Na,K-ATPase Subunit Heterogeneity as a Mechanism for Tissue-Specific Ion Regulation

Gustavo Blanco

The Na,K-ATPase comprises a family of isozymes that catalyze the active transport of cytoplasmic Na\(^+\) for extracellular K\(^+\) at the plasma membrane of cells. Isozyme diversity for the Na,K-ATPase results from the association of different molecular forms of the α (α1, α2, α3, and α4) and β (β1, β2, and β3) subunits that constitute the enzyme. The various isozymes are characterized by unique enzymatic properties and a highly regulated pattern of expression that depends on cell type, developmental stage, and hormonal stimulation. The molecular complexity of the Na,K-ATPase goes beyond its α and β isoforms and, in certain tissues, other accessory proteins associate with the enzyme. These small membrane-bound polypeptides, known as the FXYD proteins, modulate the kinetic characteristics of the Na,K-ATPase. The experimental evidence available suggests that the molecular and functional heterogeneity of the Na,K-ATPase is a physiologically relevant event that serves the specialized functions of cells. This article focuses on the functional properties, regulation, and the biological relevance of the Na,K-ATPase isozymes as a mechanism for the tissue-specific control of Na\(^+\) and K\(^+\) homeostasis.

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**KEYWORDS** Na,K-ATPase isozymes, isoforms, ouabain, digitalis, FXYD

Maintenance of an asymmetric distribution of Na\(^+\) and K\(^+\) between the cytoplasm and cell surroundings is a crucial event in the physiology of animal cells. The typical low Na\(^+\)/K\(^+\) ratio of the intracellular space depends on the activity of the Na,K-ATPase or Na pump, a membrane-associated enzyme that uses the energy from the hydrolysis of adenosine triphosphate (ATP) to transport 3 Na\(^+\) out of the cell in exchange for 2 K\(^+\) that are taken in.\(^1\) The ion gradients created by the Na,K-ATPase are necessary for many common, as well as cell-specific, processes. The enzyme is involved in maintaining cell osmotic balance and volume, the resting membrane potential of most cells, the excitability of muscle and neuronal cells, and the Na\(^+\)-coupled secondary transport of H\(^+\), Ca\(^+\), glucose, amino acids, and neurotransmitters across the plasma membrane.\(^1\) In addition, the Na pump drives the vectorial movement of water and salt across many epithelia. It plays a primary role in urine formation in the kidney,\(^2\) and is important in maintaining the electrolyte composition of particular fluid compartments, such as the aqueous humor,\(^3\) endolymph,\(^4\) and cerebrospinal fluid.\(^5\) The contribution of the Na,K-ATPase to such diverse processes requires its function to be adjusted specifically to the needs of each tissue. One of the strategies organisms have developed to confer enzymes the functional versatility that is needed to fulfill specific tasks is the expression of different molecular variants or isozymes with distinct functional capabilities. The interesting discovery that not one but multiple forms of the Na,K-ATPase exist in animal cells provided the basis for the heterogeneity of the enzyme (reviewed in \(^6\)-\(^10\)). This stimulated intense research to unveil the physiologic role of the Na,K-ATPases. The information available at present suggests that the Na,K-ATPase diversity is not a biologically redundant phenomenon.\(^9\) This article reviews the current knowledge on the Na,K-ATPase isozymes and their significance for the maintenance of cell-specific Na\(^+\) and K\(^+\) homeostasis and tissue function.

**Na,K-ATPase Molecular Structure and Subunit Composition**

The Na,K-ATPase isozymes are oligomers that result from the association of various molecular forms or isoforms of 2 major
polypeptides: the α and β subunits. A scheme of the primary structure, membrane organization, and amino acid differences among α and β isoforms are shown in Fig. 1. The α polypeptides have a molecular weight between 110 and 112 kd and are arranged in a large cytoplasmic mass, 10 membrane-spanning helices, and a small ectodomain. The α subunit is responsible for the catalytic and transport properties of the Na,K-ATPase and undergoes conformational changes (designated E1 and E2) that are coupled to the binding, occlusion, and translocation of the cations. For this, the α subunit contains the binding sites for Na⁺, K⁺, and ATP. Cardiotonic steroids such as ouabain also bind to the α subunit and inhibit the enzyme. Recently, molecular modeling based on homology to the rabbit sarcoplasmic reticulum Ca-ATPase (SERCA1a) has helped us to understand the structure-function of the enzyme. This shows that the ATP-coupled translocation of ions depends on the relative motion of 3 cytoplasmic domains in the protein. These include the actuator or A domain at the N-terminus and first intracellular loop, and the nucleotide binding (N) and phosphorylation (P) domains both located between transmembrane domains 4 and 5.

