Journal of Bioenergetics and Biomembranes, Vol. 24, No. 3, 1992

Na, K-ATPase: Isoform Structure, Function, and Expression

Jerry B. Lingrel[1]

Received February 23, 1992; accepted February 25, 1992

An interesting feature of the Na,K-ATPase is the multiplicity of α and β isoforms. Three isoforms exist for the α subunit, $\alpha 1$, $\alpha 2$, and $\alpha 3$, as well for the β subunit, $\beta 1$, $\beta 2$, and $\beta 3$. The functional significance of these isoforms is unknown, but they are expressed in a tissue- and developmental-specific manner. For example, all three isoforms of the α subunit are present in the brain, while only $\alpha 1$ is present in kidney and lung, and $\alpha 2$ represents the major isoform in skeletal muscle. Therefore, it is possible that each of these isoforms confers different properties on the Na,K-ATPase which allows effective coupling to the physiological process for which it provides energy in the form of an ion gradient. It is also possible that the multiple isoforms are the result of gene triplication and that each isoform exhibits similar enzymatic properties. In this case, the expression of the triplicated genes would be individually regulated to provide the appropriate amount of Na,K-ATPase to the particular tissue and at specific times of development. While differences are observed in such parameters as Na^+ affinity and sensitivity to cardiac glycosides, it is not known if these properties play a functional role within the cell.

Site-directed mutagenesis has identified amino acid residues in the first extracellular region of the α subunit as major determinants in the differential sensitivity to cardiac glycosides. Similar studies have failed to identify residues in the second extracellular region involved in cardiac glycoside inhibition. Further analysis of the enzymatic properties of the enzyme, understanding the regulated expression of the genes, and structure–function studies utilizing site-directed mutagenesis should provide new insights into the enzymatic and physiological roles of the various subunit isoforms of the Na,K-ATPase.

KEY WORDS: Cardiac glycosides; ouabain; α-subunits; developmental expression.

INTRODUCTION

Na,K-ATPase is a ubiquitous transmembrane enzyme which moves Na^+ out of the cell and K^+ in utilizing ATP as the driving force. This enzyme is a member of the P-type ATPases of which other members are the Ca^{2+} ATPase and H,K-ATPase (Pedersen and Carafoli, 1987). The Na,K-ATPase is found in the cells of all higher eukaryotes, including organisms such as *Drosophila*, but the enzyme is not present in lower eukaryotes such as yeast. The enzyme is composed of two subunits, a larger α subunit with a molecular weight of 112,000 and a smaller β subunit with a molecular weight of 35,000 (see reviews by Lingrel *et al.*, 1990; Sweadner, 1989; Takeyasu *et al.*, 1989). The β subunit occurs as a glycosylated protein with a molecular weight of approximately 55,000. The α subunit is responsible for the catalytic activity of the Na,K-ATPase while the β subunit is important in the maturation and transport of the enzyme to the plasma membrane (Gering, 1990). The enzyme produces an ion gradient which drives a multitude of physiological processes. It provides the energy for the Na^+-coupled transport of various nutrients into the cell including glucose, amino acids, and vitamins. The movement of ions such as Ca^{2+} and H^+ across the membrane, as well as the maintenance of osmotic balance and cell volume, also depend on the Na^+ and K^+ gradient.

[1]Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, Ohio 45267-0524.

0145-479X/92/0600-0263$06.50/0

The Na,K-ATPase plays a specialized role in many tissues; it maintains the nutrient and ion composition of cerebrospinal fluid and aqueous humor and is responsible for fluid movement across transport epithelia such as those found in the gastrointestinal tract, the nasotracheal lining, and the kidney. The electrogenic potential produced is important for the function of excitable tissues, i.e., muscle and nerve, and it is likely that the fluid reabsorption from the lungs at birth depends on the ion gradient produced by Na,K-ATPase. It is estimated that at rest ~25% of the ATP is used by this enzyme (DeWeer, 1985).

