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

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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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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

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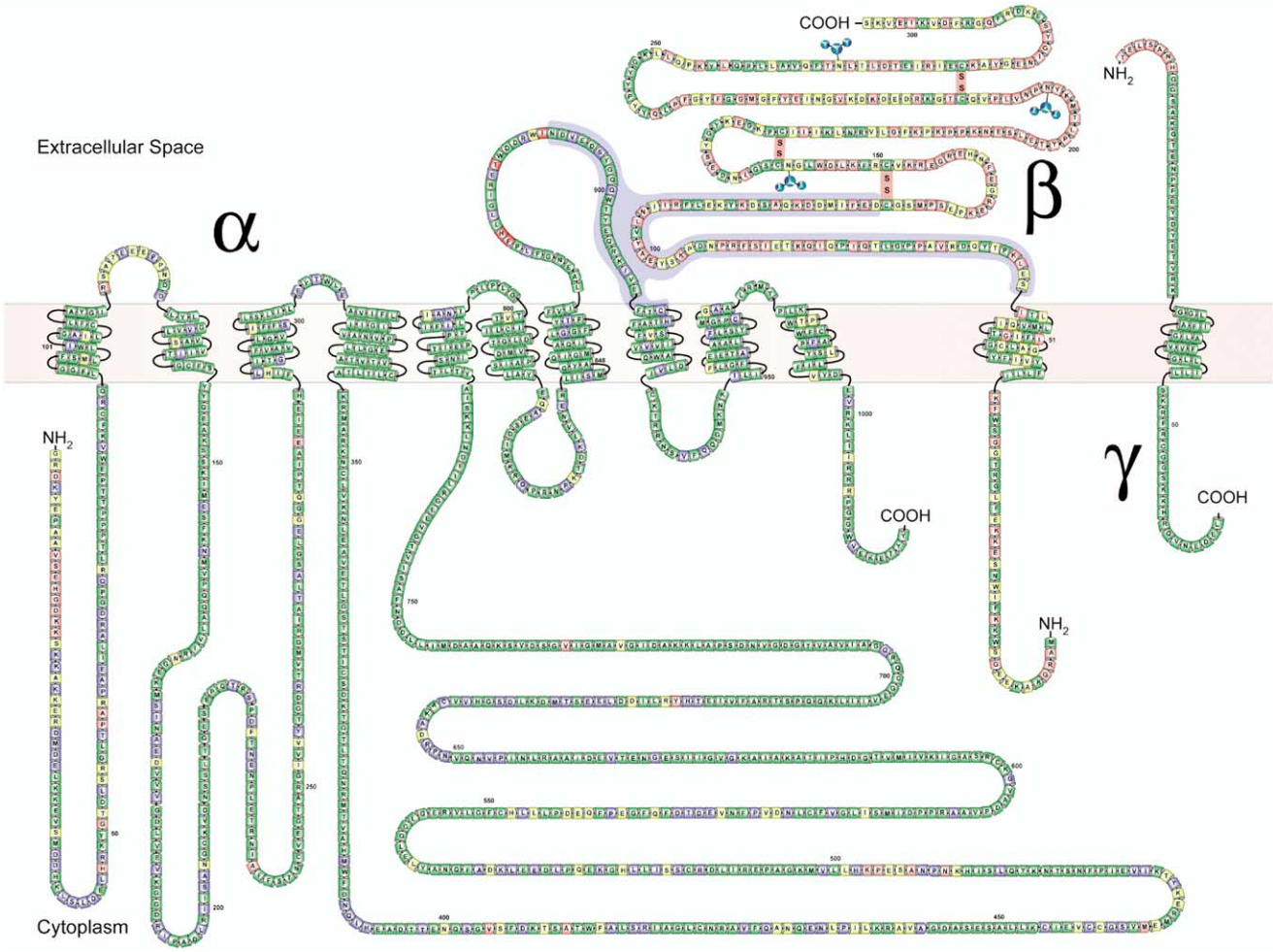