The β subunits are polypeptides that have molecular weights between 40 and 60 kd, depending on isoform- and tissue-specific differences in glycosylation. The basic structure common to all β isoforms consists of an N-terminal cytoplasmic tail, a single transmembrane region, and a large C-terminal extracellular domain comprising approximately 80% of the protein (Fig. 1). The ectodomain of all β subunits characteristically present 3 disulfide bridges and consensus sequences for glycosylation. The β subunit is essential for normal activity of the Na,K-ATPase and in vertebrates acts as a chaperone protein that assists in membrane insertion, folding, and delivery of the holoenzyme to the plasma membrane (reviewed in ). In addition, the β polypeptides also deter-
mine, in an isoform-specific manner, some of the transport and catalytic properties of the enzyme.9

Besides the α and the β subunits, other small, single-membrane–spanning polypeptides have been found to interact with the Na,K-ATPase. The first one identified, designated the γ subunit, is a hydrophobic polypeptide of approximately 7 kd that in kidney copurifies and colocalizes with the Na,K-ATPase. The first one identified, designated the α subunit.15 More recently, other polypeptides that share a FXYD signature motif with the γ subunit have been found.16 These include Phospholemman (a phosphoprotein of cardiac sarcolemma, or FXYD1), MAT-8 (for Mammary Tumor marker, or FXYD5), and the FXYD6 (phosphohippolin) and FXYD7 polypeptides from the nervous system. Although these accessory proteins are not required for activity of the holoenzyme, some of them influence the transport and kinetic properties of the Na,K-ATPase and also can operate as channels (reviewed in16).

### Subunit Diversity of the Na Pump

The first indication of the existence of various Na,K-ATPases derived from functional studies of the catalytic and transport properties of the enzyme from different tissues. Thus, dose-response curves for the inhibition of Na,K-ATPase function by cardiotonic steroids revealed that, although in kidney there was a single binding site for the compounds, in brain the curves were bimodal, suggesting the existence of an additional enzyme form significantly more sensitive to the inhibitors.17,18 Na,K-ATPase heterogeneity then was supported by the finding that the α subunit from brain migrates as 2 different bands in sodium dodecyl sulfate–polyacrylamide gels, one similar to that of the kidney or α form, and a form with less mobility, named α+.7 Subsequent biochemical studies confirmed that α+ was the isoform responsible for the high ouabain-sensitive component of the enzyme.7 Soon after, dissimilarities in trypsin digestion and in the structure of the N-terminus of α and α+ were found.19 The isolation in mammals of the complementary DNAs for 3 α isoforms and gene-mapping studies definitely established a genetic basis for the heterogeneity of the Na pump. The kidney or α form was renamed as α1, whereas α+ represented a mixture of 2 molecular variants: α2 and a third isoform, α3.20,21 More recently, the family of Na,K-ATPase α genes has expanded with the discovery of a fourth α isoform (α4) in human and rat testis.22,23 The genes coding for each Na pump isoform have been mapped to specific chromosomal regions. This, as well as the intron/exon composition of the isoforms of human, rat, and mouse, are shown in Table 1.

The Na,K-ATPase α isoforms have similar primary structures. Comparison of the amino acid sequence of the α subunit from a variety of species indicates a high degree of conservation for the isoforms. For α1 and α2, identity is approximately 92% and for α3 is greater than 96%. The α4 isoform, from rat, mouse, and human share 90% of the amino acids. Comparison of different α isoforms within the same species shows a lower degree of identity, being approximately 87% among α1, α2, and α3. In contrast, α4 shares only 76% to 78% identity with the other isoforms. The regions of highest structural variability among α isoforms include 3 main regions: (1) the N-terminal portion of the polypeptides that forms part of the A domain, (2) the extracellular loop between transmembrane segments 1 and 2 that forms part of the ouabain binding site, and (3) the isoform-specific region (ISR).26 An 11 amino acid sequence in the major loop between transmembrane domains 4 and 5 that comprises residues 489 to 499 of the rat α1 isoform. As described later, these regions are involved in some of the functional characteristics that differentiate each α isoform.

### Table 1 Chromosomal Localization and Number of Exons and Introns of Na,K-ATPase Isoform Genes From Various Species

<table>
<thead>
<tr>
<th>Isoform</th>
<th>α1</th>
<th>α2</th>
<th>α3</th>
<th>α4</th>
<th>β1</th>
<th>β2</th>
<th>β3</th>
<th>βm (β4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene nomenclature</td>
<td>ATP1A</td>
<td>ATP1A2</td>
<td>ATP1A3</td>
<td>ATP1A4</td>
<td>ATP1B1</td>
<td>ATP1B2</td>
<td>ATP1B3</td>
<td>ATP1B4</td>
</tr>
<tr>
<td>Human</td>
<td>Chromosome</td>
<td>Exon/intron</td>
<td>1p21</td>
<td>1q21-q23</td>
<td>19q13</td>
<td>1q21-q23</td>
<td>1q24</td>
<td>17p13</td>
</tr>
<tr>
<td>Rat</td>
<td>Chromosome</td>
<td>Exon/intron</td>
<td>2q34</td>
<td>13q24-q26</td>
<td>1q21</td>
<td>13q24</td>
<td>13q22</td>
<td>10q24</td>
</tr>
<tr>
<td>Mouse</td>
<td>Chromosome</td>
<td>Exon/intron</td>
<td>3F3</td>
<td>1H3</td>
<td>7A3</td>
<td>1H3</td>
<td>1H2.2</td>
<td>11B3</td>
</tr>
</tbody>
</table>

