MINIREVIEW

Endogenous Sodium Pump Inhibitors and Blood Pressure Regulation: An Update on Recent Progress (44283)

PETER A. DORIS*,[1] AND ALEXEI Y. BAGROV†

Institute of Molecular Medicine, University of Texas Health Science Center, Houston, Texas 77025; and Sechenov Institute of Evolutionary Physiology and Biochemistry,† St. Petersburg, Russia and Laboratory of Cardiovascular Science, National Institute on Aging, Baltimore, Maryland 21224*

Abstract. **Rapid progress has occurred recently in understanding the origin, chemistry, synthesis, control, and actions of endogenous materials that may be ligands for the cardiac glycoside binding site on the mammalian sodium pump (Na,K-ATPase). The present paper reviews this progress and examines in detail the evidence supporting ouabain-like and bufodienolide-like compounds as functioning in endogenous control of sodium pump activity, renal sodium excretion, blood pressure, and cardiovascular contractility. Other novel compounds identified in this search as potentially influencing natriuresis and blood pressure are also discussed.**

[P.S.E.B.M. 1998, Vol 218]

Essential hypertension is associated with altered renal function (1). Whether this change is a consequence of hypertension or whether it precedes hypertension and is necessary for its development has been a question that has been slow to elicit a satisfactory answer. Recent efforts at unraveling the genetic components of hypertension have offered new insights from which an answer is beginning to form. This paper will review the role of altered membrane ion handling in hypertension and update information on the role of endogenous sodium pump inhibitors in the pathogenesis of high blood pressure. We will also discuss and analyze recent progress in chemical characterization of such inhibitors.

This is publication #131-IMM from the Institute of Molecular Medicine for the Prevention of Human Diseases, University of Texas—Houston.

[1] To whom requests for reprints should be addressed at Institute of Molecular Medicine, University of Texas, 2121 W. Holcombe Blvd, Houston, TX 77025. E-mail: pdoris@imm2.imm.uth.tmc.edu

The authors are grateful for support from NIH DK45538 (PAD) and the National Institute on Aging, Baltimore, MD (AYB).

0037-9727/98/2183-0156$10.50/0

"Hypertension Genes" Alter Renal Sodium Absorption

In several simple, single gene forms of essential hypertension, the mechanism of hypertension has been uncovered; however, less progress has been made in the more difficult problem of identifying the genetic variations that contribute to the common polygenic form of this heterogeneous human disease. In both areas, evidence demonstrates the primacy of changes in the renal reabsorption of salt and water as the causal mechanism of hypertension. Three simple monogenetic forms of essential hypertension have been resolved at the genetic level. Each is rare, generally severe, and has an early onset, characteristics that differ from more commonly encountered polygenic forms of essential hypertension. These are: Liddle's syndrome (defects in renal sodium channels participating in sodium reabsorption) (2); syndrome of apparent mineralocorticoid excess (defect in renal 11-beta hydroxysteroid dehydrogenase that permits mineralocorticoid effects of glucocorticoids to be manifest resulting in inappropriate sodium retention) (3, 4); and glucocorticoid-remediable aldosteronism (chimerism of glucocorticoid and mineralocorticoid synthesis genes places

mineralocorticoid synthesis under glucocorticoid regulation, again resulting in inappropriate mineralocorticoid production) (5). In each case, the primary genetic defect results in increased renal sodium reabsorption.

The Milan hypertensive rat is a multigenic, complex model of human essential hypertension (6). The genetic components involved in this model may resemble more closely those that participate in at least some human hypertension. One of the genetic variations in the Milan hypertensive rat contributing to hypertension is mutation in the adducin gene (7). Adducin is a member of a family of cytoskeletal proteins. However, the mechanism by which adducin mutation contributes to hypertension still involves a primary alteration in renal sodium handling. It is accompanied by altered plasma levels of ouabain-like material (8–11). In this case, the structural protein is involved in stabilization in the cell membrane of at least one major ion transport protein, the sodium, potassium-ATPase of the sodium pump. Mutation appears to increase the V_{max} of the sodium pump perhaps by increasing the longevity of the sodium pumps resident in the cell membrane (12). This is associated with increased sodium reabsorption in the nephron and presumably with inappropriate sodium retention that is not overcome by other compensatory mechanisms (9, 13–17). Variations in adducin sequence within the human genome have now been identified, and they correspond with increased occurrence of essential hypertension (7, 18, 19).

Taken together, this new evidence of the genetic causation of hypertension has strengthened the compelling argument of Guyton and colleagues that the sole means of sustaining long-term alterations in arterial blood pressure is by a change in the normal relationship between renal perfusion pressure and sodium reabsorption (20–22). However, these genetic advances also amplify the important question at the center of this review: when renal sodium reabsorption is inappropriately increased, what mechanism leads to elevated blood pressure?

Sodium Pump Inhibitors Link Altered Sodium Reabsorption to Blood Pressure

In 1961 the important role of glomerular filtration rate in regulation of sodium balance through altered proximal sodium delivery was well understood, and the chemical identity of aldosterone and its effect on distal renal sodium absorption had come into full focus. However, the idea that other mechanisms regulating renal sodium excretion might remain to be identified persisted and found support in experiments conducted by de Wardener and colleagues suggesting a circulating natriuretic factor, widely known as "third factor" (23). At the end of the same decade and around the same period, two independent reports emerged supporting the view that the third factor might be a sodium pump inhibitor (24, 25). This view was propelled by observations of the inhibitory effect of volume expansion on epithelial ion transport (26).

Haddy and Overbeck then reported a series of experiments suggesting that a sodium pump inhibitor might participate in the pathophysiology of volume-expanded hypertension. They showed lower sodium pump activity and reduced ouabain-sensitive ATPase activity in blood vessels and cardiac tissue of volume-dependent hypertensive dogs and rats (27–30). In normal blood vessels, reduced vascular sodium pump activity could be produced by plasma from volume-expanded, hypertensive animals (31). Since similar vascular responses could be obtained with the plant-derived cardiac glycoside, ouabain, the activity was termed "ouabain-like." During this period, several clinical immunoassays used to monitor plasma levels of digitalis drugs had been developed, and most were discovered to detect materials present in individuals who had never been treated with these drugs (32, 33). One set of subjects with raised plasma digitalis immunoreactivity were volume-expanded dogs, a finding from which the term "digitalis-like" emerged to describe a possible material involved in sodium pump regulation and possessing immunological properties similar to plant cardiac glycosides (34). From this point until present a very broad range of studies emerging from many laboratories has continued to produce evidence supporting the idea that volume-expanded states and volume-expanded hypertension are associated with increased levels of circulating material that, by one assay or another, appear to resemble sodium pump inhibitors (35, 36).

Blaustein proposed a mechanism to explain the hypertensive response to endogenous sodium pump inhibitors through alteration in intracellular ionized calcium handling as a result of increased intracellular sodium following sodium pump inhibition (37). He has calculated that a small increment (8%) in intracellular sodium concentration may lead to a 1 n*M* increase in ionized intracellular calcium. This calcium increase may, in turn, be amplified 2500-fold in the resulting increase in sequestered intracellular calcium. The available pool of releasable calcium from the sarcoplasmic reticulum would consequently be augmented with important implications for contractile responses in myocardium and vascular smooth muscle (38). Experiments in isolated vascular preparations from rats have shown that very low ouabain concentrations (10^{-9} to 10^{-10} *M*) can increase caffeine-induced contractions in the presence of moderate sympathetic stimulation (38). If endogenous sodium pump inhibitors share this same property, they may, even at these very low concentrations, participate in natriuretic responses by inhibition of renal sodium transport. Natriuresis may be reinforced by increased arterial pressure occurring as the result of calcium shifts induced by the action of the same inhibitor on vascular tissues.

Progress in the Chemical Identification of Endogenous Sodium Pump Inhibitors

"Endogenous Ouabain." Substantial evidence has been developed in support of the idea that ouabain, or a molecule differing only in isomerism from ouabain, is present in human plasma (hence, "endogenous ouabain") (39,

40). Problems with ouabain as an endogenous sodium pump inhibitor were an important topic in the previous review of this subject, many remain unresolved (35).

Chemical Characterization of "Endogenous Ouabain." Recent independent verification supports the previous observation that a compound with identical mass and a mass fragmentation spectrum extremely similar, but not identical, to plant ouabain is present in mammals. Microscale analysis of material purified from bovine hypothalamus (HIF) has also resulted in the identification of a compound that is isobaric with ouabain (41, 42). A compound was released by acid digestion of the glycoside linkage of the purified HIF which, after derivatization and GC/MS analysis, was indistinguishable from L-rhamnose, which had been similarly treated. It was concluded that the sugar in HIF is L-rhamnose (42).

Naphthoylation is a derivatization technique that gives rise to fluorescent products. Derivatization with fluorescent compounds produces two important advantages; first, fluorescence detection is extremely sensitive, and second, such compounds may exhibit circular dichroism (CD) spectra. Circular dichroism is the difference in absorption between left- and right-handed circularly polarized light and is a useful technique for examining the stereochemical features of molecules. It can be extended by examination of the Cotton effect, the optical rotation of circularly polarized light.