Multiple disciplines have contributed to the understanding of this enzyme. Physiologists have long been interested in Na,K-ATPase because of its key role in the function of excitable tissues and ion and nutrient transport as well as its function in the kidney and other specialized tissues. Because this enzyme serves as a receptor for cardiac glycosides, an important group of drugs used in the treatment of congestive heart failure and arrhythmias, pharmacologists have played a significant role in understanding its structure and function. Biochemists have been fascinated by the complexity of this enzyme, namely its ability to transport an unequal number of ions across the membrane at the expense of ATP. For every three Na^+ ions removed from the cell, only two K^+ ions are transported in. Understanding the basis for the specificity, the stoichiometry, and the manner in which ATP hydrolysis is coupled to ion movement represents a formidable challenge and requires multiple approaches. Molecular genetics tools have been used to elucidate the primary structure of the enzyme as well as to define isoforms. In addition, these techniques are presently contributing to the understanding of structure–function relationships through the use of site-directed mutagenesis to alter particular amino acid residues. Molecular genetics approaches have also made it possible to isolate the genes coding for the individual α and β isoforms so their tissue- and developmental-specific expression can be studied.

A molecular genetics approach was used initially to determine the primary sequence of the α and β subunits via the cloning of *c*DNAs corresponding to the mRNA for these two proteins (Shull *et al.*, 1985; Kawakami *et al.*, 1985; Noguchi *et al.*, 1986; Shull *et al.*, 1986b). Subsequent studies described the primary sequence of α and β subunits from a variety of species (see review Lingrel by *et al.*, 1990) including the α1, α2, and α3 isoforms of the rat (Shull *et al.*, 1986a; Mercer *et al.*, 1987; Hara *et al.*, 1987; Herrera *et al.*, 1987). Three isoforms of the β subunit have been described (Martin-Vasallo *et al.*, 1989; Good *et al.*, 1990; Gloor *et al.*, 1990), and interestingly one of these appears to be the adhesion molecule AMOG. To date, the β3 isoform has only been observed during early development in *Xenopus laevis*. The availability of the primary structure has made it possible to develop potential topological relationships of the subunits with respect to the membrane, and one such model is shown in Fig. 1 (Shull and Lingrel, 1987). This model describes eight transmembrane-spanning regions in the α subunit and one in the β subunit. The model is based on defining hydrophobic regions suspected of passing through the membrane, but to date little confirming evidence for this particular structure has been obtained. The model assumes that the N-terminal and C-terminal regions are on the inside of the cell. The ATP binding site, which has been defined by chemical studies, is located on the inside of the cell, and this finding orients the transmembrane regions. There is general agreement that the first four proposed transmembrane regions are correct; however, there is uncertainty in the C-terminal half of the molecule. The Ca^{2+}-ATPase is proposed to have 10 transmembrane regions although this enzyme shows a very similar hydropathy plot to that of the α subunit of the Na,K-ATPase (MacLennan *et al.*, 1985). Careful analysis using specific monoclonal antibodies as well as incorporating cleavable sites within the α subunit should help define the structure. Nevertheless, the proposed model of Na,K-ATPase allows for the initial design of experiments to study structure–function relationships.

WHY ISOFORMS OF THE α SUBUNIT?

One of the interesting features of the Na,K-ATPase is the presence of multiple isoforms for both the α and β subunits. Even more intriguing is the finding that these isoforms are expressed in a tissue- and developmental-specific manner (Lingrel *et al.*, 1990; Sweadner 1989; Takeyasu *et al.*, 1989). The diversity of rat α mRNA expression is shown in Table I. Kidney, for example, contains only the α1 isoform and there is a small increase in its amount during development. The same is true of lung; however, lower levels are present in this tissue as compared to the kidney. In contrast, skeletal muscle contains all three isoforms, but α2 predominates during adult life. The α1 and α3 isoforms are present in smaller amounts in this tissue, with α1 remaining constant through

