The 59th Symposium of the Society of General Physiologists
Na,K-ATPase and Related Cation Pumps:
Structures, Mechanisms, and Diseases
(Organized by David Gadsby, Jack Kaplan, and Jerry Lingrel)

The 59th Symposium of the Society of General Physiologists focused on the topic of Na,K-ATPase and Related Cation Pumps: Structures, Mechanisms, and Diseases. The meeting was also the 11th International ATPase Conference, a triennial event; the previous conjunction of these two meetings occurred in 1990. The Symposium took place at the Marine Biological Laboratory in Woods Hole, MA, on September 6–11, 2005. It was organized by David Gadsby (The Rockefeller University), Jack Kaplan (University of Illinois Chicago), and Jerry Lingrel (University of Cincinnati). There were ~250 attendees representing 21 different countries. The program featured 35 invited speakers and 165 posters. As suggested by the title, the meeting covered a range of topics pertaining to the P-type ATPases, including their atomic structure, enzymology, regulation, and role in physiological and pathological processes. This summary includes contributions from the Chairs of the six symposium sessions and other participants as indicated next to the session titles. Numbers in parentheses in the text indicate the abstract number published in the Journal of General Physiology, Vol. 126, No. 1, July 2005.

Session I.
Atomic Structure
(Paul De Weer, University of Pennsylvania)
Findings presented at the first session reinforce the notion that P-type transport ATPases function according to the “alternating access” principle where the transported (or countertransported) substrate on its trip across the membrane binds at an essentially “stationary” locus whose affinity and directional accessibility are regulated by cyclical structural rearrangements.

In the session on atomic structure, Y. Sugita presented recent work from the laboratory of C. Toyoshima (University of Tokyo) on attempts to identify candidate protonation sites in the transmembrane Ca-binding loci, and to elucidate the role of proton countertransport in the operation of the SERCA1a calcium ATPase (1). Continuum electrostatic calculations on a previously solved Ca-bound structure of SERCA1a in the E1 form had shown that two acid residues in the Ca-binding region were likely to be protonated and, as confirmed by molecular dynamics simulations, to lend stability to the Ca binding site through hydrogen bonding. A new high-resolution (2.4 Å) x-ray structure of the enzyme crystallized in the E2 form in the absence of Ca2+ and presence of the inhibitors thapsigargin and dibutylhydroxybenzene was obtained. The structure reveals, besides the inhibitors, individual water molecules, several phospholipid molecules (one of them wedged between helices M2 and M4), and a new conformation for the backbone of Glu-309, which is generally accepted to form part of the cytoplasmic access gate. This high-resolution Ca2+-free structure was used to assess the protonation status of residues in the now-empty Ca-binding site. Interatomic distances, electrostatic calculations, and MD simulations all point to four protonated residues, compared with two in the Ca-bound E2 conformation. Absent knowledge about the source (cytoplasmic or SR luminal) of these protons, the transport stoichiometry remains uncertain. The Tokyo group argues that two to three protons are transported per cycle and that all P-type ATPases must effect countertransport of protons and/or other ions.

J. Møller (University of Aarhus) briefly summarized advances in overexpression of rabbit SERCA1a in Saccharomyces cerevisiae followed by biotin-based purification, functional reconstitution, crystallization, and x-ray analysis, promising tools for future structural work on recombinant Ca-ATPase (2). Turning to x-ray structures of native SERCA1a crystallized in two conformations (Ca2-E1 complexed with AMPPCP or ADP + AlF4− and Ca-free E2 complexed with AlF4− + thapsigargin), Møller offered a conformational analysis of the coupling between movements of the cytoplasmic N, P, and A domains that accompany phosphorylation–dephosphorylation steps and those of the transmembrane segment that result in Ca2+ and H+ transport. Despite minimal structural differences, the Aarhus group views the Ca2-E1 complex with ADP + AlF4− (which mimics the phosphorylation transition state), rather than the complex with AMPPCP, as being most representative of the occluded state. Nucleotide binding to Ca2-E1 causes large sequential cytoplasmic domain movements that result in the A domain pulling the long M2 helix and kinking M1 to plug cytoplasmic access to the Ca-binding sites; phosphorylation (which requires Ca2+ to be bound) then locks Ca2+ in place. After phosphoryl transfer and ADP release, the enzyme acquires the con-
formation of the dephosphorylation transition state represented by the complex with AlF$_4^-$ + thapsigargin, which has no luminal access and where a large movement of the A domain, with multiple tugs on the transmembrane segment, has destabilized the Ca$^{2+}$ binding while keeping cytoplasmic access plugged by M1-M2; to accommodate the demonstrated intervening Ca$^{2+}$/H$^+$ exchange, an intermediate state with luminal access is postulated. Phosphoenzyme hydrolysis (which requires the binding sites to be protonated) then locks the luminal door on H$^+$. Subsequent phosphate release removes the cytoplasmic plug, allowing H$^+$/Ca$^{2+}$ exchange and reinitiation of the cycle.

S.J.D. Karlish (Weizmann Institute) described the characterization, in anticipation of structural work on recombinant enzyme, of pig Na,K-ATPase obtained by large-scale expression of α and (His-tagged) β subunits in the methylotrophic yeast *Pichia pastoris* (3). The protein, detergent solubilized and purified by metal-chelate and size-exclusion chromatography, remains inactive unless prepared with added phospholipids. Solubilized enzyme, native (from kidney) as well as recombinant, sediments and chromatographs as an αβ complex. It has lower specific activity and higher ouabain sensitivity than membrane-bound native enzyme, which presumably reflects stabilization of the E1 conformation. β-Subunit deglycosylation removes activity but not with dioleoyl-phosphatidylserine present. Cholesterol, at least in the presence of dioleoylphosphatidylcholine, stabilizes the enzyme at higher temperatures.

O. Dmitriev (University of Wisconsin) described the solution-NMR structure of the 165-residue N (nucleotide binding) domain of the human P$_1$-type copper-transporting ATPase, mutations of which underlie Wilson’s disease, a severe hepato-neurological disorder caused by copper accumulation. The N domain of P$_1$-type ATPases shows little homology with that of P$_2$-type (including bacterial heavy metal) ATPases and harbors an extra insert. However, among P$_1$ members, P2-type (including bacterial heavy metal) ATPases and shares their structural organization into a seven-helix transmembrane segment and cytoplasmic A, P, and N domains. The N domain structure was found to consist of a β sheet flanked by two pairs of α helices. Nucleotide binding was found to occur by sandwiching of the purine ring by π:π and cation:π interaction between Phe-377 and Lys-395, respectively. No significant conformational changes accompanied nucleotide binding.