Figure 1 The primary structure and membrane topology of the α (α1, α2, α3, and α4) and β (β1, β2, and β3) isoforms of the Na,K-ATPase and the γ (γa and γb) polypeptides. The sequences of the rat α1 and β1 isoforms and the γa subunit are shown. Amino acid alignment among the 4 α, the 3 β, or the 2 γ polypeptides was performed using DNAstar and Megalign software (DNASTAR, Inc., Madison, WI). Each block represents an amino acid, and residues are colored to indicate the homology among the polypeptides. Green, identical residues among all the α isoforms, β subunits, or the γ polypeptides; blue, residues identical for 3 isoforms; yellow, amino acids identical for 2 isoforms; red, residues different for all isoforms and variants of the γ subunit. The area shaded in purple indicates the association domain between the α and β subunits. The S-S groups shaded in pink represent the disulfide bridges of the β isoforms.

polypeptides: the α and β subunits.[11] 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.[11,12] 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.[13] 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.[11,12]

The β subunits are polypeptides that have molecular weights between 40 and 60 kd, depending on isoform- and tissue-specific differences in glycosylation.[14] 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[14]). In addition, the β polypeptides also deter-

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

Isoform	α1	α2	α3	α4	β1	β2	β3	βm (β4)
Gene nomenclature	**ATP1A**	**ATP1A2**	**ATP1A3**	**ATP1A4**	**ATP1B1**	**ATP1B2**	**ATP1B3**	**ATP1B4**
Human								
Chromosome	1p21	1q21-q23	19q13	1q21-q23	1q24	17p13	3q22-q23	Xq25
Exon/intron	23/22	23/22	23/22	22/21	7/6	7/6	7/6	8/7
Rat								
Chromosome	2q34	13q24-q26	1q21	13q24	13q22	10q24	8q31	Xq11
Exon/intron	21/20	23/22	23/22	22/21	7/6	6/5	7/6	8/7
Mouse								
Chromosome	3F3	1H3	7A3	1H3	1H2.2	11B3	9E3.3	XA3.1
Exon/intron	23/22	23/22	23/22	22/21	7/6	7/6	7/6	8/7

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].

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 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 FXYD3), CHIF (for Corticosteroid Hormone Inducing Factor, or FXYD4), RIC (for Related to Ion Channel, 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 in[16]).

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 α*1*, 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. On the other hand, the greatest similarities among α isoforms correspond to the transmembrane hydrophobic regions, the cytoplasmic midregion around the phosphorylation site (Asp369), and the C-terminus. The striking conservation of α isoform structure and expression across species highlights the importance of the heterogeneity of the Na pump. In addition, the identification of α isoforms in organisms such as hydra,[27] crustaceans,[28] platyhelmints,[29] and zebra fish[30] suggests that the divergence of the α genes was an event that occurred early in evolution.

The discovery of the α isoforms was followed by the find-

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[14]). The differences in primary structure among β isoforms are scattered along the protein sequence and affects 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 β*m* (for muscle β), or β*4*, 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 isozymes is the association of the various α and β isoforms in different heterodimers.[8-10] 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 isozymes 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 isozymes 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.[51] Studies using Sf-9 cells indicate that when different α isoforms are expressed in the insect cells, they stably associate into oligomeric complexes.[52] 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 species[6-10,53-65] 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