Data obtained from Ensembl Genome Data Resources (www.ensembl.org)24 and the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/entrez)25.
ing of multiple forms of the β subunit, each under the control of a different gene (Table 1). Cloning of the ubiquitously expressed β1 was followed by 2 other isoforms: β2, named also adhesion molecule of glia (AMOG) for its additional function as a mediator of neuron-glia interactions,31,32 and β3.32 The β isoforms are highly conserved across species with amino acid identity scores of 94% to 96% for β1 and β2, and 75% for β3. Amino acid sequence comparisons within the same species show that the β polypeptides are more divergent than the α isoforms. Thus, β2 or β3 share with β1 34% and 39% identity, respectively, whereas β2 and β3 have 49% of their residues conserved. The major similarities among β isoforms include the transmembrane region and the 6 cysteines of the extracellular domain that participate in formation of the disulfide bridges (Fig. 1). Integrity of the disulfide bridges is important as shown by the inactivation of the enzyme after treatment with reducing agents (reviewed in 19). The differences in primary structure among β isoforms are scattered along the protein sequence and affect the glycosylation sites, which vary in location and number. Three N-linked glycosylation sites are present in β1, whereas 2 to 8 have been predicted in β2 and β3. In addition, glycosylation of the β polypeptides varies in different tissues and species. As expected from their lack of conservation, hydrocarbon content of the β subunit is not involved directly in activity of the Na,K-ATPase, and neither protein deglycosylation nor mutation of the glycosylated asparagines affect ATP hydrolysis by the enzyme (reviewed in 14).

In addition to the β1, β2, and β3 isoforms, another β subunit has been identified in human, pig, rat, and mouse muscle.34 This form, named βm (for muscle β), or β4, differs from the others by a larger N-terminal domain rich in glutamic acid, an unusual pattern of glycosylation of short high mannose and hybrid N-glycans, and a peculiar localization inside the cell.35 It still remains unknown, however, whether this isoform forms part of the Na,K-ATPase or is a component of another X,K-ATPase.

Another mechanism that contributes to Na pump subunit heterogeneity is the differential posttranscriptional processing of the α and β subunits. Thus, in canine vascular smooth muscle, alternative splicing of the α1 messenger RNA results in a polypeptide of approximately 65 kD, named α1-T.36 Despite the lack of 40% of the C-terminus, α1-T has been reported to be functional when expressed in insect, HeLa, and opossum kidney cells.37 Also, a different truncated transcript of α1, as well as a shorter form of the β1 subunit, have been found in human retinal epithelium.38 In addition, functionally active versions of α1 resulting from the use of alternative initiation codons have been described in Xenopus laevis.39 At present, the role of all these truncated subunits in the native tissues remains unknown.

The molecular diversity of the Na,K-ATPase not only involves the α and β isoforms, but also extends to the γ polypeptide. This was first suspected from the migration of γ as a doublet in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (reviewed in 40). Mass spectrometry then confirmed the existence of 2 forms (γa and γb) that, resulting from alternative splicing, differ only in few amino acids at the N-terminus (Fig. 1).41 Both γa and γb forms are present in the kidney, where they localize to different regions of the nephron.42,43 A third form of the γ polypeptide also has been reported in fetal tissues from the mouse.44

### Isozyme Heterogeneity of the Na,K-ATPase

The primary mechanism responsible for the generation of Na,K-ATPase isoforms is the association of the various α and β isoforms in different heterodimers.8,9 The co-expression of particular arrays of α and β isoforms in several tissues suggested that almost all αβ arrangements theoretically are possible.9,10 Heterologous expression of α and β isoforms in various combinations in insect and mammalian cells confirmed this and showed that association between α and β isoforms is a promiscuous event.9,45 Importantly, all αβ arrangements studied to date (α1β1, α1β2, α1β3, α2β1, α2β2, α2β3, α3β1, α3β2, α3β3, α4β1, and α4β3) resulted in catalytically competent enzymes.45-50 This indicates that multiple isoforms of the Na,K-ATPase can operate in the cell; however, it is not known if, in the native tissues, the assembly of certain α with particular β isoforms is regulated to favor formation of some isoforms over others.

The αβ isoform complexity of the Na,K-ATPase may be even more intricate and may involve the quaternary structure of the enzyme. Several lines of investigation support the idea that the Na,K-ATPase exists as an oligomer of αβ subunits.31 Studies using Sf-9 cells indicate that when different α isoforms are expressed in the insect cells, they stably associate into oligomeric complexes.32 The α/α isoform association therefore raises the possibility of multiple oligomers with a variable configuration of α and β polypeptides. At present, the functional relevance of this higher structural organization of the Na,K-ATPase remains unknown.

