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THE NA/K-ATPASE AND ITS ISOZYMES: WHAT WE HAVE LEARNED USING THE BACULOVIRUS EXPRESSION SYSTEM

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1. ABSTRACT

The use of the baculovirus expression system for heterologous protein expression in insect cells has been of immense value in understanding many different structural and functional aspects of the Na/K-ATPase, an enzyme that catalyses the active exchange of cytoplasmic Na^+ for extracellular K^+ across the plasma membrane of most cells. The advantage of using insect cells is that they provide an eukaryotic expression system able to produce large amounts of active recombinant Na/K-ATPase for biochemical studies. In addition, the host cell lines commonly used (Sf-9, Sf-21 and High Five) have very little or no Na/K-ATPase, offering an environment in which the exogenously expressed enzyme can be studied without the interference of the high Na pump background activity common to other cells. The present article reviews the advances obtained in the field of the Na/K-ATPase by using the baculovirus expression system, including the biosynthesis, assembly, intracellular trafficking of the α and β subunits of the enzyme, their interaction with other proteins, and the structure, function and regulation of the various isozymes of the transporter.

2. THE NA/K-ATPASE AND ITS STRUCTURAL HETEROGENEITY

The Na/K-ATPase, or Na pump, belongs to a class of transporters widely distributed in prokaryotic and eukaryotic cells known as the P-type ATPases. The members of this family transport a variety of ions across the plasma membrane of cells sharing a mechanism that involves the formation of a transient acylphosphate intermediate from ATP during their reaction cycle (1,2). In particular, the Na/K-ATPase uses the energy from the hydrolysis of ATP to exchange cytoplasmic Na^+ for extracellular K^+ in a 3:2 stoichiometry (3,4). The asymmetric distribution of ions created by the Na/K-ATPase play a central role in maintaining cell volume and pH, in keeping cell resting membrane potential, and in providing the chemical energy for the secondary transport

of other ions, solutes and water across the cell membrane (5). Structurally, the Na/K-ATPase is characterized by a high molecular complexity. The enzyme is constituted by two main subunits, α and β, both of which exist in different molecular variants or isoforms. At present, four distinct α polypeptides, named α1, α2, α3 and α4, and three different β subunits, known as β1, β2 and β3 are expressed in mammalian tissues in a cell type specific and developmentally regulated manner (6-11). In addition, a fourth putative isoform of the β polypeptide (βm or β4) has been described in muscle (12). The association of the various α and β subunits in different arrangements results in formation of multiple forms or isozymes of the Na/K-ATPase (10).

All α isoforms are multi-spanning membrane proteins with a molecular mass between 110-112 kDa, arranged in ten transmembrane segments, a small extracellular domain, and a large cytoplasmic region. The α subunits are responsible for the catalytic and transport properties of the Na/K-ATPase and contain the binding sites for the cations, ATP and the cardiotonic steroid ouabain (3,4). The ATP-coupled translocation of ions depends on conformational changes in the α proteins, designated E1 and E2. These changes involve the interaction of the cytoplasmic mid region of the α polypeptide that binds and hydrolyses ATP, with the transmembrane domains that coordinate and bind Na^+ and K^+ (13). Comparison with the sarcoplasmic reticulum Ca-ATPase (SERCA) for which high resolution crystals have been obtained (14,15) shows that during its reaction cycle, the Na/K-ATPase may undergo changes in the position of the transmembrane helices, and exhibit drastic movements of three major domains at the cytoplasmic side of the protein. These domains include the so called actuator or A domain placed at the N-terminus and first intracellular loop between transmembrane domains 2 and 3, The N or nucleotide binding segment, and the P or phosphorylation site, both of which are located between transmembrane domains 4 and 5 (16).

The β subunits are type II glycosylated proteins that present an N-terminal cytoplasmic tail, a single transmembrane region and a large C-terminal extracellular domain, which characteristically exhibits three disulfide bridges and consensus sequences for glycosylation. The β subunits have variable molecular weights, between 40 and 60 kDa depending on isoform and tissue specific differences in glycosylation (6). Although the β polypeptides do not directly interact with the cations or ATP, they are essential for normal activity of the Na/K-ATPase, and can determine some of the transport and catalytic properties of the enzyme. Probably more conspicuous, is the role of the β subunit during synthesis of the enzyme which consists in assisting in the correct folding and delivery of the α polypeptide to the plasma membrane (17).

In addition to the α and the β subunits, a third kind of polypeptides are associated to the Na/K-ATPase. These include small single membrane spanning proteins, the first of which was discovered in the kidney and named the γ subunit (18). Later, several other polypeptides that share a common FXYD motif with the γ subunit were identified in different tissues. These include (Phospholemman or FXYD1), MAT-8 or FXYD3, CHIF or FXYD4, RIC or FXYD5, and the FXYD6 and FXYD7 polypeptides from nervous system (19). All these accessory proteins are not required for activity of the Na/K-ATPase, but appear to influence the transport and kinetics properties of the Na/K-ATPase and can also function as ion channels (20-22).

The use of heterologous expression systems to study the Na/K-ATPase in cells in culture has been an essential tool in elucidating many structural and functional aspects of the enzyme. The subunits and isoforms of the Na/K-ATPase, as well as many different mutants, chimeras and truncated forms of the Na pump α and β polypeptides have been produced in bacteria, protozoa, yeast, amphibian, insect and mammalian cells (23-32). Each of these expression systems, with intrinsic advantages and disadvantages, have importantly contributed to our understanding of the subunit requirements of the enzyme, the ligand binding sites of the α subunit, the oligomeric structure of the transporter, the function of the different isozymes and their particular regulation. This article reviews the advances obtained in the field of the Na/K-ATPase by the exogenous expression of the enzyme in insect cells using the baculovirus expression system.