Table 2 Tissue Expression of Na,K-ATPase α and β Isoforms and FXYD Polypeptides

Tissue	Species	Isoform	FXYD
Kidney	Rat	$\alpha1$, $\beta1$	γa, γb, CHIF
Brain	Rat	$\alpha1$, $\alpha2$, $\alpha3$, $\beta1$, $\beta2$, $\beta3$	FXYD6,FXYD7
Cerebellum	Rat	$\alpha1$, $\alpha2$, $\alpha3$, $\beta1$, $\beta2$	FXYD1
Pineal gland	Rat	$\alpha1$, $\alpha2$, $\beta2$	
Pituitary gland	Rat	$\alpha1$, $\alpha3$, $\beta2$	
Choroid plexus	Rat	$\alpha1$, $\alpha2$, $\alpha3$, $\beta1$, $\beta2$	FXYD1
Peripheral nerve	Rat	$\alpha1$, $\alpha2$, $\alpha3$, $\beta1$, $\beta2$	
Heart	Rat (fetal)	$\alpha1$, $\alpha3$, $\beta1$, $\beta2$	
	Rat (adult)	$\alpha1$, $\alpha2$, $\beta1$, $\beta2$	FXYD1
	Guinea pig	$\alpha1$	
	Chicken	$\alpha1$	
	Sheep	$\alpha1$	
	Ferret	$\alpha1$, $\alpha3$	
	Dog	$\alpha1$, $\alpha3$	
	Monkey	$\alpha1$, $\alpha3$	
	Human	$\alpha1$, $\alpha2$, $\alpha3$, $\beta1$, $\beta2$	
Liver	Rat	$\alpha1$, $\beta1$, $\beta3$	
Spleen	Rat	$\alpha1$, $\beta1$, $\beta3$	
Lung	Rat	$\alpha1$, $\alpha2$, $\beta1$, $\beta3$	
Intestine, colon	Rat	$\alpha1\beta1$	CHIF
Skeletal muscle	Rat	$\alpha1$, $\alpha2$, $\beta1$, $\beta2$, βm	FXYD1
	Human	$\alpha1$, $\alpha2$, $\alpha3$, $\beta1$, βm	
Vascular smooth muscle endothelium	Rat	$\alpha1$, $\alpha2$, $\alpha3$, $\alpha1$T	
	Human	$\alpha1$, $\beta1$, $\beta2$, $\beta3$	
Adipose tissue	Rat	$\alpha1$, $\alpha2$, $\beta1$	
Cartilage	Human	$\alpha1$, $\alpha2$, $\alpha3$, $\beta1$, $\beta2$, $\beta3$	
Bone	Rat	$\alpha1$, $\beta1$, $\beta2$	
Testis	Rat	$\alpha1$, $\alpha4$, $\beta1$, $\beta3$	
Vas deferens	Rat	$\alpha1$, $\alpha2$	
	Guinea pig	$\alpha1$, $\alpha2$	
Prostate	Rat	$\alpha1$, $\beta1$, $\beta2$	
Uterus	Rat	$\alpha1$, $\alpha2$, $\alpha3$, $\beta1$, $\beta2$, $\beta3$	
Placenta	Human	$\alpha1$, $\alpha2$, $\alpha3$	
Red cells (reticulocytes)	Human	$\alpha1$, $\alpha3$, $\beta2$, $\beta3$	γ
(erythrocytes)	Human	$\alpha1$, $\alpha3$, $\beta1$, $\beta2$, $\beta3$	γ
Eye (retina)	Rat, mouse	$\alpha1$, $\alpha2$, $\alpha3$, $\beta1$, $\beta2$, $\beta3$	
(ciliary epithelium)	Rat, mouse, human	$\alpha1$, $\alpha2$, $\alpha3$, $\beta1$, $\beta2$, $\beta3$	
(lens)	Human, calf, rabbit	$\alpha1$, $\alpha2$, $\alpha3$, $\beta1$	
Ear (cochlea)	Rat, mouse, guinea pig	$\alpha1$, $\alpha2$, $\alpha3$, $\beta1$, $\beta2$	
(vestibule)	Rat	$\alpha1$, $\alpha2$, $\beta1$, $\beta2$	

Data from references[3-10,29,32,34,41,42,61,63-68].

