THE NATRIURETIC HORMONES

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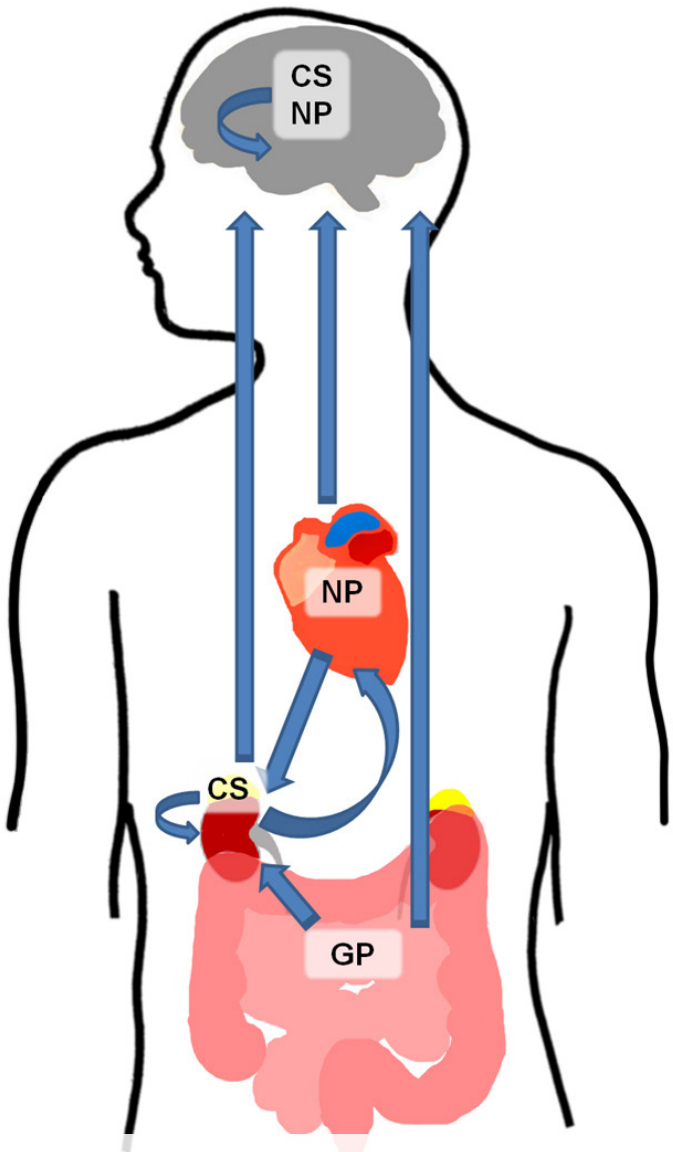

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THE NATRIURETIC HORMONES

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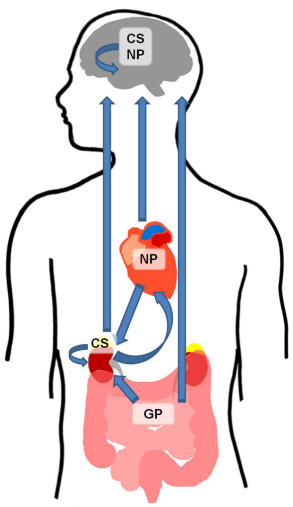

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The title follows from the original demonstration by Dr. Hugh de Wardener in 1961 that a humoral agent is produced after extracellular volume expansion which results in a vigorous diuresis and natriuresis. Thus the name of "natriuretic hormone" was coined. In the years that followed several investigators pursued the search for the hormone. What resulted, however, was the discovery of several hormones with different characteristics, all of which were natriuretic. Initially it was found that the hormone was similar in action to ouabain or digoxin, hence the appelation of ouabain-like or digoxin-like. The hormone was found to be an inhibitor of Na-K-ATPase, which would fit with it being a cardiotonic steroid. On the other hand, neither ouabain or digoxin migrated on Sephadex gel filtration in the same locus as the hormone. Other investigators claim to have identified the hormone-initially as a vanadium-diascorbate, later as bufadienolides such as marinobufagenin, yet later as a macrocylic derivative of inorganic carbon suboxide with a molecular weight of 408 Da. Some support for the latter finding was derived from an earlier report that a semi-purified Sephadex-derived compound was found to have a molecular weight of about 12,000 Da but the active compound, when split from its carrier protein, had a molecular weight of exactly 408 Da. This compound had not been further identified. As further development was the demonstration by Bricker and colleagues that a natriuretic substance could be purified from uremic urine. This turned out to be a xathurenic acid derivative. Meanwhile the focus began to turn to natriuretic peptides derived from heart (ANF and BNP). These peptides have a shorter duration of action than the cardiotonic steroid-like hormone and ANF has proved to be most useful as a measure of heart failure. It should also be stressed that marinobufagenin, like ANF, is elevated in congestive heart failure, whereas the steroid-like hormone is depressed or absent in this state. This review will attempt to describe and contrast the properties of each of the proposed natriuretic hormones, including their locus on Sephadex separation, potency, duration of action, chemical structure (if known), behavior in hypertension, renal failure, heart failure, and brain disease. As most recent work has focussed on marinobufagenin, this hormone will be brought up to date by investigators in the field.

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EDITORIAL
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Editorial: Natriuretic hormones

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Keywords: natriuretic hormones, atrial natriuretic peptides, cardiotonic steroids, hypertension, endogenous ouabain, bufodienolides

The link between renal sodium excretion and body fluid volumes has been a topic of interest since the early days of renal physiology (1). Glomerular filtration rate (GFR) and aldosterone were the first two controllers of renal sodium excretion to be recognized. A "third factor" was discovered when de Wardener and colleagues showed that volume expansion natriuresis still occurred in dogs given supramaximal doses of mineralocorticoids and without an increase in GFR (2). de Wardener et al. suggested that the natriuresis was due to a "natriuretic hormone" (NH), launching a new field of investigation. NH was thus defined as a compound that circulates in blood, the level of which is regulated appropriately by changes in sodium and water balance.

The nine chapters that follow highlight several areas in which the NH field has subsequently developed. First, de Wardener's original experiments were refined to control potential factors other than GFR and aldosterone. These studies, reviewed by Lichardus (3), confirmed the original observation, and also led to the discovery of the so-called "physical factors" that affect sodium excretion (4). Second, attempts to purify NH from various tissues and body fluids, as summarized in Table 1 by Hamlyn (5), led to the discovery of two important families of factors, atrial natriuretic peptides (ANP), and endogenous cardiotonic steroids (CTS).

The discovery of ANP led rapidly to its characterization as a family of peptides with three major components: ANP derived from cardiac tissue, BNP from brain, and CNP from endothelium (6). This complex system of natriuretic vasodilators, discussed in the chapters by Hamlyn (5), and Hodes and Lichstein (6), continues to be of interest as an NH, a neurotransmitter (6), and a factor with many other functions (7).

In sharp contrast, the identification of endogenous CTS as circulating Na, K ATPase inhibitors has been the source of considerable controversy (8). Two structural classes of CTS have been identified: authentic ouabain, and the bufodienolides, originally identified in toads (9). As discussed by Hamlyn, a bufodienolide is the more likely candidate NH, whereas ouabain in physiologic concentration appears to be antinatriuretic (5). CTS cause vasoconstriction by a mechanism proposed by Blaustein et al. (10) and endogenous CTS have been implicated in the pathophysiology of hypertension (9). Despite overwhelming evidence to the contrary, however, difficulty identifying ouabain in biological fluids continues to be reported (11, 12).

At least three other natriuretic compounds related to control of sodium balance have been identified. As discussed by Gonick (13), a natriuretic protein associated with various forms of human hypertension consists of a 408 Da compound and a 12 kDa carrier protein. The identity of these compounds has not been completed (see below). Two xanthurenic acid derivatives (MW 368 and 284), discussed by Bricker et al. (14), are potential regulators of sodium balance in chronic renal failure. Dietary sodium releases two natriuretic factors from the gastrointestinal tract identified as guanylin and uroguanylin (MW 10.3 and 1.7 kDa), discussed in the paper by Hodes and Lichstein (6).

Two other types of natriuretic compounds that inhibit Na, K ATPase, but are not natriuretic hormones as defined above, are two vanadium diascorbates (MW approximately 400), and two spherical oligo silicic acids (SOSA) (MW 408). These interesting compounds of uncertain significance are

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described in papers by their discoverers Kramer (15) and Kerek and Voicu (16), respectively. The apparent identical molecular weight of SOSA and the compound isolated by Gonick is possibly a coincidence. Alternatively, it may be related to the trace amounts of silicon in human plasma (17) and have significance beyond that implied by the serendipitous discovery of SOSA.

A third area is studies of the physiological mechanism(s) by which these factors might function as natriuretic hormones. ANP release is controlled by stretch of the cardiac atria (18), and in that regard is a classic, volume controlled, NH system as originally conceived (19). Although evidence indicates that CTS are released in response to increased sodium intake (5), no causal mechanism connecting sodium intake and CTS release has been demonstrated. As noted by Hamlyn (5), cerebrospinal fluid sodium concentration controls the release of an unidentified natriuretic factor from brain, causing "CNS natriuresis." The possible role of CTS or some other NH in this phenomenon should be explored.

A fourth area of investigation is studies of collateral, pathophysiological, effects of the various factors. Both ANP and CTS are vasoactive and have been implicated in the pathophysiology of hypertension (5, 6, 9). Since both appear to be neurotransmitters or neuromodulators (6), their role in hypertension may have both central and peripheral components. Pathological effects of excess CTS in the CNS may also include an etiologic role in mood disorders, including depression and bipolar disorder (6).

Finally, the signaling effects of CTS on Na, K ATPase not dependent on ion pumping, mediated by activation of the tyrosine kinase Src and other signaling molecules, is discussed by Xie et al. (20). This pathway is involved in the natriuretic effect of bufodienolides (21), and is implicated in pathophysiological processes, such as oxidative stress, and organ fibrosis (20).

de Wardener's original hypothesis has led to the discovery of a rich array of factors with multiple biologic activities. ANP is the only classical NH described so far. Future work, including the development of methods for antagonizing these factors, should increase our understanding of the regulation of renal sodium excretion and blood pressure and offer novel treatments for several clinical disorders.

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REVIEW ARTICLE
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Early stages of the natriuretic hormone story

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The paper reviews the early stages of the research on natriuretic hormone. The described experimental work was designed and accomplished in several internationally recognized laboratories where the author was invited to extend his projects. The cross-circulation experiments in animals with acutely increased extracellular fluid volume documented, that in the mechanism of natriuresis – besides a series of the physical natriuretic factors – there is still room for an active humoral natriuretic substance. This substance inhibited the sodium transporting enzyme, Na,K-ATPase, in the frog skin. Analogous inhibition of the renal Na,K-ATPase may be partly responsible for the increased sodium excretion. It was further shown that the extent of natriuresis is positively modulated by the concentration of sodium in the cerebrospinal fluid detected in the anterior-third ventricle region (AV3V) in the brain.

Keywords: natriuretic hormone, cross-circulation experiments, Na,K-ATPase, anterior-third ventricle region-AV3V, cerebrospinal fluid sodium concentration

The first International "Symposium on Natriuretic Hormone" was held at the Smolenice Castle, the Congress Center of the Slovak Academy of Sciences in 1969, 12 years after the suggestion of Homer Smith that some such factor could exist (1) and 8 years since the first corroborative experimental data were presented by the team of Hugh de Wardener (2). At the next symposium organized by the same Institution, a decade later we dealt rather with "Natriuretic Hormones" (3). This review is a selected account on the elaboration of the early stages of the hypothesis on the existence of a natriuretic hormone in which I was privileged to participate.

CROSS-CIRCULATION EXPERIMENTS AND "*rein au cou*"

In the experiments of de Wardener et al. (2), evidence was advanced for the transfer of a natriuretic material from the donor dog with expanded extracellular fluid volume (ECFV) by the infusion of saline to the cross-perfused recipient dog. The experiments were arranged in such a way that the provoked natriuresis and urine excretion in the recipient animal was neither a result of an increase of the glomerular filtration rate nor of the decrease of known circulating hormones, anti-natriuretic steroids, and vasopressin. Consequently, a third factor was suggested to be involved.

Our group, in pursuing this provocative idea, modified the cross-perfusion experiment in dogs. Only one kidney *in situ* in the recipient dog was cross-perfused under constant perfusion pressure by the donor's blood in order to expose the recipient kidney to a larger concentration of a natriuretic material than in cross-perfusion of the whole animals. To prevent a possible natriuretic effect of blood dilution by saline infusion rather the blood volume of the donor dog was expanded by an "artificial blood" (suspension of homologous erythrocytes in 6% bovine albumin in Ringer–Locke solution). The cross-perfused kidney increased sodium and urine excretion following the blood volume expansion of the donor dog. However, this "transferred natriuresis" was less pronounced than natriuresis provoked by infusion of saline in the previous experiment of de Wardener. It was concluded that even if the natriuretic effect of blood dilution is eliminated the cross-perfusion experiments may reveal the appearance of a natriuretic factor in blood of the donor dog following its blood volume expansion (4). It was established in other experiments that the bovine albumin in the "artificial blood" *as such* was not critical for natriuresis evoked by blood volume expansion (5).

Yet, in another experimental set up, the homologous kidney was transplanted to the neck of a dog ("*rein au cou*") in which vasopressin and creatinine were infused and DOCA was administered intramuscularly at least 3 h before the urine collection started. Subsequently, the blood volume of the dog with the transplanted kidney was expanded by infusion of homologous blood. The experimental conditions assured constant renal arterial and venous pressures in the transplanted kidney, a constant plasma oncotic pressure and constant hematocrit, glomerular filtration rate, and renal blood flow. Moderate, when compared with the renal output of the other kidney *in situ*, but significant increase in urine output and sodium excretion was observed by the transplanted kidney. Since non-hormonal and hormonal factors modulating sodium and water excretion were kept under control in the transplanted kidney, the results indicated more specifically than in previous experiments that a natriuretic humoral material might play a role in the mechanism of natriuresis provoked by blood volume expansion (6).

The operation of a blood-borne natriuretic factor in rat cross-circulation experiment was shown only when so-called sustained fluid volume expansion was achieved by urine reinfusion in the expanded donor animal. This procedure apparently intensified the natriuretic signal to the recipient animal (7).

A NATRIURETIC OR A DILUTION OF AN ANTI-NATRIURETIC SUBSTANCE?

Our next attempt was to challenge the question that emerged from the cross-circulation experiments, namely, whether the

appearance of a putative blood-borne natriuretic factor in animals with expanded ECFV was the result of an increased concentration of a natriuretic or a dilution of an anti-natriuretic substance. The experiments were performed on un-anesthetized cows, from which substantial volume of blood can be removed without reversing the effect induced by the expansion of their ECFV with 6% dextran in physiological saline (30 ml/kg b.w. at a rate of 100 ml/min). A blood sample of 1000 ml was withdrawn before the infusion and another one at the end of the infusion. The deproteinized plasma was applied intravenously to the assay rats in a volume of 0.2 ml. A non-significant tendency of control samples rather to decrease both the rate of urine flow and sodium excretion in the assay animals, was observed. On the other hand, the samples withdrawn during the ECFV expansion in cows produced statistically significant increases in urine flow, sodium excretion, and the tubular fractional sodium excretion in the assay rats. The sensitivity of the biological assay (i.e., the natriuretic activity applied in 0.2 ml of deproteinized plasma) might indicate that a separate low-molecular weight natriuretic factor might be involved, and that we are not dealing with a mere dilution effect of infusion on an anti-natriuretic activity (8).

The same plasma sample also decreased the short-circuit current representing active sodium transport in the frog skin by a transporting enzyme Na,K-ATPase (9, 10). Buckalew et al. (11) showed that an ultrafiltrate of blood from volume expanded dogs inhibited sodium transport in the toad bladder. It may be another indication for the presence of a natriuretic material in the tested plasma sample as it was proposed that the renal mechanism of natriuresis is via inhibition of the transporting enzyme in the nephron (12).

Further elaboration of the concept of an endogenous inhibitor of the Na,K-ATPase resulted in an attractive hypothesis linking the inhibitor to digoxin-like activity found in some organs, blood, and urine and to the pathogenesis of essential and low-renin arterial hypertension (13–16). Indeed, it was subsequently found that anti-digoxin serum decreased blood pressure in young rats with DOCA-salt hypertension (17). The same anti-digoxin serum, however, was ineffective in suppressing natriuresis induced by ECFV expansion with saline in rats (18). This finding illustrated at that time that the number and nature of substances represented by the endogenous digoxin-like activity had not been satisfactorily answered (19–21), which is true even now.

INVOLVEMENT OF PERIPHERAL AND CENTRAL NERVOUS SYSTEM IN THE SODIUM BALANCE

The reflex regulation of renal water excretion originating in the stretch receptors of heart atria having the vagal nerves as the afferent limb and vasopressin as an efferent limb (Gauer–Henry reflex) was proposed to be applicable also to the mechanism of renal regulation of sodium excretion. The efferent limb of the reflex was presumed to be a natriuretic factor produced in the brain. However, we found that natriuresis induced by infusion of artificial blood in dogs with either innervated or denervated kidneys was not abolished by bilateral vagotomy. Thus, the analogy of reflex renal sodium control with a modified Gauer–Henry reflex did not seem to be primarily at play (22). We, unfortunately, did not offer at that time a more creative conclusion from these experiments. It is obvious today that – 14 years before the discovery of atrial natriuretic peptides – we missed a possibility to speculate about the role of *the heart as such* in the renal sodium excretion during the ECFV expansion. Our shortcoming in judgment was partly due to the fact that others found the afferent signal to travel along afferent sympathetic nerves and the spinal cord which, of course, were not interrupted by vagotomy.

It has been shown in various species of anesthetized or conscious experimental animals that increased concentration or dilution of sodium salts in the cerebrospinal fluid (CSF) interfere, respectively, with renal sodium excretion (23). In our studies on conscious sheep, the increase in the CSF sodium concentration and the simultaneous expansion of the ECFV resulted in a much higher increase in renal sodium excretion in comparison to the effect of ECFV expansion in animals with normal CSF sodium concentration. Dilution of CSF sodium prevented completely natriuresis following the ECFV expansion. This is thus another indication of that the brain may be involved in the control of renal regulation of ECFV by monitoring sodium concentration in CSF (24).

The periventricular organs that lack the blood–brain barrier seem to be critical for changes in CSF and also in the systemic ECF sodium concentration. A critical brain area where the sodium concentration or osmolality is monitored is probably the anterior wall of the third ventricle (AV3V). This conclusion is supported by experiments in conscious sheep with ablated AV3V. The non-lesioned control sheep were in a spontaneous water balance, whereas water balance in the animals with chronically ablated AV3V region was re-established by forced application of drinking water through intrarumenal tube. It was found that ablation of AV3V region blocks natriuresis to hypertonic but not isotonic NaCl load provided the lesioned sheep is in water balance. McKinley et al. (25, 26) suggested that the AV3V region has a role in regulation of renal Na excretion in conditions where the plasma Na concentration increases. It was further proposed that increased renal Na excretion in response to hypernatremia is another cerebrally mediated osmoregulatory response. However, the AV3V region does not seem to be critical for the mechanism of natriuresis induced by ECFV expansion with isotonic saline (24, 26, 27).

The posterior hypothalamus was also found to be involved in the sodium and ECFV balance. Lesions in the posterior nucleus of the hypothalamus are followed by a renal salt wasting syndrome in rats, cats, and man. In both animal experiments and clinical cases, it was shown that this type of negative sodium balance could not be corrected by adrenal steroids. We tried to identify in rats whether the nuclei in the posterior hypothalamus react to the disturbed sodium balance. The rats were given only a 2% saline to drink for 10 days. A control group drank tap water. The hypothalami were then sectioned and the volume of 200 cell nuclei was determined in each animal of the following nuclei: posterior, ventromedialis, dorsomedialis, and arcuate. The distribution of cell nuclear volume size showed a statistically significant decrease only in the posterior hypothalamic nucleus, which might suggest that it could have been specifically influenced by increased sodium concentration in blood and/or in CSF. At the time of this experiment, changes of volume of cell nuclei in the nucleus posterior hypothalami were taken for an indication of their neuroendocrine activity in connection with salt loading (28, 29).

CONCLUSION

Using modified cross-perfusion experiments, in which the blood was not diluted, a methodology was put forward to exclude the natriuretic effect of physical factors. It was confirmed that during blood volume expansion a natriuretic or dilution of anti-natriuretic material was revealed.

It was shown, in an attempt to isolate the presumed substance playing a role in natriuresis following the blood or the whole ECF volume expansion that it may be a substance with a natriuretic activity.

The isolated substance also decreased the short-circuit current in the frog skin, which is an indication for its potential to decrease the activity of the transporting enzyme Na,K-ATPase. This action of the natriuretic substance could be its contribution to the renal mechanism of natriuresis following ECFV expansion.

It was shown in conscious sheep that the brain is involved in the control of renal regulation of ECFV by monitoring sodium concentration in CSF. A critical brain area, as indicated by others, where the sodium concentration or osmolality is monitored is the anterior wall of the third ventricle (AV3V).

Indirect evidence was presented for a neuroendocrine activity of the nucleus posterior hypothalami related to the sodium balance in rats.

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Endogenous digitalis-like factors: an overview of the history

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The sodium pump is a ubiquitous cell surface enzyme, a Na, K ATPase, which maintains ion gradients between cells and the extracellular fluid (ECF). The extracellular domain of this enzyme contains a highly conserved binding site, a receptor for a plant derived family of compounds, the digitalis glycosides. These compounds inhibit the enzyme and are used in the treatment of congestive heart failure and certain cardiac arrhythmias. The highly conserved nature of this enzyme and its digitalis receptor led to early suggestions that endogenous regulators might exist. Recent examination of this hypothesis emerged from research in two separate areas: the regulation of ECF volume by a natriuretic hormone (NH), and the regulation of peripheral vascular resistance by a circulating inhibitor of vascular Na, K ATPase. These two areas merged with the hypothesis that NH and the vascular Na, K ATPase inhibitor were in fact the same entity, and that it played a causative role in the pathophysiology of certain types of hypertension. The possibility that multiple endogenous digitalis-like factors (EDLFs) exist emerged from efforts to characterize the circulating enzyme inhibitory activity. In this review, the development of this field from its beginnings is traced, the current status of the structure of EDLFs is briefly discussed, and areas for future development are suggested.

Keywords: natriuretic hormone, digitalis-like factor, ouabain, marinobufagenin, bufodienolides, cardenolides

BACKGROUND

The regulation of salt and water excretion by the kidneys has occupied investigators since at least from the beginning of the 20th century. Highlights of these investigations are documented in early reviews of the subject, notably those by Epstein (1) and Smith (2) in the 1950s. These reviews supported the existence of a receptor-integrator-effector reflex by which changes in some component of the extracellular fluid (ECF) volume ("volume receptors") caused appropriate changes in renal sodium excretion. Both "efferent factors," the numerous hemodynamic, humoral, and neural factors known to directly affect renal sodium excretion, and "afferent factors," the stimuli that activate the efferent factors, were reviewed.

Smith, separating the factors influencing free water excretion from those affecting sodium excretion, considered the mechanism of the latter as being similar to the former. Based on these and other evolutionary considerations, he postulated that the proposed effector for sodium excretion, which he called "Hormone X", was an anti-natriuretic hormone, analogous to antidiuretic hormone, which had evolved to conserve sodium as our primitive ancestors made their "ascent through the brackish waters of the estuary/to the salt poor lakes and ponds" (Strauss) (2). Aldosterone had been identified in the early 1950s, so Smith's Hormone X was clearly proposed as an additional volume sensitive sodium retaining hormone, decreased levels of which would cause natriuresis in response to increased ECF volume.

At the time of Smith's review, it was well established that two factors were preeminent in controlling renal sodium excretion, glomerular filtration rate (GFR), and aldosterone. Thus, Smith's review set the stage for exploration for a "third factor," a term which did not originate with Smith. The earliest investigators to use the term in print, if not the first, were Bricker et al., who were searching for the mechanisms contributing to the progressive increase in the absolute rate of sodium excretion per nephron as the nephron population decreased in chronic renal failure (3). Bricker et al. were the first to recognize that similar mechanisms might contribute to both volume expansion natriuresis and the renal adaptation to chronic renal failure.

THE CONCEPT OF NATRIURETIC HORMONE

Four years after Smith's review, the mechanism of "volume expansion natriuresis" was addressed in a classic paper by deWardener et al. published in 1961 (4). They showed that natriuresis caused by saline infusion in dogs given large doses of mineralocorticoid was not abolished when GFR was reduced below initial levels by constriction of the aorta above the renal arteries. Furthermore, they showed that blood circulated from volume expanded dogs (donor) to euvolemic dogs (recipient) caused natriuresis in the recipient. Based on these studies, deWardener et al. suggested that volume expansion increased the circulating level of some natriuretic substance, and the concept of "natriuretic hormone" was born.

