Physiological role of the \( \alpha_1 \)- and \( \alpha_2 \)-isoforms of the Na\(^+\)-K\(^+\)-ATPase and biological significance of their cardiac glycoside binding site

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Larson, Iva Dostanic, John N. Lorenz, James W. Van Huyse, Jon C. Neumann, Amy E. Moseley, and Jerry B Lingrel. Physiological role of the \( \alpha_1 \)- and \( \alpha_2 \)-isoforms of the Na\(^+\)-K\(^+\)-ATPase and biological significance of their cardiac glycoside binding site. Am J Physiol Regul Integr Comp Physiol 290: R524–R528, 2006; doi:10.1152/ajpregu.00838.2005.—An interesting feature of Na\(^+\)-K\(^+\)-ATPase is that it contains four isoforms of the catalytic \( \alpha \)-subunit, each with a tissue-specific distribution. Our laboratory has used gene targeting to define the functional role of the \( \alpha_1 \)- and \( \alpha_2 \)-isoforms. While knockout mice demonstrated the importance of the \( \alpha_1 \)- and \( \alpha_2 \)-isoforms for survival, the knockin mice, in which each isoform can be individually inhibited by ouabain and its function determined, demonstrated that both isoforms are regulators of cardiac muscle contractility. Another intriguing aspect of the Na\(^+\)-K\(^+\)-ATPase is that it contains a binding site for cardiac glycosides, such as digoxin. Conservation of this site suggests that it may have an in vivo role and that a natural ligand must exist to interact with this site. In fact, cardiac glycoside-like compounds have been observed in mammals. Our recent study demonstrates that the cardiac glycoside binding site of the Na\(^+\)-K\(^+\)-ATPase plays a role in the regulation of blood pressure and that it mediates both ouabain-induced and ACTH-induced hypertension in mice. Whereas chronic administration of ouabain or ACTH caused hypertension in wild-type mice, it had no effect on blood pressure in mice with a ouabain-resistant \( \alpha_2 \)-isoform of Na\(^+\)-K\(^+\)-ATPase. Interestingly, animals with the ouabain-sensitive \( \alpha_1 \)-isoform and a ouabain-resistant \( \alpha_2 \)-isoform develop ACTH caused hypertension in wild-type mice, to a greater extent than wild-type animals. Taken together, these results demonstrate that the cardiac glycoside binding of the Na\(^+\)-K\(^+\)-ATPase has a physiological role and suggests a function for a naturally occurring ligand that is stimulated by administration of ACTH.

ouabain; adrenocorticotropic hormone; blood pressure regulation; cardiotonic steroids

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NA\(^+\)-K\(^+\)-ATPase is an integral membrane protein that uses ATP to transport Na\(^+\) out of cells and K\(^+\) into the cell. The resulting gradient drives numerous processes, such as transport of glucose into intestinal and renal epithelial cells through a glucose-sodium cotransporter, as well as the transport of other nutrients, such as amino acids, and ions, like Ca\(^{2+}\). This enzyme is also responsible for generating the resting potential of cells, which is particularly important in neuronal and muscle function.

The Na\(^+\)-K\(^+\)-ATPase is a member of the P-type family of ATPases and is closely related to the Ca\(^{2+}\)-ATPase family and the H\(^+\)-K\(^+\)-ATPase (31, 36). Na\(^+\)-K\(^+\)-ATPase is composed of two major subunits, the catalytic \( \alpha \)- and glycosylated \( \beta \)-subunit. However, other proteins, such as members of the FXYD family of proteins, interact with this enzyme in some tissues, such as heart, kidney, and brain (20, 49, 50, 52). The association of these proteins modulates cation binding affinity of the Na\(^+\)-K\(^+\)-ATPase (1, 6, 11, 57). Three isoforms of the \( \beta \)-subunit exist: \( \beta_1 \), \( \beta_2 \), and \( \beta_3 \). The \( \alpha \)-subunit has four isoforms, \( \alpha_1 \), \( \alpha_2 \), \( \alpha_3 \), and \( \alpha_4 \). While the \( \alpha_1 \)-isoform is expressed ubiquitously, the \( \alpha_2 \)-isoform is present largely in skeletal muscle, heart, brain, adipocytes, vascular smooth muscle, and eye, as well as a number of other tissues. The \( \alpha_3 \)-isoform is found almost exclusively in neurons and ovaries, but also occurs in white blood cells and heart of some species, such as humans (46, 48). The \( \alpha_4 \)-isoform is expressed in sperm and is specifically synthesized at the spermatogonia stage, where it is required for sperm motility (47, 54). It is reasonable to believe that the tissue-specific distribution of the \( \alpha \)-isoforms indicates that each

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Role of the α₁- and α₂-Isomers in Cardiac Contraction: Gene Knockout Studies

In contrast to animals lacking both copies of the α₁- or α₂-isomer, animals lacking only one copy of either gene survive and appear normal. The α₁⁺⁻⁻ animals develop normally with no observable phenotype (28). The functional role of the α₁- and the α₂-isomers in cardiac and skeletal muscle have been studied. In both cases, the particular isoform is reduced by 40% compared with wild-type, and both genotypes exhibit altered cardiac and skeletal muscle contractile functions (24, 28).

