The highly conserved cardiac glycoside binding site of Na,K-ATPase plays a role in blood pressure regulation

Iva Dostanic-Larson*, James W. Van Huysse^{†‡}, John N. Lorenz[§], and Jerry B. Lingrel^{*¶}

Departments of *Molecular Genetics, Biochemistry, and Microbiology and [§]Molecular and Cellular Physiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267; [†]Hypertension Unit, University of Ottawa Heart Institute, Ottawa, ON, Canada K1Y 4W7; and [‡]Departments of Medicine and Biochemistry and Microbiology and Immunology, University of Ottawa, Ottawa, ON, Canada K1H 8M5

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The Na,K-ATPase contains a binding site for cardiac glycosides, such as ouabain, digoxin, and digitoxin, which is highly conserved among species ranging from *Drosophila* to humans. Although advantage has been taken of this site to treat congestive heart failure with drugs such as digoxin, it is unknown whether this site has a natural function *in vivo*. Here we show that this site plays an important role in the regulation of blood pressure, and it specifically mediates adrenocorticotrophic hormone (ACTH)-induced hypertension in mice. We used genetically engineered mice in which the Na,K-ATPase $\alpha 2$ isoform, which is normally sensitive to cardiac glycosides, was made resistant to these compounds. Chronic administration of ACTH caused hypertension in WT mice but not in mice with an ouabain-resistant $\alpha 2$ isoform of Na,K-ATPase. This finding demonstrates that the cardiac glycoside binding site of the Na,K-ATPase plays an important role in blood pressure regulation, most likely by responding to a naturally occurring ligand. Because the $\alpha 1$ isoform is sensitive to cardiac glycosides in humans, we developed mice in which the naturally occurring ouabain-resistant $\alpha 1$ isoform was made ouabain-sensitive. Mice with the ouabain-sensitive "human-like" $\alpha 1$ isoform and an ouabain-resistant $\alpha 2$ isoform developed ACTH-induced hypertension to greater extent than WT animals. This result indicates that the cardiac glycoside binding site of the $\alpha 1$ isoform can also mediate ACTH-induced hypertension. Taken together these results demonstrate that the cardiac glycoside binding site of the α isoforms of the Na,K-ATPase have a physiological function and supports the hypothesis for a role of the endogenous cardiac glycosides.

adrenocorticotrophic hormone | hypertension | endogenous cardiac glycosides

Na,K-ATPase is an integral membrane protein that transports Na^+ and K^+ across the plasma membrane against their concentration gradients. The enzyme is composed of two essential subunits, α and β (1). Although the α subunit is the catalytic subunit of the enzyme, the β subunit is involved in the enzyme's maturation, localization to the plasma membrane, and stabilization of the K^+ -occluded intermediate. There are four isoforms of the α subunit ($\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 4$), each of which exhibits a unique tissue distribution (2). The $\alpha 1$ isoform is present in most tissues, whereas the $\alpha 2$ isoform is expressed in brain, heart, vasculature, and skeletal muscle. The $\alpha 3$ isoform is limited essentially to neural tissues and vascular smooth muscle and in some animals, such as humans, it is also found in the heart. The $\alpha 4$ isoform is present only in testes and specifically in spermatogonia and mature sperm (3).

The Na,K-ATPase has an evolutionarily conserved cardiac glycoside binding site, and although advantage has been taken of this site to treat congestive heart failure by drugs such as digoxin, it is unknown whether this site serves a biological function *in vivo*. Naturally occurring cardiac glycoside-like compounds have been identified in mammals (4–9). Although there have been numerous reports over the past two decades of endogenous cardiac glycosides that could, in principle, serve as ligands of Na,K-ATPase, there is no direct evidence that this occurs.

