZÁDOR,[3] ETIENNE WAELKENS,[2] LUC RAEYMAEKERS,[1] and FRANK WUYTACK,[1] [1]*Laboratory of Physiology and* [2]*Laboratory of Biochemistry, K.U. Leuven, Leuven, Belgium;* [3]*Institute of Biochemistry, University of Szeged, Szeged, Hungary* (Sponsor: David Gadsby).

In vitro, sarcolipin (SLN) reinforces phospholamban (PLB) inhibition of sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA), suggesting a new level of regulating SERCA's activity. The physiological implications of such superinhibition in vivo still need to be determined. In that respect, it was of particular interest to assess coexpression of SLN and PLB in vivo. Previous attempts to make an SLN antibody were unsuccessful, and the lack of SLN antibodies limited expression studies at the protein level. Here, we report on the first successfully generated antibody directed against SLN. These antibodies were used to assess the expression of SLN protein relative to PLB, SERCA1a, or SERCA2a in ventricle, atrium, soleus, and extensor digitorum longus (EDL) of mouse, rat, rabbit, and pig.

In pig and rabbits, SLN protein expression is limited to slow- and fast-twitch skeletal muscle correlating with SERCA1a expression. In the soleus of these animals, low levels of PLB and SERCA2a were found in addition to SLN, thus allowing possible interaction with SLN. However in rodents, SLN protein expression was confined to the atria, and no SLN was found in the ventricle or in skeletal muscle. In the atria of rodents, SLN was expressed together with high levels of PLB and SERCA2a, but not SERCA1a. Thus, the widely held view that SLN would be the natural inhibitor of SERCA1a, and PLB its counterpart for SERCA2 inhibition, is oversimplified. In addition, these data demonstrate coexpression of PLB and SLN protein in various muscles, suggesting that superinhibition of SERCA by PLB-SLN complexes could occur in vivo. For the investigated animal species, the differential pattern of coexpression in atria or slow-twitch skeletal muscle correlates with body size. [Supported by BELSPO grant P5/05; BIL grant 04/33.]

127. Up-regulation of Phospholamban Protects the Heart Against the Replacement of SERCA2a by the Higher-Ca^{2+} Affinity SERCA2b Isoform. PETER VANGHELUWE,[1] MARC TJWA,[3] AN VAN DEN BERGH,[4] WILLIAM E. LOUCH,[2] PAUL HERIJGERS,[4] EVANGELIA KRANIAS,[5] KARIN SIPIDO,[2] LUC RAEYMAEKERS,[1] and FRANK WUYTACK,[1] [1]*Laboratory of Physiology*, [2]*Laboratory of Cardiology*, [3]*CTR*, [4]*CEHA, K.U. Leuven, Leuven, Belgium;* [5]*Department of Pharmacology, University of Cincinnati, Cincinnati, OH* (Sponsor: David Gadsby)

The sarco(endo)plasmic reticulum Ca^{2+}-ATPase SERCA2a is a major determinant of cardiac relaxation and contraction. Alternative splicing of SERCA2 transcripts in the heart results in the formation of SERCA2a (98%) and of the higher Ca^{2+} affinity isoform SERCA2b (2%). In *SERCA2$^{b/b}$* mice, prevention of this alternative splicing precludes SERCA2a formation, thereby replacing SERCA2a by SERCA2b. *SERCA2$^{b/b}$* display impaired cardiac contraction/relaxation and develop left ventricular hypertrophy. Cardiac SERCA2 expression is reduced by 50% and the levels of the SERCA2 inhibitor phospholamban (PLB) protein are doubled. We tested the hypothesis that the fourfold increased PLB/SERCA ratio protects the *SERCA2$^{b/b}$* heart against the high Ca^{2+} affinity of SERCA2b. Indeed, the ratio of Ser16-phosphorylated/total PLB levels is decreased in *SERCA2$^{b/b}$*, partially reducing the Ca^{2+} affinity of SERCA2b. The effect of preventing PLB up-regulation was assessed by crossing *SERCA2$^{b/b}$* with *PLB$^{-/-}$* mice (DKO mice). Compared with *SERCA2$^{b/b}$*, DKO died sooner, were less spontaneously active, and displayed a reduced exercise capacity. PLB ablation partially improved cardiac contractile parameters (+dP/dt, E_{es}). However, left ventricular hypertrophy was exacerbated, impairing stroke volume, cardiac output (CO), and diastolic function. β-adrenergic stimulation failed to raise CO, resulting in an increased incidence of acute heart failure in DKO. Thus, spontaneous PLB up-regulation in *SERCA2$^{b/b}$* mice is beneficial by reducing SERCA2b's high Ca^{2+} affinity. Moreover, prevention of this compensation in DKO further reduced cardiac SERCA2 levels to 33% of WT, suggesting that SERCA2 down-regulation itself is also compensatory. In conclusion, this study illustrates the incompatibility of a high Ca^{2+} affinity SERCA2 pump with normal cardiac function. Adjusting SERCA and PLB expression, and PLB's phosphorylation, are compensatory mechanisms preventing excessive cytosolic Ca^{2+} removal by SERCA2b in the low micromolar Ca^{2+} concentration range. [Supported by FWO grant G.0166.04.]

128. How To Make Two-dimensional Crystals by Core Constitution of Ca^{2+}-ATPase, Phospholamban, and Sarcolipin. JENNIFER A. DOUGLAS,[1] WILLIAM J. RICE,[2] DAVID L. STOKES,[2] and HOWARD S. YOUNG,[1] [1]*Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada*; [2]*Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY* (Sponsor: David Gadsby)

The sarcoplasmic reticulum Ca^{2+}-ATPase and its regulators phospholamban (PLB) and sarcolipin (SLN) form a primary control mechanism in the recovery of resting state calcium levels in the myocardium. Defects in the regulation of Ca^{2+}-ATPase by PLB and SLN are central determinants in cardiac contractility and disease states such as hypertrophy and cardiomyopathy. Given the significance of these proteins, the structural and functional details of their regulatory mechanisms remain an important future goal for the clinical improvement of heart disease. In studying the structure and function of calcium pump regulation, coreconstitution into proteoliposomes at low lipid-to-protein ratios has been a powerful method. Using this approach, we have examined the effects of mutation on the regulatory properties of PLB and SLN in coreconstituted proteoliposomes, revealing novel insights into their regulatory function (Trieber et al., 2005. *Biochemistry.* 44:3289–3297). In addition, these same coreconstituted proteoliposomes have been used for structural studies by electron cryomicroscopy (Young et al. 1997. *Biophys. J.* 72:2545–2558; Young et al. 2001. *Biophys. J.* 81:884–894; Young and Stokes. 2004. *J. Membr. Biol.* 198: 55–63). In an attempt to better define the structural interactions between PLB, SLN, and Ca^{2+}-ATPase, we have sought methods for the production of large two-dimensional crystals suitable for high resolution electron crystallography. We previously characterized the coreconstituted proteoliposomes at low lipid-to-protein ratios and discovered procedures that produce long, tubular crystals suitable for helical reconstruction. Our new procedure comprises three steps (coreconstitution, membrane fusion, and crystallization), allowing us to produce large two-dimensional crystals suitable for high resolution structural studies. In this report, we will present our latest results characterizing the structural interaction between PLB, SLN, and Ca^{2+}-ATPase.

129. Study on the Binding Site of Acid Pump Antagonists on the Gastric Proton Pump α-Subunit Constructed by Homology Modeling. SHINJI ASANO,[1] YUSUKE KOBAYASHI,[2] ANNA MORISATO,[1] and MAGOTOSHI MORII,[2] [1]*College of Information Science and Engineering, Ritsumeikan University, Shiga, Japan*; [2]*Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Toyama, Japan* (Sponsor: Joe Hoffman)

H^+,K^+-ATPase is the proton pump responsible for gastric acid secretion. This pump is a target molecule for treatment of diseases involving gastric acid secretion, such as gastroesophageal reflux disease. When the pump is inhibited by specific inhibitors, gastric acid secretion is clinically well controlled. Acid pump antagonists (APAs), such as SCH 28080 and SPI-447, reversibly inhibit the pump, competitively with K^+, and are being developed for clinical use. Here, we studied the binding site of APAs, especially SCH 28080, by site-directed mutagenesis based on the 3-D structure of the α-subunit constructed by homology modeling and molecular dynamics simulation.

Tyr-801 on the M5 segment was replaced by alanine or serine, and the mutants were expressed in HEK-293 cells. These mutants showed 60 to 80-fold lower sensitivity to SCH 28080 in $^{86}Rb^+$ transport and K^+-ATPase assays, indicating that the side chain of this residue is important for the interaction with SCH 28080. The sensitivity to SCH 28080 seems to be dependent on the bulkiness of the side chain.

In the putative 3-D models of the α-subunit, Tyr-801 was found in the pocket of a cavity structure on the luminal surface surrounded by the M1, M4, M5, M6, and M8 segments, and the M5/M6, M7/M8, and M9/M10 loops. In the modeled E_2 and E_2P forms of the α-subunit, SCH 28080 can stably dock in this pocket. However, SCH 28080 cannot dock in the modeled E_1 form because of the pocket structure is disrupted. These results are in good agreement with the kinetic results showing that SCH 28080 specifically binds to the E_2 and E_2P forms. These results strongly suggest that this pocket is the binding site of SCH 28080 (Asano et al. 2004. *J. Biol. Chem.* 279:13968–13975).

130. Polyamines as Regulators of *Callinectes danae* Gill Na,K-ATPase. E.C. CANDIDO-SILVA, D.C. MASUI, R.P.M. FURRIEL, F. DE A. LEONE, H. BARRABIN, H.M. SCOFANO, and C.F.L. FONTES, *Universidade Federal do Rio de Janeiro, 21941-590 Rio de Janeiro, Brazil*

Euryhaline crabs *Callinectes danae* developed the capacity of self-adaptation to a broad variation in environmental salinity during the course of their evolution. The strategy adopted by these animals to keep the osmolarity of their hemolymph in a range compatible with normal cellular functions includes a series of Na^+ transporters, located in the gills, and a Na,K-ATPase located in the basolateral membranes of gill cells. However, the role of substances that control the activity of Na,K-ATPase during the self-adaptation process of *C. danae* remains unclear.

The addition of exogenous polyamines inhibited the activity of Na,K-ATPase obtained from the gills of *C. danae*. Incubation of Na,K-ATPase with spermidine, and the consecutive assays of enzyme stimulation by Na^+, K^+, and NH^+_4, showed that polyamines affected the $K_{0.5}$ for Na^+, and Vmax in the presence of Na^+, without changing the $K_{0.5}$ for K^+ or V_{max} in the presence of K^+. In addition, polyamines were able to inhibit the stimulatory effect of NH_4^+ ions, observed when the enzyme is saturated with K^+ ions. Our results also confirm that the Na,K-ATPase from *C. danae* presents one additional site for the binding of NH^+_4 ions. Phosphorylation of the Na,K-ATPase with 20 μM ^{32}P-γ-ATP was performed in the presence of different Na^+ concentrations (10 mM and 100 mM) and presence of polyamines. In the presence of 10 mM Na^+, spermidine and spermine inhibited the formation of the ATP-induced phosphointermediate. In contrast, in the presence of 100 mM Na^+, putrescine, spermidine, and spermine favored accumulation of the phosphointermediate.

We conclude that the inhibition of Na,K-ATPase is highly dependent on enzyme conformation, and on charge and size of the tested polyamines, and that the polyamines possibly interact with the cation sites of the enzyme. [Supported by CNPq and FAPESP.]

131. Characterization of ATPase Inhibition by Chelerythrine. VANESSA H. OLIVEIRA, CATARINA C.P. PRIMA, HELENA M. SCOFANO, CARLOS F.L. FONTES, and JULIO A. MIGNACO, *Laboratório de Estrutura e Regulação de Proteínas e ATPases and Laboratório de Membranas Transportadoras, Instituto de Bioquímica Médica, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*

The alkaloid chelerythrine is described as a potent and specific inhibitor of PKC activity and calcium-mediated signal transduction in cells. Chelerythrine action is also associated with induction of apoptosis, ROS generation, and K^+ efflux. All these phenomena involve the participation of calcium signaling. We tested whether chelerythrine could directly affect the activities of the Ca^{2+}-ATPases (PMCA and SERCA) and Na^+,K^+-ATPase, since these proteins are more directly involved with the homeostasis of Ca^{2+}. The ATPase activity of purified erythrocyte PMCA was measured in the presence or absence of CaM 2 μg/ml. The p-nitrophenylphosphatase (pNPPase) activity was assayed in the presence or absence of 1 μM free Ca^{2+} (SERCA) or 10 μM free Ca^{2+} (PMCA). Activity of purified Na^+,K^+-ATPase from pig kidney was assayed in presence of NaCl 120 mM, KCl 20 mM, $MgCl_2$ 2 mM, and ATP 2 mM. The concentration of chelerythrine in the medium ranged from none to 50 μM. The Ca^{2+}-coupled ATPase activity of PMCA was

inhibited by >90% by 50 μM chelerythrine, with an IC_{50} around 3–4 μM. This effect was independent of the addition of calmodulin. The Na^+,K^+-ATPase activity was inhibited to a smaller extent, reaching roughly a 30% inhibition with 10 μM chelerythrine, and SERCA activity was inhibited 70% by the same concentration of chelerythrine. The phosphorylation of SERCA by ATP was progressively decreased with increasing chelerythrine concentrations. The pNPPase activity was inhibited in the absence of calcium, but stimulated in the presence of this ion. These data demonstrate that the inhibitory effects of chelerythrine on the P-type ion-transporting ATPases are by its direct binding to the enzyme. The data of the ATPase and pNPPase activities, and the effect on phosphoenzyme levels, suggest that chelerythrine binds with high affinity to the nucleotide site of P-ATPases and perhaps with lower affinity to a second site related to the E_2 conformer of the enzyme. [Supported by CAPES, CNPq, and FAPERJ.]

132. 67-kD Melittin-like Protein from Mouse Kidney that Directly Interacts with Na,K-ATPase Seems To Be a New Protein Encoding by Gene Ak129239. NATALIYA V. DOLGOVA, ALEXANDER M. RUBTSOV, and OLGA D. LOPINA, *Department of Biochemistry, School of Biology, MV Lomonosov Moscow State University, Moscow, Russia*

Bee venom peptide melittin is known to be an inhibitor of P-type ATPases including gastric H,K-ATPase, SR Ca-ATPase, and Na,K-ATPase. It was shown earlier that melittin directly interacts with the gastric H,K-ATPase α-subunit, and the amino acid sequence of its melittin-binding site is conserved in SR Ca-ATPase and Na,K-ATPase. This implies that melittin mimics some intracellular protein(s) that interact(s) with these ATPases. The goal of this study was to find and identify melittin-like protein(s) in mouse tissue (this species' genome is now available). Immunoprecipitation of Na,K-ATPase from Triton X-100–solubilized mouse kidney homogenate by antibodies against the α-subunit of Na,K-ATPase revealed that a protein with molecular mass of ~67 ± 3 kD was among the proteins coprecipitated with the enzyme. This 67-kD protein was stained by antimelittin antibodies. The protein was purified by immunoaffinity chromatography and its NH_2-terminal sequence was determined to be *spvsn*. Fingerprint analysis of the 67-kD melittin-like protein was also performed. The 67-kD protein was shown to increase the fluorescence of FITC-labeled Na,K-ATPase only in the presence of NaCl confirming its direct interaction with Na,K-ATPase. The fingerprint data, together with NH_2-terminal sequence, were analyzed by Mascot program against NCBInr (nonredundant) database. The analysis revealed that the largest number of corresponding peptides are located in protein mKIAA0921 (*Mus musculus*) which comprises 1,552 amino acids (translated from mRNA in silico) and is homologous to neurexin. The start codon of this sequence was not identified, and residues *spvsn* were found near the middle part of protein. We suggest that the 67-kD melittin-like protein might be a product either of alternative splicing of the gene that encodes the mKIAA0921 protein, or of posttranslational proteolysis of the latter. [Partially supported by grant 01–0224 from INTAS.]

133. PST2744, an Innovative Inotropic and Lusitropic Agent, Stimulates SERCA2 Activity in Cardiac Sarcoplasmic Reticulum Preparations. PAOLO BARASSI, MARA FERRANDI, GIUSEPPE BIANCHI, and PATRIZIA FERRARI, *Prassis Research Institute Sigma-Tau, Settimo M.se and Vita Salute University, S. Raffaele Hospital, Milan, Italy*

Altered calcium cycling significantly affects both systolic and diastolic function, and triggers arrhythmias, in congestive heart failure (CHF), in which a reduced Ca^{2+} reuptake by the SR Ca-ATPase (SERCA2) and enhanced trans-sarcolemmal Ca^{2+} transport through the Na^+/Ca^{2+} exchanger contribute to a decreased SR Ca^{2+} content. Altered SR function therefore represents a new therapeutic target in CHF. PST2744 is a novel inotropic and lusitropic agent able to modulate the cardiomyocyte Ca^{2+} cycling by inhibiting the Na-K pump and stimulating the SR calcium reuptake without enhancing spontaneous Ca^{2+} efflux from the SR or increasing cAMP.

To investigate how PST2744 acts on cell Ca^{2+} cycling, SERCA2 kinetics were measured in isolated SR vesicles from normal guinea pig heart in the presence or absence of the compound. At 100 nM free calcium, PST2744 stimulated SERCA2 activity with a peak effect between 75 and 100 nM (EC50 = 77 nM). PST2744, at 100 nM, shifted the Ca^{2+}-dependent activation curve of SERCA2 leftward, significantly decreasing the K_d for Ca^{2+} (from 95 ± 6 nM to 71 ± 5 nM, $n = 4$, $P < 0.02$). V_{max} and Hill number were not modified. Digoxin, taken as a reference compound, did not affect SERCA2 kinetics.

