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Inhibition of Na/K ATPase From Rat Aorta by Two Na/K Pump Inhibitors, Ouabain and Marinobufagenin

Evidence of Interaction With Different α-Subunit Isoforms

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Recently, we have shown that mammalian plasma cross-reacts with an antibody to a bufodienolide Na/K ATPase inhibitor, marinobufagenin. In rat aorta, marinobufagenin induced vasoconstriction via inhibition of the vascular smooth muscle Na/K pump, whereas ouabain had its predominant effect on pumps localized to nerve endings.

Na/K ATPase inhibitory effects of ouabain and marinobufagenin were studied in two membrane fractions isolated from Fisher 344×BN rat thoracic aorta by sucrose density gradient centrifugation. One fraction contained predominantly the α-3 isoform of Na/K ATPase and represented membranes from the perivascular nerve endings (neuronal plasmalemma). The other membrane fraction, containing predominantly the α-1 isoform, was derived from the vascular smooth muscle sarcolemma.

The IC_{50} for inhibition of the Na/K ATPase by ouabain and marinobufagenin were 2.6 nmol/L and 0.14 μmol/L in the neuronal plasmalemma, and 50 nmol/L and 2.1 nmol/L in sarcolemma, respectively.

These results confirm the view that differential responsiveness to endogenous digitalis-like factors is a functional feature of α isoforms of Na/K ATPase. Am J Hypertens 1997;10:929–935 © 1997 American Journal of Hypertension, Ltd.

KEY WORDS: **Na/K ATPase, isoforms, inhibitors, vascular smooth muscle, ouabain, bufodienolides, marinobufagenin.**

Na/K ATPase is an integral membrane enzyme that is present in all eukaryotic cells and that couples the electrogenic countertransport of Na and K to the hydrolysis of ATP.[1] Na/K ATPase consists of two subunits (α and β) that are represented in equimolar amounts.[2] The α-subunit of Na/K ATPase is a polypeptide of $M_r \approx 100{,}000$ containing the catalytic site for ATP. The α-subunit is a specific receptor site for digitalis glycoside-like inhibitors[2] and is represented by three isoforms (α-1, α-2, and α-3). The function of the β-subunit ($M_r \approx 55{,}000$, β-1 and β-2 isoforms) is not yet fully understood.[2] Various isoforms of α- and β-subunits are distributed in mammalian tissues in a heterogenous and tissue-specific fashion.[3,4]

Mammalian plasma contains several substances with the ability to inhibit Na/K pump and to interact with various antibodies to digitalis.[5,6] Endogenous ouabain was the first endogenous digitalis-like factor (EDLF) to be purified from human plasma.[7] However, mammalian plasma was found to contain several sub-

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stances that inhibit the sodium pump and interact with digitalis antibodies.[8,9] More recent evidence suggests that at least one of the mammalian EDLF has a bufodienolide structure.[10–12] Bufodienolides are cardioactive steroids that differ from cardenolides in having a doubly unsaturated six-membered lactone ring. Bufodienolides have been previously found in various tissues of amphibia, especially in Bufonidae toads.[13] Recently, we have shown that human plasma and urine contain material that cross-reacts with antibodies against one of the amphibian bufodienolides, marinobufagenin (3β5β-dihydroxy-14,15-epoxy bufodienolide).[14,15] HPLC fractionation of chloroform extracted human urine demonstrated the presence of a compound showing maximal UV absorbance at 300 nm (which is typical to bufodienolides) and an elution profile similar to that of marinobufagenin.[14,15] Mass spectral analysis of urine-derived material showed that the purified compound was indistinguishable from toad marinobufagenin.[11] In anesthetized dogs, plasma marinobufagenin-like immunoreactive substance was responsive to acute plasma volume expansion.[16]

When vasoconstrictor properties of marinobufagenin and ouabain were compared in isolated rat aorta, two endogenous inhibitors elicited vasoconstriction via two different mechanisms.[17] Vasoconstrictor response to ouabain was blocked by phentolamine, and was associated with an apparent stimulation of the Na/K pump in aortic rings. The Na/K pump stimulating effect of ouabain was also blocked by phentolamine. At the same time, the vasoconstrictor effect of marinobufagenin was stronger than that of ouabain and was associated with a concentration-dependent inhibition of Na/K pump in isolated aortas. Neither the vasoconstrictor nor the Na/K pump inhibitory effects of marinobufagenin were sensitive to adrenoceptor blockade with phentolamine.[17]