Three problems quickly emerged after this ground-breaking study was published. First, although the cross circulation studies were careful to control the volume of the recipient dog, the possible effects of blood dilution by the saline infusion in the donor dog were not. In addition, the possibility that the natriuresis in the

recipient dog might be due to suppression of an anti-natriuretic factor as suggested by Smith had not been definitively eliminated. Each of these issues was addressed in the burst of work in other laboratories that followed the original paper by de Wardener et al. Importantly, these investigators effectively dealt with the dilution issue in a subsequent cross circulation study in which the recipient dog was infused with blood from a reservoir in which blood from both donor and recipient was in equilibrium (5). Other studies addressing the issue of dilution were published by Lichardus et al. and others (6). Studies of the effects of blood dilution on renal sodium excretion subsequently led to exploration of the so called "physical factors" on renal tubular sodium reabsorption by a number of laboratories (7).

In essentially all refinements of the cross circulation studies, the natriuresis in the recipient was much less than that in the donor animal, a finding that was never entirely explained, but some interesting observations were made. For example, response in the recipient was increased by infusing blood from the donor into the aorta just above the renal arteries (8), suggesting a short biologic half-life of the circulating natriuretic factor (9). Also, recipient response was enhanced by preventing the donor from excreting the administered volume load, suggesting some effect of "sustained" volume expansion, an interesting but poorly defined concept that has not been explored further (10).

MECHANISMS OF NATRIURESIS

The cross circulation studies did not distinguish between the presence of a natriuretic substance versus suppression of an anti-natriuretic substance. To make that distinction, a number of laboratories reported natriuretic activity in plasma, urine, and/or kidney tissue of volume expanded animals (11–13), the mechanism of which drew immediate interest. The question was whether the factor caused changes in renal hemodynamics or directly inhibited tubular sodium transport systems. The first studies suggesting the latter were performed by Bricker et al. in which inhibition of p-aminohippurate (PAH) transport by rabbit kidney cortical slices was inhibited by plasma from volume expanded subjects (14). Inhibition of transport in renal tubular epithelium was subsequently shown in isolated tubular cells (15).

Other early studies utilized anuran membranes as models of renal tubular sodium transport. Cort and Lichardus reported inhibition of sodium transport as measured by Ussing's short circuit current (SCC) technique in isolated frog skin by deproteinized, concentrated plasma extracts with very high sodium concentrations (16). In more extensive studies using plasma ultrafiltrates with physiological salt concentrations from volume expanded dogs, Buckalew et al. in 1970 showed similar effects on toad bladder SCC of *bufo marinus* (17). Ussing and others had demonstrated that the SCC in anuran membrane was due to active sodium transport, and could be inhibited by ouabain. The demonstration that the putative natriuretic hormone inhibited tubular transport and SCC set the stage for investigation of the effect of this factor or factors on Na, K ATPase. The initial attempts to relate natriuretic hormone (NH) to Na, K ATPase inhibition were unsuccessful. In the best documented studies, Katz et al. were unable to show inhibition of the enzyme in renal cortical microsomes from volume expanded dogs and rats, or an effect of plasma dialyzates from these animals on renal microsomal Na, K ATPase isolated from euvolemic animals (18). However, Gonick et al. subsequently reported that a natriuretic fraction extracted from renal tissue and plasma of volume expanded animals inhibited SCC in frog skin and ouabain sensitive Na, K ATPase isolated from whole rat kidney (19, 20).

Studies of the effect of plasma, and extracts of plasma and urine of volume expanded subjects on sodium excretion in assay animals, usually rats, demonstrated two basic patterns that differed primarily in time to peak and duration of effect. The shorter acting pattern showed an immediate onset, a peak effect in 40–60 min, and duration of about 120 min (21, 22). The longer-acting pattern exhibited a delay in onset of 10–60 min, a peak effect in 2–3 h, and duration longer than 3 h (22). Some initial purification studies indicated that the more rapidly acting factor was found in fractions containing low molecular weight substances, and the longer acting factor appeared in fractions containing high molecular weight substances (23).

NATRIURETIC HORMONE AS INHIBITOR OF Na, K ATPase

Two major developments in the late 1970s and early 1980s caused a shift in the direction of NH research. The discovery in 1981 of atrial natriuretic factor (ANF) by DeBold et al. (24) and its subsequent characterization as a peptide signaling cascade present in many organs displaced most other lines of investigation with regard to the existence and nature of a NH. Early studies did not show an effect of ANF on Na, K ATPase (25, 26); however, subsequent studies revealed a more complex situation (27–29). Nevertheless, it was clear from the early work that ANF and the natriuretic inhibitor of renal epithelial transport systems dependent on Na, K ATPase were two entirely different systems. However, very little further work on the non-ANF NH hypothesis was performed. Instead, the focus shifted to the second major development, namely, that NH might be an inhibitor of vascular Na, K ATPase that could also be a causative factor in certain types of hypertension.

The suggestion that some types of hypertension, especially those associated with ECF volume expansion, might be due to a circulating inhibitor of vascular Na, K ATPase evolved from studies of the phenomenon of potassium-induced vasodilation. Overbeck et al. showed that the dilator response to potassium, but not to other agents, was suppressed in the forelimb of the rat with two kidney, one clip hypertension and the dog with one-kidney, one-wrap hypertension (30). Subsequent studies showed that potassium-induced vasodilation was completely blocked by ouabain, leading to the hypothesis that the vasodilation was due to stimulation of vascular smooth muscle Na, K ATPase. According to this hypothesis, stimulation of the electrogenic sodium pump led to hyperpolarization, decreased voltage sensitive influx of calcium, and hence vascular relaxation (31).

Reduced serum potassium produced identical effects in the opposite direction. That is, hypokalemia was associated with vasoconstriction and suppressed Na pump activity, suggesting a cause and effect relationship. As predicted by this paradigm, vascular depolarization was found in several volume expanded hypertension models. Thus, the hypothesis was proposed that vasoconstriction leading to hypertension might be caused by generalized inhibition of vascular Na, K ATPase activity (32). In a further

refinement of the hypothesis in 1976, based on a review of then existing evidence for a humoral factor that slowly increased blood pressure in both animal models and humans with hypertension, Haddy et al. proposed that Na, K ATPase inhibition in vascular tissue, and hence vasoconstriction, might be due to a circulating factor (33). They, in fact, proposed in that review that the postulated circulating inhibitor of Na, K ATPase might be "natriuretic hormone." Thus, the two fields of ECF volume regulation and regulation of vascular tone in volume expanded models of hypertension were brought together in the search for a common, explanatory factor (**Figure 1**).

A unifying explanation for the connection between vascular tone, intracellular calcium concentration, and the sodium pump was proposed by Blaustein in 1977 (34). The model was based on the presence of a Na–Ca exchanger located in the plasma membrane, driven by the intracellular–extracellular sodium gradient. According to the hypothesis, supported by kinetic calculations (34), inhibition of the sodium pump by the NH would cause increased vasoconstriction by inhibiting the outward transport of calcium by the Na–Ca exchanger.

VOLUME EXPANDED MODELS OF HYPERTENSION

The NH hypothesis of hypertension raised the question of how volume regulation by a potentially vasoconstrictor NH occurred in normal versus hypertensive subjects. Volume expanded models of hypertension involved some manipulation that reduced the ability of the kidney to excrete sodium. This approach was based on the concept proposed by Guyton et al. (35) that all hypertension was caused by an abnormal relationship between blood pressure and renal sodium excretion. According to this hypothesis, in normal subjects, renal adaptation to increases and decreases in sodium intake occur without any or with only small changes in systemic blood pressure. However, increased blood pressure is required to maintain ECF volume regulation in the presence of impaired renal sodium excretion through the phenomenon of "pressure diuresis." Guyton postulated the rise in pressure was due to a volume induced increase in cardiac output and the consequent "long term autoregulation" (i.e., vasoconstriction) that ensued. Thus, ECF volume is maintained at the expense of increased peripheral vascular resistance and high blood pressure.

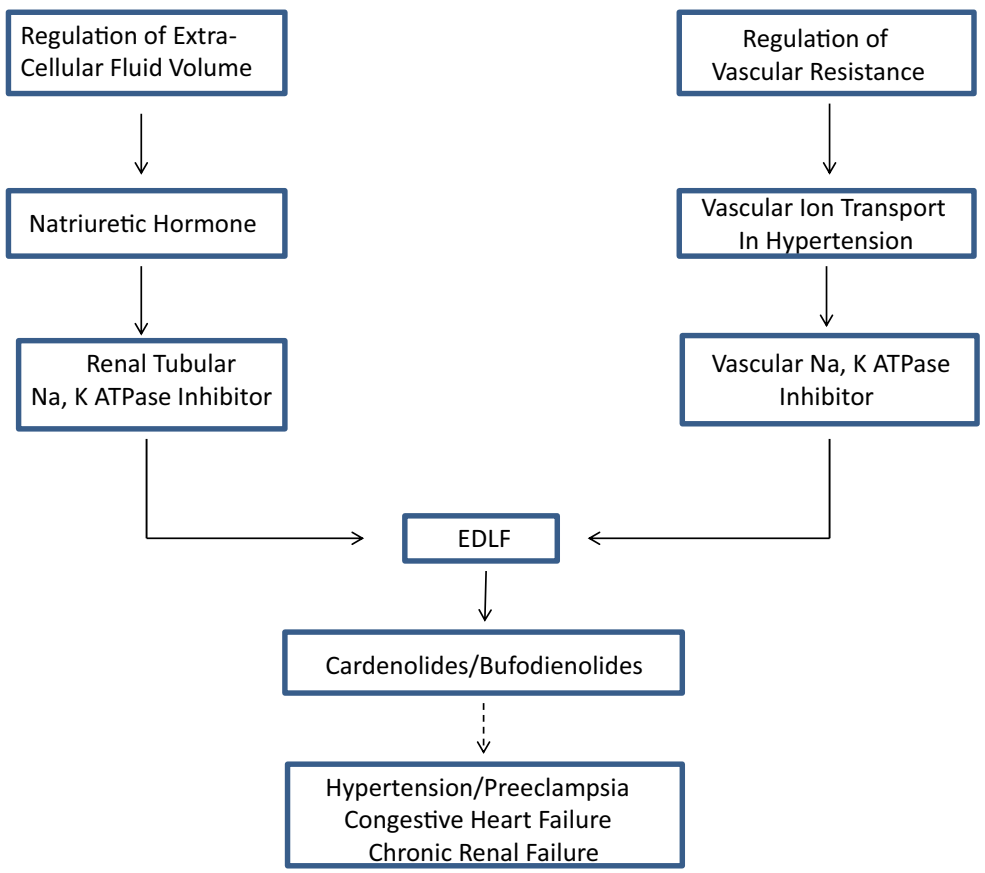

FIGURE 1 | The concept of an endogenous digitalis-like factor (EDLF) that inhibits Na, K ATPase in a manner similar to the cardiac glycosides developed from two lines of investigation (see text), the response of renal sodium excretion to extracellular fluid volume expansion, and the regulation of peripheral vascular resistance in hypertension. Research on the identity of EDLF indicates that mammalian species synthesize two classes of steroids that are either identical to, or analogs of those found in plants (cardenolides) and toads (bufodienolides). Evidence suggests that one or more of these compounds may be involved in the pathophysiology of various hypertensive disorders, chronic renal failure, and congestive heart failure.

Based on this theory, several investigators proposed a unifying hypothesis incorporating NH that explained many observations then existing in the literature (36, 37). According to this formulation, the defect in renal response to increases in sodium and water intake in hypertensive subjects leads to increases in NH, vascular Na, K ATPase inhibition, vasoconstriction, and increased blood pressure. Volume homeostasis is maintained in the presence of a defect in renal sodium excretion by both the rise in blood pressure through the mechanism of pressure natriuresis, and by the effect of the NH to inhibit renal tubular sodium reabsorption. The difference between hypertensive and normotensive subjects was, as suggested by deWardener and MacGregor, that the former would be in a "state of continuous correction of a slightly expanded extracellular volume," resulting in a sustained elevation of NH (37). While this concept may have some general validity, the role of EDLF differs in various forms of hypertension (see below).

ENDOGENOUS DIGITALIS-LIKE FACTOR

Because of the suggestion that NH might be an inhibitor of Na, K ATPase, it was subsequently referred to as "ouabain-like" or "digitalis-like." This terminology became more than nomenclature as the field turned to proving the true digitalis-like nature of the circulating factor.

THE CONCEPT OF ENDOGENOUS DRUG-LIKE COMPOUNDS

The demonstration that the specificity and actions of some drugs were due to drug binding to stereospecific receptors had led to speculation that naturally occurring endogenous compounds existed that bound specifically to these receptors (38). The discovery of endogenous opioids was a direct result of this hypothesis (39). In 1976, Ginzler et al. proposed, as an extension of this concept, that antigen–antibody binding specificity might be analogous to drug-receptor binding specificity (40). That is, an antibody specific for a drug might recognize the same structure as the specific receptor for that drug, and could act as a "surrogate" receptor. This hypothesis had at least two interesting implications. First, antibodies to drugs might recognize endogenous compounds that utilize the same receptor as the drug; and second, antibodies to drugs (or endogenous compounds) might be used to block the effects of those compounds by displacing them from their receptor. The second possibility had already been anticipated by a number of investigators including the demonstration that digoxin antibodies would reverse the clinical manifestations of digoxin intoxication (41).

Based on these concepts, Gruber et al. showed in 1980 that plasma of volume expanded dogs but not euvolemic dogs contained a factor that cross reacted with digoxin antibodies in a specific fashion; i.e., the dose response curve in the digoxin radioimmunoassay (RIA) of the endogenous factor was parallel to that of authentic digoxin (42). Furthermore, plasma extracts containing the digoxin immunoreactive compound inhibited Na, K ATPase, providing further evidence for a true EDLF that had some structural and functional similarity to digoxin. The finding also suggested that digoxin RIAs could be used to study plasma levels of this factor and numerous studies of mammalian "digoxin-like" factor were soon published (43). However, studies using this approach are subject to non-specific cross reactivity of various interfering substances in the digoxin RIA and have led to some confusion (44, 45). Interestingly, the first demonstration of an endogenous substance that cross reacts with digoxin antibodies was in newborns, who were suspected of having been poisoned with digoxin (46).

CHARACTERIZATION OF EDLF

Subsequent to the work briefly described above, numerous attempts have been made to purify and identify the principal factor responsible for the digitalis-like factor demonstrated in volume expanded subjects. According to the initial hypothesis, a truly endogenous, natriuretic, hypertension promoting digitalis-like factor would have the following characteristics. First of all, it would be synthesized endogenously, and secreted under the control of relevant physiological or pathophysiological stimuli. Second, it would inhibit renal and vascular Na, K ATPase in a "ouabain-like" fashion; that is, it would bind to the same receptor and have similar effects on the enzyme as ouabain. Thirdly, its inhibition of renal tubular and vascular Na, K ATPase would cause natriuresis and vasoconstriction, respectively. Unfortunately, attempts to identify such a factor have been complicated by the fact that inhibition of the enzyme in various assay systems is a non-specific effect of many diverse compounds (47). As a result, numerous candidate structures have been identified, including steroids, lipids, peptides, and a variety of other novel compounds (45, 48, 49). A complete review of these reports is beyond the scope of this paper. Rather, we have chosen to focus on a class of compounds that are "digitalis-like" steroids, and which meet the theoretical criteria outlined above, with one exception. They have not been shown unequivocally to be "endogenous" since their synthetic pathway has not been completely elucidated, although preliminary studies suggest they are synthesized in the adrenal gland (50–53).

In 1991, Hamlyn et al. reported purification of a compound indistinguishable from ouabain by mass spectroscopy from 300 l of human plasma (54). Subsequent work seemed to confirm this observation and indicated that mammalian ouabain is present in multiple body fluids and tissues. However, the issue of whether mammalian tissues contain authentic ouabain has remained highly controversial (55–57) despite substantial evidence in support of this finding (58).

Amphibian species have been known for many years to synthesize a number of different steroids called bufodienolides that inhibit Na, K ATPase in a manner similar to the cardenolides (59). Dienolides differ from cardenolides in the structure of the lactone ring, which contains six members and two unsaturated double bonds compared to five members and one double bond in the cardenolides (43). Both cardenolides and dienolides have a 14 β hydroxyl group and a cis tertiary configuration of the C/D ring junction. Lichstein et al. identified a bufodienolide in toad skin and plasma as resibufogenin (60). They also demonstrated that the concentration of dienolides in toad skin was regulated by the salt content and osmolality of its aquatic environment (61, 62).

Bagrov et al. purified a digitalis-like compound from toad venom (63), which they subsequently identified as a previously described bufodienolide marinobufagenin (MBG) (64). Subsequently, purification of a substance from urine of patients after an acute myocardial infarction by high pressure liquid

chromatography confirmed a structure indistinguishable from authentic MBG (65). Using a polyclonal antibody to toad MBG, they demonstrated increased concentration of a compound recognized by that antibody in plasma of volume expanded dogs (66) and rats (67), and patients with preeclampsia (68). Using antibodies specific for ouabain and MBG, they demonstrated that mammalian plasma contains both ouabain-like and MBG-like compounds (66). Subsequent work has demonstrated that the MBG-like compound meets essentially all the criteria originally postulated for the EDLF-type NH described above (69, 70).

Yoshika et al. have shown that MBG immunoreactivity secreted by adrenomedullary derived cells in tissue culture is composed of at least two compounds, MBG and a related compound marinobufotoxin (MBT) (71). MBT was shown to increase blood pressure when administered intraperitoneally to rats (71).

EDLF AND HYPERTENSION

Using multiple assays for EDLF, numerous studies have attempted to show some correlation between plasma EDLF levels and the blood pressure in human and experimental hypertension, details of which have been previously reviewed and are beyond the scope of this paper (69, 72–80) Many of these studies have relied on measurements using RIA technology with antibodies raised against the compound(s) of interest. As noted, these studies are subject to cross reactivity with compounds other than those to which the antibody was raised. Despite these problems, it seems likely that some EDLF(s) are elevated in some forms of human and experimental hypertension and may play a role in its pathophysiology.

Although most studies of the role of EDLF in hypertension have focused on the circulating factors, it seems likely that endogenous ouabain plays a role in certain types of hypertension through a pathway in the central nervous system (CNS). EDLF has been demonstrated in hypothalamic and pituitary extracts of rats, a compound (or compounds) that crossreacts with a polyclonal anti-ouabain antibody (81). Extensive studies by Huang et al. have shown increases in this compound in the hypothalamus of Dahl salt-sensitive rats (82), spontaneously hypertensive rats (SHR) (83), and normal rats in which blood pressure is increased by an increase in cerebrospinal fluid sodium concentration (84). The critical role of brain EDLF in each of these models was demonstrated by prevention of the rise in blood pressure by CNS administration of a commercially available antigen binding fragment (FAB) of an antidigoxin antibody known to cross react with EDLF (Digibind®) (see below). A further complexity in the hypertension promoting CNS EDLF system has been demonstrated by studies of central infusion of angiotensin II in rats. This hypertension provoking maneuver causes an increase in circulating endogenous ouabain through activation of a neuronal pathway involving central aldosterone (85).

An integrated role for both endogenous cardenolides and bufodienolides in hypertension in Dahl salt-sensitive rats is suggested by studies showing that release of MBG is controlled by the CNS ouabain pathway discussed above (86). Further studies on the role of CNS pathways in the pathophysiology of hypertension, and in controlling circulating endogenous digitalis-like factors (EDLFs) and blood pressure should be of interest.

REVERSAL OF EDLF EFFECTS BY FUNCTIONAL ANTAGONISTS

Several functional antagonists of EDLF have been reported to reverse the effects of Na, K ATPase inhibition in various clinical and experimental situations, among which are anti-dogoxin and anti-ouabain antiserum, and two steroid compounds, rostafuroxin and resibufagenin, that may be receptor antagonists of one or another component of EDLF.

Digibind® is a purified FAB of a sheep anti-digoxin antibody developed for the treatment of digoxin intoxication that is no longer available commercially. Studies using Digibind® as a probe to assess the possible role of EDLF in hypertensive subjects assume that it will cross react with EDLF, and that in large enough doses will displace EDLF from its receptor, analogous to its effect in digoxin toxicity. A number of studies are compatible with this formulation. Krep et al. showed that Digibind® reduced blood pressure in the DOCA-salt rat model (87). Kaide et al. obtained the same results in a 5/6 reduced renal mass model (88). In the latter study, no effect of Digibind® on blood pressure was observed in sham-operated controls, suggesting that the blood pressure reduction was not due to some non-specific or toxic effect of Digibind® such as an anaphylactoid reaction. Mann et al. had suggested the latter, but their studies were done with commercial preparations other than Digibind® (89). In addition to these *in vivo* studies, Krep et al. showed that Digibind® reversed the contraction response of isolated aorta to an EDLF isolated from peritoneal dialysis fluid (90). Digibind® has also been reported to reduce blood pressure in several hypertension models when given directly into the CNS (84, 91), to block the natriuresis of saline infusion in dogs (66), and to improve neonatal outcomes in fetuses born to patients with preeclampsia (92).

Antibodies against other glycosides have also been shown to lower blood pressure in animal models. Anti-ouabain antibodies had no effect on blood pressure in normal rats (93). However, immunization against ouabain prevented the development of hypertension in Dahl salt-sensitive rats (94), and reduced sodium excretion in normal rats (93). Also, administration of MBG antibodies lowered blood pressure in Dahl salt-sensitive rats (95). These studies suggest that whatever EDLF might be, whether single or multiple compounds, it cross reacts with antibodies against several candidate EDLFs.

In addition to the work with antibodies, two possible receptor antagonists of EDLF have been reported. Rostafuroxin® is a digitoxigenen derivative that selectively displaces ouabain from the Na, K ATPase receptor (96). The compound lowered blood pressure in Milan hypertensive rats (97), but failed to lower blood pressure in clinical trials in essential hypertension in humans (77). Resibufogenin (RBG) is a bufodienolide isolated from toad skin (60) and the traditional Chinese medication Chan Su made from dried toad venom (98). RBG has a structure that only differs from MBG by one oxygen atom on the 5-position of the steroid nucleus (99). Although RBG has "digitalis-like activity" (inhibits *in vitro* Na, K ATPase activity and ouabain binding) (60), RBG has been shown to lower blood pressure in rat models of preeclampsia and DOCA-salt hypertension (100), both of which have elevated levels of MBG. These data suggest RBG antagonizes at least some effects of MBG, but the exact mechanism has not been elucidated.

Although similar in structure, different cardenolides and bufodienolides have surprising and unpredictable species and tissue differences in their biological actions (101–103), including antagonism of each other's effects (104–107). The mechanism of the latter phenomenon has been extensively studied by Song et al. (105). They proposed a complex set of models in which α and β subunits of Na, K ATPase can function as tetraprotomers with varying degrees of aggregation and pump inhibition. This concept may lead to an entirely new method of manipulating sodium pump function that could have clinical implications.

It should also be noted that ACTH induced hypertension in rats can be prevented by making the α-2 Na, K ATPase receptor for cardiac glycosides resistant to those compounds through genetic manipulation (108). This clearly implicates endogenous Na, K ATPase inhibitor(s) in the etiology of this type of experimental hypertension.

SUMMARY IN RETROSPECT AND FUTURE DIRECTIONS

The search for a factor that regulates renal sodium excretion in response to increased blood volume, a NH, stimulated by the experiments of deWardener et al. (4) has produced a huge body of literature, which can no longer be reviewed in a single article. This review, an update of an earlier one (109), emphasizes how the search for a NH converged with studies of the mechanism of increased vascular resistance in hypertension, resulting in the discovery of EDLF(s) (**Figure 1**). This important discovery has widespread physiologic and pathophysiologic implications and explains, at least in part, the highly conserved nature of the ouabain binding site on membrane Na, K ATPase.

The fact is that, after all the work briefly summarized here, an amazing degree of complexity to a relatively simple if naïve concept has emerged. Regarding the original NH proposal of deWardener et al., no single entity has emerged that fits their hypothesis, and new physiologically relevant natriuretic factors may yet be discovered (9). Atrial natriuretic peptides are clearly volume sensitive natriuretic factors that likely play some role in the renal response to acute volume expansion. ANPs have multiple effects including vasodilation, and one or more of these peptides probably play some role in the pathophysiology of hypertension and congestive heart failure (110). Interactions between ANP and several EDLFs have been demonstrated, which have potentially important physiologic and pathophysiologic implications (27, 111, 112), and further work in the area can be anticipated.