Reactive hydroxyls present both on ouabain and on HIF were naphthoated and analyzed by HPLC, and CD spectra were obtained. HPLC elution times of the naphthoated derivatives of ouabain and HIF were different (42). Furthermore, ouabain showed a split CD curve with a positive Cotton effect. However, HIF demonstrated no Cotton effect. Since three hydroxyl groups on rhamnose are naphthoated, and this structure alone produces a large Cotton effect, this suggests that the remaining naphthoates cancel the positive split CD of the rhamnose derivative (43). This intriguing evidence suggests that HIF is either a regio- or stereoisomer of ouabain. However, it is unclear if the hydroxyl groups on the aglycone, ouabagenin, are in an unusual spatial arrangement to cancel the rotation produced by the rhamnose derivative or if the rhamnose residue is coupled at a different location on the genin that the C3 position of ouabain (See Figure 1 for examples).

At present, the most feasible means to identify the correct stereochemistry may be to synthesize all possible stereo- and regioisomers to identify those with an identical CD spectrum to the endogenous mammalian compound. If only one such isomer has this spectrum, and if its derivative shares identical HPLC elution properties, then the problem will be solved. This is not a trivial undertaking since there is a large number of possible isomers that are isobaric and that retain the same essential structural features as ouabain (steroid nucleus, lactone side group at C17, rhamnose sugar residue, and 5 hydroxyl groups on the steroid nucleus). Finally, it should be noted that it is possible, though unlikely considering the relatively mild conditions, that isomerism occurred during the purification of HIF and is not a property of the native compound.

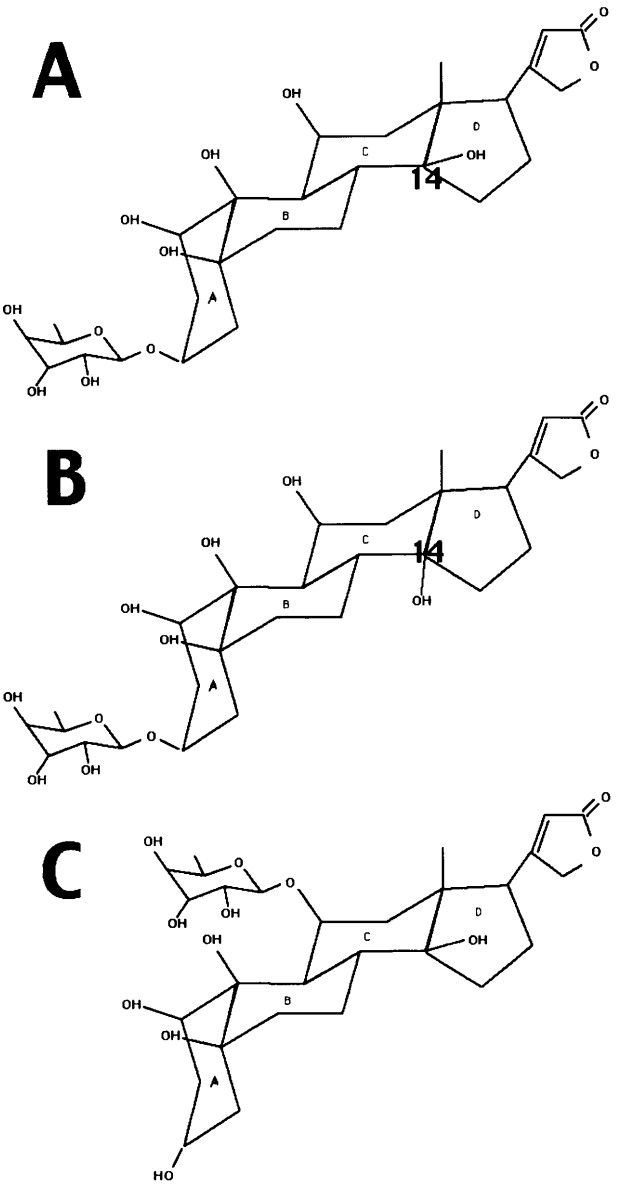

Figure 1. (A) Structure of ouabain showing lactone group at C17, steroid nucleus with A/B and C/D rings in *cis* conformation and rhamnose residue; (B) A stereoisomer of ouabain. Isomerism is in the spatial arrangement of the hydroxyl group at the C14 of the steroid nucleus; (C) A regioisomer of ouabain. Isomerism is in the location of the attachment of the rhamnose residue to the steroid nucleus.

Comparisons of HIF with the human plasma-derived "endogenous ouabain" have now been made. Interestingly, both the human-derived material and HIF form pentanaphthoates that are different from those formed by ouabain (42). Both mammalian-derived pentanaphthoates coelute on HPLC and have a different elution time from ouabain-pentanaphthoate. Thus, two separate groups using two different source materials have now purified what appears to be the same compound. This compound is not only isobaric with ouabain, but also clearly isomeric: "iso-ouabain."

This is important evidence that, as it grows in strength and clarity, may begin to answer some of the challenging problems posed by ouabain isomers as endogenous regulators of mammalian sodium pumps. The next vital piece of evidence required is confirmation that both HIF and human "endogenous ouabain" can be synthesized *in vivo* and that they do not result from environmental sources.

This exciting new progress must be tempered with the important problems that remain before iso-ouabain can be accepted as a biologically important regulator of mammalian sodium pump function. Hansen has questioned whether the secretion rate suggested for ouabain is sufficient to have any effect on the vast number of receptors present, estimating that an output corresponding to 7.2 years of the reported secretory rate would be necessary to occupy all receptors (44). However, until an exact structure of the endogenous material is known, no suitable standard is available to quantify release rates or circulating levels accurately, and their actual molar concentration cannot be determined.

Indeed, these considerations bear on another important issue: the ability of some investigators, but not others, to confirm the presence of ouabain in mammalian samples. It has been suggested that antibody selectivity is critical to the ability to identify iso-ouabain using antibodies raised against plant ouabain (45). This argument presumes a large regioisomeric difference between ouabain and iso-ouabain. The available mass spectral data do not provide much assistance in clarifying this possibility (40, 41). All "endogenous ouabain" antibodies raised to date have been raised against plant ouabain. It is therefore difficult to imagine that either the conjugation chemistry used to generate the immunogen (reductive amination) results in profound differences between the antibodies raised or that polyclonal antibodies raised against plant ouabain could be so divergent in their ability to detect endogenous ouabain.

Problems arising from the chemical properties of iso-ouabain remain. The same problems exist for plant ouabain (see earlier review (35)). They include the polarity of this molecule (a characteristic that isomerism would be unlikely to change) and the unique structure of the plant sugar residue (rhamnose is not known to be involved in any other mammalian carbohydrate metabolism and is not normally absorbed across the gut). Early descriptions of HIF included properties incompatible with the high polarity of ouabain or its isomer, namely, the ability to access the extracellular binding site in inside-out liposomes (46). Ouabain has extremely low permeability through such membranes, and permeability of the isomer would be expected to differ only to a small extent (47–49). This raises questions about whether the earlier descriptions refer to the same compound that was ultimately identified as a ouabain isomer. Further, this earlier material has different properties in its interaction with the sodium pump than ouabain. For example, its ligand binding requirements differ significantly from plant ouabain, and HIF does not support phosphorylation of the sodium pump from inorganic phosphate. Potency is also estimated to be greater than ouabain (50). It is possible that some, but not all, of these differences might be characteristics of the isomer. However, those differences not attributable to isomerism await a satisfactory explanation.

Biosynthesis and Regulation of "Endogenous Ouabain." Further evidence has come to light involving synthesis of "endogenous ouabain" by the adrenal gland. Evidence of release from the adrenal in intact animals has been generated (51, 52). However, in humans, plasma ouabain immunoreactivity was not different after bilateral adrenalectomy (53). Evidence of biosynthesis is essential to support a role for the compound in control of the mammalian sodium pump. Cell culture approaches provide a means of closely controlling the environment and so have been useful to examine whether the production of "endogenous ouabain" is in fact endogenous. Cultured cells from bovine adrenocortical regions suggest that the zona glomerulosa may be the region where such compounds are synthesized (57). However, we have not been able to demonstrate by HPLC purification and ouabain immunoassay that the material released by cultured murine adrenocortical cells, which we have previously shown to have numerous cardiac glycoside-like properties, is similar to ouabain (49, 54, 55). Furthermore, stimulation of adrenal steroidogenesis with cholesterol analogs was not accompanied by increased production of such material. Likewise, inhibition of cholesterol side-chain cleavage and further metabolism of the product of this reaction (pregnenolone) did not reduce output of the material. In contrast, Laredo and colleagues reported that 90% or more of the material extracted from conditioned bovine adrenocortical cultures co-eluted with authentic ouabain (56). Accumulation of ouabain immunoreactivity in conditioned medium from a mixed adrenocortical cell culture (derived from all three adrenocortical zones) occurred at a similar rate for ouabain as aldosterone. Cortisol accumulation was approximately 100-fold greater. Ouabain immunoreactivity appeared at high levels in cultures stimulated with both angiotensin II and ACTH (57). Recent work suggests that type II angiotensin receptors mediate the stimulation by angiotensin II, whereas stimulation of aldosterone and cortisol by angiotensin II is independent of type II angiotensin receptors (58).