to $\beta1$, the $\beta2$ and $\beta3$ isoforms, respectively.[64] The $\beta3$ polypeptide is also an abundant isoform of retinal photoreceptor cells.[65] The isoform with the most limited pattern of expression is $\alpha4$, the polypeptide is selectively expressed in male germ cells of the testis.[62] Recently, very low levels of a transcript corresponding to the human and mouse $\alpha4$ orthologs also have been detected in heart, pancreas, liver, and placenta.[66] In addition, a larger transcript (7.5 kb compared with 4 kb) encoding $\alpha4$ has been found in skeletal muscle.[66] The expression of $\alpha4$ 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.[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 occurs.[68] A remarkable example of the plasticity in the expression of isoforms is found in rat heart. In this tissue, the $\alpha3$ isoform, present during fetal life, is repressed and replaced by the adult form, $\alpha2$. Interestingly, this switch coincides with changes in the action potential and excitability of the myocardiocytes, suggesting the importance of the Na,K-ATPases in determining the electrical properties of the heart.[69] Changes in isoform expression also appear to occur in neuronal cells, as suggested by the recent finding that newborn mice express $\alpha2$ instead of the predominant $\alpha3$ of the adult animals.[70] Tissue-specific regulation of Na,K-ATPase isoform expression also is influenced

by endocrine status (reviewed in[71,72]), it is regulated during exercise,[73] and is altered with disease.[74] All these changes suggest the importance of Na,K-ATPase isozymes in regulating Na^+ and K^+ balance to tissue physiologic demands or for re-establishing ion homeostasis lost during pathologic states.

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 $\alpha1\beta1$ 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, Sweadner[75] showed that the $\alpha1\beta1$ 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 $\alpha2$ and $\alpha3$. 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 co-expression 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 in[9]). One included the determination of the kinetic behavior of the Na,K-ATPase from tissues enriched in particular $\alpha\beta$ isoform combinations. This, for example, helped determine the enzymatic characteristics of the Na,K-ATPases from adipose tissue (containing $\alpha1\beta1$ and $\alpha2\beta1$)[77] or pineal gland (composed of $\alpha1\beta2$ and $\alpha3\beta2$).[78] Another approach consisted of studying the properties of the Na,K-ATPase at different stages of development in tissues that exhibited changes in isoform expression, such as heart and brain.[7,9] The finding that in rats the $\alpha1$ isoform is 100-fold more resistant to ouabain than $\alpha2$ and $\alpha3$ was an essential tool in all these studies to functionally separate $\alpha1$ from mixed populations of $\alpha2$ or $\alpha3$.[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 $\alpha1$, the K_i of the ouabain-sensitive $\alpha2$ and $\alpha3$ isoforms was determined to be approximately 40 and 80 nmol/L, respectively.[79,80] Later, Jewell and Lingrel[81] studied the kinetic properties of $\alpha1$, $\alpha2$, and $\alpha3$ 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 $\alpha2$ and $\alpha3$, these investigators made the isoforms ouabain resistant. Then, the mutated isoforms, and the naturally resistant rat $\alpha1$, 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 $\alpha1\beta1$ and $\alpha2\beta1$ have affinities for Na^+, K^+, and ATP that are different from those of the $\alpha3\beta1$ 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 isoform function. This allowed the study of α isoforms without having to modify their ouabain sensitivity and permitted the analysis of isozymes with different $\alpha\beta$ 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 $\alpha1$ or rat $\alpha3$ isoforms with the β subunit of the related H,K-ATPase or chimeric Na,K-, H,K- 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 isozymes to ouabain. It was shown that $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ have a high affinity for the cardiotonic steroid, with $\alpha3\beta1$ exhibiting a slightly higher K_i 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 isozymes to Rb^+ indicated higher values for $\alpha1\beta1$ than $\alpha1\beta2$ or $\alpha1\beta3$.[48] Geering et al also used this system to determine the transport properties of human isozymes resulting from all possible combinations of the $\alpha1$, $\alpha2$, and $\alpha3$ with $\beta1$, $\beta2$, and $\beta3$.[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 isozymes. Insect cells produce, assemble, and target to the plasma membrane Na pump $\alpha\beta$ complexes that are competent catalytically. By using this system, Blanco et

