Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function

GUSTAVO BLANCO AND ROBERT W. MERCER
Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

Blanco, Gustavo, and Robert W. Mercer. Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. Am. J. Physiol. 275 (Renal Physiol. 44): F633–F650, 1998.—The Na-K-ATPase is characterized by a complex molecular heterogeneity that results from the expression and differential association of multiple isoforms of both its α- and β-subunits. At present, as many as four different α-polypeptides (α1, α2, α3, and α4) and three distinct β-isoforms (β1, β2, and β3) have been identified in mammalian cells. The stringent constraints on the structure of the Na pump isozymes during evolution and their tissue-specific and developmental pattern of expression suggests that the different Na-K-ATPases have evolved distinct properties to respond to cellular requirements. This review focuses on the functional properties, regulation, and possible physiological relevance of the Na pump isozymes. The coexistence of multiple α- and β-isoforms in most cells has hindered the understanding of the roles of the individual polypeptides. The use of heterologous expression systems has helped circumvent this problem. The kinetic characteristics of different Na-K-ATPase isozymes to the activating cations (Na$^+$ and K$^+$), the substrate ATP, and the inhibitors Ca$^{2+}$ and ouabain demonstrate that each isoform has distinct properties. In addition, intracellular messengers differentially regulate the activity of the individual Na-K-ATPase isozymes. Thus the regulation of specific Na pump isozymes gives cells the ability to precisely coordinate Na-K-ATPase activity to their physiological requirements.

ouabain; sodium pump; protein kinases

THE DISCOVERY THAT MANY ENZYMES in the cell are expressed in multiple molecular forms has become one of the most fascinating and intriguing phenomena in biology. In 1959, Markert and Möller (134) first used the term “isozymes” to describe the separate proteins that catalyze the same biochemical reaction. Since then, researchers have looked for structural variants of enzymes to understand their physiological function. In some cases, duplication of genes or alternative posttranscriptional processing of the gene message results in isozymes with unique biological properties. The development of multiple isozymes often provides the functional versatility cells need to fulfill their physiological requirements. This review covers the present status in the continuously growing field of the isozymes of the Na-K-ATPase, emphasizing the functional properties and regulatory mechanisms of the individual Na pump polypeptides.

The Na-K-ATPase, or Na pump, is a membrane-bound protein that establishes and maintains the high internal K$^+$ and low internal Na$^+$ concentrations typical of most animal cells. By using the energy from the hydrolysis of one molecule of ATP, it transports three Na$^+$ out in exchange for two K$^+$ that are taken in. The electrochemical gradient the Na-K-ATPase generates is critical in maintaining the osmotic balance of the cell, the resting membrane potential of most tissues, and the excitable properties of muscle and nerve cells. In addition, the Na$^+$ gradient provides the energy that fuels the Na-coupled transporters. These secondary transport systems mediate the translocation of ions (H$^+$, Ca$^{2+}$, Cl$^-$, PO$_4^{3-}$, SO$_4^{2-}$), substrates (glucose and amino acids), and neurotransmitters across the plasma membrane (83, 84, 109, 190, 191). In the kidney, the Na-K-ATPase plays a primary role in driving the reabsorption of Na$^+$ and water. Thus the enzyme is essential in the maintenance of body fluid and electrolyte homeostasis (83, 109).

The Na-K-ATPase or Na pump belongs to a widely distributed class of P-type ATPases that are responsible for the active transport of a variety of cations across cell membranes (127, 171). P-type ATPases are found in both prokaryotic and eukaryotic cells, and are responsible for transporting H$^+$, Na$^+$, Mg$^{2+}$, K$^+$, Ca$^{2+}$,
Cu$^{2+}$, and Cd$^{2+}$ (127). All these enzymes use the hydrolysis of ATP to drive the transport of cations against an electrochemical potential. The P-type designation refers to the unique characteristic of these enzymes in forming a transient, phosphorylated aspartyl residue during the catalytic cycle. Accompanying the phosphorylation-dephosphorylation process, the P-type ATPases bind, occlude, and transport ions by cycling between two different cation-dependent conformations, called E1 and E2 (167, 171, 191). The precise molecular mechanisms that couple the hydrolysis of ATP to the conformational changes and the translocation of ions remain unknown.

In addition to having a common reaction mechanism, P-type ATPases also display comparable tertiary structures, equivalent membrane topological organization, and several highly conserved protein domains (127). The region around the phosphorylated aspartate, the TGES/A (threonine, glycine glutamine, serine/alanine) motif between transmembrane domains two and three, and several regions involved in ATP binding exhibit a high degree of amino acid homology. In addition to implying a common evolutionary ancestor, the similarities in the structure and reaction mechanism of these diverse proteins often make conclusions drawn from one transporter relevant to the others (46).

The Na-K-ATPase is an oligomer composed of stoichiometric amounts of two major polypeptides, the α- and the β-subunits. The primary structures and membrane organization of the Na-K-ATPase α- and β-subunits are depicted in Fig. 1. The α-subunit is a multispansing membrane protein with a molecular mass of ~112,000 Da that is responsible for the catalytic and transport properties of the enzyme. The α-subunit contains the binding sites for the cations, ATP, and the inhibitor, ouabain (reviewed in Refs. 120, 147, 160, 165). The β-subunit is a polypeptide that crosses the membrane once and, depending on the degree of glycosylation in different tissues, has a molecular weight between 40,000 and 60,000 Da. The β-subunit is essential for the normal activity of the enzyme (31, 52, 139), and it appears to be involved in the occlusion of K$^+$ (126) and the modulation of the K$^+$ and Na$^+$ affinity of the enzyme (33, 35, 62, 64, 102). In addition, in vertebrate cells, the β-subunit may act as a chaperone, stabilizing the correct folding of the α-polypeptide to facilitate its delivery to the plasma membrane (reviewed in Refs. 52, 139).

A third protein, termed the γ-subunit, has also been identified in purified preparations of the enzyme (166). The γ-subunit is a small, hydrophobic polypeptide of 8–14 kDa that was considered to be a contaminant of purification, until it was shown that it could be covalently labeled by photoaffinity derivatives of ouabain (78, 123, 169). Other evidence that the γ-subunit is a component of the Na-K-ATPase is that the subunit colocalizes with the α-subunit in nephron segments and coimmunoprecipitates with αβ-complexes (148). Also, the high degree of homology among γ-subunits from several species suggests that the subunit may be important in Na-K-ATPase function.

Expression studies have shown that the γ-subunit is not required for normal Na-K-ATPase activity (59, 91, 176). However, recently it was shown that the γ-subunit can modify the voltage dependence of K$^+$ activation of the α1β1-isozyme when expressed in Xenopus oocytes (20). In addition, it appears that the γ-subunit can stabilize the E1 conformation of the enzyme (204) and may be required for cavitation in mouse embryos (108). Interestingly, the γ-subunit belongs to a family of small membrane proteins involved in the passage of ions across the plasma membrane. This family of proteins, which includes phospholemman (158), channel inducing factor (CHIF; Ref. 12), and Mat-8 (mammary tumor, 8 kDa; Ref. 152), induces ion channel activity when expressed in Xenopus oocytes (150). Consistent with these findings, the human γ-subunit induces cation-selective channels when expressed in Xenopus oocytes (150). The physiological significance of this activity and whether it requires the other Na-K-ATPase subunits is unknown. Although there is increasing evidence that the γ-subunit can modify Na-K-ATPase function, the exact role of the subunit in Na-K-ATPase function awaits further experimentation.