Whereas mice lacking one copy of the α₂-isomeric gene exhibit an increase in the force of cardiac and skeletal muscle contraction, animals lacking one copy of the α₁-isomeric gene have decreased contraction in both cardiac and skeletal muscle (24, 28). These different phenotypes were unexpected and suggest that the α₁- and α₂-isomers play different roles in the regulation of muscle contractility. The increase in contractile force of the α₂-isomeric heterozygous knockout mice fit with the proposed model that inhibition of Na⁺-K⁺-ATPase activity results in an increase in intracellular Na⁺ levels, which, in turn, causes an increase in Ca²⁺ through the reverse mode of the Na/Ca exchanger. However, the hypotonicity in the α₁-isomeric heterozygous knockout mice is opposite of the proposed model. Subsequent studies showed that the hypotonicity in the α₁⁺⁻⁻ hearts is not the result of calcium overload, which could have been responsible for the phenotype (42).

To help address why the α₁-haplo-insufficient animals exhibit unexpected cardiac hypotonicity, mRNA microarray analysis was carried out on heart tissue from wild-type and α₁⁺⁻⁻ animals (40). Interestingly, the expression of a number of genes is altered, and these could be grouped into various clusters, including metabolic and ion transport pathways. The expression of a number of genes that are involved in enhancing cardiac contractility, such as atrial natriuretic peptide, are increased. Furthermore, the changes in gene expression in heart provided evidence of altered kidney and adrenal gland function. These indicate that multiple organs in these animals are compromised with the loss of one copy of the α₁-isomeric, and this raised the possibility that the reduced cardiac contractility in α₁-heterozygous mice may be due, in part, to compensatory changes that accumulate during development. Therefore, it may be difficult to draw conclusions about α₁-isomeric function from animals lacking one copy of the α₁-isomeric gene.

Role of the α₁- and α₂-Isomers in Cardiac Function: Isoform Specific Pharmacological Inhibition

Whereas the ablation and reduction of the α₁- or α₂-isomers show the importance of these isoforms, they failed to definitively demonstrate a physiological function for them. Therefore, a different approach was used to examine the specific function of the α₁- and α₂-isomers of the Na⁺-K⁺-ATPase.

An approach for investigating the role of each α-isomeric, without the long-term compensation that occurs during development in knockout animals, is to develop animals where only one isoform can be pharmacologically inhibited at a time and analyze the physiological consequence of this inhibition. All α-isomers of the Na⁺-K⁺-ATPase in most species, except for a few, such as the rat and mouse α₁-isomeric, are inhibited by low concentrations of ouabain. We took advantage of our earlier studies defining the amino acids conferring differential sensitivity to ouabain. The basis for the relative sensitivity of the mouse and rat α₁-isomers to ouabain resides in two amino acids in the first extracellular region of the α₁-subunit (44). The mouse α₂-isomeric, which is sensitive to ouabain, has a leucine at position 111 and an asparagine occurs at position 122. This is in contrast to the α₁-isomeric, which has low affinity for ouabain, where arginine and aspartic acid appear at these sites. The positive charge of the latter two amino acids appears to confer resistance to ouabain (44). By using the mice with the combination of sensitive and insensitive isoforms, we can inhibit only one isoform by ouabain without altering the activity of other α-isomers.

With the use of a Cre-loxP gene-targeting strategy, mice were developed where the leucine and asparagine of the α₂-isomeric gene were converted to arginine and aspartic acid, i.e., L111R and N122D (15). These animals express a portion of the α-wild-type gene replaced with a sequence conferring ouabain resistance and are referred to as knockin mice.

The resulting knockin animals show no difference in the levels of expression and tissue distribution of the α₁-, α₂-, or α₃-isomers (15). However, the muscle and heart of the α₂-ouabain-insensitive animals exhibit a loss of ouabain binding, as expected, because in wild-type mice, both tissues have a sensitive α₂-isomeric in addition to the relatively insensitive α₁-isomeric. This loss of ouabain binding indicates that the targeted α₂-isomeric is successfully modified to a ouabain-resistant isoform. The gene-targeted knockin animals are born in normal Mendelian ratios and show no outward phenotype. Furthermore, their baseline cardiovascular hemodynamics are normal (15). The animals also respond normally to the β-adrenergic agonist dobutamine. Therefore, the conversion of the α₂-isomeric to a ouabain-resistant protein does not alter essential properties of the Na⁺-K⁺-ATPase.