Although several studies have shown a correlation between elevated endogenous cardiac glycosides and certain pathological conditions, the physiological function of endogenous cardiac glycoside-like compounds is still uncertain (10–20). Plasma levels of endogenous cardiac glycosides are high in several animal models of hypertension, as well as in human essential hypertension and preeclampsia (10–17). In addition, marked increases in endogenous ouabain-like compounds occur in congestive heart failure, both in animal models and human patients (18–20). One concern raised with respect to endogenous cardiac glycoside-like compounds is that they occur in levels which may not significantly inhibit the Na,K-ATPase. Interestingly, a number of studies have shown that exogenous cardiac glycosides, specifically ouabain, at low concentrations initiate a signaling cascade (via Src/Ras/mitogen-activated protein kinases) (21–26), as well as increase Na,K-ATPase activity *in vitro* (27).

The $\alpha 2$, $\alpha 3$, and $\alpha 4$ isoforms of mice are naturally sensitive to cardiac glycosides, and these isoforms could serve as receptors for endogenous ligands. Because the $\alpha 1$ isoform is ouabain-resistant in mice, it is unlikely that it is a receptor for such ligands. However, in humans the $\alpha 1$ isoform is sensitive to cardiac glycosides and may be responsive to a naturally occurring ligand (28, 29).

We tested whether the cardiac glycoside binding site of the $\alpha 1$ and $\alpha 2$ isoforms of Na, K-ATPase has a biological function *in vivo* by using genetically engineered mice with modified cardiac glycoside binding affinity of the $\alpha 1$ and $\alpha 2$ Na,K-ATPase isoforms. The present study demonstrates that the cardiac glycoside binding site plays a key role in regulating blood pressure; specifically it mediates adrenocorticotrophic hormone (ACTH)-induced hypertension in mice.

Materials and Methods

Animals. Development of mice expressing the cardiac glycoside-resistant $\alpha 2$ isoform and generation of animals expressing the high-affinity $\alpha 1$ isoform and low-affinity $\alpha 2$ isoform were described (30, 31). Genotypes were determined by PCR analysis of DNA from tail biopsies, as described in refs. 30 and 31. The use of mice in these experiments was approved by the University of Cincinnati Animal Care and Use Committee.

Administration of Saline and ACTH. Mice were administered saline or 500 $\mu\text{g}/\text{kg}$ ACTH (fragment 1–24, Sigma) every 8 h by s.c. injection for 5 days. Tail-cuff measurements of systolic blood pressure (SBP) were obtained 3 h after administration, i.e., 5 h before the next injection.

Administration of IgG γ , Digibind, and KB-R7943. Mice were i.v. administered IgG γ or 30 $\mu\text{g}/\text{kg}$ Digibind (GlaxoSmithKline)

Abbreviations: ACTH, adrenocorticotrophic hormone; SBP, systolic blood pressure.

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[¶]To whom correspondence should be addressed. E-mail: jerry.lingrel@uc.edu.

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every day during the ACTH treatment 2 h before tail-cuff measurement. The inhibitor of the reverse mode of the Na/Ca-exchanger, KB-R7943 (Tocris, Ellisville, MO), was administered i.v. 10 $\mu\text{g/kg}$ 2 days before and every day during the 3-day ACTH treatment; saline (vehicle alone) was administered i.v. as a control. Before each i.v. administration, mice were anesthetized by isoflurane. After injection mice were allowed to fully recover before their blood pressure was measured.

Tail-Cuff Blood Pressure Measurements. Tail-cuff measurements were obtained by using a Visitech (Apex, NC) system computerized apparatus, as described in ref. 32. In brief, for each measurement, 10 preliminary blood pressure measurements were obtained to acclimate the mice to the apparatus, and these were followed by 10 recorded SBP and heart rate measurements. There was little delay between measurements. The blood pressure waveform was carefully observed for movement artifacts during both preliminary and measurement cycles. Data were accepted if SBP was identified by the computer in at least five of the 10 measurements and was >80 or <200 mmHg (1 mmHg = 133 Pa). Recordings not meeting these criteria ($<1\%$ of the recordings) were discarded.