In vivo experiments also demonstrate differential responses to cardenolides and bufodienolides. Thus, Pamnani et al have shown that bufalin produced greater pressor response and positive inotropic and natriuretic effects in rats as compared to ouabain.[18] More recently, from analyzing nonhomogenous distribution of the sodium pumps in various tissues and their interactions with Na/K ATPase inhibitors, Beauge suggested that the main functional feature of the α-isoforms of Na/K ATPase is their differential response to cardioactive steroid inhibition.[19]

Given that in our previous experiments in rat aortic rings, effects of two Na/K ATPase inhibitors (the cardenolide ouabain and the bufodienolide marinobufagenin) appeared to be due to the inhibition of Na/K ATPase in intravascular nerve endings and vascular smooth muscle, respectively, we decided to investigate whether the effects of two Na/K ATPase inhibitors in rat aorta are due to interaction with different isoforms of the sodium pump.

METHODS

Preparation of Membrane Fractions Twenty-four Fisher 344xBN (Charles River Labs, Wilmington, MA) male rats (250 $\pm$ 35 g) were killed with an overdose of sodium pentobarbital (64.8 mg/kg intraperitoneally).

Aortas were removed from the surrounding tissues, and sarcolemmal membrane fraction was purified as described by Allen et al,[20] with minor modifications: 2 to 3 cm segments of aortas were excised from the surrounding tissues at t = 4°C in physiological salt solution (in mmol/L: NaCl, 130; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 1; glucose, 5.4; KH_2PO_4, 1.1, $NaHCO_3$, 24; pH = 7.4; t = 4°C), repeatedly washed by the solution of the same composition, and cut into 1 to 2 mm rings.

A substantial portion of Na/K ATPase in cardiovascular tissues is associated with adrenergic neural endings.[21] To denervate the vascular segments, aortic segments from each rat were divided into two equal parts; one of them was treated with 6-hydroxydopamine (6′OHDA), as reported by Aprigliano and Hermsmeyer.[22] 6′OHDA (300 μg/mL) was applied to the vascular rings twice for 10 min with a 30-min interval. Because 6′OHDA treatment is associated with a temporary exposure of the tissue to a low pH (4.9),[22] the remaining parts of the aortas were put through the same procedure, except that no 6′OHDA was added to the medium.

Next, the aortic rings were placed into flasks containing 250 mmol/L sucrose and 5 mmol/L histidine (t = 4°C; pH = 7.4) minced by scissors and processed with Polytron 20S homogenizer (Kinematica, Basel, Switzerland). The tissue was further homogenized in a glass homogenizer (Glas-Col, Terre Haute, IN) with a tight-fitting Teflon pestle. The homogenized tissue was centrifuged (6,000 *g* for 15 min, t = 4°C) in a Sorvall RC-5B centrifuge (Du Pont Instruments, Wilmington, DE). Then, the pellet was once again homogenized in a glass–Teflon homogenizer and added to the supernatant. The combined supernatant was respun at 20,000 *g* for 30 min at 4°C, using the same centrifuge. The resultant supernatant was centrifuged in a Beckman (Fullerton, CA) L8-N centrifuge (148,000 *g*, 90 min, 4°C) and the resultant pellet was suspended in a homogenizing medium. The mixture was applied to discontinous sucrose gradients consisting of 0.32, 0.8, 1.0, 1.2, and 1.4 mol layers buffered with 5 mmol/L histidine (pH = 7.4) and centrifuged at 148,000 *g* for 90 min (Beckman L8-N SW28, t = 4°C). Two bands appeared at the 0.8 mol (fraction of sarcolemma) and 1.2 mol (neural endings plasmalemma) interface and were aspirated with a Pasteur pipette. Two membrane fractions were sedimented by the centrifugation at 148,000 *g* for 90 min, the pellets were

resuspended in 1 mL of histidine-sucrose buffer and stored in liquid nitrogen for 5 to 10 days.