**Session II.**

**Ion Binding Sites and Transport Mechanisms**

(Luis Beaugé, Instituto M. y M. Ferreira)

The contributions in this session dealt with structure-function relationships in P$_i$ATPases associated with binding and translocation of the transported ions. A variety of plasma membrane ATPases were featured, such as those involved in the coupled transport of Na$^+$ and K$^+$, Ca$^{2+}$ and H$^+$, or H$^+$ and K$^+$, as well as bacterial heavy metal transporting enzymes. Most presentations included site-specific mutations followed by analysis of transport function parameters or, in one case, of ion movement in a pump transformed into an ion channel.

The Ca-ATPase now has high-resolution structures associated with several different states of the enzyme, as discussed above. J.P. Andersen and colleagues (University of Aarhus) discussed the functional analysis of site-directed mutations with respect to these structures (8). In particular, they identified residues important in E1-E2 conformational changes, Ca$^{3+}$ and nucleotide binding, and K-dependent dephosphorylation of the E2-P conformation. These studies are forging an understanding of the nature of the interactions within the protein, and with ligands, that drive the transport cycle in these pumps.

Although the atomic structure of the Na$_i$K-ATPase has not been determined directly, general structural features have been inferred using homology modeling based on the Ca-ATPase. Various steps in the ion translocation pathway were discussed at new levels of detail. P.L. Jørgensen (University of Copenhagen) described events involved in binding of Na$^+$ from the cytoplasm (4). Na$^+$ is thought to promote its own entry into the transport pathway by opening the cytoplasmic gate controlling access to cation binding sites. The gate is then closed by ATP binding and subsequent phosphorylation, so occluding the ions. The open state of the gate is associated with an increase in the rate of chymotryptic cleavage of the α-subunit of the pump at Leu-266, indicating a conformational change that makes this residue more accessible.

The binding sites at which Na$^+$ resides after passing through the cytoplasmic gate were extensively discussed. H.-J. Apell (Universität Konstanz) described the ion-binding events as sequential, with the occupancy of sites triggering changes in protein conformation that allow the pump transport reaction to cycle (6). An analysis of
the electrical events associated with the cycle suggested that most of the voltage dependence of the pumping rate arises from the electrodiffusion of ions through the associated pore to and from the binding sites, rather than from the conformational changes of the protein or the phosphorylation/dephosphorylation reactions that underlie the gating process. The overall net charge movement by the pump ultimately results from the asymmetry in the number of Na$^+$ (3) and K$^+$ (2) ions translocated with each cycle. While the structure of the Ca-ATPase suggests the position of two of the ion-binding sites in the Na,K-ATPase, the location of the third site for Na$^+$ ions is less clear. J.D. Horisberger and colleagues (Université de Lausanne) used mutagenesis to search for residues that affect Na$^+$ but not K$^+$-dependent kinetics of the pump (39). They identified a putative binding site in a cavity bounded by the transmembrane (TM) segments 5, 6, 8, and 9. Similar work presented by Taniguchi et al. (40) assigned interactions with Thr-774 in TM5 as an important component in the binding of the third Na$^+$ ion. Horisberger et al. also identified parts of the second and third extracellular loops of the α-subunit that are possibly linked to the gate that controls movement of ions between their binding sites and the extracellular fluid.

The nature of this part of the Na$^+$ translocation pathway was further investigated by P. Artigas and colleagues (The Rockefeller University). The marine toxin palytoxin opens gates in the Na,K pump, turning it into ouabain-inhibitable ion channel. Mutations and MTS modifications at position 131 in TM2 and other residues at the extracellular ends of TMs 1, 2, and 6 alter the single-channel conductance in a charge-dependent manner, suggesting these aspects of the protein form the outer mouth of the channel. Modifications at Thr806, at the top of TM6, decrease conductance irrespective of charge, indicating steric effects in a narrowed part of the pore.

Ion-binding sites have also been identified in heavy-metal transporting (type P$_{109}$) ATPases, as described by J. Argüello (Worcester Polytechnic Institute). As in the other ATPases, these binding sites are complex, involving coordination of the metals by amino acids in TMs 6, 7, and 8 (corresponding to TMs 4, 5, and 6 of the P$_{10}$-ATPases). Site-directed mutagenesis studies revealed that these binding sites conferred the heavy-metal specificities that characterize this class of pumps.

Session III.
Cation Pump Subunit Interaction
(Kazuya Taniguchi, Hokkaido University)
Cation pump subunit interactions presented in the conference fell into two categories, one related to interactions between protomers, αβ or αβγ in monovalent cation pumps or conformational variants in SR-Ca-ATPase, and the other to interactions between the glycosylated β-chain and catalytic α-chain. Although the speakers mainly concentrated on the latter category, interactions between protomers, presented mainly in posters, may be important for a full understanding of pump function.

The oligomericity of Na/K-ATPase was proposed more than 30 years ago (Repke and Schon, 1973; Stein et al., 1973). Recent experimental evidence to show oligomericity has been reviewed (Askari, 1987, 2000; Schoner et al., 1994; Taniguchi et al., 2001). Studies of ATP binding to Na,K-ATPase and H,K-ATPase showed the simultaneous presence of EP:EATP in the membrane-bound Na/K- (9, 93) and H/K-ATPase (9, 90). One mole of P$_i$ is liberated from each 0.5 mol of EP and 0.5 mol of EATP simultaneously during the H-ATPase reaction, providing strong functional evidence for the oligomericity of the enzyme. Single molecule detection techniques for FITC-treated H/K-ATPase (76) and high-performance liquid chromatography of Na/K-ATPase (83) showed that the soluble tetrameric structure is fully active in both Na,K-ATPase and H,K-ATPase. Hayashi et al. (83) showed that the stoichiometry of ouabain binding to each protomer, diprotomer, and tetraprotomer is close to 1:1:0:75, respectively, and that the fraction of tetraprotomer in C12E8-solubilized Na,K-ATPase increased by >50% in the presence of phosphatidylserine at acidic pH. Kutcha and Thomas (86) showed the presence of diprotomer, tetraprotomer, and much larger oligomers in membrane Na,K-ATPase after treatment with subsolubilizing levels of SDS using time resolved phosphorescence anisotropy. Gable et al. (77) suggested that the binding of SDS to the enzyme increased the clustering of protomers through α-α contacts at discrete domains. Nakamura et al. (44) proposed the existence of two conformational variants in SR-Ca-ATPase molecules that bind two calcium ions, noncooperatively and positive-cooperatively, respectively, in the absence of high concentrations of ATP. Glycerol changed the calcium-sensing characteristics of SR-Ca-ATPase, implying the participation of water molecules in calcium binding and/or subunit interactions. In summary, these results suggest that Na,K-ATPase and related pumps may operate not as independent monomers, as implied by the Post-Albers scheme, but as higher-order structures containing interacting catalytic units.