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

Isozyme	Na^+ Activation $K_{0.5}$ (mmol/L)	K^+ Activation $K_{0.5}$ (mmol/L)	ATP Activation K_m (mmol/L)	Ouabain Inhibition K_i (mol/L)	Ca^{2+} Inhibition K_i (mol/L)
$\alpha1\beta1$	16.4 ± 0.7	1.9 ± 0.2	0.46 ± 0.10	$4.3 \pm 1.9 \times 10^{-5}$	$1.0 \pm 0.2 \times 10^{-4}$
$\alpha2\beta1$	12.4 ± 0.5	3.6 ± 0.3	0.11 ± 0.01	$1.7 \pm 0.1 \times 10^{-7}$	$7.3 \pm 4.6 \times 10^{-6}$
$\alpha2\beta2$	8.8 ± 1.0	4.8 ± 0.4	0.11 ± 0.02	$1.5 \pm 0.2 \times 10^{-7}$	
$\alpha3\beta1$	27.9 ± 1.3	5.3 ± 0.3	0.09 ± 0.01	$3.1 \pm 0.3 \times 10^{-8}$	$1.9 \pm 1.0 \times 10^{-5}$
$\alpha3\beta2$	17.1 ± 1.0	6.2 ± 0.4	0.07 ± 0.02	$4.7 \pm 0.4 \times 10^{-8}$	
$\alpha4\beta1$	13.5 ± 1.3	5.9 ± 1.1	0.19 ± 0.04	$6.4 \pm 2.1 \times 10^{-9}$	
$\alpha4\beta3$	12.9 ± 0.6	5.0 ± 0.3	0.18 ± 0.04	$1.8 \pm 0.7 \times 10^{-8}$	

NOTE. Apparent affinities ($K_{0.5}$), K_m, and inhibition constant (K_i) parameters were calculated from dose-response curves of Na,K-ATPase activity for the indicated ligands. Values represent the mean ± standard error.

al[9,48,91] determined the kinetic parameters for Na^+, K^+, ATP, ouabain, and Ca^{2+} of various Na,K-ATPases. The results, summarized in Table 3, show that the rat Na,K-ATPase isozymes 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 $\alpha\beta$ enzyme composition. However, several general conclusions can be drawn. For example, the enzymatic properties of the Na pump isozymes mainly are dependent on the α isoform; the β subunits being secondary modulators of the interaction of the enzyme with the cations. Isozymes containing the $\alpha3$ isoform exhibit much lower apparent affinity for Na^+ than those composed of $\alpha1$ and $\alpha2$, and the $\alpha1$ isoform has lower ATP affinity than $\alpha2$ and $\alpha3$. 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 isozymes and species corresponds to the reactivity to ouabain. In the rat, the sensitivity to the cardiotonic steroid varies with K_{is} for $\alpha1$, $\alpha2$, and $\alpha3$ that are in the millimolar, micromolar, and nanomolar range, respectively.[9] 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 $\alpha1$, $\alpha2$, and $\alpha3$, results have been controversial and both single as well as multiple binding sites for ouabain have been reported.[92,93] Crambert et al[50] have shown that human Na,K-ATPases do differ in the ouabain association and dissociation rates, being more rapid for $\alpha2$ than for $\alpha1$ and $\alpha3$. Also, K^+ antagonism of ouabain binding affects $\alpha1$ more than $\alpha2$ or $\alpha3$. 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 $\alpha2$ and $\alpha3$ 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 Ca^{2+} levels, which is taken up by the Ca-ATPase into the sarcoplasmic reticulum. Because of the increase in intracellular stores, more Ca^{2+} can be released on myocardial cell depolarization, enhancing the force of contraction of the heart and the cardiac output.[94] The existence of Na pump isozymes 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.[95] 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.[94] Interestingly, rat Na pump isozymes expressed in insect cells also differ in their sensitivity to Ca^{2+}, $\alpha2$ and $\alpha3$ isoforms but not $\alpha1$ are inhibited by physiologic concentrations of Ca^{2+}.[9] This may be relevant for the overall inotropic effect because the increase in Ca^{2+} levels elicited by the cardiotonic steroids may be enhanced further by inhibition of the ouabain-sensitive $\alpha2$ and $\alpha3$ isoforms.