PST2744 was also tested on cardiac SR vesicles from human failing hearts obtained from CHF patients who underwent cardiac transplantation. At 100 nM free Ca^{2+}, 3–10 nM PST2744 stimulated the SERCA2 activity by 45–70% in four different preparations. These results indicate that also in human failing hearts, PST2744 may stimulate SERCA2 activity with high potency.

PST2744 is the first example of a compound that, by directly stimulating SERCA2 activity, favors SR Ca^{2+} reuptake with consequent improvement of relaxation during diastole and of contraction during systole. The SERCA2 stimulatory effect of PST2744 may therefore account for both its inotropic and lusitropic activities.

134. *Bufo marinus* Bladder H,K-ATPase Is Not Sensitive to Palytoxin. S. GUENNOUN, R.F. RAKOWSKI, and J.-D. HORISBERGER, *Department of Pharmacology and Toxicology of the University of Lausanne, CH-1005 Lausanne, Switzerland; Department of Biological Sciences, Ohio University, Athens, OH 45701* (Sponsor: J.-D. Horisberger)

"Non-gastric" H,K-ATPase is a group IIc P-ATPase closely related (~75% amino acid identity) to Na,K-ATPase and to gastric H,K-ATPase. It is expressed in the apical membrane of colonic and collecting duct cells and its expression is strongly increased by K^+ depletion. *Bufo marinus* (bladder), rat (colonic), and human (ATPAL1) "non-gastric" H,K-ATPases are moderately sensitive to ouabain with a K_I on the order of 0.2–0.5 mM (similar to the K_I for *Bufo* Na,K-ATPase), and can be inhibited by high concentrations of SCH-28080. Although the transport stoichiometry is not precisely known, "non-gastric" H,K-ATPases most probably exchange equivalent numbers of intracellular H^+ and Na^+ ions for extracellular K^+ ions, resulting in net electroneutral transport. Palytoxin (PTX) binds to Na,K-ATPase and transforms it into a nonspecific cation channel (Artigas and Gadsby. 2003. *Proc. Natl. Acad. Sci. USA.* 100:501–505). The current study was designed to determine whether palytoxin has an effect on nongastric H,K-ATPase. *Bufo marinus* bladder H,K-ATPase and *Bufo* α1 Na,K-ATPase were expressed with *Bufo* β2 (bladder) subunit in *Xenopus* oocytes, and the response to palytoxin exposure was monitored after inhibition of the endogenous *Xenopus* Na,K-ATPase with 50 μM ouabain. Expression of both *Bufo* ATPases was confirmed by measurements of rubidium uptake. Exposure to 10 nM palytoxin induced a large increase of membrane conductance (~30-fold, from 5.3 ± 1.3 to 145 ± 40 μS) in oocytes expressing the *Bufo* Na,K-ATPase, but no significant change (5.6 ± 1.0 to 5.7 ± 1.0 μS) in the oocytes expressing the H,K-ATPase, nor in those expressing the β subunit alone. We are presently investigating the effect of palytoxin on the rat colonic H,K-ATPase expressed in HeLa cells to verify that this observation can be generalized to mammalian "non-gastric" H,K-ATPases. [Supported by the Swiss National Fund grant 31-65441.01 to J.-D. Horisberger and NIH grant NS-22979 to R.F. Rakowski.]

135. α3 Na^+/K^+-ATPase Is the neuronal receptor for Agrin in the CNS. LUTZ G.W. HILGENBERG,[1] HAILING SU,[1,2] DIANE K. O'DOWD,[1,2] and MARTIN A. SMITH,[1] [1]*Department of Anatomy and Neurobiology and* [2]*Department of Cell and Developmental Biology, University of California, Irvine, CA 92697* (Sponsor: Diane K. O'Dowd)

The extracellular matrix protein agrin serves as an essential catalyst of postsynaptic differentiation at the vertebrate neuromuscular junction by activating the receptor tyrosine kinase MuSK. Agrin is also highly expressed in the brain where it has been implicated in playing a role in the formation and stability of synapses, growth and patterning of neuronal processes, and calcium homeostasis, but by a mechanism that is independent of MuSK. Thus, understanding how agrin mediates these diverse functions in the brain requires identification of the neuronal receptors with which it interacts.

Previously, using fragments of agrin as probes, we demonstrated that agrin binding sites are concentrated at synapses formed between CNS neurons. Here we show that immunoblots of these agrin fragments chemically cross-linked to mouse cortical neurons contain a single reactive band that, by mass spectrometry, consists of a mixture of agrin and the α3 subunit of the Na^+/K^+-ATPase. Consistent with this observation, this agrin–agrin receptor complex can be immunoprecipitated from detergent extracts of agrin cross-linked neurons with either an agrin antiserum or α3 Na^+/K^+-ATPase monoclonal antibody.

Agrin binding inhibits α3 Na^+/K^+-ATPase function. Consistent with the neuron-specific expression of α3 Na^+/K^+-ATPase in brain, fluorescence ratio imaging shows that agrin induces a rapid and reversible increase in intracellular $[Na^+]$ in neurons but not nonneuronal cells. Agrin also triggers rapid and reversible neuron depolarization, similar to the effects of ouabain, a well-characterized Na^+/K^+-ATPase inhibitor. Moreover, bath application of C-Ag15, an agrin fragment that antagonizes agrin's effects, blocks ouabain-induced neuron depolarization; this is direct evidence that agrin binds to and inhibits the α3 Na^+/K^+-ATPase. Agrin's ability to modulate activity of the α3 Na^+/K^+-ATPase suggests a key role in controlling activity-dependent processes in neurons and provides a molecular framework for agrin function in the central nervous system. [Supported by NIH grants NS033213 and NS27501.]

136. Heterologous Expression of Four Different SERCAs in *Saccharomyces cerevisiae* Show Dissimilar Sensitivities to Thapsigargin. HILARY P. KRUSE, PHILLIP D.

WEEKS, DAVID L. WILLIAMS, and CRAIG GATTO, *Division of Biomedical Sciences and Department of Biological Sciences, Illinois State University, Normal, IL* (Sponsor: Jack H. Kaplan)

Recent publications of the high-resolution SERCA structure from Toyoshima's lab have outlined various domains required for activity, including binding sites for ATP, phosphate, Ca ions, as well as the binding site for the inhibitor thapsigargin. We are interested in the evolutionary conservation of the thapsigargin-binding site. To determine the thapsigargin sensitivity of SERCAs from different species, we used yeast genetics to examine several different SERCAs within a common background. Specifically, we expressed a vertebrate, plant, and two parasite SERCAs in a yeast strain devoid of endogenous Ca pumps (i.e., strain K616; Cunningham and Fink. 1996. *Mol. Cell. Bio.* 16:2226–2233). These SERCAs were cloned into the pYES vector (containing *URA*) and transformed into competent K616 cells and selected by growth in the absence of uracil. Protein expression was under the GAL1 promoter and induced by growth in the presence of 2% galactose. The K616 yeast strain is unable to grow in the presence of EGTA due to the inability to supply sufficient Ca to the ER and Golgi. Thus, one can easily determine functional expression of heterologous pumps by complementation of strain K616 on EGTA-containing medium. We found that all four clones were able to rescue K616 in the presence of EGTA. Next, we determined the ability of these four different SERCAs to facilitate complementation in the presence of increasing concentrations of the known SERCA inhibitors, cyclopiazonic acid (CPA) and thapsigargin. Not surprisingly, CPA significantly reduced yeast complementation with an IC_{50} of ~100 μM for all four SERCA pumps. In contrast, we found interesting differences between the sensitivities of these SERCAs toward thapsigargin. Specifically, the vertebrate and one parasite isoform had similar thapsigargin sensitivities with an IC_{50} of ~1 μM, whereas the plant and the other parasitic SERCA were much more resistant to thapsigargin, with only a slight growth reduction at 10 μM thapsigargin. [Supported by USDA-CREES and NSF-CAREER grants to C. Gatto; NIH grant AI061671 to D.L. Williams.]

137. Modulation of the Kinetic Properties of P-type ATPases by Curcumin. YASSER A. MAHMMOUD, *Institute of Physiology and Biophysics, Aarhus University, Aarhus, Denmark*

Curcumin, the major constituent of turmeric, is an important nutraceutical that has been shown to be useful in the treatment of many diseases like cellular transformation and Alzheimers disease. Indeed, curcumin modulates the activity of many cellular proteins. As an inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase, curcumin was suggested to correct cystic fibrosis defects in some model systems (Egan et al. 2004. *Science.* 304:600–602), whereas others have reported no or little effects on cystic fibrosis after curcumin treatment (Song et al. 2004. *J. Biol. Chem.* 279:40629–40633), suggesting that the curcumin effect is complicated and not only due to inhibition of the Ca^{2+}-ATPase. We tested the hypothesis that curcumin may modulate other members of the P_2-type ATPase superfamily by studying the effects of curcumin on the activity and kinetic properties of the Na,K-ATPase. Curcumin treatment inhibited Na,K-ATPase activity in a dose-dependent manner ($K_{0.5} \sim 14.6$ μM). Curcumin decreased the apparent affinity of Na,K-ATPase for K^+ and increased it for Na^+ and ATP. Kinetic analyses indicated that curcumin induces a threefold reduction in the rate of E1P→E2P transition, thereby increasing the steady-state phosphoenzyme level. Curcumin treatment significantly abrogated K^+ occlusion in the enzyme as evidenced from kinetic and proteolytic cleavage experiments. Curcumin also significantly decreased the vanadate sensitivity of the enzyme. Thus, curcumin partially blocks the K^+ occlusion site, and induces a constitutive shift in the conformational equilibrium of the enzyme toward the E1 conformation. The physiological consequences of curcumin treatment previously reported in different epithelial model systems may, at least in part, be related to the direct effects of curcumin on Na,K-ATPase activity. Furthermore, curcumin was found to inhibit the gastric H,K-ATPase, with a $K_{0.5}$ for curcumin inhibition of ~5 μM. Thus, curcumin is a modulator of P-type ATPases (Mahmmoud, Y.A. 2005. *Br. J. Pharmacol.* 145:236–245). [Supported by the Danish Research Academy grant 22–01-0499.]

138. Effect of Extracellular Na^+ and K^+ Concentrations on Ouabain Affinity in Wild-type and Mutant Na,K-ATPases. R. RADKOV, S. KHAROUBI-HESS, N. MODYANOV, K. GEERING, and J.-D. HORISBERGER, *Department of Pharmacology and Toxicology of the University of Lausanne, CH-1005 Lausanne, Switzerland*; *Department of Pharmacology, Medical College of Ohio, Toledo, OH* (Sponsor: J.-D. Horisberger)

Ouabain binding to Na,K-ATPase is known to be favored by the E2 conformation of the protein, and the apparent competitive inhibition between ouabain and extracellular potassium can be explained by the activation by K^+ of the dephosphorylation step and the transition to the E1 states. The role of cation site occupancy

is however not completely understood. Resistance to ouabain in rat α1 isoform is known to be related to the presence of charged residues in the first extracellular loop while the low sensitivity of the related "nongastric" H,K-ATPase (ATP1AL1 in human) must be due to other primary structure differences as this protein does not have charged residues at homologous locations. To understand the role of specific residues that are divergent between Na,K-ATPase and ATP1AL1 and the role of cation site occupancy, we have studied ouabain inhibition in wild-type and mutant Na,K-ATPase proteins in which H,K-ATPase–specific residues were introduced in the Na,K-ATPase sequence (E314K, E319R, P785A, and V927E). We measured the steady-state Na,K-pump K^+-activated current at −50 mV after exposure to ouabain under various ionic conditions. When compared with the control (Na 100 mM, K 10 mM), apparent ouabain affinity was approximately threefold increased in the absence of K^+ in the wild type and all mutants except P785A, for which an increase of ouabain K_I was observed. Removing extracellular Na^+ resulted in a ∼10-fold increase in apparent affinity (in the absence of extracellular K^+) in the wild type and all mutants. Mutant E319R had an approximately twofold lower affinity for ouabain under all conditions, suggesting either a role of this residue in ouabain binding or a strong effect on the E1/E2 equilibrium that remains to be studied. [Supported by the Swiss National Fund grant 31-65441.01 to J.-D. Horisberger and by NIH grant HL36573 to N. Modyanov.]

139. Palytoxin (PTX)-induced Cell Death Cascade in Bovine Aortic Endothelial Cells (BAECs). WILLIAM P. SCHILLING, WILLIAM G. SINKINS, DEBORAH SNYDER, and MARK ESTACION, *Rammelkamp Center for Education and Research, MetroHealth Medical Center, and the Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, OH*

Maitotoxin (MTX) initiates a cell death cascade in BAECs that reflects three sequential changes in plasmalemma permeability. Initially, MTX activates Ca^{2+}-permeable nonselective cation channels that produce an increase the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) as monitored by fura-2. This is followed by the opening of large pores that allow molecules <800 D to enter the cell. Pore formation can be followed by uptake of the vital dye, ethidium bromide (EB). Finally, the cells lyse, not by rupture of the membrane, but rather by the activation of a glycine-sensitive "death channel" that lets large macromolecules such as lactate dehydrogenase (140 kD) or GFP (28 kD) leave the cell. Previous studies have shown that PTX-induced channels are permeable to Ca^{2+}. Thus, the purpose of the present study was to evaluate the effect of PTX on $[Ca^{2+}]_i$ and the downstream mechanisms associated with cell death in BAECs. In extracellular buffer containing 2 mM Ca^{2+}, PTX (3–100 nM) produced a graded increase in $[Ca^{2+}]_i$ that was enhanced by increasing Ca^{2+} to 10 mM, and blocked by removal of extracellular Ca^{2+} or by the presence of ouabain. The PTX-induced increase in $[Ca^{2+}]_i$ could be reversed by addition of ouabain at various times after PTX, demonstrating that the rise in $[Ca^{2+}]_i$ is related to interaction of PTX with the Na^+ pump. PTX also produced a concentration-dependent, biphasic increase in EB uptake that was blocked by ouabain. The first phase of EB uptake reflects activation of the dye-permeable pore. The second phase of EB uptake, which was blocked by glycine (5 mM), was associated with release of GFP and hence reflects cell lysis. Time-lapse videomicroscopy showed that PTX-induced membrane blebbing was primarily associated with the lytic phase. These results demonstrate that PTX initiates a cell death cascade that is similar to that induced by MTX. [Supported by NIH grant HL65323.]

140. Characterization of MCS Factors as Nontoxic, Highly Potent Inhibitors of the Na,K-ATPase and of Other P-type ATPases. FRANZ KEREK, *Max-Planck Institute For Biochemistry, Martinsried, Germany*

Recently identified inorganic macrocyclic carbon suboxide (MCS) factors are reported to inhibit in the nanomolar range the Na,K-ATPase, SR Ca-ATPase, and other P-type ATPases. The initially suggested structure of the MCS factors as macrocyclic carbon suboxide derivatives is revised.

The actual chemical structure of the inhibitory factors was proven through chemical synthesis, and by chromatographic and spectroscopic data coupled with chemical derivatization and mass spectrometry. The previously reported, highly potent inhibition of the Na,K-ATPase activity was widely confirmed with the synthetically prepared MCS factors. Results of recently completed investigations of the mechanism suggest that these novel inhibitory factors bind to the intracellular side of the Na,K-ATPase (Stimac et al., unpublished data).

It was shown further that MCS and ouabain binding are not competitive, and thus MCS factors are not cardiac steroid–like inhibitors of the Na,K-ATPase.

Electrophysiological experiments revealed that MCS factors cause a significant relaxation of vascular smooth muscle by contractions produced by potassium loading or electrical excitation. By systemic administration in rats, MCS produces a linear decrease (of up to 30%) of

the peak isometric tension by striated muscle contraction, reaching a minimum value at ~70–80 min after a single i.m. administration of the substance.

In contrast to their potent enzymatic and physiological activity, the MCS factors manifest a surprisingly low-level toxicity, established "in vitro" with various types of cell cultures. Immune cells (e.g., T-lymphocytes) treated with MCS show a significantly reduced activity without primary manifestation of substance-related toxic phenomena. Results of "in vivo" toxicity studies in mice and rats confirmed the low level systemic toxicity of the MCS factors.

141. Effects of Ouabain on the α2 Isoform of Na,K-ATPase, Electrogenesis, and Contractility of Rat Diaphragm. I.I. KRIVOI,[1] T.M. DRABKINA,[2] A.N. VASILIEV,[1] V.V. KRAVTSOVA,[1] and F. MANDEL,[2] [1]*Department of General Physiology and* [2]*A.A. Ukhtomski Institute of Physiology, St. Petersburg State University, St. Petersburg, 199034, Russia*

The effects of different concentrations of ouabain on skeletal muscle electrogenesis and contractility were studied using isolated neuromuscular rat diaphragm preparations. Resting membrane potentials (V_m) and action potentials (AP) of muscle fibers were recorded using intracellular microelectrodes. In addition, isometric muscle contractions upon direct stimulation were observed. Ouabain concentrations that block the α2 but not the α1 isoform (≤1 μM) of rat Na,K-ATPase, depolarized V_m by 4.3 ± 0.7mV (from −78 to −74 mV, $P < 0.01$) in a dose-dependent manner with $K_{0.5}$ of 131 ± 19 nM. Complete block of α2-Na,K-ATPase activity by 1 μM ouabain decreased AP amplitudes by ~17% and prolonged rise times and half-decay times by up to 20 and 10%, respectively. Such changes would be expected to cause impairment in excitation–contraction coupling. In contrast, 1 μM ouabain increased twitch and tetanic (50/s for 1 s) contraction forces by 5–10%. Furthermore, partial (<10%) α2-Na,K-ATPase blockade by 10 nM ouabain increased twitch tension by 16.4 ± 5.3% ($P < 0.01$). Similar positive inotropy (15.4 ± 4.2%, $P < 0.01$) was observed with 1 nM marinobufagenin a second Na,K-ATPase ligand. Lower ouabain concentrations (1–2 nM) increased contractile force by 10%, without affecting V_m and AP. This suggests that the positive inotropic effects occur via a different mechanism than the changes in V_m and AP. Ouabain did not significantly affect the time course of muscle fatigue (2/s for 5 min or tetanic stimulation 50/s each 10 s for 15 min). In conclusion, complete block of the α2-Na,K-ATPase produces minor changes in muscle electrogenesis but does not impair muscle contractility, consistent with the idea that the α2 isoform is not essential for maintenance of basic ionic homeostasis and membrane potentials in skeletal muscle. On the other hand, the positive inotropic effects of Na,K-ATPase ligands suggest that α2-Na,K-ATPase may play a role in the regulation of skeletal muscle performance, perhaps mediated by circulating endogenous digitalis-like factors. [Supported by RFBR grant 04–04-49535.]