3. RECOMBINANT BACULOVIRUSES FOR THE STUDY OF THE NA/K-ATPASE

The baculovirus expression system is an attractive eukaryotic system for the study of P-type ATPases and specifically the Na/K-ATPase. The system uses the baculovirus, *Autographa californica* nuclear polyhidrosis virus (AcNPV) to infect insect cells, commonly Sf-9 and Sf-21 cell lines that derive from the ovary of the fall armyworm, *Spodoptera frugiperda* and *Trichoplusia ni* or High Five cells (33,34). Expression in the cells is directed by recombinant viruses in which the gene of interest is placed under the control of a promoter that naturally directs the synthesis of polyhedrin, the protein that coats AcNPV. Because the promoter is highly active at late stages of the infective cycle, the peak of protein production is usually achieved between 24 and 72h after infection (33,34). A major advantage of this system for studying the Na/K-ATPase is that, in contrast to cells from most vertebrates, the Sf-9, Sf-21 and High Five cells have very little or no endogenous Na/K-ATPase, allowing expression of the Na pump polypeptides in an environment relatively free of any major contaminating Na/K-ATPase activity (35,36). Insect cells carry out most of the co- and post-translational protein modifications that mammalian cells perform, including core glycosylation, proteolytic processing, signal peptide cleavage, phosphorylation, and lipid modification (34). The insect cells, however, have restricted capacity to perform complex glycosylation, a fact that, for the Na/K-ATPase, is reflected in the faster electrophoretic mobility of the Na/K-ATPase β polypeptides (35). Because, as will be described, complex

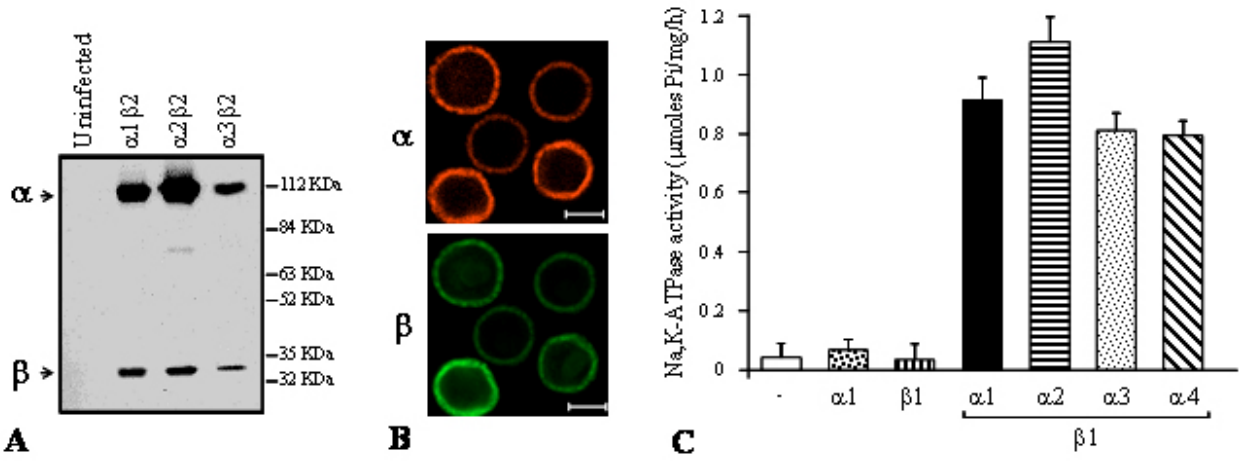

Figure 1. Functional expression of Na/K-ATPase α and β isoforms in Sf-9 insect cells. A) Expression and assembly of the α and β polypeptides in Sf-9 cells. After 48 h infection, cells metabolically labeled with ^{35}S-methionine were lysed with 1% CHAPS in 150 mM NaCl, 25 mM Hepes pH 7.4 and subjected to immunoprecipitation using 5α, an antibody that recognizes all α isoforms. Immunoprecipitated proteins were then separated by SDS/PAGE and analyzed by autoradiography. B) Immunolocalization of α1 and β1 polypeptides in Sf-9 cells. Cells grown in 24 well plates on glass cover-slips were infected for 48 hs. After fixation with paraformaldehyde immunofluorescence was performed using C464-6B antibody and a polyclonal affinity purified anti-β antiserum to detect the Na/K-ATPase α and β subunits respectively. As secondary antibodies, a rhodamine-conjugated goat anti-mouse and a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit were used. Scale bars = 10 μm. C) Na/K-ATPase activity levels for different Na/K-ATPase isozymes expressed in Sf-9 cells. Activity represents the hydrolysis of ATP sensitive to 1 mM ouabain, measured in medium containing: 120 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 3 mM [γ-^{32}P]ATP-cold ATP, 30 mM Tris-HCl (pH 7.4).

glycosylation has minimal consequences for the intracellular processing or function of the Na pump (17), this characteristic does not represent a limitation for studying the Na/K-ATPase. Importantly, insect cells are able to express high amounts of the Na/K-ATPase polypeptides, they can accurately assemble the α and β isoforms, and are able to efficiently deliver catalytically active Na/K-ATPase molecules to the plasma membrane of the cells. This is shown in Figure 1 for different Na/K-ATPase α and β polypeptides. The ability to produce functional Na/K-ATPase molecules in infected insect cells has proven the baculovirus expression system as an excellent tool to investigate different aspects of the Na/K-ATPase. These are discussed in the following sections.