In the symposium session, E. Bamberg (Max-Planck-Institut für Biophysik) proposed that the α-β subunit interaction has a direct role in catalytic activity assessed using the techniques of cysteine-scanning mutagenesis and voltage-clamp fluorimetry (61), permitting the kinetic effects of [Na$^+$]-dependent steps on electrogenic partial reactions of the Na pump to be examined. The results underlined the role of the Na,K-ATPase β-subunit as a modulating cofactor in the enzymatic properties of the holoenzyme (80). Bamberg also presented measurements of fluorescence resonance energy transfer
between the α and β subunits showing movement of defined residues during the Na,K-ATPase reaction (79). J. Kaplan (University of Illinois Chicago) proposed the β-subunit–dependent polarization of the Na,K-ATPase (87), and the extent of glycosylation of the β2-subunit appeared to play a significant role in Na pump plasma membrane delivery in renal cells (78). O. Vagin et al. (University of California Los Angeles) had previously demonstrated that a reduction in β-subunit glycosylation in the case of H,K-ATPase reduced apical localization, increased ER retention, and increased degradation of H,K-ATPase. Further studies (94) showed that six of the seven N-glycosylation sites in the H,K-ATPase β-subunit are essential for its apical sorting in LLC-PK1 cells.

Data obtained from site-directed mutations introduced into Na,K-ATPase β chains to increase the number of N-glycosylation sites show that the N-glycans linked to the β-subunit isoform of P2-type ATPases contain apical sorting information.

Rajasekaran et al. (11, 89, 155) provided evidence that Na,K-ATPase, the protein serine/threonine phosphatase PP2A, the phospholipid-binding protein annexin II, and occludin form a novel apical junctional complex involved in the regulation of tight-junction permeability in epithelial cells. They also showed that the β-subunit is involved in the suppression of carcinoma cell motility by a mechanism involving an interaction between the two subunits of Na,K-ATPase and PI-3-kinase signaling pathway. The decreased cell motility of β-expressing cells is due to an increased attachment of these cells to the substratum, where the activation of the focal adhesion kinase by Src is involved.

Further studies to clarify the role of subunit interactions during active transport of cations as well as in the regulation of cation pumps are eagerly awaited.

Session IV.
Inherited Dysfunction of Cation Pumps
(Carolyn Slayman, Yale University)

Genetic studies of membrane transport have increased in recent years with the introduction of powerful new methods to map, clone, and manipulate mammalian genes. On the one hand, large-scale genome sequencing and haplotype mapping have made it relatively straightforward to pinpoint genes that correspond to known, transport-related phenotypes. In parallel, it is now easier than ever before to introduce desired mutations into the mouse genome for in vivo functional analysis of individual transporters. Session IV provided five excellent examples of studies in this broad area.

In the first talk, S. Lutsenko (Oregon Health and Sciences University) described the function and regulation of two human copper-transporting ATPases known as the Menkes disease protein (MNKP) and Wilson’s disease protein (WNDP) (Barnes et al., 2005) (15). These ATPases, which are 60% identical in amino acid sequence, were originally discovered by cloning the genes for inherited neurodegenerative disorders. They have since been shown to pump copper from the cytoplasm into the secretory pathway, contributing to the biosynthesis of secreted copper-dependent enzymes and helping to maintain the intracellular copper concentration. Lutsenko and her colleagues have used high-resolution fluorescent imaging to explore the cellular and subcellular distribution of MNKP and WNDP in the mouse cerebellum, where both are expressed. WNDP is localized in Purkinje neurons, where it provides copper for the biosynthesis of secreted proteins such as ceruloplasmin, while MNKP switches its location from Purkinje neurons in early development to closely associated Bergmann glial cells in the adult animal. Interestingly, inactivating the WNDP gene leads to a shift in ceruloplasmin expression from Purkinje neurons to Bergmann glia, where MNKP can now provide the required copper. Thus, there is clearly crosstalk between the two cell types in the regulation of copper distribution.

The second talk was part of a long-term study by investigators at the University of Cincinnati, who are using gene-knockout techniques to dissect the physiological roles of the four α-subunit isoforms of mammalian Na,K-ATPase (14). The general approach has been to construct mice that lack either one copy (heterozygous animals) or both copies (homozygous null animals) of each of the isoform genes. Although homozygous knockouts for the α1, α2, and α3 subunits have proved to be lethal before or at birth, all three heterozygotes are viable and fertile. A. Moseley has now examined the behavior of these animals using a variety of standard methods to assess anxiety (“zero maze” test) and spatial learning and memory (Morris water maze). While α1−/− mice appeared normal, α2−/− mice had reduced locomotor activity and heightened anxiety, and both α2−/− and α3−/− mice showed evidence of impairment in learning and memory. Taken together, the results point to a role of Na,K-ATPase α isoforms in the modulation of behavior.