Serum Cardiac-Glycoside-Like Immunoreactivity. Serum preparation and ELISA experiments were performed as described in ref. 32 by using Digibind (GlaxoSmithKline) as primary Ab. Although extraction with 25% acetonitrile yields mostly hydrophilic (ouabain-like) cardiac glycoside compounds, small amounts of hydrophobic compounds could have been extracted and detected in ELISA, because Digibind cross-reacts with digoxin, ouabain, and marinobufagenin (albeit with a 20-fold lower affinity than ouabain) (33, 34). Thus, the activity in this assay is referred to as the cardiac glycoside-like immunoreactivity.

Statistical Analysis. Student's *t* test was used to compare the experimental group of mice to the corresponding control mice. Two-way ANOVA for time and treatment with repeated measurements was performed, and a post hoc test of Fisher's least significant difference was obtained.

Results

The Cardiac Glycoside Binding Site Has No Apparent Biological Role Under Resting Laboratory Conditions. To determine whether the cardiac glycoside binding site of the Na,K-ATPase $\alpha 2$ isoform plays a biologically important role, we developed mice where the $\alpha 2$ isoform is resistant to these compounds (30). These mice express the naturally ouabain-resistant $\alpha 1$ isoform and are designated as $\alpha 1^{R/R}\alpha 2^{R/R}$ mice. The $\alpha 2$ isoform was converted to low affinity by introducing L111R and N122D amino acid substitutions, which abolished the high affinity binding of cardiac glycosides but without altering enzymatic activity (28, 30). The R111 and D122 amino acids occur naturally in the ouabain-resistant $\alpha 1$ isoform of Na,K-ATPase. The overall cardiovascular performance in $\alpha 1^{R/R}\alpha 2^{R/R}$ mice was normal (30), and their heart to body weight ratio (4.9 ± 0.16 mg/g) did not differ from WT (5.1 ± 0.2 mg/g). Also, the hearts from $\alpha 1^{R/R}\alpha 2^{R/R}$ mice appeared histologically normal. The average survival rate of ouabain-resistant $\alpha 2$ mice (23.5 ± 0.01 months) did not differ from WT animals (22.9 ± 0.02 months). These findings indicate that the cardiac glycoside binding site of the $\alpha 2$ isoform is not important for growth, development, survival, or basal cardiovascular function under standard laboratory conditions. This is expected because endogenous cardiac glycoside-like compounds, which have the ability to interact with this site and may be natural ligands of Na,K-ATPase, occur at very low levels under basal laboratory conditions (4–9).

Fig. 1. The cardiac glycoside-resistant $\alpha 2$ isoform mice do not develop ACTH-induced hypertension. (A) SBP in WT $\alpha 1^{R/R}\alpha 2^{S/S}$ mice and cardiac glycoside-resistant $\alpha 2$ isoform $\alpha 1^{R/R}\alpha 2^{R/R}$ mice during prolonged treatment with saline ($n = 15$ for each genotype) and ACTH ($n = 25$ for each genotype). (B) Change in SBP from baseline (control day 0) of each animal during saline and ACTH treatment. Values are represented as averages $\pm$ SE. Control days, -1 and 0. *, $P < 0.01$ vs. saline-treated WT mice; #, $P < 0.01$ vs. ACTH-treated WT mice.

Mice Expressing the Ouabain-Resistant $\alpha 2$ Isoform of Na,K-ATPase Do Not Develop ACTH-Induced Hypertension. We next determined whether the cardiac glycoside binding site of the $\alpha 2$ isoform plays a role under conditions that are associated with increased plasma levels of endogenous cardiac glycoside-like compounds. Chronic administration of ACTH induces hypertension that is accompanied by an increase in endogenous ouabain-like, digoxin-like, and marinobufagenin-like compounds (35, 40–44). Administration of ACTH also results in the secretion of endogenous ouabain from bovine adrenocorticotrophic cells and rat adrenal gland (36–39). Furthermore a marked increase in circulating endogenous ouabain-like compounds correlates with elevated blood pressure in human ectopic ACTH syndrome (45). Thus, we analyzed the effect of ACTH on SBP and heart rate in WT ($\alpha 1^{R/R}\alpha 2^{S/S}$) and $\alpha 1^{R/R}\alpha 2^{R/R}$ mice.