The presence of isomeric ouabain in bovine hypothalamus (HIF) raises the question of whether the latter is a site of synthesis or a target site that accumulates iso-ouabain from another source. Some early evidence of release of cardiac glycoside-like material from cultured hypothalamic cells has been reported (59, 60) though more selective tools directed at the known structural features of HIF now need to be used to assess whether synthesis and release of HIF also take place in the hypothalamus.

Pharmacology of "Endogenous Ouabain." Evidence that ouabain is able to influence regulation of arterial blood pressure or renal sodium excretion is controversial, raising questions about whether the identification of an "endogenous ouabain" represents the culmination of the search

for a third factor with hypertensinogenic properties. Studies have been reported in humans, sheep, and rats with mixed findings (61–68), possibly related to the mode of administration. In rats, observations of ouabain-induced elevation of blood pressure have been obtained. In this species, the relatively glycoside-resistant cardiac sodium pump permits higher doses of ouabain to be tolerated (64–66). However, even in the rat, studies in which ouabain fails to elevate blood pressure have been reported in spite of doses administered that have been near toxic levels and infusions extending over a prolonged period (67–69). Evidence suggests that ouabain may require the amplifying effect of reduced renal excretory capacity to elevate blood pressure (69). No evidence of amplification of the pressor response to angiotensin II or norepinephrine has been observed in humans or sheep (61–63, 70). Of course, all such experiments hinge on the untested assumption that the plant-derived ouabain administered has the same effects as iso-ouabain.

Endogenous Materials with Homology to Amphibian Cardiotonic Steroids. Along with evidence indicating a *cardenolide* structure of endogenous sodium pump inhibitors, an increasing body of evidence supports an endogenous mammalian *bufodienolide* (EB). Bufodienolides are cardioactive steroids originally described in Bufonidae toads and, unlike cardenolides, are C24 steroids. They possess a six-membered, doubly unsaturated lactone ring and demonstrate maximal UV absorbance at 295–300 nm (71, 72). Bufodienolides were introduced into clinical practice more than 1000 years ago. The dried skin of toads, which contains various bufodienolide derivatives, comprises one of the main active principles of the traditional Chinese remedy Sen-So (or Ch'an Su), which has been (and continues to be) used in the treatment of congestive heart failure.

Elucidation of the chemical structure of bufodienolides began in several laboratories in the 1920s. Within three decades it was established that skin and paratoid glands of various toad species contain several cardioactive steroids, which exist both in nonconjugated (bufogenins) and conjugated (bufotoxins) forms. Unlike plant-derived cardenolides, which are conjugated with a sugar residue to form cardiac glycosides, bufotoxins are steroids conjugated with suberylarginine and sulfates (71, 73). Positive inotropic effects of bufodienolides have been studied extensively *in vitro* and *in vivo* in many laboratories (74).

Flier and co-workers demonstrated that toad skin and plasma contain a material that inhibits Na,K-ATPase, cross-reacts with several digitalis antibodies, and competes with tritiated ouabain for the digitalis receptor site on the sodium pump (75, 76). Flier *et al.* also demonstrated that bufodienolides are widespread in frogs (77). Remarkably, the highest levels of bufodienolides were detected in those frog species that migrate back and forth from dry to aquatic environments (77). Considering that the skin is a major organ for regulation of water and electrolyte homeostasis in amphibia, these authors proposed an appealing hypothesis that the sodium pump and bufodienolides (sodium pump inhibitors) in the skin represent a system that regulates fluid balance in amphibia. In the course of evolution, this system has also become protective against predators. Indeed, cases of lethal intoxication after ingestion of toads have been described in the literature (78).

Later, the hypothesis that toad bufodienolides are natural ligands of the digitalis receptor site on Na,K-ATPase was advanced by Lichtstein's group. Lichtstein and co-workers have identified EB in plasma and skin of *Bufo viridis* as resibufagenin (3-beta hydroxy 14,15-epoxy bufodienolide) (79) and have shown that this compound displays positive inotropic effects *in vitro* (80, 81). Subsequently, they demonstrated the presence of bufodienolide in toad brain and have shown that, in *Bufo viridis,* brain and skin concentrations of bufodienolide change according to environmental salinity (82).

Although the chemistry of bufodienolides has been studied extensively for decades, the biosynthetic pathways are practically unknown. However, in plants, bufodienolides were shown to originate from pregnenolone (83, 84). Using ^{14}C-cholesterol, Siperstein *et al.* have shown that cholesterol is a precursor in *Bufo marinus* (85).

Evidence for Existence of Bufodienolides in Mammals. Results demonstrating that amphibian bufodienolides represent an endogenous material involved in water and electrolyte homeostasis prompted a search for a mammalian bufodienolide. First, Kieval and co-workers found a material in the extracts of human bile that cross-reacted with a polyclonal antibody against bufalin (3-beta, 14-beta dihydroxy bufodienolide) (86). In 1991, Goto *et al.* detected the presence of two fractions with ouabain-displacing properties in human urine; one of those compounds reacted with bufalin antibody (87). Naomi *et al.* (88) using several digoxin antisera and several substances that were potential EBs (including bufalin), established immunological profiles of plasma from different groups of patients and suggested that a circulating digoxin-like substance may have a bufodienolide nature. The presence of bufalin-like immunoreactive material in human plasma has also been reported by Panesar *et al.* (89) and Numazawa *et al.* (90).

Lichtstein's group has demonstrated the presence of digitalis-like material in the eye lenses of several mammalian species (91). The highest levels of digoxin-like immunoreactive material was found in human cataractous lenses. This material, purified by HPLC, demonstrated maximal UV absorbance at 298 nm, which is typical for bufodienolides. Mass-spectrometrically it has been established that the purified compound has a molecular mass of 270 representing bufalin-peptide derivatives (91).

Schoner reported that human plasma and bovine adrenals contain material that cross-reacts with antibody against a plant-derived bufotrienolide, proscillaridin A (3-beta rhamnosido-14-beta-hydroxy bufotrienolide) (92). Later,

Schoner and associates purified three fractions with the ability to inhibit the sodium pump and to react with proscillaridin antibody from plasma of a group of 101 human subjects consisting of healthy volunteers and patients with hypertension and heart failure (93). Plasma levels of proscillaridin-like immunoreactivity from a less hydrophobic fraction correlated with the systolic, diastolic, and pulse pressure, the latter correlation being the strongest (93). Proscillaridin-like immunoreactive material has been also detected in Y-1 murine adrenocortical cell culture medium (94).

Bagrov and colleagues have demonstrated that the venom of *Bufo marinus* contains a potent vasoconstrictor that reacts with digoxin antibody, but is different from bufalin and resibufagenin (95). Subsequently, this compound has been identified as a previously described steroid, marinobufagenin (Figure 2, 3-beta, 5-beta dihydroxy 14,15-epoxy bufodienolide) (96). Polyclonal antibody raised against marinobufagenin cross-reacted with the material from human, porcine, and canine plasma and urine (97–100). When chloroform-extracted human urine was fractionated on reverse-phase HPLC columns, the fraction containing maximum marinobufagenin immunoreactivity eluted later than ouabain-like material and demonstrated no separation from a standard of marinobufagenin purified from the venom of *Bufo marinus* (97, 99). The material purified from human urine was identical in mass spectroscopy to marinobufagenin from *Bufo marinus* venom (101). Plasma levels of marinobufagenin immunoreactivity were increased in acute plasma volume expansion in dogs and rats (98, 101) and during two other states associated with increased plasma volume: voluntary hypoventilation in healthy humans (97) and pre-avoidance hypoventilation in micropigs (100).

Recently, Hilton and co-workers purified a bufodienolide compound with sodium pump inhibitory activity from human placentae. Using fast-atom bombardment mass spectroscopy, this substance was characterized as 3-beta, 14-alpha, 20:21 bufenolide (102).

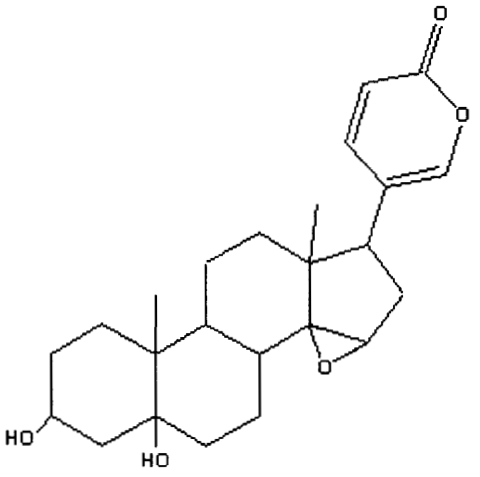

Figure 2. Structure of marinobufagenin.