Na Pump Isoform Diversity

As with many other essential proteins in the cell, the Na-K-ATPase is expressed as several isozymes. Indeed, there are different genes encoding distinct molecular forms of both the α- and β-polypeptides (reviewed in Refs. 69, 117, 120, 121, 196, 197). Evidence for the existence of Na pump isozymes can be traced to experiments that analyzed the cardiotonic steroid sensitivity of Na-K-ATPase preparations from rodents. Marks and Seeds (135) found heterogeneous ouabain inhibition curves for the Na-K-ATPase from mouse brain. The activity could be resolved into two components of high (K$^+$ = 10$^{-7}$ M) and low (K$^+$ = 10$^{-4}$ M) affinity for the inhibitor. This was in clear contrast to the single inhibition profile for enzyme preparations from the kidney (135). The earliest structural evidence for variants of the Na pump was found in brine shrimp. In this crustacean, the α-subunit could be resolved into two distinct forms on SDS-polyacrylamide gels (163). The first direct demonstration of Na-K-ATPase isoforms in mammals was accomplished by Sweadner (194), who found two forms of the α-subunit, the already identified renal α-form and a brain form that was termed α+. This novel catalytic subunit of the enzyme had a slower migration in SDS-polyacrylamide gels and a higher sensitivity to ouabain (194). This pioneering work was followed by studies that focused on the biochemical properties of both isoforms. Thus α+ was shown to display a higher reactivity toward N-ethylmaleimide (208), a higher sensitivity to the vitamin B derivative pyrithiamine (137), and an increased resistance to trypsin digestion compared with α (209). These properties suggest distinct structural differences between the isoforms. Later, the demonstration of dissimilarities at the NH$_2$ terminus of α and α+ (129) suggested a genetic basis for the isoform difference. The advent of
Fig. 1. Scheme of the membrane topology of the α- and β-isoforms of the Na-K-ATPase. Sequences of rat α1- and β1-isoforms are shown. Residues are colored to indicate the amino acid homology among the different α-isoforms (α1, α2, α3, and α4) or β-isoforms (β1, β2, and β3).
molecular biological techniques resulted in the identification of at least three α-polypeptides in vertebrates, presently known as α1, α2, and α3 (186, 187, 193). More recently, Shamraj and Lingrel (185) identified a fourth α-isofrom (α4) in rat testis.

The α-isofroms of the Na-K-ATPase have been cloned from several mammalian species. An analysis of the phylogenetic distribution of the α-proteins using antibodies directed to conserved and specific regions of the isoforms indicates that isoforms appear to exist in all mammals, including both placentant and marsupial species (164). The identifications of Na-K-ATPase α-isofroms in the chicken (70) and teleost fishes (164) imply their broad presence among vertebrates (201). Moreover, the finding of α-isofroms in crustaceans (14) and possibly in hydra (45) and platyhelminths (159) indicates that the divergence of Na-K-ATPase genes occurred early in evolution.

Further studies have shown that the Na pump molecular diversity also extends to its β-subunit. At present, three different Na-K-ATPase β-isofroms have been identified. Two of the isoforms, β1 and β2, have been found in different tissues of mammals (82, 136) and birds (115), whereas β3 has been detected in amphibians (85), mouse (27), rat, and human (82, 136). Moreover, in the zebra fish, an Na-K-ATPase β-subunit highly homologous to β3 has been isolated (7). This species, however, a detailed characterization of other β-isofroms has not been accomplished, making it difficult to establish a correlation with the subunits of other species.

Both the α- and β-isofroms of the Na pump exhibit a tissue-specific pattern of expression. The α1-isofrom in association with the β1-subunit is found in nearly every tissue (reviewed in Refs. 69, 117, 119, 121, 196). In addition, α1β1 is the principal isofrom of the kidney, a tissue that has classically been used as the source of Na-K-ATPase (reviewed in Refs. 83, 84, 109, 191). The presence of other Na-K-ATPase isoforms in the kidney has been a matter of debate (2, 8, 51, 73, 88, 104, 140, 206). The α2- and α3-isofroms have been detected in renal cortex, medulla, and papilla using both reverse-transcription followed by polymerase chain reaction amplification and [3H]ouabain titration analysis (125).

Measurement of mRNA and protein levels indicates that the α2- and α3-isofroms correspond to only 0.1% of the α1β1 enzyme of the kidney (125). In contrast to the broad tissue distribution of α1 and β1, the other α- and β-polypeptides are more restricted in their expression. The pattern of Na-K-ATPase isoform expression, at both the mRNA and protein level, has been studied with most detail in the rat. The α4-isofrom is a testis-specific isoform (185). The α2-isofrom predominates in adipocytes (130), muscle (99, 114), heart (215), and brain (143), and α3 is abundant in nervous tissues (95, 143). In the nervous system, the expression of Na-K-ATPase isoforms reaches its greatest intricacy. Neuronal cells may express a unique isozyme or multiple isoforms consisting of the various αβ-heterodimer combinations (117, 119, 121, 196). Whereas neurons are the principal source of the α3-polypeptide (reviewed in Refs. 117, 198), glial cells preferentially express α2 (40, 43, 76). Also, the β-isofroms are distributed in a tissue-dependent manner. The β2-isofrom is found in skeletal muscle (114), pineal gland (189), and nervous tissues (162), whereas β3 is present in testis, retina, liver, and lung (11, 133). In addition, the pattern of expression of the Na-K-ATPase isoforms is subjected to developmental as well as hormonal regulation and can be altered during disease (39, 49, 50, 68, 217).

Additional Mechanisms Involved in the Production of Na-K-ATPase Isozymes

In addition to the expression of different genes for the α- and β-subunits, several other mechanisms are involved in the generation of Na-K-ATPase isoforms. For example, variations in the transcriptional processing of the Na pump subunit messages occur in some cells. This is the case for a truncated form of the α1-subunit, named α1-T, which has been found in canine vascular smooth muscle (146). Alternative splicing of the α1 mRNA results in a polypeptide of ~65,000 Da. The α1-T includes the first 554 amino acids of α1 and terminates with a peptide of 27 amino acids from the retained intron sequence. Although α1-T lacks 40% of the COOH-terminal sequence, it retains the ATP binding and phosphorylation site. This, added to the observation that cells containing α1-T show undetectable levels of the other α-isofroms, has led to the suggestion that the truncated isofrom may function as an ATPase (4, 146). However, it remains unknown whether α1-T is catalytically competent and, if so, whether it is able to support the transport of ions. Interestingly, truncated transcripts of both the α1- and β1-isofroms have also been found in human retinal epithelium. The truncated α1 sequence in this tissue encodes the first 681 amino acids of the α1-polypeptide. The deletion produces a shift in the open-reading frame of the message to produce a unique COOH terminus of 44 residues, which surprisingly displays similarity to the COOH-terminal sequence of the sarcoplasmic reticulum (SR) Ca-ATPase. The truncated β-polypeptide of retina is two amino acids shorter at the COOH terminus than the typical β1-isofrom. In addition, the last two COOH-terminal residues differ from those of the full-length subunit (170).