Before treatment there was no significant difference in baseline SBP (Fig. 1A) or heart rate (data not shown) between WT ($\alpha 1^{R/R}\alpha 2^{S/S}$) and $\alpha 1^{R/R}\alpha 2^{R/R}$ animals. The diastolic blood pressure in these mice could not be determined accurately by using the tail-cuff method of measurement. There was no significant difference in SBP (Fig. 1A) or heart rate (data not shown) between saline-treated $\alpha 1^{R/R}\alpha 2^{S/S}$ and $\alpha 1^{R/R}\alpha 2^{R/R}$ animals. However, after 5 days of treatment with ACTH, a significant difference in SBP was observed between $\alpha 1^{R/R}\alpha 2^{S/S}$ (WT) and $\alpha 1^{R/R}\alpha 2^{R/R}$ mice (Fig. 1A). ACTH had no effect on heart rate in mice of either genotype (data not shown). On the second day of ACTH treatment, the SBP in WT mice increased significantly compared to saline control. A gradual increase in SBP occurred during the next 3 days and reached a maximum by the fourth day of treatment. In contrast, no effect on SBP in $\alpha 1^{R/R}\alpha 2^{R/R}$ mice after 5 days of ACTH treatment. The change in SBP, relative to basal blood pressure, clearly demonstrates a significant elevation in WT $\alpha 1^{R/R}\alpha 2^{S/S}$ mice with no

Fig. 2. ACTH induced the elevation of endogenous cardiac glycoside-like compounds, and their neutralization abolishes hypertension. (A) Elevated ouabain-like immunoreactivity in serum from WT $\alpha 1^{R/R} \alpha 2^{S/S}$ and $\alpha 1^{R/R} \alpha 2^{R/R}$ mice on day 5 of saline ($n = 10$ for each genotype) and ACTH ($n = 13$ for each genotype) treatment. *, $P < 0.05$ vs. saline. (B) Digibind reduced SBP in ACTH-treated WT $\alpha 1^{R/R} \alpha 2^{S/S}$ ($n = 10$) and had no effect on $\alpha 1^{R/R} \alpha 2^{R/R}$ ($n = 10$) mice. (C) Sheep IgG ($n = 8$ for each genotype), as a negative control, had no effect on SBP in ACTH-treated WT $\alpha 1^{R/R} \alpha 2^{S/S}$ and $\alpha 1^{R/R} \alpha 2^{R/R}$ mice. Values are averages $\pm$ SE. Control days, -2 , -1 , and 0 . #, $P < 0.01$ vs. $\alpha 1^{R/R} \alpha 2^{R/R}$ ACTH.

effect in $\alpha 1^{R/R} \alpha 2^{R/R}$ animals over the course of ACTH treatment (Fig. 1B). These data demonstrate that the cardiac glycoside binding site of the $\alpha 2$ isoform mediates ACTH-induced hypertension in mice and suggest that an endogenous ligand may exist, which interacts with this site. Logical candidates are endogenous cardiac glycosides, which have been postulated to play a role in the elevation of blood pressure (4–17).