Pharmacology of Bufodienolides and the Physiological Significance of Mammalian Bufodienolide(s). Inotropic effects of various cardiotonic bufodienolide steroids had been extensively studied even before it was shown that these compounds inhibit the sodium pump (reviewed in Ref. 74). Indications of a possible role of mammalian bufodienolide as an endogenous inotrope include observations of a digitalis-like effect of bufalin-immunoreactive material from human bile on isolated canine Purkinje fibers (86), and correlation of proscillaridin-like immunoreactivity with the pulse pressure in humans (93).

During acute volume expansion in anesthetized dogs, the initial rise in plasma concentration of marinobufagenin-like immunoreactivity occurred in parallel with an increase in left ventricular (LV) dP/dt (97). Pretreatment of saline-infused animals with digoxin antiserum, which binds bufodienolides including marinobufagenin, prevented the increase in LV dP/dt and decreased urinary release of sodium and marinobufagenin-like (but not ouabain-like) material. Although these observations showed a parallelism between plasma levels of EB immunoreactive substance and natriuresis, it is unclear whether or not EB has direct natriuretic activity.

Data on the natriuretic activity of bufalin are conflicting. Brownlee *et al.* reported that bufalin is a potent inhibitor of canine renal Na,K-ATPase, but a weak natriuretic (103). Eliades *et al.* investigated acute hemodynamic effects of bufalin in anesthetized dogs and found that although intravenous infusion of bufalin (5–50 μg/min) produced a positive inotropic effect and rise of systemic blood pressure, natriuresis and diuresis were not observed (104). At the same time, when anesthetized male Wistar rats were infused intravenously with approximately the same doses of bufalin, positive inotropic and pressor responses were associated with a prominent natriuretic effect, which was much stronger compared with the effect of equimolar concentrations of ouabain (105). To our knowledge, no comparisons of the effects of chronic administration of bufalin with those of ouabain have been reported. Yates and McDougall (106) studied the effect of direct injection of bufalin (500 μg/hr) into the renal artery in conscious sheep and found that bufalin increased sodium excretion; however, this increase was not associated with the changes in glomerular filtration rate and in effective renal plasma flow. When bufalin was given intravenously to anesthetized male Sprague-Dawley rats (10 mg/min for 40 min), glomerular filtration rate, renal blood flow, and urinary sodium excretion were lower than in controls although blood pressure in bufalin-treated animals was elevated (107). These observations do not provide any evidence toward the direct natriuretic effect of bufalin, and suggest that the observed natriuretic effects (104–106) could be due to the hemodynamic effects of this bufodienolide.

As can be seen from the data on acute administration of bufalin to experimental animals, this substance raises the systemic blood pressure (104–107). Mechanisms of the va-

soconstrictor and pressor effects of bufalin have been investigated in several studies. Eliades *et al.* (104) have shown that intrabrachial infusion of bufalin rapidly increases perfusion pressure, augments the pressor response to norepinephrine, and diminishes potassium-induced vasodilation. Cress *et al.* showed that bufalin (10 mmoles/l) increased vascular tone, and electric stimulation induced norepinephrine outflow in isolated dog saphenous vein (108). Since tetrodotoxin inhibited the effects of bufalin on norepinephrine outflow, it was concluded that an effect on the intravascular nerve endings is important for bufalin's vasoconstrictor action (108). This is in keeping with an observation by the same group that intravenous infusion of bufalin in rats is associated with a positive chronotropic effect (105). Later, these observations were confirmed in isolated rat aortae where the vasoconstrictor effect of bufalin was also blocked by phentolamine (95). Interestingly, in rat aortic rings the vasoconstrictor action of bufalin appeared to stimulate the sodium pump. No such stimulatory effect was observed in vascular rings pretreated with phentolamine (24). Similar stimulation due to norepinephrine release caused by nanomolar doses of digitalis was observed in isolated guinea pig hearts (109).

Bufalin given to rats (14.8 μg/kg/day) for 6 weeks did not change blood pressure, whereas higher doses (29.6 μg/kg/day) increased systolic blood pressure by 30 mm Hg (110). Another bufodienolide, resibufagenin, caused vasoconstriction in an isolated rabbit pulmonary artery in micromolar concentrations. Similar to the action of bufalin in isolated dog saphenous vein and rat aorta, this effect was also associated with the release of norepinephrine from nerve terminals (111).

In isolated human resistance arteries, nanomolar concentrations of bufalin caused concentration-dependent potentiation of the tone in vessels precontracted with norepinephrine and inhibited endothelium-dependent relaxation (112). In human blood vessels marinobufagenin was less effective, evoking contractile responses in isolated human pulmonary arteries at concentrations of 10–100 nmol/l (99).

Bagrov and coworkers have compared the mechanisms of vasoconstrictor effects of ouabain and marinobufagenin in isolated rat aortae (96). Marinobufagenin-induced vasoconstriction was phentolamine-resistant, whereas ouabain had its predominant effect on pumps localized to nerve endings. The vasoconstrictor effect of ouabain, similar to that of bufalin, was associated with a stimulation of the sodium pump (96). Subsequently, Na,K-ATPase inhibitory effects of ouabain and marinobufagenin were studied in two membrane fractions isolated from rat thoracic aorta by sucrose density gradient centrifugation (113). One fraction contained predominantly the α-3 isoform of Na,K-ATPase and represented membranes from the perivascular nerve endings. The other membrane fraction, containing predominantly the α-1 isoform, was derived from the plasmalemmal membrane. The IC_{50} for inhibition of Na,K-ATPase by ouabain and marinobufagenin were 1 nmol/l and 0.5 μmol/l in the neuronal membrane fraction, and 0.1 μmol/l and 10 nmol/l in sarcolemma, respectively. Therefore, in rat aorta marinobufagenin preferentially inhibits Na,K-ATPase in vascular smooth muscle membranes, and ouabain demonstrates higher affinity toward Na,K-ATPase from vascular nerve endings. With respect to isoform specificity, marinobufagenin resembles the Na,K-ATPase inhibitor purified from human peritoneal dialysate by Graves and colleagues (see below) (114).

A separate line of evidence indicates a possible role of endogenous bufodienolides as regulators of cell differentiation and proliferation. In 1982 Schreiber and Kolbel hypothesized that a digoxin-like immunoreactive adrenal steroid participates in the development of cardiac hypertrophy (115). Zhang *et al.* have shown that low concentrations (10 nmol/l) of bufalin produce differentiation-inducing activity in three human leukemia-derived cell lines (promyelocytic HL60, monoblastic U937, and myeloblastic ML1) (116). Later, bufalin was reported to induce apoptosis in human leukemia U937 cells (117) and inhibit the *in vitro* proliferation of cultured endothelial cells (118). Interestingly, the action of bufalin on cell differentiation was attributed to the Na,K-ATPase inhibitory effect of this steroid (119). When the effect of a fraction from human plasma with the ability to cross-react with bufalin antibody and to displace ^{3}H-ouabain was studied in THP-1 leukemia cells, the endogenous factor induced cell differentiation (120).

Although the above observations compose an incomplete picture, they favor a hypothesis that at least some endogenous digitalis-like activity in mammals is attributable to a bufodienolide compound(s). However, the mixed picture illustrated above is reflected in recent work suggesting that variation in actions may be due to the presence of several endogenous materials. Butler *et al.* studied plasma bufodienolides in *Bufo marinus* (120). Plasma from *Bufo marinus* contains several bufodienolides, which exist in nonconjugated form, as well as bufotoxins (conjugates with suberylarginine and sulfates). These compounds displayed different abilities to inhibit Na,K-ATPase and ^{3}H-ouabain binding and different retention times from reverse-phase HPLC columns. Systematic studies of bufodienolide pharmacology and standardization of methods of measurement of bufodienolide compounds will lead to better understanding of the importance of mammalian bufodienolides in the regulation of the sodium pump, cardiovascular control, and pathogenesis of hypertension.

Other Areas of Progress in the Search for Endogenous Regulators of the Sodium Pump

Possible Natriuretic Factors Derived from Human Uremic Urine. Following de Wardener's original idea that the third factor should principally be a natriuretic compound, Wechter and colleagues embarked on a series of studies to identify compounds in human uremic urine using an *in vivo* assay system that detects the natriuretic property of isolated materials (121–123). Using this system, a num-

ber of compounds have been identified. One is a metabolite of the calcium channel blocker, diltiazem, which is produced by oxidative deamination and deacetylation of the parent compound (124). The mechanism of action of this exogenous compound is not known.

However, the same group has made further progress in identifying another compound in uremic urine as 2,7,8-trimethyl-2-(beta-carboxymethyl)-6-hydroxychroman (123, 125). It is produced *in vivo* by oxidative metabolism of gamma-tocopherol, a member of the vitamin E complex. As such, it differs somewhat from common expectations of an "endogenous" compound. However, the authors have provided some preliminary indication that levels may be elevated in uremic urine compared to normal. More impressive is the elucidation of the mechanism of action of this compound. It appears to be an inhibitor of potassium channels in the ascending limb of the loop of Henlé. Blockade at this site may limit the recycling of potassium ions that provide substrate to the Na-K-Cl co-transporter, the major mechanism by which sodium is reabsorbed in this nephron region. Consequently, distal sodium delivery increases and natriuresis may result. Much further work will be required to determine whether this interesting new compound participates in physiological regulation of natriuresis (and blood pressure) and how dietary intake of tocopherols may impinge upon normal regulation of salt and water balance.