Other short variants of the α1-polypeptide have been found in Xenopus laevis embryos. In Xenopus, deletions at the NH2 terminus of the protein result from the alternative initiation of translation at codons encoding either Met34 or Met43. Sequential mutation of each of these residues and expression of the corresponding cRNAs in Xenopus oocytes shows that both truncated subunits are catalytically competent when coexpressed with the β1-isofrom. Moreover, the α-subunit with Met33 at the NH2 terminus exhibits a lower affinity for K+ than the Met34 truncated or full-length polypeptides (42). These results support the hypothesis that the NH2 terminus of the α1-subunit, which is rich in lysine and contains an excess of positively charged amino acids, forms a cation-selective gate (187, 210).
Another mechanism that contributes to the heterogeneity of the Na pump is the association of the $\alpha$- and $\beta$-isoforms into different $\alpha$$\beta$-heterodimers. The possibility that combinations of different heterodimers could be formed was suggested from the expression of multiple isoforms in several tissues and cells (43, 117, 162, 181, 182, 221). For example, in the pineal gland, both the $\alpha_1\beta_2$ and $\alpha_1\beta_2$ Na-K-ATPase isozymes are present (189). The $\alpha_3\beta_2$-isoform combination is also found in retinal photoreceptor cells (181, 182), whereas the cells of the choroid plexus express the $\alpha_1\beta_1$- and $\alpha_2\beta_1$-isozymes (221). Specific cell populations within the central nervous system (117, 198), the ciliary epithelial cells of the eye (81), the sensory organ of the ear (202), and the choroid plexus (221) express several isoforms with the potential of multiple $\alpha$ and $\beta$ combinations. The promiscuous association between $\alpha$- and $\beta$-isoforms was confirmed in insect cells expressing different combinations of the rat Na-K-ATPase polypeptides. In this expression system, each $\alpha$-isoform could properly assemble with either the $\beta_1$- or the $\beta_2$-subunit into catalytically active complexes (30, 33, 35, 36). Similarly, nonspecific $\alpha\beta$ association was found for the Na-K-ATPase chicken isoforms exogenously expressed in mammalian cells (116). In this manner, multiple Na-K-ATPase isozymes ($\alpha_1\beta_1$, $\alpha_1\beta_2$, $\alpha_2\beta_1$, $\alpha_2\beta_2$, $\alpha_3\beta_1$, and $\alpha_3\beta_2$) can result from the pairing of the various $\alpha$- and $\beta$-polypeptides (33, 35).

The complexity of the Na-K-ATPase may also extend to the oligomeric structure of the enzyme. We have recently demonstrated that the different $\alpha$-isoforms are able to specifically and stably associate into oligomeric complexes (32). The existence of multiple sets of oligomers raises the possibility of an even greater degree of intricacy in the molecular heterogeneity of the Na pump. However, whether the quaternary structure of the Na pump influences the functional properties of the enzyme is still unclear.

Structure of $\alpha$- and $\beta$-Isoforms

The complete amino acid sequence of the $\alpha$-isoforms has been deduced from the cDNAs coding for the polypeptides from rat (180, 186), chicken (70), and human (121, 193). The $\alpha$-subunits in the rat vary slightly in length; the $\alpha_3$-isoform is the smallest with 1,014 amino acids, $\alpha_1$ possesses 1,024, $\alpha_2$ possesses 1,021, and $\alpha_4$ is the largest with 1,028 residues. The amino acid sequence, site-specific labeling, and immunologic and proteolytic digestion studies have provided some insight into the possible transmembrane orientation of the $\alpha$-subunit (9, 44, 120, 122, 151, 160, 199, 210, 213). These studies predict an NH$_2$-terminal segment with four transmembrane spanning domains, a large cytoplasmic domain consisting of roughly one-third of the polypeptide, and a carboxy-terminal region containing six membrane spanning domains (Fig. 1). This membrane topography is also similar to the models suggested for the Ca$,^+$, H$,^+$, and H-K-ATPases (44, 46).

Across species the degree of identity for the $\alpha_1$- and $\alpha_2$-isoforms is $\sim$92% and is over 96% for $\alpha_3$. There is also a high degree of identity ($\sim$87%) among the $\alpha_1$, $\alpha_2$, and $\alpha_3$-isoforms. In contrast, $\alpha_4$ is the most divergent, sharing a 78% identity with the $\alpha_1$-isoform. As shown in Fig. 1, the highest structural variability among the isoforms occurs at the NH$_2$ terminus, the extracellular ouabain binding site between transmembrane segments 1 and 2, and the cytoplasmic region between amino acids 403 and 503. In contrast, the greatest similarities occur in the cytoplasmic middle region where the ATP binding and phosphorylation sites are located, the transmembrane hydrophobic regions and the COOH-terminal region (reviewed in Refs. 117, 120, 147).

The $\alpha$-subunit undergoes translational or posttranslational modifications, some of which appear to be isoform specific. In the mature $\alpha_1$- and $\alpha_2$-polypeptides, the first 5 amino acids are cleaved (129). It is not known whether the $\alpha_3$-isoform is processed in a similar fashion. A tissue-specific, posttranslational modification has been suggested for the $\alpha_3$-isoform. An isoform-specific antibody that reacts with a region near the ATP binding site identifies $\alpha_3$ in the brain but does not react to the same isoform from heart. This lack of reactivity has been interpreted as a blockage of the antibody epitope by a modification of the protein during synthesis (10). The nature of this modification, as well as its occurrence in other tissues and $\alpha$-isoforms, is unknown.

The amino acid sequence of the $\beta$-isoforms has been deduced from the rat, human, chicken, Xenopus, and mouse cDNAs (82, 115, 133, 136). In the rat, the $\beta_1$-isoform has 304, the $\beta_2$-isoform has 290, and the $\beta_3$-isoform has 279 amino acids. All $\beta$-isoforms share a common basic structure (Fig. 1). The $\beta$-isoforms consist of a short NH$_2$-terminal cytoplasmic region, a transmembrane spanning segment, and a large extracellular domain. The homology of the $\beta_1$- and $\beta_2$-isoforms across mammalian species is $\sim$95%. This value drops to 60% when nonmammalian species are included in the comparison. The homology among different $\beta$-isoforms is lower than that found for the catalytic subunit (Fig. 1). Compared with $\beta_1$, the $\beta_2$-polypeptide exhibits 58% similarity (34% identity, 24% favored substitutions), whereas the $\beta_3$-subunit is 68% homologous, with an identity of 39%. The similarity between $\beta_2$ and $\beta_3$ reaches 61%, with 49% of the residues conserved (133). Interestingly, the $\beta_2$-subunit primary structure is more closely related to the H-K-ATPase $\beta$-isoform than to the $\beta_1$-isoform, suggesting that the $\beta_2$- and H-K-ATPase $\beta$-isoform genes diverged more recently than $\beta_1$ and $\beta_2$ (188). The transmembrane domain of the $\beta$-subunit is the most highly conserved region both among isoforms and species.

All $\beta$-isoforms are heavily glycosylated. The $\beta_1$-isoform from mammals has three N-linked glycosylation sites. The putative N-linked glycosylation sites for the $\beta_2$-isoform vary depending on the species. Thus the chicken subunit has four potential glycosylation sites, the rat has seven, the human has eight, and the mouse $\beta_2$-polypeptide has nine (reviewed in Ref. 52). It is not known whether all the sites are used. Inhibition of glycosylation of the Na-K-ATPase $\beta_1$-subunit with tunicamycin results in catalytically competent Na pumps...
with normal affinity for ouabain (200, 218, 219). Expression of an enzyme in which all β-subunit N-linked glycosylation sites are mutated also renders an active enzyme with conserved K⁺ and ouabain affinities. However, the reduction in the ability of the nonglycosylated β-subunit to assemble with the α-subunit and the higher sensitivity of the enzyme to proteolysis suggest that glycosylation may play a role in protein folding (16).