Expression in insect cells also has helped to determine the function of the $\alpha4$ isoform. Because the $\alpha4$ 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 $\beta1$ subunit, $\alpha4$ displays the catalytic, phosphorylation, and transport characteristics typical of a Na,K-ATPase and, in addition, shows enzymatic properties that are unique.[48] Thus, compared with the other isozymes, $\alpha4\beta1$ has a high apparent affinity for Na^+, the lowest apparent affinity for K^+, an intermediate K_m for ATP, and the highest sensitivity to ouabain (Table 3). Expression of $\alpha4$ 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.[23-96] The $\alpha4$ isoform can assemble with both β isoforms expressed in the testis ($\beta1$ and $\beta3$), rendering isozymes with equal functional properties.[48] Importantly, $\alpha4$ also is active catalytically in the native tissue and represents 50% of the total Na,K-ATPase of the male gonad.[9-48] Activity of $\alpha4$ is

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.[97]

Accessory Polypeptides as Modulators of Na Pump Isozyme Function

Besides the α and β isoform 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 isoform specific. For example, the γ subunit associates with $\alpha1\beta1$ in kidney and modifies some of the functional characteristics of the enzyme, such as the voltage dependence of K^+ activation, the K_m for ATP, the conformational E1/E2 equilibrium, the apparent affinities for Na^+ and K^+, and the K^+/Na^+ antagonism of the enzyme.[40] Both the γa and γb 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 γ polypeptides.[98] Other FXYD members also influence the Na,K-ATPase. Phospholemman can interact with the $\alpha1$, $\alpha2$, and $\alpha3$ isoforms in cerebellum and with $\alpha1$ and $\alpha2$ in heart, and when expressed in oocytes, it decreases the apparent affinities of the isoforms for Na^+ and K^+.[54,99] Opposite of the effect of γ and Phospholemman, CHIF lowers the $K_{0.5}$ of $\alpha1\beta1$ for Na^+ when expressed in oocytes and HeLa cells.[100] Finally, FXYD7 is an isozyme-specific regulator of the Na,K-ATPase that has been shown to associate with $\alpha1\beta1$ in brain and with $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ in *Xenopus* oocytes. FXYD7 decreases the apparent affinity of only the $\alpha1\beta1$ and $\alpha3\beta1$ isozymes for K^+ and, interestingly, it does not interact with isozymes containing $\beta2$.[101] In conclusion, the FXYD accessory polypeptides represent an additional level of Na,K-ATPase function control, finely tuning the intrinsic properties of the isozymes.