Endogenous Cardiac Glycoside-Like Compounds Are Elevated in ACTH-Treated Mice. Chronic administration of ACTH resulted in 3- to 4-fold higher levels of circulating cardiac glycoside-like compounds in the blood serum from both WT $\alpha 1^{R/R} \alpha 2^{S/S}$ and $\alpha 1^{R/R} \alpha 2^{R/R}$ mice (Fig. 2A). These findings are in agreement with previous studies describing an increase in circulating levels of these compounds in ACTH-induced hypertension (40–45). Thus, the difference in blood pressure between WT and $\alpha 1^{R/R} \alpha 2^{R/R}$ mice after the ACTH administration is not due to differences in levels of circulating endogenous cardiac glycoside-like compounds. Although our extraction method recovered hydrophilic, i.e., ouabain-like, compounds, small amounts of hydrophobic (digoxin-like) compounds may have also been extracted and detected by ELISA by using the Digibind Ab.

To establish a possible correlation between the endogenous cardiac glycoside compounds and elevation of blood pressure, we neutralized circulating cardiac glycosides with Digibind, an anti-cardiac glycoside Ab, and analyzed this effect on ACTH-induced hypertension. i.v. infusion of Digibind prevented ACTH-induced rise in blood pressure in WT mice (Fig. 2B). These results are consistent with previous studies by using Digibind in rat models of hypertension (46–52). As a negative control, i.v. infusion of non-specific sheep IgG had no effect on the development of the ACTH-induced hypertension (Fig. 2C). These data provide evidence that the rise in blood pressure in ACTH-treated mice is due to cardiac glycoside-like compounds, and importantly, that these compounds mediate a biological effect through the cardiac glycoside binding site of the $\alpha 2$ isoform.

ACTH-Induced Hypertension Depends on the Reverse Mode of the Na/Ca-Exchanger. We and others (31, 53) have shown that the Na,K-ATPase $\alpha 2$ isoform is functionally coupled and colocalized with the Na/Ca-exchanger in heart and aortic vascular smooth muscle. Because cardiac output and vascular resistance, which depend on Na/Ca-exchanger activity, play a major role in regulating blood pressure, we tested whether the cardiac glycoside binding site of the $\alpha 2$ isoform is mediating the ACTH-induced hypertension through functional coupling with the Na/Ca-exchanger. i.v. infusion of KB-R7943, an inhibitor of the reverse Na/Ca-exchanger mode, before administration of ACTH did not alter SBP in WT $\alpha 1^{R/R} \alpha 2^{S/S}$ and $\alpha 1^{R/R} \alpha 2^{R/R}$ mice (Fig. 3). This indicates that KB-R7943 does not affect basal blood pressure in awake, restrained animals. The administration of KB-R7943, however, abolished the ACTH-induced hypertension in WT $\alpha 1^{R/R} \alpha 2^{S/S}$ mice (Fig. 3A) but had no effect on $\alpha 1^{R/R} \alpha 2^{R/R}$ mice; SBP remained normal in $\alpha 1^{R/R} \alpha 2^{R/R}$ mice over the course of ACTH treatment (Fig. 3B). Thus, the ACTH-induced hypertension is mediated not only by the cardiac glycoside binding site of the $\alpha 2$ isoform, but also is mediated by the reverse mode of the Na/Ca-exchanger.

The Ouabain-Sensitive $\alpha 1$ Isoform of Na,K-ATPase Can Also Mediate ACTH-Induced Hypertension. In humans, unlike rodents, the Na,K-ATPase $\alpha 1$ isoform is sensitive to cardiac glycosides (28, 29). Thus, the cardiac glycoside binding site of the $\alpha 1$ isoform may play a biological role in hypertension similar to that of the $\alpha 2$ isoform. We tested whether the ouabain sensitive α isoform plays a biological role in ACTH-induced hypertension by using genetically engineered mice expressing an ouabain-sensitive $\alpha 1$ isoform and an ouabain-resistant $\alpha 2$ isoform ($\alpha 1^{S/S} \alpha 2^{R/R}$). The $\alpha 1$ isoform was converted to a high affinity subunit by introducing R111L and D122N amino acid substitutions, which enhanced binding of cardiac glycosides ≈ 100 -fold without altering enzymatic activity (31). The mice were developed by using the Cre/loxP strategy and were mated to animals with an ouabain-resistant $\alpha 2$ isoform (31). The homozygous $\alpha 1^{S/S} \alpha 2^{R/R}$ mice were born in expected Mendelian ratio and exhibited normal development and growth. Birth weight