Na/K ATPase Activity Activity of Na/K ATPase in membrane fractions was measured using an enzyme-linked assay as previously reported,[20] except addition of alamethicin as an ionophore (0.5 mg/1 mg sarcolemmal protein) and NaN_3 (5 mmol/L, to block mitochondrial ATPase) to the incubation medium, as recommended by Dixon et al,[23] for cardiac sarcolemmal preparation. ATP hydrolysis was assessed spectrophotometrically by measuring NADH oxidation at 340 nm using the linked enzyme system, pyruvate kinase (PK)-lactate dehydrogenase (LDH). The system contained 3 mmol/L $MgCl_2$ 0.2 mmol/L ATP, 100 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L phosphoenolpyruvate, and suspension of PK (2 units/mL) and LDH (95.5 units/mL). Na/K ATPase was determined as the difference between oxidation of NADH in the presence and absence of 0.1 mmol/L ouabain and expressed as micromoles of ADP/milligram of protein/hour. Protein was maintained between 10 and 20 μg/cuvette in a volume of 1.0 mL.

Western Blotting Solubilized membrane protein from both fractions and rat kidney, brain, and heart as a control (10 to 50 μg of protein/lane) were separated by 8% Tris-Glycine polyacrylamide gel electrophoresis in sodium dodecyl sulfate buffer (Novex, Novel Experimental Technology, San Diego, CA)[24] and the protein transferred to a nitrocellulose membrane (0.45 mm pore size, Novex) essentially as described previously.[25] The blots were blocked in 5% dried nonfat milk (wt/vol) and 0.1% Tween-20 in Tris-buffered saline (TBS) overnight at 4°C, and incubated for 1 h with isoform specific anti-α-1 and anti-β-2 (Upstate Biotechnologies, Lake Placid, NY) and anti-α-3 (gift of Dr. Thomas A. Pressley, Texas Tech University, Lubbock, TX) antibodies raised in rabbits against fusion proteins, at a dilution of 1:1,000, and antisynaptophysin monoclonal mouse antibody (Sigma Chemicals, St. Louis, MO, 1:200). The specificities of antibodies against isoforms of Na/K ATPase in Western blotting analysis have previously been demonstrated in detail.[26] After washing (TBS, 0.1% Tween 20), the blots were incubated with corresponding peroxidase-conjugated affinity-purified goat anti-rabbit IgG (Amersham Corp., Amersham, Buckinghamshire, England; ECL kit) or anti-mouse antiserum (Sigma Chemicals) at 1:1,000 for 1 h. Immunoreactivity was detected by enhanced chemiluminescence (ECL) (Amersham Corp.). The reacted blots were exposed to Hyperfilm-ECL for times ranging from 1 to 10 min.

Miscellaneous As reported previously, marinobufagenin was purified from the venom of *Bufo marinus* toad in the Laboratory of Pharmacology, Sechenov Institute of Evolutionary Physiology and Biochemistry, St. Petersburg, Russia.[17] Chemicals were obtained from Sigma Chemicals (St. Louis, MO) unless otherwise indicated. Statistical analyses (Student *t* test) and analyses of concentration-response curves (ANOVA, Bonferroni test, and nonlinear regression analysis) were performed using GraphPad Instat and GraphPad Prism (San Diego, CA).

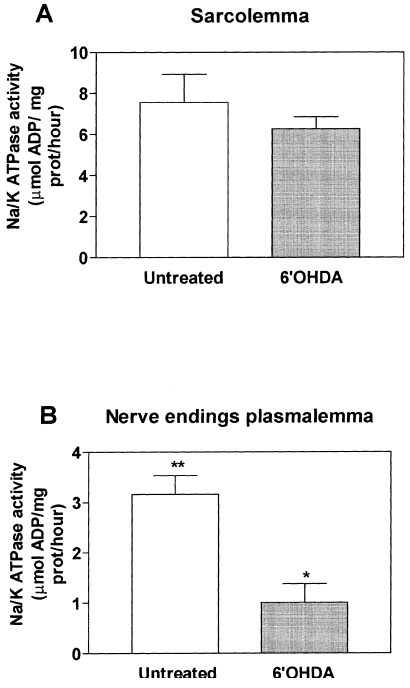

FIGURE 1. *Activity of Na/K ATPase in membrane fractions from rat thoracic aorta.* **(A)** *Sarcolemma.* **(B)** *Neuronal plasmalemma. Open bars, membranes from untreated tissue; filled bars, membranes from aortas pretreated with 6'OHDA. Means ± SEM from six to nine experiments.* *P < .01 v *activity of Na/K ATPase in the neuronal plasmalemma from untreated aortas;* **P < .02 v *activity of Na/K ATPase in sarcolemma from untreated aortas. Unpaired two-tailed* t *test.*