142. Chronic Nicotine Exposure Affects the Ouabain-sensitive (α_2) Isoform of Na,K-ATPase in Rat Diaphragm Muscle. F. MANDEL,[1] T.M. DRABKINA,[1] A.N. VASILIEV,[2] V.V. KRAVTSOVA,[1] I.I. KRIVOI,[2] [1]*A.A. Ukhtomski Institute of Physiology and* [2]*Department of General Physiology, St. Petersburg State University, St. Petersburg, 199034, Russia*

The deleterious effects of cigarette smoking are well known, and numerous studies have investigated the physiological, pharmacological, and behavioral effects of chronic nicotine exposure. Although literally thousands of studies have examined the effects of nicotine on the brain, in contrast, scarcely any recent citations are available concerning the effects of nicotine on skeletal muscle. In particular, the effect of nicotine on Na,K-ATPase isoforms and the related membrane electrogenesis is unknown. Recently, we (Mandel et al., 2005. *Biophys. J.* 68:624a) observed a functional interaction between α2-isoform of Na,K-ATPase and the nicotinic acetylcholine receptor (nAChR) in rat diaphragm muscle that results in hyperpolarization of the resting membrane potential (V_m) due to 100 nM concentrations of nAChR agonists (ACh, nicotine, carbamylcholine). Those results suggest that circulating cholinergic ligands including nicotine might influence α2-pump function and skeletal muscle electrogenesis. The present study examined the effects of chronic nicotine administration on V_m of skeletal muscle fibers and on the activity of the α2-isoform of the Na,K-ATPase. Control and experimental rats (seven per group) were subcutaneously injected twice daily for a period of 2–4 wk with vehicle (0.9% NaCl) ± nicotine (6 mg/kg/d). The rats were killed and the V_m were recorded intracellularly in isolated diaphragm muscle. In experimental rats V_m (−71.6 ± 0.4 mV) was significantly different ($P < 0.01$) from control (−73.7 ± 0.4 mV). Histograms of the distribution of V_m were normal in both cases but shifted from each other. Upon addition of 1 μM ouabain, a concentration that selectively and completely inhibits the α2-isoform of rat Na,K-ATPase, V_m of both groups was depolarized to ~68 mV. Thus, the contribution of the α2-isoform to V_m is 6.0 ± 0.6 mV and 3.2 ± 0.6 mV, in control and experimental rats, respectively.

Furthermore, after α2-pump blockade, the V_m histograms become superimposable, indicating that chronic nicotine exposure decreases the α2-pump contribution to V_m. [Supported by RFBR grant 04–04-49535.]

143. The Effect of Thyroid Hormones on the Calcium-mediated Contractile Responses of the Rat Diaphragm Muscle. KAYODE O. ADENIYI and DEBORAH SAMBO, *University of Papua New Guinea, Papua New Guinea*; *Family Practice at Burpengary Plaza, Burpengary, Australia*

It has been demonstrated that contraction and relaxation in skeletal muscle occurs primarily by means of intracellular Ca^{2+}, and that there is only a minimal dependence on extracellular Ca^{2+} (Takamori et al. 1971. *Arch. Neurol.* 25:535–546). The functional state of the diaphragm is very important for the normal functioning of the respiratory system. Thyroid gland secretes calcitonin, a hormone that is known to have significant influence on intracellular Ca^{2+} store and transport, and on the magnitude of extracellular Ca^{2+} in the body (Parthmore, J.G., and L.J. Deftos. 1978. *J. Clin. Endocrinol. Metab.* 47:184–188). Thyroxine and triiodothyronine are also produced by the thyroid gland, and they are known to be involved in protein metabolism and therefore are of great importance in determining the functional status of most skeletal muscle (Adeniyi et al. 1989. *Am. J. Physiol.* 256:G975–G978). The present study therefore examines the role the thyroid hormones play in the Ca^{2+}-mediated contractility of the diaphragm muscle of Wistar albino rats weighing 150–200 g. The results show that thyroidectomy significantly affected Ca^{2+} flux and contractility of the diaphragm muscle.

144. Thyroid Hormones Differently Regulate the Expression and Function of Ca^{2+}-ATPase in Light and Heavy Sarcoplasmic Reticulum. ANA PAULA ARRUDA, DENISE P. CARVALHO, and LEOPOLDO DE MEIS, *Instituto de Bioquímica Médica, ICB,UFRJ, Rio de Janeiro, Brazil* (Sponsor: Giuseppe Inesi)

The sarcoplasmic reticulum (SR) is composed of two fractions: the heavy fraction, which contains proteins involved in calcium release, and the light fraction, which is enriched in Ca^{2+}-ATPase (SERCA), an enzyme responsible for the Ca^{2+} transport from the cytosol to the lumen of the SR, using ATP hydrolysis. In addition to its role in Ca^{2+} transport, SERCA 1 but not SERCA 2 is also able to hydrolyze ATP without pumping Ca^{2+}, producing more heat per mole of ATP cleaved than the heat released during the ATPase activity coupled with Ca^{2+} transport (de Meis, L. 2001. *J. Biol. Chem.* 276: 25078–25087). Thyroid hormones regulate SERCA expression and function. In red muscles, hyperthyroidism increases SERCA 1 and decreases SERCA 2 expression (Van Hardeveld et al. 1996. *Am. J. Physiol.* 271:C1908–C1919), and this effect was accompanied by an increase in the rate of heat production (Arruda et al. 2003. *Biochem. J.* 375:753–759). We now show the effects of hypo- and hyperthyroidism on the light and heavy SR fractions of red and white skeletal muscle from rabbits. We observed that the expression and the kinetic parameters of SERCA 1 were not altered by hypothyroidism in white muscle. In red muscle, however, low T3 produced a 50% decrease in the SERCA 1 isoform expression, which was accompanied by a 46% decrease of both the ATPase activity and heat production. Interestingly, we observed that the effects of hypothyroidism were restricted to the light SR fraction, while the major differences in hyperthyroidism were found in the heavy SR fraction. We conclude that thyroid hormones regulate SERCA thermogenic activity and also modify the pattern of protein expression in the different regions of SR. This was found to be more pronounced in red than in white skeletal muscle. [Supported by CNPq (A.P. Arruda), FAPERJ, and PRONEX.]

145. Regulation of the Na,K-ATPase Cellular Traffic: Role of ERK1/2 MAP Kinase. ALEXANDER V. CHIBALIN, OLGA KOTOVA, and HIROKI TSUCHIDA, *Section of Integrative Physiology, Department of Surgical Sciences, Karolinska Institutet, SE-171 77 Stockholm, Sweden* (Sponsor: David Gadsby)

Insulin induces translocation of skeletal muscle Na,K-ATPase to the plasma membrane from intracellular stores, subsequently increasing Na,K-ATPase activity. The molecular signaling mechanism by which insulin stimulates Na,K-ATPase activity still remains to be elucidated. We have recently reported that phosphorylation of Na,K-ATPase α-subunit by ERK1/2 is an essential signal for translocation of Na,K-ATPase to the plasma membrane in human skeletal muscle (Al-Khalili et al. 2004. *J. Biol. Chem.* 279:25211–25218) and kidney (Zhong et al. 2004. *Cell. Mol. Life. Sci.* 61:2782–2790). These results indicate that ERK1/2 may serve as a universal trigger of the sodium pump activation in different human tissues. However, whether this phosphorylation triggers the exocytosis of the pump, or reduces the rate of endocytosis, remains to be determined. The aim of this study was to investigate the effects of Na,K-ATPase phosphorylation by ERK1/2 on interaction of the pump with proteins involved in membrane traffic, and clarify the mechanisms of Na,K-ATPase translocation to

the plasma membrane in skeletal muscle. Stimulation of human skeletal muscle cells with 100 nM insulin leads to increased abundance of Na,K-ATPase α_1- and α_2-subunits at the cell surface and phosphorylation of their Thr-Pro sequence motif, specific for ERK1/2. A predicted phosphorylation site of Na,K-ATPase α-subunits by ERK1/2 is Thr81, located in the poly-proline–rich motif -^{80}LTPPPTTPE^{88}-. This amino acid sequence has been shown to be involved in regulation of receptor-mediated endocytosis of Na,K-ATPase. Interaction of Na,K-ATPase α-subunit and clathrin adaptor protein 2 is disturbed in cells treated with insulin, while the interaction is intact in cells pretreated with 20 μM MEK1/2 inhibitor PD98059. In contrast, insulin leads to increased binding of cofilin, an actin-depolymerizing protein, to the α-subunit of Na,K-ATPase. The endocytosis of cell surface–biotinylated Na,K-ATPase was reduced by insulin and the effect was abolished by PD98059. Our findings suggest that insulin-stimulated phosphorylation of Na,K-ATPase α-subunit on a threonine residue by ERK1/2 arrests the formation of an endocytic complex consisting of Na,K-ATPase, adaptor protein 2, and clathrin, thereby preventing Na,K-ATPase endocytosis and leading to increased plasma membrane α-subunit abundance due to constitutive exocytosis. [Supported by grants from Swedish Medical Research Council, the Swedish Heart and Lung Foundation, and the Novo Nordisk Foundation.]

146. Regulation of the Na,K-ATPase by Protein Kinase C in Prostate Epithelial Cells. MARIE J. DURAN, LAUREN D. PARISH, and THOMAS A. PRESSLEY, *Department of Physiology, Texas Tech University HSC, Lubbock, TX*

Human prostate epithelial cells display multiple endogenous forms of the α and β Na,K-ATPase isoforms (e.g., α1, α3, β1, β2, and β3), which make them an interesting model for the study of regulatory diversity by protein kinase C (PKC). In the present study, three human prostate cell lines of varying metastatic potential, PNT-2 (benign), DU-145 (weakly metastatic), and PC-3 (highly metastatic), were challenged with 1 μM phorbol myristate acetate (PMA) for 5 min, and then Na,K-ATPase–mediated transport was measured. While the pump-mediated transport was increased in PNT-2 and DU145 cells (15 and 18% respectively), a decrease in transport (−16%) was observed for PC-3 cells in response to PMA, suggesting a cell context difference in the response to PKC stimulation. The use of different concentrations of PMA revealed that the Na,K-ATPase is inhibited in a dose-dependent manner in PC-3 cells. As expected, inactive analogs of PMA did not induce any alteration in pump-mediated transport. Also, no changes in intracellular ATP and Na^+ concentrations were observed after exposure to phorbol esters. To examine a possible explanation for the differences in response, a careful evaluation of the various PKC isozymes expressed in these three cell lines was performed. Amplification of mRNAs by RT-PCR revealed a unique expression pattern for PKCβ and PKCμ that are present only in metastatic cells. These results suggest that regulation of the Na,K-ATPase by PKC is achieved by interaction of a matrix of PKC isozymes with the Na,K-ATPase isoforms. [Supported by NIH grant RR-19799; Ms. Parish is an HHMI undergraduate fellow.]

147. The Mechanism of Hormonal Regulation in Cells Lacking Ser-18 in the NKA α1. RIAD EFENDIEV and CARLOS H. PEDEMONTE, *College of Pharmacy, University of Houston, Houston, TX*

We have previously demonstrated that the hormonal regulation of Na,K-ATPase, in a proximal tubule cell culture model, is mediated by translocation of Na,K-ATPase molecules between intracellular compartments and the plasma membrane. In opossum kidney cells stably transfected with the rat Na,K-ATPase α1 subunit, hormones like dopamine and angiotensin II either inhibit or stimulate, respectively, the Na,K-ATPase activity. While Ser-18 of the rat Na,K-ATPase α1 is essential for dopamine-induced inhibition of proximal tubule Na,K-ATPase, both Ser-11 and Ser-18 are essential for angiotensin II stimulation of Na,K-ATPase activity. Actually, hormones induce the phosphorylation of these amino acids, and this might be the mechanism that triggers either endocytosis or membrane recruitment of Na,K-ATPase molecules. In contrast to the rat α1, the Na,K-ATPase α1 subunits of human, pig, and mouse cells do not have a Ser-18. We tested the hypothesis that, in these cells, hormones may exert opposite effects on the Na,K-ATPase activity through a common pathway involving the phosphorylation of Ser-11. We observed that in native LLCPK1 cells, which are a model of proximal tubule epithelia of pig origin, dopamine or PMA treatment produced either inhibition or activation, respectively, of the Na,K-ATPase activity. The same results were observed in LLCPK1 cells transfected with rat S18A α1. However, Ser-11 is essential for both inhibition and stimulation of Na,K-ATPase since mutation of this amino acid to alanine precluded any regulation of Na,K-ATPase activity. Treatment of LLCPK1 with either dopamine or phorbol ester produced phosphorylation of α1, and a reduction or increase, respectively, of Na,K-ATPase molecule abundance at the plasma membrane. A model of the molecular mechanism in-

volved in the hormonal regulation of Na,K-ATPase in proximal tubule cells will be discussed.

148. β-Arrestins and Spinophilin Associate with the Na^+,K^+-ATPase and Regulate Na^+,K^+-ATPase Trafficking. TOHRU KIMURA,[1] PATRICK B. ALLEN,[2] ANGUS C. NAIRN,[2] and MICHAEL J. CAPLAN,[1] [1]*Department of Cellular and Molecular Physiology and* [2]*Department of Psychiatry, Yale University School of Medicine, New Haven, CT* (Sponsor: Joseph F. Hoffman)

The Na^+,K^+-ATPase is expressed ubiquitously in virtually all tissues and plays a key role in the maintenance of intracellular electrolyte homeostasis. Renal Na^+,K^+-ATPase activity and trafficking are regulated by several hormones, including dopamine and adrenergic hormones through the action of G protein–coupled receptors (GPCRs). Spinophilin is an ubiquitously expressed protein that appears to play a critical role in regulating signaling through GPCRs. Spinophilin is an actin binding protein and is linked to the actin cytoskeleton. In addition, it serves as a targeting subunit for protein phosphatase I, linking this enzyme to a variety of substrate phosphoproteins. In polarized epithelial cells, spinophilin colocalizes with the Na,K-ATPase at the lateral membranes. β-Arrestins regulate the signaling and trafficking of GPCRs in a multifaceted way. Both β-arrestins and spinophilin interact with activated GPCRs and modulate the duration and magnitude of receptor signaling. Arrestin induces endocytosis and down-regulation of activated receptors, whereas spinophilin antagonizes arrestin binding and prolongs signaling by preventing receptor internalization. We found that both arrestin and spinophilin directly associated with the Na^+,K^+-ATPase and that the association with arrestins was blocked in the presence of spinophilin. In LLC-PK1 cells that stably expressed β-arrestins, the Na^+,K^+-ATPase was localized more intracellularly in comparison to mock-transfected cells or spinophilin-expressing cells. Furthermore, expression of spinophilin appeared to slow the rate of Na^+,K^+-ATPase endocytosis, whereas overexpression of β-arrestins accelerated internalization of the Na^+,K^+-ATPase. The interaction between the Na^+,K^+-ATPase and β-arrestins was increased in the presence of PMA and calyculin A, which activate PKC phosphorylation. In spinophilin knockout mice, the Na^+,K^+-ATPase was found mainly in intracellular compartments in epithelial cells of the choroid plexus. Taken together, it appears that association with β-arrestins and spinophilin may be important modulators of Na^+,K^+-ATPase trafficking. [Supported by NIH grant GM42136 (M.J. Caplan) and MH40899 (A.C. Nairn).]

149. The Last Residue of the Na,K-ATPase Catalytic Subunit Isoform-specific Region Plays a Critical Role in the Isoform-specific Response to Protein Kinase C. SANDRINE V. PIERRE,[1] MARIE-JOSEE DURAN,[2] DEBORAH L. CARR,[2] and THOMAS A. PRESSLEY,[2] [1]*Department of Pharmacology and Therapeutics, Medical College of Ohio, Toledo, OH*; [2]*Department of Physiology, Texas Tech University HSC, Lubbock, TX*

To explain the physiological relevance of subunit diversity in the Na,K-pump, one must look to the regions of structural variability, rather than conservation, for the origin of functional differences. The primary structures of the four isoforms of the α-subunit are nearly identical, with the exception of the amino terminus and the isoform-specific region (ISR) located near the center of the molecule. Earlier work has shown that replacing the rat α1 ISR (K^{489}NPNASEPKHL^{499}) by the α2 sequence (E^{487}REDSPQS-HV^{496}) increases the response of α1 to protein kinase C (PKC) after heterologous expression in opossum kidney cells. This suggests that the ISR plays a key role in isoform-specific regulation. In several cell culture models, PKC regulation of Na,K-ATPase activity occurs by membrane recycling via clathrin-coated vesicles (CCV). The specificity of CCV-mediated trafficking is achieved by adaptor proteins, which recognize specific target sequences within cargo proteins, such as the di-leucine (LL) motif. In the α1 sequence, L^{499} is immediately followed by another L, making it a potential target for adaptor protein binding. Moreover, this site is disrupted by replacement of the α1 ISR with that of α2, providing a potential explanation for the altered regulatory response originally observed. We therefore evaluated the response to PKC of a L499V mutant of α1, and compared it to wild-type α1 and the α1α2α1 chimera after expression in opossum kidney cells. Using pump-mediated $^{86}Rb^+$ uptake as a measure of enzyme function, we found a doubling of the response to kinase activation with phorbol esters in cells expressing the mutant when compared with wild-type α1, comparable to the increase in transport seen with the α1α2α1 chimera. This result suggests that the two leucines spanning the end of the ISR may indeed form a motif critical to the isoform-specific response of the Na,K-pump to PKC activation. [Supported by NIH RR-19799.]