Fig. 3. The Na/Ca-exchanger plays a role in ACTH-induced hypertension. (A) KB-R7943 prevented the development of ACTH-induced hypertension in WT mice ($n = 6$). Saline (KB-R7943 vehicle) had no effect on the development of ACTH-induced hypertension in WT $\alpha 1^{R/R} \alpha 2^{S/S}$ mice ($n = 7$). (B) Both saline-administered ($n = 5$) and KB-R7943-administered ($n = 7$) $\alpha 1^{R/R} \alpha 2^{R/R}$ mice did not develop ACTH hypertension. Values are averages $\pm$ SE. Control days, -1 and 0 . #, $P < 0.01$ vs. $\alpha 1^{R/R} \alpha 2^{S/S}$ ACTH plus KB-R7943.

(1.13 ± 0.12 g) and their weight at 6 weeks of age (30 ± 1.2 g) were similar to that of WT (1.06 ± 0.08 g and 31 ± 0.4 g, respectively). The average survival rate of $\alpha 1^{S/S} \alpha 2^{R/R}$ mice (22.5 ± 0.05 months) did not differ from WT animals (22.9 ± 0.02 months). Basal cardiovascular hemodynamics and β -adrenergic cardiac response were indistinguishable between anesthetized WT and $\alpha 1^{S/S} \alpha 2^{R/R}$ animals (31).

Before treatment with ACTH or saline, the SBP in the awake, restrained homozygous $\alpha 1^{S/S} \alpha 2^{R/R}$ mice did not differ from that of WT $\alpha 1^{R/R} \alpha 2^{S/S}$ mice (Fig. 4A). There was also no difference in SBP between saline-treated WT $\alpha 1^{R/R} \alpha 2^{S/S}$ and $\alpha 1^{S/S} \alpha 2^{R/R}$ mice. Administration of ACTH induced hypertension in both $\alpha 1^{S/S} \alpha 2^{R/R}$ and WT $\alpha 1^{R/R} \alpha 2^{S/S}$ mice (Fig. 4A). Whereas SBP gradually increased in WT $\alpha 1^{R/R} \alpha 2^{S/S}$ mice, knock-in $\alpha 1^{S/S} \alpha 2^{R/R}$ animals responded quickly to ACTH treatment, increasing blood pressure by 15 mmHg on the first day. Change in SBP from baseline clearly showed that the $\alpha 1^{S/S} \alpha 2^{R/R}$ mice displayed more rapid and a greater change in blood pressure than WT $\alpha 1^{R/R} \alpha 2^{S/S}$ animals (Fig. 4B). These results demonstrate that ACTH-induced hypertension can be mediated by the ouabain-sensitive $\alpha 1$ isoform. As shown in Fig. 5, administration of ACTH increased circulating levels of endogenous cardiac glycoside-like compounds in mice expressing the high affinity $\alpha 1$ isoform and neutralization with Digibind abolished development of hypertension after ACTH administration.

Discussion

The present study demonstrates that the highly conserved cardiac glycoside binding site of the Na,K-ATPase is a key

Fig. 4. Mice with an ouabain-sensitive $\alpha 1$ isoform and an ouabain-resistant $\alpha 2$ isoform develop ACTH-induced hypertension. (A) SBP in WT $\alpha 1^{R/R} \alpha 2^{S/S}$ and $\alpha 1^{S/S} \alpha 2^{R/R}$ mice during prolonged treatment with saline ($n = 12$ for each genotype) and ACTH ($n = 30$ for each genotype). (B) Change in SBP from baseline during saline and ACTH treatment. Values are represented as averages $\pm$ SE. Control days, -1 and 0 . #, $P < 0.05$ vs. $\alpha 1^{R/R} \alpha 2^{S/S}$ ACTH.