### Cell and Tissue Expression of the Na Pump Polypeptides

With the availability of the nucleotide sequences and the production of specific antibodies, the search began to gain insight into the distribution of the various Na,K-ATPase isoforms in different cells and tissues. It soon was found that the α and β isoforms exhibit a complex tissue-specific pattern of expression. Expression of isoform messenger RNA and protein has been surveyed for a wide variety of tissues and species6,10,33,65 and the data have been summarized in Table 2. As shown, while α1β1 is the isozyme most widely expressed and the predominant Na,K-ATPase of the kidney, the other α and β isoforms exhibit a much more restricted pattern of expression (reviewed in 6-10). The central nervous system and retinal photoreceptors exhibit the broadest spectrum of isoforms, expressing all α and β polypeptides except α4. Within the nervous tissue, mature neuronal cells are the main source of the α3 polypeptide, whereas glial cells preferentially express α2.8 Also, many neurons can express β1 and β2,8 whereas astrocytes and oligodendrocytes contain in addition...
to β1, the β2 and β3 isoforms, respectively.\textsuperscript{54} The β3 polypeptide is also an abundant isoform of retinal photoreceptor cells.\textsuperscript{63} The isoform with the most limited pattern of expression is α4, the polypeptide is selectively expressed in male germ cells of the testis.\textsuperscript{64} Recently, very low levels of a transcript corresponding to the human and mouse α4 orthologs have also been detected in heart, pancreas, liver, and placenta.\textsuperscript{65} In addition, a larger transcript (7.5 kb compared with 4 kb) encoding α4 has been found in skeletal muscle.\textsuperscript{66} The expression of α4 in tissues different from testis still remains to be determined at the protein level. In addition to the tissue differences, there is some variability in isoform expression among species; this is particularly characteristic in cardiac tissue.\textsuperscript{67}

Several other factors are involved in the regulation of Na,K-ATPase isoform expression. For example, in most tissues, development is accompanied by an increase in the expression of 1 or more isoforms. In few other tissues, changes in isoform pattern of expression occur.\textsuperscript{68} A remarkable example of the plasticity in the expression of isoforms is found in rat heart. In this tissue, the α3 isoform, present during fetal life, is repressed and replaced by the adult form, α2. Interestingly, this switch coincides with changes in the action potential and excitability of the myocardocytes, suggesting the importance of the Na,K-ATPases in determining the electrical properties of the heart.\textsuperscript{69} Changes in isoform expression also appear to occur in neuronal cells, as suggested by the recent finding that newborn mice express α2 instead of the predominant α3 of the adult animals.\textsuperscript{70} Tissue-specific regulation of Na,K-ATPase isoform expression also is influenced

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Species</th>
<th>Isoform</th>
<th>FXYD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>Rat</td>
<td>α1, β1</td>
<td>γa, γb, CHIF</td>
</tr>
<tr>
<td>Brain</td>
<td>Rat</td>
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<td>FXYS6,FXYD7</td>
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<tr>
<td>Cerebellum</td>
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<td>FXYD1</td>
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<tr>
<td>Pineal gland</td>
<td>Rat</td>
<td>α1, α2, β2</td>
<td></td>
</tr>
<tr>
<td>Pituitary gland</td>
<td>Rat</td>
<td>α1, α3, β2</td>
<td></td>
</tr>
<tr>
<td>Choroid plexus</td>
<td>Rat</td>
<td>α1, α2, α3, β1, β2</td>
<td>FXYD1</td>
</tr>
<tr>
<td>Peripheral nerve</td>
<td>Rat (fetal)</td>
<td>α1, α3, β1, β2</td>
<td>FXYD1</td>
</tr>
<tr>
<td>Heart</td>
<td>Guinea pig</td>
<td>α1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chicken</td>
<td>α1</td>
<td></td>
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<tr>
<td></td>
<td>Sheep</td>
<td>α1</td>
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</tr>
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<td>Ferret</td>
<td>α1, α3</td>
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<td></td>
<td>Dog</td>
<td>α1, α3</td>
<td></td>
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<td></td>
<td>Monkey</td>
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<td></td>
</tr>
<tr>
<td></td>
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<td>FXYD1</td>
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<td>Rat</td>
<td>α1, β1, β3</td>
<td></td>
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<tr>
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<td>Rat</td>
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<tr>
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<td>α1, α2, β1, β3</td>
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<tr>
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<td>α1β1</td>
<td>CHIF</td>
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<td>FXYD1</td>
</tr>
<tr>
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<td>FXYD1</td>
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<tr>
<td>Adipose tissue</td>
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<tr>
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<tr>
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<tr>
<td>Placenta</td>
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<tr>
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<td>γ</td>
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<tr>
<td>(erythrocytes)</td>
<td>Human</td>
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<td>γ</td>
</tr>
<tr>
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<tr>
<td>(ciliary epithelium)</td>
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<td>α1, α2, α3, β1, β2, β3</td>
<td>FXYD1</td>
</tr>
<tr>
<td>(lens)</td>
<td>Human, calf, rabbit</td>
<td>α1, α2, α3, β1</td>
<td>FXYD1</td>
</tr>
<tr>
<td>Ear (cochlea)</td>
<td>Rat, mouse, guinea pig</td>
<td>α1, α2, α3, β1, β2</td>
<td>FXYD1</td>
</tr>
<tr>
<td>(vestibule)</td>
<td>Rat</td>
<td>α1, α2, β1, β2</td>
<td></td>
</tr>
</tbody>
</table>