The analysis of cardiac contractility in intact mice and in ex vivo isolated heart preparations demonstrates that ouabain, at concentrations that inhibit the sensitive α₂-isomeric in wild-type mice, fail to induce positive cardiac inotropy in knockin hearts. This is in contrast to the increased cardiac contractility observed in the wild-type heart following administration of the same concentrations of ouabain. This clearly indicates that the α₂-isomeric is responsible for the ouabain-induced positive cardiac inotropy and demonstrates the importance of the α₂-isomeric in the regulation of cardiac contractility (15). How-
ever, these studies do not provide information on the role of the α1-isoform in these processes.

The role of the α1-isoform in cardiac contractility was determined by developing animals with a ouabain-sensitive α1-isoform (in rodents, the α1-isoform is naturally ouabain insensitive) and a resistant α2-isoform (16). The ouabain sensitivity of the α1-isoform was conferred by introducing R111Q and D122N amino acid substitutions. The ouabain-sensitive α1-isoform mice develop normally and show no visible phenotype. The expression and tissue distribution of each α-isoform is also not altered by modification of the genotype. However, the ouabain sensitivity of the Na\(^{+}\)-K\(^{+}\)-ATPase in tissues, and specifically in kidney, where wild-type animals express only a ouabain-resistant α1-isoform, is enhanced as expected in the gene-targeted animals. These animals were then mated to those with a ouabain-resistant α2-isoform to give animals with the genotype α1\(^{SS}\) α2\(^{RR}\), where S and R indicate sensitive and resistant to ouabain, respectively. In these animals only the α1-isoform is inhibited by low concentrations of ouabain and the function of this isoform in cardiac contractility can be determined without inhibiting the α2-isoform.

When ouabain is administered to α1\(^{SS}\) α2\(^{RR}\)-mice, a very large increase in cardiac contractility occurs (16). This indicates that the α1-isoform, similar to the α2-isoform when inhibited by ouabain, elicits an increase in cardiac contraction. It should be pointed out that the magnitude of the increase is much greater when α1 is inhibited compared with inhibiting the α2-isoform, which is likely because the α1-isoform is more abundant than the α2-isoform in the mouse heart.

Whereas these studies suggest that both α1- and α2-isoforms play a similar role in cardiac contractility, they do not address the mechanism by which these two isoforms regulate cardiac function. It has been postulated that the Na\(^{+}\)-K\(^{+}\)-ATPase, particularly the α2-isoform, modulates Ca\(^{2+}\) levels during contraction through functional coupling with Na/Ca exchanger (28, 29, 51). Thus, we tested whether the α1- or α2-isoform functionally couples and physically interacts with the Na/Ca exchanger (16). Pretreatment with KB-R7943, an inhibitor of the reverse mode of the Na/Ca exchanger, abolished the cardiotoxic effects of ouabain in isolated wild-type and the ouabain-sensitive α1-isoform hearts. Also, immunoprecipitation analyses demonstrated that both the α1- and α2-isoforms colocalize with the Na/Ca exchanger in the heart. These studies clearly demonstrate that both the α1- and α2-isoforms regulate cardiac contraction through the functional and physical coupling with the Na/Ca exchanger. In summary, both isoforms colocalize with the Na/Ca exchanger, and their inhibition by ouabain leads to enhanced contractility.

**Physiological Role of the α2- Isoform in Blood Pressure Regulation**

The importance of the α2-isoform in regulation of cardiac contractility and the fact that the cardiac output and vascular resistance regulate blood pressure, led us to investigate the physiological role of the α2-isoform in the regulation of blood pressure (17). Both wild-type and ouabain-resistant α2-isoform mice were administered ouabain (once a day by intraperitoneal injection) over an extended period of time, and their blood pressure was measured daily via tail cuff. Chronic administration of ouabain induced hypertension in wild-type but not in knockin mice, indicating that the α2-isoform is involved in the blood pressure response to ouabain. In addition, acute administration of ouabain increased blood pressure in wild-type but not in α1\(^{RR}\) α2\(^{RR}\)-mice. This shows that the α2-isoform plays a role in maintenance of the overall cardiovascular hemodynamics and is further supported by data demonstrating that heterozygous α2-isoform knockout mice are hypertensive (56).