regulator of blood pressure. Specifically, it mediates the development and maintenance of ACTH-induced hypertension. Interestingly, both the $\alpha 1$ and $\alpha 2$ isoforms of the Na,K-ATPase can regulate blood pressure via their ouabain-sensitive cardiac glycoside binding site. Although previously it was suggested that the $\alpha 1$ and $\alpha 2$ isoforms may play a differential role (54), the present study is in agreement with previous analysis demonstrating that these two isoforms play a similar role in cardiac contractility (31). The finding that the high affinity of the $\alpha 1$ isoform cardiac glycoside binding site can mediate ACTH-induced hypertension is particularly important because this isoform is sensitive to cardiac glycosides in humans. Thus, the $\alpha 1$ isoform along with the $\alpha 2$ isoform may regulate blood pressure in humans. Interestingly, other species besides rodents have a cardiac glycoside-resistant $\alpha 1$ isoform. These include some Bufo species (55) and Monarch butterflies (56). This resistance could have developed for a number of reasons. For example, Monarch butterflies concentrate cardiac glycosides to protect themselves against predators and as such need an ouabain-resistant Na,K-ATPase. Also, because rodents may feed on plants, which could contain cardiac glycosides, their ouabain-resistant $\alpha 1$ isoform may protect them from being poisoned by these compounds.

Our finding that the cardiac glycoside binding site of the Na,K-ATPase functions in regulating blood pressure strongly suggests that an endogenously occurring ligand may be interacting with this site. Logical candidates are the endogenous cardiac glycoside-like compounds observed by numerous laboratories (4–9), which we show are elevated after 5 days of ACTH treatment in mice. Several laboratories have identified and observed a correlation between ACTH-induced hypertension and a specific type of endogenous cardiotonic steroid, including ouabain-like, digoxin-like, and marinobufagenin-

Fig. 5. Elevation of endogenous cardiac glycoside-like compounds and their neutralization with Digibind in $\alpha 1^{S/S} \alpha 2^{R/R}$ mice after ACTH treatment. (A) Cardiac-glycoside-like immunoreactivity in the blood serum from saline administered $\alpha 1^{S/S} \alpha 2^{R/R}$ mice ($n = 10$) and ACTH administered $\alpha 1^{S/S} \alpha 2^{R/R}$ mice ($n = 13$) on day 5 of daily treatment. *, $P < 0.05$ vs. saline control. (B) The effect of Digibind ($n = 8$) and sheep IgG γ ($n = 7$) on SBP in ACTH-treated $\alpha 1^{S/S} \alpha 2^{R/R}$ mice. Infusion of Digibind abolished development of hypertension, whereas sheep IgG γ had no effect. Values are averages $\pm$ SE. Control days, -2, -1, and 0. #, $P < 0.01$ vs. $\alpha 1^{R/R} \alpha 2^{S/S}$ ACTH plus Digibind.

like compounds (35, 40–43). This is in agreement with the previous postulation that ACTH-induced hypertension is mediated by the unique adrenocortical steroid (57, 58). Although our studies demonstrated an *in vivo* function of the cardiac-glycoside binding site of the Na,K-ATPase and strongly suggested that an endogenous ligand for this site must exist, information on the exact nature of the ligand is unknown. The abolition of ACTH-induced hypertension by Digibind indicates that endogenous cardiotonic steroids are potential ligands. However, because Digibind interacts with most subtypes of cardiotonic steroids (although with different affinities) (33), it is unknown whether a specific cardiotonic steroid is an essential natural regulator of Na,K-ATPase *in vivo*.