Sodium Pump Inhibitor Derived from Peritoneal Dialysate of Chronic Renal Failure Patients. Efforts to select suitable starting materials for enrichment of endogenous inhibitors of the sodium pump have led to investigations of peritoneal dialysate from chronic renal failure patients. Graves and colleagues have studied ultrafiltrates of this material by HPLC coupled with both digoxin immunoreactivity and a sensitive sodium pump bioassay examining the hydrolysis of radiolabelled inorganic phosphate from ATP (126–128). Using this approach they have identified a fraction that is clearly dissimilar in its elution profile from ouabain and digoxin (127, 129). This material appears to be chemically unstable, and activity is lost even from frozen samples, another property quite distinct from cardiac glycosides.

Extensive analysis of the interaction of this material with the sodium pump has been performed (129). Interaction with Na,K-ATPase has been compared with that of ouabain, and some important distinctions have emerged. The labile factor appears to inhibit Na,K-ATPase by the same mechanism and with similar kinetics as ouabain. Inhibition of the enzyme activity in vascular smooth muscle by the factor can be blocked by Fab fragments of digoxin antibody (130). Remarkably, this factor appears to have maximum potency on the ouabain-resistant, alpha-1 isoform of the enzyme (114). Rat alpha-1 isoform is highly resistant to cardiac glycosides. Kidney microsomes were prepared to represent a relatively pure source of this isoform. Microsomal preparations from muscle (predominantly alpha-2 isoform) and fetal brain (predominantly alpha-3) were also examined. The factor was a relatively potent inhibitor of all isoforms in rat, including alpha-1. No measurement of the molecular mass of the applied inhibitor was available so a direct measure of molar potency was not available. However, 50% inhibition of the brain enzyme was achieved with 47 μg of ouabain whereas similar inhibition could be obtained with less than 10 ng of purified material. These observations argue strongly that pump inhibitors may exist and may be considerably more potent than plant cardiac glycosides. Such inhibitors may be endogenous and involved in the response to volume expansion of renal failure. Structural information, as yet undetermined for this material, will be difficult to obtain until stabilization of the lability and purification of increased quantities are achieved.

A Co-Transport Inhibitor (Na:K:2Cl) Present in Urine and Plasma. Garay and colleagues have produced evidence suggesting that both the sodium pump and the sodium, potassium, chloride co-transporter may be regulated by endogenous inhibitors (131). Crude preparations of rat urine from salt-loaded animals demonstrated an increased ability to inhibit the co-transporter in human erythrocytes (132). The same activity is present in humans in both plasma and urine (133). It is increased by salt loading, but no difference in the activity present in urine or plasma between normotensive and hypertensive subjects was observed. However, congestive heart failure was strongly associated with increased plasma and urinary activities of the inhibitor (134). The source of this material may be the posterior pituitary (135).

Attempts at purification have been made and have led to the conclusion that two compounds are responsible for the co-transport inhibitory activity. One of these compounds has been identified as equol, an isoflavanoid that may be derived from bacterial metabolism in the gut (136). Although excretion of this material in urine increases in volume-loaded rats (137), it is unclear if this reflects increased synthesis or simply the consequence of increased renal filtration on excretion. However, urinary levels of equol may be mildly natriuretic. The identity of the additional, presumably more potent material possessing co-transport inhibitory activity has not yet been reported.

Summary

Progress continues in the effort to identify endogenous ligands and understand their role in regulation of Na,K-ATPase at the cardiac glycoside binding site. Important advances include the identification of iso-ouabain; however, further efforts are being made to resolve the stereochemistry of this compound. With such information, it will be possible to begin to assess biological aspects of iso-ouabain's function. Evidence indicates that bufodienolide-like molecules are also candidate mammalian compounds, and the role of such compounds in controlling cardiovascular and renal function is being explored actively. Other candidate compounds remain, and insight into their structure and function continues to be refined. Finally, as a by-product of studies

directed toward control of renal sodium excretion, several interesting, new molecules have been identified or partially purified. These add new insight into potential regulatory mechanisms influencing integration of cardiovascular and renal function.

Note added in proof: Schoner and colleagues have recently reported the purification of 20μg of material indistinguishable from plant ouabain (i.e., not iso-ouabain) from bovine adrenals (138). Analysis of the purified material included mass and nuclear magnetic resonance spectroscopy. In addition, another new report independently indicates the presence of ouabain in bovine adrenal tissue and the release of similar material into cell culture supernatants (139).

1. Cowley AW Jr., Roman RJ. The role of the kidney in hypertension. JAMA **275:**1581–1589, 1996.
2. Hansson JH, Schild L, Lu Y, Wilson TA, Gautschi I, Shimkets R, Nelson-Williams C, Rossier BC, Lifton RP. A *de novo* missense mutation of the beta subunit of the epithelial sodium channel causes hypertension and Liddle syndrome, identifying a proline-rich segment critical for regulation of channel activity. Proc Natl Acad Sci USA **92:**11495–11499, 1995.
3. Walker BR, Edwards CRW. 11-beta-hydroxysteroid dehydrogenase activity in hypertension and renal disease. Adv Nephrol **22:**329–347, 1993.
4. Seckl JR, Brown RW. 11-beta-hydroxysteroid dehydrogenase: On several roads to hypertension. J Hypertens **12:**105–112, 1994.
5. Lifton RP, Dluhy RG, Powers M, Rich GM, Cook S, Ulick S, Lalouel JM. A chimaeric 11 beta-hydroxylase/aldosterone synthase gene causes glucocorticoid-remediable aldosteronism and human hypertension. Nature **355:**262–265, 1991.
6. Bianchi G, Ferrari P, Salvati P, Salardi S, Parenti P, Cusi D, Guidi E. A renal abnormality in the Milan hypertensive strain of rats and in humans predisposed to essential hypertension. J Hypertens **4:**S33–36, 1986.
7. Bianchi G, Tripodi G, Casari G, Salardi S, Barber BR, Garcia R, Leoni P, Torielli L, Cusi D, Ferrandi M, Pinna LA, Baralle FE, Ferrandi P. Two point mutations within the adducin genes are involved in blood pressure variation. Proc Natl Acad Sci USA **91:**3999–4003, 1994.
8. Ferrandi M, Tripodi G, Salardi S, Florio M, Modica R, Barassi P, Parenti P, Shainskaya A, Karlish S, Bianchi G, Ferrari P. Renal Na,K-ATPase in genetic hypertension. Hypertension **28:**1018–1025, 1996.
9. Parenti P, Villa M, Hanozet GM, Ferrandi M, Ferrari P. Increased Na-pump activity in the kidney cortex of the Milan hypertensive rat strain. Febs Lett **290:**200–204, 1991.
10. Ferrandi M, Minotti E, Salardi S, Florio M, Bianchi G, Ferrari P. Ouabain-like factor in Milan hypertensive rats. Am J Physiol **263:**F739–F748, 1992.
11. Ferrandi M, Minotti E, Salardi S, Florio M, Bianchi G, Ferrari P. Characteristics of a ouabain-like factor from Milan hypertensive rats. J Cardiovasc Pharmacol **22:**S75–78, 1993.
12. Tripodi G, Valtorta F, Torielli L, Chieregatti E, Salardi S, Trusolino L, Menegon A, Ferrari P, Marchisio P, Giuseppe B. Hypertension-associated point mutations in the adducin alpha and beta subunits affect actin cytoskeleton and ion transport. J Clin Invest **97:**2815–2822, 1996.
13. Boberg U, Persson AE. Increased tubuloglomerular feedback activity in Milan hypertensive rats. Am J Physiol **250:**F967–974, 1986.
14. Ferrari P, Torielli L, Cirillo M, Salardi S, Bianchi G. Sodium transport kinetics in erythrocytes and inside-out vesicles from Milan rats. J Hypertens **9:**703–711, 1991.
15. Persson AE, Bianchi G, Boberg U. Evidence of defective tubuloglomerular feedback control in rats of the Milan hypertensive strain (MHS). Acta Physiol Scand **122:**217–219, 1984.
16. Melzi ML, Bertorello A, Fukuda Y, Muldin I, Sereni F, Aperia A. Na,K-ATPase activity in renal tubule cells from Milan hypertensive rats. Am J Hypertens **2:**563–566, 1989.
17. Melzi ML, Syren ML, Assael BM, Sereni F, Aperia A. Increased renal tubular Na-K-ATPase activity in Milan hypertensive rats in the prehypertensive period. Pediatr Nephrol **5:**700–703, 1991.
18. Cusi D, Barlassina C, Azzani T, Casari G, Citterio L, Devoto M, Glorioso N, Lanzani C, Manunta P, Righetti M, Rivera R, Stella P, Troffa C, Zagato L, Bianchi G. Polymorphisms of alpha-adducin and salt sensitivity in patients with essential hypertension. Lancet **349:**1353–1357, 1997.
19. Bianchi G, Tripodi MG, Casari G, Torielli L, Cusi D, Barlassina C, Stella P, Zagato L, Barber BR. Alpha-adducin may control blood pressure both in rats and humans. Clin Exp Pharmacol Physiol **1:**S7–9, 1995.
20. Guyton AC. Blood pressure control: Special role of the kidney and body fluids. Science **252:**1813–1816, 1991.
21. Guyton AC. Kidneys and fluids in pressure regulation. Hypertension **19**(Suppl. I):I2–I8, 1992.
22. Hall JE, Guyton AC, Brands MW. Pressure-volume regulation in hypertension. Kidney Int **49:**S35–S41, 1996.
23. De Wardener HE, Mills IH, Clapham WF, Hayter CJ. Studies on the efferent mechanism of the sodium diuresis which follows the administration of intravenous saline in the dog. Clin Sci **21:**249–258, 1961.
24. Kramer HJ, Gonick HC, Paul WL, Lu E. Third factor: Inhibitor of Na-K-ATPase? In: Awall N, Burglund F, Josephson B, eds. Proceedings of the 4th International Congress of Nephrology. Basel: Karger, 1970, p373.
25. Buckalew VMJ, Martinez FJ, Green WE. The effect of dialysates and ultrafiltrates of plasma of saline-loaded dogs on toad bladder sodium transport. J Clin Invest **49:**926–935, 1970.
26. Haddy FJ, Pamnani MB. Evidence for a circulating endogenous Na^+-K^+ pump inhibitor in low-renin hypertension. Fed Proc **44:**2789–2794, 1985.
27. Clough DL, Pamnani MB, Overbeck HW, Haddy FJ. Decreased myocardial Na,K-ATPase in rats with one-kidney, Goldblatt hypertension. Fed Proc **36:**491, 1977.
28. Clough DL, Pamnani MB, Haddy FJ. Decreased myocardial Na,K-ATPase in one-kidney, one-clip hypertensive rats. Am J Physiol **245:**H244–H251, 1983.
29. Haddy FJ, Overbeck HW. The role of humoral agents in volume expanded hypertension. Life Sci **19:**935–948, 1976.
30. Overbeck HW, Pamnani MB, Akera T, Brody TM, Haddy FJ. Depressed function of a ouabain-sensitive sodium-potassium pump in blood vessels from renal hypertensive dogs. Circ Res **38:**II-48–II-52, 1976.
31. Pamnani MB, Huot S, Buggy J, Clough D, Haddy FJ. Demonstration of a humoral inhibitor of the Na-K pump in some models of experimental hypertension. Hypertension **3:**II96–II101, 1981.
32. Delfert DM, Valdes R. Impact of digoxin-like immunoreactive factors on digoxin measurements by immunoassays. J Clin Immunoassay **8:**157–164, 1985.
33. Pudek MR, Seccombe DW, Jacobson BE, Whitfield MF. Seven different digoxin immunoassay kits compared with respect to interference by a digoxin-like immunoreactive substance in serum from premature and full-term infants. Clin Chem **29:**1972–1974, 1983.
34. Gruber KA, Whitaker JM, Buckalew VM. Endogenous digitalis-like substance in plasma of volume-expanded dogs. Nature **287:**743–745, 1980.
35. Doris PA. Regulation of Na,K-ATPase by endogenous ouabain-like materials. Proc Soc Exp Biol Med **205:**202–212, 1994.
36. Haddy FJ, Buckalew VM. Endogenous digitalis-like factors in hypertension. In: Laragh JH, Brenner BM, Eds. Hypertension: Pathophysiology, Diagnosis, and Management, 2nd ed. New York: Raven Press, Vols 1:1055–1067, 1995.
37. Blaustein MP, Hamlyn JM. Role of a natriuretic factor in essential hypertension: An hypothesis. Ann Intern Med **98:**785–792, 1983.
38. Blaustein MP. Physiological effects of endogenous ouabain: Control of intracellular Ca-2+ stores and cell responsiveness. Am J Physiol **264:**C1367–C1387, 1993.
39. Hamlyn JM, Blaustein MP, Bova S, DuCharme DW, Harris DW, Mandel F, Mathews WR, Ludens JH. Identification and characterization of a ouabain-like compound from human plasma. Proc Natl Acad Sci USA **88:**6259–6263, 1991.
40. Mathews WR, DuCharme DW, Hamlyn JM, Harris DW, Mandel F,