Ouabain, MBG, and other bufodienolides continue to be investigated as putative physiological regulators of renal and cardiovascular function, but no clear integrating hypothesis has yet emerged. MBG appears to fit the criteria for the circulating factor proposed by the original NH hypothesis better than ouabain (72), but ouabain is clearly involved in the regulation of sodium excretion by the CNS and further work on this system is anticipated (9, 73). The intrarenal mechanism by which bufodienolides cause natriuresis, which involves the recently discovered signaling function of Na, K ATPase, should be of ongoing interest (113). The synthetic pathways and tissue(s) origin for the various EDLFs have not yet been completely determined, and high priority should be given to this project (58). If EDLFs play a role in normal physiology and some hypertensive states, as current evidence indicates, interference with their synthesis or antagonism of their effects should provide further insights, and possibly new targets for antihypertensive drugs.

Anti-digoxin antibodies interact with a broad range of EDLFs (114) and have been utilized in both experimental animals and man, with some interesting results (87, 88, 115, 116). Although the commercial preparation used in most of these studies is no longer available, another commercially available preparation of digoxin antibodies (DigiFab®) has similar if not identical cross reactivity with EDLF (116–118). Finally, the ability of individual cardiotonic steroids (CTS) to interfere with the effects of other CTS has added another layer of complexity and suggests another novel approach to the study of and possible therapy of various conditions in which CTS may be involved (105).

Despite ongoing controversy regarding some of the details (58), the hypothesis that an endogenous regulator(s) of the ouabain binding site on the Na, K ATPase enzyme is involved in control of the cardiovascular system has proved to be immensely fertile. Further investigation of these endogenous regulators holds great promise for a better understanding of cardiovascular physiology, the pathophysiology of a diverse set of clinical disorders, including hypertension, preeclampsia, chronic renal failure, congestive heart failure, and cancer (70), and the intricate complexities of the ouabain binding site (108, 119, 120).

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The trade-off between dietary salt and cardiovascular disease; a role for Na/K-ATPase signaling?

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It has been postulated for some time that endogenous digitalis-like substances, also called cardiotonic steroids (CTS), exist, and that these substances are involved in sodium handling. Within the past 20 years, these substances have been unequivocally identified and measurements of circulating and tissue concentrations have been made. More recently, it has been identified that CTS also mediate signal transduction through the Na/K-ATPase, and consequently been implicated in profibrotic pathways. This review will discuss the mechanism of CTS in renal sodium handling and a potential "trade-off" effect from their role in inducing tissue fibrosis.

Keywords: cardiotonic steroids, digitalis-like factors, fibrosis, sodium pump, signaling, renal failure, hypertension

INTRODUCTION

Increased dietary sodium chloride (NaCl) intake has been implicated in cardiovascular and renal diseases for some time (1), and this implication has recently become fairly solid (2). This relationship between dietary sodium intake and cardiovascular disease is demonstrated in several large scale studies, such as the international study of salt and blood pressure (INTERSALT) (3) and the dietary approaches to stop hypertension (DASH) (4). With this relationship so demonstrated, understanding the specific mechanisms underlying the deleterious effects of NaCl becomes timely and relevant to clinical management.

This review will focus on one of the factors linking dietary NaCl to cardiovascular and renal disease. We will specifically discuss the role of digitalis-like factors, also known as endogenous cardiotonic steroids (CTS), which function as innate inhibitors of the Na/K-ATPase (5). Although the existence of these endogenous factors has been controversial (6–8), this is no longer the case. Some of these recent breakthroughs include the chemical identification of specific CTS in experimental animals and humans (9, 10), establishment of normal and pathological concentrations for these substances as well as defining possible roles for CTS in animal models of and human disease states (11–13). We would also stress that the discovery of the cell signaling functions of the Na/K-ATPase and its role in molecular cellular biology (14–16) has also been quite relevant to this field. Here, we will emphasize the role of trade-off with respect to CTS signaling and Na homeostasis.

Abbreviations: Ca, calcium; Cl, chloride; CTS, cardiotonic steroid; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transformation; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; Fli-1, friend leukemia integration 1 transcription factor; GFR, glomerular filtration rate; Grb2, growth factor receptor-bound protein-2; MBG, marinobufagenin; Na, sodium; Na/K-ATPase, sodium potassium ATPase; NAC, N-acetyl cysteine; NHE3, sodium-hydrogen exchanger 3; PI(3)K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; ROS, reactive oxygen species; SERCA, sarcoplasmic endoplasmic reticulum calcium ATPase; Shc, Src homology-2 domain containing protein; SOS, Son of Sevenless protein; TCB, telecinobufagin; TGF, transforming growth factor.

RENAL SALT REABSORPTION AND THE EVIDENCE FOR "THIRD FACTOR"

The microscopic architecture of the kidney involves the attachment of vascular filtering units called glomeruli with tubules that modulate the quantity, electrolytes, and acid-base content of tubular fluid, which ultimately becomes urine. Simplistically, the tubules can be roughly broken down into proximal, where 60–80% of all Na and water reabsorption occur and distal, the nephron segments responsible for the fine tuning of what is excreted as urine.

Clearly the renin–angiotensin–aldosterone system, vasopressin and the sympathetic nervous system are critically important in mammalian volume regulation as well as to the maintenance of blood pressure in the face of a hypovolemic insult (17). However, it is very clear that perturbations in these systems cannot explain natriuretic responses to acute or chronic expansion of blood volume (18). This point was first demonstrated in 1961 in a classic paper by de Wardener and colleagues (19). This study showed that natriuresis induced by saline infusion occurred even if renal perfusion pressure and glomerular filtration rate (GFR, factor 1) and aldosterone concentrations (factor 2) were prevented from changing. This so called "third factor," which we now understand is (are) CTS, was a "hot" topic in the 1960s and 1970s, and was even incorporated into Guyton's model for circulatory

homeostasis (20). Cort and Lichardus observed that a circulating substance in animals subjected to carotid artery occlusion induced natriuresis in different mammals and inhibited sodium transport in frog skin (21). Buckalew showed that an ultrafiltrate of volume-expanded dogs inhibited sodium transport in toad bladders. They went on to propose that the active substance was an inhibitor of the Na/K-ATPase (22). Gonick and coworkers showed that volume expansion in rats, in fact, produced a chemical which did inhibit the ATPase activity of rat kidneys (11). In 1980, Gruber and Buckalew noted that elevated levels of circulating digoxin-like material was seen in volume-expanded dogs (23). Other important contributions were made in the laboratory of Schrier and de Wardener over the next decade (24–26). However, doubt as to the validity of Na/K-ATPase inhibitors developed during the 1980s and 1990s because of inconsistencies in the reported results. In particular, prevailing CTS assays were based on cross-reactivity of CTS with antibodies to digoxin. This cross-reactivity of the commercially employed anti-digoxin antibodies to CTS varied considerably (27–32). Probably, the most important inconsistency was that digitalis did not appear to be natriuretic in normal subjects (33). On this background, atrial (and brain) natriuretic peptide(s) were discovered, were obviously natriuretic, and their concentrations (which could be easily measured) were increased in volume-expanded states (34–38). Undoubtedly, these points deflected interest from the study of CTS. However, enthusiasm was renewed in the recent past for the following reasons. First, several CTS have been isolated from experimental animals and humans and chemically characterized. Specifically, marinobufagenin (MBG) as well as telecinobufagin (TCB) have been isolated from plasma and urine (9). Ouabain has also been identified although there is still some debate as to whether this is ouabain or something distinct, which also reacts to anti-ouabain antibodies (10, 39). The concentrations of ouabain (or ouabain like compound) and MBG appear to be in the range of 200–2700/min in humans, depending on whether disease is present (5, 40, 41). Plasma levels of TCB and bufalin are less well defined at present. Also, quite importantly, a signal cascade has been identified, which does not appear to involve enzymatic inhibition of the Na/K-ATPase. This signaling pathway involves CTS binding of the caveolar Na/K-ATPase in the company of Src and the EGFR and the elaboration of a signal cascade, which involves the generation of reactive oxygen species (ROS) (14, 16). Both of these concepts have been extensively reviewed (42–44).

"TRADE-OFF" CONCEPT, A HISTORICAL PERSPECTIVE

The concept of "trade-off" plays an extremely powerful role in physiology. This is perhaps best described by Neal Bricker who postulated that in renal disease, the hormonal forces driving nephrons to maintain fluid and electrolyte homeostasis would be complicated by the untoward consequences of these elevated hormones mediating other effects, essentially creating the signs, symptoms, and pathophysiologic changes associated with the uremic syndrome (45, 46). As sodium (Na) handling is so critical to volume balance, electrolyte homeostasis, and acid-base status, it is not surprising that Bricker formulated this hypothesis to involve the Na/K-ATPase.

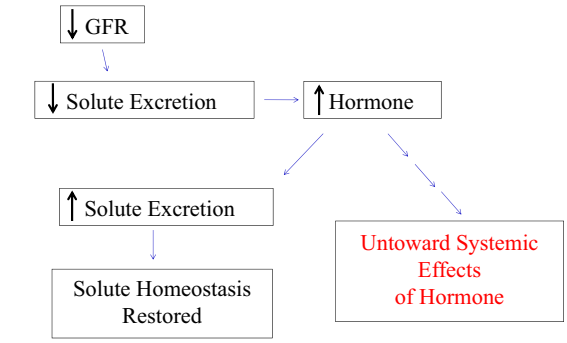

FIGURE 1 | The schematic in figure demonstrates Bricker's proposed trade-off mechanism by which physiologic changes such as reduced glomerular filtration rate (GFR) leading to the increased generation of a hormone can produce the desired effect in solute homeostasis, but with untoward effects in renal and other tissues.

Bricker speculated that an inhibitor of the Na/K-ATPase would circulate in increased concentration as a response to decreased GFR in order to maintain Na homeostasis (45). This inhibition would subsequently lead to decreased renal Na reabsorption, hence the maintenance of Na homeostasis (**Figure 1**). Unintended effects of higher concentrations of this Na/K-ATPase inhibitor would be responsible for some of the symptoms, signs, and abnormal laboratory results seen with chronic renal failure as well as potentially contribute to the progressive nature of chronic kidney disease (45, 47–50). As we will detail in this review, a potential consequence of increases in natriuretic hormone levels, specifically elevated CTS levels may be the profibrotic effects of these molecules (51). Before we address this, however, it may be useful to briefly discuss the evolution of our understanding of the Na/K-ATPase (45, 46), which had been described and characterized several decades before (52).

DISCOVERY OF THE Na/K-ATPase, ITS ROLE IN SIGNALING CASCADES VS. ION TRANSPORTATION

The Na/K-ATPase was discovered by Skou in 1957 (53). This protein was demonstrated to be responsible for the electrogenic exchange of sodium and potassium (54). The Na/K-ATPase, also called the sodium pump, is present in all living cells (55). Although there has been some evolutionary modification of the sodium pump, in all multicellular animal cells, the sodium pump consists of (at least) a dimer of an alpha and beta subunit and is considered a member of P-type ATPases (43). Different isoforms of the alpha and beta subunits have been identified and are believed to have functional differences, a topic which has been extensively reviewed (56). Genes encoding the alpha-1 and alpha-2 isoforms reside on the chromosome 1 whereas alpha-3 appears to be coded for on chromosome 19 and alpha-4 (present only in sperm) is mapped to chromosome 13 in humans (57). The act of pumping sodium and potassium is accompanied by changes in conformation and phosphorylation state (43). It also requires energy provided by the hydrolysis of ATP as was initially identified also by Skou (58).The work of Skou was ultimately matured into the currently accepted Post-Albers model

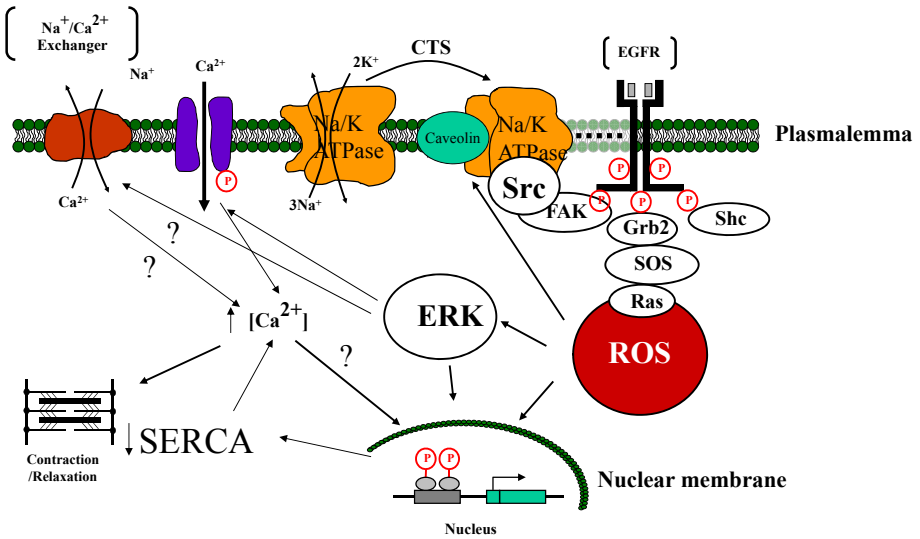

FIGURE 2 | A schematic illustrating the involvement of cardiotonic steroid (CTS) – induced Na/K-ATPase signal cascade initiated by the Na/K-ATPase mediated activation of Src tyrosine kinase and subsequent downstream targets eventually leading to the development of reactive oxygen species (ROS). Specifically, we postulate that in the microdomain of caveolae, the Na/K-ATPase functions as a scaffolding protein, interacting with CTS and changing conformation so as to active Src. Src then trans-activates the EGFR which leads to a signal cascade involving FAK, Shc, Grb2, and SOS resulting in the generation of ROS which in turn activates additional Na/K-ATPase molecules as well as causes downstream activation of ERK as well as effects on the nuclear transcription (43). ERK activation has effects on both L-type channels and possibly the Na/Ca exchanger with net effect to increase cytosolic Ca in some tissues (15). Nuclear effects in myocardial tissue include downregulation of SERCA transcription and translation (70). Abbreviations: EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; Shc, Src homology-2 domain containing protein; Grb2, growth factor receptor-bound protein-2; SOS, son of sevenless protein; ERK, extracellular-signal-regulated kinase; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase.

for Na/K-ATPase pumping function (43). The alpha 1 subunit of the Na/K-ATPase has 11 transmembrane domains as well as several well defined cytosolic regions referred to as the N, P, catalytic, and A domains (43). Interestingly, the development and maintenance on an evolutionary scale of caveolin and Src binding motifs, which are scattered throughout these cytosolic domains appeared to occur between single celled animal structures and slime mold (59).

In the late 1990s, the laboratory of Dr. Zijian Xie added a significant wrinkle to this understanding. While it is certainly possible that some signaling does occur through the chemical inhibition of the plasmalemmal Na/K-ATPase, it does appear that other mechanisms must be proposed to explain the signaling. In fact, it appears that the specific Na/K-ATPase molecules responsible for the greatest amount of signaling in response to the binding of CTS are actually not involved in pumping sodium or potassium (60). In the late 1990s, Dr. Xie and colleagues observed that in neonatal cardiac myocytes, ouabain caused increases in ROS measured with CMDCF (14). It was further noted that some of the downstream effects of ouabain were blocked by *N*-acetyl cysteine (NAC) or vitamin E. These increases in ROS could be demonstrated even when cytosolic calcium was maintained low by removal of extracellular calcium (16). It was further noted that Ras activation appeared to be necessary to see increases in ROS (16). Other studies determined that interactions between the Na/K-ATPase and Src appeared to initiate the signal cascade. The alpha 1 subunit of the Na/K-ATPase binds Src and appears to maintain it in an inactive state. However, binding a CTS appears to alter the Na/K-ATPase structure allowing Src to became activated which, in turn, trans-activates the EGFR, and begins the signal cascade which causes increases in ROS (61–64). The Na/K-ATPase–Src complex appears to function similar to a receptor tyrosine kinase. Downstream activation of PLC, PI(3)K, and PKC has also been established (15, 65–68) (**Figure 2**). The role of ROS in pump signaling has been extensively reviewed elsewhere (14, 16, 51, 69).

Although inhibition of the Na/K-ATPase is certainly one possible mechanism by which digitalis and related molecules might "signal," it is important to emphasize that even transporting epithelia typically have a redundancy of Na/K-ATPase pumping units given that cytosolic Na levels live within a range ideally suited to regulate Na/K-ATPase activity. While it is possible that certain compartments of the cell see higher local concentrations of Na with modest inhibition of Na/K-ATPase pump activity, we emphasize that physiological and even pharmacological concentrations of digitalis do not demonstrably increase cytosolic Na concentrations in physiologically relevant preparations (42). We would further point out that most studies, including those from our lab, which demonstrate inhibition of the Na/K-ATPase by circulating substances do so with strategies to control for the cytosolic Na concentration (71–74).

Approximately one decade ago, a further analogy of Na/K-ATPase signaling to the signaling of receptor tyrosine kinases was established with the observation that CTS binding to the Na/K-ATPase in renal tissues triggers endocytosis of

the CTS-Na/K-ATPase complex (75). Subsequent studies have demonstrated that this internalization is associated with endosomal accumulation of the Na/K-ATPase and its caveolar signaling partners, and that the process requires both caveolin (and caveolar structure) and clathrin (76, 77). We have gone on to demonstrate that this process appears to also regulate the expression of the apical sodium transporter, NHE3, as well as impact renal salt excretion *in vivo* (78–80). Recent data from the laboratory of Dr. Lingrel utilizing novel genetic manipulations of the different alpha 1 isoforms in mice indicate that it is the alpha 1 subunit, which can be considered the functional receptor for these CTS. Interestingly, the amount of Na/K-ATPase alpha 1 subunit as well as it is affinity for CTS appear to both positively correlate with the magnitude of the signaling effect (81–84).

Recently, we have made several observations that bring the consideration of ROS in the context of Na pump signaling in a new light. First, we found that the Dahl salt-resistant (R) strain of rats had a natriuretic response to a high salt diet, which did not require substantial increases in blood pressure (hence the term "salt resistant") and was accompanied by activation of Src and ERK as well as redistribution in the renal proximal tubule cells of the basolateral Na/K-ATPase and apical NHE3. This was previously observed with the wild type Sprague Dawley animals (which were used as a founder population to generate Dahl R and salt sensitive, S, rats). In contrast, the Dahl S rats did not have this redistribution. Isolated proximal tubules from young Dahl R and S rats maintained on a low salt diet demonstrated ouabain sensitivity and insensitivity, respectfully, in terms of Src and ERK activation as well as redistribution of the NaK-ATPase and NHE3 (85). Moving back to LLC-PK1 cells, we noted that the signaling observed with ouabain or other CTS could be duplicated by exposure to an ROS generation system (Glucose Oxidase + Glucose), blocked by anti-oxidants (e.g., *N*-acetyl cysteine) and was accompanied by specific carbonylation of two amino acids in the A domain portion of the alpha 1 subunit (86). Given that the proximal tubules of Dahl S rats demonstrate considerable carbonylation of plasma proteins including the Na/K-ATPase prior to exposure to high salt *in vivo* or ouabain *in vitro* (unpublished data), this suggests that chronic oxidation of the Na/K-ATPase may lead to impaired signal transduction in the proximal tubule and a form of oxidant "fatigue." Perhaps of even greater importance, the protein oxidation seen with both ouabain and glucose oxidase/glucose was found to be reversible in a biochemical rather than a physiological sense since removing ouabain or glucose oxidase/glucose led to the return to non-carbonylated proteins regardless of whether new protein synthesis or protein degradation were inhibited. In addition, signaling through the Na/K-ATPase appeared to impact the amount and degree of protein carbonylation induced by glucose oxidase/glucose suggesting a role for the Na/K-ATPase as both a receptor and amplifier of ROS (86). We had seen *in vivo* data supporting this concept in earlier studies discussed below. Although a feed-forward system (which this appears to be) suggests ongoing amplification, it seems clear that endocytosis of this molecular machinery would be an effective termination mechanism (87). Whether the oxidatively modified Na/K-ATPase is a trigger for endocytosis is a topic we are actively investigating at present.

On this background, it is useful to consider whether a CTS is effectively natriuretic *in vivo*. This discussion began many years ago regarding the CTS pharmacological agent, digoxin, or digitalis, which was noted to effect natriuresis in patients with congestive heart failure but not normal subjects (88). Currently, there remains debate as to whether a CTS such as ouabain is, in fact, natriuretic (89). Although clearly this is important in understanding the physiological relevance of the molecular mechanisms described above, we would caution the reader that the answer to this question may be different depending on the physiological state of the experimental animal or subject at the time of the study (80, 85, 90). That said, we would certainly concede that a correlation between renal Na/K-ATPase signaling or inhibition and natriuresis may not always be present.

ROLE IN CARDIAC AND RENAL FIBROSIS WITH EXPERIMENTAL RENAL FAILURE

Concern that CTS signaling through the Na/K-ATPase might be profibrotic grew from several studies. First, we observed that experimental renal failure produced cardiac fibrosis in both rat and mouse (91). We would stress that human uremic cardiomyopathy is believed to also be complicated by fibrosis. When we performed active immunization prior to induction of experimental renal failure, the cardiac fibrosis was markedly attenuated. In a separate group of animals, infusion of MBG designed to achieve similar plasma levels of MBG as seen with experimental renal failure also caused cardiac fibrosis. Evidence for Na/K-ATPase signaling (e.g., Src and ERK activation) was see in both animals subjected to experimental renal failure or MBG infusion whereas active immunization against the MBG-Albumin conjugate attenuated this in the experimental renal failure group (51, 70, 91, 92). In addition, blockade of Na/K-ATPase signaling with active (or passive) immunization as well as pharmacologic blockade (see below) dramatically attenuated the oxidant stress in tissues seen with experimental renal failure (51, 91, 93, 94). Based on these animal studies, we next examined how CTS affected fibroblasts grown in culture. We noted that CTS (e.g., MBG, ouabain) induced increases in fibroblast collagen production as evidenced by either increased labeled proline incorporation or procollagen expression determined with Western blot. Evidence for Na/K-ATPase signaling (e.g., Src or ERK activation) could be observed as well. Moreover, ROS scavenging or pharmacological or molecular biological Src inhibition prevented increases in proline incorporation and collagen production seen with CTS. An increase in transcription was identified as we saw substantial increases in both mRNA for collagen as well as luciferase in cells transfected with a reporter construct following exposure to CTS. However, we did not see evidence for increased TGF beta signaling in these cells although pharmacological antagonism of the TGF beta system did block CTS stimulated collagen production (51). We next examined how CTS affected Fli-1 expression, stimulated by work performed by Watson and colleagues. Fli-1 is a negative regulator of collagen synthesis (95), and we noted that CTS induce decreases in Fli-1 expression in several types of fibroblasts (cardiac, renal, and dermal). We also observed that decreases in Fli-1 appear to be necessary for MBG to induce increases in collagen. Additional work showed that CTS induce translocation of PKCdelta from the cytosol to the nucleus in a PLC

dependent manner. It appears that the translocation of PKCdelta causes Fli-1 phosphorylation and subsequent degradation (94).

These studies next led to work examining the effects of mineralocorticoid antagonists. We should first say that Finotti and colleagues reported 30 years ago that spironolactone and canrenone were antagonists of ouabain binding to the Na/K-ATPase (96). We looked at whether this observation was applicable to our system. *In vitro*, we saw that both spironolactone and canrenone could attenuate MBG-induced increases in collagen production in cardiac fibroblasts. Interestingly, we could not see a substantial effect of aldosterone on cardiac collagen production. Our *in vitro* observations were extended to *in vivo* studies where we saw that administration of spironolactone to rats with experimental renal failure markedly attenuated the observed cardiac fibrosis (94). This suggests the Na/K-ATPase signaling cascade may be a useful target for therapeutic drug development.

Further studies have demonstrated that the effects of MBG (and other CTS) are not specific for cardiac fibroblasts. We have noted that renal fibroblasts have a very similar response as cardiac fibroblasts, suggesting a potential pathological role for MBG in producing renal fibrosis and progressive renal failure. Using MBG infusion in the rat, we saw that such infusion was associated with the induction of Snail, a transcription factor known to be involved in epithelial–mesenchymal transformation (EMT). In LLC-PK1 cells grown in culture, MBG induces EMT in a dose and time dependent way (97).

TRADE-OFF WITH RESPECT TO CTS

With the aforementioned data, we would suggest that the CTS signal cascade through the Na/K-ATPase fits the concept of "trade-off." Specifically, CTS concentrations increase in response to volume expansion and/or salt loading. These CTS mediate increases in urinary Na excretion, maintaining Na homeostasis, but the endocytosis machinery may fatigue with ongoing stimulation. Moreover, there are other consequences of the elevated CTS concentrations, namely vasoconstriction and hypertension along with fibrosis, which was described above (**Figure 3**). The fibrosis may lead to further renal insensitivity in terms of natriuresis, and the combination of events cascading to produce progressive cardiovascular disease.