Another important feature in the structure of the β1-subunit is the presence of three disulfide bridges, which in the rat polypeptide occur between Cys125—Cys148, Cys158—Cys174, and Cys212—Cys275. All the cysteines, but not their relative positions within the sequences, are conserved in the β2- and β3-isolomers, suggesting similarities in the tertiary structure of the polypeptides. Treatment of the Na-K-ATPase with reducing agents results in inactivation of Na-K-ATPase, suggesting that the disulfide bonds are required for enzyme function. The removal of just one of the disulfide bonds by site-directed mutagenesis of the involved cysteines has been shown to be sufficient to abolish proper assembly of the αβ-subunits (16). The location and role of sulfhydryl bridges in the other Na pump β-isolomers are unknown.

Enzymatic Properties of Na-K-ATPase Isozymes

Understanding of the enzymatic properties of the individual isozymes may help in determining the basis for the complex molecular diversity that characterizes the Na-K-ATPase. The characterization of the functional properties of the different α- and β-polypeptide combinations has been a difficult task. Often more than one isoform is expressed in the same cell, complicating the analysis of the individual isoforms. Because it constitutes nearly all the Na pump in the kidney, the functional properties of the α1β1-isozymes have been extensively analyzed. With its high levels of Na-K-ATPase activity, the kidney has been of immense value in understanding the structure, biochemical reaction mechanism, and kinetic properties of the Na-K-ATPase. In contrast, the lack of natural sources for each of the other Na pump isoforms has made their characterization more difficult. The first attempt to evaluate the catalytic properties of the Na pump isoforms was the comparison of the substrate affinities of the enzymes from kidney and brain (208). This early work suggested that the Na-K-ATPase isoforms have distinct differences in their affinities to Na⁺, K⁺, and ATP. Later, Sweadner (195) compared the properties of Na-K-ATPases from rat axolemma and rat kidney. The renal α1β1-isozyme displayed a lower ATP, similar K⁺, and higher Na⁺ affinities than the neuronal enzyme composed of the α2- and α3-subunits. Also, differences in Na⁺ or K⁺ apparent affinities have been found between Na-K-ATPases partially purified from brine shrimp (55, 56) or different tissues of the rat (41, 72, 211). Moreover, the transport properties of rat kidney- and axolemma-derived enzyme delivered into dog erythrocytes by membrane fusion differ in their dependence on Na⁺ and K⁺ (154). In whole adipocytes from rat, it was found that the α1-isozyme has a $K_m$ for Na⁺ that is threefold lower than that of the α2-isozyme (128, 130). This difference, however, was not retained when membranes from the same source were prepared (41). Such discrepancies were also encountered for the Na⁺ affinity of α1 and α2 from entire or disrupted rat brain synaptic vesicles (41). Other differences in isoform enzymatic properties were found for the α3β2 Na-K-ATPase from the pineal gland, which exhibits a higher Na⁺ affinity than the renal α1β1 (189). On the other hand, similar Na⁺ and K⁺ kinetics were identified in cerebellar or hippocampal Na-K-ATPases at different stages of development, despite variations in the amount of isoforms expressed (13, 24, 25, 28).

Although several studies suggested differences in the enzymatic behavior of the Na pump isoforms, discrepancies in the reported kinetic parameters led to uncertainties in the functional characteristics of the isoforms. In contrast, the reactivity of the Na pump isoforms toward ouabain is less controversial. Because of their broad sensitivities to cardiac glycosides, it is particularly evident for the rodent Na-K-ATPase isoforms. In the rat, for example, α1 was reported to be 100-fold more resistant to ouabain than α1 + (α2 + α3) (67, 100, 135, 194, 195). On the basis of the differential sensitivities to trypsin, early experimental evidence also suggested that the α3-isoform has a high affinity for ouabain (209). Working with partially purified Na-K-ATPase from rat brain stem, researchers identified three populations of Na-K-ATPase with different sensitivities to ouabain, possibly corresponding to α1, α2, and α3 (22, 29). In rabbit, pig, dog, and human, the differences in the cardiac glycoside affinity may not be as marked, since the α1-isoform in these species is much more sensitive to ouabain (23, 88, 90, 92, 104, 131, 179).

With the introduction of molecular biology techniques to the Na pump field, a better understanding of the function of each isoform became possible. The availability of the cDNAs for the different Na pump isoforms allowed studies in heterologous expression systems. Consequently, several investigators have used mammalian cells to separately express each Na pump isoform. Thus, when expressed in cultured rodent cells, the rat α2- and α3-isoforms have been shown to display $K_v$ values for ouabain of ~40 and 80 nM, respectively (89, 113). Through study of the molecular basis for the difference between primate and rodent cells in their sensitivities to the cardiac glycosides, it was shown that the rat α1-isoform could confer ouabain resistance to ouabain-sensitive CV-1 cells (65, 113). Using the differences in ouabain sensitivities between rodents and other mammals, J ewell and Lingrel (105) studied the kinetic properties of the rat α1-, α2-, and α3-subunits. By changing two residues in the extracellular loop between the first and second transmembrane segments, they converted the rat α2 and α3 into ouabain-resistant forms. The mutated isoforms were stably transfected into HeLa cells by their ability to support growth in 1 mM ouabain (105). Through this method, the expressed isoforms could be functionally separated.
from the endogenous ouabain-sensitive Na-K-ATPase. Analysis of the individual isoforms demonstrated that the αβ1 and αβ2 display similar affinities for Na\(^+\), K\(^+\), and ATP, whereas the αβ3-isoyme exhibits a lower affinity for Na\(^+\) compared with αβ1 and αβ2. Subsequently, the highly ouabain-sensitive Na-K-ATPase from rat brain or dog heart, ascribed to αβ3, was also reported to have a lower apparent affinity for Na\(^+\) than the intermediate and resistant components (α1 and α2) from the same tissues (23, 80). To identify the structural basis for the difference in the Na\(^+\) requirements between the isoforms, a series of chimeric α1/α3-subunits were expressed in HeLa cells. Analysis of the Na\(^+\) affinities of each chimera did not reveal a region clearly responsible for the differences in Na\(^+\) dependence between the isoforms, indicating that multiple residues spanning the α-polypeptide may be cooperating in the binding and transport of the cation (106).

Expression of hybrid Na-K-ATPase polypeptides between α- and β-isoforms from different species in Xenopus oocytes has also helped in elucidating the characteristics of the Na pump isoforms. Through this system, the α1β1 Na-K-ATPase has been shown to exhibit a higher activation by Rb\(^+\) than the α1β2- and α1β3-isoymes (102, 177). Utilizing heterologous expression in yeast, which lack endogenous Na-K-ATPase activity, Farley and colleagues (97, 98) successfully obtained catalytically competent Na pump molecules in cultured Sf-9 insect cells. As shown, the apparent affinity for Na\(^+\) varies with a rank of order α2β2 > α2β1 > α1β1 = α3β2 > α3β1. Also, the apparent affinity for K\(^+\) differs among the isoymes, following the sequence α1β1 > α2β1 = α2β2 > α3β1 = α3β2. For the activation by ATP, the enzymes composed of the α2- and α3-isoymes display equivalent K\(_m\) values, which are approximately four times lower than that of the α1β1. Altogether, these results indicate that the major kinetic differences occur among Na-K-ATPases that differ in the α-subunit composition. These observations are in reasonably good agreement with the kinetic properties reported for the rat α1-, α2-, and α3-isoymes expressed in HeLa cells (57, 58, 105, 154, 203). The only difference between our results and those obtained in HeLa cells is the K\(^+\) affinity of the α3-isoymes, which we found to be lower than that of the α1 or α2. The differences in the membrane environment of mammalian and invertebrate cells, the analysis of hybrid Na pump molecules in the HeLa cells, or the alteration of the isoymes by mutation of their ouabain binding site may be responsible for the disparity in results.