Data from references 3-10, 29, 32, 41, 42, 63-68.
Kinetic Properties of the Na,K-ATPase Isozymes

Another difference among Na pump isozymes is their enzymatic properties. Because it constitutes nearly all the Na,K-ATPase in the kidney, the functional characteristics of the α1β1 isozyme were the first and most extensively characterized. Na,K-ATPase from other tissues showed intriguing kinetic differences. Thus, by comparison of the enzymes from kidney and axolemma, Sweadner9 showed that the α1β1 isozyme of the renal tissue not only has a higher resistance to ouabain, but also a lower ATP, similar K+, and higher Na+ affinities than the neuronal Na,K-ATPase, abundant in α2 and α3. Differences in response to the cations also were encountered for the transport properties of the kidney and axolemma enzymes delivered by membrane fusion into dog erythrocytes.76 This confirmed that the functional dissimilarities actually depended on the presence of individual Na,K-ATPases, and not only on the lipidic environment surrounding the Na,K-ATPase. Additional studies using other tissues and different cell types in culture also suggested isoform-specific functional differences.9 However, the frequent coexpression of several α and β isoforms in most cells complicated the analysis of Na pump isozyme kinetics. To circumvent this problem, several approaches were used (reviewed in10). One included the determination of the kinetic behavior of the Na,K-ATPase from tissues enriched in particular αβ isoform combinations. This, for example, helped determine the enzymatic characteristics of the Na,K-ATPases from adipose tissue (containing α1β1 and α2β1)77 or pineal gland (composed of α1β2 and α3β2).78 Another approach consisted of studying the properties of the Na,K-ATPase at different stages of development in tissues that exhibited changes in isozyme expression, such as heart and brain.7,9 The finding that in rats the α1 isozyme is 100-fold more resistant to ouabain than α2 and α3 was an essential tool in all these studies to functionally separate α1 from mixed populations of α2 or α3.9 Although this observation provided fundamental information of the functional heterogeneity of the Na,K-ATPase, characterization of the enzymatic properties of each Na,K-ATPase required their isolation. This was obtained by heterologous expression of the different α polypeptides in cells in culture. Thus, when expressed in rodent cells that normally contain α1, the K+ of the ouabain-sensitive α2 and α3 isoforms was determined to be approximately 40 and 80 nmol/L, respectively.79,80 Later, Jewell and Lingrel81 studied the kinetic properties of α1, α2, and α3 from rat in HeLa cells, taking advantage of the ouabain affinity differences between the exogenous isoforms and the sensitive Na,K-ATPase of the human cells. By changing 2 amino acids from the first extracellular loop of α2 and α3, these investigators made the isoforms ouabain resistant. Then, the mutated isoforms, and the naturally resistant rat α1, were transfected into HeLa cells and stable clones expressing the various α polypeptides were selected with ouabain. The function of each isoform finally was distinguished from the endogenous Na,K-ATPase with ouabain. By using this approach, it was shown that α1β1 and α2β1 have affinities for Na+, K+, and ATP that are different from those of the α3β1 isozyme.81 More recent studies in HeLa cells indicated differences in isoform interaction with extracellular Na+ and K+.82 In addition, the group of Pressley, by using Cos-1 and kidney opossum cells,24,83 and Daly et al.84 working with HeLa cells, showed that the N-terminal portion of the α polypeptides is important in defining the K+ kinetic differences among isoforms. This last group also determined that the mechanistic basis for these dissimilarities depend on isozyme differences in E1/E2 conformational equilibrium, as well as on the rates of partial reactions associated with the translocation of Na+ and K+.85