Our findings are of importance from the aspect of ectopic ACTH syndrome (45). Hypertensive patients with this syndrome have elevated endogenous cardiac glycosides, specifically ouabain-like compounds. Our data clearly implicates the interaction of the Na,K-ATPase and its natural ligand, via the conserved cardiac glycoside binding site, in the pathobiology of ectopic ACTH syndrome. Elevated levels of ACTH and sustained hypertension are also symptomatic of Cushing syndrome (59). Further analysis of this syndrome could also implicate the Na,K-ATPase in the epidemiology of this disease. Previous studies have established a very close correlation between hypertension and elevation of endogenous cardiac glycosides (4–17), which was postulated even before the discovery of endogenous cardiac glycoside-like compounds (60–63). High levels of these ligands also occur in humans with mineralocorticoid-induced hypertension (38, 45, 49–52), glucocorticoid-induced hypertension (45, 35, 40–44), and in a large number of patients with essential hypertension (10–13).

However, whether interaction of these compounds with Na,K-ATPase plays a role in development of these forms of hypertension needs to be determined.

The finding that only nanomolar levels of endogenous cardiac glycosides are found in serum has raised the question of whether these concentrations are sufficient to inhibit significant amounts of the Na,K-ATPase. Our previous analysis (32) demonstrated that in mice, administration of exogenous ouabain resulted in nanomolar concentrations of this compound in blood serum and caused hypertension. The development and maintenance of this hypertension, i.e., the ouabain-induced hypertension, was also mediated by the cardiac glycoside binding site of the $\alpha 2$ Na,K-ATPase isoform (32). This is compatible with the present study and together support the physiological role of the cardiac glycoside binding site of the Na,K-ATPase in the regulation of blood pressure. Nevertheless, it is still important to determine whether this physiological role is mediated through alteration (inhibition or activation) of Na,K-ATPase activity or is the result of a signaling cascade mediated by this enzyme.

The dependence of ACTH-induced hypertension on the reverse mode of the Na/Ca-exchanger suggests that inhibition of Na,K-ATPase activity is involved. This is in agreement with previous studies that have demonstrated the importance of the Na/Ca-exchanger in salt-dependent hypertension, which is also accompanied by increased levels of endogenous cardiac-glycoside-like compounds (62). Inhibition of the Na,K-ATPase raises intracellular Na^+ , which reverses the mode of the transport by the Na/Ca-exchanger. The influx of Ca^{2+} through the reverse Na/Ca-exchanger increases intracellular Ca^{2+} concentrations, which results in increased contractility. Because, the Na,K-ATPase mediates influx of Ca^{2+} through the Na/Ca-exchanger is a major mechanism for the positive inotropic effect in heart and the vascular system, our studies suggest that enhanced vascular resistance and cardiac output may play a role in the development of ACTH-induced hypertension (30, 32). This is in agreement with previous analysis showing that the increase in the vascular resistance plays a major role in the development of the ACTH-induced hypertension in rat (63). Although the low levels of endogenous ligand that are detected in circulation may inhibit only a small number of Na,K-ATPase molecules, this could still result in relatively localized increase in intracellular Ca^{2+} concentrations. The $\alpha 2$ isoform accounts for only 15 and 5% of total Na,K-ATPase in vasculature and heart tissues, respectively, and it is known that a 50% reduction in these amounts enhances cardiac and vasculature contraction (31, 32, 54). It is important to note, that the involvement of the reverse mode of the Na/Ca-exchanger in ACTH-induced hypertension is also compatible with the Na,K-ATPase mediating a signaling cascade. It is known that low concentrations of ouabain can induce signaling in a Ca^{2+} -dependent manner *in vitro* (25).

In summary, the present data provide conclusive evidence that the cardiac glycoside binding site, which mediates the pharmacological effects of digitalis and related drugs used in the treatment of congestive heart failure, is also the receptor for endogenous ligands involved in the regulation of cardiovascular function *in vivo*. Such studies support the hypothesis that a steroid hormone may exist that regulates blood pressure through the interaction with the Na,K-ATPase.

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