The remainder of the session explored the way in which human mutations have begun to shed light on the role of Na,K-ATPase in brain function. K. Swendener (Massachusetts General Hospital) began by reviewing recent work in this field (12). Six different mutations of the Na,K-ATPase α3-subunit in eight unrelated families have now been linked to a dominant disorder known as rapid onset dystonia Parkinsonism (RDP), in which metabolic stress leads to involuntary muscle contractions (de Carvalho Aguiar et al., 2004) (12). In parallel, 15 mutations of the Na,K-ATPase α2-subunit in 16 families have been linked to a different disorder known as familial hemiplegic migraine type 2 (FHM2). As pointed out by Swendener, it is probably significant that the α3-subunit is expressed only in neurons, where it makes a
small hyperpolarizing contribution to membrane potential, while the α2-subunit is expressed preferentially in astrocytes, where an impairment of K\(^+\) clearance might be expected to lead to migraines and epilepsies. Sweadner and her coworkers have mapped the mutations associated with RDP and FHM2 onto structural models of the α-subunit and found that most of the affected amino acid residues are predicted to be buried within the protein at important intramolecular contact points. Indeed, when this idea was tested by transfecting several of the mutant forms into oocytes, reduced expression was seen, consistent with defects in protein folding and/or stability. B. Vilsen (University of Aarhus) next presented the results of detailed studies on two mutations of the Na,K-ATPase α3-subunit leading to RDP: F785L, located near the end of membrane-spanning segment 5, and T618M, located within the P domain. Neither mutation completely destroys ATPase function. Rather, both lead to complex sets of kinetic alterations, with a reduction in the affinity of the E, form of the ATPase for Na\(^+\) but no change in the affinity of the E,P form for K\(^+\). Similarly, R. Blostein (McGill University) described three mutations of the Na,K-ATPase α2-subunit that result in familial hemiplegic migraine type 2 (Segall et al., 2004): T345A, R689Q, and M731T (13). All three mutant ATPases display measurable, although abnormal, activity. In one case (T345A), K\(^+\) affinity is decreased, while the remaining two mutants display a reduction in catalytic turnover. As work progresses on both RDP and FHM2, it seems likely that useful insights will emerge into the structure–function relationships of the α2 and α3 isoforms and the role of both isoforms in the brain.

Session VA.

Regulation of Cation Pumps: FXYD proteins
(Haim Gary, Weizmann Institute)

FXYD proteins are a group of seven short transmembrane proteins named after the invariant extracellular motif F-X-Y-D. The various members of this family have been shown to interact specifically with the Na,K-ATPase and alter its kinetic properties. Since each of them has a characteristic tissue distribution, it is assumed that FXYD proteins are tissue-specific modulators or auxillary subunits of the Na, K-ATPase, which adapt its kinetic properties to the physiological needs of a particular tissue, cell type, or physiological state, without affecting it elsewhere (for a recent review see Garty and Karlish, 2005). The best studied family members are phospholemman (FXYD1), the major phosphoprotein in heart and muscle; the γ-subunit of Na,K-ATPase (FXYD2), a kidney-specific auxiliary subunit of the pump; CHIF (FXYD4), an aldosterone-induced protein expressed in kidney and colon; and FXYD10, a phospholemman-like protein from shark rectal gland. The four speakers in this session addressed various aspects of the structural and functional interactions between FXYD proteins and the α-subunit of the Na,K-ATPase. The major conclusions, supported by complementary data from different laboratories are as follows. (a) The transmembrane domain of FXYD proteins plays a key role in their effects on the apparent affinity of the pump for cytoplasmic Na\(^+\) and extracellular K\(^+\). (b) This segment is located in a groove formed between the second, fourth, and ninth transmembrane domains (TM2, TM4, and TM9) of the α-subunit of Na,K-ATPase. (c) The FXYD transmembrane domain is also critical for the high affinity interactions with α, but the residues involved are different from those mediating the functional effects.

K. Geering (Université de Lausanne) reported a mutation analysis of TM9, identifying residues involved in the interactions with FXYD proteins (16). One conclusion is that different amino acids mediate the efficient association with FXYD proteins and the functional effects of these FXYD proteins on the K\(^+\) affinity of Na,K-ATPase. This conclusion was confirmed by tryptophan-scanning mutagenesis analysis of the transmembrane domain of FXYD7. The amino acids required for efficient association with α appear to be clustered in a region that contains two well conserved glycines. In the second part of her presentation Geering reported the characterization of FXYD3, a poorly studied member of the family. This FXYD protein was found to have two unique properties. First, unlike FXYD1, FXYD4, and FXYD5, the putative signal peptide of FXYD3 is not cleaved and therefore it may have two, rather than one, transmembrane domains. Second, FXYD3 appears to be the only member of the family that interacts with the H,K-ATPase, in addition to the Na,K-ATPase.

Structural aspects of FXYD proteins were addressed further by F. Marassi (Burnham Institute). Her group expressed FXYD1, FXYD3, and FXYD4 in E. coli and studied the structure of the purified proteins in detergent micelles and lipid bilayers by NMR spectroscopy. The transmembrane domains of the three proteins were found to be “classical” α helices with a periodicity of 3.6 residues. The transmembrane helix is flanked by two additional short helical regions in the extracellular and intracellular domains and a fourth helical segment is found near the COOH terminus. Interestingly, the boundaries between the helical regions and the connecting segments coincide with the positions of intron–exon junctions in the corresponding genes. This may account for the puzzling observation that in spite of the small size of FXYD proteins (<100 amino acids), each of them is coded by at least six exons, and the conserved 35-amino acid segment is made up of three separate exons. The above data suggest that the FXYD proteins may have been assembled from small discrete structural modules through exon shuffling.

The third presentation by F. Cornelius (University of Aarhus) addressed the structure of FXYD10 and the
phosphorylation-induced conformational change of the cytoplasmic segment of this protein (17). The structural interactions between shark Na,K-ATPase and the cytoplasmic domain of FXYD10 were investigated using chemical cross-linking, which has placed the COOH terminus of FXYD10 in the vicinity of Cys-254 in the A domain of α. Such interactions within the A domain could explain the inhibitory effects of FXYD10 and the effects of its phosphorylation. Experiments with NH₂-terminal truncation of α and addition of exogenous FXYD10 indicate an interaction between these moieties in shark enzyme, but not in pig kidney enzyme.