The use of other expression systems, such as yeast, Xenopus laevis oocytes, and insect cells in which the host cells have minimal or no Na,K-ATPase, provided another excellent alternative to analyze isozyme function. This allowed the study of α isoforms without having to modify their ouabain sensitivity and permitted the analysis of isoforms with different αβ composition in an environment free of contaminating endogenous Na,K-ATPase. By using expression in yeast, the group of Farley determined the function of hybrid enzymes composed of sheep α1 or rat α3 isoforms with the β subunit of the related H,K-ATPase or chimeric Na+, K+, H-β subunits. This work helped to established the subunit requirements for Na,K-ATPase activity and the role of the β subunit in modulating the interaction of Na+ and K+ with the enzyme.86-88 More recently, the yeast expression system was used to study the response of the Na,K-ATPase human isoforms to ouabain. It was shown that α1β1, α2β1, and α3β1 have a high affinity for the cardiotonic steroid, with α3β1 exhibiting a slightly higher K+ value for ouabain.89 Expression of α and β isoforms from different species in Xenopus oocytes also have been instrumental in understanding the assembly and functional properties of Na,K-ATPases. Through this system, the activation kinetics of rat isoforms to Rb+ indicated higher values for α1β1 than α1β2 or α1β3.48 Geering et al also used this system to determine the transport properties of human isoforms resulting from all possible combinations of the α1, α2, and α3 with β1, β2, and β3.50 The human α isoforms differentially influence the voltage dependence, Na+ apparent affinities, and turnover rates of the enzyme and, as it occurs in the rat, the β isoform modulates the response of the enzyme to K+.50 In addition, coincident with the experiments in yeast, all human α isoforms produced in oocytes exhibited a sensitivity for ouabain in the nanomolar range.50 The baculovirus system and the expression of Na,K-ATPase polypeptides in Sf-9 cells also have been very valuable in determining the enzymatic properties of the Na pump isoforms. Insect cells produce, assemble, and target to the plasma membrane Na pump αβ complexes that are competent catalytically. By using this system, Blanco et
have an important physiologic role. This is relevant particu-
larly in pathological conditions used, or in species differences in 
expression of various Na,K-ATPases. The results, summarized in 
Table 3, show that the rat Na,K-ATPase isoforms significantly differ in their reactivity to the ligands. Comparison of the results obtained from different expression systems is difficult because of differences in the membrane environment of vertebrate and insect cells, in the experimental conditions used, or in species differences in αβ enzyme composition. However, several general conclusions can be drawn. For example, the enzymatic properties of the Na pump isoforms mainly depend on the α isoform; the β subunits being secondary modulators of the interaction of the enzyme with the cations. Isozymes containing the α3 isoform exhibit much lower apparent affinity for Na⁺ than those composed of α1 and α2, and the α1 isoform has lower ATP affinity than α2 and α3. Although the isozyme kinetic parameters do not indicate large differences among the Na,K-ATPases, they are sufficient to generate changes in intracellular cation levels that could impact on the membrane potential, excitability, and contractility of cells.

The most conspicuous kinetic difference among isoforms and species corresponds to the reactivity to ouabain. In the rat, the sensitivity to the cardiotonic steroid varies with Kᵦ, for α1, α2, and α3 that are in the millimolar, micromolar, and nanomolar range, respectively. In species other than rodents, isoform differences in ouabain sensitivity are much less marked, a fact that has complicated the functional discrimination between the α polypeptides. In human heart, for example, a tissue known to express α1, α2, and α3, results have been controversial and both single as well as multiple binding sites for ouabain have been reported. Crambert et al have shown that human Na,K-ATPases do differ in the ouabain association and dissociation rates, being more rapid for α2 than for α1 and α3. Also, K⁺ antagonism of ouabain binding affects α1 more than α2 or α3. This suggests that although the intrinsic inhibition constants of the human Na,K-ATPases to ouabain are similar, at physiologic K⁺ the cardiotonic steroid may target predominantly the α2 and α3 isoforms. Differences in reactivity to cardiotonic steroids may have an important physiologic role. This is relevant particularly for the control of inotropism in the heart. By inhibiting the Na,K-ATPase, the cardiotonic steroids elicit a transient increase in intracellular Na⁺. This induces via the Na/ Ca exchanger a secondary increase in Ca²⁺ levels, which is taken up by the Ca-ATPase into the sarcoplasmic reticulum. Because of the increase in intracellular stores, more Ca²⁺ can be released on myocardial cell depolarization, enhancing the force of contraction of the heart and the cardiac output. The existence of Na pump isoforms with different affinity for digitalis is important because it allows a fraction of the total enzyme to be inhibited, preventing the toxicity that would be caused by total Na,K-ATPase inactivation. The discovery that digitalis-like compounds including ouabain are synthesized in mammals further supports the relevance of cardiotonic steroids as endogenous regulators of excitability and contractility in heart. A mechanism similar to that of heart may be operating in vascular smooth muscle for the control of vascular peripheral resistance and blood pressure, and in skeletal muscle cells for regulation of contractility. Interestingly, rat Na pump isoforms expressed in insect cells also differ in their sensitivity to Ca²⁺, α2 and α3 isoforms but not α1 are inhibited by physiologic concentrations of Ca²⁺. This may be relevant for the overall inotropic effect because the increase in Ca²⁺ levels elicited by the cardiotonic steroids may be enhanced further by inhibition of the ouabain-sensitive α2 and α3 isoforms.