As mentioned before, the most conspicuous kinetic difference among the isoymes corresponds to the reactivity toward ouabain, with α3β1 and α3β2 displaying a high, the α2β1 and α2β2 an intermediate, and α1β1 a relatively free from contaminating Na-K-ATPase activity (36, 59). In addition, the recombinant viruses can be used independently and in combination to study assembly and activity of the various Na-K-ATPase isoforms.

The insect cells are able to correctly assemble the various α- and β-isoforms into functional enzyme (30, 33, 35, 36, 59). Infecting cells with the various recombinant baculoviruses has demonstrated that all α-isoforms can stably assemble with either the β1- or the β2-polypeptides. This is consistent with the results of other investigators (1, 116, 177). Although all possible α and β pairs result in active enzyme, some combinations, such as α1β2, exhibit lower activity, suggesting that not all associations are equally favored. Evidence for a selective interaction of certain α- and β-subunits has been reported using Xenopus oocytes (178). The importance of this preferential assembly in directing isozyme expression is unknown.

By analyzing the dose-response curves toward Na\(^+\), K\(^+\), ATP, and the inhibitor ouabain, the enzymatic properties of the various Na pump isoymes were determined. Table 1 summarizes the kinetic parameters for the rat Na-K-ATPase isoymes expressed in the insect cells. As shown, the apparent affinity for Na\(^+\) varies with a rank of order α2β2 > α2β1 > α1β1 = α3β2 > α3β1. Also, the apparent affinity for K\(^+\) differs among the isoymes, following the sequence α1β1 > α2β1 = α2β2 > α3β1 = α3β2. For the activation by ATP, the enzymes composed of the α2- and α3-isoymes display equivalent K\(_m\) values, which are approximately four times lower than that of the α1β1. Altogether, these results indicate that the major kinetic differences occur among Na-K-ATPases that differ in the α-subunit composition. These observations are in reasonably good agreement with the kinetic properties reported for the rat α1-, α2-, and α3-isoymes expressed in HeLa cells (57, 58, 105, 154, 203). The only difference between our results and those obtained in HeLa cells is the K\(^+\) affinity of the α3-isoymes, which we found to be lower than that of the α1 or α2. The differences in the membrane environment of mammalian and invertebrate cells, the analysis of hybrid Na pump molecules in the HeLa cells, or the alteration of the isoymes by mutation of their ouabain binding site may be responsible for the disparity in results.

As mentioned before, the most conspicuous kinetic difference among the isoymes corresponds to the reactivity toward ouabain, with α3β1 and α3β2 displaying a high, the α2β1 and α2β2 an intermediate, and α1β1 a

**Table 1. Kinetic characteristics of the rat Na-K-ATPase isoymes expressed in Sf-9 insect cells**

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Na(^+) Activation (K_{05}), mM</th>
<th>K(^+) Activation (K_{05}), mM</th>
<th>ATP Activation (K_{05}), mM</th>
<th>Ouabain Inhibition (K_i), mM</th>
<th>Calcium Inhibition (K_i), mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native α1β1</td>
<td>17.5 ± 0.4</td>
<td>2.1 ± 0.7</td>
<td>0.32 ± 0.04</td>
<td>9.8 ± 0.9 (\times) 10(^{-5})</td>
<td>1.0 ± 0.2 (\times) 10(^{-4})</td>
</tr>
<tr>
<td>α1β1</td>
<td>16.4 ± 0.7</td>
<td>1.9 ± 0.2</td>
<td>0.46 ± 0.10</td>
<td>4.3 ± 1.9 (\times) 10(^{-5})</td>
<td>7.3 ± 4.6 (\times) 10(^{-6})</td>
</tr>
<tr>
<td>α2β1</td>
<td>12.4 ± 0.5</td>
<td>3.6 ± 0.3</td>
<td>0.11 ± 0.01</td>
<td>1.7 ± 0.1 (\times) 10(^{-7})</td>
<td>1.9 ± 1.0 (\times) 10(^{-5})</td>
</tr>
<tr>
<td>α2β2</td>
<td>8.8 ± 1.0</td>
<td>4.8 ± 0.4</td>
<td>0.11 ± 0.02</td>
<td>1.5 ± 0.2 (\times) 10(^{-7})</td>
<td>4.0 ± 1.2 (\times) 10(^{-8})</td>
</tr>
<tr>
<td>α3β1</td>
<td>27.9 ± 1.3</td>
<td>5.3 ± 0.3</td>
<td>0.09 ± 0.01</td>
<td>3.3 ± 0.3 (\times) 10(^{-8})</td>
<td>4.7 ± 0.4 (\times) 10(^{-8})</td>
</tr>
<tr>
<td>α3β2</td>
<td>17.1 ± 1.0</td>
<td>6.2 ± 0.4</td>
<td>0.07 ± 0.02</td>
<td>3.3 ± 0.3 (\times) 10(^{-8})</td>
<td>4.7 ± 0.4 (\times) 10(^{-8})</td>
</tr>
</tbody>
</table>

Values are means ± SE. Apparent affinities \(K_{05}\), \(K_{i}\), and inhibition constant \(K_i\) parameters were calculated from Na-K-ATPase activity dose-response curves for the indicated effectors. For comparison, the native α1β1 isoyme from the kidney has been included.
low sensitivity to the cardiotonic steroid (36, 155). Interestingly, the rat α1β1 enzyme expressed in insect cells is over 2-fold more sensitive and the α3β1 approximately 20-fold less sensitive to ouabain than the native enzyme. This difference in sensitivity between the native and expressed Na-K-ATPase may be a result of the different lipid environments of the enzyme. Alternatively, the sensitivity to ouabain may be influenced by the β-subunit or by the oligomeric structure of the α-subunit.

At present, the enzymatic properties of Na-K-ATPases composed of the α4-isofrom have not been analyzed. To determine the ouabain sensitivity of the α4-isofrom, we performed dose-response curves for the ouabain inhibition of Na-K-ATPase activity in membrane preparations from rat testis, the only tissue known to contain this isoform. As shown in Fig. 2A, the experimental data is best fitted assuming the existence of two isozymes with different sensitivities to the cardiotonic steroid. One of the components corresponding to ~45% of the total Na-K-ATPase has a calculated K_i of 1.4 ± 0.3 × 10^{-4} M and very likely represents the α1-isofrom. The second component represents ~55% of the total activity and exhibits a high affinity for the inhibitor, with a K_i of 1.8 ± 1.0 × 10^{-5} M. As previously shown (185), immunoblot analysis of the testis preparation showed that the tissue does not express the α2- or α3-isofoms (Fig. 2B). A complete characterization of the β-subunits in the rat testis has not been performed. However, β1 and β3 mRNAs have been detected in the gonad (133). The possibility that α1β3 represents the component of high affinity for ouabain is unlikely, since the other β-subunits do not influence the kinetics of the enzyme toward the cardiotonic steroids. Therefore, the strongly ouabain-sensitive Na-K-ATPase encountered probably corresponds to α4. It is unknown which β-isofrom naturally associates with α4. Consequently, the highly ouabain-inhibitable ATPase activity could correspond to α4β1 or α4β3. Undoubtedly, a complete determination of the kinetic characteristics of this isoform will provide insight into its role in Na-K-ATPase function.