3.1. Subunit requirement for the Na/K-ATPase

Studies in insect cells, as well as in other cell systems, indicate that co-expression of both the α and β subunits are required for function of the Na/K-ATPase (35,37,38). Thus, co-infection of Sf-9 cells with α and β viruses results in catalytically competent Na/K-ATPase that undergoes the normal cycle of reactions of the native enzyme (35,39). This is reflected by: a) a Na^+- and K^+-dependent, ouabain sensitive hydrolysis of ATP, b) binding of [^{3}H]-ouabain to the enzyme, c) phosphorylation of the α subunit from ATP that is dependent on Mg^{2+} and Na^+, sensitive to K^+, and blocked by ouabain, d) phosphorylation of the α subunit by Pi that is stabilized by ouabain, and e) ouabain-sensitive transport of ^{86}Rb and ^{22}Na (35,36,39).

In contrast, when Sf-9 cells are infected with only the α subunit, Na/K-ATPase activity is no longer attained, and instead, a hydrolysis of ATP that is different from that of the holoenzyme is observed in the cells (40). This activity is dependent on Mg^{2+} and is inhibited by EGTA or high ionic strength. Along with this particular activity, the phosphorylation pattern of the unassociated α changes. α alone phosphorylation can only be obtained from ATP, is dependent on the presence of Mg^{2+}, does not require Na^+ or K^+ and is unaffected by ouabain. Phosphorylation is greatly reduced by EGTA by a poorly understood mechanism that does not involve Ca^{2+} chelation, and is susceptible to increased ionic strength secondary to an augment in the concentrations of Na^+, K^+, Tris or choline. As in the holoenzyme however, the phosphointermediate formed in the absence of the β subunit exhibits all the chemical properties consistent with phosphorylation at the normal aspartyl residue (Asp369) in the α subunit. Interestingly, β independent phosphorylation is not restricted to the α1 polypeptide, since also the α2 and α3 isoforms exhibit it. Whether the catalytic and phosphorylating properties of α alone are accompanied by the translocation of ions remains unknown. The importance of the β polypeptide in Na/K-ATPase function agrees with observations made in other expression systems that showed that β is required for the correct structural folding of the α polypeptide, that it influences the K^+ activation of the enzyme, and that is important in forming the cation occluding complex of the Na/K-ATPase (17,28,41). On the other hand, the catalytic function of the unassociated α polypeptide may be of

physiological significance and associated to the finding that Na/K-ATPase α and β subunits can exists separate from each other in some tissues (42-44).

Besides helping understand the α and β subunit requirement of the Na/K-ATPase, the baculovirus expression system has also been useful to explore the role of the γ subunit of the enzyme. When expressed in Sf-9 cells, the γ polypeptide associates with the α subunit, and does not require the α and β polypeptides to be delivered to the plasma membrane of the cells (45). Functionally, the γ subunit is not necessary for overall activity of the Na/K-ATPase, however, it is able to modify the ouabain affinity of the α1β1, as well as the α2β1 and α3β1 isozymes (Blanco et al. unpublished observations). This suggests that γ functions as an accessory subunit that modulates Na/K-ATPase activity. The role of γ has also been studied in kidney cells and *Xenopus* oocytes, where the subunit is able to influence the transport properties and Na^+ and K^+ affinities of the enzyme (46-48). In addition, γ expression in insect cells, and in *Xenopus* oocytes results in induction of large inward Na^+ and K^+ currents and the uptake of the ions by the cells (45). This indicates an independent role for the subunit as an ion channel. At present, the functional significance of association of the active transport of Na^+ and K^+ catalyzed by the Na/K-ATPase and the channel activity of γ remains unknown.

3.2. Biosynthesis and assembly of the Na pump α and β subunits

The first information regarding the biosynthesis of the Na/K-ATPase derived from experiments in Xenopus oocytes and mammalian cells. This showed that the endoplasmic reticulum (ER) is the cell compartment in which all essential processing of the Na/K-ATPase takes place (17,49,50). In the ER the Na pump subunits are synthesized, the first 5 amino acids of the α subunit are cleaved, the β subunit becomes glycosylated, and both α and β polypeptides are assembled (17,51). The finding that most of the α polypeptide that remains unassembled in the cells is retained in the ER, stays in a conformation that is more sensitive to trypsin, and is rapidly degraded, provided evidence for a role of the β subunit in Na/K-ATPase synthesis (17,52). A series of subsequent studies concluded that the β subunit functions as a chaperone protein that helps in the folding and correct membrane topogenesis of the α polypeptide to allow delivery of the mature holoenzyme to the plasma membrane. According to a model proposed by Geering, et *al*. (17), the role of β in Na pump folding is particularly important for the last 6 C-terminal transmembrane domains of the α subunit, which being less hydrophobic, make membrane insertion intrinsically less efficient. Studying the structural and functional characteristics of the α and β subunits along their maturation pathway in High Five insect cells, the group of Kaplan confirmed the importance of the ER in the quality control and "editing" of the newly synthesized Na/K-ATPase (53). Supporting observations made in mammalian cells, these authors showed that in the ER the Na/K-ATPase gains its functional capability (53,54). In addition, these authors found that even eliminating the activity of the α subunit, by mutating the phosphorylation site, Asp369 to Ala, the Na/K-ATPase is still properly assembled and targeted to the plasma membrane suggesting that a functional enzyme is not a prerequisite for plasma membrane delivery (53). This, however, appears to vary depending on the cell expression system used since intracellular retention of inactive Na/K-ATPase does not take place in yeast (55) or mammalian cells (56), but occurs in *Xenopus* oocytes (57,58). These differences may be reconciled by the existence of distinct mechanisms for recognition of correct protein folding and assembly in the ER of each cell type. In any case, this highlights the importance of the ER in Na/K-ATPase heterodimerization and cell trafficking.