Clark MA, Ludens JH. Mass spectral characterization of an endogenous digitalis-like factor from human plasma. Hypertension **17:**930–935, 1991.
41. Tymiak AA, Norman JA, Bolgar M, DiDonato GC, Lee H, Parker WL, Lo LC, Berova N, Nakanishi K, Haber E, Haupert GTJ. Physicochemical characterization of a ouabain isomer isolated from bovine hypothalamus. Proc Natl Acad Sci USA **90:**8189–8193, 1993.
42. Zhao N, Lo LC, Berova N, Nakanishi K, Tymiak AA, Ludens JH, Haupert GT. Na,K-ATPase inhibitors from bovine hypothalamus and human plasma are different from ouabain: Nanogram scale CD structural analysis. Biochemistry **34:**9893–9896, 1995.
43. Nakanishi K, Berova N, Lo LC, Zhao N, Ludens JH, Tymiak AA, Warrack B, Haupert GT Jr. Search for an endogenous mammalian cardiotonic factor. Adv Exp Med Biol **404:**219–224, 1996.
44. Hansen O. Do putative endogenous digitalis-like factors have a physiological role? Hypertension **24:**640, 1994.
45. Hamlyn JM, Hamilton BP, Manunta P. Endogenous ouabain, sodium balance, and blood pressure: A review and a hypothesis. J Hypertens **14:**151–167, 1996.
46. Anner BM, Rey HG, Mossmayer M, Meszoely I, Haupert Jr, G.T. Hypothalamic Na^+-K^+-ATPase inhibitor characterized in two-sided liposomes containing pure renal Na^+-K^+-ATPase. Am J Physiol **258:**F144–F153, 1990.
47. Cook JS, Will PC, Proctor WR, Brake ET. Turnover of ouabain-binding sites and plasma membrane proteins in HeLa cells. In: Cook JS, Ed. Biogenesis and Turnover of Membrane Macromolecules. New York: Raven Press, 1976.
48. Will PC, Longworth JW, Brake ET, Cook JS. Analysis of intracellular drug (ouabain) sequestration as a mechanism of detoxification. Mol Pharmacol **13:**161–171, 1977.
49. Doris PA, Hayward-Lester A, Bourne D, Stocco DM. Ouabain production by cultured adrenal cells. Endocrinology **137:**533–539, 1996.
50. Hallaq HA, Haupert GT. Positive inotropic effects of the endogenous Na/K-transporting ATPase inhibitor from the hypothalamus. Proc Natl Acad Sci **86:**10080–10084, 1989.
51. Boulanger BR, Lilly MP, Hamlyn JM, Laredo J, Shurtleff D, Gann DS. Ouabain is secreted by the adrenal gland in awake dogs. Am J Physiol **264:**E413–E419, 1993.
52. Ludens JH, Clark MA, Robinson FG, DuCharme D.W. Rat adrenal cortex is a source of a circulating ouabain-like compound. Hypertension **19:**721–724, 1992.
53. Naruse K, Naruse M, Tanabe A, Yoshimoto T, Watanabe Y, Kurimoto F, Horiba N, Tamura M, Inagami T, Demura H. Does plasma immunoreactive ouabain originate from the adrenal gland? Hypertension **23:**I102–105, 1994.
54. Doris PA, Kilgore MW, Durham D, Stocco DM. An endogenous digitalis-like factor derived from the adrenal gland: Studies of adrenocortical tumor cells. Endocrinology **125:**2580–2586, 1989.
55. Doris PA, Jenkins LA, Stocco DM. Is ouabain an authentic endogenous mammalian substance derived from adrenal gland? Hypertension **23:**632–638, 1994.
56. Laredo J, Hamilton BP, Hamlyn JM. Ouabain is secreted by bovine adrenocortical cells. Endocrinology **135:**794–797, 1994.
57. Laredo J, Hamilton BP, Hamlyn JM. Secretion of endogenous ouabain from bovine adrenocortical cells: Role of the zona glomerulosa and zona fasciculata. Biochem Biophys Res Commun **212:**487–493, 1995.
58. Laredo J, Shah JR, Lu ZR, Hamilton BP, Hamlyn JM. Angiotensin II stimulates secretion of endogenous ouabain from bovine adrenocortical cells *via* angiotensin type 2 receptors. Hypertension **29:**401–407, 1997.
59. Kendall JM, Thomas SE, Spurlock G, Mir MA. An active sodium transport inhibitor released from spontaneously hypertensive and normotensive rat fetal hypothalamic cells in culture. Am J Hypertens **1:**83S–87S, 1988.
60. Morgan K, Lewis MD, Spurlock G, Collins P, Foord SM, Southgate K, Scanlon M, Mir MA. Characterization and partial purification of the sodium-potassium-ATPase inhibitor released from cultured rat hypothalamic cells. J Biol Chem **260:**13595–13600, 1985.
61. Pidgeon GB, Richards AM, Nicholls MG, Charles CJ, Rademaker MT, Lynn KL, Bailey BR, Lewis LK, Yandle TG. Chronic ouabain infusion does not cause hypertension in sheep. Am J Physiol **270:**E386–E392, 1996.
62. Pidgeon GB, Richards AM, Nicholls MG, Bailey RR, Lynn KL, Lewis LK, Yandle TG. Effect of ouabain on pressor responsiveness in normal man. Am J Physiol **30:**E642–E647, 1994.
63. Pidgeon GB, Richards AM, Nicholls MG, Lewis LK, Yandle TG. Acute effects of intravenous ouabain in healthy volunteers. Clin Sci **86:**391–397, 1994.
64. Yuan CM, Manunta P, Hamlyn JM, Chen S, Bohen E, Yeun J, Haddy FJ, Pamnani MB. Long-term ouabain administration produces hypertension in rats. Hypertension **22:**178–187, 1993.
65. Manunta P, Rogowski AC, Hamilton BP, Hamyln JM. Ouabain-induced hypertension in the rat: Relationships among plasma and tissue ouabain and blood pressure. J Hypertens **12:**549–560, 1994.
66. Huang BS, Huang XF, Harmsen E, Leenen FHH. Chronic central versus peripheral ouabain, blood pressure, and sympathetic activity in rats. Hypertension **23:**1087–1090, 1994.
67. Yasujima M, Abe K, Tanno M, Kohzuki M, Kasai Y, Sato M, Omata K, Takeuchi K, Yoshinaga K, Masugi F, Ogihara T. Effects of ouabain on blood pressure regulation in rats. J Hypertens **4:**597–601, 1986.
68. Li M, Martin A, Wen C, Turner SW, Lewis LK, Whitworth JA. Long-term ouabain administration does not alter blood pressure in conscious Sprague-Dawley rats. Clin Exp Pharmacol Physiol **22:**919–923, 1995.
69. Sekihara H, Yazaki Y, Kojima T. Ouabain as an amplifier of mineralocorticoid-induced hypertension. Endocrinology **131:**3077–3082, 1992.
70. Pidgeon GB, Lewis LK, Richards AM, Nicholls GM. Studies on ouabain: Plasma levels and pressor effects. Hypertension **24:**385, 1994.
71. Fieser LF, Fieser M. Steroids. New York: Reinhold, pp727–809, 1959.
72. Barbier M, Shroter H, Meyer K, Schindler O, Reichstein T. Die Bufogenine des Paratoidensekrets von Bufo marinus (L) Schneider. Helv Chim Acta **42:**2486–2506, 1959.
73. Meyer K, Linde H. Collection of toad venoms and chemistry of toad venom steroids. In: Bucherl W, Buckley E, Eds. Venomous Animals and their Venoms. New York: Academic Press, pp521–556, 1971.
74. Chen KK, Kowarikowa A. Pharmacology and toxicology of toad venom. J Pharmacol Sci **56:**1535–1542, 1967.
75. Flier JS. Ouabain-like activity in toad skin and its implications for endogenous regulation of ion transport. Nature **274:**285–286, 1978.
76. Flier JS, Maratos-Flier E, Pallotta JA, McIsaac D. Endogenous digitalis-like activity in the plasma of the toad *Bufo marinus.* Nature **279:**341–343, 1979.
77. Flier JS, Edwards MW, Daly JW, Myers CW. Widespread occurrence in frogs and toads of skin compounds interacting with the ouabain site of $Na,^+$ K^+-ATPase. Science **208:**503–505, 1980.
78. Lutz B. Venomous frogs and toads. In: Bucherl W, Ed. Venomous Animals and Their Venoms. London: Academic Press, pp423–473, 1970.
79. Lichtstein D, Kachalsky S, Deutsch J. Identification of a ouabain-like compound in toad skin and plasma as a bufodienolide derivative. Life Sci **38:**1261–1270, 1986.
80. Shimoni Y, Gotsman M, Deutsch J, Kachalsky S, Lichtstein D. Endogenous ouabain-like compound increases heart muscle contractility. Nature **307:**369–371, 1984.
81. Shimoni Y, Gotsman M, Epstein M, Kachalsky S, Deutsch J, Lichtstein D. Further characterisation of the inotropic effect of a bufodienolide glycoside—an endogenous ouabain-like compound. Cardiovasc Res **20:**229–239, 1986.
82. Lichtstein D, Gati I, Babila T, Haver E, Katz U. Effect of salt acclimation on digitalis-like compounds in the toad. Biochim Biophys Acta **1073:**65–68, 1991.
83. Heftmann E. Biochemistry of Steroids. New York: Reinhold Pub. Co, 1960.
84. Heftmann E. Steroid hormones in plants. Lloydia **38:**195–209, 1975.
85. Siperstein MD, Murray AW, Titus E. Biosynthesis of cardiotonic sterols from cholesterol in the toad, *Bufo marinus.* Arch Biochem Biophys **67:**154, 1957.
86. Kieval RS, Butler VP Jr., Derguini F, Bruening RC, Rosen MR. Cellular electrophysiologic effects of vertebrate digitalis-like substances. J Am Coll Cardiol **11:**637–643, 1988.
87. Goto A, Yamada K, Ishii M, Sugimoto S, Yoshioka M. Immunoreactivity of endogenous digitalis-like factors. Biochem Pharmacol **41:**1261–1263, 1991.