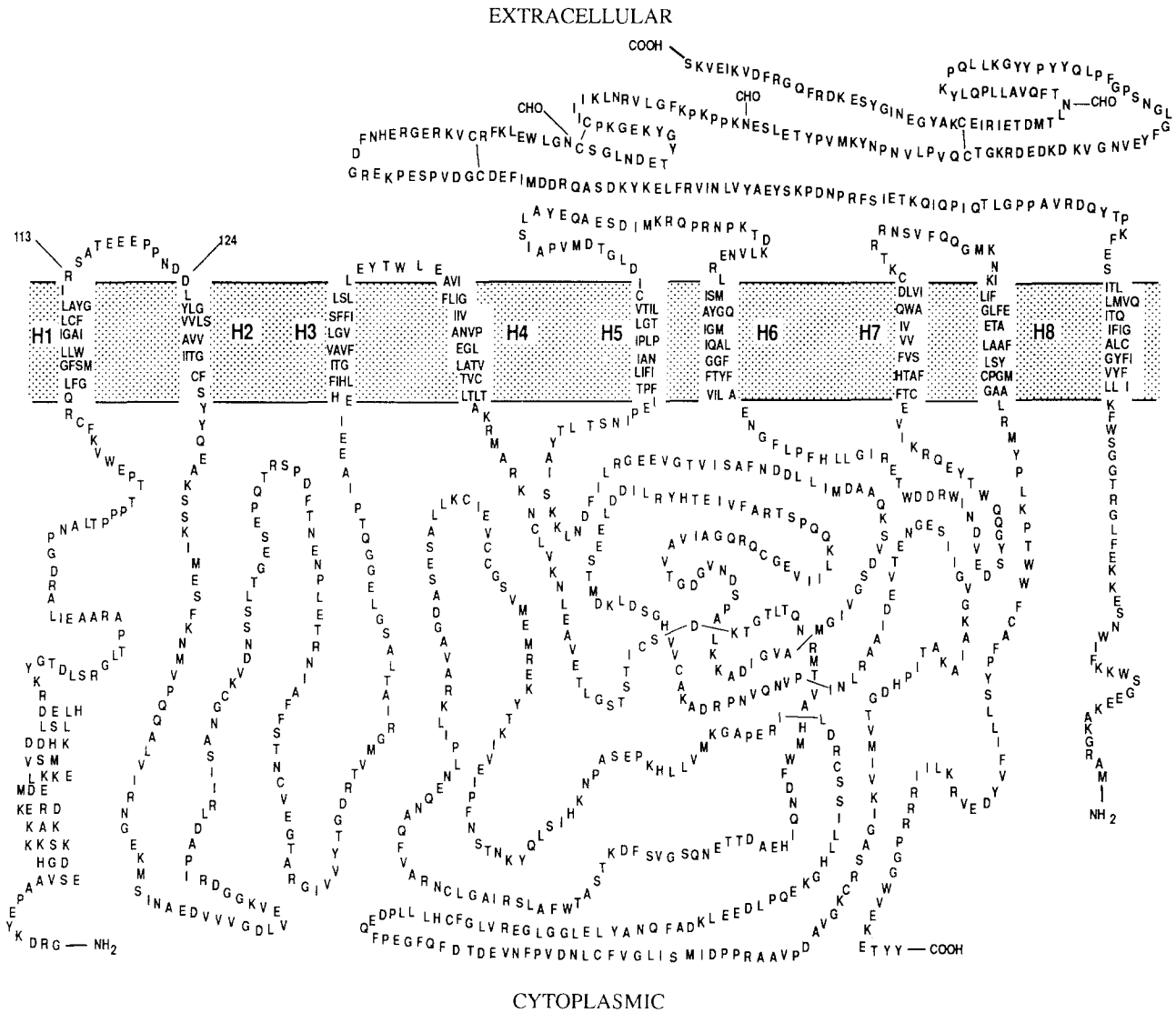

Fig. 1. Transmembrane model of the α and β subunits of the Na,K-ATPase. The model is based on hydropathy analysis and regions known to reside in the cytoplasm (Shull *et al.*, 1985, Shull and Lingrel, 1987; Kirley, 1989).

development while the α3 isoform follows a developmental pattern similar to that of α2. Brain contains all three isoforms, with each increasing during development, although the increase of α1 and α2 lags behind that of α3. Rat heart follows yet another developmental pattern: α1 is present at all stages but α2 and α3 exhibit a reciprocal switch, with α3 being present during fetal life and α2 replacing α3 during adult life (Orlowski and Lingrel, 1988; Shyjan and Levenson, 1989). The α2 and α3 isoform mRNAs are enriched in the conduction system of the rat heart (Zahler *et al.*, 1992). The pattern of expression of the α isoforms in the human heart differs from the rat (Table I). In human heart, all three isoform mRNAs are present in adult ventricle in approximately equal amounts (Shamraj *et al.*, 1991), while α1 and α3 mRNAs predominate in fetal heart (Gilmore-Hebert *et al.*, 1989). No data are available for neonatal heart. Although the abundances cited above are based on mRNA concentrations, there appears to be general agreement between these values and the actual amount of protein present in tissues as determined by immunological analysis (Lucchesi and Sweadner, 1991). The tissue-specific expression of the chicken α and β isoforms has