Studies in insect cells have also served to investigate the role of N-linked glycosylation and disulfide bonds in the function of the β subunit. Site-directed mutagenesis disruption of glycosylation in the β subunit does not interfere with αβ heterodimer formation or delivery of an active Na/K-ATPase to the plasma membrane of High Five cells (59). Similar observations have been made in oocytes (60), epithelial (61) and neuronal (62) cells, in which protein glycosylation had been prevented by site directed mutagenesis or using tunicamycin. Although the hydrocarbon chains are of minimal importance for formation, cell trafficking and activity of the Na/K-ATPase, nonglycosylated β subunits are more prone to degradation suggesting that the sugars may play a role in the folding and structural stability of the subunit (59). Also, glycosylation may restrict β assembly with specific α isoforms since a nonglycosylated β2 subunit efficiently associates with the α2 polypeptide but not with α1 (63).

The role of the disulfide bridges of the β subunit, have been studied in insect cells by analyzing the assembly properties of β subunits where the Cys residues in the extracellular domain were substituted with Ala (59). This showed that disulfide bonds are not required for Na/K-ATPase αβ heterodimerization; however, disulfide bridges, in particular the two closest to the C-terminus, are necessary for correct trafficking of the Na/K-ATPase to the plasma membrane. ER retention is due to recognition of misfolding of the mutant β ectodomain since sequential removal of the protein ectodomain at each sulfhydryl bridge enabled some targeting of the β subunit (59). This implies that chaperones are involved in folding control and exit of the β polypeptide from the ER. Results in other expression systems show an even more drastic effect of Cys-deficient β subunits in αβ association and Na pump stability; therefore, it is clear that disulfide bridges are required for normal expression of functional enzyme at the cell surface (17).

Expression of different truncated variants of the β polypeptide in the insect cells also helped revealing the sites involved in association with the α subunit. This showed that the transmembrane and intracellular segments of the β polypeptide are sufficient for heterodimer assembly and expression of the holoenzyme at the plasma

membrane (59). While other reports using cross-linking and chimeras between the Na,K- and H,K-ATPase β subunits also support this observation (64-67), studies using yeast two-hybrid analysis showed that the extracellular 63 amino acids adjacent to the C-terminus of the β transmembrane domain are also important for αβ interaction, and specifically with the M7/M8 extracellular loop of the α subunit (68). It is possible that the concerted action of several regions in the β subunit is involved in αβ heterodimerization.

While assembly with β is important for delivery of the α subunit to the plasma membrane of some cell types, Sf-9 cells are able to target the individual Na/K-ATPase α and β subunits to the cell surface. This is not a consequence of the inability of the cells to recognize and retain the free α and β proteins in the ER, since other multimeric proteins expressed by them are delivered to their final destinations only after proper assembly (35). A6 epithelial cells also target unassembled Na pump polypeptides to the plasma membrane (69). Therefore, it appears that Na/K-ATPase processing may depend on cell specific mechanisms and production of functional αβ complexes may alternatively occur in cell compartments different from the ER. To determine the site of Na/K-ATPase assembly in Sf-9 cells, DeTomaso, et *al*. (70) used an original approach based on the ability of baculovirus infected Sf-9 cells to fuse when exposed to media with low pH. In this manner, when cells separately expressing the α and β subunits were plated together and subjected to acidic shock, the α and β polypeptides were found to associate within the large plasma membrane syncytia formed after fusion. This indicates that association of the Na pump polypeptides can take place not only at the ER, but also at the plasma membrane of the cells. Although unassociated α and β polypeptides may be leaving the ER in some tissues (42-44), the physiological significance of this alternative processing of the Na/K-ATPase is still unknown.