As shown in Table 1, the β-isofrom composition does not influence the kinetic properties of the Na-K-ATPase isozymes as much as the α-polypeptides do. However, the β-subunit is able to influence the Na^+ requirement of the enzyme. Thus, for the α2- and α3-isofoms, the apparent affinity for Na^+ increases when associated with β2. A modulatory effect of the β-isofrom on the catalytic properties of the α-subunit has also been shown using Xenopus oocytes. Hybrid α1β1- and α1β2-polypeptides consisting of a Torpedo α- and a rat β-subunit, have different affinities for Ra^+ (102, 103, 177). Moreover, comparison of results from transfected HeLa cells (57, 105) with native enzyme from pineal gland (189) suggests an effect of the β-isofrom on the Na^+ dependence of the rat α3-isofrom. At present, the role the β-subunit plays in the reaction cycle of the Na-K-ATPase is uncertain. Characterization of the kinetics of hybrid Na-K-ATPases composed of the α1- or α3-isofoms and chimeric β-subunits containing the NH_2 terminus of β1 and the COOH terminus of the H-K β-subunit suggests that the β-subunit influences the interaction of the enzyme with Na^+ and reduces the ability of Na^+ in forming the phosphoenzyme complex from ATP (64). It also appears that the β-subunit can influence the K^+ affinity of the Na-K-ATPase (62). With use of the Xenopus oocytes as an expression system, the α1β2 enzyme exhibits a lower affinity for K^+ than α1β1 (102, 103, 177). Similarly, this trend was maintained in Sf-9 cells where the α2- and α3-isofoms in combination with β2 exhibit a slightly lower affinity for K^+ than the β1 counterparts (33, 35). In addition, the β-subunit has been shown to be actively involved in forming or stabilizing the K^+-occluding complex of the enzyme (126). Because in insect cells the β-isofoms are mainly...

![Fig. 2. A: dose-response curves for the ouabain inhibition of Na-K-ATPase from rat testis membranes. Na-K-ATPase activity was determined after preincubation of the samples for 30 min at 37°C in the reaction mixture containing 120 mM NaCl, 30 mM KCl, 3 mM MgCl_2, 0.2 mM EGTA, 30 mM Tris·HCl (pH 7.4), and the indicated ouabain concentrations. The reaction was started by the addition of [γ-32P]ATP. Values are expressed as percentage of maximal activity in the absence of the inhibitor. Curves represent the best fit of the data assuming the existence of two enzyme populations with different ouabain affinities. Each value is the mean, and error bars represent the standard errors of the mean of 4 experiments performed in triplicate. B: immunoblot analysis of the Na-K-ATPase α-subunit expression in rat testis. Testis proteins (30 μg) were separated by SDS-PAGE (7.5% gel) and transferred to nitrocellulose. Na-K-ATPase α-polypeptides were detected using isoform-specific antibodies as before (32). A membrane preparation from rat brain (20 μg) is shown as control.]

expressed in their core-glycosylated forms (36, 59), the difference in Na\(^+\) affinity between isoforms containing \(\beta1\) or \(\beta2\) most likely depends on the dissimilar amino acid structure of the \(\beta\)-isoforms rather than the sugar composition. Additional evidence of the importance of the \(\beta\)-subunit in modulating the activity of the Na-K-ATPase has been suggested from the kinetic analysis of the human \(\alpha1\beta3\) Na-K-ATPase expressed in insect cells. The \(\beta3\)-isoform slightly influences the Na\(^+\), K\(^+\), and ouabain affinities of the enzyme (214).

A physiological role for the difference between Na-K-ATPases composed of \(\beta1\) and \(\beta2\) is difficult to infer. Although \(\beta2\) confers \(\alpha2\) and \(\alpha3\) with a similar apparent affinity for Na\(^+\) as the \(\alpha1\beta1\) enzyme, \(\alpha2\beta2\) and \(\alpha3\beta2\) still retain the particular K\(^+\), ATP, and ouabain reactivities of their counterpart \(\beta1\)-containing isoforms. The importance of the \(\beta2\)-polypeptide in Na-K-ATPase function may be related to other properties intrinsic to this subunit. For example, an unexpected functional property of the \(\beta2\)-isoform is that, in addition to its role as a constituent of the Na pump, it serves as a cell adhesion molecule that mediates Ca\(^{2+}\) independent neuron-astrocyte interactions (82, 177). In addition, \(\beta2\) has been shown to be involved in neuronal migration and neurite outgrowth (5, 82, 153). Coinciding with its role in cell-cell interaction, the decrease in \(\beta2\) mRNA levels in human kidney, lung, and liver carcinomas suggests that this subunit may be involved in tumorigenesis (3). Although the link between cell recognition and ion transport is unknown, it is conceivable that \(\beta2\) might modulate Na pump function to provide the ionic environment required in such specific cellular processes. The importance of the \(\beta2\) has recently been demonstrated in mice carrying a targeted deletion of the gene for this isoform (132). The \(\beta2\)-deficient animals exhibit motor incoordination at 15 days of age, develop tremors and paralysis of extremities, and subsequently die at 17–18 days after birth. The observation that at different areas of the nervous system there is swelling and vacuolization of astrocyte processes suggested the requirement of \(\beta2\) in maintaining ionic homeostasis. However, the cause of death in these rodents is unclear, since \(\beta2\) is not necessary for the long-term survival of cultured telencephalic grafts obtained from the transgenic mice (101).

**Physiological Relevance of Na Pump Isozymes**

It seems plausible that the existence of Na-K-ATPase isoforms is based, at least in part, on their specific kinetic characteristics (30, 33, 35, 57, 105, 107). The subtle differences in their affinities for cations and ATP may be essential in adapting cellular Na-K-ATPase activity to specific physiological requirements. Because of its ubiquitous expression, the \(\alpha1\beta1\)-Isozyme may function as the housekeeping Na-K-ATPase in the cell, whereas the other isoforms may mediate tissue-specific roles. For example, in neurons where all the isoforms are present, while the \(\alpha1\)- and \(\alpha2\)-isoforms are maintaining the basal ionic gradients, \(\alpha3\), because of its low affinity for the cations, will be operating very slowly. With depolarization and the repeated firing of action potentials, the Na\(^+\) and K\(^+\) gradients are dissipated. Under these conditions, while the \(\alpha1\) and \(\alpha2\)-isoforms are working at saturation, the \(\alpha3\)-isoform will be activated. In this manner, \(\alpha3\) functions as a spare pump to help restore the resting membrane potential. Also, the high affinity for ATP endows \(\alpha3\) with the ability to utilize the low nucleotide concentrations occurring near the cell membrane after intense neuronal activity. Moreover, the higher Na\(^+\) affinity of the \(\alpha2\)-isoform gives it a steady working capability, since Na\(^+\) will be rate limiting only at very low concentrations. This is important in glial cells, where after neuronal activity, \(\alpha2\) can efficiently clear the high extracellular K\(^+\) to prevent further depolarization. In addition, as discussed below, multiple Na-K-ATPase isozymes with different apparent affinities for Na\(^+\) may be physiologically important in secondary control of intracellular Ca\(^{2+}\) levels. These variations in cytoplasmic Ca\(^{2+}\) are important in the regulation of contraction, secretion, and excitability (37).