In the final talk, H. Garty (Weizmann Institute) discussed structural and functional features of the transmembrane domains of a number of FXYD proteins (18). Chemical cross-linking has located Cys-49 in the transmembrane segment of FXYD4 at a distance of 3–6 Å from Cys-140 in the TM2 domain of α. Similar interactions have been demonstrated for the corresponding FXYD1 and FXYD2 residues (Phe-48 and Phe-36, respectively), suggesting that the general disposition of the three FXYD proteins with respect to TM2 is similar. The fact that FXYD2 and FXYD4 have opposite effects on the apparent affinity of the Na,K-ATPase for cell Na⁺ has been used to analyze the structure–function relationships of these proteins. Examining the functional effects of FXYD2/FXYD4 chimeras as well as mutants in which individual amino acids were exchanged between the two proteins has underscored the key role of several transmembrane residues in determining the apparent affinity of the pump for cell Na⁺.

Together, these four presentations provided valuable new insights into the structure and structure–function relationships of FXYD proteins and established the role of this family in the physiological regulation of the Na,K-ATPase.

Session VB.

Regulation of Cation Pumps: Phospholamban, Sarcolipin and Receptor-mediated Regulation

(Lawrence G. Palmer, Cornell University)

The fifth session of the symposium focused on the regulation of Na,K-ATPase and Ca-ATPase, particularly by phosphorylation-dependent events. There are at least two such pathways. One involves phosphorylation-dependent movement of pump proteins to and from the plasma membrane. The other entails the modulation by phosphorylation of the interactions of pumps with accessory membrane proteins.

A. Bertorello (Karolinska Institute) described the trafficking of Na,K-ATPase, particularly in response to signals from G protein–coupled receptors. These events are clearly important in regulating pump activity in epithelial cells (21). In renal cells, activity is increased by angiotensin II and decreased by dopamine and parathyroid hormone. This regulation correlates well with changes in transepithelial Na⁺ reabsorption by the renal proximal tubule in response to these hormones, and reflects insertion of pumps into and retrieval from the plasma membrane, respectively. Lung epithelial cells also regulate the number of pumps on their surface, although the signal transduction pathways are different. Dopamine, for example, which decreases pump activity in the kidney, actually increases it in the lung. C. Pedemonte (University of Houston) discussed the involvement of phosphorylation in these events, particularly the roles of two phosphorylation sites in the α1-subunit of Na,K-ATPase, Ser-18, and Ser-11 (147). When epithelial cells were transfected with pumps carrying the S11A mutation, both stimulation of activity by angiotensin II and phorbol ester and inhibition by dopamine were abolished. Ser-18 was also essential for regulation of pump activity in opossum kidney cells. Phosphorylation of these sites appears to be required for regulated trafficking of the protein rather than for activity per se.

The sarcoplasmic/endooplasmic reticulum Ca-ATPase (SERCA) and its interaction with the accessory protein phospholamban (PLB) provide an example of the second type of regulation. D. Thomas (University of Minnesota) used several biochemical approaches to study this phenomenon (19). FRET analysis indicated that the two membrane proteins bind to each other with high affinity. PLB inhibits pump activity, and this inhibition, but not binding itself, is relieved either by high Ca²⁺ concentrations or by phosphorylation of PLB at Ser-19. NMR and EPR techniques were used to determine the structure of PLB in lipid micelles. The cytoplasmic domain of the protein can adopt several conformations with increasing degrees of disorder and extension, with phosphorylation favoring the ordered state. Thomas proposed that the most extended and disordered form of PLB is responsible for inhibition of the pump, acting by preventing ATP-induced changes in the P-domain of SERCA. Phosphorylation of PLB would then relieve inhibition through stabilization of the ordered state of the cytoplasmic tail.

A similar scheme may account for modulation of Na,K-ATPase in cardiac myocytes by β-adrenergic signals. D. Bers (Loyola University Chicago) and colleagues showed that the β-adrenergic agonist isoproterenol stimulated pump activity by decreasing the apparent K⁺m of the pump for intracellular Na⁺ (123). This regulation involves the FXYD-protein phospholemman (PLM) as it is absent in PLM-knockout mice. FRET analysis indicated a direct interaction between Na,K-ATPase and PLM in the membrane of HEK293 cells (122); this interaction was reduced by activation of either PKA or PKC. This suggests that hormones such as noradrenaline may increase pump activity by phosphorylating PLM and reversing the increased K⁺m for Na⁺ that results from PLM binding.
Regulation of Cation Pumps: Specific P-type ATPase Inhibitors (Robert Farley, University of Southern California)

Symposium session V continued the theme of regulation of cation pumps with two presentations dealing with specific P-type ATPase inhibitors. Perhaps the best known P-type ATPase inhibitors are the cardiac glycosides such as ouabain and digitalis, which inhibit Na,K-ATPase. The inhibition of the sodium pump by these molecules was originally discovered by Hans Schatzmann who published this result in 1953. Schatzmann was looking to obtain evidence in erythrocytes to support the suggestion by W. Wilbrandt that mineralocorticoids might stimulate Na° transport in the kidney tubule because they chelate the ion and function as a mobile Na° carrier, or as the Na°-binding site in a pump protein. Frustrated by the failure to observe the expected stimulation of Na° transport, Schatzmann decided to try another steroid, ß-strophantoside, which produced a clear inhibition of the pump. An account of this discovery is provided in Schatzmann’s recollection published in 1995 (Schatzmann, 1995). It quickly became apparent that rodent Na,K-ATPase is far less sensitive to inhibition by cardiac glycosides than other species. The explanation for this observation was provided in 1988 by Price and Lingrel (1988), who showed that two charged amino acids in the Na,K-ATPase α-subunit at the border between the membrane and the extracellular loop connecting TM 1 and 2 were responsible for the relative resistance of the rodent pump. These amino acids are not charged in sensitive species. The difference in these amino acids between sensitive and resistant species is not sufficient to explain the specificity of cardiac glycosides for Na,K-ATPase, however, because the gastric H,K-ATPase, which is not inhibited by these molecules, also has the uncharged amino acids at these positions. Many reports that describe effects of site-directed mutations on cardiac glycoside affinity or inhibition of the sodium pump have been published from different labs in an effort to define the essential amino acids in the binding site for these molecules. The conclusions drawn from these reports are limited, however, because they describe loss-of-function mutations. In his presentation in this session, J.J. de Pont (Radboud University) described the use of chimeric Na,K-/H,K-ATPase polypeptides and point mutations to confer high-affinity ouabain binding to the gastric H,K-ATPase (20). In the end, gastric H,K-ATPase containing only seven amino acids from Na,K-ATPase in the extracellular loops connecting TM 3 and 4, and TM 5 and 6, bound ouabain with an affinity comparable to that of native Na,K-ATPase. Similar high-affinity ouabain binding was conferred to the ouabain-insensitive nongastric H,K-ATPase using the same approach. Of course, additional amino acids are probably involved in the recognition of the cardiac glycosides by the protein, but they are the same in the two ATPases. A homology model was described for Na,K-ATPase, based on the E2P crystal structure of Ca-ATPase, in which the amino acids identified by de Pont and his collaborators, and several other amino acids identified earlier in loss-of-function mutants, are important for cardiac glycoside binding.