3.3. Oligomeric structure of the Na/K-ATPase

Although there has been general agreement that the Na/K-ATPase consists of α and β subunits in equimolar amounts, the quaternary structure of the enzyme has been a matter of long controversy. A large number of studies have been directed to establish the structural organization of the Na/K-ATPase. Evidence resulting from two-dimensional crystallography and the finding that extensive detergent treatment renders a monomeric solubilized enzyme that maintains its normal activity, favored the notion that the Na/K-ATPase exists as an αβ protomer (71-75). In contrast, molecular weigh determinations using analytical ultracentrifugation, low angle laser light scattering coupled with high performance gel chromatography, fluorescence resonance energy transfer, radiation inactivation, and evidence from cross-linking and ligand binding studies, all support oligomeric models for the Na/K-ATPase, with the enzyme consisting either in an αβ heterodimer with interacting α subunits, or a higher order oligomer (76-80). Experiments in insect cells using baculoviruses have provided another way to analyze subunit associations between the α polypeptides of the enzyme. Blanco, et *al*. (81) showed that when two different α isoforms are coexpressed in Sf-9 cells, immunoprecipitation using an antibody against one of the isoforms is also able to pull down the other α isoform. This showed that α1/α2, α1/α3 and α2/α3 are able to associate forming detergent-stable complexes. Moreover, this association is specific and not just the result of overcrowding of the expressed proteins, as determined by the inability of the Na/K-ATPase α isoforms to oligomerize with the α subunit of the structurally related H,K-ATPase. Interestingly, the α/α association in these experiments did not require the simultaneous expression of the β polypeptide. Importantly, the oligomers formed are not limited to the baculovirus directed polypeptides, and is also found in the native environment of the brain (81). The ability of various Na/K-ATPase isoforms to oligomerize suggests the possibility of association of αβ isoforms in different arrays, providing a higher level of intricacy to the structural complexity of the enzyme.

In a series of subsequent experiments, Koster, et *al*. (82) delineated the domain in the α subunit necessary for α/α assembly. This was achieved constructing deletion mutants of the Na/K-ATPase α1 subunit, and chimeras in which the N-terminal, the cytoplasmic mid region, and the C-terminal segments of the α subunits of the Na/K-ATPase were exchanged with those of the H,K-ATPase that is unable to oligomerize with the Na pump. All constructs were then coexpressed with the wild type Na/K-ATPase α1 subunit and analyzed for α/α specific association. This approach showed that the interaction domain between α subunits resides in the cytoplasmic mid region of the α polypeptide between Gly554 and Pro785. Further chimeras targeted to the cytoplasmic mid region narrowed down the association domain of the α subunit to a segment between Pro561 and Gln709. The prevalence of hydrophobic residues in this region may provide the forces needed in forming the interface of the extramembranous α/α contact site (83). The role of the cytoplasmic region in α/α association is consistent with previous reports of cross-linking studies on proteolytic fragments of the Na/K-ATPase α polypeptide. However, different from the immunoprecipitation experiments in insect cells, association from cross-linking required phosphorylation of the α subunit and αβ molecules that are catalytically active (77). Accordingly, the cytoplasmic M4-M5 loop of the α subunit produced in bacteria is able to interact with purified full-length Na/K-ATPase in a MgATP dependent manner (84). In addition, using recombinant Na/K-ATPase expression in insect cells, Kaplan and colleagues demonstrated that association between α1 subunits that have been tagged with different epitopes for recognition takes place as early as in ER membranes (85). Interestingly, in these experiments, a deletion mutant of the α subunit, lacking approximately 65% of the cytoplasmic P and N domains was still able to undergo α/α association with the wild type enzyme. These results, combined with those of Koster, et *al*. (82), indicate that 78 amino acids at the C-terminal portion of the M4-M5 loop may be sufficient for interaction. Alternatively, the β subunit may be participating in maintaining enzyme oligomerization.

Formation of oligomers is not a property unique to the Na/K-ATPase and has also been reported for the α subunit of the Ca- and H,K-ATPases (86,87). At present, the functional significance of the higher order organization of the Na/K-ATPase remains unresolved.

3.4. Cation binding sites in the Na/K-ATPase α subunit

Understanding the mechanisms of Na/K-ATPase action has presented a major challenge to researchers working in the field. A central goal in this respect has been establishing the structure and identifying the binding sites for the various ligands in the catalytic subunit of the enzyme. Several different approaches have contributed to this purpose, including the use of carboxyl modifying agents, protease digestion, epitope accessibility and mutagenesis of specific residues in the enzyme (3,4,88). This last alternative has been the most widely used and characterization of numerous mutants of the Na/K-ATPase α subunit in mammalian cells, yeast and *Xenopus* oocytes have identified amino acids involved in the binding of Na^+, K^+, ATP and ouabain (reviewed in 88). The baculovirus system has also revealed important structure-function features of the enzyme. For example, it has helped to ascertain the membrane topology of the α subunit. Thus, by using a combined approach consisting in introduction of Cys residues into predicted extracellular loops of the α polypeptide and surface labeling of the protein with a membrane impermeable Cys-specific reagent, the 10-transmembrane model organization of the polypeptide was confirmed (89). Also, expression in insect cells have shown that substitution of all the native 23 Cys of the Na pump α subunit by Ala or Ser results in an enzyme with slightly altered apparent affinities for Na^+ and K^+, but with an overall molecular activity similar to the wild type enzyme (90). This indicated that Cys residues have no essential role for the catalytic function of the enzyme and that the Na/K-ATPase inactivation that is caused by sulfhydryl reacting agents (91) is probably due to introduction of bulky moieties into the α polypeptide rather than interfering with specific Cys residues. The Cys-less mutant, however, is retained more in intracellular stores, associates less efficiently with the β polypeptide, and is less stable to degradation suggesting that although the Cys are not important for function, they play a role in protein folding (90).

Baculovirus infected cells have also importantly contributed in deciphering the role of specific residues in Na/K-ATPase function. For example, Koster, et *al*. (92), found that Glu781 in the fifth transmembrane domain of the rat Na/K-ATPase α subunit plays a critical role in cation coordination and selectivity of the Na/K-ATPase. In addition, Glu781 mutants result in altered affinity for ATP, suggesting that the fifth transmembrane segment is an important communicative link in transmitting information between the ATP binding domain and the cation transport region of the α subunit. The importance of Glu 781 is also supported by studies in Hela and Cos cells (93-95).