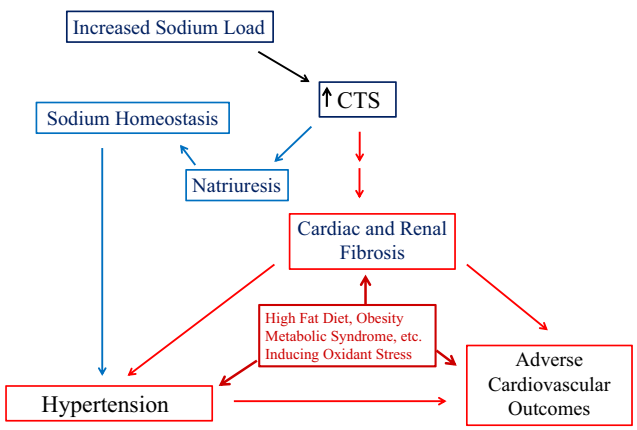

FIGURE 3 | The schematic shown in figure illustrates the balance between the natriuretic effect of cardiotonic steroids (CTS) and the trade-off of inducing Na/K-ATPase-mediated signal transduction leading to cardiac and renal fibrosis, eventually contributing to the development of hypertension and adverse cardiovascular outcomes. In addition, chronic metabolic states resulting in the production of reactive oxygen species (ROS) creating oxidative stress may exacerbate the progression of cardiac and renal disease.

FUTURE DIRECTIONS

As we better understand the role of CTS signaling through the Na/K-ATPase, several therapeutic targets come to mind, which may provide novel and effective therapy for different chronic diseases. First, there is the interaction of the CTS with the Na/K-ATPase. This has been addressed experimentally in our laboratory with both active and passive immunization (51, 91, 93, 98) as well as pharmacologically with several different approaches (94, 99). Other groups have developed different substances which can loosely describe as "ouabain antagonists" which we have recently reviewed (5). Rostafuroxin has been very well characterized and appears to have potential for the treatment of hypertension (100, 101). Recently, our laboratory has begun to develop strategies to alter the interaction between the Na/K-ATPase alpha 1 subunit and Src (102). However, it is clear that the aforementioned signaling cascade affords a number of possible sites for intervention including but not limited to the generation of ROS (69), activation of Src and activation of ERK. Unfortunately, these molecular targets will also fit under the general rubric of "trade-off." Although some aspects of CTS and signaling through the Na/K-ATPase may be maladaptive as we have discussed in this review, it is almost certain that that inhibition of this CTS-Na/K-ATPase pathway may have deleterious effects which need to be navigated.

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Natriuretic hormone: the ultimate determinant of the preservation of external sodium balance

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The present manuscript focuses on a putative natriuretic hormone. It includes the history of a long-term search for the pure molecule, ranging from partial purification to synthesis. It includes a description of seven different bioassay systems used, a resume of the sequential steps in purification, and a summary of a series of experimental protocols employed in the effort to define the biologic properties of the inhibitor of sodium (Na) transport. Two closely related molecules were purified and synthesized. Both are xanthurenic acid derivatives (xanthurenic acid 8-O-β-D-glucoside and xanthurenic acid 8-O-sulfate). It is concluded that one or both of these two low molecular weight compounds (MW: 368 and 284) meet many of the criteria for the final modulator of Na excretion.

Keywords: natriuretic hormone, sodium transport, ENaC, xanthurenic acid derivatives, synthesized NH

INTRODUCTION

It is our assumption that there is a single natriuretic hormone that serves to modulate the renal excretion of sodium (Na) so as to preserve an ongoing equality between consecutive 24 h Na excretion by the kidneys and the contemporaneous intake of Na. The focus on what we presume to be a hormone was initiated from the classic experiments of DeWardener et al. (1–3). The present symposium provides a contemporary review of the state-of-the-art of natriuretic hormone research by key investigators who have pursued the identity and biologic properties of several different putative natriuretic hormones.

Because of the design of DeWardener's protocol, it appeared likely that all of the factors then known to affect the renal excretion of Na could be excluded as the definitive control element. These included changes in glomerular filtration rate (GFR) (so-called "first factor") and changes in mineralocorticoid hormone activity ("second factor"). We coined the term "third factor" (4), which subsequently was replaced by "natriuretic factor."

Throughout a long period of time, a multinational group of investigators has sought to isolate and characterize the natriuretic hormone. To this point in time, despite considerable progress, the ultimate goal remains elusive. Hence, the present symposium.

Our interest in an endogenous natriuretic hormone arose out of long-term studies on the pathologic physiology of chronic progressive renal disease (CRD) (5). It was the pattern of Na excretion that evolved as nephron destruction proceeded.

What is a remarkable change in the estimated Na excretion rate per nephron from the beginning to the end of CRD is presented in **Table 1**. In constructing this table, the intake of Na per 24 h was maintained constant (e.g., at 120 mEq/day) and at all levels of GFR, external Na balance was preserved (the latter is not unusual down to a GFR as low as 10% of normal.).

At a normal GFR of 120 ml/min in an 80 kg person, Na balance is achieved by the excretion of 1/2 of 1% of the filtered load of Na. A fall in GFR to 60 ml/min mandates the excretion of 1% of the filtered Na. A further reduction of GFR (to 30 ml/min) due to the progression of the underlying renal disease requires the excretion of 2% of the filtered Na. And at a GFR of 15 ml/min, external Na balance is achieved by the excretion of 4% of the filtered Na. Thus, per unit of Na entering the extracellular fluid on a constant Na intake, the excretion rate per milliliter of residual GFR with no time delay increases by eightfold. Finally, in many forms of CRD, single nephron GFR (SNGFR) doubles as nephron loss advances. Na excretion rate per residual nephron per milliequivalents of Na intake, approaches 16 times the value in the normal subject.

BIOASSAY SYSTEMS

Seven different bioassay systems were used in both the sequential steps in the isolation, purification, and synthesis of NH and the studies of the biologic effects of the test materials. Each assay system allowed for a quantitative measure of the inhibitory effects of a test sample on Na transport either *in vitro* or *in vivo*.

The primary *in vitro* systems (frog skin and toad bladder) (6, 7) involve polar epithelial cells that transport Na from the serosal surface of the membrane across the mucosal surface. Na transport is quantified by the short-circuit current across the isolated membrane (in an Ussing chamber). Activity of partially purified to pure NH was detectable only when the inhibitor was added to the serosal surface of the membrane.

In micropuncture studies (8), isolated cortical collecting tubules, dissected from normal rabbits were perfused with partially purified test material from the urine of uremic patients. The perfusate was delivered into the lumen of each nephron segment and the peritubular surface was bathed in a solution of known composition (see Experimental Data).

The *in vivo* assays were performed in rats (9). The test materials were delivered intravenously, intraarterially, or via a gastric tube.

Table 1 | Adaptation in Na excretion per nephron in advancing CRD on a constant salt intake.

GFR ml/mn	Na in mEq/day	Na out mEq/day	μl SNGFR % of normal	Na out per nephron nEq	Magnification per nephron
120	120	120	100	700	1
60	120	120		1,400	2
30	120	120		2,800	4
15	120	120	200	5,600	8
7 1/2	120	120		11,212	16

See text in reference to the increase in SNGFR. Column 6 depicts the exponential increase in sodium excretion per nephron per unit of sodium intake.

In the rat assay, the rats employed had: (1) two normal kidneys; (2) one remnant kidney (75% reduction of renal mass) and a contralateral normal kidney; or (3) a solitary remnant kidney following removal of the normal kidney.

PURIFICATION OF NH

Twenty-four hour collections of urine were reduced from their original volume to approximately 20 ml of sludge by lyophilization. The sludge was then redissolved in isotonic saline to 25 ml components each equal to 6 h of original urine. Each sample then was chromatographed through Sephadex G-25 with online monitoring of UV absorption at 290 nm and electrical conductivity (10).

Biologic activity was limited to the "post-salt" peaks using the frog skin assay (11). Short-circuit current decreased from 40–50 to 20–30 μAmp/cm^2. These samples were purified using high performance liquid chromatography (HPLC) runs. The active fractions were purified further by two consecutive HPLC runs. The eluate was monitored by fluorescence and UV absorbance.

After further concentration, a bioassay was performed by infusion of the test material into the uremic rat (11). A strong and sustained natriuresis was recorded.

The natriuretic fractions, consistently showed two peaks with strong fluorescence (excitation 332 nm; emission 430 nm) and characteristic UV absorption (UV max at 338 nm). These spectroscopic signatures were used as the main pooling criteria for further HPLC-based purification of NH (11). Additional steps in isolation, purification, and synthesis of the two molecules: xanthurenic acid 8-O-β-D-glucoside and xanthurenic acid 8-O-sulfate are described in a separate publication (11).

EXPERIMENTAL DATA

UREMIC VS. CONTROL SUBJECTS

Based on the adaptation in Na excretion in advancing CRD (see **Table 1**), bioassays were performed on normal rats using partially purified urine samples from 17 patients with advanced CRD (mean GFR 8.7 ml/min) and 14 normal control subjects. The assays from the normal subjects were negative [i.e., no significant increase in either absolute Na excretion ($U_{Na}V$), or the fraction of filtered Na excreted (FE_{Na}) (12)]. The uremic fractions produced a highly significant increase from baseline levels in both parameters of Na excretion. With more concentrated samples of the uremic urine fractions, values for ΔFE_{Na} rose to levels as high as 12%.

ADVANCED CRD WITH A SUPERIMPOSED EDEMA-FORMING STATE: (THE NEPHROTIC SYNDROME)

Eight patients with advanced CRD and the nephrotic syndrome were studied (12). Assays were performed on normal rats using partially purified fractions of serum in all eight studies. Fractions of urine were also used in three studies. Values for $U_{Na}V$ decreased from control levels (by an average of 0.97 μEq/min). The mean value for FE_{Na} also decreased (1.35%).

Danovitch et al. (13) studied a group of five patients with far advanced CRD (GFR 5.2–16.0 ml/min) who initially were in Na balance on controlled metabolic diets containing from 58 to 342 mEq of Na per day. In each patient, while under close observation (clinical and laboratory), the Na content of the diet was reduced at intervals of 1 week or longer over 4–14 weeks. Four of the patients exhibited a salt-losing state, wherein Na excretion exceeded Na intake. Two of these patients required intravenous salt replacement. At the completion of the studies, all patients maintained external Na balance while ingesting a mean of 5.0 ± 2.9 mEq of Na/day. Thus, in contrast to patients with advancing CRD who maintain Na balance on a constant salt intake and a progressively decreasing nephron population, these patients were subjected to a progressive reduction of Na intake with an unchanging nephron population. (GFR remained constant throughout the studies.) The adaptive increase in Na excretion in advancing CRD (**Table 1**), thus was reversed with slow (and cautious) serial reductions in salt intake. The salt-losing tendency of CRD was reversed.

MICROPUNCTURE STUDIES

As described under Bioassay Systems, partially purified natriuretic factor was infused into the lumen of isolated cortical collecting tubules of normal rabbits. No effect was observed with intraluminal infusions. However, when the natriuretic material was added to the solution bathing the peritubular surface, the effects were rapid in onset and highly significant. Net Na flux (measured isotopically) from luminar to peritubular surface of the nephrons, decreased from 6.29 to 3.20 pmol/s ($p < 0.001$) with no change in Na flux in the opposite direction. Potential difference rose rapidly from −22.5 to −12 mV. Control studies, using the same fraction from normal subjects, had no effect (8).

STUDIES ON NORMAL HUMAN BEINGS EXPOSED TO WATER IMMERSION

Epstein et al. (14) have established water immersion to the neck as a reliable and reproducible stimulus evoking natriuresis in normal subjects. The experiments were performed on 12 normal adults (15). Each was studied twice – once under control conditions sitting in a chair and again during water immersion. The paired studies were performed at the same time of day. Partially purified urine samples were assayed for natriuretic activity in normal rats.

$U_{Na}V$ and FE_{Na} did not change from the baseline values in the control studies. During water immersion $U_{Na}V$ rose (1.27 ± 0.28 μEq/min) and FE_{Na} increased from pre-immersion baseline values by 1.29 ± 0.21%. Both changes were highly significant ($p < 0.001$) (15).

STUDIES IN DOGS

Normal dogs were maintained on a Na intake varying from 3 to 258 mEq/day, with and without fludrocortisone. Urine was partially purified and assayed for natriuretic effect in normal rats and for inhibition of Na transport in isolated toad bladders. In dogs on the 258 mEq Na diet and 0.2 mg fludrocortisone/day, both a statistically significant natriuretic response and inhibition of short-circuit current ($p < 0.001$) were observed. In dogs fed 3 mEq of Na per day with fludrocortisone, no significant effect was found in either assay system (16).

END-ORGAN RESPONSIVENESS

The effects of nephron loss on the natriuretic response (9) to partially purified natriuretic factor were studied in three groups of rats, each on a normal salt diet.

1. Group 1: normal rats: intraarterial infusion into one renal artery produced a unilateral natriuresis.
2. Group 2: intraarterial infusion into the renal artery of a unilateral remnant kidney in rats with a contralateral normal kidney produced an increase in FE_{Na}, which was equal bilaterally. Intravenous infusion of the natriuretic fraction also produced comparable increments of FE_{Na} in the remnant and the normal kidneys.
3. Group 3: in uremic rats with a solitary remnant kidney (no contralateral kidney), the intraarterial infusion of natriuretic factor produced an increase in FE_{Na} that was significantly greater than in remnant kidneys of group 2 rats or normal kidneys of group 1.

NATRIURETIC RESPONSE TO SYNTHESIZED (PURE) NH

Synthesized preparations of xanthurenic acid 8-O-β-D-glucoside (NH) and xanthurenic acid 8-O-sulfate (NH-1) were bioassayed in normal rats (11).

The intravenous infusion of NH (range 0.14–16.4 nmol) and NH-1 (0.7–4.21 nmol) was studied in eight and five normal rats, respectively. In the NH group, $\Delta U_{Na}V$ averaged 3.68 ± 0.55 μEq/min; in the five experiments in which NH-1 was the test substance, $\Delta U_{Na}V$ averaged 4.33 ± 0.71 μEq/min (1).

In five additional studies, a combination of NH (1.4–3.41 nmol) and NH-1 (1.75–5.44 nmol) was assayed. $\Delta U_{Na}V$ averaged 5.0 ± 0.89 μEq/min.

STUDIES BY HOFFMAN AND ASSOCIATES

In a recent publication, Hoffman et al. (17) studied the effects of xanthurenic acid 8-O-β-D-glucoside on Na excretion in adult male Sprague-Dawley rats. Each rat was given two consecutive incremental doses ($6.3 + 31.5$ nmol) of NH (designated as XAG). Values for $\Delta U_{Na}V$ were 3.21 ± 1.12 and 3.99 ± 0.95 at the two dosages, respectively. Values for ΔFE_{Na} increased significantly (1.63 ± 0.46) during the second dose of XAG.

In these studies, GFR (inulin clearance) remained unchanged. Mean arterial pressure (MAP) and total renal blood flow were recorded electronically every 5 min for 60 s during the control periods and for 30–40 min periods during the XAG infusions. All hemodynamic values remained stable (17).

Two important new observations were made by Hoffman and coworkers:

1. In rats pretreated with amiloride, an inhibitor of $E_{Na}C$, the epithelial Na channel in the distal tubule, the natriuretic effects of XAG were completely abolished (17).
2. In rats subjected to chronic blockade of the NO system, the natriuretic response to XAG was diminished suggesting to these investigators that the renal effects of XAG could be mediated in part by activation of the renal NO system (17).

DISCUSSION

A large number of factors, both humoral and physical are known to influence the renal tubular transport of Na. But none of these, including changes in GFR or mineralocorticoid hormone activity (see Introduction) is believed to be the final modulator of net Na transport and thus of Na excretion. We believe that natriuretic hormone fulfills this role. It thus would serve as the definitive element of a sophisticated biologic control system that is charged with the preservation of Na balance and with the constancy of the extracellular fluid volume. But, while there may be virtual unanimity of opinion about the existence of natriuretic hormone, there is no such unanimity about the nature of this hormone.

In this manuscript, we have reviewed the properties of a natriuretic factor, which we have pursued for a long period of time. The activity was obtained from both serum (or plasma) and urine using material that ranged from partially purified to pure, chemically synthesized molecules (11). We believe that the experimental data reviewed in the manuscript meet at least some of the criteria for natriuretic hormone. These include:

1. The rapid and reversible inhibition of net Na transport across polar epithelial cell systems, including the distal portion of the nephron.
2. The foregoing biologic activity is present only when the inhibitor is added to the peritubular surface of the nephron or the serosal surface of equivalent *in vitro* models.
3. The natriuretic effect of the purified and synthesized material is completely blocked by prior administration of amiloride, an inhibitor of $E_{Na}C$ activity in the distal portion of the nephron.

   The mechanism of this action could relate either to a direct effect on the number and/or the activity of $E_{Na}C$ channels, or to a change in the relation between open vs. closed $E_{Na}C$ channels.
4. Inhibition of Na transport in the distal nephron associated with a rapid shift in transepithelial electric potential difference (i.e., a less negative intraluminal potential).

   This change would favor the excretion of Cl with Na as opposed to increased secretion of K.
5. No evidence for a fixed coupling ratio between the inhibition of Na transport and K secretion.
6. Increase in natriuretic activity in advancing CRD in purified serum or urine samples.
7. An increase in end-organ responsivity to the inhibitor associated with nephron loss.
8. Lack of natriuretic effect of the inhibitor in patients with advanced CRD and a superimposed edema-forming state (the nephrotic syndrome).
9. Reversal of salt-losing state in advanced CRD by progressive slow reduction of salt intake to very low levels.

Table 2 | A comparison of several natriuretic substances.

	Ouabain (OLS)	Marinobufogenin (MFG)	Vanadium diascorbate (VD)	Atrial natriuretic peptides			Xanthurenic acid 8-O-β-D-glucoside (XAG)
				ANP	BNP	CNP	
Isolation	Plasma and adrenal cortex and hypothalamus (18–23)	Plasma, urine, and adrenal cortex (24, 25)	Urine and plasma (26)	Atria, heart, and kidney (27)	Brain, heart, and kidney (27)	Brain, heart, and vasculature (27, 28)	Plasma and urine from uremic patients (6, 8, 10, 29)
Stimulus	AII, ACTH, ↑BP positive sodium balance or intake; ↑ serum K+ up to 5 mEq/l	Same as OLS	Salt loading, aldosteronism, and volume expansion (26)	Stretch of cardiac wall – especially the atria, ET adrenergic stimuli (30)	Same as ANP (30)	Same? as ANP (30)	Normal and uremic patients and animals (10, 15)
M.W.	584.6 (31)	387? 600? (31)	403	2,000–3,000± (32)	2,000–3,000± (32)	2,000–3,000± (32)	368 (glucoside) 284 (sulfate) (11)
Structure	Steroid (31)	Steroid (31)	Vanadate diascorbate from ascorbic acid (26)	28 AA (32)	32 AA (32)	22 AA (32)	8-O-(-D-glucoside 8-O-sulfate of xanthurenic acid from tryptophan (11)
Site of Action	α2α3 NaK ATPase primarily (18–23, 25, 26, 33–43) acts on basolateral membrane of PCT and NHE3 in PCT (36, 37)	α1 NaK ATPase in PCT (24, 44)	NaK ATPase in PCT acts on basolateral membrane(26)	Blocks ENaC, ↑ GFR, blocks NaK ATPase AII in PCT (30, 45, 46) ↓ H_2O absorption in CT, ↓ urine concentration (47, 48)	Similar to ANP	Direct vasodilator and simulator to ANP (47)	Blocks ENaC acts from basolateral surface (17)
Natriuresis	Variable from no natriuresis to mild natriuresis (21, 25, 33, 36, 38, 39)No effect on α1-NaK ATPase	Variable natriuresis (24, 44)	Moderate natriuresis (26)	Moderate through CGMP (28, 30, 32, 46)	Same as ANP(28, 30, 32, 46, 49)	None (28, 47)	Eliminated by blocking ENaC (17)
RBF	↓ (40)	?	?	↑ RBF → ↑ GFR (30, 45, 50)	Variable (50)	No	No effect (17)
GFR	↓ Or no change (40)	?	?	↑ GFR dilates afferent arteriole and constricts efferent (49)	Same as ANP (49)	No	No effect (17)
K excretion	↓	↓	↓	↑	↑	↑	Minimal (11)
Vasoactivity	↑ BP, vasoconstriction (21, 22, 33, 34, 36, 40–43) ↑ Ca influx and Na influx into vessel wall (22, 34, 42–44)	↑ BP, vasoconstriction (24, 44)	↑ BP (27) ↑ Ca and Na influx into vessel wall (25, 27)	↓ BP (32, 47)	↓ BP (32, 47)	?	None (17)

10. Presence of natriuretic activity in urine of normal subjects during water immersion – an experience known to produce central hypervolemia.
11. Increased natriuretic activity in normal dogs on a high salt diet and superimposed mineralocorticoid hormone.
12. No natriuretic activity in normal dogs on a low salt diet and superimposed mineralocorticoid hormone.

OTHER "NATRIURETIC" FACTORS

Four other categories of putative natriuretic hormones are considered in separate papers in this symposium. These compounds include: (1) ouabain [or ouabain-like substances (OLS)]; (2) marinobufogenin (MFG); (3) vanadium diascorbate (VD); and atrial natriuretic peptides (ANP).

A summary of key properties of each is shown in **Table 2** and a brief description of some relevant characteristics follows.

OUABAIN (OLS)

A small molecule (MW 584.6) isolated from plasma (18), urine, adrenal cortex, and hypothalamus (19–23). The primary and relevant effects are the inhibition of NaK ATPase and the cross reaction with ouabain antibodies (21).

But they are inconsistently natriuretic (21, 22, 33, 34), presumably because ouabain has little effect on the α1 subunit of NaK ATPase (22, 35). Indeed, there also recent evidence that OLS may actually cause Na retention (26).

MARINOBUFOGENIN

Also a small molecule (MW between 387 and 600) (31), which is isolated from plasma, urine, and the adrenal cortex (24, 44). No studies have shown that administration of MFG causes natriuresis in assay animals. However, administration of anti-MBG antibodies reduces Na excretion (24, 44). Exogenous administration of bufalin, a very closely related compound, injected into the renal artery of sheep has been shown to produce natriuresis. No similar studies have as yet been published using MFG (51).

VANADIUM DIASCORBATE

A small molecule (MW 403) isolated from plasma and urine (26). Inhibits NaK ATPase in the proximal convoluted tubule. Produces moderate natriuresis, may produce influx of Ca^{++} into vessel walls and thereby increase BP.

ATRIAL NATRIURETIC PEPTIDES

Molecular weight 2,000–3,000± (32). The natriuretic peptides, while producing natriuresis, also increase GFR and increase glomerular pressure by dilating the afferent arteriole, and constricting the efferent arteriole (30, 45, 50). It may cause progressive renal failure in animal models.

If ANP is the final modulator of Na balance, there is a paradox. In advanced cirrhosis, there is an increase in preload and a decrease in central volume and EAV. ANP levels are elevated (50). Likewise in CHF there is a decrease in EAV and an increase in preload and again ANP levels are elevated. Physiologically both situations should invoke Na retention due to the decrease in the EAV, which in turn should shut off natriuresis, but in both of these situations ANP is elevated.

The arterial natriuretic peptides are primarily vasoactive and act on multiple sites of the nephron including the proximal tubule.

CONCLUSION

The cumulative data presented in this paper lend support to the view that xanthurenic acid 8-O-β-D-glucoside and xanthurenic acid 8-O-sulfate could be the long sought after and elusive natriuretic hormone. But to validate, or refute this thesis, additional experimental evidence is required. A partial list of key areas of future study includes:

1. A sensitive assay system for quantifying the levels of the putative hormone in body fluids.
2. Establishing the site of production.
3. The character of the signaling process (including the nature of the receptors), involved in initiating and controlling the induced natriuresis.
4. The enzymes involved in the synthesis.