88. Naomi S, Graves S, Lazarus M, Williams GH, Hollenberg NK. Variation in apparent serum digitalis-like factor levels with different digoxin antibodies. Am J Hypertens **4:**795–801, 1991.
89. Panesar NS. Bufalin radioimmunoassays: In search of the endogenous digitalis-like substance. J Immunoassay **15:**371–391, 1994.
90. Numazawa S, Honma Y, Yamamoto T, Yoshida T, Kuroiwa Y. A cardiotonic steroid bufalin-like factor in human plasma induces leukemia cell differentiation. Leuk Res **19:**945–953, 1995.
91. Lichtstein D, Gati I, Samuelov S, Berson D, Rozenman Y, Landau L, Deutsch J. Identification of digitalis-like compounds in human cataractous lenses. Eur J Biochem **216:**261–268, 1993.
92. Sich B, Kirch R, Antolovic R, Schoner W. Demonstration of inhibitors of the sodium pump in human plasma and bovine adrenals cross-reacting with proscillaridin A antibodies. In: Schoner W, Bamberg V, Eds. The Sodium Pump. Stuttgart: Steinkopff Eds, pp767–770, 1994.
93. Sich B, Kirch U, Tepel M, Zidek W, Schoner W. Pulse pressure correlates in humans with a proscillaridin A immunoreactive compound. Hypertension **27:**1073–1078, 1996.
94. Rasheed N, Doris PA. Production of cardiac glycoside-like material by Y1 cells in serum-free conditions. FASEB J **9:**A637, 1995.
95. Bagrov AY, Roukoyatkina NI, Fedorova OV, Pinaev AG, Ukhanova MV. Digitalis-like and vasoconstrictor effects of endogenous digoxin-like factor(s) from the venom of Bufo-Marinus toad. Eur J Pharmacol **234:**165–172, 1993.
96. Bagrov AY, Roukoyatkina NI, Pinaev AG, Dmitrieva RI, Fedorova OV. Effects of two endogenous Na,$^+$ K$^+$-ATPase inhibitors, marinobufagenin and ouabain, on isolated rat aorta. Eur J Pharmacol **274:**151–158, 1995.
97. Bagrov AY, Fedorova OV, Austin-Lane JL, Dmitrieva RI, Anderson DE. Endogenous marinobufagenin-like immunoreactive factor and Na,K-ATPase inhibition during voluntary hypoventilation. Hypertension **26:**781–788, 1995.
98. Bagrov AY, Fedorova OV, Dmitrieva RI, French AW, Anderson DE. Plasma marinobufagenin-like and ouabain-like immunoreactivity during saline volume expansion in anesthetized dogs. Cardiovasc Res **31:**296–305, 1996.
99. Bagrov AY, Dmitrieva RI, Fedorova OV, Kazakov GP, Roukoyatkina NI, Shpen VM. Endogenous marinobufagenin-like immunoreactive substance: A possible endogenous Na,K-ATPase inhibitor with vasoconstrictor activity. Am J Hypertens **9:**982–990, 1996.
100. Fedorova OV, French AW, Anderson DE. Inhibition of erythrocyte Na,K-ATPase activity during anticipatory hypoventilation in micropigs. Am J Hypertens **9:**1126–1131, 1996.
101. Bagrov AY, Fedorova OV, Dmitrieva RI, Howald W, Hunter AP, Kuznetsova EA, Shpen VM. Characterization of a urinary bufodienolide NA$^+$, K$^+$-ATPase inhibitor in patients after acute myocardial infarction. Hypertension **31:**1097–1103, 1998.
102. Hilton PJ, White RW, Lord GA, Garner GV, Gordon DB, Hilton MJ, Forni LG, McKinnon W, Ismail FMD, Keenan M, Jones K, Morden WE. An inhibitor of the sodium pump obtained from human placenta. Lancet **348:**303–305, 1996.
103. Brownlee AA, Lee G, Mills IH. Marked inhibition of canine renal Na/K ATPase by a bufodienolide but weak natriuretic activity in the rat. J Physiol **390:**94P, 1987.
104. Eliades D, Swindall B, Johnston J, Pamnani MB, Haddy FJ. Hemodynamic effects of bufalin in the anesthetized dog. Hypertension **13:**690–695, 1989.
105. Pamnani MB, Chen S, Bryant HJ, Schooley JJ, Eliades DC, Yuan CM, Haddy FJ. Effects of three sodium-potassium adenosine triphosphatase inhibitors. Hypertension **18:**316–324, 1991.
106. Yates NA, McDougall JG. Effects of direct renal arterial infusion of bufalin and ouabain in conscious sheep. Br J Pharmacol **108:**627–630, 1993.
107. Patel AR, Kurashina T, Granger JP, Kirchner KA. Acute Na,$^+$ K$^+$-ATPase inhibition with bufalin impairs pressure natriuresis in the rat. Hypertension **27:**668–671, 1996.
108. Cress LW, Freas W, Haddy F, Muldoon SM. Effects of bufalin on norepinephrine turnover in canine saphenous vein. Hypertension **18:**516–522, 1991.
109. Hougen TJ, Spicer N, Smith TW. Stimulation of monovalent cation active transport by low concentrations of cardiac glycosides. Role of catecholamines. J Clin Invest **68:**1207–1214, 1981.
110. Pamnani MB, Chen S, Yuan CM, Haddy FJ. Chronic blood pressure effects of bufalin, a sodium-potassium ATPase inhibitor, in rats. Hypertension **23:**I-106–I-109, 1994.
111. Oberfrank F, Vizi ES, Baker PF, Samuelov S, Lichstein D. Comparison of the effects of a bufodienolide and ouabain on neural and smooth muscle preparations. Neurosci Res **10:**235–244, 1991.
112. Woolfson RG, Graves J, LaBella FS, Templeton JF, Poston L. Effect of bufalin and pregnanes on vasoreactivity of human resistance arteries. Biochem Biophys Res Commun **186:**1–7, 1992.
113. Fedorova OV, Bagrov AY. Inhibition of Na/K ATPase from rat aorta by two endogenous Na/K-pump inhibitors, ouabain and marinobufagenin: Evidence of interaction with different alpha-subunit isoforms. Am J Hypertens **10:**929–935, 1997.
114. Tao QF, Hollenberg NK, Price DA, Graves SW. Sodium pump isoform specificity for the digitalis-like factor isolated from human peritoneal dialysate. Hypertension **29:**815–821, 1997.
115. Schreiber V, Kolbel F. Does an endogenous digoxin-like immunoreactive factor participate in the development of cardiomegaly? Cor et Vasa **24:**228–232, 1982.
116. Zhang LS, Nakaya K, Yoshida T, Kuroiwa Y. Bufalin as a potent inducer of differentiation of human myeloid leukemia cells. Biochem Biophys Res Commun **178:**686–693, 1991.
117. Watabe M, Masuda Y, Nakajo S, Yoshida T, Kuroiwa Y, Nakaya K. The cooperative interaction of two different signaling pathways in response to bufalin induces apoptosis in human leukemia U937 cells. J Biol Chem **271:**14067–14072, 1996.
118. Lee DY, Yasuda M, Yamamoto T, Yoshida T, Kuroiwa Y. Bufalin inhibits endothelial cell proliferation and angiogenesis *in vitro.* Life Sci **60:**127–134, 1996.
119. Numazawa S, Inoue N, Nakura H, Sugiyama T, Fujino E, Shinoki M, Yoshida T, Kuroiwa Y. A cardiotonic steroid bufalin-induced differentiation of THP-1 cells: Involvement of Na,$^+$ K$^+$-ATPase inhibition in the early changes in proto-oncogene expression. Biochem Pharmacol **52:**321–329, 1996.
120. Butler VP Jr., Morris JF, Akizawa T, Matsukawa M, Keating P, Hardart A, Furman I. Heterogeneity and lability of endogenous digitalis-like substances in the plasma of the toad, *Bufo marinus.* Am J Physiol **271:**R325–R332, 1996.
121. Benaksas EJ, Murray EDJ, Rodgers CL, Pham T, Bigornia AE, DeWind SA, Giebel R, Brubacher ES, Wechter WJ. Endogenous natriuretic factors 1: Sodium pump inhibition does not correlate with natriuretic or pressor activities from uremic urine. Life Sci **52:**1045–1054, 1993.
122. Levine BH, Murray ED, Bigornia AE, Dewind SA, Wechter WJ. Endogenous natriuretic factors-2: Characterization of natriuretic and vasopressive substances from human uremic urine. J Cardiovasc Pharmacol **22:**S63–S68, 1993.
123. Murray ED, Kantoci D, Dewind SA, Bigornia AE, Damico DC, King JG, Pham T, Levine BH, Jung ME, Wechter WJ. Endogenous natriuretic factors 3: Isolation and characterization of human natriuretic factors LLU-alpha, LLU-beta(1), and LLU-gamma. Life Sci **57:**2145–2161, 1995.
124. Kantoci D, Murray ED Jr., Quiggle DD, Wechter WJ. Endogenous natriuretic factors. 5: Synthesis and biological activity of a natriuretic metabolite of diltiazem and its derivatives. J Med Chem **39:**1196–1200, 1996.
125. Wechter WJ, Kantoci D, Murray ED Jr., D'Amico DC, Jung ME, Wang WH. A new endogenous natriuretic factor: LLU-alpha. Proc Natl Acad Sci USA **93:**6002–6007, 1996.
126. Tao QF, Soszynski PA, Hollenberg NK, Graves SW. Sensitive assay for sodium pump inhibition. Clin Chem **40:**1595–1596, 1994.
127. Graves SW, Glatter KA, Lazarus JM, Williams GH, Hollenberg NK. Volume expansion in renal failure patients: A paradigm for a clinically relevant [Na,K]ATPase inhibitor. J Cardiovasc Pharmacol **22:**S54–S57, 1993.
128. Glatter K, Graves S, Hollenberg N, Soszynski P, Tao Q, Frem G, Williams G, Lazarus J. Sustained volume expansion and [Na-,K]ATPase inhibition in chronic renal failure. Am J Hypertens **7:**1016–1025, 1994.
129. Tao QF, Soszynski PA, Hollenberg NK, Graves SW. Specificity of the volume-sensitive sodium pump inhibitor isolated from human peritoneal dialysate in chronic renal failure. Kidney Int **49:**420–429, 1996.
130. Krep HH, Graves SW, Price DA, Lazarus M, Ensign A, Soszynski PA, Hollenberg NK. Reversal of sodium pump inhibitor induced