150. Oxygen-induced Regulation of the NA/K ATPase in Dissociated Rat Cerebellar Granule Cells. ANNA BOGDANOVA,[1] IRINA PETRUSHANKO,[1,2] NIKOLAY BOGDANOV,[1,3] ALEXANDER BOLDYREV,[3] and MAX GASSMANN,[1] [1]*Institute of Veterinary Physiology, Vetsuisse Faculty, University of Zurich, Zur-*

ich, Switzerland; [2]*Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia*; [3]*Department of Biochemistry, M.V. Lomonosov Moscow State University, Moscow, Russia*

Our previous studies revealed that the activity of Na/K ATPase in hepatocytes, cardiac myocytes, and erythrocytes is a function of oxygen concentration. The molecular mechanisms of oxygen sensitivity of the Na/K ATPase, however, remain unknown. We have followed oxygen-induced regulation of the Na/K ATPase in dissociated cerebellar granule cells obtained from 9–10-d-old rats. Exposure of the cells to oxygen concentrations that were previously reported to be physiological in rat cerebellum (3–5 kPa) resulted in up-regulation of both transport and hydrolytic function of the enzyme as compared with "hypoxic" (0.5–1 kPa) or hyperoxic (21 kPa) conditions. Notably, increase in activity of the enzyme at 3–5 kPa was not followed by changes in cellular ATP levels. Oxygen-induced responses could be observed already after 7 min of equilibration at predefined pO_2 and became more pronounced after 30–60 min of incubation. Activation of the Na/K ATPase observed at 3–5 kPa was followed by a characteristic pattern of free radical production after 30–60 min of treatment. Furthermore, increase in transport activity of the enzyme could be completely inhibited by treating the cells with the NO synthase inhibitor L-NAME. Incubation of cells under hyperoxia (21 kPa) for 30–60 min resulted in a gradual increase in free radial production, depletion of reduced glutathione stores, suppression of transport activity of the Na/K ATPase, and a consequent decrease in cell viability. Prolonged hypoxic exposure (0.5–1 kPa) was associated with cell survival, did not affect cellular redox state, and caused minor decreases in ATP levels. Na/K ATPase activity remained low over 60 min of incubation under hypoxic conditions. Our observations demonstrate that activity of the Na/K ATPase in rat cerebellar granule cells crucially depends on the oxygen concentration. Furthermore, our data suggest that free radical formation and NO synthases are involved in oxygen-induced regulation of the Na/K ATPase function.

151. Na/K-pump Decreases the Level of Reactive Oxygen Species in Neuronal Cells, Protecting Brain Against Oxidative Stress. ALEXANDER A. BOLDYREV, *Center for Molecular Medicine and International Biotechnological Center* (*Department of Biochemistry*), *M.V. Lomonosov Moscow State University, Moscow, Russia*

Brain Na/K-ATPase is more sensitive to reactive oxygen species (ROS) than kidney Na/K-ATPase. ROS treatment results in oxidation of SH-groups of Na/K-ATPase and their reduction results in proportional restoration of enzyme activity. Oxidative modification results also in modification of kinetic properties of the enzyme. After partial oxidation of SH-groups of the enzyme by free radicals, the complex (non-Michaelis) kinetics of ATP hydrolysis that is characteristic of native enzyme is replaced by a nearly Michaelis dependence with negative cooperativity for ATP, which points out the participation of some SH-groups in the formation of functional oligomers. Differences in sensitivity to oxidative attack of Na/K-ATPase from various tissues are based on the amount and localization of sulfhydryls in the α1, α2, and α3 isoforms of the enzyme. We have used flow cytometry to measure ROS levels in isolated cerebellar neurons incubated with agonists of the different glutamate receptors. Activation of NMDA-type glutamate receptors was found to stimulate intracellular ROS production and inhibit α(2+3) Na/K-ATPase isoforms. Stimulation of metabotropic (group II+III) glutamate receptors enhanced the toxic effect of NMDA, whereas stimulation of metabotropic group I receptors both prevented the increase in ROS levels and protected the Na/K-ATPase of the neurons, which then survived. At the same time, application of ouabain contributed to generation of ROS following inhibition of α2+α3 (ouabain-sensitive) isoforms. Analysis of combined effects of ouabain and NMDA-receptor antagonists suggested that α(2+3) isoforms (preferably in E1 conformation) might suppress ROS production derived from NMDA receptor activation, and that calcium ions from both extracellular space and endoplasmic reticulum were responsible for the excitotoxic action of NMDA. Using specific membrane-permeable inhibitors of the signal transduction pathway, the following chain of regulatory reactions was demonstrated: NMDA-R → Ca^{2+}_{in} → Pk-C → NADPH oxidase → ROS → MAP kinase, with ouabain-sensitive Na/K pumps being responsible for protecting neurons from the excitotoxic effect of NMDA receptor activation. [Supported by RF grant 1760.2003.4.]

152. Search for Intermediates of Na^+,K^+-ATPase-mediated $[Na^+]_i/[K^+]_i$-independent Death Signaling Triggered by Cardiotonic Steroids. OLGA A. AKIMOVA,[1,2] OLGA D. LOPINA,[2] PAVEL HAMET,[1] and SERGEI N. ORLOV,[1,2] [1]*Centre de Recherche, Centre Hospitalier de l'Université de Montréal* (*CHUM-Hôtel-Dieu*), *Montreal, PQ, Canada*; [2]*Faculty of Biology, M.V. Lomonosov Moscow State University, Moscow, Russia* (Sponsor: David Gadsby)

Previously, we reported that ouabain and other cardiotonic steroids (CTS) kill renal epithelial and vascular

endothelial cells via their interaction with the Na^+,K^+-ATPase α-subunit, but independently of elevation of the $[Na^+]_i/[K^+]_i$ ratio. In distinct cell types, side-by-side with inhibition of Na^+,K^+-ATPase–mediated ion fluxes, CTS trigger $[Ca^{2+}]_i$ oscillation, activation of Ras, mitogen-activated protein kinases (MAPK), phosphoinositide-3 kinase (PI3K), and protein kinase C, as well as the production of reactive oxygen species and cytoskeleton reorganization. In the first part of this study we examined the potential involvement of the above-listed intermediates in death signaling triggered by ouabain in epithelial cells (C7-MDCK). We did not observe any effect of extra- (EGTA) and intracellular (BAPTA) Ca^{2+} chelators, $[Ca^{2+}]_i$-raising compounds (thapsigargin and ATP), inhibitors of Ras signaling (α-hydroxyfarnesyl-sulphosphoric acid), or of PI3K (wortmannin), or of MAPK ERK1/2 kinase (PD98059), or of tyrosine kinases (genistein), nor any effect of activators (4β-PMA and forskolin) or inhibitors (calphostin) of serine-threonine kinases on the death of ouabain-treated cells. Ouabain did not affect cellular redox state and did not produce superoxide anion or hydroperoxide. Neither N-acetylcysteine nor reduced glutathione suppressed the death of ouabain-treated cells. Thus, our results show that none of the above-listed signaling systems plays a major role in the development of Na^+_i,K^+_i-independent death machinery triggered by CTS interaction with the Na^+,K^+-ATPase α-subunit. In the second part, we studied proteins that interact with Na,K-ATPase and so may be involved in the signaling that leads to ouabain-induced cell death. Immunoprecipitation, followed by separation of proteins by 2D-PAGE, revealed proteins with molecular weights 70, 64, 55, 40, and 34–35 kD, that interacted with Na,K-ATPase in the presence of 3 μM ouabain. The role of these proteins in CTS-induced cell death signaling is currently under investigation. [Supported by the Canadian Institutes for Health Research and the Kidney Foundation of Canada.]

153. Different Action of Ouabain and Digoxin at Low Concentration on Sodium Pump. SILVANA BALZAN, GIUSEPPINA D'URSO, GIUSEPPINA NICOLINI, FRANCESCA FORINI, and UMBERTO MONTALI, *CNR Institute of Clinical Physiology, Department of Human and Environmental Sciences, University of Pisa, Pisa, Italy*

The classic cellular action of cardiac glycosides is the inhibition of the cation active transport mediated by the Na^+/K^+ATPase. Nevertheless, some studies suggest that very low ouabain concentrations can stimulate the activity of the sodium pump in isolated cardiac myocytes, involving the α_2 and α_3 isoforms (Gao et al. 2003. *J. Gen. Physiol.* 119:297–312).

In this study we investigate the effect of ouabain and digoxin on the Na^+/K^+ATPase activity in human erythrocytes, which represent a classical model for membrane studies. The activity of the sodium pump was measured via the ouabain-sensitive ^{86}Rb uptake, using drug concentrations ranging from 10^{-11} M to 10^{-4} M. Low ouabain concentrations, $<10^{-9}$ M, significantly stimulated ^{86}Rb uptake, and the maximal increase of Rb transport above baseline values was 18 ± 5% (mean ± SEM, $n = 13$, $P < 0.01$) at 0.1 nM ouabain. No stimulation by digoxin was observed under the same conditions. Analysis of expression of the α isoforms of the sodium pump, by immunoblotting, revealed the presence of both α_1 and α_3 isoforms.

The sodium pump activation by ouabain is in agreement with the previous results and might be correlated with the presence of the α_3 isoform. On the other hand, the different behavior of digoxin indicates that ouabain (but not digoxin) might interact with a membrane site near, or in close association with, the Na^+/K^+ATPase, and might thereby positively modulate the sodium pump. Moreover, this observation suggests a novel important role for an endogenous factor, similar or identical to ouabain, detected at low concentrations in plasma (Hamlyn et al. 1991. *Proc. Natl. Acad. Sci. USA.* 88:6259–6263).

154. Human RBC Na,K-ATPase Is Inhibited by Prophylactic γ-Irradiation. LEANDRO A.O. BARBOSA, OTACILIO C. MOREIRA, VANESSA H. OLIVEIRA, JULIO A. MIGNACO, CARMEN M. NOGUEIRA, ANTONIO A.C. ARAÚJO, and CARLOS F.L. FONTES, *Instituto de Bioquímica Médica, UFRJ, Rio de Janeiro, Brazil*

Blood transfusion can cause graft vs. host disease. To prevent that, it is customary to irradiate blood products before transfusion, but storage of irradiated blood can result in some damage to red blood cells (RBC). In this work we investigate if two plasma membrane enzymes (Na,K-ATPase and plasma membrane Ca-ATPase [PMCA]) could be modified by γ-radiation.

Blood was collected in AS-1 bags and RBC were separated from platelets and plasma by centrifugation and then treated with γ-radiation (25 Gy). The plasma ion levels were measured by flame photometry, and the LDH levels were measured by clinical laboratory protocols. Na,K-ATPase and PMCA activities were measured by hydrolysis of [γ-^{32}P]ATP in RBC ghosts. The RBC permeability was assessed through $^{45}Ca^{2+}$ uptake by inside-out vesicles (IOV).

Samples were collected 1, 7, 14, and 28 d after irradiation treatment. As a control, nonirradiated blood was used. No difference in plasma level of LDH, pH, or Ca

was found between control and irradiated blood. However, the plasma K concentration was progressively increased with storage time, from 4.45 ± 0.15 to 38.35 ± 1.35 mmol/liter (control), and from 8.1 ± 1.8 to 56.5 ± 5.15 mmol/liter (irradiated). A corresponding decrease of plasma Na was observed, from 147.5 ± 1.5 to 129 ± 4.0 mmol/liter (control), and from 145.5 ± 0.5 to 109 ± 0.1 mmol/liter (irradiated). There was no significant difference in PMCA activity. On the other hand, the Na,K-ATPase activity was strongly inhibited by radiation, by 14 ± 3.1% on day 1, 51.6 ± 7.3% on day 14, and 43.4 ± 5.9% on day 28. Although PMCA activity was unchanged, Ca uptake was analyzed. Surprisingly, the Ca content in IOV from irradiated samples was about threefold lower than from control samples, suggesting a substantial damage to the plasma membrane of irradiated RBC. Our results suggest a dual effect of γ-radiation on RBC: an inhibition of the Na,K-ATPase, and an effect on the plasma membrane that leads to an increase in passive ion permeability.

155. Na,K-ATPase–mediated Cell-Substratum Attachment Involves FAK and Src Signaling. SONALI P. BARWE, SIGRID A. RAJASEKARAN, and AYYAPPAN K. RAJASEKARAN, *Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA* (Sponsor: Ayyappan K. Rajasekaran)

Na,K-ATPase is a ubiquitous membrane-bound enzyme consisting of two essential noncovalently linked subunits, a 110-kD α-subunit (Na,K-α) and a 55-kD β-subunit (Na,K-β). Na,K-ATPase pump activity catalyzes an ATP-dependent transport of three sodium ions out of, and two potassium ions into, the cell per pump cycle, thereby generating transmembrane sodium and potassium gradients. The Na,K-α is the catalytic subunit, whereas the Na,K-β plays a regulatory role and is required for the maturation of Na,K-α. Recently, we showed that Na,K-β is involved in the suppression of cell motility in carcinoma cells by a novel mechanism involving a cross talk between the two subunits of Na,K-ATPase and the members of the PI3-kinase signaling pathway. We found that Na,K-ATPase is localized to the lamellipodia, an actin-rich microdomain generally found in cells with increased cell motility. In this study, we present evidence that decreased cell motility of Na,K-β–expressing cells is due to increased attachment of these cells to the substratum. Furthermore, we found that activation of focal adhesion kinase (FAK) by Src is involved in the increased attachment of Na,K-β–expressing cells to the substratum. These results reveal a novel role for Na,K-β in cell–substratum attachment in epithelial cells and suggest that decreased expression of this protein in carcinoma cells is associated with increased cell motility. [Supported by NIH grant DK56216.]

156. The Na,K-ATPase α4 Isoform from Humans Has Distinct Enzymatic Properties and Is Important for Sperm Motility. GLADIS SANCHEZ, BRADY TIMMERBERG, JOSEPH S. TASH, and GUSTAVO BLANCO, *Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, KS 66160*

Among the various α isoforms that constitute the Na,K-ATPase, α4 shows the most restricted pattern of expression. The isoform is present in testis, where it is abundant in male germ cells. In the rat, α4 exhibits enzymatic properties that are different from those of the other Na,K-ATPase α polypeptides. In addition, activity of α4 is important for rat sperm motility (Woo et al. 2000. *J. Biol. Chem.* 275:20693–20699). At present, the function of α4 in species other than rat is unknown. We explored its role in human spermatozoa. Similar to the rat, we found that human male gametes express not only α4, but also the α1, β1, and β3 isoforms. Ouabain inhibition curves of Na,K-ATPase activity in sperm homogenates, showed a biphasic response, supporting the expression of two functional α polypeptides in the cells. To determine the kinetic characteristics of the human α4 isoform in more detail, we used the baculovirus expression system. Expression of α4 in combination with the β1 and β3 subunits in Sf-9 cells resulted in active enzymes with similar kinetics. However, α4β1 and α4β3 differed from the human α1β1 isozyme, exhibiting higher affinity for Na^+, lower affinity for K^+, and higher sensitivity to ouabain. The difference in ouabain affinity between the two α isoforms allowed us to explore the role of α4 in sperm motility. Using computer-assisted sperm motility analysis, ouabain concentrations of 10^{-8} M that inhibit only α4 significantly decreased percent motility, but did not affect sperm straight line, curvilinear, or average path velocity. This suggests that α4 is involved in the on–off mechanisms of flagellar motility rather than in regulation of sperm velocity. Altogether, our results suggest that, similar to the rat orthologue, the human Na,K-ATPase α4 isoform has distinct enzymatic properties, which are important for sperm physiology. [Supported by NIH grant HD043044-01.]

157. Ouabain Stimulates Glycogen Synthesis in Skeletal Muscle via ERK1/2 and c-Src–dependent

Mechanisms. OLGA KOTOVA, DANA GALUSKA, SARA TALIA, CATHERINE HOOK, and ALEXANDER V. CHIBALIN, *Section of Integrative Physiology, Department of Surgical Sciences, Karolinska Institutet, SE-171 77 Stockholm, Sweden* (Sponsor: David Gadsby)

Ouabain is a cardiac glycoside that specifically binds to Na,K-ATPase and inhibits its activity. Recent evidence indicates that in addition to ion transport function, Na,K-ATPase can sense low concentrations of ouabain and play an important role as a signal transducer. Moreover, ouabain has been described as a member of the new class of steroid hormones, endogenously produced in mammalian adrenal glands. The aim of this study was to investigate the effects of ouabain on glucose metabolism in rat soleus muscle and human skeletal muscles cells (HSMC), and to clarify the mechanisms of ouabain signal transduction. Soleus muscles from male Wistar rats were incubated ex vivo to assess glycogen synthesis and glucose oxidation with and without 200 μM and 2 mM ouabain, in basal and insulin-stimulated (12 nM) conditions in the presence or absence of the MEK1/2 inhibitor PD98059 or the c-Src inhibitor PP2. Ouabain stimulated glycogen synthesis at both concentrations. This effect was additive to that of insulin and abolished in the presence of PD98059 or PP2. Similarly, in HSMC, 100 nM ouabain increased glycogen synthesis. In isolated soleus muscle, ouabain exposure reduced glucose oxidation, and this effect was reversed in the presence of PP2. Ouabain treatment resulted in increased interaction of α_1- and α_2-subunits of Na^+,K^+-ATPase with c-Src, as assessed by coimmunoprecipitation with c-Src. Phosphorylation of ERK1/2, p90rsk, and GSK 3α/β was increased in response to ouabain in both HSMC and isolated soleus muscle, and this effect was prevented in the presence of PD98059 and PP2. Ouabain also stimulated p90rsk activity. Incubation of HSMC with 100 nM ouabain increased phosphorylation of the α-subunit of Na-pump on a MAP kinase–specific Thr-Pro motif; however, the abundance of α-subunits on the cell surface was not affected. In conclusion, our studies indicate that in skeletal muscle, the cardiac glycoside ouabain mediates increased glycogen synthesis, additively to insulin. This increase in glycogen synthesis is mediated by activation of c-Src-, ERK1/2-, p90rsk-, and GSK3-dependent signaling pathway. [Supported by grants from Swedish Medical Research Council, the Swedish Heart and Lung Foundation, and the Novo Nordisk Foundation.]