The second group of specific P-type ATPase inhibitors discussed in this session were the inhibitors of the gastric H,K-ATPase. Two classes of molecules have been found to be important and useful inhibitors of this enzyme. The first class is the pyridylmethylsulfinyl benzimidazoles such as omeprazole. These molecules were shown to inhibit gastric acid secretion in the late 1970s and the H,K-ATPase in the early 1980s. They are weak bases that accumulate in acid space of the secretory canalculus where they are acid activated to form cationic thiophilic intermediates. There they react with one or more cysteine sulphydryls at the luminal surface of the acid pump to form disulfide bonds with the protein. These covalent inhibitors of the H,K-ATPase have had widespread commercial success, with omeprazole sold over the counter as Prilosec, and lansoprazole (Prevacid) being the no. 3 prescription drug sold in the USA in 2004 with over $4 billion in sales. The second class of inhibitors is the substituted imidazo [1,2a]pyridines, which are K°-competitive inhibitors of the H,K-ATPase. The best known example of these molecules is SCH28080, and although several derivatives of this drug have been developed, they are not yet on the market. These drugs were serendipitously discovered and shown to inhibit gastric H,K-ATPase in the early 1980s with submicromolar IC50 values. G. Sachs (University of California Los Angeles) delivered a comprehensive presentation about the two classes of H,K-ATPase inhibitors and their interaction with the pump. A homology model for the H,K-ATPase was developed based on the crystal structure of Ca-ATPase, and the effects of many point mutations in the H,K-ATPase on omeprazole or SCH28080 affinity were explained using this model. A mechanism of hydronium ion transport by the ATPase was proposed, and a molecular dynamics simulation for the path of K° binding to the pump showed the importance of several amino acids in this process. This simulation also demonstrated the importance of these amino acids in the competitive inhibition of K° binding by SCH28080.

Session VD.

Regulation of Cation Pumps: Signaling via Na,K-ATPase (Amir Askari, Medical University of Ohio)

Though this session was listed under the heading “Regulation of Cation Pumps,” the topics covered represent the other side of the regulation coin, i.e., regulation by Na,K-ATPase of the functions of other cellular proteins. This is a relatively new area of research. Of course, it has been known for a long time that ion pumping by Na,K-ATPase is essential to the life of most mammalian...
cells, and that some specialized cell functions (e.g., cardiac contractility) are regulated by changes in intracellular ion concentrations that result from alterations of the pumping function of this enzyme. What is new about Na,K-ATPase and signaling is the recent realization (Xie and Askari, 2002) that many of the regulatory effects of Na,K-ATPase on cellular functions are not the consequence of changes in its ion pumping function, but rather due to interaction of pump proteins with the neighboring membrane proteins, the ligand-induced changes in these protein–protein interactions, and the resulting alterations in the network of signaling cascades that are known to be linked to these pump neighbors. The invited speakers of this symposium covered selected topics in this growing area of research.

The introductory remarks by the chair (A. Askari, Medical University of Ohio) dealt with the existence and mechanism of amplification within the signaling pathways that are linked to Na,K-ATPase, and are activated in response to ouabain and related cardiac glycosides that are also considered to be the endogenous physiological ligands of this enzyme/receptor (Schoner and Scheiner-Bobis, 2005). In a number of recent studies on different types of intact cells, it has been noted that ouabain-induced signaling and its varied downstream effects (e.g., stimulation or inhibition growth) are obtained at such low ouabain concentrations that little or no inhibition of the pumping function of Na,K-ATPase is measurable. Such findings have often raised questions, either by the investigators doing the work or by critics, as to whether these low-dose effects may be due to ouabain binding to receptors other than Na,K-ATPase. The speaker suggested signal amplification, a well recognized phenomenon in receptor biology, as a more reasonable explanation (164). He presented data showing that in human breast cancer cells, where low and nonlethal concentrations of ouabain inhibit proliferation through transactivation of Src/EGFR, sustained activation of ERK1/2, and the resulting induction of p21Cip1 there is indeed such amplification, and that occupation of <10% of the receptors (Na,K-ATPase) by the signal (ouabain) is sufficient to yield >90% response (growth arrest) (Kometiani et al., 2005). Regarding the most likely mechanism of this gain in sensitivity, it was suggested, by analogy with the extensively studied amplification in bacterial chemotaxis, that clusters/teams of the oligomeric Na,K-ATPase and its signaling partners within the restricted space of caveolae may be responsible for this signal amplification (164).

In the presentation by Z. Xie (Medical University of Ohio), emphasis was on the interaction of Na,K-ATPase with Src. He reviewed his published data on ouabain-induced signaling in caveolae of the renal epithelial cells, indicating that an early event in the signaling cascade is the activation of Src. New evidence was then presented to show that this is indeed due to the direct interaction of Src with the α-subunit of Na,K-ATPase. Using purified kidney Na,K-ATPase, recombinant Src, and GST- and His-tagged fusion products of the various Src and α-subunit domains, the results of in vitro binding assays showed the following. (a) Src interacts with α-subunit at two sites, one involving the binding of Src’s SH2 domain to the cytoplasmic domain of α-subunit that connects transmembrane helices 2 and 3, and the other, the binding of Src’s kinase domain to the cytoplasmic domain connecting transmembrane helices 4 and 5 of α-subunit. (b) The latter interaction causes Src inhibition, and ouabain disrupts this interaction, disinhibiting the bound Src. FRET and BRET analyses in intact cells transfected with tagged constructs of Src and α-subunit were then presented that supported the conclusions of the binding assays. This is the first definitive identification of an immediate signaling partner of Na,K-ATPase, allowing further studies on the molecular details of Src interaction with Na,K-ATPase. Xie also presented FRET data indicating that inactive mutants of α-subunit (D369N and D369E) also interact with Src, providing further support for the independence of the signaling function of Na,K-ATPase from its catalytic and pumping functions.