Table I. Relative Abundance of α Isoform mRNAs of Na,K-ATPase[a]

Tissue	α isoform mRNA		
	α1	α2	α3
Rat			
Kidney			
Fetal	+++	−	−
Neonatal	++++	−	−
Adult	+++++	−+	−
Lung			
Fetal	++	−	−
Neonatal	++++	−	−
Adult	++	−+	−
Muscle			
Fetal	+	−+	−
Neonatal	+	++	+
Adult	+	++++	+
Brain			
Fetal	+	+	++
Neonatal	++	++	+++
Adult	+++	+++	+++
Heart			
Fetal	+++	−	++
Neonatal	+++	+	++
Adult	+++	++	−
Human			
Heart			
Fetal	++		++
Neonatal			
Adult	+++	+++	+++

[a]Based on the data from Orlowski and Lingrel (1988), Shamraj *et al.* (1991) and Gilmore-Hebert *et al.* (1989).

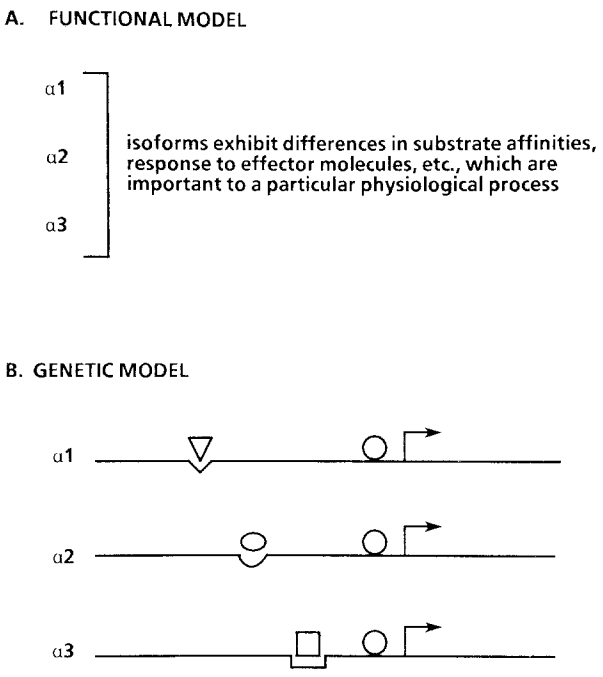

Fig. 2. Models of α isoform diversity. Model A is based on each α isoform conferring different functional properties on the Na,K-ATPase and that these differences play a significant role in the particular physiological process to which they provide a Na^+ and K^+ gradient. Model B assumes that functional differences among the α isoforms either do not exist or that, if they do, these differences play no significant physiological role. Rather the multiple isoforms result from triplication of the α subunit gene with each resulting gene evolving in its own tissue and developmental specific regulation. The end result is the correct amount of Na,K-ATPase in each tissue without regard to the activities of the α isoforms present. →, Transcriptional start site; ○, transcriptional factor present in all cells; □, ⬡, and ▽, developmental or tissue-specific transcription factors.

also been examined (Takeyasu *et al.*, 1989) and found to follow a pattern similar to that of the rat. Therefore, it is likely that while some differences may exist among species, the general pattern of developmental- and tissue-specific expression will probably be the same and may have been preserved to couple particular isoforms to certain physiological processes.

It is possible that the distribution of isoforms in various tissues and during development is coupled to the specific physiological function for which the Na,K-ATPase provides the ion gradient. This model predicts that the catalytic or regulatory properties of each of the isoforms differ and isoforms with a particular functional property would be matched to specific physiological functions. For example, isoforms could vary in their Na^+ affinity, and an isoform exhibiting a specific affinity would be expressed in a tissue or cell type where this particular property is required (Fig. 2A). Likewise, it is possible that different α isoforms respond differently to hormones, other effector molecules, or ion concentrations. Support for a functional basis for isoforms depends on the identification of enzymatic or other properties that differ among the isoforms. Alternatively, it is possible that all three isoforms have very similar properties and even if these properties differ, these differences are not functionally significant. In this case, the existence of multiple isoforms may have its basis in regulating the amount of Na,K-ATPase activity. Rather than overlaying a very complicated gene regulation mechanism on a single gene, organisms may have triplicated their α subunit genes and divided the overall regulatory requirements among these genes (Fig. 2B). According