158. Signaling Along the P→A Mechanism Involves Changes in Phosphorylation State and Localization of Cell Adhesion Proteins. DAVID FLORES-BENÍTEZ, CATALINA FLORES-MALDONADO, ISABEL LARRÉ, FABIÁN GARCÍA-SANTÍN, LIORA SHOSHANI, MARCELINO CEREIJIDO, and RUBÉN G. CONTRERAS, *Department of Physiology, Biophysics, and Neurosciences, Center for Research and Advanced Studies, Mexico City, Mexico*

In a previous work, we described a P→A mechanism that transduces ouabain binding to the Na^+,K^+-ATPase (P = pump) into changes in phosphorylation and retrieval of cell–cell adhesion and cell–substrate attaching (A) molecules, causing a release of the cell from the monolayer. It also releases β-catenin from the submembrane scaffold and readdresses it to the nucleus. P→A involves activation of protein tyrosine kinases (PTKs) and extracellularly regulated kinases (ERK1/2), as well as an increase in cell content of $p190^{Rho\text{-}GAP}$. Here we investigate primarily the order in which the cascade proceeds, until it reaches cell adhesion molecules, modifies their phosphorylation status, and retrieves them from the plasma membrane. We observe that one PTK activated by ouabain is c-Src, whose inhibition with PP2 prevents cell detachment. The activation of PTKs is necessary for the phosphorylation of ERK1/2, a step that can be blocked by genistein. Phosphorylated ERK1/2 are in turn necessary for RhoA cell content decrease; this last effect can be efficiently prevented by the ERK inhibitor UO126. Tracking the effect of ouabain from the Na^+,K^+-ATPase to cell detachment, we observe that inhibition of c-Src or ERK1/2 prevents retrieval of occludin, β-catenin, and focal adhesion kinase (FAK), which are conspicuous members of tight junctions, adherens junctions, and focal adhesions, respectively. Retrieval of β-catenin appears to be associated with an increase of its tyrosine phosphorylation that is blocked by PD98059, indicating the participation of ERK1/2. FAK instead is dephosphorylated on tyrosine 397, a process in which c-Src is necessary. The P→A mechanism operates through a chain of reactions that involves, sequentially, ouabain binding to the Na^+,K^+-ATPase, activation of c-Src, activation of ERK1/2, and both inhibition and cell content decrease of RhoA. Ouabain induces changes in phosphorylation and causes retrieval of cell adhesion molecules.

159. Cloning and Expression of Na,K-ATPase Isoforms from the Euryhaline Bullshark, *Carcharhinus leucas*. LARA MEISCHKE and GORDON CRAMB, *School of Biology, University of St. Andrews, St. Andrews, Fife, KY16 9TS, UK* (Sponsor: David Gadsby)

α and β subunit isoforms of the Na,K-ATPase were cloned from the euryhaline bullshark (*Carcharhinus leucas*) and their tissue-specific expression characterized in both freshwater (FW) and sea water (SW) acclimated

sharks. The β subunit isoform encodes a 305–amino acid protein of 35.2 kD, although Western blot analyses reveal the presence of several species ranging from 45–55 kD, indicative of variable states of glycosylation. The protein exhibits 61, 32, and 30% amino acid identity with human β1, β2, and β3 isoforms, respectively, suggesting that this shark gene is the paralogue of mammalian β1 isoforms. The α isoforms (initially designated A, B, and C) encode proteins ranging from 1,118 to 1,025 amino acids. Western blot analysis using an A isoform–specific antibody detected a single protein migrating at 95 kD on SDS-PAGE. The α subunit isoforms exhibit the following amino acid identities to human α1, α2, α3 and α4 isoforms, respectively: A isoform, 88.1, 84.6, 84.7, and 79.1%; B isoform, 87.6, 88.1, 86.3, and 79.3%; C isoform, 83.7, 78.6, 88.4, and 72.2%. These homologies suggest that the A isoform is the paralogue of α1, the B isoform the paralogue of α2, and the C isoform the paralogue of the α3 mammalian isoforms. Northern blotting and RT-PCR indicate that all isoforms are expressed in the gill, gut, kidney, and rectal gland. Although SW acclimation has been reported to decrease Na, K-ATPase activity in the kidney and increase activity in the rectal gland, there was no significant change in the abundance of mRNA for any isoform nor any change in the α1/α-A or β(1) subunit protein expression following transfer of fish to SW. These results suggests that the reported tissue-specific changes in enzyme activity following FW/SW transfer are not associated with either the transcriptional regulation of any of the Na,K-ATPase α or β isoforms nor changes in α1 or β1 protein abundance. [Supported by NERC grant NER/A/S/2000/01270.]

160. Hypoxia-mediated Na,K-ATPase Endocytosis Is Regulated by the Actin Cytoskeleton and the Small RhoA GTPase. LAURA A. DADA, EVA NOVOA, EMILIA LECUONA, LYNN WELCH, and JACOB I. SZNAJDER, *Pulmonary and Critical Division, Feinberg School of Medicine, Northwestern University, Chicago, IL* (Sponsor: Jack Kaplan)

During ascent to high altitude, or pulmonary edema, alveolar epithelial cells (AEC) are exposed to low oxygen concentrations. Hypoxia inhibits alveolar fluid reabsorption and Na,K-ATPase activity in AEC by triggering its endocytosis. This process increases mitochondria-generated reactive oxygen species (ROS) and serine phosphorylation of the α1 subunit through PKC-zeta. Recent evidence indicates a causal relationship between the endocytic process and the organization of the actin cytoskeleton. Here, we sought to investigate the role of the actin cytoskeleton and the RhoA GTPase-mediated signaling pathway in the hypoxia-mediated Na,K-ATPase endocytosis. To determine whether actin organization is required for the internalization of Na,K-ATPase, we applied lantrunculin B and phallacidin, which induce actin cytoskeleton depolymerization and stabilization, respectively. The Na,K-ATPase endocytosis was assessed by measuring Na,K-ATPase abundance at the plasma membrane by biotin labeling. Preincubation with lantrunculin B and phallacidin prevented the hypoxia-mediated Na,K-ATPase internalization. Rho GTPases regulate actin dynamics and they play a role in vesicular trafficking. Hypoxia led to RhoA activation, and Na,K-ATPase endocytosis could be prevented by expressing a dominant negative form of RhoA. The hypoxia-mediated Na,K-ATPase endocytosis was regulated by the Rho-associated kinase (ROCK), as preincubation with the specific inhibitor Y-27632, or transfection with dominant negative ROCK, prevented the hypoxia-induced endocytosis. During hypoxia, the mitochondrial electron transport is partially inhibited, causing redox changes in the electron carriers that result in the generation of superoxide anions and hydrogen peroxide (H_2O_2), which can act as second messengers upon entering the cytosol. The effect of hypoxia on RhoA activation was mimicked by exogenous H_2O_2. Also, AEC transfected with RNAi against Rieske iron sulfur protein (a member of the mitochondrial complex III) blocked the hypoxia-mediated RhoA translocation. Accordingly, we provide evidence that the actin cytoskeleton and RhoA are necessary for the internalization process during hypoxia–ROS-mediated Na,K-ATPase endocytosis. [Supported by NHLBI PO1HL071643.]

161. Na^+ Pump α1 and α2 Isoform Targeting of Ca^{2+} Reporter Protein Reveals Spatially Distinct Sub-plasma Membrane Ca^{2+} Signals. MOO YEOL LEE,[1] HONG SONG,[1] JUNICHI NAKAI,[2] MASAMICHI OHKURA,[3] MICHAEL I. KOTLIKOFF,[4] and MORDECAI P. BLAUSTEIN,[1] [1]*Department of Physiology, University of Maryland School of Medicine, Baltimore, MD*; [2]*Laboratory for Memory and Learning, RIKEN Brain Science Institute, Wako-shi, Saitama 351-0198, Japan*; [3]*First Department of Pharmacology, School of Pharmaceutical Sciences, Kyushu University of Health and Welfare, Yoshino, Nobeoka, Miyazaki 882-8508, Japan*; [4]*Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY*

PLasmERosomes are Ca^{2+} transport/signaling complexes that consist of certain plasma membrane (PM) microdomains, the subjacent "junctional" sarco-/endoplasmic reticulum (jS/ER), and the tiny volume of cyto-

sol in the junctional space (JS) between them. Some ion transporters such as Na^+ pumps with α2 or α3 subunits, Na/Ca exchangers, and store-operated Ca^{2+} channels (SOCs) are confined to PM microdomains that overlie jS/ER. In contrast, Na^+ pumps with α1 subunits and the PM Ca^{2+} pump are more uniformly distributed in the PM, but may be excluded from the junctional microdomains. Accordingly, we propose that PLasmERosomes are specialized for the local trafficking of Ca^{2+} between the extracellular fluid and the S/ER lumen without direct diffusion of Ca^{2+} into the bulk cytosol. Hence, we postulate that the local Ca^{2+} concentration in the JS ($[Ca^{2+}]_{JS}$) of PLasmERosomes may be regulated differently from the Ca^{2+} concentration in bulk cytosol ($[Ca^{2+}]_{CYT}$).

To test our hypothesis, we employed G-CaMP2, an improved version of the Ca^{2+} sensor, G-CaMP (Nakai et al. 2001. *Nat. Biotechnol.* 19:137–141), for the measurement of $[Ca^{2+}]_{JS}$ and $[Ca^{2+}]_{CYT}$. Our strategy was to construct fusion proteins to target G-CaMP2 to different cytosolic domains: bulk cytosol, the "nonspecific" near-PM space, and the PLasmERosome JS. In the latter two cases, we took advantage of the differential distribution of α1 and α2 subunits of Na^+ pumps. This report describes the construction of α1(f)G-CaMP2 and α2(f)G-CaMP2 fusion proteins and the signals obtained from these two chimeras when they are expressed in arterial smooth muscle cells. Preliminary results indicate that α2(f)G-CaMP2 reports Ca^{2+} signals related to SOCs, whereas α1(f)G-CaMP2 reports agonist-evoked Ca^{2+} signals; fura-2 reports both types of signals. Our results support the hypothesis that $[Ca^{2+}]_{JS}$ is regulated differently from bulk cytosolic Ca^{2+}, and are direct evidence for localized Ca^{2+} signaling in the JS where Na^+ pumps with α2 subunits reside. [Supported by NIH grants HL-45125 and NS-16106.]

162. The Role of Endosomal Na^+,K^+-ATPase in the Regulation of Endocytosed Membrane Traffic. DAVID LICHTSTEIN,[1] TOMER FELDMANN,[2] VALDIMIR GLUCKMANN,[2] ELLA FRIEDMAN,[2] URI SHPOLANSKY,[1] and HAIM ROSEN,[2] [1]*Department of Physiology and* [2]*The Kuvin Center for the Study of Infectious and Tropical Diseases, Institute of Microbiology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel*

The plasma membrane Na^+,K^+-ATPase is a key player in ion homeostasis and numerous consequent processes. This enzyme is specifically inhibited by a family of compounds known as cardiac steroids (CS). We recently demonstrated that CS, by interacting with the Na^+,K^+-ATPase induce changes in the recycling of the plasma membrane in human NT2 cells (Rosen et al. 2004. *Mol. Biol. Cell.* 15:1044–1054). The goal of the present study was to elucidate the molecular mechanisms involved in this phenomenon. Using fluorescent microscopy, immunocytochemistry, in vivo pH measurements and ion transport assays, we show that (1) CS and monensin induce identical and synergistic changes in endocytosed membrane traffic; (2) the inhibition of plasma membrane Na^+,K^+-ATPase activity by lowering extracellular K^+ does not induce these changes; (3) similarly, the commonly used steroid ouabain, unlike digoxin or bufalin, fails to induce these changes and even antagonizes those induced by other CS; (4) ouabain and bufalin differentially affect endosomal acidification; (5) endosomal acidification is required but not sufficient for CS-induced changes in membrane traffic; (6) CS cause the retention of transferrin in the early endosome. Collectively, these results provide evidence for the participation of endosomal Na^+,K^+-ATPase in the regulation of endosomal pH and consequently in intracellular endocytosed membrane traffic. We propose that the endosomal pH, which determines the stability of endocytosed ligand–receptor complexes and the route of the endocytosed membrane, is established by H^+-ATPase activity but is finely tuned by Na^+,K^+-ATPase activity. Upon transfer to the early endosome, the endocytosed Na^+,K^+-ATPase–CS complex leads to reduced Na^+,K^+-ATPase activity, increased acidification, and retention of cargo in this compartment. This study establishes a novel physiological role for intracellular Na^+,K^+-ATPase activity. [Supported by the Israel Science Foundation grant 269/04.]

163. Signaling Pathways Involving the Sodium Pump and Insulin Receptor Stimulate NO Production in Endothelial Cells. ALEXANDER EVA and GEORGIOS SCHEINER-BOBIS, *Institut für Biochemie und Endokrinologie, Fachbereich Veterinärmedizin, Justus-Liebig-Universität Giessen, Giessen, Germany*

The cardiac steroid ouabain, a known inhibitor of the sodium pump or Na,K-ATPase, has been shown to release endothelin from endothelial cells when used at concentrations below those that inhibit the pump. The present study addresses the question of which signaling pathways are activated by ouabain in endothelial cells.

Our findings indicate that ouabain, applied at low concentrations to human umbilical artery endothelial cells (HUAECs), induces a reaction cascade that leads to translocation of endothelial nitric oxide synthase (eNOS) and to activation of phosphatidylinositol 3-kinase (PI3K). These events are followed by phosphorylation of Akt (also known as protein kinase B, or

PKB) and activation of eNOS by phosphorylation. This signaling pathway, which results in increased nitric oxide (NO) production in HUAECs, is inhibited by the PI3K-specific inhibitor LY294002. Activation of the reaction cascade is probably not due to endothelin-1 (ET-1) binding to the ET-1 receptor B (ET_B), since application of the ET_B -specific antagonist BQ-788 did not have any effect on Akt or eNOS phosphorylation.

The sodium pump appears to be in close contact with the insulin receptor (IR), as demonstrated in coimmunoprecipitation experiments. This suggests that ouabain leads to IR activation upon its binding to the sodium pump. This results in the activation of the proliferation and survival pathways involving Akt activation, which then stimulates eNOS and NO production in HUAECs. [Supported in part through DFG, Sche 307/5-2.]

164. Digitalis-induced Growth Arrest in Human Breast Cancer Cells: On the Importance and Mechanism of Amplification of Digitalis Signal through Na/K-ATPase. LIJUN LIU and AMIR ASKARI, *Department of Pharmacology, Medical College of Ohio, Toledo, OH*

Therapeutic concentrations of digitalis drugs inhibit the proliferation of MDA-MB-435s cells by inducing the interaction of Na/K-ATPase with Src/EGFR, activation of ERK1/2, and the resulting up-regulation of cell cycle inhibitor $p21^{Cip1}$ (Kometiani et al. 2005. *Mol. Pharmacol.* 67:929–936). Extending this work, we made a quantitative comparison of ouabain dose–response curves for growth arrest and pump inhibition (active $^{86}Rb^+$ uptake) in intact cells. We found that the ratio of Ki (pump)/Ki (proliferation) = 7.2. Such large gains in sensitivity are characteristic of several signal transducing pathways of other receptors. Making the reasonable assumption that Na/K-ATPase is the only receptor for ouabain, the large amplification factor clearly shows that occupation of a small fraction of pumping Na/K-ATPase (say <10%) by digitalis drugs, or endogenous digitalis-like factors, is sufficient to cause a large inhibition (say >80%) of cell growth. Based on the known properties of Na/K-ATPase, and by analogy to the known mechanisms of sensitivity gain in other receptor systems, the likely causes of the large amplification factor in the signaling function of Na/K-ATPase include (a) interactions among multiple α,β-protomers of the functional Na/K-ATPase in the plasma membrane, and (b) induced clustering of Na,K-ATPase oligomers with neighboring proteins such as Src, EGFR, and caveolins. While the relative contributions of these mechanisms to the amplification of digitalis signal remain to be explored, the upstream location of both mechanisms suggests that similar amplifications also occur in other cell types with different digitalis downstream effects, e.g., stimulation of proliferation or hypertrophy. [Supported by NIH grant HL36573.]