More recently, insect cells have been used to delineate the structural determinants involved in the cation selectivity of the Na/K-ATPase (96). For this, the function of a series of mutants were characterized in which residues in the membrane spanning segments of the α subunit of the Na/K-ATPase, highly conserved across species and isoforms, were changed to the corresponding residues common to the gastric H,K-ATPases of different species. The reasoning being that the divergent amino acids contained within the cation path across the membrane in these two P-type ATPases could be responsible for restricting the type of ion (Na^+ or H^+) transported by each enzyme across the lipid bilayer. The results indicated that transmembrane domains 4 and 6 (TM4 and TM6), and to a lesser extend 5 (TM5), are important for discrimination of Na^+ over H^+. This agrees with several other studies that have targeted individual amino acids in those regions of the protein (97-105), and supports the predictions from homology modeling of the Na/K-ATPase (16,106). Although, the simultaneous replacements in all transmembrane domains exacerbated the inability of the enzyme to select the correct cation, this was not sufficient to convert the Na/K-ATPase into a H,K-ATPase, and the resulting enzyme behaved as a hybrid Na/H,K-ATPase (96). This supports the notion that other regions outside the membrane bilayer participate in the particular properties of each P-type ATPase (98). Besides their importance in cation discrimination, TM4, TM5 and TM6 also participate in the conformational transitions associated with the binding of ions, influencing the E1-E2 conformational equilibrium of the enzyme (96). In conclusion, TM4, TM5 and TM6 play an essential role in the specificity of cation translocation by the Na/K-ATPase.

Besides helping understand the structure-function of the Na/K-ATPase, recombinant protein expression in Sf-9 cells has been important in uncovering the structural and catalytic characteristics of other P-type ATPases (107-110).

3.5. Isozyme kinetic properties

The ability to express solely and in various combinations the Na/K-ATPase polypeptides has immensely helped understanding the assembly properties and function of the various α and β isoforms. A particular example is represented by the α4 polypeptide, which function could not be predicted from its structure, approximately equally distant from the Na/K-ATPase α isoforms and the catalytic subunit of the H,K-ATPase (111). Coexpression of α4 with the Na/K-ATPase β1 subunit in Sf-9 cells results in an enzyme that is inhibited by ouabain, that requires Na^+, K^+ and Mg^{2+}, and that is sensitive to pHs below 7.2, indicating the ability of the polypeptide to function as a catalytic subunit of the Na/K-ATPase. Further characterization of α4 showed that the isoform is sensitive to vanadate, as expected for a P-type ATPase, but is insensitive to thapsigargin or Sch-28080, compounds that are specific inhibitors of the sarcoplasmic reticulum Ca-ATPase and gastric H/K-ATPase respectively. Also, α4 is phosphorylated by ATP in a ouabain-sensitive, Na^+ dependent and K^+ sensitive manner, typical for a Na/K-ATPase, and is able to catalyze the transport of ^{86}Rb inside the cells in a ouabain sensitive fashion (112). Evidence for the function of α4 as an additional Na/K-ATPase isoform also came from studies in

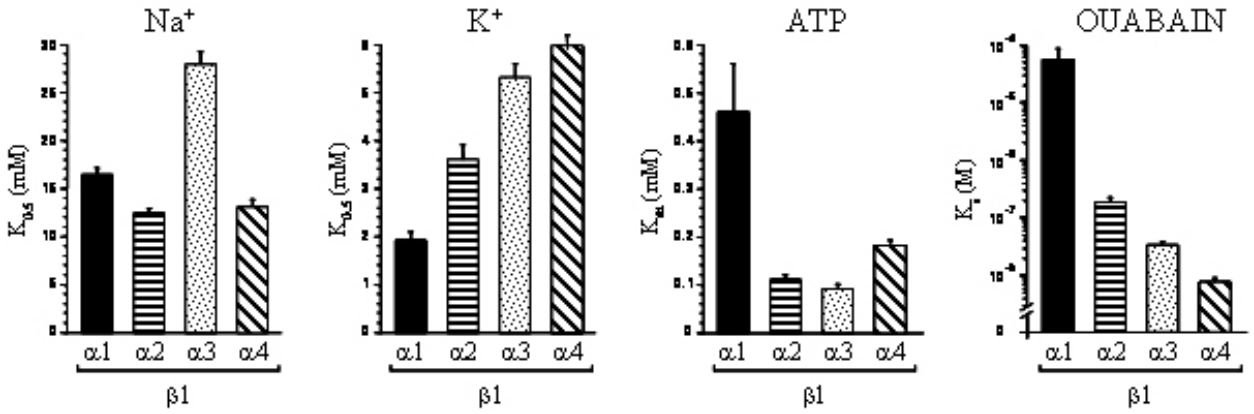

Figure 2. Kinetic parameters of rat Na/K-ATPase isozymes expressed in Sf-9 insect cells. Apparent affinities ($K_{0.5}$), K_m and inhibition constant (K_i) of isozymes composed of different α isoforms and the β1 subunit were calculated from dose-response curves of Na/K-ATPase activity for the indicated ligands. Values represent the mean ± standard error.

fibroblasts and in human kidney cells (113,114). Analysis of the kinetic parameters of α4 indicated a particularly high sensitivity of the isoform to ouabain (112,113). This property was used as a tool to explore the function of α4 in the native tissue. The α4 polypeptide is the Na/K-ATPase isoform with the most restricted pattern of expression, being expressed in the testes (111). In the gonad, the highly ouabain sensitive activity of α4 represents approximately half of the total Na/K-ATPase of the tissue, the remaining Na/K-ATPase corresponding to the ubiquitous α1 isoform (115). Activity of α4 is present in both the diploid and haploid germ cells of the testis, and its function is upregulated during gametogenesis, comprising in spermatozoa approximately two-thirds of the Na/K-ATPase of the cells (116). Expression of the isoform is confined to the mid region of the flagellum, where it is important for the motility of the gametes (117).