to this scheme, only the overall level of Na,K-ATPase in a particular cell or at a specific developmental stage is important. Attaining the levels of enzyme is accomplished through the combined regulation of the three α isoform genes. As depicted in Fig. 2B, some of the regulatory events, i.e., the presence of *cis*-acting elements and *trans*-acting factors, could be shared by the α isoform genes while others would be dedicated to the expression of a specific isoform gene. It is known that the expression of the three α isoform genes is under independent control but it is not known whether this is to provide differential expression of three functionally different isoforms or simply to provide the appropriate levels of functionally equivalent isoforms.

One approach to addressing this question is to define the functional properties of Na,K-ATPase containing the α1, α2, and α3 isoforms, respectively. As several tissues contain only the α1 isoform, i.e., kidney, intestine, and lung, determining the properties of the enzyme containing this isoform has been straightforward. However, as cells expressing only α2 or α3 isoforms do not naturally occur, the properties of Na,K-ATPase with these isoforms has been more difficult to assess.

One functional difference observed among the isoforms of Na,K-ATPase relates to ouabain binding. Tissues from many species possess a sensitive enzyme but rodents, i.e., rats and mice, have both high- and low-affinity forms. The enzyme from rat kidney, which is composed exclusively of the α1 isoform, exhibits low affinity for ouabain. The high-affinity form has been attributed to the α2 and α3 isoforms (see review by Sweadner). Again the difficulty in determining differences in sensitivity of enzyme containing the α2 and α3 isoforms has been the inability to identify tissues or cells that express one or the other separately. The two high-affinity forms were analyzed as separate components in rat brain, and computed I_{50} values of 0.023 and 0.46 μM were obtained for ouabain (Berrebi-Bertrand *et al.*, 1990). The α1 containing enzyme in the same analysis gave an I_{50} value of 320 μM. The differential sensitivity to trypsin of α2 and α3 isoforms in the rat brain stem axolemma membrane has been taken advantage of to study ouabain inhibition of enzyme with the α3 isoform (Urayama and Sweadner, 1988). Following treatment with trypsin, enzyme with α2 is inactivated while that with α3 is not. From these studies, it is concluded that Na,K-ATPase with the α3 isoform is highly sensitive to ouabain (I_{50}, 0.13 μM). Examination of enzyme containing the rat α3 isoform was performed following expression of this isoform in 3T3 cells using an expression vector carrying the rat α3 isoform cDNA (Hara *et al.*, 1988). A K_i value for ouabain of 0.08 μM was obtained using this approach, while measurements of Na,K-ATPase containing the α3 isoform of rat pineal glands where α3 is the predominant isoform yielded a K_i value of 1.6 μM (Shyjan *et al.*, 1990).

Early studies which compared kidney enzyme to that from various brain preparations demonstrated a higher Na^+ affinity of the brain Na,K-ATPase with little difference in K^+ affinity (Skou, 1962; Urayama and Nakao, 1979; Sweadner, 1985). This suggests that differences in the substrate affinity of the various isoforms exist, but because the brain contains all three isoforms (or two isoforms in axolemma preparation), it is difficult to associate a particular affinity with individual subunits. In adipocytes, Lytton (1985) reported an affinity for Na^+ of 52mM for α(+) as compared to 17 mM for α1 containing enzyme. Differences in isoforms were also obtained when comparing rat brain, heart, and kidney enzyme under various conditions (Feige *et al.*, 1988). Advantage has been taken of the fact that rat pineal gland contains only α1 and α3 subunits in neonatal rats and a small amount of α1 and α3 in adult glands to measure the affinity for Na^+ (Shyjan *et al.*, 1990). Based on these studies, it was concluded that the α3 isoform had a lower K_m for Na^+ compared to α1 and that enzyme containing α3 did not show positive cooperativity for Na^+ activation. In this case, the α3 isoform appears to be associated with the β3 subunit.