165. The Influence of Ouabain on Capacitative Calcium Entry in C6 Cells. N.A. DELAMERE,[1,2] Y. HOU,[1] and A.K. HARTFORD,[2,3] [1]*Department of Pharmacology and Toxicology and* [2]*Department of Ophthalmology and Visual Sciences, University of Louisville, Louisville, KY;* [3]*Molecular Medicine Laboratory, Royal College of Surgeons in Ireland, Dublin, Ireland*

In previous studies ouabain was shown to increase capacitative calcium entry (CCE) in cultured rat and mouse astrocytes (Hartford et al. 2004. *Glia.* 45:229–237). Astrocytes derived from knockout mice that lack the Na,K-ATPase α2 isoform did not respond to ouabain, suggesting that interaction between ouabain and Na,K-ATPase α2 may be necessary for ouabain to stimulate CCE. Here, we examined the response to ouabain in C6 cells, a rat glioma cell line. Western blot analysis of C6 cells revealed abundant Na,K-ATPase α1. In contrast, Na,K-ATPase α2 and α3 were undetectable. The cells were exposed to ouabain at a concentration of 1 μM. Previous studies have shown that 1 μM ouabain concentration is not sufficient to inhibit activity of the rat Na,K-ATPase α1 isoform. Cytosolic calcium was detected using Fura-2 and CCE was measured by quantifying peak height of the calcium rise observed on calcium addition to calcium-depleted cells. Consistent with calcium entry via CCE, the calcium rise was suppressed by 2-APB. The calcium rise was significantly increased in C6 cells exposed to 1 μM ouabain. Baseline cytoplasmic calcium concentration was not changed by 1 μM ouabain. Cell sodium content, measured by atomic absorption spectrophotometry, also was unchanged in ouabain-treated cells. Lack of a sodium increase supports the notion that the CCE response to 1 μM ouabain takes place without a cell-wide change of Na,K-ATPase activity. The findings illustrate the ability of ouabain to alter CCE in cells that do not express a detectable amount of the Na,K-ATPase α2 isoform. [Supported by NIH grant EY014069, RPB Inc., the James Graham Brown Cancer Center and the KY Lions Eye Foundation.]

166. Characterization of Second Isoform of Secretory Pathway Ca^{2+}/Mn^{2+}-ATPase. RUSLAN I. DMITRIEV,[1] NIKOLAY B. PESTOV,[1,2] TATYANA V. KORNEENKO,[1] MARIA B. KOSTINA,[1] MIKHAIL I. SHAKHPARONOV,[1] [1]*Shemyakin-Ovchinnikov Institute of Bioorganic*

Chemistry, Russian Academy of Sciences, Moscow, Russia; [2]*Medical College of Ohio, Toledo, OH* (Sponsor: Jack Kaplan)

The secretory pathway calcium ATPase (SPCA) is the third member of the Ca^{2+}-ATPase family that also includes SERCA and PMCA. SPCA is unique in its ability to transport both Ca^{2+} and Mn^{2+}. We have determined the correct primary structure of SPCA2 using RACE amplification from rat distal colon RNA. The deduced amino acid sequence of SPCA2 has 62% of its residues identical with SPCA1. Analysis of tissue-specific expression of SPCA2 using RT-PCR in rat, pig, and human tissues demonstrated that SPCA2 is present in the gastrointestinal and airway tracts (with the highest level in the distal colon), and in the skin of all species. In the pig, the strongest signal was observed in the bulbourethral gland. Also, expression of SPCA1 and SPCA2 in rat mammary gland was compared, and it was found that SPCA2 level increases during lactation whereas SPCA1 decreases. Immunohistochemical detection of SPCA2 in human intestine demonstrated it to be prominent in goblet cells. A SPCA2-gfp chimera is localized predominantly in the Golgi of transiently transfected cultured cells. SPCA1 was also previously shown to be a resident of the Golgi. However, we performed fractionation of membranous compartments of rat liver followed by Western blotting with specific antibodies, and, unexpectedly, SPCA1 was detected not only in the Golgi but also in plasma membranes. These results indicate that physiological functions of SPCAs remain only poorly understood. Detection of SPCA2 in goblet cells, as well as in some exocrine glands, suggests that this enzyme may be important for correct formation of certain secretory components, such as mucins. Interestingly, database searches revealed that SPCA2 is present in various tetrapod species, including mammals, birds, and amphibians, whereas fishes and invertebrates possess only one SPCA isoform. Therefore, it is logical to speculate that SPCA2 radiated from SPCA1 during adaptation to terrestrial habitats. [Supported by RFBR grants 03–04-49059, 04–04-49413, and 03–04-49046.]

167. Expression and Function of α_3 Na^+,K^+-ATPase in Primary Afferent Neurons of Vertebrate Animals. MAXIM DOBRETSOV,[1] VALERIE K. HAFTEL,[2] DMITRY ROMANOVSKY,[1] and IGOR SIZOV,[1] [1]*Department of Anesthesiology, University of Arkansas for Medical Sciences, Little Rock, AR*; [2]*Department of Biology, Morehouse College, Atlanta, GA* (Sponsor: Michael L. Jennings)

Functional Na^+,K^+-ATPase is a complex of α and β protein subunits. The α subunit of mammalian Na^+,K^+-ATPase enzyme has four isoforms, which are expressed in a tissue- and cell-specific manner. Thus, α_3 Na^+,K^+-ATPase is abundant in some, but not all, neurons and not abundant in any other cells of adult vertebrates. The reasons and functional consequences of nonuniform and neuron-specific expression of α_3 Na^+,K^+-ATPase are not known. Recent studies have shown that across the variety of types of rat primary sensory afferent neurons, only stretch receptor afferents express α_3 Na^+,K^+-ATPase (Dobretsov et al. 2003. *Neuroscience.* 116:1069–1080). Here we provide evidence that similar, stretch receptor–associated expression of α_3 Na,K-ATPase isozyme is also a feature of populations of mouse and human peripheral sensory neurons. Furthermore, in vivo recordings of the activity of rat stretch receptor afferents suggest that electrogenic activity of α_3 Na^+,K^+-ATPase in these neurons is an important determinant of their ability to generate and transmit high-frequency coded messages. Finally, the stretch receptor of vertebrates is a low-threshold mechanoreceptor unique among peripheral neurons in its capacity to maintain high-frequency slowly adapting discharge over the seconds of continuous activity. Computer simulations conducted in our laboratory suggest that expression of α_3 Na^+,K^+-ATPase, which according to the literature has relatively low affinity for intracellular Na^+, may be an obligatory prerequisite for this functional uniqueness of the stretch receptor. Numerical simulation predicts that, unlike α_3 Na^+,K^+-ATPase–expressing neurons, the neuron expressing the enzyme with high affinity for intracellular Na^+ (α_1 Na^+,K^+-ATPase) has higher voltage threshold, faster frequency accommodation, and is not capable of continuous discharge in response to sustained depolarization. [Supported by NIH grant DK067284.]

168. The Physiological Role of the α1 and α2 Isoforms of Na,K-ATPase. IVA DOSTANIC,[1] JAMES VAN HUYSSE,[2] JOHN N. LORENZ,[3] and JERRY B. LINGREL,[1] [1]*Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH*; [2]*University of Ottawa Heart Institute, Hypertension Unit, Ottawa, Ontario, Canada;* [3]*Department of Molecular and Cellular Physiology, University of Cincinnati College of Medicine, Cincinnati, OH*

The primary objective of this study was to examine the functional role of the α1 and α2 isoforms of the Na,K-ATPase in the regulation of cardiovascular function. Previous studies in knock-out mice lacking one copy of the α1 or α2 gene suggested that these two isoforms may play a differential role in the regulation of cardiac contractility. The hearts from the $\alpha1^{+/-}$ mice

were hypocontractile, while those from the $\alpha 2^{+/-}$ mice were hypercontractile. To directly analyze the role of the α1 isoform, we developed knock-in mice expressing an ouabain-sensitive α1 isoform and a ouabain-resistant α2 isoform, and analyzed cardiovascular physiology following selective inhibition of the α1 isoform by ouabain. The function of the α2 isoform was determined by analyzing knock-in mice expressing wild-type α1 isoform and a ouabain-resistant α2 isoform. In wild-type mice, the α1 isoform is relatively resistant to cardiac glycosides, while the α2 isoform is sensitive to these compounds. Here we demonstrate that inhibition of either α1 or the α2 isoform results in positive cardiac inotropy and elevated blood pressure.

Using our knock-in mice, which express different combinations of ouabain-sensitive and ouabain-resistant α isoforms, we examined whether the highly conserved cardiac glycoside binding site has a biological significance in vivo. Although this site has been used as a pharmacological target for cardiac glycosides in the treatment of congestive heart failure, its normal function is unknown. The present study demonstrates that highly conserved cardiac glycoside binding sites of both the α1 and α2 isoforms play an important function in the regulation blood pressure. This is the first evidence that natural regulation of the Na,K-ATPase via this site has biological significance. [Supported by the National Institute of Health grants R01 HL28573 (J.B. Lingrel), R01 HL 66062 (J.B. Lingrel), and R01 DK57552, and by Heart and Stroke Foundation of Ontario Grant NA-5102 (JWV).]

169. Hypertension-related Adducin Polymorphism Activates Signal Transduction Pathways via Na-K ATPase within Renal Caveolae in a Congenic Rat Model. MARA FERRANDI,[1] ISABELLA MOLINARI,[1] PATRIZIA FERRARI,[1] and GIUSEPPE BIANCHI,[2] [1]*Prassis sigma-tau, Settimo Milanese, Milan, Italy;* [2]*San Raffaele Hospital, Vita e Salute University, Milan, Italy*

Polymorphism of the cytoskeletal protein adducin is associated with hypertension and organ complications in humans and in Milan hypertensive rats (MHS). Adducin pressor effect is related to a constitutive enhanced renal sodium reabsorption, mediated by an increased expression and activity of renal Na-K ATPase both in MHS rats and in renal adducin-transfected cells. In a cell-free system, mutated adducin stimulates Na-K ATPase with higher affinity than the wild-type variant and affects integrin cell surface exposure in cells. Recently, the integrin and Na-K ATPase–activated signaling pathways within caveoale have been described in cardiac remodeling and hypertension. Here we investigated whether adducin polymorphism affects caveolar Na-K ATPase–Src and integrin cascade in a congenic adducin rat model (NA), in which MHS α-adducin locus (*Add1)* has been introduced into the homogeneous genetic and hormonal background of normotensive MNS controls.

Compared with MNS, NA showed a significant ($P < 0.05$) increase of blood pressure (+15 mmHg) and renal Na-K ATPase activity (15%). Renal caveolae, prepared by a detergent-free purification method (200 mM Na^+ carbonate followed by a sucrose gradient) from NA ($n = 6$), showed a significant ($P < 0.05$) enrichment of α-adducin-Ser^{724} (+40%), α1 (+13%), and β1 (+23%) Na-K ATPase isoforms, Src (+25%), Src-Tyr^{418} (+37%), EGFr (+22%), αv-integrin (+20%) and ERK (+30%), but similar caveolin 1 content.

These findings indicate that the cross-talk between Src-EGFr and integrin signaling is influenced by adducin mutation in rat renal caveolae. The Na-K ATPase and ERK activation observed in the presence of mutated adducin provide a molecular explanation both for the enhanced renal tubular sodium reabsorption, and for the organ complications associated with the hypertension-related adducin polymorphism.

170. Vascular NCX1 Isoforms Mediate Ouabain-induced Hypertension. SATOMI KITA,[1] JIN ZHANG,[2] MORDECAI P. BLAUSTEIN,[2] YUJI ARAI,[3] and TAKAHIRO IWAMOTO,[1] [1]*Department of Pharmacology, School of Medicine, Fukuoka University, Fukuoka, Japan*; [2]*Department of Physiology, University of Maryland School of Medicine, Baltimore*, MD; [3]*Department of Bioscience, National Cardiovascular Center Research Institute, Osaka, Japan* (Sponsor: Takahiro Iwamoto)

Ouabain is known to induce hypertension in experimental animals, but the mechanism is not clearly understood. Recently, we observed an antagonistic interaction between ouabain and SEA0400, a specific inhibitor of Na^+/Ca^{2+} exchanger type 1 (NCX1), in vascular contractile function. Infusion of ouabain (0.5 μg/kg/min) into the femoral arteries of anesthetized beagles reduced the femoral blood flow (i.e., caused vascular constriction). The additional infusion of SEA0400 (50 μg/kg/min) then restored blood flow to the basal level. In contrast, intrafemoral infusion of SEA0400 alone did not affect the blood flow. Furthermore, a low dose of ouabain (100 nM) increased fluo-4 fluorescence (i.e., intracellular Ca^{2+} concentration) by ~12% and myogenic tone by 20–25% in intact, pressurized mouse small mesenteric arteries. SEA0400 (300 nM) abolished these effects induced by nanomolar ouabain. We created transgenic mice that specifically express

NCX1.3 in smooth muscle ($N1.3^{Tg/Tg}$). Chronic administration of ouabain (300 μg/kg/d) produced more severe hypertension in $N1.3^{Tg/Tg}$ mice than in wild-type mice. Oral administration of SEA0400 (10 mg/kg) suppressed ouabain-induced hypertension in these two kinds of mice (to the same level). These findings indicate that ouabain-induced vasoconstriction and hypertension are mediated by Ca^{2+} entry via NCX1 in vascular smooth muscle. [Supported by Grants-in-Aid for scientific research (14570097, 16590213) from the Ministry of Education, Science, and Culture of Japan, a grant from the Salt Science Research Foundation (No. 02), and NIH grant HL-45125.]

171. Uncoupling of Ca^{2+}-ATPase from Marlin Heater Organ and Tuna Deep Red Muscle by Temperature. ANA MARIA LANDEIRA-FERNANDEZ,[1] DANIELLY C. FERRAZ DA COSTA,[1] JEFFERY MORRISSETTE,[2] and BARBARA BLOCK,[2] [1]*Instituto de Bioquimica Medica, CCS/UFRJ*, Rio de Janeiro, *Brasil*; [2]*Hopkins Marine Station of Stanford University, Pacific Grove, CA*

Although most fish are ectothermic, the ability to elevate body temperature by internal heat generation (endothermy) has been documented in one group of teleosts, the suborder Scombroidei. In this group, regional endothermy has developed along two different paths: one typified by tunas that are able to warm their bodies by conserving metabolic heat released during digestion and red muscle contraction, and another in billfishes, where only the cranial cavity is kept warm by a specialized heater organ. Derived from eye skeletal muscle, heater organ expresses a unique thermogenic phenotype with cells void of contractile filaments, but retaining the cellular components involved in energy metabolism and Ca^{2+} mobilization, suggestive of a mechanism for heat production involving Ca^{2+} fluxes from internal stores. The sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) maintains a low cytoplasmic Ca^{2+} concentration by pumping Ca^{2+} from the cytosol into the SR lumen at the expense of ATP. The aim of this work was to investigate the activity of SERCA from blue marlin (*Makaira nigricans*) heater organ and albacore tuna (*Thunnus alalunga*) red muscle over the range of temperatures the fish experience in the wild. The rate of ATP hydrolysis catalyzed by SERCA from these tissues is highly temperature dependent. In contrast, the rate of Ca^{2+} uptake displayed a low temperature dependence, having nearly the same rate of Ca^{2+} transport at all temperatures tested (10–30°C). Calculations of the ratio between the ATP hydrolysis and Ca^{2+} transport revealed that the pump is highly coupled at low temperatures with values ~2 (5–15°C). However, at temperatures of 15–30°C, this ratio decreases to ~0.5. These results are in contrast to rabbit skeletal muscle SERCA for which ATP hydrolysis and Ca^{2+} uptake are both highly temperature dependent. We propose that the inefficiency of the SERCA pump from blue marlin heater tissue and albacore deep red muscle may be involved in thermogenesis. [Support by NSF and FAPERJ.]

172. Localization and Expression of 70-kD Protein in Goat Spermatozoa Having Na^+,K^+-ATPase Inhibitory and Aryl Sulfatase A Activities. MADHUMOULI CHATTERJEE,[1] PRADEEP DAS,[2] ARINDAM MAZUMDER,[2] SUBIR K. NAGDAS,[3] and PARIMAL C. SEN,[1] [1]*Department of Chemistry, Bose Institute, Kolkata 700 009, India;* [2]*National Institute of Cholera and Enteric Diseases, Kolkata 700 010, India*; [3]*Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN*

We have previously isolated and purified a goat sperm protein of 70 kD molecular weight, designated as P70, and characterized it as an inhibitor of Na^+,K^+-ATPase (Mandal et al. 2001. *Mol. Cell. Biochem.* 223:7–14). Our recent studies reveal that the first 10 amino acid residues from the NH_2-terminal end of P70 have a high degree of homology with aryl sulfatase A from mice, pig, and human. Indirect immunofluorescence study shows the presence of the protein on the goat sperm surface. Furthermore, live goat sperm, and the extract of peripheral sperm plasma membrane proteins, exhibit aryl sulfatase A's desulfation activity. The P70 remains on the head surface of in vitro capacitated cauda epididymal sperm, as shown by positive immunofluorescence staining of cauda sperm. Immunoblot and flow cytometric studies corroborate the above findings. The presence of P70 on capacitated cauda sperm surface suggest a possible role of this protein in sperm zona-pellucida binding. In the present report, we demonstrate aryl sulfatase A–like activity in P70 and describe its localization and expression in goat sperm.