Regulatory Mechanisms for the Control of Na Pump Activity and Expression

Another reason for the existence of Na pump isozymes 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.[71,72] Recent evidence suggests that all these mechanisms may operate in an isoform- and tissue-specific manner to regulate Na^+ and K^+ balance. Because the isozymes 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.[9] Isoform-specific changes in expression and shifts in $\alpha\beta$ cell compartmentalization represent an important mechanism for regulation of Na pump composition at the plasma membrane. In muscle cells, $\alpha2$ is translocated to the cell surface on insulin stimulation.[102] In contrast, in neostriatal neurons, the same isoform is endocytosed to intracellular stores by dopamine.[103] The effect of dopamine is blocked by glutamate, which instead elicits targeting of $\alpha1$ to the neuronal plasmalemma.[103] Data from several laboratories indicate that a complex intracellular signaling network that involves protein kinases (PK) and phosphatases, through reversible phosphorylation of the α subunit, plays a major role in the regulation of the Na,K-ATPase amounts and activity at the cell plasma membrane. This has been studied mostly in the kidney for the $\alpha1\beta1$ isozyme.[71,72] Less information is available for isozymes different from $\alpha1\beta1$. Nevertheless, protein kinase C (PKC) activation has been shown to produce a larger inhibition of the activity of $\alpha2$ and $\alpha3$, than $\alpha1$ in neuronal cells.[104] Studies in baculovirus-infected cells also showed isoform-specific changes in response to PK activation. Phorbol esters that activate PKC lead to inhibition of all isoforms; the protein kinase A activator, dibutyryl cyclic adenosine monophosphate, stimulates the activity of $\alpha3\beta1$ and decreases that of $\alpha1\beta1$ and $\alpha2\beta1$. Finally, activation of protein kinase G diminishes the activity of $\alpha1\beta1$ and $\alpha3\beta1$, without altering that of $\alpha2\beta1$. The response of the isozymes to PK is concurrent with the phosphorylation of all α isoforms.[105] In $\alpha1$, PKA induces phosphorylation of Ser943, a residue highly conserved among isoforms. In contrast, for PKC 2 serines, Ser11 and Ser18 at the N-terminus of $\alpha1$ have been recognized as targets for phosphate incorporation.[72] The lack of conservation of these sites in $\alpha2$, and the presence of only Ser18 in $\alpha3$, suggest that other residues or regions may be involved in regulation of these isoforms. Pierre et al have shown that the ISR region is important for the isoform-specific changes in function elicited by PKC. Thus, exchange of the amino acid sequence of the ISR between the $\alpha1$ and $\alpha2$ isoforms results in chimeras with a response to phorbol esters that is different from that of the wild-type enzymes. While the ISR from $\alpha2$ acts as a stimulatory signal for the PKC response of $\alpha1$, the ISR of $\alpha1$ confers $\alpha2$ with a PKC nonresponsive phenotype.[26] It is unknown at present whether the ISR by itself is sufficient to mediate the effect of PKC or if interactions with other cytoplasmic motifs in the α subunit are required.

Physiologic Relevance of Na Pump Isozymes

The unique expression, function, and regulation of the Na pump isozymes strongly suggest their physiologic importance. Accordingly, evidence for a biological role of the izozymes in cell- and tissue-specific processes gradually is coming to light. Because of its ubiquitous expression, the $\alpha1\beta1$ isozyme most likely functions as the housekeeping Na,K-ATPase that maintains the basal Na^+ and K^+ gradients in the cell. Its prevalence in kidney also suggests that $\alpha1\beta1$ is best adapted for salt and water reabsorption across the epi-

thelium. In the renal tissue, activity of the isozyme at the basolateral membrane of tubular epithelial cells is regulated quantitative and qualitatively by the concerted action of several hormones and intracellular messengers.[2] In addition, interaction predominantly with γ in thick ascending limb and CHIF in collecting ducts modifies $\alpha1\beta1$ function.[98,101] 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 $\alpha1\beta1$ 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 isozymes different from $\alpha1\beta1$. This may be the role of the neuronal $\alpha3$-containing isozymes. Because of the low apparent affinity of $\alpha3$ for Na^+, the isoform operates at slow rates in the cells at rest. When the ion gradients are dissipated after depolarization, intracellular Na^+ levels increase and $\alpha3$ becomes activated. In this manner, isozymes composed of $\alpha3$ function as spare pumps to help the ubiquitous $\alpha1\beta$ pumps restore the resting membrane potential of the cells. Also, the high affinity of $\alpha3$ for ATP allows it to function at the low nucleotide concentrations occurring near the cell membrane during intense neuronal activity.