Expression in insect cells also has helped to determine the function of the α4 isoform. Because the α4 polypeptide has approximately the same degree of homology with the other Na,K-ATPase α isoforms and with the catalytic subunit of the H,K-ATPase, its function as a Na pump required demonstration. When co-expressed with the β1 subunit, α4 displays the catalytic, phosphorylation, and transport characteristics typical of a Na,K-ATPase and, in addition, shows enzymatic properties that are unique. Thus, compared with the other isoforms, α4+β1 has a high apparent affinity for Na⁺, the lowest apparent affinity for K⁺, an intermediate Kₘ for ATP, and the highest sensitivity to ouabain (Table 3). Expression of α4 in fibroblasts and HEK 293 cells showed similar results, except for the affinity for K⁺ that was reported to be higher, and the sensitivity to ouabain that was shown to be slightly lower.

The α4 isoform can assemble with both β isoforms expressed in the tests (β1 and β3), rendering isozymes with equal functional properties. Importantly, α4 also is active catalytically in the native tissue and represents 50% of the total Na,K-ATPase of the male gonad. Activity of α4 is

Table 3 Kinetic Characteristics of Different Isozymes of the Rat Na,K-ATPase Expressed in Sf-9 Insect Cells

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Na⁺ Activation Kᵦ (mmol/L)</th>
<th>K⁺ Activation Kᵦ (mmol/L)</th>
<th>ATP Activation Kᵦ (mmol/L)</th>
<th>Ouabain Inhibition Kᵦ (mol/L)</th>
<th>Ca²⁺ Inhibition Kᵦ (mol/L)</th>
</tr>
</thead>
<tbody>
<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 × 10⁻⁵</td>
<td>1.0 ± 0.2 × 10⁻⁴</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 × 10⁻⁷</td>
<td>7.3 ± 4.6 × 10⁻⁶</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 × 10⁻⁷</td>
<td>1.9 ± 1.0 × 10⁻⁵</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>3.1 ± 0.3 × 10⁻⁸</td>
<td>1.9 ± 1.0 × 10⁻⁵</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>4.7 ± 0.4 × 10⁻⁸</td>
<td>1.9 ± 1.0 × 10⁻⁵</td>
</tr>
<tr>
<td>α4β1</td>
<td>13.5 ± 1.3</td>
<td>5.9 ± 1.1</td>
<td>0.19 ± 0.04</td>
<td>6.4 ± 2.1 × 10⁻⁹</td>
<td>1.8 ± 0.7 × 10⁻⁸</td>
</tr>
<tr>
<td>α4β3</td>
<td>12.9 ± 0.6</td>
<td>5.0 ± 0.3</td>
<td>0.18 ± 0.04</td>
<td>6.4 ± 2.1 × 10⁻⁹</td>
<td>1.8 ± 0.7 × 10⁻⁸</td>
</tr>
</tbody>
</table>

NOTE: Apparent affinities (Kᵦ), Kᵦ, and inhibition constant (K) parameters were calculated from dose-response curves of Na,K-ATPase activity for the indicated ligands. Values represent the mean ± standard error.
present in both the diploid and haploid germ cells of the testis and its function increases during cell differentiation to constitute approximately two thirds of the Na,K-ATPase of the spermatozoa.

**Accessory Polypeptides as Modulators of Na Pump Isozyme Function**

Besides the \(\alpha\) and \(\beta\) isofrom composition, the properties of the Na,K-ATPase depend on the interaction with the FXYD family of polypeptides. This interaction appears to be tissue, as well as isofrom specific. For example, the \(\gamma\) subunit associates with \(\alpha_1\beta_1\) in kidney and modifies some of the functional characterisics of the enzyme, such as the voltage dependence of \(K^+\) activation, the \(K_m\) for ATP, the conformational \(E_1/E_2\) equilibrium, the apparent affinities for \(Na^+\) and \(K^+\), and the \(K^+/Na^+\) antagonism of the enzyme. Both the \(\gamma_a\) and \(\gamma_b\) variants of the polypeptide can affect the response of the Na,K-ATPase to the cations; however, the effect is dependent on posttranslational modifications of the \(\gamma\) polypeptides. Other FXYD members also influence the Na,K-ATPase. Phospholemman can interact with the \(\alpha_1\), \(\alpha_2\), and \(\alpha_3\) isofoms in cerebellum and with \(\alpha_1\) and \(\alpha_2\) in heart, and when expressed in a cell, it decreases the apparent affinities of the isofoms for \(Na^+\) and \(K^+\). Opposite of the effect of \(\gamma\) and Phospholemman, CHIF lowers the \(K_0.5\) of \(\alpha_1\beta_1\) for \(Na^+\) when expressed in oocytes and HeLa cells. Finally, FXDY7 is an isofrom-specific regulator of the Na,K-ATPase that has been shown to associate with \(\alpha_1\beta_1\) in brain and with \(\alpha_1\beta_1, \alpha_2\beta_1,\) and \(\alpha_3\beta_1\) in Xenopus oocytes. FXYD7 decreases the apparent afinity of only the \(\alpha_1\beta_1\) and \(\alpha_3\beta_1\) isofoms for \(K^+\) and, interestingly, it does not interact with isofoms containing \(\beta_2\). In conclusion, the FXYD accessory polypeptides represent an additional level of Na,K-ATPase function control, finely tuning the intrinsic properties of the isofoms.