Once it is realized that Na,K-ATPase may function as a signal transducer due to interaction with neighboring signaling proteins, it is evident that there will be significant diversity in the nature of the connected pathways and the downstream consequences in different cell types. The presentation by G. Scheiner-Bobis (Justus-Leibig-Universität) exemplified such diversity (163). This laboratory had already shown that in endothelial cells of human umbilical artery, low ouabain concentrations that certainly do not inhibit the pump significantly, and may indeed induce activation of ERK1/2, promote transcription of the endothelin-1 gene and stimulate cell proliferation. Continuing this work, Scheiner-Bobis reported new data showing that in these endothelial cells, the same low ouabain concentrations activate Akt (protein kinase B), a prominent member of the “cell survival” squad of protein kinases, and that this is preceded by the activation of PI3-kinase and followed by the activation of eNOS and increased generation of nitric oxide. Evidence was also presented to suggest that the above activated cascades may be linked to ouabain-induced transactivation of the insulin receptor; adding this receptor tyrosine kinase to the list of candidates as direct interacting neighbors of Na,K-ATPase.

The last speaker of the session, R.G. Contreras (Center for Research and Advanced Studies, Mexico) focused on the regulation of cell–cell adhesion and cell-substrate attachment by Na,K-ATPase (91, 158). This research team had already reported on the nature of the signaling pathways that link Na,K-ATPase to cell attachment. They showed that ouabain and other known inhibitors of the enzyme alter intracellular ionic content and detachment-related signals, but that there is no
causal association between the signaling events and pump inhibition. Reviewing these findings and presenting new data, Contreras concluded that the order in which the signaling cascade proceeds to reversible cell detachment is as follows: (1) ouabain binding to Na,K-ATPase, (2) Src activation, (3) ERK1/2 activation, (4) inhibition/down-regulation of RhoA, and (5) release of occludin, β-catenin, and FAK from tight junctions, adherens junctions, and focal adhesions, respectively. In addition to providing insight into the complex mechanisms of cell attachment and their relation to Na,K-ATPase, these results are of further significance; thus, when considered along with the findings of other laboratories on the signal transducing effects of Na,K-ATPase in renal epithelial cells (Wang et al., 2004), the inescapable conclusion is that even in studies of the same cell type, each laboratory must be looking, by choice or necessity, at only a limited segment of a signaling network that is enormously complicated. Perhaps it only seems to be so at this early phase of the field’s development.

Session VI.
Physiological Roles of Cation Pumps
(Mordecai Blaustein, University of Maryland)

The final session of the conference was devoted to the physiological roles of Na,K-ATPase and Ca-ATPases, with emphasis on knowledge gained from null or point mutations, or altered physiological conditions. M.P. Blaustein (University of Maryland) began the session by emphasizing the functional significance of the Na+ pump α-subunit isoforms. His laboratory’s studies using NH2-terminal 90–120 amino acid segments revealed that the sorting signal for the α2 isoform, but not α1, is a 10–20 amino acid sequence close to the NH2 terminus. This sequence targets α2 to plasma membrane (PM) microdomains that overlie “junctional” sarco-/endoplasmic reticulum (S/ER) in astrocytes and arterial smooth muscle cells (ASMCs). These cells normally express α1 and α2 Na+ pumps in ~4:1 ratio. Full-length α1 and α2 isoforms with a protein Ca2+ sensor (GCaMP2) attached to the COOH termini were transfected into these cells. The expressed α2 fusion protein, but not the α1 construct, detected the Ca2+ entry through store-operated channels (SOCs) that are associated with S/ER Ca2+ store refilling. Thus, α1 and α2 are expressed in different, nonoverlapping PM domains; only α2 is confined to the PM microdomains at PM-S/ER junctions. What, then, is the difference in the function of these 90% identical proteins?

This question was addressed by comparing arterial function in wild-type (WT) mice and mice with a null mutation in one α1 or one α2 gene (α1+/- and α2+/-; lines generated in J.B. Lingrel’s lab). Myogenic tone is the spontaneous constriction of intact arteries when intraluminal pressure is raised above 10–20 mm Hg. The greater the tone, the greater the resistance to blood flow and the higher the blood pressure in intact animals. Low dose (1–100 nM) ouabain, which inhibits only the α2 Na+ pumps in rodents, elevated cytoplasmic Ca2+ and augmented myogenic tone in small (120–140 μm diameter) mesenteric arteries. Isolated mesenteric arteries from α1+/- mice had normal myogenic tone. In contrast, arteries from α2+/- mice, like those from WT mice treated with low dose ouabain, had significantly more myogenic tone at intraluminal pressures between 20 and 100 mm Hg. The increased vascular tone (in vitro) was reflected by a significantly higher blood pressure in the α2+/- mice than in the α1+/- or WT mice. Thus, α2 Na+ pumps control myogenic tone and long-term blood pressure; reduced α2 activity increases myogenic tone and blood pressure.

J.B. Lingrel (University of Cincinnati) continued with this theme of the role of Na+ pump α-subunit isoforms in the pathogenesis of hypertension (22). His aim is to elucidate the role of the cardiotonic steroid binding site on the α-subunit isoforms. He and his colleagues have studied two mouse hypertension models: the hypertension induced by chronic administration of low-dose ouabain and that induced by the administration of ACTH, which is known to stimulate the synthesis and secretion of endogenous ouabain by adrenal glomerulosa cells. The Lingrel group generated mice with a targeted mutation in the α2-subunit ouabain-binding site that converts it from a high ouabain affinity to a low affinity site (i.e., ouabain resistant). In contrast to the effect in WT mice, blood pressure did not rise in the mice with this α2 mutation when ouabain was administered chronically. ACTH administration also failed to elevate blood pressure in mice with this α2 mutation. In addition, the ACTH-induced elevation of blood pressure in WT mice was antagonized by Digibind, an antibody preparation that is used clinically to treat digitalis intoxication; Digibind binds cardiotonic steroids including digoxin and ouabain. These seminal observations demonstrate, convincingly, that there is a circulating ligand (a ouabain-like compound) for the cardiotonic steroid binding site, and that it plays a role in the pathogenesis of some forms of hypertension. The data also may resolve a long-standing puzzle about the “missing hormone” in ACTH-induced hypertension.