A differential function for the Na-K-ATPase isoforms is suggested by the stringent regulation of the expression of the \(\alpha\) - and \(\beta\)-polypeptides under various physiological conditions. During development, a change in the relative amount of Na pump isoforms occurs in several tissues (157). A remarkable example of regulation of Na-K-ATPase isoform expression is found in the developing rat heart. In this tissue, between the second and third week of life, there is a switch from the \(\alpha3\)-isoform to \(\alpha2\)-isoform, which becomes the predominant isoform of the adult myocardium (124). Interestingly, this event coincides with important changes in the electrophysiological properties of the myocardium (121, 124). The Na pump isoforms may also be differentially regulated by hormones (reviewed in Ref. 68). Hormones can elicit their action by modulating the expression of a particular isoform or by directly affecting the activity of an individual Na-K-ATPase (68). For example, in rat skeletal muscle, insulin is able to produce a rapid translocation of preexisting \(\alpha2\) Na-K-ATPase from intracellular stores to the plasma membrane (99). This results in the recruitment of additional functional Na pumps to the cell surface and increased Na-K-ATPase activity. In addition, the normal expression of Na-K-ATPase isoforms can be altered by pathological conditions. For instance, in several cardiac diseases, the Na-K-ATPase isoform composition of the heart is modified. These changes may reflect the cellular attempt to reestablish the altered homeostasis associated with the disease. Consequently, it seems conceivable that the preferential regulation or modification of a specific isoform may be important in finely adjusting cellular ionic homeostasis.

The difference among rodent Na-K-ATPase isoforms in ouabain affinity is intriguing, although its physiological relevance remains unknown. It is possible that differences in ouabain binding may have a role in the regulation of Na-K-ATPase activity. Only rodents are known to have Na-K-ATPases that vastly differ in their ouabain affinities. In other species, the \(\alpha1\beta1\)-isozyme is much more sensitive to the cardiotonic steroids, making the isoform differences in ouabain sensitivity less
A detailed study of the ouabain binding constants for species other than rodents has not been accomplished. Nevertheless, more than one ouabain affinity site has been reported in non-rodent species (66, 67, 88, 90, 104, 131). In humans, both single as well as complex ouabain binding have been found in the heart (66, 67, 184), which is known to express the α1-, α2-, and α3-isoforms of the Na pump (216). In the heart, the existence of multiple isoforms is undoubtedly important in mediating the action of the cardiotonic steroids. It is clear that cardiotonic steroids act through their ability to inhibit the Na-K-ATPase. This inhibition results in a transient increase in intracellular Ca\(^{2+}\), which induces through the Na/Ca exchange system an increase in intracellular Ca\(^{2+}\). The rise in internal Ca\(^{2+}\) is taken up by the SR via an SR Ca-ATPase. Because of the elevated Ca\(^{2+}\) content in the SR, more Ca\(^{2+}\) can be released when the cardiocyte is stimulated, leading to an augmented force of contraction and an increased cardiac output (37, 48, 118, 141, 145, 205). The relevance of the cardiotonic glycosides as Na-K-ATPase modulators is supported by the finding of endogenous, digitoxin-like compounds in mammals (reviewed in Refs. 37, 38, 60, 61, 111, 168). An isomer of ouabain has been found to be produced in the bovine hypothalamus (93, 94, 220), and ouabain itself is present in the adrenal glands of several species including humans (37, 38, 86, 87). Thus endogenous ouabain may regulate cardiovascular toxicity by inhibiting a discrete number of Na-K-ATPase pumps to modulate cellular excitability and heart and vascular muscle contractility.

Studying the effect of Ca\(^{2+}\) on the activity of the different rat Na-K-ATPase isoforms expressed in Sf-9 cells, we found that the cation differentially affects the function of each isoform (34). As has been shown before, the α1β1-isozyme is quite resistant to Ca\(^{2+}\) (15). In contrast, the α2β1- and α3β1-isozymes display K\(_i\) values 10- and 100-fold lower than that of the α1β1, respectively (Table 1). Similarly, in rat axolemma, myometrium, and skeletal muscle, the Na-K-ATPase activity that has a high sensitivity to ouabain is tantamount in mediating the action of the cardiotonic steroids. Interestingly, in contrast to the α1-isoform, α2-isoform in astrocytes and α3-isoform in neurons and myocytes exhibit a reticular distribution on the plasma membrane which parallels the underlying endoplasmic reticulum or SR (110). This distribution is identical to that of the Na/Ca exchanger. Thus, while α1 is regulating bulk cytosolic Na\(^{+}\), α2 and α3 may adjust Na\(^{+}\) concentrations in the restricted cytosolic space between the plasma membrane and reticulum to indirectly control Ca\(^{2+}\) (110).

Regulation of Na-K-ATPase Isozymes

Regulation of the Na-K-ATPase activity occurs by different cellular mechanisms and can be achieved by modulation of the number of enzyme molecules present at the plasma membrane or by influencing the activity of the Na-K-ATPase already at the cell surface. For example, the amount of the enzyme at the plasma membrane can be modified by changes in the rate of synthesis or degradation of the individual Na pump polypeptides as well as by mobilization of Na pump molecules from the endosomal pools to the cell surface (reviewed in Refs. 68, 138). Alternatively, the activity of the Na pump at the cell surface can be directly regulated (26, 68, 138, 149), providing a rapid adjustment in Na pump function. Several effectors have been implicated in this acute response; the primary one is the intracellular Na\(^{+}\) concentration, which at steady state is rate limiting. Also, as mentioned before, endogenous ouabain and intracellular Ca\(^{2+}\) are potential short-term modulators of Na pump activity (34, 37, 212).

Recent evidence suggests that intracellular messengers can also affect Na-K-ATPase activity. Depending on the tissue, activation of protein kinases can induce an increase or decrease in Na pump activity (26, 54, 68, 138, 149, 172). Agents that increase cellular cAMP, as well as exogenous derivatives of cAMP, lead to Na-K-ATPase inhibition in the medullary thick ascending limb of the loop of Henle and the cortical collecting duct (18, 26, 96, 149, 172, 174) and to Na-K-ATPase stimulation in the proximal convoluted tubules (47, 74). This response seems to be mediated, at least in part, by protein kinase A (PKA)-directed phosphorylation of Ser\(^{943}\) of the Na pump α-subunit (77). Phosphorylation of the Na pump α-subunit is reversible, as demonstrated by the decrease in Na-K-ATPase activity after activation of a dopamine- and cAMP-regulated phosphoprotein (DARPP-32), an endogenous inhibitor of protein phosphatase 1 (PP1) (6). This suggests that a phosphorylation/dephosphorylation event may dynamically regulate the activity of the Na pump. Moreover, in the proximal nephron and in cultured canine kidney cells, the Na-K-ATPase is inhibited by phorbol esters or diaacylglycerol analogs, in a process that involves protein kinase C (PKC) activation (79, 172, 183) and, possibly, the phosphorylation of the Na pump α-subunit at Ser\(^{16}\) (17). In addition, several other mechanisms have been postulated concerning the way by which protein kinases exert their effects on the Na pump. For example, in amphibian nephron cells, PKC produces Na-K-ATPase inhibition by increasing cell endocytosis and internalization of Na pump molecules (21), and in isolated nephron segments, PKA may stimulate phospholipase A\(_{2}\) (PLA\(_{2}\)) and the production of arachidonic acid and its metabolites (173). Moreover, in the kidney another kinase, protein kinase G (PKG), and the production of cGMP appear to mediate the loss of
salt and water produced by acetylcholine, bradykinin, and atrial natriuretic peptide (144, 175). In contrast, in the duck salt gland cGMP stimulates ouabain-sensitive \( p \)-nitrophenylphosphatase hydrolysis by the Na pump (192).