**Regulatory Mechanisms for the Control of Na Pump Activity and Expression**

Another reason for the existence of Na pump isofoms may be related to the possibility of specific regulation of Na,K-ATPase function in different tissues. Na,K-ATPase regulation can be achieved by direct modification of Na pump activity, or by altering the amount of enzyme at the plasma membrane of the cell through changes in the rate of synthesis/degradation or redistribution with intracellular stores. Recent evidence suggests that all these mechanisms may operate in an isoform- and tissue-specific manner to regulate \(Na^+\) and \(K^+\) balance. Because the isofoms have distinct affinities for \(Na^+\) and \(Ca^{2+}\), the cations acting intracellularly can modulate them differentially. Also, ouabain and endogenous digitalis-like compounds binding extracellularly can affect the Na,K-ATPases differentially.

**Physiologic Relevance of Na Pump Isozymes**

The unique expression, function, and regulation of the Na pump isofoms strongly suggest their physiologic importance. Accordingly, evidence for a biological role of the isofoms in cell- and tissue-specific processes gradually is coming to light. Because of its ubiquitous expression, the \(\alpha_1\beta_1\) isofyme most likely functions as the housekeeping Na,K-ATPase that maintains the basa\(l\) \(Na^+\) and \(K^+\) gradients in the cell. Its prevalence in kidney also suggests that \(\alpha_1\beta_1\) is best adapted for salt and water reabsorption across the epi-
thelium. In the renal tissue, activity of the isoform at the basolateral membrane of tubular epithelial cells is regulated quantitatively and qualitatively by the concerted action of several hormones and intracellular messengers. In addition, interaction predominantly with γ in thick ascending limb and CHIF in collecting ducts modifies α1β1 function. Altogether, these effectors provide the fine adjustment needed for the control of Na\(^+\) and K\(^+\) exchange along the nephron and the regulation of natriuresis. The particular influence of γ in increasing α1β1 affinity for ATP also may be important in allowing the isozyme to function more efficiently under the low-oxygen and energy-compromised environment of the renal medulla. In conclusion, in the kidney, Na,K-ATPase function is achieved mainly through multiple regulatory mechanisms targeting one isozyme rather than favoring the expression of different Na,K-ATPases. In other tissues, Na,K-ATPase diversity is favored instead, and ion transport is controlled through the activity of functionally different isoforms. For example, in neurons the rapid changes in Na\(^+\) and K\(^+\) concentrations accompanying nerve activity may require the fast adjustments provided by isoforms different from α1β1. This may be the role of the neuronal α3-containing isoforms. Because of the low apparent affinity of α3 for Na\(^+\), the isoform operates at slow rates in the cells at rest. When the ion gradients are dissipated during depolarization, intracellular Na\(^+\) levels increase and α3 becomes activated. In this manner, isoforms composed of α3 function as spare pumps to help the ubiquitous α1β1 pumps restore the resting membrane potential of the cells. Also, the high affinity of α3 for ATP allows it to function at the low nucleotide concentrations occurring near the cell membrane during intense neuronal activity.

The properties of α2 suggest it is important for the function of gial, neuronal, and muscle cells. The high affinity for ATP and Na\(^+\) provides α2 with a steady working capability. This allows the isozyme to clear effectively the high K\(^+\) activity may require the fast adjustments provided by isoforms different from α1β1. This may be the role of the neuronal α3-containing isoforms. Because of the low apparent affinity of α3 for Na\(^+\), the isoform operates at slow rates in the cells at rest. When the ion gradients are dissipated during depolarization, intracellular Na\(^+\) levels increase and α3 becomes activated. In this manner, isoforms composed of α3 function as spare pumps to help the ubiquitous α1β1 pumps restore the resting membrane potential of the cells. Also, the high affinity of α3 for ATP allows it to function at the low nucleotide concentrations occurring near the cell membrane during intense neuronal activity.

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ralysis in animals in which the $\beta_2$ gene was deleted, and by the ability of $\beta_2$ to promote cell adhesion and reduce the invasive characteristics of glioma cells. At present, the relationship between cell adhesion/interaction and Na*, K* transport in the nervous system or other tissues where $\beta_2$ is expressed is unknown.

**Conclusions**

Twenty-five years after their discovery, knowledge about the isoforms of Na,K-ATPase has broadened considerably. The information gathered on the distinct cell expression, enzymatic properties, and regulation of the various Na,K-ATPases, as well as the evidence emerging from studies on transgenic mice suggests that the structural heterogeneity of the enzyme is of physiologic importance. Many questions remain concerning the role of the Na pump isoforms and their particular regulation in cell-specific processes. Future studies aimed to understand better these important aspects of the Na,K-ATPase may help develop pharmacologic and genetic means for the tissue-selective exogenous control of Na* and K* balance.

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