An expression system has recently been used to examine the differential properties of Na,K-ATPase containing α1, α2, and α3 isoforms (Jewell and Lingrel, 1991). HeLa cell lines expressing the rat α1, α2, and α3 isoforms separately were developed. In the case of rat α1, which confers resistance to ouabain when expressed in sensitive HeLa cells, the properties of enzyme containing this isoform could easily be determined in the presence of 1 μM ouabain which inhibits the sensitive endogenous or HeLa cell enzyme. The α2 and α3 isoforms are sensitive to cardiac glycosides and their properties cannot be analyzed directly using this approach. However, as the differential sensitivity between the α1 and the α2 and α3 containing enzymes resides in two amino acids at the border of the first extracellular region of the α3 subunit, these two amino acids in α2 and α3 were converted to those present in the rat α1 isoform, producing a resistant form. The expression of these α subunits in HeLa cells gave enzyme which was not inhibited by ouabain and

allowed the rat α2 and α3 containing enzyme to be analyzed directly. Again the endogenous enzyme was inhibited by the addition of 1 μM of ouabain, a concentration which has little or no effect on the enzyme containing the rat α2 or α3 isoforms. Thus, three cell lines were produced, each expressing a different rat α isoform under conditions where the endogenous enzyme could be inhibited by ouabain, allowing the properties of Na,K-ATPase carrying the α1, α2, or α3 subunits to be analyzed. A major advantage of this approach is that the enzyme activities are from the same cell type and include identical membrane environments. These studies indicate that the apparent affinity for Na^+ of α1 and α2 containing enzymes is similar ($K_{0.5} \simeq 3.5$ mM) while that containing α3 is two- threefold higher ($\simeq 8$ mM). Also, the apparent affinity of the α3 isoform for K^+ appears to be slightly lower. The discrepancies among the studies mentioned above are likely to be due to differences in cell type, isolation procedures, and assay conditions.

From the above discussion, it is evident that properties such as Na^+ and perhaps K^+ affinity vary with the nature of the isoform as does the response to cardiac glycosides. The variation in Na^+ and K^+ affinities may or may not be significant with respect to overall physiology as the cellular concentration of Na^+ ions, for example, may be higher than the K_m. However, as the isoforms are differentially distributed among cell types, it is possible that these differences in affinity have significant physiological consequences. In order to resolve this question, it will be necessary to investigate in detail cellular ion concentrations and whether, for example, replacing one isoform for another via homologous recombination and gene targeting will actually affect the physiology of the animal. This is also true of the differential cardiac glycoside effect. If there is an endogenous digitalis-like factor which regulates Na,K-ATPase, the marked resistance of the rodent α1 isoform to ouabain compared to this same isoform in other species is difficult to reconcile and the physiological significance is unclear. It is possible that the α1 isoform of the rat has become resistant to provide some survival value and that the activity of this isoform and possibly the others have been altered to compensate for this difference. It is also possible that the cardiac glycoside receptor site is not used *in vivo*, i.e., there is no physiological ligand that interacts with this receptor to modulate the activity of the enzyme. Again, it is likely that proof of this will come either from identifying the ligand or from homologous recombination and gene targeting studies. The latter approach can be used to alter the cardiac glycoside binding site *in vivo* and a determination made as to whether this affects the physiology of the animal. Replacing the cardiac glycoside binding site of the rat α1 by that of α2 would result in an animal with all three isoforms responsive to this class of drugs. If no physiological effect is observed, then it is possible that this site does not play a significant role in the biology of the animal. Alternatively, it will be interesting to convert the α2 or α3 isoform genes to ones that code for resistant subunits. Again, the lack of a physiological effect would argue against the receptor site being used *in vivo*, while a significant deviation from normal physiology would suggest the presence of a natural ligand interacting with the Na,K-ATPase.