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Natriuretic hormones, endogenous ouabain, and related sodium transport inhibitors

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The work of deWardener and colleagues stimulated longstanding interest in natriuretic hormones (NHs). In addition to the atrial peptides (APs), the circulation contains unidentified physiologically relevant NHs. One NH is controlled by the central nervous system (CNS) and likely secreted by the pituitary. Its circulating activity is modulated by salt intake and the prevailing sodium concentration of the blood and intracerebroventricular fluid, and contributes to postprandial and dehydration natriuresis. The other NH, mobilized by atrial stretch, promotes natriuresis by increasing the production of intrarenal dopamine and/or nitric oxide (NO). Both NHs have short (<35 min) circulating half lives, depress renotubular sodium transport, and neither requires the renal nerves. The search for NHs led to endogenous cardiotonic steroids (CTS) including ouabain-, digoxin-, and bufadienolide-like materials. These CTS, given acutely in high nanomole to micromole amounts into the general or renal circulations, inhibit sodium pumps and are natriuretic. Among these CTS, only bufalin is cleared sufficiently rapidly to qualify for an NH-like role. Ouabain-like CTS are cleared slowly, and when given chronically in low daily nanomole amounts, promote sodium retention, augment arterial myogenic tone, reduce renal blood flow and glomerular filtration, suppress NO in the renal vasa recta, and increase sympathetic nerve activity and blood pressure. Moreover, lowering total body sodium raises circulating endogenous ouabain. Thus, ouabain-like CTS have physiological actions that, like aldosterone, support renal sodium retention and blood pressure. In conclusion, the mammalian circulation contains two non-AP NHs. Identification of the CNS NH should be a priority.

Keywords: salt, sodium, urine, excretion, sodium pump, ouabain, hormone

INTRODUCTION

Natriuretic hormones (NHs) can be defined as substances whose circulating levels and effects fluctuate in a parallel manner with dietary sodium intake (1). NHs have long been implicated in sodium balance and are likely to be of the most significance in western acculturated societies where sodium intake typically is >100 meq/day (2). Indeed, ingestion of high salt meals raises the osmolarity of the circulation, stimulates secretion of antidiuretic hormone (ADH), and raises the natriuretic activity of the blood. In principle, the mode of action of NHs includes suppression of primary active sodium transport in the kidney and/or damping of secondary active transport systems involving sodium (1) or even potassium (3), effects on renal vascular tone and glomerular filtration rate (GFR), and activation of intrarenal natriuretic factors, such as prostaglandins, nitric oxide (NO), or dopamine. This article presents a personal and condensed overview of known and unknown non-atrial NHs and addresses the role of endogenous sodium pump inhibitors as NHs.

SEARCHING FOR NATRIURETIC HORMONES

It is well accepted that sodium balance is not fully explained by the up and downregulation of glomerular filtration and mineralocorticoid-stimulated reabsorption (4, 5). The first clear evidence for a "third factor" arose from the pioneering experiments of deWardener in which dogs that received excess mineralocorticoid and vasopressin increased their urinary sodium excretion in response to blood volume expansion with saline at a time when glomerular filtration was being lowered experimentally (6). Thus, the increase in sodium excretion was mediated by diminished tubular reabsorption of sodium and water. Cross-circulation studies, as well as work using isolated kidney studies in dogs and rats (6–10) excluded significant alterations in the composition of the blood, changes in renal nerve activity, glomerular filtration, renal blood flow, or renal perfusion pressure as mediators. A humoral "NH" was required.

The discovery of the atrial peptides (APs) and their natriuretic activity initially promised to explain some of the outstanding functions of an NH (11–13). APs augment sodium excretion (14–16) and saline infusions raise plasma AP (17, 18). However, in dogs, the effects of physiological changes in plasma APs and low dose infusions on sodium excretion were less obvious and, under certain experimental conditions, circulating APs and sodium excretion changed diametrically or, were temporally unconnected (19–21). Thus, some other NH was required.

The search for humoral agents that trigger salt excretion has relied on a variety of assays that range from isolated enzymes all the way to whole kidneys and animals (22). **Table 1** lists some tissues and fluids from which a variety of natriuretic factors

Table 1 | An overview of sources and characteristics of natriuretic factors.

Source for isolation	Characteristics	References
Adrenal	No short acting factors described	(8, 28, 29)
	Ouabain,[a] proscillaridin A-like compound[b]	(30)
Blood	Rapid onset, chymotrypsin-sensitive	(31–34)
	Rapid sustained natriuresis, MW < 500–700	(35)
	Trpysin sensitive, slow onset	(36)
	Precursor? slow onset	(37)
	Leucine aminopeptidase-sensitive, chymotrypsin-resistant	(38–41)
	Ouabain[a]	(42)
Hypothalamus/ pituitary	ADH, Oxytocin, MSH	See text
	Ouabain[a]	(43)
Intestine	Guanylin (small heat stable peptide)	(26, 44)
Kidney	High MW, release PGE_2 dependent	(45–48)
	Urodilatin[c] (ANP 95–126) Small peptide	(24, 49, 50)
Liver	Long acting, high MW (bound?), hepatic blood > portal blood	(51–59)
Urine	Low MW, Chymotrypsin-sensitive peptide	(33)
	Low MW, non-peptidic, acidic, Sephadex post salt fraction	(60–62)
	LLU-α[d]	(3)
	High MW, slow onset	(36, 46, 63–65)
	Marinobufagenin[e]	(66)
	Prolidase-sensitive peptide	(61)
	Urodilatin[c] (small peptide)	See kidney
	Uroguanylin (small heat stable peptide)	(27, 44)
	Xanthurenic acid β-glucoside and xanthurenic acid sulfate	(25, 67)

MW, molecular weight.

All materials listed with high MW are likely proteins.

[a] *Natriuretic at supraphysiological and pharmacological doses.*

[b] *Expected to have similar natriuretic activity as the bufadienolides (68, 69).*

[c] *Not likely to circulate in significant amounts.*

[d] *LLU-alpha; 2,7,8-trimethyl-2-(pcarboxyethyl)-6-hydroxychroman.*

[e] *Immunoreactivity present in the circulation (70) but not isolated from blood. The natriuretic effect of MBG per se has not been reported but is inferred from studies with bufalin and closely related steroids (68).*

were obtained. It is a significant accomplishment that numerous factors with natriuretic activity including guanylin, uroguanylin, urodilatin, LLu-α, xanthurenic acid, and a number of steroidal sodium pump inhibitors have been isolated and identified (14, 22–27). These materials likely account for some of the bioactivity in some, but not all, studies where natriuretic activity has been demonstrated. It is less clear that any of these materials fits the physiological profile expected for a NH as will be apparent from the discussion that follows.

NATRIURETIC HORMONES: HOW MANY?

Other than the APs, there are numerous hormones and endogenous materials that are known natriuretic agents. These include melanocyte stimulating hormone, dopamine, certain phospholipids, prostaglandins, kinins, and parathyroid hormone (71). These are not discussed here.

Evidence based upon pharmacological interventions, as well as an analysis of the kinetics of salt excretion mentioned below, suggests there are at least two major NH mechanisms unrelated to the APs. One mechanism is activated by the central nervous system (CNS) and the other involves maneuvers that increase atrial stretch. Pharmacological inhibition of renal NO blunts the magnitude of saline natriuresis (72) and both specific and non-selective dopamine antagonists attenuate volume expansion and water immersion (i.e., atrial stretch mediated) natriuresis but not that activated by CNS sodium (73–77). Yet another key factor that distinguishes these two NH systems is their kinetics; the rates of the decline in sodium excretion when the natriuretic stimuli are abruptly removed differ markedly for CNS- and atrial distention natriuresis. The kinetic features are potentially diagnostic; they can be used to evaluate candidate NHs.

The atrial distention arising from balloon inflation requires intact cardiac but not renal nerves, the stretch can be reversed in seconds, and the evoked natriuresis declines rapidly (21). Critically, the kinetics of the decline in natriuresis are uncontaminated by residual volume that typically would remain following a saline load (78). The second experimental paradigm is the natriuresis evoked by infusion of hypertonic saline into the brain. As the flow rates in the cerebral ventricles are much higher than the rates at which hypertonic stimuli are typically infused, simply stopping the infusion exposes the kinetics of the decline in salt excretion. Accordingly, **Figure 1** compares the decline in renal sodium excretion evoked by either atrial distension or CNS sodium. Three points are apparent: (1) the decay kinetics in both instances are first order; for CNS natriuresis, they remain linear for well over 1 h. The kinetics demonstrate that a single reaction likely is the dominant rate limiting step for the natriuresis evoked by each stimulus. (2) The CNS natriuresis, when activated by hypertonic saline (79–83), dehydration (84), or norepinephrine (85), produces similar rate constants with no major species differences. (3) The rate constants for the decline in CNS natriuresis are ~2–3-fold less (slower) than that evoked by atrial distension. Thus, the combined evidence derived from the sensitivity to pharmacological agents and the kinetic observations indicate that CNS- and atrial distension natriuresis must be mediated by different mechanisms.

Compensatory mechanisms might conceivably alter the kinetics in **Figure 1**, especially if significant salt and water loss were to occur along with declining blood pressures. During the 40 min atrial distention in **Figure 1**, blood pressure increased modestly. Plasma renin was suppressed in one set of experiments but not another. Following the distension, in one set of experiments, blood pressure remained elevated even though the natriuresis declined rapidly and aldosterone was unchanged or increased. Nevertheless, changes in aldosterone would have been too slow to have had impact. Under the conditions used, and among the measured hormonal and hemodynamic variables, the only changes

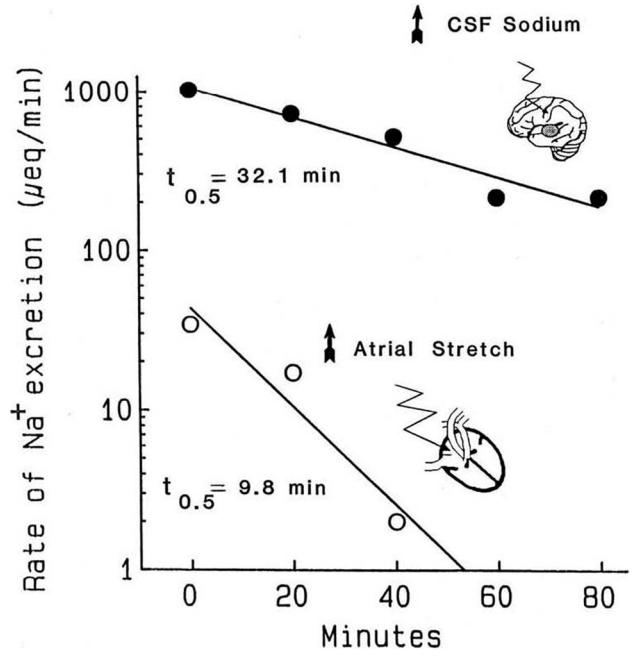

FIGURE 1 | Kinetics of the decline in natriuresis following abrupt removal of the natriuretic stimulus. Reproduced from Ref. (71) with permission. Data are adapted from Ref. (21) in conscious dogs and Ref. (86) in conscious goats. Reported values for the $t_{1/2}$ of the decline in CNS natriuresis range from 24 to 32 min in the goat, 32 min in the sheep, and 15 min in the rat (71). These half times are likely to represent the clearance of their respective humoral mediators from the circulation.

convincingly associated with the decline in natriuresis following atrial distension were the return of left, right, and pulmonary pressures (i.e., cardiac nerve activity) to normal. With regard to CNS natriuresis, the decline in natriuresis is an extended first-order process; the absence of curvature over the time course implies no major influence by a compensatory process.

Among the candidate NHs in **Table 1**, there is, unfortunately, no readily interpretable information regarding the halftimes for the decline in their natriuretic effects. Most of the unidentified materials were impure, with variable onset times, and, reminiscent of urodilatin (49), some produced a natriuresis that lasted many hours following infusion. The absence of kinetic information is understandable; the primary experimental emphasis was the demonstration of natriuresis *per se*. And for decay kinetics to be informative, a near steady-state natriuresis would ideally be desirable prior to stimulus removal. This is not always an easy condition to meet. Regarding the recently identified materials in **Table 1**, no kinetic information is available. However, among all the materials, urodilatin shows a most interesting physiological correlate; in human beings, urinary urodilatin excretion closely paralleled the circadian rhythm for sodium excretion over many days (49). As urodilatin itself is not found in the circulation, it is not, by definition, an NH; although the unknown substance (?) that presumably links sodium intake with urinary urodilatin and sodium excretion could be. Thus, for all listed materials in **Table 1**, there is currently no compelling evidence that their behaviors fit the definition of a physiologically relevant NH given in the introduction.

Hereafter, I focus primarily on CNS natriuresis and consider the potential role of sodium pump inhibitors as NHs.

CNS NATRIURESIS

The brain, via an unknown humoral NH, mediates the natriuresis evoked by increased plasma sodium concentration, intracerebroventricular (icv) sodium, and dehydration (79, 81, 87). The natriuresis may be damped but is not eliminated by renal denervation (88), is activated by small increases of plasma sodium (1–2 mM). The CNS NH may have a dominant influence in postprandial natriuresis (89) and is a blood-bourne factor distinct from APs (90, 91), ADH (92), or dopamine (74).

Central nervous system natriuresis can be activated by the elevation of either blood-bourne or cerebrospinal fluid sodium; both dehydration and postprandial natriuresis are blocked or reversed by hyponatremic CSF (93–95) or rehydration (96). Push–pull perfusion techniques suggest a discrete area of the third ventricle is near the sodium sensing apparatus (97). Further, the ablation of central structures, including the anteroventral and posterior hypothalamus in a variety of species, or decapitation, profoundly influence the ability to regulate osmotic balance, tolerate hyperosmotic challenge, and excrete sodium (80, 83, 98–106). The lesioned areas have included the median eminence, medial preoptic nucleus, organum vasculosum of the lamina terminalis, and the periventricular preoptic area. The consequences of these lesions are impaired thirst and ADH secretion, reduced renal natriuretic response, and hypernatremia. In contrast, this same system, when overactivated, can lead to profound hyponatremia. This phenomenon, sometimes termed "cerebral salt wasting," and resembling some of the features of the syndrome of inappropriate ADH secretion, has been noted in some CNS disorders (107–111).

The observation that dehydration results in hypernatremia and provokes a compensatory natriuresis in the face of reduced extracellular fluid volumes, and that the natriuresis subsides with rehydration, suggests that the tendency to hypernatremia during dehydration and following a high salt meal is actively opposed by an unknown osmotically sensitive mechanism [see in Ref. (84)]. In each instance, the natriuretic response to these stimuli is present in animals with denervated kidneys but absent in animals with hypothalamic lesions (84, 112). Further, CNS natriuresis is not explained by blood pressure changes and persists when renal artery pressures are servo controlled (92, 113).

In each of the aforementioned situations, changes in circulating ADH have been implicated as the efferent mediator of CNS natriuresis (114, 115). Indeed, CNS natriuresis is either absent or slowed in rats congenitally deficient in AVP (116, 117), is absent in hypophysectomized rats but reappears in rats pretreated with large amounts of ADH and in rats given a dD-AVP analog (88). ADH certainly contributes to the control of sodium excretion in rats, dogs, and man (84, 89, 118–123). ADH infusions are natriuretic, and specifically implicated in CNS (114, 115) but not saline natriuresis. However, ADH is not sufficient to account for CNS natriuresis (92, 117), although it may be permissive (124, 125). For example, AV3V-lesioned sheep and dehydrated normal sheep both lost similar amounts of body water, although the hypernatremia

was much worse in the lesioned animals (112). Thus, something other than ADH was lacking in the lesioned animals to explain the greater hypernatremia with the same overall water loss.

Little is known about the chemical nature of the CNS NH other than it appears to be heat stable (126). Its actions have an interesting temporal association with ADH and/or oxytocin (92, 127). For example, plasma ADH rises during the prehypertensive period associated with mineralocorticoid escape; a period when increased CNS NH would be expected (128). Consistent with the latter supposition, urinary sodium excretion in sheep given 3–4 day infusions of aldosterone was almost entirely blocked by the acute CNS administration of a low sodium cerebrospinal fluid during mineralocorticoid escape (129). Further, mineralocorticoids also augment the osmotic sensitivity of ADH secretion (130).

Oxytocin also has a role in renal sodium excretion (131–134) and restores the ability of hypophysectomized dogs and rats to excrete sodium at a brisk rate during saline expansion (20, 135). Yet other humoral factors implicated during CNS and ADH natriuresis include an inhibitor of prostacyclin synthesis (136) and a humoral substance that inhibits active sodium transport in toad bladder (137). Hemorrhage, paradoxically, also evokes a natriuresis that depends on the CNS (118). The natriuresis is blocked when intrarenal prostaglandin synthesis is inhibited (119). The simplest interpretation is that activation of intrarenal V1 receptors stimulates prostaglandin synthesis and the resultant products influence sodium reabsorption at distal tubular sites (138). Overall, the phenomenon ascribed to CNS natriuresis has complex interdependencies and is associated with the diminution of renal tubular sodium transport.

Of significant relevance, CNS-mediated natriuresis depends upon the prevailing level of dietary sodium intake. In sodium depleted dogs, infusion of hypertonic saline into the carotid artery is not natriuretic (139). Moreover, the phenomenon of postprandial natriuresis in the sheep is activated only when dietary sodium intakes reach a threshold of 50–75 mmol of sodium/24 h (140), i.e., when plasma renin and aldosterone are largely suppressed. Thus, the CNS NH system is likely of great physiological relevance; it is appropriately integrated with other key factors that govern long-term sodium balance.

The CNS also has a permissive role in the response to saline expansion of blood volume (79, 102, 103). Hypophysectomy reduces saline natriuresis; the deficit is reversed partially by administration of oxytocin and ADH (141). Furthermore, the application of a constricting vice to the neck of anesthetized dogs so as to exclude the brain and pituitary factors from the circulation impairs saline natriuresis (102). In view of the abovementioned role of the CNS, it is surprising that remarkably little attention has been focused on the natriuretic activity associated with extracts from brain and pituitary (**Table 1**). The little that is known is that the bioactivity of natriuretic extracts from hypothalamus persists following treatment with thioglycollate (to exclude oxytocin or ADH), and that an unidentified tridecapeptide was found in bioactive fractions from the posterior pituitary (142, 143).

ARE SODIUM PUMP INHIBITORS NATRIURETIC HORMONES?

There is much evidence linking sodium pump inhibitors with salt balance and cardiovascular and renal disease (144, 145). The Na,K-ATPase inhibitory activity of plasma from normal individuals on a high sodium diet was 25 times greater than that when the individuals were on a low sodium intake (146). Further, the plasma from individuals on high sodium diets, purified natriuretic material from urine, and ouabain, all stimulated glucose-6-phosphate dehydrogenase (G6PD) activity. G6PD activity is claimed to be inversely related to Na,K-ATPase activity (147) and related to inhibition of proximal tubular Na,K-ATPase (148), although the G6PD assay is not considered a surrogate method for the Na,K-ATPase.

Nevertheless, increased blood levels of sodium pump inhibitors, as measured by traditional well-accepted means, have been repeatedly associated with acute volume expansion, high dietary salt, mineralocorticoid excess, chronic renal failure, and CNS natriuresis (31, 32, 35, 38, 149–155). Haddy and coworkers using animal models of low renin hypertension observed that sodium pump inhibition could be reproduced in normal animals given a rapid volume expansion and that this effect could be transferred to the arteries of another animal via the plasma (156). Further, in acutely saline-expanded dogs, the plasma levels of a polar Na,K-ATPase inhibitor and a digoxin immunoreactive material were elevated at a time when endogenous ouabain (EO) was unchanged (37, 157). Moreover, the plasma of dogs undergoing atrial distension strongly inhibited the ouabain-sensitive ^{86}Rb uptake into human red cells. Notably, the bioactivity of the plasma declined substantially when retested a few days later, and was undetectable after 10 days (Hamlyn and Goetz, unpublished observations). This indicates that the inhibitor is unstable in plasma and is reminiscent of the labile digoxin-like material described by Graves et al. (158). Other work implicated the CNS in the control of humoral sodium pump inhibitors; Buckalew et al. (159) found that the jugular effluent inhibited active sodium transport to a greater extent than the blood from the femoral vein. Further, increased levels of circulating sodium pump inhibitors depend upon the integrity of hypothalamic structures within the AV3V area (103, 160). Moreover, the lesion sites overlap those whose integrity is required for CNS natriuresis. Thus, the interrelationship between increased circulating sodium pump inhibitors and natriuresis continues to be of interest. When taken together, there is no doubt that the circulation contains inhibitors of sodium transport, but what are these materials, do their levels change appropriately with salt, and are they natriuretic? Below we focus on sodium pump inhibitors that have been isolated and that have been previously linked with the aforementioned criteria.

IDENTIFICATION OF SODIUM PUMP INHIBITORS

Starting from either human plasma or urine (brain, adrenal, and the eye are not discussed here), four groups isolated sodium pump inhibitors and identified them as ouabain- (42, 161, 162), digoxin- (163), marinobufagenin- [MBG, (66)], and telocinobufagin-like steroids (162, 164), respectively. There are altered levels of these materials in numerous experimental and clinical studies (70, 164–169). All these steroids inhibit the sodium pump and, when bound, at least one evokes biased signaling in a manner strikingly reminiscent of the β-adrenergic receptor (168, 170–172). These cardiotonic steroids (CTS) typically are natriuretic and variably kaliuretic when infused acutely at pharmacological (micromolar) doses into anesthetized animals or the renal artery and, in the

case of ouabain, selectively inhibit sodium transport in the distal tubules (68, 69, 173, 174). The natriuretic response is linearly related to the inhibition of Na pumps in the dog (175). But are they physiologically relevant NHs?

WHAT DO THE KINETICS OF THE DECLINE IN NATRIURESIS TELL US ABOUT THE ROLE OF KNOWN SODIUM PUMP INHIBITORS?

By comparing the circulating half lives of any putative NH with the half times in **Figure 1**, it is possible to determine whether it is a plausible mediator of natriuresis. Here, I examine the circulatory half lives of a number of well-known sodium pump inhibitors and compare them with the information in **Figure 1**. For example, in the dog, the plasma half lives for intravenous ouabain, digoxin, resibufagenin, and bufalin were ~18 h, ~30 h, 21 min, and 25 min, respectively [Ref. (176–178)]. In the rat, the circulating half lives for intravenous cinobufagin, resibufagenin, and bufalin were 44, 42, and 25 min, respectively (179). Therefore, it is apparent that, among these known steroidal sodium pump inhibitors all, with the exception of bufalin, are simply cleared too slowly from the circulation to be kinetically plausible humoral mediators of CNS natriuresis. In the case of atrial distention natriuresis, the kinetic analysis reveals that none of the abovementioned sodium pump inhibitors are likely primary humoral mediators. With regard to CNS natriuresis, only the clearance of bufalin is sufficiently fast in both dogs and rats to warrant further investigation. The kinetic analysis does not prove bufalin as the humoral mediator in CNS natriuresis, but simply suggests that this steroid (or those that are closely related but for which no clearance data are available, e.g., MBG) cannot, as yet, be excluded. A lingering concern with bufalin, or any CTS sodium pump inhibitor, as a NH is the potentially serious conceptual problem that their acute vasoconstrictive action within the renal vasculature will oppose their tubular effects (180).

RENAL SODIUM PUMP ISOFORMS: IS THEIR OUABAIN SENSITIVITY IMPORTANT?

Nearly all mammalian tissues express the α-1 catalytic subunit of the sodium pump; muscle and muscle and nerve also express sodium pump isoforms with α-2 and α-3 subunits (181). In the rat kidney, sodium pumps with the α-1 catalytic subunit are insensitive to micromolar ouabain but are somewhat sensitive to bufalin and marinobufagenin; the acute natriuretic effect of bufalin is greater than that of ouabain (69). For many years, it was believed that the kidney expressed only the α1 isoform even though the ouabain sensitivity of the renal Na pump increases progressively along the nephron (182); the distal tubules are believed to be ~50–100-fold more sensitive than their proximal tubule counterparts. More recently, small numbers of highly ouabain-sensitive α-2 sodium pumps have been detected in rat kidney and they are functionally significant. For example, in response to acute low doses of ouabain, the α2 sodium pumps trigger enhanced Ca^{2+} signaling and NO generation in the descending vasa recta (183). It is not known if these signaling effects extend to the renal epithelia, but if they do then the acute natriuretic effects of ouabain could involve short-term NO-mediated events. In contrast, the acute natriuretic effects of bufalin and other bufadienolides are thought to be mediated by inhibition of α1 sodium pumps (184).

In the kidney, the renal ouabain-insensitive α-1 sodium pumps far outnumber their ouabain-sensitive α-2 cousins. Interestingly, saline natriuresis was augmented when rodent α-1 sodium pumps were made highly ouabain-sensitive (185). Further, the augmented component of the natriuresis was blocked by digoxin antibody fragments (Fab). However, the kinetic analysis in **Figure 1** makes it clear that neither ouabain nor digoxin are viable mediators of atrial distention (saline) natriuresis; the digoxin Fab fragments must, therefore, have interacted with an unknown material that preferred ouabain-sensitive sodium pumps. Thus, occupation of the ouabain binding site by this material can contribute to, but does not fully account for, the phenomenon of saline natriuresis.