Although the effect of intracellular messengers on the function of the \( \alpha1\beta1 \)-isozyme has been well documented (26, 68, 138), the regulation of the other Na-K-ATPase isozymes by protein kinases is poorly understood. We studied the effect of different protein

![Diagram](image-url)
kinases on the rat Na-K-ATPase isoforms expressed in Sf-9 cells. Similar to mammalian cells, insect cells glycosylate, phosphorylate, acylate, and perform most other posttranslational modifications dictated by the primary amino acid sequence. In addition, they display a complex signal transduction machinery comparable to that of their mammalian counterparts (156). As shown in Fig. 3, activation of PKA, PKC, and PKG, using dibutyryl-cAMP, phorbol 12-myristate 13 acetate (PMA), or dibutyryl-cGMP, respectively, is able to differentially modify the function of the Na-K-ATPase isoforms. Specificity of the response is demonstrated by the reversibility of the effect by the respective protein kinase competitive inhibitors. As shown, PKC activation leads to inhibition of all isoforms, PKA activation stimulates the activity of the Na-K-ATPase \( \alpha_3\beta_1 \) and decreases that of the \( \alpha_1\beta_1 \) and \( \alpha_2\beta_1 \)-isozymes. Finally, activation of PKG diminishes the activity of the \( \alpha_1\beta_1 \) and \( \alpha_3\beta_1 \)-isozymes, without altering that of \( \alpha_2\beta_1 \). The regulation of the activity of the Na pump isoforms elicited by PKA and PKC does not depend on changes in the rate of synthesis or degradation of the Na-K-ATPase polypeptides, but is rather a result of changes in the molecular activity of the Na-K-ATPases (34).

The mechanisms by which protein kinases regulate the Na-K-ATPase isoforms are not well characterized. Direct phosphorylation of the Na pump \( \alpha \)-subunit by PKA and PKC was originally demonstrated in vitro for the purified enzyme from kidney (6). Although it has been more difficult to demonstrate, phosphorylation of the endogenous Na pump has also been shown in intact cells (reviewed in Refs. 26, 68, 149). In the insect cells, we found a PKA- and PKC-dependent phosphorylation of \( \alpha_1 \), as well as the \( \alpha_2 \)- and \( \alpha_3 \)-isoforms. Figure 4 shows that competitive inhibitors of PKC and PKA partially prevent the phosphorylation of the Na pump \( \alpha \)-isoforms by treatment with PMA and dibutyryl-cAMP respectively. This implies that PKC and PKA are able to specifically phosphorylate the \( \alpha_1 \), \( \alpha_2 \), and \( \alpha_3 \)-polypeptides. However, it has not been ruled out that the PKA- and PKC-dependent phosphorylation involves another downstream messenger that may induce the phosphorylation or inhibit the dephosphorylation of the enzyme. In any case, phosphorylation appears to be at least one of the mechanisms by which PKA and PKC affect Na-K-ATPase isozyme activity. In agreement with these results, Beguin et al. (19) have shown that PKC is able to phosphorylate the rat \( \alpha_2 \)-isoform, although less efficiently than the \( \alpha_1 \)-isoform. Also, it appears that rat

![Fig. 4. Phosphorylation of the Na-K-ATPase \( \alpha_1 \), \( \alpha_2 \), and \( \alpha_3 \)-isoforms by PKA and PKC. Sf-9 were grown in 6-well plates and infected with baculovirus containing the different rat Na-K-ATPase isoforms. Forty hours after infection, cells were starved for 1 h in phosphate-free medium and labeled for 1 h with \( ^{32} \)Porthophosphate (0.3 mCi/well). Then, for PKC phosphorylation (A), cells were treated either with or without H7 (200 nM) for 30 min and PMA (1 \( \mu \)M) for an additional hour. For PKA phosphorylation (B), cells were treated with or without 8-bromoadenosine cyclic monophosphothioate, Rp isomer (8-BrcAMP50, 50 \( \mu \)M) for 30 min and dibutyryl-cAMP (DBcAMP, 2 mM) for another hour. Cells expressing only the \( \beta \)-subunit and treated with PMA or dibutyryl-cAMP are shown as a control. In all cases, cells were lysed and samples were subjected to immunoprecipitation using monoclonal antibodies as described (32). Precipitated proteins were separated by SDS-PAGE (7.5%), gels were dried, and radioactivity was quantified using a PhosphorImager SI scanner and the ImageQuant NT software (Molecular Dynamics, Sunnyvale, CA). A representative experiment is shown, and bars represent the intensity of labeling as a percentage of the phosphorylation obtained in absence of protein kinase inhibitors.](http://ajprenal.physiology.org/content/181/4/F644/Fig4)
α3-isoform is phosphorylated by PKC, whereas the same isoform from other species is not. In the α1-isoform, phosphorylation by PKC was originally mapped to Ser61 (17). Recently, Ser23 has also been identified as a potential site for phosphorylation (75). Furthermore, Pedemonte et al. (161) have shown that an α1 deletion mutant, which lacks the first 31 amino acids, fails to respond to PKC. The region containing the NH2 terminus of the α-subunit exhibits the most sequence divergence, both among species and isozymes. For example, Ser61 is present only in the α1-isoform, whereas Ser23 is present in the α1-subunit of the rat and Torpedo californica as well as in the rat α3-isoform. Phosphorylation of the α2-isoform suggests that additional residues might also be involved. The similar decrease in activity found after PKC stimulation for α2β1 and α3β1 indicates that phosphorylation of other serine residues might be occurring within the same protein domain in these isozymes. In contrast, phosphorylation of the α1-isoform by PKA takes place at Ser943 (77) in a highly conserved cytoplasmic region between transmembrane segments 8 and 9. Thus the same residue might be involved in the phosphorylation of α1 and α3 by this protein kinase. Interestingly, if Ser943 is involved in the effect of PKA on all isozymes, then the incorporation of Pγ must somehow alter α3β1 in a different manner, to increase Na-K-ATPase activity in this isozyme.

A physiological explanation for these isozyme-specific effects is difficult to infer; nevertheless, the observation provides additional evidence for a role for the Na pump isozymes. For example, the Na-K-ATPase α3β1-isozyme has the distinct kinetic characteristics of a low affinity for Na+ and K+ and a high affinity for ATP (Table 1). The specific activation of α3β1, concomitant with the inhibition of the other two Na pump isozymes, could lead to subtle variations in the Na+ and K+ ionic gradients and hence to changes in the membrane potential and cellular excitability. This could be particularly important in neuronal cells, where α3 is predominantly expressed.

It is clear that the activity of the Na-K-ATPase is under the control of a variety of intracellular messengers that are able to modulate the function of the particular isozymes in a specific fashion. Because the Na-K-ATPase isozymes have kinetic properties that are unique, isozyme-specific regulation may be important in adapting Na pump function to the requirements of each cell.

Conclusions

Since the Na-K-ATPase isozymes were identified in 1979 (194), our knowledge regarding their expression, function and regulation has significantly broadened. The structural heterogeneity that characterizes the enzyme does not represent simple redundancy, but rather it is an evolutionary refinement that contributes to the diversity in Na pump function. It is therefore not surprising that stringent regulatory mechanisms have developed to adjust their expression and activity to serve the requirements of each cell under various physiological and pathological conditions. Future studies to clarify the mechanisms important in Na-K-ATPase isoform regulation and to precisely link isozyme function to physiological processes will provide a better understanding of the role of the Na-K-ATPase isozymes in cellular functions.

Address for reprint requests: G. Blanco, Dept. of Cell Biology and Physiology, Washington Univ. School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110.

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