vascular smooth muscle contraction with Digibind: Stoichiometry and its implications. Am J Hypertens **9:**39–46, 1996.
131. Dagher G, Brossard M, Feray JC, Garay RP. Modulation of erythrocyte Na transport pathway(s) by excess Na intake. Life Sci **37:**243–253, 1985.
132. Garay RP, Alda O, Soler A, Pares I, Lou M, Gimenez I, Nazaret C, Hannaert P. A potent inhibitor of the $Na,^{+}$ $K,^{+}$ Cl^{-} cotransport system in urine from salt-loaded rats. J Hypertens **11:**S266–267, 1993.
133. Pares I, de la Sierra A, Coca A, del Mar Lluch M, Urbano-Marquez A, Garay R. Detection of a circulating inhibitor of the $Na,^{+}$ $K,^{+}$ Cl^{-} cotransport system in plasma and urine after high salt intake. Am J Hypertens **8:**965–969, 1995.
134. Dubois-Rande JL, Montagne O, Alvarez-Guerra M, Nazaret C, Crozatier B, Gueret P, Castaigne A, Garay RP. Endogenous sodium-potassium-chloride cotransport inhibitor in congestive heart failure. J Am Coll Cardiol **28:**1464–1470, 1996.
135. Alda JO, Alvarez-Guerra M, Lou M, Gimenez I, Soler A, Garay RP. Site of origin of a urinary Na-K-Cl cotransport inhibitor. Miner Electrolyte Metab **21:**403–410, 1995.
136. Alda JO, Mayoral JA, Lou M, Gimenez I, Martinez RM, Garay RP. Purification and chemical characterization of a potent inhibitor of the Na-K-Cl cotransport system in rat urine. Biochem Biophys Res Commun **221:**279–285, 1996.
137. Alvarez-Guerra M, Vargas F, Alda JO, Garay RP. Endogenous inhibitor of Na-K-Cl cotransport system in inbred Dahl rats. Am J Physiol **272:**F356–363, 1997.
138. Schneider R, Wray V, Nimtz M, Lehmann WD, Kirch U, Antolovic R, Schoner W. Bovine adrenals contain, in addition to ouabain, a second inhibitor of the sodium pump. J Biol Chem **273:**784–92, 1998.
139. Perrin A, Brasmes B, Chambaz EM, Defaye G. Bovine adrenocortical cells in culture synthesize an ouabain-like compound. Mol Cell Endocrinol **126:**7–15, 1997.