RESULTS

Na,K-ATPase Inhibition in Membrane Fractions As presented in Figure 1, the activity of Na,K-ATPase in the fractions of sarcolemma and neuronal plasmalemma was 7.55 ± 1.41 and 3.16 ± 0.37 μmol ADP/mg protein/h, respectively. The activity of Na/K ATPase in sarcolemma and in neuronal plasmalemma comprised 50% and 33% of the total ATPase activity, respectively. As also shown in Figure 1, pretreatment of the aortas with 6′OHDA resulted in a threefold decrease in the activity of Na/K ATPase in the neuronal plasmalemma (1.01 ± 0.35 μmol ADP/mg protein/h), whereas 6′OHDA treatment did not affect activity of Na/K ATPase in sarcolemma (6.2 ± 0.61 μmol ADP/mg protein/h).

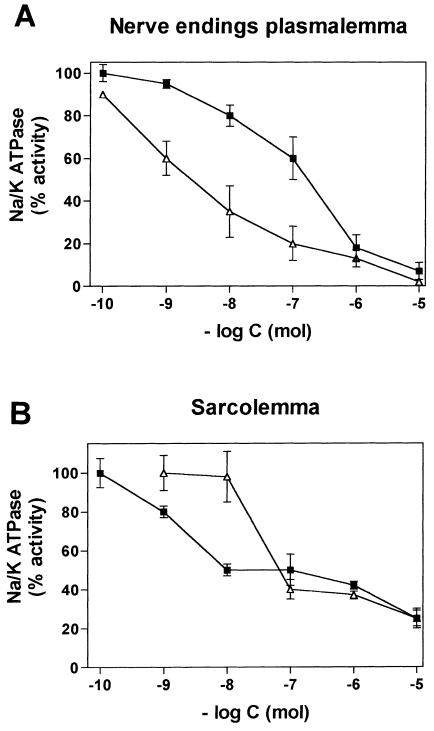

FIGURE 2. *Na/K ATPase inhibitory effects of ouabain (filled squares) and marinobufagenin (open triangles) in the membrane fractions from rat thoracic aorta.* **(A)** *Neuronal plasmalemma.* **(B)** *Sarcolemma. Means ± SEM from four to six experiments.*

Figure 2 shows concentration–response curves for the inhibition of Na/K ATPase by ouabain and marinobufagenin in both membrane fractions. The IC_{50} to marinobufagenin and ouabain in the fraction of sarcolemma were 2.1 and 50 nmol/L, respectively. In the neuronal plasmalemma fraction, ouabain and marinobufagenin caused a 50% inhibition of Na,K-ATPase activity at concentrations of 2.6 and 140 nmol/L, respectively.

Western Blotting Analysis Figure 3 shows that synaptophysin-like immunoreactive material was present in neuronal plasmalemma but not in sarcolemma. As presented in Figure 4, an α-1 isoform of Na/K ATPase was detected in sarcolemma but not in neuronal plasmalemma. At the same time, α-3 isoform immunoreactivity was present in neuronal plasmalemma but was undetectable in the fraction of sarcolemma (Figure 4). As shown in Figure 5, the β-2 isoform of Na/K ATPase demonstrated the same distribution between the two fractions as did the α-3 isoform.

DISCUSSION

The major observation of this study is that two Na/K ATPase inhibitors, marinobufagenin and ouabain, display greater affinity to Na/K ATPase localized to sarcolemma and plasmalemma of the neural endings in rat aorta, respectively. Western blot analysis demonstrated that aortic sarcolemma contains predominantly the α-1 isoform of Na/K ATPase, whereas α-3 and β-2 isoforms predominate in the neural plasmalemma.