Expression in Sf-9 cells has also served to demonstrate the function of variants of the α subunit, such as α1T, a truncated version of the Na/K-ATPase α polypeptide that naturally occurs in smooth muscle cells and that lacks 40% of the carboxy terminal domain of the protein (118).

In addition, the baculovirus expression system has provided essential information for the function of the isozymes of the Na/K-ATPase. This showed that assembly of different α and β isoforms is a promiscuous event and that most αβ arrangements are catalytically active (119-122). However, the lower activity of certain αβ pairs produced, such as α1β2, suggests that isoform association is not equally efficient for all αβ combinations and some heterodimers may be favored in the native tissues that simultaneously express several isoforms. Evidence for specific αβ isoform interaction has been reported in Xenopus oocytes (63).

The analysis of the enzymatic properties of rat Na/K-ATPase isozymes produced in insect cells showed functional differences to the activating cations Na^+ and K^+, ATP and the inhibitor ouabain, the major kinetic dissimilarities corresponding to Na/K-ATPases that differ in the α subunit (10). In contrast, the β subunit isoform composition does not drastically affect the properties of a particular α polypeptide, inducing only minimal changes in the Na^+ requirement of the enzyme (10). The functional parameters of the Na/K-ATPase α isoforms is presented in Figure 2. As shown: a) α2 exhibits the highest apparent affinity for Na^+, b) α3 is the isoform with lower apparent affinity for Na^+ and K^+, c) both α2 and α3 display equivalent K_m values for ATP, which are approximately four times lower than that of the α1β1, d) the α4 isoform has a high affinity for Na^+ and low affinity for K^+, and e) the most prominent kinetic difference among the Na pump isozymes corresponds to their reactivity to ouabain, with a rank of sensitivity following the sequence α4 > α3 > α2 > α1. The differences in ouabain reactivity are typical of rat, being less marked for the isozymes from other species (27,28). Essentially, the kinetic differences between the rat Na/K-ATPase isozymes determined in insect cells coincide with those found in other expression systems. Rodent and human cells have been used after changing the ouabain affinity of the α isoforms to allow their distinction from the endogenous pumps of the cells (30,31,123). *Xenopus* oocytes and yeast offered another excellent alternative to analyze isoform activity, without the complication of endogenous Na/K-ATPase activity (27,28,124-126).

In sum, the various Na/K-ATPase isozymes have different functions. This, combined with the particular tissue expression the α and β polypeptides exhibit suggests that the heterogeneity of the Na/K-ATPase is of physiological importance. In this manner, while the ubiquitous α1β1 is most likely functioning to maintain the basal Na^+ and K^+ gradients in the cell, the other isozymes may be playing tissue specific roles. This conclusion is being supported by recent evidence emerging from genetic manipulation of α1 and α2 expression in transgenic mice (127-131).

3.6. Mechanisms for Na pump isozyme regulation

An important amount of work has been devoted to understand the factors that regulate Na/K-ATPase function. Many stimuli including ions, hormones and neurotransmitters, as well as association with the FXYD polypeptides, have been found to modulate the enzyme (reviewed in 132). Also, intense research has been directed to unveil the mechanisms involved in Na/K-ATPase regulation. Function of the Na/K-ATPase can be controlled by direct adjustment of the activity of the enzyme at the surface of the cell, or via changes in expression at the plasma membrane through regulation of synthesis/degradation or redistribution of the enzyme with intracellular stores. These mechanisms require regulation at different levels, including ligand interaction with the enzyme, the control of gene transcription and translation, and the phosphorylation of the Na/K-ATPase α subunit at specific Ser residues (132). Na pump phosphorylation is a reversible event that is mediated by a complex, cell-specific signaling network that involves activation of protein kinases (PK) and phosphatases. In kidney epithelial cells for example, dopamine inhibits the enzyme function through a mechanism that involves phosphorylation of the α1 isoform via activation of PKC and phosphatidylinositol 3 kinase (PI3K-I_A). This allows binding of the adaptor proteins, AP-2 to the α subunit, which in term serves as a signal for Na/K-ATPase clathrin-coated vesicle endocytosis (133-135). Serotonin and isoproterenol instead, through activation of another PKC isoform, promote Na pump targeting to the plasma membrane (136). Most of the studies to date have focused on the α1β1 isozyme, in which the sites modified by phosphorylation have been mapped to Ser16 and Ser23 for PKC and to Ser943 for PKA (137-139).