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**Fig. 1.** Ouabain-sensitive Na\(^{+}\)-K\(^{+}\)-ATPase mediates ACTH-induced hypertension. Chronic administration/exposure to ACTH releases naturally occurring ligand(s) for the Na\(^{+}\)-K\(^{+}\)-ATPase. Ouabain-sensitivity of the Na,K-ATPase determines its reactivity towards this endogenous ligand. The ouabain-sensitive Na\(^{+}\)-K\(^{+}\)-ATPase binds the ligand via the cardiac glycoside binding site. This interaction results in the development of hypertension. In contrast, ouabain-resistant Na\(^{+}\)-K\(^{+}\)-ATPase cannot interact with the endogenous ligand, and ACTH-induced hypertension does not occur. Q, glutamine; N, asparagine; R, arginine; and D, aspartic acid.
Physiological Role of the Cardiac Glycoside Binding Site

One of the interesting features of the Na\(^{+}\)-K\(^{+}\)-ATPase is that it possesses a binding site for cardiac glycosides, which have been used for centuries in treatment of congestive heart failure. The observation that the cardiac glycoside binding site of the Na\(^{+}\)-K\(^{+}\)-ATPase is conserved through evolution, from hydra to human (10), suggests that this site may play an important biological role. The finding of endogenous cardiac glycoside-like compounds in mammals further strengthens this hypothesis (4, 8, 9, 14, 21–23, 37–39, 43, 45). Although the exact number and structure of endogenous cardiac glycosides are still unknown, elevated levels have been correlated with an increase in blood pressure (4, 13, 21, 22, 26, 32, 37–39, 53) and development of congestive heart failure (3, 25).

Our approach in determining whether the ouabain binding site plays a biological role, and, thereby, testing the physiological relevance of endogenous ligands, was to utilize the knockin mice described above in which the cardiac glycoside binding site of the \(\alpha_1\) and \(\alpha_2\)-isoforms is altered. As indicated earlier, \(\alpha_1^{R/R}\) \(\alpha_2^{R/R}\) animals show no observable phenotype under normal laboratory conditions nor do animals with a sensitive \(\alpha_1\)-isoform. These animals are born in normal Mendelian ratios, their weight gain is normal, litter sizes are normal, and their life span is unchanged from wild-type animals. On the surface, these studies suggest that the ouabain binding site does not play a significant biological role under normal conditions. However, endogenous ouabain levels are present at very low concentrations under resting conditions but increase under stress, such as exercise (4) and ACTH administration (19, 21, 34, 35, 55) or a high-salt diet (26, 53). Therefore, animals with an ouabain-resistant \(\alpha_2\)-isoform and \(\alpha_1^{R/R} \alpha_2^{R/R}\), were compared with wild-type animals (\(\alpha_1^{T/S} \alpha_2^{S/S}\)) following daily administration of ACTH (18). In wild-type animals, blood pressure began to increase on the second day of injection and plateaued at about 5 days. In animals that have the resistant \(\alpha_2\)-isoform, this increase in blood pressure does not occur, indicating that ACTH-induced hypertension is mediated through the ouabain binding site of \(\alpha_2\)-isoform of Na\(^{+}\)-K\(^{+}\)-ATPase. This suggests a role for this binding site, at least in this hypertensive condition, and further implicates the presence of an endogenous ligand that would interact with this site. During ACTH-induced hypertension, there is an increase in endogenous ouabain-like compounds, suggesting involvement of these compounds (18). Furthermore, it has been shown that adrenocortical cells secrete ouabain-like material following stimulation by ACTH (5, 33).

In humans, both the \(\alpha_1\) and \(\alpha_2\)-isoforms are sensitive to ouabain, and it was of interest to determine whether the ouabain binding site of the \(\alpha_1\)-isoform could also play a role in ACTH-induced hypertension in mice. There is a robust increase in blood pressure associated with administration of ACTH in \(\alpha_1^{S/S} \alpha_2^{R/R}\) in contrast to no increase in blood pressure in \(\alpha_1^{R/R} \alpha_2^{R/R}\) animals. This indicates that the \(\alpha_1\)-isoform can also mediate ACTH-induced hypertension and suggests that in humans, where the \(\alpha_1\)-isoform is sensitive to cardiac glycosides, regulation of the Na\(^{+}\)-K\(^{+}\)-ATPase through the \(\alpha_1\)-isoform could also have physiological significance in the regulation of blood pressure.

Although these studies do not identify the ligand that is interacting with the Na\(^{+}\)-K\(^{+}\)-ATPase, they do demonstrate that the cardiac glycoside binding site is important and that an endogenous ligand must exist (Fig. 1). It is likely that the cardiac glycoside binding site of this enzyme will be found to play an important role in a number of biological functions.

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