The evidence that the membrane fraction containing the α-3 isoform represented plasmalemma of the neural endings was confirmed by the following observations. First, pretreatment of aortic rings with 6′OHDA, which causes destruction of neural endings in the tissue, resulted in a dramatic decrease in the activity of Na/K ATPase in this fraction, while not affecting the activity in sarcolemma. Second, the fraction of neuronal plasmalemma, but not the sarcolemma, contained synaptophysin immunoreactivity, which is specific to the nervous tissue.[27] Third, Western blotting analysis of neural plasmalemma fraction revealed the presence of α-3 and β-2 isoforms of Na/K ATPase. The combination of α-3 and β-2 isoforms of the Na/K ATPase subunit comprises a functional dimer that is typical for axolemma.[28]

Adams et al investigated the vasoconstrictor effects of ouabain in dog mesentery artery, and suggested the existence of two mechanisms: direct, associated with inhibition of Na/K ATPase in vascular smooth muscle cells; and indirect, associated with the effect of digitalis on Na/K ATPase from adrenergic endings in the vascular wall.[21] Analyzing the vasoconstrictor effects of digitalis, Adams and coworkers measured the activity of Na/K ATPase in various membrane fractions from dog mesentery artery. In that study, the fraction of sarcolemma was obtained at 0.6 to 1.0 mol of sucrose (0.8 mol of sucrose in our experiment). In our study, the fraction of neuronal plasmalemma was obtained at 1.2 mol of sucrose. The activity of Na/K ATPase in this fraction was less than in sarcolemma. This observation is in agreement with the data of Adams et al, which also showed that activity of Na/K ATPase in the fractions obtained at 1.0 to 1.5 mol of sucrose, was lower than in the sarcolemma.

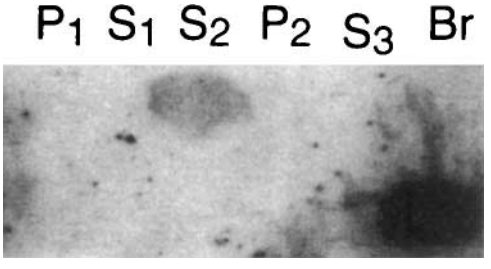

FIGURE 3. *Western blot analysis of synaptophysin-like immunoreactivity (38 kDa band) in membrane fractions from rat thoracic aorta. P_1 and P_2, fraction of sarcolemma (10 and 50 μg/lane, respectively). S_1, S_2, and S_3, fraction of neuronal plasmalemma (10, 20, and 50 μg per lane, respectively). Br, rat brain Na/K ATPase standard (50 μg/lane; Upstate Biotechnology).*

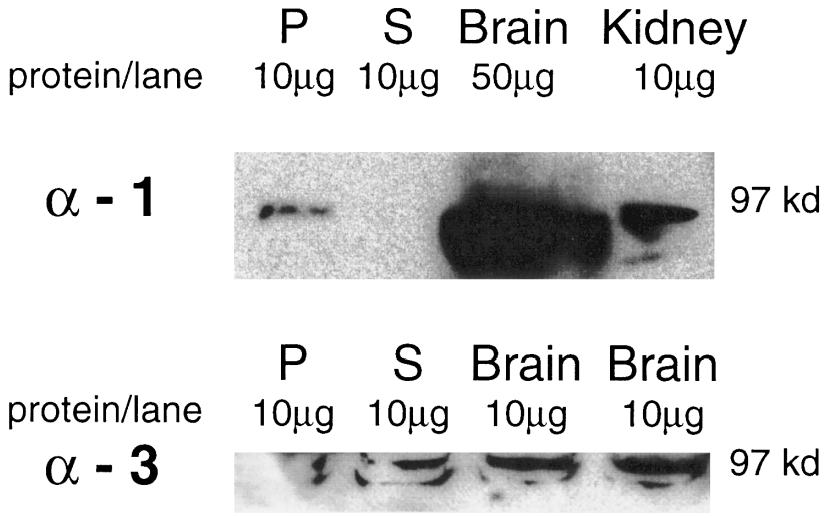

FIGURE 4. *Western blot analysis of Na/K ATPase α-1- and α-3–subunit expression in membrane fractions from rat thoracic aorta. P, fraction of sarcolemma; S, fraction of neuronal plasmalemma. Brain and kidney, standards of Na/K ATPase from rat brain and kidney (Upstate Biotechnology).*

The fact that ouabain displayed a high capacity to inhibit Na/K ATPase from the fraction of neuronal plasmalemma (IC_{50} = 2.6 nmol/L) is in accord with previous reports of high sensitivity of the α-3 subunit to ouabain.[29] Previously, Na/K ATPase from Purkinje fibers was shown to be more sensitive to inhibition by ouabain, as compared with cardiomyocytes.[30] Later, Zahler et al showed that α-3 subunit of Na/K ATPase in rat hearts is associated with the sites of cardiac and neuromuscular impulse transmission.[31] Our observations that Na/K ATPase from rat aortic neuronal plasmalemma is highly sensitive to ouabain and contains α-3 isoform is consistent with the results that Zahler et al obtained in the rat heart.[31]