Evidence for a specific regulation of the Na/K-ATPase isozymes derives from experiments in Sf-9 insect cells. The differences in Na^+ affinity indicate that intracellular fluctuations of the cation can directly regulate the α isoforms in a specific manner (10). The finding that Ca^{2+} differentially inhibits α1, α2 and α3 suggests this is another mechanism for fast selective modulation of Na pump isoform activity (140). Also, the dissimilar reactivity to ouabain include the digitalis compounds as an additional factor in isoform specific control (10). The finding that ouabain is endogenously produced in mammals further suggests a role for this compound as a natural modulator of Na^+ and K^+ transport (141). Another characteristic of the Na/K-ATPase α isoforms is their dissimilar sensitivity to oxidants, which suggests they may differently respond to oxidative stress (142).

In addition to revealing the effect of ligands, studies using baculoviruses have helped exploring the role of PK on the function of the different Na/K-ATPases. In this manner, in Sf-9 cells, dibutyryl cyclic AMP and activation of protein kinase A (PKA) stimulates the activity of the Na/K-ATPase α3β1 isozyme and decrease that of α1β1 and α2β1. In contrast, activation of protein kinase G (PKG) with dibutyryl cyclic GMP diminishes the activity of the α1β1 and α3β1 isozymes, without altering that of α2β1. Finally, phorbol esters and activation of protein kinase C (PKC) inhibits all isozymes (143). Different from α1 and α3, the effect of PKA and PKC on the α2 isoform occurs via the phospholipase 2 (PLA2) pathway, and the formation of arachidonic acid or its metabolites (143). The PK induced changes in Na/K-ATPase activity in insect cells are secondary to PKA and PKC mediated phosphorylation of the Na/K-ATPase α subunits. Interestingly, not only α1, but also the α2 and α3 polypeptides are substrates for PK dependent phosphate incorporation (143). Although with different efficiencies, phosphate incorporation into different α isoforms has also been reported after *in vitro* phosphorylation experiments (144). Studies of Na/K-ATPase regulation by dopamine and glutamate in neostriatum neuronal cells also provide evidence for an isoform selective regulation (133). However, while in neurons the changes in Na/K-ATPase isozyme activity correlate with variations in α isoforms at the cell surface, in the insect cells, regulation rather depends on changes in the molecular activity of the Na/K-ATPases (133,142). This suggests two different alternative ways for the phosphorylation-mediated regulation of the Na/K-ATPase; one affecting the function and the other the number of pump units at the plasma membrane of cells.

Altogether these results indicate that the Na/K-ATPase is under the control of a variety of intracellular messengers that, in a cell specific manner, selectively modulate the activity of the different isozymes. Because the various Na/K-ATPases possess unique catalytic function, their differential regulation may be essential in controlling cellular cation homeostasis to the particular needs of each cell.

3.7. Interaction of the Na/K-ATPase with other proteins

An exciting line of investigation in the recent years has been the finding that the Na/K-ATPase does interact with a variety of soluble and membrane bound proteins in the cell. Thus, binding of ouabain to the Na pump elicits the scaffolding of a group of proteins in caveolae to constitute a signaling module. This signalosome transmits signals via multiple pathways, characteristically Src kinase and an intracellular cascade of phosphorylating events that include the mitogen activated MEK and MAPK kinases. This ultimately results in expression of a series of genes and cell growth (145). In this manner, the Na/K-ATPase, besides its role as an ion pump, functions as a receptor and signal transducer of ouabain effects.

In addition, other non-canonical roles of the Na/K-ATPase may emerge from the identification of proteins that interact with the Na pump. The use of the yeast two-hybrid screen analysis in a human kidney library employing as bait the N and P domains, or the A domain of the Na/K-ATPase α subunit, provided evidence for novel interacting partners of the enzyme. These include protein phosphatase 2A; CD81, a member of the tetraspan superfamily that regulates signaling and sorting processes; rat lin10 that may link the Na pump to a variety of molecules involved in signal transduction and protein trafficking; SNARE-associated protein that mediates

membrane fusion events, and Polycystin 1 (PC1), a protein involved in signaling and Ca^{2+} transport in the kidney (146).

Nguyen, et *al*. (147), explored the interaction and functional consequence of Na/K-ATPase association with PC1 and polycystin 2 (PC2) in Sf-9 cells. When the eleven transmembrane domains and C-tail of PC1, or the full length PC2, were expressed with the rat Na/K-ATPase α1 isoform, immunoprecipitation analysis showed physical interaction with PC1 but not with PC2. Analysis of the Na/K-ATPase kinetic properties detected no important changes in the apparent affinities of the enzyme to Na^+ and K^+, both in the presence of PC1 and PC2. However, PC1 induced an increase in sensitivity of the Na/K-ATPase to ouabain. Characterizing proteins that affect the function of the Na/K-ATPase opens a challenging new field of investigation that will be important in understanding how Na^+ and K^+ homeostasis is regulated in specific tissues.

4. FINAL REMARKS

Since it was first introduced to study the Na/K-ATPase over 10 years ago (35), the baculovirus expression system has importantly helped understanding several aspects of the enzyme. Insect cells provide a system with a high capacity for protein expression in an environment similar to that of mammalian cells, but devoid of the interference of endogenous Na pump. This offers a unique opportunity to study Na/K-ATPase α and β isoforms alone, in different combinations, or after manipulating their structures to investigate the cell biology and biochemical characteristics of the resulting enzymes. Many essential questions remain concerning the Na/K-ATPase mechanisms of function, regulation and interaction with other proteins. The baculovirus expression system will continue providing an excellent tool to advance our understanding of the Na^+ and K^+ active transport system, as well as of other members of the P-type family of ATPases.

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