OUABAIN AS A SALT RETAINING STEROID

In contrast to the well-accepted acute natriuretic effects of high doses of sodium pump inhibitors, the chronic effects of low concentrations can be diametrically opposite. In the case of ouabain, the prolonged daily administration of low nanomole amounts in the rat suppresses Ca^{2+} signaling and NO generation in the endothelium of the descending vasa recta, reduces renal blood flow and glomerular filtration, raises sympathetic nerve activity, directly augments vascular myogenic tone and contractility, and raises blood pressure (186–193). Further, chronically reduced total body sodium in human beings is associated with elevated circulating levels of EO (194, 195), i.e., the chronic relationship between plasma EO and salt intake is, like aldosterone and renin, roughly "L"-shaped (196). In addition, and as might be anticipated from the above noted chronic observations, clinical studies have shown that among salt-loaded EH patients, renal tubular sodium reabsorption was highest in the group with elevated circulating EO (197). Thus, the behavior of circulating EO under physiological circumstances, as well as its long-term vascular and renal tubular actions, all appear to favor sodium retention.

Dramatic increases in circulating EO have been reported during exercise, a state associated with increased sympathetic activity and a decline in renal blood flow (198). The circulating levels of EO rise acutely in response to the stress of cardiac surgery (199) and the preoperative plasma levels of EO enhance the identification of those patients who will develop acute kidney injury postsurgery (200). Once again, the behavior and actions of EO in these stressful situations is associated directly or indirectly with salt and water retention, rather than salt excretion. When taken together, the current evidence strongly favors the view that EO is a physiologically relevant hormone with a variety of interesting actions that augment vascular tone and promote renal sodium retention.

In summary, the hunt for NHs has led recently to the complete identification of numerous natriuretic materials. In spite of these notable successes, none of the materials seems to fit the anticipated physiological profile for a mammalian NH. Much evidence indicates there are two major non-AP NHs that remain to be isolated and identified. It may be argued that identification of the CNS NH should be a priority in view of its broad physiological relevance, relationship to dietary sodium intake, and the implication

of a profound role in salt balance in a number of pathological disorders.

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Natriuretic hormones in brain function

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Natriuretic hormones (NH) include three groups of compounds: the natriuretic peptides (ANP, BNP and CNP), the gastrointestinal peptides (guanylin and uroguanylin), and endogenous cardiac steroids. These substances induce the kidney to excrete sodium and therefore participate in the regulation of sodium and water homeostasis, blood volume, and blood pressure (BP). In addition to their peripheral functions, these hormones act as neurotransmitters or neuromodulators in the brain. In this review, the established information on the biosynthesis, release and function of NH is discussed, with particular focus on their role in brain function. The available literature on the expression patterns of each of the NH and their receptors in the brain is summarized, followed by the evidence for their roles in modulating brain function. Although numerous open questions exist regarding this issue, the available data support the notion that NH participate in the central regulation of BP, neuroprotection, satiety, and various psychiatric conditions, including anxiety, addiction, and depressive disorders. In addition, the interactions between the different NH in the periphery and the brain are discussed.

Keywords: atrial natriuretic peptide, cardiac steroids, ouabain, guanylin, brain function

INTRODUCTION

Natriuretic hormones (NH) are compounds that act in an endocrine or paracrine fashion to regulate extracellular fluid volume and blood pressure (BP) through the stimulation of sodium excretion by the kidney. Three groups of compounds fall into this broad definition: the natriuretic peptides (NP: ANP, BNP, and CNP), the guanylin peptides (GP), and the endogenous cardiac steroids (CS: ouabain, digoxin, and marinobufagenin). A large body of evidence supports the notion that in addition to their natriuretic effects, these hormones participate in numerous brain functions. Our goal is to review the established information on the biosynthesis, release, and physiological roles of NH, with particular focus on the brain. The available literature on the interactions between the different NH families in the periphery and in the brain is also addressed.

NATRIURETIC PEPTIDES

The first demonstration of an endocrine link between the heart and kidneys came from the pioneering experiments of De Bold, which led to the discovery of atrial NP (ANP), the founding member of the family of NP. De Bold and his colleagues found that injecting rats with an atrial homogenate caused significant natriuresis and diuresis (1). Additional members of this family of peptides were purified over the course of the following years: B-type NP (BNP) (2) and C-type NP (CNP) (3). ANP, BNP, and CNP are expressed as pre-pro-hormones and are proteolytically processed to form the mature peptides. The three peptides share a similar structure consisting of two cysteine residues flanking a 17-residue disulfide-linked ring that is essential for biological activity (3). The main inducer of ANP release is atrial wall stretch (4). BNP is released from the atrium, as is ANP, but its main sources are the ventricles, where BNP is transcriptionally regulated by cardiac wall stretch resulting from volume overload (5). There are three known NP receptors: NP receptor-A (NPR-A), or guanylyl cyclase A (GC-A), which binds ANP and BNP (6); NPR-B (GC-B), which is highly specific for CNP (7); and NPR-C. NPR-A and NPR-B are membrane-bound receptors consisting of an extracellular ligand binding domain, a single transmembrane region and an intracellular GC domain that rapidly releases cyclic guanosine monophosphate (cGMP) in response to the NP binding (8). The cGMP then acts as a second messenger that activates protein kinase-G (PKG) and subsequent cellular signaling cascades (9). A third receptor, NPR-C, contains only a short intracellular fragment and has no GC activity. The main function of NPR-C is to clear NP through receptor-mediated internalization and degradation (10).

PHYSIOLOGICAL ROLES OF NP

Atrial natriuretic peptide has a major role in the regulation of BP. In the kidney, ANP induces natriuresis and diuresis by increasing the glomerular filtration rate (GFR) and inhibiting sodium and water reabsorption (11). ANP acts as a functional antagonist of the renin–angiotensin–aldosteron system by inhibiting renin secretion from the kidney and aldosterone production in the adrenal glands (12). It stimulates smooth muscle cell relaxation in blood vessels, causing vasorelaxation (13). It also regulates the intravascular volume by increasing endothelium permeability (14). In accordance with these effects, it was found that ANP knockout mice developed salt-sensitive hypertension (15). ANP also directly affects the heart by inhibiting cardiac hypertrophy (16). BNP activates the same receptor as ANP but its precise functional significance is not well understood. Studies on mice with targeted disruption of BNP (17) and on cultured cardiac fibroblasts (18) established BNP as an antifibrotic factor that plays a role in ventricular remodeling. Indeed, high concentrations of BNP were found in the ventricles following congestive heart failure or myocardial infarction, rendering it an important biomarker for

these conditions (19). Unlike the other two family members, CNP acts in an autocrine/paracrine fashion. Although NPR-B is present in the kidney, CNP has little natriuretic or diuretic effect, and it is a much more potent cardiovascular effector (20). CNP is produced by the endothelium and induces vasorelaxation (21). It also participates in vascular remodeling following injury (22). In addition to their cardiovascular and renal effects, NP show a wide-spread effect throughout the body (8): They act as bronchodilators and vasorelaxants in the lungs (23), elicit anti-inflammatory effects in the immune system (24), and have metabolic effects on the adipose tissue (25) and on long bones (26).

NP IN THE BRAIN

The ANP, BNP, and CNP and their receptors are expressed in the brain, which implies a possible role for these peptides in brain function. CNP is the most abundantly present NP in the brain (27), suggesting that it acts as a neurotransmitter or neuromodulator rather than a cardiac hormone (28). Accordingly, the CNP-specific receptor – NPR-B is widely spread throughout the brain: NPR-B mRNA was detected in the cerebral cortex, the limbic area, preoptic-hypothalamic regions, motor nuclei, and the brainstem (29). ANP and BNP are also present in the brain and have interesting neuromodulatory functions. ANP expression was first found in the hypothalamus (30), which is the main source of NP in the brain (31, 32). ANP is present in neurons and glia in the cerebral cortex (33) and in the cerebellum (34). ANP was also described in neurons and fibers in the limbic area, olfactory bulb, thalamus, and striatum (31, 35–37). BNP was found in the hypothalamus (38) and cerebral cortex (33). Unlike ANP and CNP, no BNP mRNA was detected in the brain (39), suggesting the peripheral origin of this peptide. Interestingly, ANP and BNP were described in some of the circumventricular organs in the brain – the highly vasculated structures in the hypothalamus that allow endocrine communication between the periphery and CNS (40). Considerable ANP-like immunoreactivity was found in nerve fibers of the vascular organ of the lamina terminalis and the subfornical organ in rat brain (31). Neurons in the subfornical organ were shown to respond to ANP by increased cGMP production (41). Neurons of the circumventricular organs express receptors for the majority of the cardiovascular hormones (42), including NP receptors: NPR-A and NPR-B were found in the vascular organ of lamina terminalis, the subfornical organ, area postrema, and the choroid plexus (43).

NP in central regulation of BP

The presence of NP and their receptors in the brain, and in the circumventricular organs in particular, led to the postulation that NP, either locally produced in the brain or arriving via the peripheral circulation, might affect neuronal pathways that centrally regulate BP. However, the results are inconsistent. Intracerebroventricular (i.c.v.) administration of ANP was reported to cause a decrease in BP in normal and spontaneously hypertensive conscious rats, but only at concentrations 10 times higher than the physiological level (44). Low concentrations of ANP were shown to have a depressor effect in anesthetized rats with sinoartic denervation, leading to a decrease in BP and sympathetic outflow (45, 46). A study performed on conscious sheep showed that CNP, but not ANP, decreased BP upon i.c.v. administration (47). Numerous studies found no change in BP upon central administration of ANP (48–52) or BNP (53). However, there are reports describing a decrease in vasopressin secretion following central ANP infusion, suggesting that ANP and vasopressin may interact to attenuate the central pressor effects of vasopressin (49, 51–54). Pretreatment of rats with i.c.v. BNP was also shown to suppress vasopressin secretion (53). In several studies it was postulated that ANP acts in the brain by partially inhibiting the angiotensin II (ANG II) pathway. ANP injection prevented the pressor effect of centrally administered ANG II (46, 51). On the behavioral level, centrally administered ANP was shown to inhibit water intake induced by ANG II or dehydration in rats (55). It was also found to attenuate salt appetite in spontaneously hypertensive rats (SHR) (48). These results suggest that ANP may not be directly involved in central regulation of BP, but rather act as a secondary modulator of other mechanisms, perhaps, similar to its peripheral effect, by counteracting to the effects of vasopressin and ANG II.

NP in neuroprotection

Natriuretic peptides were shown to exert a neuroprotective effect in cultured cells and *in vivo*. Cortical spreading depression (CSD) is a wave of depolarization followed by transient suppression of electrical activity in the brain (56). Rats preconditioned with an evoked episode of CSD were protected from neuronal damage following cerebral ischemia (57). Wiggins and his colleagues found that an acute episode of CSD caused an elevation in ANP mRNA and peptide levels in the rat cortex. The elevation was prolonged, overlapped the time window for CSD-induced neuroprotection and accompanied by ANP-dependent activation of cGMP signaling cascades (58). Increased cGMP levels were previously implicated in the neuroprotective mechanism of CSD (59). This notion is supported by studies showing that ANP and BNP caused an elevation in cGMP levels and inhibited apoptosis of PC12 cells (60). However, there is no direct evidence of this effect in the brain. A neuroprotective effect was also demonstrated in rat retinal neurons, where ANP was shown to ameliorate NMDA-induced neurotoxicity, presumably in a dopamine-dependent manner (61). It was postulated that the ANP neuroprotective effect is mediated via the cerebral blood flow. Indeed, an increased number of ANP-immunoreactive astrocytes and other glial cells were found in the white matter surrounding an infarction area in rats (62). This neuroprotective effect may be modulated by cGMP signaling, since cGMP-phosphodiesterase inhibitor was found to have a protective effect in a focal brain injury model in rats (63). In ischemic brain edema induced in rats, intravenous (i.v.) administration of ANP proved to have a beneficial effect. At pharmacological doses, the peptide significantly suppressed the elevation of the brain's water and sodium content and reduced the area of edema, as revealed by magnetic resonance imaging (MRI) (64). ANP was beneficial even after delayed administration, and reduced brain edema when injected i.c.v. 4 h after induction of hemorrhagic brain injury in rats (65). BNP too was implicated in neuroprotection following brain injury. James and colleagues demonstrated that i.v. administration of BNP improved cerebral blood flow and reduced inflammation in brain injury models in mice, as manifested by reduced neurodegeneration and improved functional outcome (66). Although these experiments were performed using

high doses of exogenous human recombinant BNP (nesiritide), endogenous BNP may play a role in recovery from brain injury, as elevated BNP levels have been associated with this condition. Elevated plasma BNP levels were described in patients with traumatic brain injury (67, 68), stroke (69), and other brain injuries (70, 71). Elevated BNP levels were also reported in the cerebrospinal fluid (CSF) of brain trauma patients (67). These changes, however, correlated with a poor clinical outcome in trauma and stroke patients (72, 73). This may indicate an insufficiency of the endogenous neuroprotective mechanism. The mechanism of NP neuroprotection could be mediated through immunomodulation, as was demonstrated in the periphery (74). All these clinical and pre-clinical observations lead to the premise that ANP and BNP are part of an endogenous protective mechanism in the brain against injury or damage.

NP in behavior

Natriuretic peptides modulate the function of the hypothalamic–pituitary–adrenal (HPA) axis and influence anxiety and addictive behavior. NP regulate the HPA-axis at several levels: ANP inhibits the release of corticotrophin (ACTH) and corticortrophin releasing hormone (CRH) (75, 76), which, in turn, stimulate ANP release, acting in a feedback loop (76). ANP also directly inhibits cortisol secretion, whereas CNP exerts the opposite effect (77). Central or peripheral administration of ANP decreased anxiety-associated behavior in rats (78). In humans, lower levels of ANP were described in patients with anxiety-related disorders, including panic disorder (79) and posttraumatic stress disorder (80), and high ANP levels were associated with lower anxiety levels in patients recovering from cardiac failure (81). Experimentally induced panic attacks were followed by an increase in plasma ANP levels, which was faster and more pronounced in panic disorder patients (79, 82). These observations suggest a therapeutic potential for ANP agonists in the treatment of anxiety-related disorders (83). Indeed, pretreatment with i.v. ANP significantly reduced the number of experimentally induced panic attacks in panic disorder patients and in healthy individuals (84, 85). The effects of ANP on anxiety are presumed to be mediated through inhibition of the HPA-axis. In healthy individuals, pretreatment with ANP was able to partially block the sympathetic activation induced by a bolus injection of CRH (86). However, further investigation is needed to fully understand the interplay between ANP and the HPA-axis.

B-type natriuretic peptide, like ANP, was found to have an anxiolytic effect (87). On the other hand, CNP enhances cortisol secretion (77) and has an anxiogenic effect in rodents and humans (88, 89). However, it is worth mentioning that high doses of CNP (up to 5 μg), were used in these experiments; at low doses (100 ng), CNP reduced anxiety-like behavior in rats (87). At doses similar to those used for ANP, CNP increased the levels of anxiety-related behavior when administered i.c.v. in rats (88). This effect was abolished by a CRH antagonist, pointing toward an HPA-axis related mechanism (89). In humans, pretreatment with CNP enhanced the emotional effect of the anxiogenic agent CCK-4, and increased the release of ACTH following this treatment (90). CNP was also shown to stimulate cortisol and prolactin release (91). All these finding indicate that CNP is a potent anxiogenic substance that acts by stimulating the HPA-axis. It is therefore that CNP antagonists were considered in anti-anxiety therapy (83).

Atrial natriuretic peptide may modulate alcohol withdrawal-related anxiety. In alcohol-dependant patients, abrupt cessation of alcohol consumption is accompanied by an array of symptoms known as alcohol withdrawal (92). ANP is involved in some of the neurobehavioral aspects of alcohol withdrawal, including anxiety and alcohol craving (93). In mice, i.p. ANP administration attenuated anxiety-like behavior following alcohol withdrawal (94). Handling-induced convulsions resulting from withdrawal were reduced by i.c.v. infusion of ANP, whereas anti-ANP antibodies had the opposite effect (95). Consistently, NPR-A knockout mice showed increased stress-related alcohol consumption and aggravated withdrawal symptoms (96). In humans, acute alcohol consumption elevated plasma ANP levels in healthy individuals (97). In patients with alcohol-dependence, plasma ANP levels were lower during detoxification compared with those in non-drinking individuals (93). The lower levels correlated with alterations in promoter DNA methylation, which was significantly reduced as compared with that in healthy controls (98). On the emotional level, lower ANP levels were associated with increased anxiety and alcohol craving during withdrawal (93, 99). It was postulated that the mechanism of ANP involvement in withdrawal-related stress is mediated through the HPA-axis (100). However, although the HPA-axis stress response affects the patient's recovery from alcohol addiction (101) as well as relapse rate (102), cortisol and ACTH levels do not correlate with those factors affected by ANP, such as alcohol craving (102) and perceived stress (99). ANP involvement in alcohol dependence is supported by recent genetic studies. A genome-wide association study (GWAS) revealed alcohol dependence to be associated with a single-nucleotide polymorphism located in gene GATA4, which encodes a transcription factor regulating ANP (103, 104). This finding was confirmed by a candidate association study, which found GATA4 to be linked to alcohol dependence at the gene level (105). This genetic variation in GATA4 was also shown to be associated with an increased relapse rate in patients (106), and greater reactivity in the amygdala to alcohol-related images, as shown by functional MRI (107). These results suggest that NP, possibly by modifying the stress response of the HPA-axis, are involved in the pathological states of anxiety disorders and alcohol dependence.

FUTURE CHALLENGES

As described above, there is evidence for the involvement of NP in several brain functions. These observations open exciting new venues for research and drug development. However, many open questions remain to be clarified. In all the cases described above, it appears that NP do not regulate brain functions directly, but rather modulate other endocrine pathways, such as ANG II in BP regulation, or the HPA-axis in anxiety-related disorders. As for their neuroprotective qualities, those could be mediated via other mechanisms activated by brain injury, such as the immune system. The intricate interactions between NP and other cellular systems need to be studied in depth. On the more basic level, although the control of NP biosynthesis and release in the periphery is well established, not much is known about locally produced NP in non-cardiac tissues, the brain in particular. Information is lacking as to

the specific cell types in the brain that produce NP, the factors regulating NP production and release, the modes of their elimination, and the neuronal signaling pathways that they affect. Electrophysiological studies are necessary to establish the effects of NP on neuronal excitation and channel activation. It is possible that NP do not directly regulate neuronal activity, but rather modulate it via their effect on glial cells. Studies on the NP effect on calcium release and neurotransmitters uptake in glial cells may help elucidate this point. Also, the interactions between NP and known neurotransmitters and their receptors may be of importance, and should be addressed.

GUANYLIN AND UROGUANYLIN

Dietary sodium leads to increased natriuresis in an aldosterone-independent manner. This observation led to the postulation that an additional NH is released from the intestine in response to salt intake (108). Such a hormone was discovered in 1992 – the previously unknown endogenous ligand of the intestinal receptor GC-C, activated by bacterial enterotoxins (109), and termed guanylin. Guanylin was purified from rat jejunum, and it was shown to activate GC-C in T84 human intestinal cells (109). One year later, a second endogenous ligand of GC-C was purified from the urine and intestinal mucosa of opossums, and named uroguanylin (110). More recently, additional members of the family, such as lymphoguanylin and renoguanylin were identified (111, 112). GP are expressed as pre-pro-hormones and are proteolytically cleaved to produce the biologically active peptides (113). They share a similar structure two pairs of cysteine residues forming disulfide bonds in conserved positions that are essential for their biological function (114, 115). Like NP, guanylin and uroguanylin bind to a single-membrane-spanning receptor GC (116). GC-C has a similar GC-C domain architecture to GC-A and GC-B and elicit an increase in cellular cGMP (8), which may account for the similar function of the two peptide families. GC-C is mainly expressed in the intestine, but GC-C transcripts were also found in the adrenal gland, kidney, lung, reproductive system, lymphatic organs, and brain (117, 118).

PHYSIOLOGICAL FUNCTION OF GP

Guanylin peptides are produced in the intestine after oral sodium intake and are secreted into the intestinal lumen (119). The resulting increase in enterocyte cGMP stimulates chloride and bicarbonate secretion and inhibits sodium absorption, causing greater secretion of fluids into the intestine (113). Guanylin and uroguanylin also exert long-term effects on the intestine by regulating intestinal cell proliferation (120). In the kidney, these peptides cause increased natriuresis, kaliuresis, and diuresis without changes in GFR or renal blood flow (121). The renal effects of GP are maintained in GC-C null mice (122), suggesting the existence of an additional receptor whose identity is yet to be discovered. Indeed, novel members of the receptor GC family were described in specific cell types in the olfactory system (123, 124).

GP IN THE BRAIN

There are few reports on the expression of GP in the brain (118). Their main effect in the CNS is likely endocrine: guanylin and uroguanylin are secreted from the gut and enter the circulation, mainly as prohormones (125, 126), and subsequently affect extra-intestinal tissues such as the kidney (127) and the brain (128). GC-C, the main intestinal receptor for GP, was found in the midbrain (129) and the hypothalamus (128).

Uroguanylin in satiety control

The intestine is an important endocrine organ, secreting hormones that centrally regulate satiety and food intake (130). The intestinal hormones are vigorously studied as possible therapeutic targets for the growing public health concern regarding obesity and metabolic diseases (131). Valentino and colleagues identified uroguanylin as a novel satiety hormone and showed that food intake causes increased intestinal prouroguanylin secretion in fasting individuals and mice (128). Administration of bacterial enterotoxins (a GC-C agonist) i.v. or i.c.v. (but not orally) reduced food intake in fasting mice, and i.v. administration of anti-prouroguanylin antibodies blocked this response (128). The receptor GC-C is expressed in the mouse hypothalamus. However, uroguanylin expression was not found in this region, suggesting an endocrine rather than a paracrine mode of regulation (128). To strengthen this postulation, Valentino showed an increase in cGMP in the hypothalamus in response to treatment with prouroguanylin, suggesting that this prohormone is cleaved in the hypothalamus by an unknown enzyme to produce the active peptide form (128). Mice lacking GC-C exhibited impaired satiety that resulted in increased food intake, obesity, and metabolic syndrome. In these animals, as opposed to the normal controls, i.v. administration of bacterial enterotoxins did not reduce food intake (128). Although further validation of this new endocrine pathway is necessary, the study provides strong evidence that uroguanylin is a central mediator of food intake, and it may provide a new therapeutic target for obesity and metabolic diseases (132).

GP in behavior

Guanylate cyclase C is expressed in dopaminergic neurons in the midbrain, and GC-C knockout was associated with behavioral changes in mice (129). Gong and collogues showed the expression of the GC-C protein in the ventral tegmental area and substantia nigra compacta in mice (129). Voltage clamp recordings from mouse dopaminergic neurons revealed that guanylin and uroguanylin significantly increased the neuronal activation evoked by metabotropic and muscarinic receptor agonists. This effect was abolished by blocking PKG signaling downstream from GC-C, and it was absent in GC-C knockout mice (129). The knockout mice exhibited increased locomotor activity, high levels of novelty seeking behavior and impulsivity. Such behavior was attenuated by low doses (1 mg/kg) of amphetamine, used to treat attention deficit hyperactivity disorder (ADHD) in humans. It is widely accepted that the dopaminergic system in the midbrain is involved in the etiology of ADHD (133). The GC-C knockout mice were described by the authors as a new animal model for ADHD, as they exhibit some of the symptoms related to this condition (129, 134). However, as the behavioral changes described could mimic several human conditions, further validation of the model is needed, and evidence of the involvement of GC-C in ADHD in humans is required. This pathway can provide new therapeutic targets for diseases involving the dopamine system, such as ADHD and schizophrenia.

FUTURE CHALLENGES

Unlike NP and CS, it appears that GP are not synthesized in the brain, but rather arrive via the circulation from the intestine. However, the link between the intestine and the brain is not clear. Which brain-derived factors, if any, induce GP release from the intestine, and what signaling pathways they regulate require further investigation. The humoral or neuronal pathways that mediate the differential endocrine and paracrine effects of GP on remote organs such as the brain and kidney need to be established. Additionally, new members of the GC receptor family have been recently described in specific sensory neurons (135). There is a strong possibility that there are additional receptors which mediate GP functions in specific brain areas.

CARDIAC STEROIDS

Cardiac steroids are a group of compounds that bind to and inhibit Na^+, K^+-ATPase. These compounds, originally discovered in plants and toad skin, have been used for centuries in Eastern and Western medicine to treat cardiac failure (136). Investigation into these substances started with the search for a missing "third factor" in the regulation of sodium homeostasis, as described in the classic work by de Wardener et al. (137). Although the interest in endogenous CS as the "third factor" has subsided with the finding of the NH, these studies paved the way for the recognition of CS in mammalian tissues and circulation. Rat brain extracts were shown to inhibit Na^+, K^+-ATPase activity and ouabain binding (138–140). Consequently, ouabain (141, 142), digoxin (143), and several bufadienolides (144–148) were identified in mammalian tissues, urine and plasma. CS are considered to be produced in the adrenal cortex and hypothalamus (149, 150), although their complete synthetic pathway is unknown. CS are subdivided into cardenolides, such as ouabain and digoxin, and bufadienolides, including bufalin and marinobufagenin. All CS have a steroid nucleus with a lactone ring at position C-17, and a hydroxyl group at C-14. The 5-member- and 6-member lactone rings are essential for the biological function of the cardenolides and bufadienolides, respectively (151). The only established receptor for all CS is the catalytic α subunit of the plasma membrane Na^+, K^+-ATPase. In addition to the inhibition of the Na^+, K^+-ATPase pumping function (152), the binding of CS results in the activation of signaling transduction cascades, including the Src-kinase, the MAP-kinase, and the PKC signaling pathways (153, 154).