The properties of $\alpha2$ suggest it is important for the function of glial, neuronal, and muscle cells. The high affinity for ATP and Na^+ provides $\alpha2$ with a steady working capability. This allows the isoform to clear effectively the high K^+ left at the extracellular space after depolarization, even at decreased cytosolic concentrations of Na^+ and ATP. In this manner, $\alpha2$ is important in preventing K^+-induced depolarization to maintain excitability in cells. The role of $\alpha2$ has been shown in mice in which the expression of the isoform was knocked out. These mice exhibited akinesia and died soon after birth because of irregular breathing caused by an abnormal rhythmic firing of the neurons of the respiratory center.[70] Another important role of $\alpha2$ derives from its ability to regulate intracellular Ca^{2+} levels. This has been shown by gene-targeting studies in excitable tissues of mice. Animals lacking one copy of the $\alpha2$ gene show half levels of expression of the isoform and abnormal heart and skeletal muscle function.[106,107] In the transgenic animals the heart characteristically is hypercontractile and skeletal muscle exhibits a greater force of contraction than in normal controls. Conversely, in mice in which expression of $\alpha1$ is perturbed, cardiac and muscle tissue are hypocontractile.[106,107] Thus, it appears that regulation of the $\alpha1/\alpha2$ ratio at the plasma membrane is important for muscle contractility. This observation may explain the increase of $\alpha2$ at the surface of skeletal muscle cells during exercise.[73] The hypercontractility induced by $\alpha2$ in the transgenic animals[106] has been attributed to larger calcium transients in the cells, caused by a primary increase in cytoplasmic Na^+ levels, which secondarily augments Ca^{2+} levels via the Na/Ca exchanger. This mechanism in the $\alpha2$-deficient mice is supported by the abnormally increased intracellular Ca^{2+} levels in astrocytes from these animals.[108,109] Interestingly, Juhaszova and Blaustein[110] found that in glial cells and myocytes $\alpha2$ colocalizes with the Na/Ca exchanger and the underlying endoplasmic reticulum, suggesting that these components act as a functional unit to regulate cytoplasmic Na^+ and Ca^{2+}. In this manner, regulation of the cations can be limited to microdomains preventing global cell ionic changes. Coincidental with its role in controlling cytoplasmic Ca^{2+} is the high binding capacity of $\alpha2$ for ouabain. This property has led to the idea that the isoform may be responsible for mediating the inotropic effect of digitalis in the heart. In support of this, reduction of $\alpha2$ activity in hearts of transgenic animals mimics the effect of the cardiotonic steroids.[106] The role of $\alpha2$ as the digitalis effector has been explored by using Cre-Lox technology to develop homozygous knock-in mice that express a mutated ouabain-insensitive $\alpha2$ isoform. Measurements of cardiac contractility showed that the ouabain-induced inotropy typical of normal mice is abolished in these animals.[111] Although these results strongly suggest the relevance of $\alpha2$ for the cardiotonic effect in rodents, it is unclear whether the isoform plays a similar role in other species including humans, in whom the differences in digitalis binding is not as marked. Another function of $\alpha2$ is related to the ability of the isoform to regulate body kalemia controlling K^+ exchange between extracellular and muscle stores. Evidence for such a role derives from experiments showing a relationship between dietary K^+ and plasma membrane levels of $\alpha2\beta2$ in skeletal muscle.[112]

A specific function also has been ascribed to the $\alpha4$ isoform of spermatozoa. The difference in affinity for ouabain between the resistant $\alpha1$ and sensitive $\alpha4$ isoforms was used to discriminate between both isoforms of the male gametes from rat. Ouabain inhibition of $\alpha4$ results in impairment of the motility of spermatozoa.[113] This suggested the importance of $\alpha4$ in maintaining membrane potential and excitability of the cells. Subsequent experiments showing that the ouabain-inhibited motility is recovered by ionophores that allow the exit of H^+ from the cells suggest that $\alpha4$ is involved in control of gamete intracellular pH via the Na/H exchanger. The functional coupling of $\alpha4$ to the Na/H exchanger is supported by the colocalization of both polypeptides to the midpiece of the spermatozoon flagellum.[113,114]

Although there is some evidence for a biological function of the α isoforms, the role of the β subunits is more obscure. Beyond the requirement of the β subunits for Na,K-ATPase maturation and modulation of activity,[9,14] an unforeseen function has been reported for $\beta2$. The isoform present in glia is a recognition molecule that mediates neuron-glia interactions and is important for cell adhesion, neuronal migration, and neurite outgrowth. These properties of $\beta2$ are supported by the observation of motor incoordination, tremor, and pa-

ralysis in animals in which the $\beta 2$ gene was deleted,[115] and by the ability of $\beta 2$ to promote cell adhesion and reduce the invasive characteristics of glioma cells.[116] 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 isozymes 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 isozymes 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.[105]

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