DEFINING THE CARDIAC GLYCOSIDE BINDING SITE

As indicated above, the Na,K-ATPase containing the rat α1 isoform exhibits a marked insensitivity to cardiac glycosides, while enzyme containing the α2 or α3 isoform is sensitive to this class of compounds. This natural resistance was utilized to define regions of the α subunit responsible for this differential sensitivity (Price and Lingrel, 1988). In initial studies, chimeras were made between the sensitive sheep α1 and insensitive rat α1 isoforms. A convenient *Bam*HI site occurs at an identical position in both *c*DNAs and allows for exchange of the two halves. The chimera containing the N-terminal half of the rat α1 isoform and the C-terminal half of the sheep α1 isoform confers resistance to ouabain when expressed in sensitive cells. The alternative construct, i.e., the N-terminal half of sheep α1 isoform and C-terminal half of rat α1, does not confer resistance to sensitive cells. These results indicated that determinants for differential ouabain sensitivity are located in the N-terminal portion of the α1 subunit. Site-directed mutagenesis was used to identify two amino acid residues which are responsible for the difference between the sensitive and resistant isoforms. These amino acids are located at positions 113 and 124 in the rat α1 subunit. In the resistant form, these positions are occupied by Arg and Asp, respectively, while in the sensitive sheep isoform, uncharged amino acids Gln and Asn occupy these two sites. Examination of other sensitive forms indicates that uncharged amino acids are present at these positions as well, suggesting that charged amino acids confer the resistant phenotype (Emanuel *et al.*,

1989). The introduction of various combinations of charged amino acids in these two positions converted the sensitive α1 isoform to one that confers resistance; however, the I_{50} varies based on the particular amino acid combination (Price *et al.*, 1990). Replacement with uncharged amino acids did not yield subunits able to confer resistance. Substitutions at other positions within this region such as replacement of Asp (corresponding to 123 on the rat α1 isoform) with other amino acids were found to confer the resistance property to the sheep α1 (Price *et al.*, 1989). Other amino acid substitutions within this region have not converted the sensitive form to an insensitive one, however, it has not yet been determined whether modest alterations in ouabain sensitivity occur. The possibility exists that amino acids within the adjacent transmembrane regions are involved in ouabain sensitivity. It is difficult to distinguish between amino acid residues that are directly involved in binding and those that alter the ability of ouabain to translate its inhibitory effect to the active site of the enzyme. Additional mutants should help to define residues involved in both binding and in the transducing effect.

Amino acid substitutions have also been made in the second extracellular region of the sheep α1 isoform and in no case have these substitutions resulted in resistant enzyme (Lingrel *et al.*, 1991). This is somewhat surprising as this site has some similarity to a steroid binding site and therefore represents a potential ouabain binding region. The inability of a particular substitution to convert a sensitive to an insensitive form must be viewed with caution because not only must the enzyme fail to be inhibited to ouabain, but it must also retain activity with the substituted amino acid. Enzymatic activity is required to give the resistant phenotype. This possibility has been explored for some of the amino acid substutitions in the second extracellular region by simultaneously introducing these changes into a resistant form and determining if the resulting subunits retain their ability to confer resistance to sensitive cells (Lingrel *et al.*, 1991). In this case, amino acid replacements in the second extracellular region, which do not confer resistance to ouabain, were introduced into the sheep α1 isoform which carried mutations in the first extracellular region known to prevent ouabain inhibition. The changes included the substitution of the Trp residue by Phe, Pro, or Cys and substitution of the first Glu for Gly and the second Glu for Gln. These results demonstrate that the amino acid substitutions tested in the second extracellular region do not alter ouabain sensitivity nor significantly inhibit the activity of the enzyme. The availability of this plus-minus phenotypic selection procedure will greatly aid in the identification of amino acid residues affecting ouabain sensitivity and will allow the continued assessment of the role of various amino acid residues in drug binding. At the present time, it is difficult to predict the nature of the receptor binding site. It is possible that only the first extracellular regions involved in binding ouabain, but, more likely, other regions, either extracellular or lying within the transmembrane regions, will be found to alter cardiac glycoside sensitivity. It is interesting to note that two Cys residues (Cys 367, Cys 656) located in the cytoplasmic portion of the α1 subunit have been shown to alter ouabain sensitivity (Kirley *et al.*, 1991). In addition, an antibody which interacts with the first extracellular region increases the sensitivity of Na,K-ATPase to ouabain rather than preventing inhibition as would be expected if cardiac glycosides bind to this site (Arystarkhova *et al.*, 1992).

Future studies should characterize the cardiac glycoside binding site in detail and identify amino acid residues involved in the transport of ions as well as in coupling ATP hydrolysis to this process. Also, the properties of the enzyme containing each of the isoforms must be investigated further and the regulated expression of the isoform genes described in detail.

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