173. Multiple Alleles of Duck Na^+,K^+-ATPase May Suggest Selective Expression. DWIGHT W. MARTIN and JOHN R. SACHS, *Department of Medicine, Stony Brook University, Stony Brook, NY*

We have sequenced the α and β subunits of the duck Na^+,K^+-ATPase. The α subunit of the duck Na^+,K^+-ATPase differs from the α subunit of the chicken ATPase by 29 amino acid substitutions. The duck β chain differs from the chicken β chain by 34 substitutions. Using the time of divergence of chickens and ducks (65E6

yr), we calculated the rate of base substitution per site per year. For α chains, the rate was 0.57 E-9 and for β chains the rate was 0.84 E-9. Base substitutions at synonymous sites were calculated to occur at a rate of 1.16E-9 in α chains and 1.58E-9 in β chains. We determined that the mutation rates for the Na^+,K^+-ATPase α and β chains are comparable to those of hemoglobin β and α chains, respectively. Sequencing cDNA from multiple ducks, we found that there were three different alleles for α chains (1, 2, and 3). Allele 1 and 2 differed from each other at 15 synonymous sites. Using the above rate of substitutions at synonymous sites, the divergence of alleles 1 and 2 occurred 20.4 E6 yr ago. Allele 3 appears to be a crossover product of 1 and 2 combining the 5′ end of 1 and the 3′ end of 2. Crossover occurred 7.5 E6 yr ago. Genetic drift in separate populations is one possible explanation for the fixation of these alleles. However, fixation could also have occurred by selection. Since the amino acid sequences of these alleles are identical, selection could not be based on enzyme function. However, since allele 3 was fixed with the 5′ end of allele 1, it seems possible that selection occurred because of a difference in the promoter region, with expression rates of allele 1 and 3 being more responsive to stimulation induced by salt adaptation. [Supported by NIH-DK19185.]

174. Differential Expression of the Na^+,K^+-ATPase Subunit Isoforms in Dorsal Root Ganglion Cells after Axotomy and During Regeneration. MARIA F. ARTEAGA, RICARDO GUTIÉRREZ, JULIO AVILA, ALI MOBASHERI, LUCIO DÍAZ-FLORES, and PABLO MARTÍN-VASALLO, *Bioquímica y Biología Molecular y Anatomía Patológica, Universidad de La Laguna, La Laguna, Tenerife, Spain; Veterinary Preclinical Sciences, University of Liverpool, Liverpool, UK*

Neural injury triggers changes in the expression of a large number of gene families. The expression of the Na^+,K^+-ATPase subunit isoforms (α, β, and γ) at the protein level was studied in dorsal root ganglion (DRG) of the rat in normal conditions, after axotomy, and during regeneration. To this end, we performed double-label (ATPase/cell-specific markers) indirect immunofluorescence on frozen sections of DRG using isoform-specific antibodies. In normal DRG, α1 and α2 were expressed in the plasma membrane of all cell types, while there was no detectable signal for α3 in most DRG cells. After axotomy, α1 and α2 expression decreased evenly in all cells, while there was a remarkable onset in α3 expression, with a peak about day 3, which gradually disappeared throughout regeneration (day 7). β1 was restricted to the nuclear envelope and plasma membrane of neurons and satellite cells. Immediately after injury, β1 showed a homogeneous distribution in the soma of neurons. No β2 expression was found. β3-specific immunofluorescence appeared in all neurons, although it was brightest in the smallest, diminishing progressively after injury until day 3 and, thereafter, increasing in intensity, until it reached normal levels. FXYD7 was expressed weakly in a few DRG neurons (<2%). It increased intensely in satellite cells immediately after axotomy, and in all cell types at day 3. Transient switching of members of the Na^+,K^+-ATPase isoform family elicited by axotomy suggests variations in the sodium pump isozymes with different affinities for Na^+, K^+, and ATP from those in intact nerve. This adaptation may be important for regeneration. [Supported by grants BFI2002-04653 from MCYT-DGI and PI2001/002 from Gobierno de Canarias, Spain.]

175. Tissue Distribution of the AQP-4 Water Channel: A Study Using Normal Human Tissue MicroArrays. ALI MOBASHERI, DAVID MARPLES, IAIN YOUNG, CHRISTOPHER MOSKALUK, and ANTONIO FRIGERI, *Faculty of Veterinary Science, University of Liverpool, Liverpool, UK; School of Biomedical Sciences, University of Leeds, Leeds, UK; Department of Pathology, Department of Biochemistry, and Department of Molecular Genetics, University of Virginia Health System, Charlottesville, VA; Department of General and Environmental Physiology and Centre of Excellence in Comparative Genomics, University of Bari, Bari, Italy*

Aquaporin water channels are a family of membrane proteins that facilitate water movement across biological membranes. Aquaporin-4 (AQP-4) is the predominant aquaporin found in the brain. However, the distribution of AQP-4 in many normal human tissues is still unknown. In this study immunohistochemistry was used to determine the tissue distribution and relative expression of AQP-4 in normal human tissues using NIH/CHTN Tissue MicroArrays. Immunohistochemistry was performed using a rabbit polyclonal AQP-4 antibody raised against rat AQP4 (Chemicon International). AQP-4 was present throughout the central nervous system; AQP-4 was abundantly expressed in the cerebral cortex, cerebellar cortex, choroid plexus, ependymal cell layer, hippocampus, and spinal cord motor neurons. Lower levels were detected in white matter and meninges. AQP-4 was moderately expressed in other locations, including seminiferous tubules, prostate, lung, collecting tubules in the inner renal medulla, and parietal and chief cells at the base of gastric glands. Very low expression was noted in the renal proximal tubules, adrenal cortex, cardiac and skeletal

muscle, and pancreas. These findings confirm the abundance of AQP-4 in the nervous system and its presence in the stomach. AQP-4 was also detected in new locations, including the prostate where it may be involved in prostatic fluid formation. [Supported by grants from Novartis and the Medical Research Council.]

176. Distribution of the Aquaporin Water Channels AQP1, AQP3, AQP4, and AQP9 in the Human Choroid Plexus. ALI MOBASHERI, DAVID MARPLES, and ANTONIO FRIGERI, *Faculty of Veterinary Science, University of Liverpool, Liverpool, UK; School of Biomedical Sciences, University of Leeds, Leeds, UK; Department of General and Environmental Physiology and Centre of Excellence in Comparative Genomics, University of Bari, Bari, Italy*

Aquaporins control the permeability of endothelial and epithelial barriers by facilitating water movement across cell membranes. The aquaporin family consists of 12 small integral membrane proteins (AQP0-AQP11) (Agre et al. 2002. *J. Physiol.* 542:3–16). Aquaporins are found throughout the body, but in the brain they are intimately involved in the production of cerebrospinal fluid and the control of water movement at the blood–brain barrier (Nicchia et al. *Neuroscience.* 2004. 129:935–945). Aquaporins may contribute to the development of brain edema formation after acute cerebral insults such as ischemia or traumatic injury. In this study, we determined the expression and distribution of AQPs 1–4 and AQP9 in the human choroid plexus. Polyclonal antibodies to AQP1, AQP2, AQP3, AQP4, and AQP9 were used to determine the expression of these water channels in formalin-fixed samples of choroid plexus on human Tissue MicroArrays by immunohistochemistry as described in a recent paper (Mobasheri and Marples. 2004. *Am. J. Physiol. Cell Physiol.* 286:C529–C537). Normal human Tissue MicroArrays (TMAs) were obtained from the Cooperative Human Tissue Network (CHTN) of The National Cancer Institute (NCI). AQP1 was abundantly expressed at the ventricular-facing (apical) surface of the choroid plexus epithelium. Expression of AQP2 was not detected in the choroid plexus. Expression of AQP3 and AQP9 was intracellular and not polarized. AQP4 was found in some, but not all, choroid plexus epithelial cells. The data suggest that AQP1, AQP3, AQP4, and AQP9 contribute to water and small solute transport in the choroid plexus during cerebrospinal fluid secretion. [Supported by grants from Novartis and the Medical Research Council.]

177. Uterine Na,K-ATPase Isoform Switching During the Progression of Pregnancy in the Rat and Mouse. RACHEL FLOYD, ALI MOBASHERI, PABLO MARTÍN-VASALLO, and SUSAN WRAY, *Departments of Physiology and Veterinary Preclinical Sciences, University of Liverpool, Liverpool, UK; Departamento de Bioquímica y Biología Molecular, Universidad de La Laguna, La Laguna, Tenerife, Spain*

Uterine contraction depends on the electrogenic action of Na,K-ATPase, which creates cation gradients across the cell membrane to facilitate propagation of contraction and return of the membrane to resting potential. Na, K-ATPase function relies on the catalytic properties of its α subunits and the presence or absence of β and FXYD constituents, all of which are expressed as multiple isoforms (4α, 3β, and 7FXYD). Little is known about uterine Na,K-ATPase subunit isoform expression, cellular localization, or pregnancy-related changes. In this study, we have used RT-PCR and Western blotting to monitor the expression of Na,K-ATPase isoforms throughout the course of pregnancy in the rat. Mouse uterine Na,K-ATPase expression was monitored by RT-PCR. We also used immunohistochemistry to determine the distribution of Na,K-ATPase isoforms in the rat uterus. Expression of α1, β1, and β3 isoforms was consistent throughout pregnancy. Expression of the α2 and of α3 isoforms peaked in early pregnancy (day 9/10) and decreased toward term. The β2 isoform was detectable only in the early stages of pregnancy (day 9/10 and day 16/17). Transcripts a and b of FXYD2 were present throughout pregnancy (FXYD2c was not detected). Immunohistochemistry using a pan α antibody (mAb 9A7) demonstrated high Na,K-ATPase expression in the outer longitudinal and inner circular smooth muscle layers and in the epithelial lining of the endometrium. Immunohistochemistry using isoform-specific antibodies revealed that the dominant isoform in the uterus is α1, although α2 and α3 expression was observed in both smooth muscle layers. These results suggest that there are shifts in Na,K-ATPase expression during pregnancy which may affect intracellular $[Na^+]_i$ and hence $[Ca^{2+}]_i$. This may explain the increase in uterine smooth muscle contraction during the latter stages of pregnancy. [Supported by grants from The Wellcome Trust and the Medical Research Council.]

178. Nongastric H,K-ATPase in Rodent Anterior Prostate. NIKOLAY B. PESTOV,[1,2] TATYANA V. KORNEENKO,[1,2] ROSSEN RADKOV,[1] RUSLAN I. DMITRIEV,[2] GARY SHULL,[3] and NIKOLAI N. MODYANOV,[1] [1]*Medical College of Ohio, Toledo, OH;* [2]*Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia;* [3]*University of Cincinnati, Cincinnati, OH* (Sponsor: Jack Kaplan)

Nongastric (colonic) H,K-ATPase is present at the highest level in epithelium of rodent anterior prostate, a specialized gland important for proper sperm coagulation. RT-PCR, immunohistochemistry, and coprecipitation experiments demonstrate that Na,K-ATPase is represented by α1β1 and α1β3 complexes and resides in basolateral membranes, whereas the nongastric H,K-ATPase α-subunit (αng) is located exclusively in apical membranes in association specifically with β1 isoform. The lack of colocalization of αng with β3 indicates that αβ interactions between X,K-ATPase are regulated in vivo in a highly specific manner. Gene targeting truncation of αng induces a number of changes in the gland's transcriptome and proteome. Expression levels of several transcripts were found to be dramatically affected as observed with the use of PCR-mediated cDNA differential display. Most of them encode uncharacterized proteins such as experimental autoimmune prostatitis antigen 2 (EAPA-2) and a novel protein termed here hyprotin. Proteomic analysis using electrophoresis, trypsin digestion and LC-MS-MS has shown the mutants' secretions to be significantly enriched in two proteins, a chaperone GRP78/BiP and IgG Fc-binding protein. The increase in secreted GRP78/BiP indicates that gene targeting derangement of the ATPase induces a peculiar stress response in prostate epithelial cells. Apical polarization of the H,K-ATPase in prostate epithelium provides an important argument that this enzyme is responsible for active potassium uptake from the secretion in exchange for protons. However, no differences were found between the mutants and wild-type control animals in concentrations of potassium, sodium, and other cations in their secretions as measured by inductivity-coupled plasma mass spectrometry (ICP-MS). This lack of effect on the ionic composition shows that physiological importance of nongastric H,K-ATPase remains insufficiently understood and allows us to hypothesize that this enzyme may be important for functions other than potassium reabsorption. [Supported by NIH grant HL-36573 and RFBR grant 03–04-49046.]

179. The Second Sodium Pump: Identification and Characterization. F.J. ROMERO, M.A. ROCAFULL, L. THOMAS, L. CARIANI, and J.R. DEL CASTILLO, *Laboratorio Fisiología Gastrointestinal, Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas (IVIC), Caracas, Venezuela* (Sponsor: G. Whittembury)

Enterocytes transport Na by two different active mechanisms: the ouabain-sensitive Na/K pump and the second Na pump, associated with the ouabain-insensitive Na-ATPase. Although these pumps and their ATPases have multiple functional differences (del Castillo et al. 1985. *Biochim. Biophys. Acta.* 812:402–412; del Castillo et al. 1985. *Biochim. Biophys. Acta.* 812:413–422; del Castillo et al. 2003. *Arch. Biochem. Biophys.* 419:190–197), the biochemical entity related to the second sodium pump has not been isolated so far. To identify it, basolateral plasma membranes of guinea pig enterocytes were solubilized with C12E9. The solubilized fraction, which contained the Na-ATPase (181 ± 6.5 nmol Pi/mg/min) and the Na/K-ATPase (381 ± 8.4 nmol Pi/mg/min), was filtered in Sepharose 6B. The Na/K- and Na-ATPases copurified. These ATPases were separated by concanavaline-A–sepharose affinity chromatography. The Na-ATPase, with specific activity of 1650 ± 104 nmol Pi/mg/min, was further purified by ion exchange chromatography in Hi Trap Q Sepharose 4 Fast Flow. SDS-PAGE and 2D-PAGE of it showed α/β subunits of 100 and 45 kD, respectively. Polyclonal antibodies against Na/K-ATPase did not recognize the purified Na-ATPase (Western blot analysis). Kinetic properties of the purified enzyme were similar to those of the native membrane-bound enzyme, indicating that it had not been substantially altered during the purification procedure. Purified Na-ATPase was phosphorylated from [^{32}P]-ATP. Phosphorylated intermediate was Mg dependent, vanadate sensitive, stimulated by Na and furosemide, and insensitive to ouabain. Phosphorylated Na-ATPase was sensitive to alkaline pH and hydroxylamine, suggesting an acyl-phosphate bond, which was associated with the 100-kD polypeptide of the enzyme. Finally, to clone the Na-ATPase of enterocytes, suppression PCR cDNA subtractive hybridization, using colonocyte cDNA as a driver, and PCR screening with degenerated oligonucleotides, which recognize P-type ATPase phosphorylation and Mg-ATP–binding motives, were used. A differentially expressed double-strand cDNA fragment corresponding to a putative Na-ATPase was obtained. Thus, the basolateral plasma membrane Na-ATPase is structurally and functionally dissociable from the Na/K-ATPase. It appears as a new member of the P-type ATPases.

180. Expression of Na Pump Isoforms in Neural Cells Derived from Mouse and Monkey Embryonic Stem Cells. TOMOAKI SAI,[1,2] MASAHIRO OTSU,[1] MOTOHIRO HARADA,[3] TOMOKO MOMOKI-SOGA,[1] TAKASHI NAKAYAMA,[4] YUTAKA SUZUKI,[5] YASUSHI KONDO,[5] HISATAKA KASAI,[5,6] and NOBUO INOUE,[1] [1]*Laboratory of Regenerative Neurosciences, Tokyo Metropolitan University Faculty of Health Sciences, Tokyo, Japan*; [2]*Department of Orthopaedic Surgery, The Jikei University School of Medicine, Tokyo, Japan*; [3]*School of Science and Engi-*

neering, Aoyama Gakuin University, Tokyo, Japan; [4]*Department of Biochemistry, Yokohama City University School of Medicine, Yokohama City, Japan*; [5]*Advanced Medical Research Laboratories, Tanabe Seiyaku Co. Ltd., Osaka, Japan*; [6]*Department of Applied Chemistry, Kogakuin University, Tokyo, Japan* (Sponsor: David Gadsby)

Na pump isoforms are essential for neural functions. We investigated expression of the isoforms during in vitro differentiation of mouse and monkey embryonic stem (ES) cells into neural cells. The neural cells were produced from ES cells by neural stem sphere (NSS) method, consisting of two stages in culture. In stage 1, cultured in astrocyte-conditioned medium (ACM) under free-floating conditions, colonies of undifferentiated ES cells gave rise to NSSs with a periphery of neural stem cells. In stage 2, culturing the NSSs on an adhesive substrate in ACM promoted differentiation of the cells exclusively into neurons (Nakayama et al. 2003. *Neurosci. Res.* 46:241–249). Quantitative real-time RT-PCR analysis of the isoforms of α and β subunits demonstrated that colonies of mouse and monkey ES cells expressed mainly gene α1 and β1. In contrast, gene expression of α2, α3, β2, and β3 was very low. The NSSs derived from ES cell colonies in stage 1 also expressed mainly α1 and β1. During neurogenesis in stage 2, expression of α3 and β1 was remarkably up-regulated, whereas increase in expression of α1, α2, β2, and β3 was subtle. Mouse and monkey neural stem cells, which were prepared by the NSS method (Nakayama et al. 2004. Neuroreport. 15:487–491), expressed mainly α1 gene. When the neural stem cells were differentiated into neurons by culture in ACM, expression of α3 was up-regulated and became comparable to that of α1. In contrast, expression of α1 and α2 increased slightly during differentiation. Immunofluorescence analysis showed that ES cells and neural stem cells were positive for α1 isoform but neurons were positive for both α1 and α3 isoforms. These results support physiological importance of α3 isoform in neurons. [Supported by Selective Research Fund of Tokyo Metropolitan Universities.]