PHYSIOLOGICAL FUNCTION OF CS

Na^+, K^+-ATPase is an essential enzyme expressed in all mammalian cells. CS have widespread effects in different types of cells, including cardiac myocytes, smooth muscle cells, epithelial cells, and neurons (153, 154). CS play important roles in many physiological and pathological processes, among them sodium homeostasis (155), cardiac function (156), BP (157), cell growth (158), and behavior (159). CS form the link between dietary sodium intake and salt-sensitive hypertension (155). As described below, CS regulate BP and hypertension by their effects in the periphery and in the CNS. Given their presence in the brain and CSF, these substances were postulated to act as neurotransmitters or neuromodulators, and they were shown to be involved in psychiatric conditions such as depressive disorders (159). On the cellular level, CS were found to function in cell growth and proliferation (158) as well as in cell migration (160) and they may be associated with the development of cancer (161).

CS IN THE BRAIN

Based on their ability to inhibit ouabain binding and Na^+, K^+-ATPase activity, CS were identified in bovine hypothalamus (140), rat brain (138), and CSF from humans (162, 163). Immunohistochemical studies of mammalian brains revealed high concentrations of CS in the paraventricular nucleus and the supraoptic nucleus (164). Cultured rat hypothalamic neurons were shown to secrete CS *in vitro* (164, 165), supporting the premise that the hypothalamus is the source of endogenous brain CS. The only established CS receptor, Na^+, K^+-ATPase, is expressed throughout the brain. Three isoforms of this enzyme are expressed in the brain: α1, α2, and α3. They display a complex expression pattern: neurons are the principal source of the α3 isoform (166) [although some express α2, especially in the neonate (167)], whereas glial cells predominantly express α2 (168). The α1 isoform is expressed in all cell types, and considered a house keeping protein. The different subunit isoforms vary in their sensitivity to CS and may mediate differential functions of these substances.

CS in central regulation of BP

It is widely accepted that excess dietary sodium is an extremely important factor in essential hypertension (169), although the mechanism by which sodium elevates BP is not clear. A large body of evidence links endogenous CS to the regulation of BP and hypertension. In patients with essential hypertension, plasma levels of ouabain and marinobufagenin were increased in about 40%, with a high correlation with BP (170–175). The plasma levels of these substances in hypertensive patients and in rats increased with sodium intake (176–178). Several animal models for hypertension showed increased circulating levels of CS (178–180). Furthermore, prolonged infusion of ouabain produced hypertension in animals (181–183), but had no effect in genetically modified ouabain-insensitive mice (183, 184). In transgenic mice, a greater natriuretic response to sodium loading was demonstrated in animals expressing a highly CS-sensitive Na^+, K^+-ATPase α1 subunit (185). Studies on mice carrying mutations in the gene encoding α2 showed that ouabain-induced elevation of BP in rodents was mediated via this isoform: reduction of the expression level of α2 was associated with increased BP (186). In contrast, animals overexpressing α2 were hypotensive (187). Treatment of hypertensive rats with anti-digoxin antibodies (185, 188) or anti-marinobufogenin antibodies (178) administered to rats on a high sodium intake, resulted in a marked reduction in BP. Endogenous ouabain was put forward as a putative target for the treatment of hypertension; the ouabain inhibitor rostafuroxin showed promising results in hypertensive rats (189). Studies by Leenen and colleagues indicated that CS involvement in BP regulation is partially mediated by their effect in the CNS. The first indication of brain involvement came from experiments in SHR, in which adrenalectomy did not prevent the increase in CS levels following high sodium intake (177). Lesions in the most anteroventral part of the third ventricle (AV3V) showed that this region is essential in mediating the pressor effects of increased CSF sodium concentration via endogenous

ouabain (190, 191). The effects of both acute and prolonged ouabain infusion in sodium-loaded rodents were abolished by administration of ANG II type 1 receptor blockers such as losartan (192, 193), as well as in transgenic rats with reduced brain renin-angiotensin pathway activity (194). These results pointed to the involvement of this pathway in the effect of ouabain. All of these finding led to a unifying hypothesis regarding the role of CS in sodium-induced hypertension: sodium loading increases the levels of ouabain in salt-sensitive individuals (195, 196). In addition to induction of vasoconstriction in the periphery, ouabain also acts in the brain, where it activates the renin-angiotensin pathway, causing sympathetic activation, vasoconstriction and consequently, an elevation in BP.

CS in depressive disorders

Mood disorders include major depression and dysthymia, characterized by depressive episodes, and bipolar disorder (BD) marked by both depressive and manic episodes. These conditions pose a growing public health concern in the Western world. The etiology of these diseases is not completely understood. Early reports of the psychiatric effects of CS came from doctors describing a syndrome termed "foxglove frenzy" or "digitalis delirium" in patients with digitalis intoxication (197). More recently, a comprehensive hypothesis was put forward, linking brain CS levels and Na^+, K^+-ATPase activity with BD (198, 199). BD has consistently been associated with abnormalities in Na^+, K^+-ATPase activity in erythrocytes (200, 201). A significant mood-related decrease in the enzyme's activity was found in manic BD patients (202). Furthermore, Na^+, K^+-ATPase density was significantly lower in BD patients than in major depressed and schizophrenic patients (159). The plasma levels of endogenous CS were found to be significantly decreased in manic individuals as compared with those in normal controls (203, 204). Conversely, the levels of these compounds were higher in the parietal cortex of BD patients (159). More recently, it was found that there is a reduction in brain Na^+, K^+-ATPase α1 isoform expression in mice treated with the mood stabilizer lithium (205). An allelic association between BD and a Na^+, K^+-ATPase α subunit gene (ATP1A3) was reported (206). We have demonstrated the prominent linkage to BD of six single-nucleotide polymorphisms (SNPs) in the three genes of the Na^+, K^+-ATPase α isoforms. Haplotype analysis of the α2 isoform showed the significant association of two loci haplotypes with BD (207). A genetic knockdown of the neuron-specific Na^+, K^+-ATPase α3 isoform induced manic-like behavior in mice (208). Numerous studies have demonstrated that i.c.v. injection of ouabain induces hyperactive behavior in rats (159, 209), which could be ameliorated by administration of mood stabilizing drugs such as lithium (210). Reduction of the endogenous brain CS level by i.c.v. injection of anti-ouabain antibodies had anti-depressive effects in rats (159, 211). This was reflected by significant changes in catecholamine metabolism in the hippocampus and ventral tegmentum, two regions known to be associated with mood disorders (211). The molecular pathway underlying the CS behavioral effect is unknown. Ouabain injected i.c.v. elicited the activation of the ERK and Akt signaling pathways in the brain (212, 213), which are known to be activated via Na^+, K^+-ATPase. Other effects of ouabain include a reduction in brain-derived neurotrophic factor (BDNF) (214), activation of mammalian target of rapamycin (mTOR) signaling (213) and an increased level of oxidative stress (215). These findings strongly link the Na^+, K^+-ATPase and CS system to the etiology of depressive disorders, and BD in particular, and suggest their potential application in future drug development.

FUTURE CHALLENGES

Despite the identification of cardenolides and bufadienolides in mammalian tissue in many independent studies (see Cardiac steroids), some still question the validity of these findings. Recently, it was claimed that ouabain, the most studied cardenolide, could not be detected in human plasma (216). This issue must be clarified. An additional major missing piece of information for the establishment of CS as neurotransmitters or neuromodulators is the elucidation of their biosynthetic pathway in the adrenal gland and brain. Although the available literature supports the notion that these steroids are synthesized in mammals, the key enzymes involved have not been identified. This issue was recently reviewed in Ref. (217). Several CS were identified in the human body. It was postulated that the different α isoforms of the Na^+, K^+-ATPase serve as receptors for the different CS. Which of the CS are involved in brain functions, and which isoform combinations they activate are topics for future research.

INTERACTIONS OF ANP WITH CS AND GP

Mutual interactions exist between CS and NP in the periphery and in the brain. Ouabain and digoxin were shown to cause increased ANP expression and secretion in rat and rabbit atria (218–221), and in anesthetized dogs (222). In patients with congestive heart failure, i.v. administration of digitalis increased the plasma levels of ANP and BNP (223). Indeed, ANP regulates the secretion of CS in the brain (224–226). ANP decreased the release of CS from rat brain extract when added to the tissue, or when administered i.v. to the animals prior to their sacrifice (224). On the other hand, another study showed that i.c.v. injection of synthetic ANP increased blood CS levels, whereas i.v. administration or incubation with this peptide had no effect (225). The effect of ANP on CS release was abolished by lesions in the AV3V region (226), the area in the hypothalamus that is thought to mediate CS central regulation of BP (191). In addition to secretion regulation, NP and CS interact at the functional level. ANP differentially modulates the effect of marinobufogenin in the rat heart and kidney (227). Ouabain was shown to antagonize the effect of ANP on vasorelaxation in rabbit aorta and in dogs (228, 229), whereas ANP abolished an ouabain-induced increase in aldosteron secretion (230). Administration of anti-ouabain antibodies caused increased sensitivity to ANP-induced vasodilation in rat aorta (231). In rat heart muscle preparations, ANP was shown to attenuate several effects of ouabain, including ouabain-induced increase in contractility, Na^+, K^+-ATPase and ERK phosphorylation (232). ANP also interacts with GP. Santos-Neto and colleagues showed synergism between ANP, guanylin, and uroguanylin in the kidney (233). They demonstrated in an isolated perfused rat kidney that pretreatment with ANP significantly enhanced the natriuretic, kaliuretic, and chloriuretic responses to low doses of guanylin and uroguanylin (233). Low doses of ANP enhanced GP induced diuresis, and vice versa

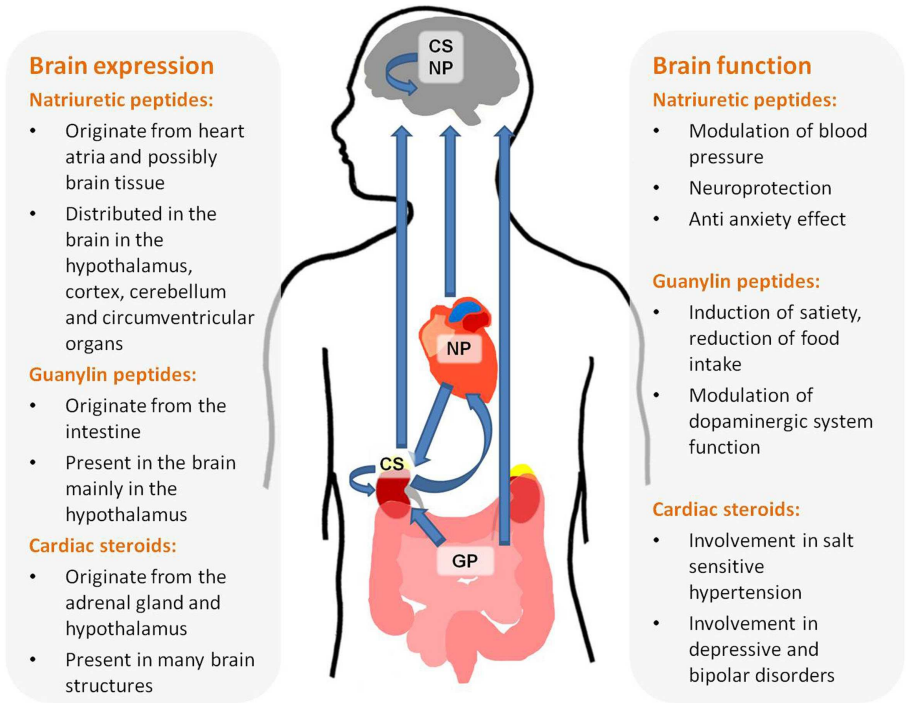

FIGURE 1 | Origin, brain distribution, and function of natriuretic hormones.

(233). Since GP and NP activate GC receptors, their interaction may be mediated through the shared second messenger, cGMP (6, 116, 234). These initial studies suggest the physiological crosstalk between ANP and CS, particularly in the cardiovascular system and in the brain, and between ANP and GP in the kidney. More studies are needed to deepen our understanding of the nature of these interactions, which may be of significance in the regulation of peripheral and central functions of the NH.

CONCLUSION

This review summarizes the available data implicating NH in brain function. There is a vast amount of data supporting the assessment that the three families of NH, NP, Guanylins, and endogenous CS are present in the brain and participate in high brain functions (see **Figure 1**). These include central regulation of BP, satiety, neuroprotection, and behavior. In depth research of these effects will not only increase our thorough understanding of brain function but may also lead to new treatments for brain-related diseases.

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Identification of putative natriuretic hormones isolated from human urine

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This brief review describes some representative methodological approaches to the isolation of putative endogenous inhibitors of epithelial sodium transport – i.e., as ouabain-like factors (OLF) that inhibit the sodium transport enzyme Na-K-ATPase or inhibit the epithelial sodium channel (ENaC). Gel chromatography and reverse-phase (RP)-high performance liquid chromatography (HPLC) of lyophilized and reconstituted 24 h-urine from salt-loaded healthy humans led to two active fractions, a hydrophilic OLF-1 and a lipophilic OLF-2, whose mass (Ms)-spectroscopic data indicate a M_r of 391 (1, 2). Further identification was attempted by Ms-, infrared (IR)-, ultraviolet (UV)-, and ^{1}H-NMR-spectroscopy. OLF-1 and OLF-2 may be closely related if not identical to (di)ascorbic acid or its salts such as vanadium (V)-V^v-diascorbate with M_r 403 (3) and V^{IV}-diascorbate. OLF-1 and V^v-diascorbate are about 10-fold stronger inhibitors of Na-K-ATPase than OLF-2 and V^{IV}-diascorbate, respectively. In conscious rats, i.v. infusion of OLF-1 and OLF-2 resulted in a strong natriuresis. In a similar study, Cain et al. (4) isolated a sodium transport inhibitor from the urine of uremic patients by gel chromatography and RP-HPLC. In uremic rats, a natriuretic response to the injection of the active material was found. Xanthurenic acid 8-O-β-D-glucoside (M_r 368) and xanthurenic acid 8-O-sulfate (M_r 284) were identified as endogenous inhibitors of sodium transport acting, e.g., by ENaC blockade. No definite relation to blood pressure, body fluid volume, or sodium balance has been reported for any of these above factors, and further studies to identify the natriuretic and/or ouabain-like compound(s) or hormone(s) will be needed.

Keywords: sodium transport, natriuretic hormone, human urine, endogenous inhibitors, epithelial sodium transport

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Introduction – Background

With this brief review, some methological aspects of the isolation of putative endogenous membrane transport inhibitor(s) and natriuretic factor(s) will be described, and the results compared with those of similar attempts by other groups of investigators.

In 1969 (5), and in more detail in 1974 (6) and 1977 (7), we demonstrated for the first time that acute extracellular fluid volume (ECFV)-expansion in rats may release a natriuretic factor or "hormone," which was postulated to act through inhibition of the sodium pump. Thus, ECFV-expansion was accompanied by a decrease in Na-K-ATPase in the renal cortex and the appearance of an inhibitor of Na-K-ATPase in the serum of these rats, respectively. Using gel chromatography, this inhibitory activity was also detected in the post-salt fraction of serum from ECFV-expanded dogs and in the serum and urine of salt-loaded humans. Besides the *in vitro*-assay of the inhibitory activity, we also demonstrated the inhibitory effect of this serum fraction on epithelial sodium transport, i.e., on

short-circuit current (SCC) and potential difference (PD) in the isolated frog skin (8). This fraction of serum or urine was also found to cause natriuresis in a rat bioassay. We concluded that a natriuretic factor emerges in the circulation with excessive salt load whose mechanism of action was to modulate the Na-K-ATPase enzyme in the vasculature as well as in the renal tubule.

Attempts to identify this humoral inhibitor(s), however, remained unsuccessful despite the use of extensive methodologic approaches. Besides an endogenous ouabain (9), several bufodienolides (10) have been identified using the methods described in this paper of which two compounds with M_r of around 400 daltons will be considered in the present mini-review, namely vanadium (V)-diascorbate(s) and derivatives of xanthurenic acid (4). The compounds were assumed to be present in the circulation, and therefore may be excreted in the urine.

Ouabain-Like Factor(s) as Endogenous Sodium Transport Inhibitors

As source for isolation and identification of a putative natriuretic hormone and/or endogenous epithelial sodium transport enzyme inhibitor, we pooled large quantities (50–100 L) of urine from salt-loaded healthy humans, which was lyophilized to dryness and reconstituted with 0.01M acetic acid, and then subjected to gel chromatography using Sephadex G-25 and Sephadex G-10 columns.

Ouabain-Like Factors and Vanadium-Diascorbic Acid: Effects on Na-K-ATPase

To detect the serum and urine fractions with the active compound(s), we employed an *in vitro*-assay of Na-K-ATPase using a purified commercially available hog cerebral enzyme preparation. We also used this Na-K-ATPase membrane fraction as marker to follow-up activity with purification steps during the subsequent chromatographic steps. To detect the potential natriuretic activity, all fractions were screened for their natriuretic effect using a bioassay in conscious rats (11).

The transport enzyme Na-K-ATPase inhibitory and natriuretic activity(ies) eluted from the Sephadex G-25 column in a post-salt fraction. When this fraction was then subjected to gel chromatography on Sephadex G-10, a strongly active enzyme inhibitory material eluted in a late fraction (1). This late fraction also showed a significant natriuretic action (11). This enzyme inhibitory and natriuretic fraction was subjected to high performance liquid chromatography (HPLC), and subsequently to thin layer chromatography (TLC). Characterization of the active material was attempted by mass (M_r)-, nuclear magnetic resonance (^{1}H-NMR)-, infrared (IR)-spectroscopy (1), and ultraviolet (UV)-fluorescence/absorbance. The natriuretic activity was also studied by bioassay to identify the active compounds after gel filtration, reverse phase (RP)-HPLC, and amino acid analysis for its potential peptidic character (11).

Reverse-phase HPLC of this highly active late fraction from Sephadex G-10 resulted in two subfractions with significant Na-K-ATPase enzyme inhibition. They were named ouabain-like factors (OLF); one eluted in the water phase as the more polar hydrophilic OLF-1; the second eluted in a later phase at 20% acetonitrile as the more apolar lipophilic OLF-2. These fractions also produced a significant natriuresis (see below).

Analysis of Chemical Structure

Both compounds showed signals for hydroxyl and carboxyl groups as well as criteria for esters or lactones (a precursor of ascorbic acid in plants and animals is L-gulono-y-lactone, and 2,3-diketogulonic acid is an oxidation product of ascorbic acid). No signals for aromatic, aliphatic, heterocyclic, or steroid structures were found. Whereas the IR-spectrum of OLF-1 is different from that of OLF-2 (1), UV-, M_r-, and ^{1}H-NMR-criteria were similar and fluorescence of both compounds when separated by TLC required the presence of a dicarboxylic acid-like conformation; dicarboxylic acid [see also Ref. (11): Asp, Glu as carboxylic acids] is an organic compound containing two carboxyl functional groups (–COOH). IR- and ^{1}H-NMR spectra of OLF-1 and OLF-2 suggest a chemical structure resembling a sugar or sugar derivative. However, sugars are not fluorescent as are the OLF recovered from TLC. Therefore, these data suggest the unknown compounds to be identical with ascorbic acid or its salts such as V^{v}-diascorbate and V^{IV}-diascorbate, respectively, with M_r 403 (3). The superscript roman numbers indicate the oxidative state of vanadium (V): V^{IV} oxide (V_2O_5), the most stable oxygen combination, and V^{IV} oxide (VO_2) represent two of the four oxygen states of vanadium. V-diascorbates elute from the RP-HPLC column at similar elution times and acetonitrile gradients as the hydrophilic and lipophilic OLF-1 and OLF-2, respectively. V^{IV}-diascorbate also showed the same UV-maximum as we found for OLF. Thus, ascorbic acid seems to be an important cornerstone of the structure of the yet unknown humoral ATPase inhibitor.

It is noteworthy that the water solubility of the individual ascorbic acid salts of metals varies remarkably, and it may be assumed that V^{v}- and V^{IV} diascorbates with their different water solubility elute from the RP-HPLC column at similar elution times as the OLF-1 and OLF-2, respectively. V-diascorbates also show the same UV-maximum as the OLF and are strong candidates for the urinary hydrophilic OLF-1 and lipophilic OLF-2, respectively.

Effects on Enzyme Kinetics

These active subfractions, containing OLF-1 and OLF-2, were further purified by two-dimensional preparative TLC to single compounds, whose mass spectroscopic (MS) data suggested a M_r of around 400. Actually, OLF-2, which dose-dependently inhibited Na-K-ATPase, was found to have a M_r of 391 (1). With respect to the effects of OLF-1 and OLF-2 and of V^{v}- and V^{IV}-diascorbates on Na-K-ATPase enzyme activity and kinetics, *in vitro* studies showed that OLF-1 and OLF-2 inhibited the enzyme in its E2 configuration. In analogy to the polar OLF-1, which revealed an approximately 10-fold stronger enzyme inhibition (IC_{50} 1.5×10^{-5} M) than the apolar OLF-2 (IC_{50} 1.5×10^{-4} M), we found that V^{v}-diascorbate (IC_{50} 2×10^{-6} M) is a significantly stronger inhibitor of Na-K-ATPase than V^{IV}-diascorbate (IC_{50} of 9×10^{-5} M) (3, 5, 12). In this context, I should mention that we found previously that certain trace metals are strong inhibitors of this enzyme (13).

Renal and Vascular Mechanisms of Action of OLF

Regarding the potential mechanism of the physiological and pathological effects of OLF-1 and OLF-2 on vascular smooth muscle cells (VSMCs) and inner medullary collecting duct cells (IMCD cells), we found in an *in vitro*-assay that OLF-1 and OLF-2 enhanced

VSMC contractility by increasing intracellular Ca^{2+} similar to the effect of ouabain (14, 15). Similar effects were found with OLF-1 and OLF-2 on intracellular Ca^{2+} in IMCD cells, suggesting inhibition of tubular Na-reabsorption and thus regulating renal excretion, i.e., to enhance Na-excretion (16).

Ouabain-Like Factors and V-Diascorbates: Natriuretic Effects

For demonstration of the natriuretic activity, we used a bioassay in conscious rats (12). As mentioned above, in our assay system, the post-salt fraction IV from Sephadex G-25 was applied to Sephadex-G-10 and resulted in a late fraction, which was applied to RP-HPLC. When administered i.v., OLF-1 resulted in an immediate, eightfold rise in natriuresis from approximately 1 to 8 μEq/min/mg, whereas the apolar OLF-2 caused a natriuresis of slower onset reaching its maximum after 60 min and lasting for more than 180 min. This was confirmed also by injection of the active fractions obtained by quantitative TLC.

Natriuretic Factor Unrelated to OLF

Finally, I should mention that we described previously a natriuretic compound, which we suggested to be a peptide. Thus, when the pooled post-salt natriuretic urine fraction obtained by gel chromatography (see above) was subjected to repetitive RP-HPLC, a late eluting fraction showed strong natriuretic activity in the bioassay and was associated with a fluorescence peak when treated with o-phthaldialdehyde as a marker for primary amines (11). Amino acid analysis before and after total acid hydrolysis suggested a peptide tentatively containing the amino acids (AA) Asp, Glu, Gly, Phe, and Ser (1, 11). The natriuretic activity was lost after incubation with chymotrypsin, which splits bonds with aromatic AA (2). We found, in addition, that several synthetic (mono-) peptides of di- and tri-AA are significantly natriuretic when injected i.v. (unpublished data).

Xanthurenic Acid 8-O-β-D-Glucoside and Xanthurenic Acid 8-O-Sulfate as Endogenous Sodium Transport Inhibitors

Cain et al. (4) followed a protocol very similar to that of Kramer et al. for isolation of the natriuretic activity except that they used the urine of uremic patients as source of the inhibitor and a bioassay in (conscious?) uremic rats. As marker for the active material, Cain et al. used changes of the SCC of the isolated frog skin – as we described in 1977 (8) – for monitoring transepithelial sodium transport inhibitory activity. For monitoring its natriuretic effect, the above mentioned bioassay in uremic rats was used. A direct *in vitro*-assay for inhibition of the Na-K-ATPase enzyme by the natriuretic factor or "hormone" was not employed. The authors rather speculate that the natriuretic hormone may act via other sodium pumps in the kidney, e.g., the epithelial sodium channel (ENaC) in the distal tubule.