In contrast, Na/K ATPase from aortic sarcolemma was more sensitive to inhibition by marinobufagenin. These observations are consistent with our earlier findings of the vasoconstrictor effects of ouabain and marinobufagenin in rat aorta.[17] In rat aorta, ouabain-induced contractions were blocked by phentolamine and, therefore, must have been due to the action of ouabain on intravascular neural endings and to the release of norepinephrine. Norepinephrine, in turn, is known to stimulate the activity of vascular sodium pump.[32] Indeed, in our previous study, effects of vasoconstrictor concentrations of ouabain in rat aortas were associated with an apparent stimulation of the sodium pump. This stimulating effect was also antagonized by phentolamine.[17]

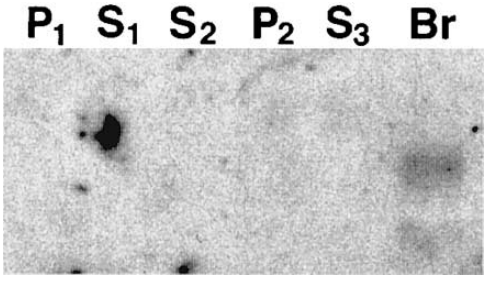

FIGURE 5. *Western blot analysis of Na/K ATPase β-2–subunit expression in membrane fractions from rat thoracic aorta. P_1 and P_2, fraction of sarcolemma. S_1, S_2, and S_3, fraction of neuronal plasmalemma. Br, standard of Na/K ATPase from rat brain (Upstate Biotechnology).*

Previously, stimulation of Na/K pump activity due to release of norepinephrine was described in guinea pig myocardium to explain the mechanism of the positive inotropic effect of low concentrations of ouabain.[33,34] The positive inotropic action of the low (nanomolar) concentrations of digitalis was attributed to this neuromodulatory effect whereas, at higher concentrations, the positive inotropic action of digitalis was due to the inhibition of the sodium pump in cardiomyocytes.[34] Our previous experiments showed that similar mechanisms of action of Na/K ATPase inhibitors exist in rat aorta and may be mediated by two different sodium pump inhibitors, ouabain and marinobufagenin.[17]

Differential responsiveness of two membrane fractions, containing α-1 and α-3 isoforms of the sodium pump, to marinobufagenin and ouabain seems to be in agreement with the distribution of the levels of ouabain-like and marinobufagenin-like immunoreactivities between plasma and cerebrospinal fluid. Thus, in the Fisher 344xBN rat, levels of marinobufagenin-like and ouabain-like immunoreactivity in extracted plasma were 0.2 to 0.5 nmol/L and 0.1 nmol/L,

respectively.[35] In rat plasma, therefore, marinobufagenin-like immunoreactive material is present in concentrations that could affect the activity of Na/K ATPase in vascular smooth muscle (α-1 isoform). In the rat, α-3 and α-2 isoforms of Na/K ATPase are specific to the excitable tissues, including brain. In our recent experiments, marinobufagenin-like immunoreactivity in the cerebrospinal fluid of male Wistar rats was almost undetectable, whereas concentration of ouabain-like immunoreactivity comprised 0.602 ± 0.211 nmol/L (Bagrov et al, in preparation).

Our previous experiments also indirectly suggest that marinobufagenin interacts with α-1 subunit of the sodium pump in the kidneys. In acute plasma volume expanded dogs, an increase in urinary release of marinobufagenin-like immunoreactive material occured in parallel with the maximal natriuretic response.[16] Pretreatment of the animals with digoxin antibody reduced the natriuretic response and decreased the urinary output of marinobufagenin-like, but not the ouabain-like, material. Previously, it has been shown that α-1 is a major isoform of Na/K ATPase in the kidneys of various species, including dogs.[36]

In conclusion, our present results suggest that vasoconstrictor effects of physiologically realistic (nanomolar) concentrations of two endogenous Na/K pump inhibitors, ouabain and marinobufagenin, are mediated via interactions with different isoforms of α-subunit of Na/K ATPase. The relevance of these observations to pathogenesis of hypertension remains to be clarified.

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