181. An NH_2-terminal Signal Targets Na^+ Pump α2 Subunits to Plasma Membrane–ER Junctions. HONG SONG, MOO YEOL LEE, STEPHEN P. KINSEY, and MORDECAI P. BLAUSTEIN, *Department of Physiology, University of Maryland School of Medicine, Baltimore, MD*

Na^+ pumps (Na/K-ATPase) are expressed as αβ dimers. In mouse astrocytes, Na^+ pumps with the α1 isoform of the catalytic (α) subunit are uniformly distributed in the plasma membrane (PM). Those with the α2 isoform are confined to PM microdomains that overlie "junctional" endoplasmic reticulum (jER), where they colocalize with Na/Ca exchangers. Our objective was to determine how α2 targets to its appropriate PM destination, and to examine its function. We generated α1–α2 chimeras (e.g., NH_2-terminal α1 segment and COOH-terminal α2 segment, and vice versa) and NH_2-terminal 90–130 amino acid (AA) truncations. These constructs, tagged with Flag (COOH terminal) or GFP (green fluorescence protein; NH_2 terminal), were transfected into primary cultured mouse astrocytes. Immunocytochemistry of cells expressing the chimeras indicated that the α2 targeting signal was contained within the NH_2-terminal 130 AA. The α2/α1 chimeras targeted to the PM at PM-ER junctions and down-regulated native, full-length α2 (i.e., the constructs were "dominant negative"). NH_2-terminal (120 or 90 AA) truncated α2, but not α1, peptides also targeted to the PM-ER junctions, and were dominant negative for full-length α2. Furthermore, although α3 is not normally expressed in astrocytes, NH_2-terminal truncated (120 AA) α3 also localized to PM-ER junctions and was dominant negative for α2 in astrocytes. Truncated α2 constructs also were "functionally dominant negative." Ca^{2+} signals (Fura-2 measurement) in cells transfected with truncated α2 were comparable to those in cells from α2 null mutants ($\alpha2^{-/-}$): 1 μM ATP-evoked Ca^{2+} transients were augmented, and the response to 100 nM ouabain was abolished. These data demonstrated that the α2 targeting/tethering signal is contained within the NH_2-terminal 90 AA and that α2 and α3, but not α1, use the same targeting/tethering signal. [Supported by NIH grants NS-16106 and HL-45215, and by a KOSEF Fellowship to M.Y. Lee.]

182. Activation of $(Na^+ + K^+)$-ATPase. KAI Y. XU, *Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD*

$(Na^+ + K^+)$-ATPase (NKA) catalyzes Na^+/K^+ ion active transport and regulates cardiac contraction. Since the discovery of NKA, activation of NKA and its biological influences remain unknown. Our studies reveal that native catalytic power of NKA can be further accelerated when protein–protein interaction occurs at an effective site on the extracellular surface of α-subunit of the enzyme. Both the rate of MgATP hydrolysis and Na^+/K^+ ion active transport are enhanced without changing the reaction conditions. The maximum turnover of NKA is approximately two times faster than that of the controls for both ouabain-resistant (rat) and ouabain-sensitive (dog) enzymes. Studies of [^{3}H]ouabain binding and immunofluorescent labeling suggest that the effective site of NKA is not a conventional digitalis-binding site. Acti-

vated NKA directly affects cellular activities by regulating intracellular Ca^{2+} transients and inducing a positive inotropic effect in isolated rat cardiac myocytes. For half a century, scientists have only known that inhibition of NKA induces a positive inotropic effect. Activation of NKA markedly augmenting cardiac contraction is an unexpected finding, which broadens our view of NKA and opens a new chapter of investigation. Moreover, congestive heart failure is an important cause of death worldwide. Understanding of molecular activation of NKA may lead to new types of therapy to benefit heart failure patients. [Supported by NIH grant HL52175 and Johnson&Johnson's Focused Giving Program.]

183. Cytotoxic Comparative Effects of Iopamidol, Cyclosporin, Tacrolimus, and Amikacin on Human and Rat Renal Na,K-ATPase Activities. P.P.R. ROCHA JR., M.C. BARNESE, C.A. MARTINS, F.G. BINO, M.V. CASTRO-FARIA, and M. YOUNES-IBRAHIM, *Lab Integrado de Nefrologia, Universidade do Estado do Rio de Janeiro, UERJ, Rio de Janeiro, Brazil* (Sponsor: D.C. Gadsby)

The importance of the impairment of human renal tubular Na,K-ATPase activity induced by several nephrotoxins has been suggested in the literature on the basis of animal experiments. To contribute further to the understanding of human tubular cytotoxic mechanisms, we studied comparative in vitro effects of four nephrotoxic drugs (iopamidol, cyclosporin A, tacrolimus, and amikacin) on rat and human Na,K-ATPase enzymes. The enzymes were purified from kidneys (cortical and medullar portions) of both species by a modified Jorgensen's method. Rat enzymes were extracted from adult Wistar rat tissues. Human tissues were obtained from cadaver donor organs, which could not be used in transplant operations. Na,K-ATPase preparations had specific activities between 65 and 253 μmol/Pi/h/mg protein. Enzyme activities under Vmax conditions were measured by colorimetric assay in the presence or absence of varied concentrations of nephrotoxic agents. Based on the inhibitory effect on human and rat enzymes observed for each drug, it was possible to characterize three different cytotoxic agent profiles: (1) similar dose-dependent inhibition curves for human and rat Na,K-ATPase, as exemplified by amikacin; (2) a higher inhibitory sensitivity of the human enzyme, as found with iopamidol ($P < 0{,}001$); and (3) a selective species-dependent inhibition of human Na,K-ATPase as found with cyclosporin A and tacrolimus, neither of which affected rat enzyme. We conclude that (1) under our experimental conditions, all four of the drugs showed their highest dose-dependent inhibitory effects on human renal cortex Na,K-ATPase; (2) inhibition of the human enzyme by these drugs started at concentrations comparable to those found in usual therapeutic conditions; and (3) the choice of an experimental model for useful studies on these nephrotoxins must take into consideration the Na,K-ATPase response to these drugs of the relevant animal model.

184. Ligand-mediated Graded Recruitment of Single $InsP_3$ Receptor Ca^{2+} Release Channels. LUCIAN IONESCU,[1] DON-ON DANIEL MAK,[1] CARL WHITE,[1] and J. KEVIN FOSKETT,[1,2] [1]*Department of Physiology and* [2]*Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA*

Modulation of the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) by receptor-mediated generation of inositol 1,4,5-trisphosphate ($InsP_3$) and activation of its receptor ($InsP_3R$), a Ca^{2+} release channel in the endoplasmic reticulum, is a ubiquitous cellular signaling mechanism. A fundamental aspect of $InsP_3$-mediated signaling is the phenomenon of "quantal release," the ability of cells to have graded release of Ca^{2+} from intracellular stores in response to incremental levels of extracellular agonist or cytoplasmic $InsP_3$ concentrations ($[InsP_3]$), enabling intracellular Ca^{2+} stores to act as increment detectors of stimuli. The mechanisms that underlie quantal Ca^{2+} release have remained obscure, but likely involve variable recruitment of $InsP_3R$ channels with heterogeneous sensitivity to $[InsP_3]$. Here, we demonstrate that mechanisms intrinsic to the single $InsP_3R$ channel itself generate heterogeneous sensitivity to both of its primary ligands, $InsP_3$ and Ca^{2+}. Patch clamp electrophysiology of nuclei isolated from insect Sf9 cells revealed a consistent and high probability of detecting functional endogenous $InsP_3R$ channels, enabling the average number of activated channels in the membrane patch (N_A) to be accurately quantified. Unexpectedly, N_A was found to be a graded function of both $[Ca^{2+}]_i$ and $[InsP_3]$. A molecular model involving channel inactivation can account for these results and demonstrates that heterogeneous ligand sensitivity can be generated in a homogeneous population of $InsP_3R$ channels, providing a mechanism for quantal Ca^{2+} release that is intrinsic to the $InsP_3R$ Ca^{2+} release channel itself.

185. The Endoplasmic Reticulum Gateway to Apoptosis: Bcl-X_L Modulation of the $InsP_3$ Receptor. CARL WHITE,[1] CHI LI,[3] JUN YANG,[1] NATALIYA B. PETRENKO,[1] CRAIG B. THOMPSON,[3] and J. KEVIN FOSKETT,[1,2] [1]*Department of Physiology,* [2]*Department of Cell and Developmental Biology, and* [3]*Department of Cancer Biology, and Abramson Family Cancer Research Institute, Univer-*

sity of Pennsylvania, Philadelphia, PA (Sponsor: J. Kevin Foskett)

Members of the Bcl-2 protein family modulate outer mitochondrial membrane permeability to control apoptosis. However, these proteins also localize to the endoplasmic reticulum (ER), the functional significance of which is controversial. Here we provide evidence that anti-apoptotic Bcl-2 proteins regulate the inositol 1,4,5-trisphosphate receptor ($InsP_3R$) ER Ca^{2+} release channel resulting in increased cellular apoptotic resistance. We identified a biochemical interaction between anti-apoptotic Bcl-X_L and the carboxy terminus of the $InsP_3R$ by coimmunoprecipitation of endogenous proteins and in vitro pull-down assays. Single channel patch clamp electrophysiology of the $InsP_3R$ in native ER membranes revealed a functional interaction. Recombinant Bcl-X_L in the pipette or when overexpressed increased the sensitivity of single $InsP_3R$ channels to low [$InsP_3$], enhancing the ligand-dependent regulation of channel activity. Pro-apoptotic Bax and tBid antagonized this effect by blocking the biochemical interaction of Bcl-X_L with $InsP_3R$. At the whole cell level, increased Bcl-X_L expression increased survival in the presence of apoptotic stimuli, an effect that was dependant on $InsP_3R$. These data support a novel model in which Bcl-X_L is a direct effector of the $InsP_3R$, increasing its sensitivity to $InsP_3$ and enabling ER Ca^{2+} release to be more sensitively coupled to extracellular signals. As a consequence, cells are protected against apoptosis by a more sensitive and dynamic coupling of ER to mitochondria through Ca^{2+}-dependent signal transduction that serves to preserve cellular bioenergetics and survival. [Supported by NIH grants (C.B. Thompson and J.K. Foskett), an NIH training grant (C. Li), an American Heart Association Fellowship (C. White), and the Abramson Family Cancer Research Institute.]

186. Na^+ Pumps with α2 Subunits Control Myogenic Tone and Blood Pressure. JIN ZHANG,[1] MOO YEOL LEE,[1] MAURIZIO CAVALLI,[1] LING CHEN,[2] ROBERTO BERRA-ROMANI,[1] C. WILLIAM BALKE,[1,2,3,4] GIUSEPPE BIANCHI,[5] PATRIZIA FERRARI,[5] JOHN M. HAMLYN,[1,3] TAKAHIRO IWAMOTO,[6] JERRY B. LINGREL,[7] DONALD R. MATTESON,[1,3] W. GIL WIER,[1,3] and MORDECAI P. BLAUSTEIN,[1,2,3] [1]*Department of* Physiology, [2]*Department of Medicine, and* [3]*Center for Heart, Hypertension, and Kidney Disease, University of Maryland School of Medicine, Baltimore, MD*; [4]*Department of Medicine, Department of Physiology, and Institute for Molecular Medicine, University of Kentucky College of Medicine, Lexington, KY*; [5]*Prassis Instituto Ricerche Sigma-Tau, Milan, Italy*; [6]*Department of Pharmacology, Fukuoka University School of Medicine, Fukuoka, Japan*; [7]*Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH* (Sponsor: Mordecai P. Blaustein)

A key question in hypertension is: How is long-term blood pressure (BP) controlled? A clue is that excess dietary salt and salt retention are responsible for >50% of human essential hypertension (EH). This raises the possibility that Na^+ pumps are a critical link between salt and hypertension. Indeed, the Na^+ pump inhibitor, ouabain, induces hypertension in rodents. Moreover, animals with salt-dependent hypertension and ~40% of patients with EH have high endogenous ouabain (EO) levels. However, the mechanisms by which ouabain and Na^+ pumps control BP are unresolved. We hypothesized that salt retention elevates EO and induces hypertension mediated by smooth muscle Na^+ pumps and Na^+/Ca^{2+} exchangers (NCX). Indeed, overexpression of NCX-type 1 in smooth muscle increases salt sensitivity and elevates BP (Iwamoto et al. 2004. *Nature Med.* 10:1193–1199). Here we study BP and isolated small arteries of mice with genetically reduced expression of Na^+ pump α1 ($\alpha1^{+/-}$) or α2 ($\alpha2^{+/-}$) catalytic subunits. Both low dose ouabain (1–100 nM; inhibits only α2) and high dose ouabain (≥1 μM; inhibits α1) elevate myocyte Ca^{2+} and constrict arteries from $\alpha1^{+/-}$, as well as $\alpha2^{+/-}$ and wild-type mice. Nevertheless, only mice with reduced α2 activity ($\alpha2^{+/-}$), but not α1 ($\alpha1^{+/-}$), have elevated BP. Also, isolated, pressurized arteries from $\alpha2^{+/-}$, but not $\alpha1^{+/-}$, have increased myogenic tone. Ouabain antagonists (PST 2238 and canrenone) and NCX blockers (SEA0400 and KB-R7943) normalize myogenic tone in ouabain-treated arteries, but only the NCX blockers work in $\alpha2^{+/-}$ arteries. All four agents lower BP in salt-dependent and ouabain-induced hypertension. Thus, chronically reduced α2 activity ($\alpha2^{+/-}$ or chronic ouabain) apparently regulates myogenic tone and long-term BP, whereas reduced α1 activity ($\alpha1^{+/-}$) plays no persistent role. Accordingly, in salt-dependent hypertension, EO increases vascular resistance and BP by reducing α2 Na^+ pump activity and promoting Ca^{2+} entry via NCX in myocytes. [Supported by NHLBI/NIH grants to C.W. Balke, M.P. Blaustein, J.B. Lingrel, and W.G. Wier, and postdoctoral fellowships from the AHA Mid-Atlantic Affiliate (to J. Zhang) and the Korea Science and Engineering Foundation, KOSEF (to M.Y. Lee).]

187. Mutational and Gel-based Analysis of Cation–Protein Interactions Involved in Tetramer Stability of the KcsA K^+ Channel. MANOJ N. KRISHNAN, PATRICK TROMBLEY, SIEW HWEE LEE, and ED-

WARD MOCZYDLOWSKI, *Department of Biology, Clarkson University, Potsdam, NY*

Crystallographic studies indicate that K^+ binds to seven discrete sites in the central pore of the KcsA K^+ channel. We have shown that K^+ and congener inorganic cations function in stabilizing the quaternary structure of KscA as revealed by the cation dependence of temperature-induced tetramer dissociation monitored by SDS-PAGE. Cation titrations of tetramer protection at high temperature (85°C or 90°C) show that K^+, Rb^+, Cs^+, Tl^+, and NH_4^+ stabilize the tetramer with affinity constants ($K_{0.5}$) ranging from 1.1 to 9.6 mM and Hill coefficients (N) ranging from 1.7 to 3.3. In contrast to the latter cooperative behavior indicative of multisite binding, Ba^{2+} and Sr^{2+} stabilize the tetramer with single-site behavior ($N \simeq 1$) and $K_{0.5}$ values of 210 nM and 11 μM, respectively. Correspondingly, Ba^{2+} has been shown to bind to a single S4 site in the selectivity filter by crystallographic analysis. To examine the contribution of the S4 site to tetramer stability, we studied mutations of Thr75, a residue involved in K^+ coordination via the side chain hydroxyl group. Compared with wild-type KcsA (wt), the T75C mutant exhibited a 5–30°C reduction of the denaturation temperature ($T_{0.5}$) for various cations, indicating reduced thermal stability. T75C also exhibited a 0.8- to 13.8-fold change in $K_{0.5}$ for K^+, Rb^+, Cs^+, and Tl^+ relative to wt, whereas $K_{0.5}$ for Ba^{2+} increased by a factor of 13,000. Substitution of Thr75 by Ala resulted in similar titration behavior to the Cys mutant, while mutation of this residue to Ser or Asn severely impaired tetramer formation. In summary, mutation of Thr75 greatly alters cation binding to the selectivity filter. These results also confirm a major role of the S4 site in Ba^{2+} binding and tetramer stability. [Supported by NIH grant P01 NS42202 and AHA 0150058N.]

188. A Novel K Channel on the Apical Membrane of the Proximal Tubule. JULIE NICOLE, TOBIN J. HOPPES, and ALAN S. SEGAL, *Department of Medicine, University of Vermont, Burlington, VT*

The major function of the renal proximal tubule is the bulk reabsorption of isotonic saline. Entry of sodium (Na^+) across the apical membrane via cotransporters depolarizes the cell, constantly diminishing the driving force for continued reabsorption. We hypothesized that a potassium (K) conductance exists in the apical membrane of the proximal tubule. The opening of such channels would generate a counteracting hyperpolarizing force to maintain transcellular transport. Heretofore, the presence of a brush border has hindered direct recording from apical ion channels. Using a preparation of native proximal tubule cells isolated from *Ambystoma tigrinum*, we have overcome this obstacle. We are able to form giga-ohm seals on the apical membrane with a 75% success rate. Recording from apical membrane patches reveals the presence of several channels, one of which is an inwardly rectifying K channel. The inward slope conductance of this channel is ~30 pS. The channel is highly selective for K^+, and is not observed when patch pipettes are filled with Na^+, Li^+, Cs^+, Rb^+, or NH_4^+. However, like some other K channels, the apical K channel does conduct thallium (Tl^+). Channel activity is blocked when 5 mM barium is added to the pipette. Addition of nigericin (to induce intracellular acidosis) during cell-attached recordings decreases channel activity, consistent with the idea that channel behavior is linked to the apical Na^+/H^+ (NHE3) antiporter. Stimulation of transcellular transport increases channel activity. In conclusion, we have identified a novel inward rectifier K channel in the apical membrane of native proximal tubule cells. Channel activity is influenced by intracellular pH and the level of transport. We speculate that this K channel helps maintain the driving force for continued Na^+ reabsorption across the apical membrane, and may also represent a K^+ secretory pathway in the proximal tubule. [Supported by NIH grant DK064325.]

INDEX TO AUTHORS OF ABSTRACTS

Abstract number follows name