Taken together, the observations from the Lingrel and Blaustein labs suggest that reduced α2 Na+ pump activity plays a key role in some forms of hypertension in rodents, in which the α1 isoform has an unusually low affinity for the drug. In humans, however, α1 as well as α2 have a high affinity for ouabain. Therefore, to examine the role of α1 Na+ pumps, Lingrel and colleagues generated mice with mutated α1 (high ouabain affinity) and α2 (low ouabain affinity). ACTH very rapidly (within 24 h) induced a severe, Digibind-sensitive hypertension in these mice. Thus, it appears
that inhibition of either α1 or α2 can cause hypertension. But this appears to contradict the evidence (described above) that α1+/− mice have normal blood pressure, even though expression of the predominant α1 isoform is reduced by ~50%. A possible explanation is that there may be compensation for prolonged (e.g., congenital) reduction of α1 activity (or expression), whereas reduced α2 activity (or expression) may not be compensated. The resolution to this dilemma has important implications for understanding the pathogenesis of at least some forms of human (essential) hypertension.

The central role of renal epithelial (α1) Na+ pumps in salt (NaCl) and water reabsorption is well documented; indeed excessive salt reabsorption/ retention is widely recognized to trigger hypertension. Less well recognized is the evidence that α1 Na+ pumps in the basolateral membrane of the pulmonary alveolar epithelial cells (AEC) play a critical role in fluid reabsorption from the alveoli. Hypoxia, which may have a variety of causes, including the ascent to high altitude, and lung pathologies, including pulmonary fibrosis and chronic obstructive pulmonary disease, reduces the clearance of alveolar fluid by AEC. The accumulation of fluid in the alveoli (pulmonary edema) is due to a decreased number of Na+ pumps in the AEC plasma membrane. J.I. Szajdier (Northwestern University) described the mechanism(s) responsible for the hypoxia-induced retrieval of Na+ pumps from the plasma membrane. Within 6 to 24 h after acute hypoxia (8% O2), there is as much as a 50% decrease in the Na+ pumps on the surface. The total Na,K-ATPase activity in the AEC is not reduced, however. Instead, the Na+ pumps are retrieved from the surface by endocytosis and are retained within endosomal vesicles; these pumps are available for recycling to the surface upon reoxygenation.

A hypoxia-inducible factor, HIF-1α, appears to be the master controller for this recycling. Hypoxia, via ubiquination of HIF-1α, stimulates mitochondria to produce reactive oxygen species (ROS). ROS promote the retrieval of Na+ pumps into endosomes by activating protein kinase C-ε (PKC-ε)-mediated phosphorylation of the Na+ pump α1-subunit on Ser-18. Antioxidants, a PKC-ε antagonist (bisindolylmaleimide I, Bis), and mutation of Ser-18 to Ala all prevented the hypoxia-induced retrieval of the α1 Na+ pumps.

G. Shull (University of Cincinnati) focused on some pathophysiology of Ca2+ transport; he discussed the tumorigenic effects of haploinsufficiency of two different Ca-ATPases, SERCA2 and SPCA1 (23). SERCA2 is the S/ER Ca2+ pump found in the heart, slow twitch skeletal muscle, smooth muscle, and nonmuscle cells including adult skin epidermis. In humans, missense mutations of SERCA2 have been found in Darier disease, which is manifested by keratotic lesions of the skin and nails. Haploinsufficiency of SERCA2 predisposes mice to squamous cell tumors, and represents a novel mode of cancer susceptibility. Shull found that 89% of SERCA2+/− mice developed squamous cell carcinomas and papillomas (there were 107 tumors in 49 mice), whereas only 1 out of 100 WT mice had a tumor. The tumors were heavily keratinized, and were associated with increased expression of keratins 6 and 17 as well as increased p53 and H-ras in the forestomach tumors. These effects were apparently not the result of SERCA2 insufficiency due to loss of one allele because the remaining allele was up-regulated and SERCA2 expression was as robust in the WT animals. Perhaps it is the factors that promote up-regulation of the WT allele in the heterozygotes that induce the tumorigenesis.

Hailey-Hailey disease, another dermatosis characterized by keratin overexpression, is associated with missense mutations in SPCA1. This is the secretary pathway Ca2+ pump that is found in most cell types and is especially abundant in keratinocytes and in the kidney. Mice with haploinsufficiency of SPCA1 (SPCA1+/−) also develop squamous cell carcinomas and papillomas that overexpress keratin; tumors were observed in 6 of 26 SPCA1+/− mice, but in none of the 23 controls. These tumors are, however, later in onset and tend to be located in different places than those induced in SERCA2+/− mice.

Both SERCA and SPCA affect protein processing and trafficking. Shull raised the possibility that the increase in cytoplasmic Ca2+ concentration that occurs with the haploinsufficiency of either of these pumps may, by altering protein processing, exert a permissive effect on tumorigenesis. This may provide novel insight into an important pathway that regulates cell proliferation.

In sum, these four reports elucidate the central role that certain widely distributed ATP-driven ion pumps play in some very common pathophysiological processes.

The Na/K pump represents a cornerstone of general physiology, creating the ion concentration differences essential for cell excitability, cell volume control, and transepithelial ion and water transport. How the protein transduces the chemical energy of ATP into that stored in ion gradients remains a critical unsolved problem. However, this symposium showed that an understanding of this process at a molecular level may be close at hand. Perhaps the most dramatic progress in recent years is the elucidation of the atomic structure of the closely related Ca-ATPase at different stages of the pump cycle; similar structures of the Na,K-ATPase have been inferred by homology and actual crystal structures of the Na pump should come soon. This information interacts synergistically with increasingly detailed physiological and biochemical measurements of pump function. At the same time, new and
sophisticated genetic and cell biological approaches are revealing the roles of the pump in regulating cellular processes. The symposium showed that focusing on a single protein family through the lenses of different disciplines can reveal much about the workings of transporters, cells, and organs.

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