Xanthurenic Acid Derivatives: Effects on Epithelial Sodium Transport

Epithelial sodium transport was measured as changes of SCC and PD in the isolated frog skin. For isolation and identification of the transport inhibitor, one gel chromatographic step and three consecutive HPLC steps were applied. Final identification was achieved by mass (Ms)-, IR-, UV-, and NMR-spectroscopy. Purification of the activity was estimated from UV peak with a characteristic spectrum at 338 nm. Xanthurenic acid 8-O-β-D-glucoside (M_r 368) and xanthurenic acid 8-O-sulfate (M_r 284) were identified as the endogenous sodium transport (ENaC, Na-K-ATPase) inhibitors.

Xanthurenic Acid Derivatives: Natriuretic Effects

The material (M_r 368) obtained from two HPLC runs was tested for natriuretic effect in their uremic rat bioassay. Urinary sodium excretion rose immediately and reached its maximum approximately 40 min after intra-arterial infusion (5). Urinary volume increased slightly and then decreased to below baseline, i.e., a decrease in urine volume with a rise in urinary osmolality.

A pathophysiological role of xanthurenic acid, a tryptophane derivative, is difficult to envisage as this uremic toxin may inhibit transmembranous sodium transport independent of a potential role as specific circulating natriuretic or sodium transport inhibiting "hormone." Thus, although Bricker et al. showed that the natriuretic action of the isolated inhibitor paralleled the changes in renal function, as an alternative explanation, it may be reasonable to assume that with the progressive decrease in renal function and the accumulation of toxic metabolites, the rise in fractional sodium excretion may parallel the urinary concentration of the xanthurenic derivatives.

Summary

Although there is no doubt that an as yet unidentified natriuretic compound can be isolated from human urine by gel filtration and RP-HPLC, whose activity changes in parallel with salt (sodium chloride) intake, i.e., it correlates with salt-balance (low or high salt intake). Therefore, the activity may be related to an as yet unidentified "natriuretic hormone" that is assumed to play a crucial role in the fine-tuning of renal tubular sodium handling and may thus be involved in the long-term body fluid and blood pressure regulation.

We found two Na-K-ATPase inhibitors, the hydrophilic OLF-1 and the lipophilic OLF-2 (1). The hydrophilic form was more potent than the lipophilic one. The lipophilic compound was moderately natriuretic but strong ATPase inhibitor. Both compounds showed UV fluorescence/absorbance of lower intensity in the hydrophilic (hydrated) form. Both enzyme inhibitors showed UV-absorbance, which requires the presence of a dicarboxylic acid-like arrangement (1). In addition, from our data the unknown compound(s) most likely fulfill(s) the criteria for *lactones*.

Unfortunately, for none of the three classes of endogenous sodium transport inhibitors, a physiologic or pathophysiologic role was demonstrated, i.e., no correlation to body fluid and sodium balance or blood pressure was documented. Thus, further studies are required to confirm the structures of the various endogenous factors; final identification of their physiological and pathophysiological significance must await urgent results of additional well-designed studies.

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Evidence for a 12 kDa "carrier protein" for natriuretic hormone

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The search for the elusive Na-K-ATPase-inhibiting natriuretic hormone continues. In this review, evidence is presented that isolating the carrier protein for natriuretic hormone from hypertensive plasma is a necessary first step before splitting off the final hormone. The carrier protein has a molecular weight of 12 kDa while the final hormone has a molecular weight of 408 Da. Both compounds inhibit Na-K-ATPase but the compound containing the carrier protein predominates. The question has been raised as to whether the carrier protein is in actuality proANF, a 17 kDa protein that can be split between a 14 kDa protein (the presumptive proANF) and the 3 kDa ANF.

Keywords: natriuretic hormone, carrier proteins, hormones, hypertension, review, Anf, proANF

INTRODUCTION

Circulating inhibitors of sodium-potassium adenosine triphosphatase (Na-K-ATPase) have been shown to be of possible pathogenetic importance in the mechanism of essential hypertension (1–3). Although previous studies have demonstrated the presence of both high-molecular weight (HMW), ranging from 11 to 70 kDa (4–8) and low-molecular weight (LMW) either natriuretic or Na-K-ATPase inhibitors, no previous attempts had been made to ascertain whether HMW or LMW forms predominate in hypertension. This review summarizes the steps taken by our laboratory to first identify the HMW form, and then split off the final LMW form of the hormone. We have in the process determined the approximate molecular weight of the HMW form and the precise molecular weight of the LMW form. Unfortunately, while awaiting the identification of the latter compound, it was lost due to freezer failures in two different laboratories a continent apart. This review is presented in intricate detail in the hopes of encouraging subsequent investigators to pursue the final identification of the LMW natriuretic hormone, as well as the identity of the "carrier protein."

PREDOMINANCE OF HMW PLASMA Na-K-ATPase INHIBITOR IN HYPERTENSION

In an initial study (9), plasma samples obtained from 26 patients with essential hypertension, 12 normotensive controls, and 6 normotensives with a family history of hypertension were separated into HMW and LMW moieties by passage through a 1 kDa Amicon membrane. The LMW moiety was separated on C-18 Sep-Pak cartridges, applying a 10% stepwise acetonitrile trifluoroacetic acid gradient. The HMW moiety was further separated on Sephadex G-75. Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis revealed that the fraction with inhibitory activity contained a distinct 12 kDa protein band, with staining intensity depending on the presence or absence of hypertension (**Figure 1**). Na-K-ATPase inhibitory activity was found in several LMW fractions, but differences between hypertensives and normotensives were observed in only the 50% acetonitrile fraction (0.29 ± 0.12 SD versus 0.11 ± 0.12 μmol/L ouabain equivalents, $p < 0.01$). Na-K-ATPase inhibitory activity in the HMW fraction was 38 times the inhibitory activity in the LMW fraction and was significantly increased in hypertensives as compared to normotensive controls (10.9 ± 8.9 versus 1.3 ± 0.8 μmol/L ouabain equivalents, $p < 0.01$). Inhibitory activity in both HMW and LMW factions correlated positively with mean blood pressure ($r = 0.42$, $p < 0.05$ and $r = 0.35$, $p < 0.05$). The inhibitory activity in the HMW fraction, but not the LMW fraction, also correlated positively with diastolic blood pressure and inversely with the natural log of plasma renin activity ($r = 0.40$, $p < 0.01$). These results indicate that the HMW moiety is the predominant circulating form of the Na-K-ATPase inhibitor in hypertension.

DISSOCIATION OF THE LMW Na-K-ATPase INHIBITOR FROM THE HMW PROTEIN INHIBITOR

Pooled blood samples from 10 patients with well-documented essential hypertension, not taking any medications for at least 3 weeks, were collected into chilled vacutainers containing sodium ethylenediamine tetraacetic acid (EDTA) and Trasylol (10). Individual samples were also collected from patients with primary aldosteronism, congestive heart failure (CHF), before and after treatment, and normal controls. The treatment of congestive failure employed diuretics and vasorelaxants but avoided digitalis glycosides.

SDS-PAGE was performed according to the procedure described by Laemmli (11).

Plasma samples were also passed through a series of Amicon membranes, the initial ultrafiltration step employing a 1 kDa (YM-2) membrane. The retentate was reconstituted in distilled water and heated for 10 min at 70°C in the presence of 4% beta-mercaptomethanol and 1 mol/L formic acid. The solution was cooled down and subsequently placed on a 30 kDa (YM-30) membrane. The resulting filtrate, containing the dissociated protein, was lyophilized and subjected to further purification.

The dissociated protein was adsorbed onto a SEP-PAK C-18 cartridge. Interfering compounds, e.g., small peptides, hydrophobic

substances, etc., were retained on the SEP-PAK C-18 cartridge. The protein of interest was eluted off the SEP-PAK C-18 cartridge with distilled water. This fraction was lyophilized, reconstituted in 1 mL of distilled water, and subsequently separated on Sephadex G-75. The plasma preparation was eluted off the column with 10 mmol/L ammonium acetate, pH 6.5. Fractions containing the Na-K-ATPase inhibitory material (12 kDa protein), which eluted after the albumin peak, were pooled, lyophilized, and subjected to a series of assays.

Duplicate bioassay procedures for the natriuretic response of the 12 kDa protein were performed according to the method described by Purdy et al. (12). Outcomes were averaged. Results are displayed in **Figure 2**.

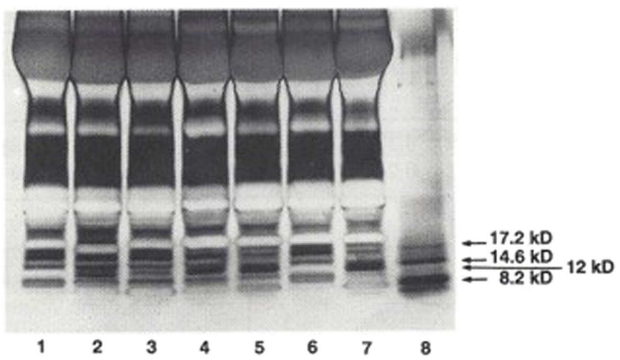

FIGURE 1 | SDS-PAGE separation of protein bands from plasma from hypertensives and normotensives. Bands were stained with silver nitrate. Myoglobin protein markers of 17.2, 14.6, and 8.2 kDa molecular weight and the locus of the 12 kDa protein are shown in Lane 8. Lane 1: Normotensive control. Lanes 2 and 3: normotensives with family history of hypertension. Lanes 4 and 5: hypertensives. Lane 6: normotensive control. Lane 7: hyperaldosteronism. Plasma samples applied in lanes 2 and 3 were obtained from the two individuals shown to possess elevated levels of HMW Na-K-ATPase inhibitor. From Ref. (9) with permission from the editors.

An Econosphere C-18 column was packed with Econosphere C-18 silica, 5 μm particle size. The reversed phase C-18 column was equilibrated with triple distilled water. The LMW plasma Na-K-ATPase inhibitor (p-NKAI) was eluted off the column with a linear acetonitrile gradient (0–100% over a period of 30 min). The eluate was continuously monitored at 210 nm. One minute fractions were collected, lyophilized, and subsequently tested for the presence of Na-K-ATPase inhibitory activity.

P-NKAI was further purified by HPLC separation combined with electrochemical detection using a Model 5100A Coulocomb Detection System. On reversed phase C18 chromatography, p-NKAI appeared at 4% acetonitrile, co-eluting with a urinary inhibitor. P-NKAI was ultrafiltrable through an Amicon YM-05 membrane and thus has a presumed molecular weight of less than 500 Da. Rechromatography of active fractions on a 3 μm C-18 column monitored electrochemically yielded two active compounds, p-NKAI-1 and p-NKAI-2, both of which were inhibitors of the Na-K-ATPase enzyme system (**Figure 3**). P-NKAI-1 caused 50% inhibition and p-NKAI-2 caused 8% inhibition of Na-K-ATPase in a volume of inhibitor corresponding to 187 μL of original plasma. The remaining fractions were without inhibitory activity.

The mass spectrum of p-NKAI-1 showed a fairly intense protonated molecular ion at mass 409 and also the sodium and potassium adduct ions at masses 431 and 447, respectively. This would indicate that the molecular weight of p-NKAI-1 is 408 Da (**Figure 4**).

A purified hog cerebral cortex Na-K-ATPase preparation was employed for Na-K-ATPase and K-pNPPase inhibition assays. The tubes were preincubated with either the 12 kDa protein or the purified LMW plasma factor for 5 min at 37°C. The enzymatic reaction was initiated by adding 0.025 mL enzyme preparation (25 mg/mL). The reaction was stopped by adding 1.0 mL ice cold 10% trichloroacetic acid after an incubation time of 15 min. After centrifugation, 0.5 mL of supernatant was assayed for inorganic phosphate according to the procedure described by Fisk and Subbarow (13). Both the 12 kDa protein and the LMW plasma factor

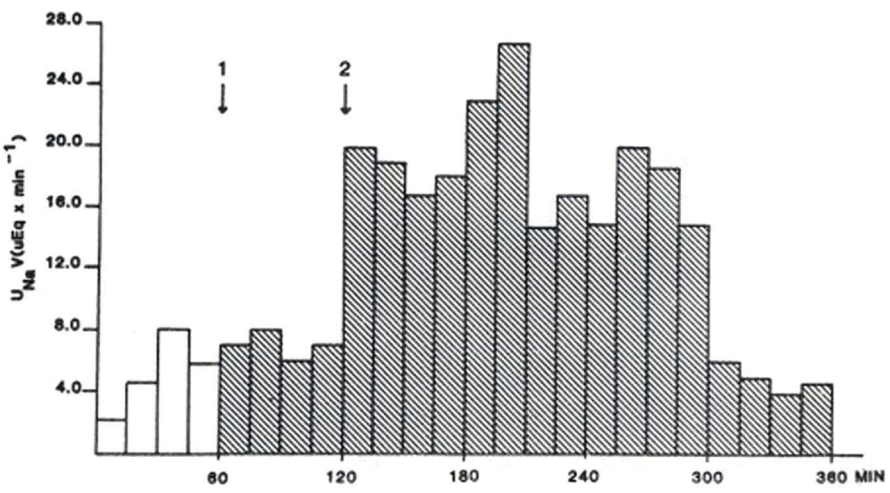

FIGURE 2 | Rat natriuretic bioassay of semi-purified 12 kDa protein representing 2.5 mL of original plasma. First arrow designates time of injection of control solution. The second arrow designates the time of injection of the 12 kDa protein. From Ref. (10) with permission from the editors.

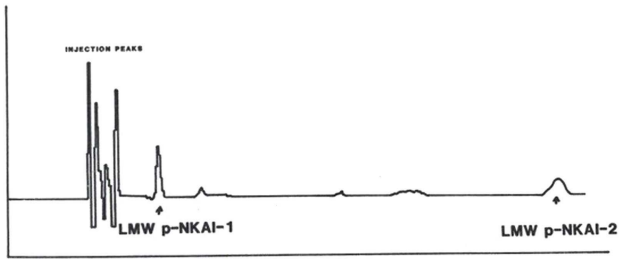

FIGURE 3 | Elution of semi-purified LMW p-NKAI, monitored electrochemically. Arrows indicate the loci of p-NKAI-1 and p-NKAI-2. From Ref. (10) with permission from the editors.

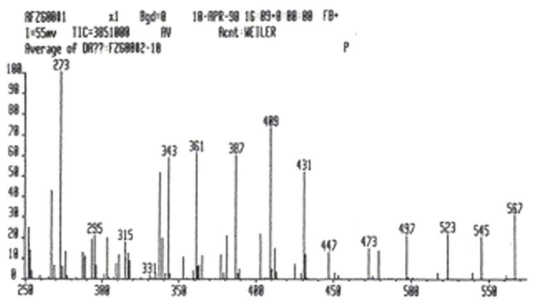

FIGURE 4 | Mass spectroscopy analysis of p-NKAI-1. Note peaks at masses 409, indicating hydrogen adduct, 431, indicating sodium adduct, and 447, indicating potassium adduct. The mass of p-NKAI-1 is, therefore, 408. From Ref. (10) with permission from the editors.

(p-NKAI) were shown to inhibit the Na-K-ATPase and K-pNPPase enzyme systems in a dose-related manner, analogous to ouabain. The IC_{50} for inhibition of Na-K-ATPase by p-NKAI corresponds to 8×10^{-7} mol/L ouabain equivalents.

P-NKAI-1 was also tested for its vasoactive properties according to the procedure described by Purdy and Weber (14). Isolated femoral artery segments from New Zealand White rabbits were sectioned into 3 mm segments, then mounted in a 30 mL tissue bath containing Krebs-bicarbonate solution aerated continuously with 95% O^2/5% CO_2 at 37°C. Subsequently, p-NKAI-1 was assayed for its vasoactive behavior in the presence and absence of norepinephrine. A dose–response curve was established for p-NKAI-1; the concentration of p-NKAI-I yielding 1% contractile response was selected for the studies of synergy with norepinephrine. One hundred microliters of p-NKAI-1 produced a 1% contractile response, 300 μL produced a 5% contractile response and 600 μL of p-NKAI-1 produced an 18% contractile response. Similarly, the addition of 100 μL of p-NKAI-1 to a bath containing 10^{-8} mol/L norepinephrine increased the contractile response from 60 to 86%.

The dose–response curve for Na-K-ATPase inhibition of the semi-purified 12 kDa protein paralleled the dose–response curve for ouabain; 50% inhibition of Na-K-ATPase, corresponding to 5×10^{-6} mol/L ouabain, was produced by the 12 kDa inhibitor in a fraction containing 2.7 mg/mL Lowry protein.

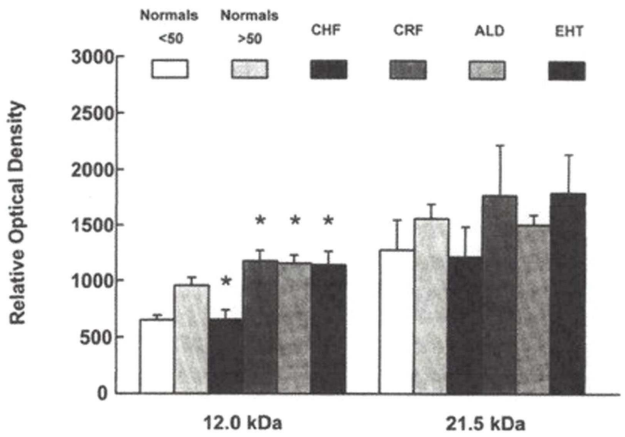

FIGURE 5 | Relative optical densities of 12 and 21 kDa bands. $*p < 0.05$ for CRF, Aldo, EHT compared to normals < 50 and for CHF compared to normals > 50. From Ref. (14) with permission.

The ^{3}H ouabain displacement assay revealed that the 12 kDa protein fraction displaces ^{3}H-ouabain from its receptor in a dose-related manner, similar to ouabain. There was no cross-reactivity with digoxin antibody.

COMPARISON OF 12 kDa PROTEIN, MARINOBUFAGENIN, AND OUABAIN IN VARIOUS DISEASE STATES

In a third study (15), plasma from 101 patients were examined [25 normals (N) < age 50, 13N > age 50, 7 with acute CHF, 24 with chronic renal failure (CRF), on dialysis, 5 with idiopathic hyperaldosteronism (PA), and 27 with essential hypertension, untreated (EHT)]. Plasma was extracted with 32% acetonitrile, and analyzed by fluoroimmunoassay (DELFIA) for marinobufagenin and ouabain. In addition, from 32 patients (6N < 50, 6N > 50, 5 CHF, 5 CRF, 6 EHT, and 4 PA), SDS gradient gels were obtained. The 12 kDa bands were extracted, analyzed for Na-K-ATPase inhibition, marinobufagenin, and ouabain, and compared to 14 and 21 kDa bands. Marinobufagenin was found to be elevated in CRF, EHT, PA, and CHF. Ouabain was increased only in PA. When the relative optical densities of 12 and 21 kDa bands were contrasted, CRF, PA, and HT were found to be increased and CHF to be decreased in the 12 kDa band, with no discernible changes in the 21 kDa bands (**Figure 5**). Following extraction of the bands, Na-K-ATPase inhibitory activity measured 38% in 16 pooled 12 kDa bands, with essentially no activity found in the 14 kDa or 21 kDa bands. SDS-PAGE separation of plasma proteins confirmed that the 12 kDa band was elevated in primary aldosteronism, diminished in CHF, with return toward normal after treatment (**Figure 6**). Thus, only the 12 kDa band possessed all of the attributes of natriuretic hormone.

DISCUSSION

Following an initial flurry of activity, which utilized natriuresis as an index of hormone activity, most subsequent studies of natriuretic hormone have utilized Na-K-ATPase inhibition as a more rapidly obtained index (1–3). Digitalis-like (EDLF) or

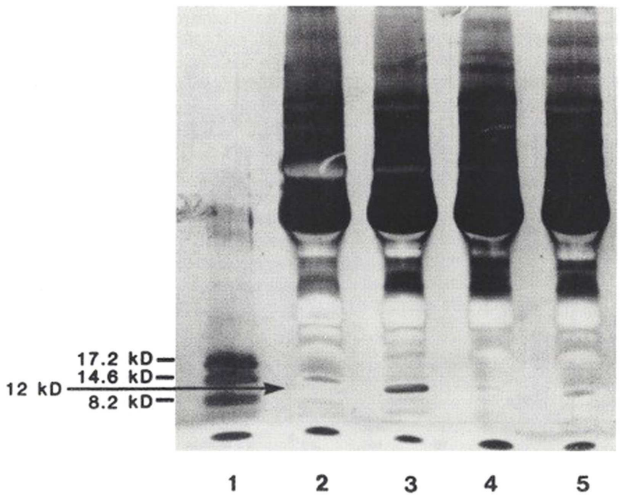

FIGURE 6 | SDS-PAGE separation of protein bands from the plasma of patients with various volume-related disease states. Myoglobin protein markers of 17.2, 14.6, and 8.2 kDa are shown in lane 1. Lane 2: normal control. Lane 3: hyperaldosteronism. Lane 4: congestive heart failure, pre-treatment. Lane 5: congestive heart failure, post-treatment. These gel separations are from the plasma of individual patients. From Ref. (10) with permission from the editors.

ouabain-like activities (OLF), measured by radioimmunoassay, were also initially employed as measures of natriuretic hormone. But digoxin-like immunoreactivity was found to be non-specific (16), while the radioimmunoassay for OLF did not prove reliable when measured by HPLC followed by ELISA (17), or by ultrasensitive UPLC-MS/MS (18). The lower limit of quantification by the latter method was 1.7 pmol/L, while ouabain was non-detectable. The suggestion that the presence of endogenous ouabain in human beings is non-detectable has been vigorously debated by Blaustein (19). For the moment, therefore, we must consider this an unresolved matter. Thus, we are left with the Na-K-ATPase inhibition assay as presumably the most reliable as well as the most rapid assay of EDLF activity.

The remaining ouabain-like hormone, which has been suspected to be the putative natriuretic hormone, is marinobufagenin (20), for which studies of activity in several diseases have appeared, including volume-expanded normals (20), CHF (21), CRF (22), essential hypertension (23), primary aldosteronism (15), and pre-eclampsia (24). The one noteworthy discrepancy between natriuretic hormone determined by marinobufagenin radioimmunoassay and natriuretic hormone, as determined by the Na-K-ATPase assay, are the findings in CHF [see above and Ref. (25)]. Urinary sodium values are low in CHF, LMW urinary Na-K-ATPase inhibitors are also lower than normal (25), and arterial central volume is diminished rather than increased. Kramer and Kruck (26) found that a natriuretic substance present in an ultrafiltrate of normal urine from volume-expanded individuals was absent in the urine of patients with edema related to cirrhosis with ascites or with nephrotic syndrome, edematous states physiologically similar to CHF. Furthermore, they also demonstrated that plasma and urine fractions of normal individuals following Sephadex G-25 separation consistently reduced short-circuit current when applied to the serosal surface of frog skin (anti-natriferic effect) (26), whereas plasma and urine fractions from patients with edema lacked this effect. In addition, we have shown previously that although the LMW Na-K-ATPase inhibitor in human urine has less activity than normal in CHF, the activity reverts toward normal as CHF improves (25).

The radioimmunoassay for marinobufagenin has been recently validated by high resolution mass spectrometry but has been measured only in CRF, where it is elevated (27). Until the CHF results are similarly verified, it is not possible to be sure that radioimmunoassay results for marinobufagenin in disease states other than CRF also reflect the true status.

We suspect that the HMW Na-K-ATPase inhibitor may be a carrier protein for the LMW inhibitor since the latter can be split off by use of beta-mercaptoethanol, an agent known to cleave S-S bonds, plus heat and formic acid, properties employed by Lindner et al. (28) to dissociate oxytocin and vasopressin from their neurophysin carrier. It is also pertinent that Morich and Garthoff (5) found that both salt-sensitive (DS) and salt-resistant (DR) rats displayed two protein bands in their plasma on SDS-PAGE, in the molecular weight range of 14–15 kDa. When DS rats were given salt and developed hypertension, the upper band diminished but the lower band became more intense. The difference in molecular weight between the two bands was estimated to be between 300 and 400 Da. Mass spectrometry of the first of the LMW inhibitors in the present study (NKAI-1) revealed a molecular weight of 408 Da, as shown by the hydrogen adduct of 409 Da, the sodium adduct of 431 Da, and the potassium adduct of 447 Da. The molecular weight of 408 is identical to that described by Kerek (29), a biochemist, for an initially identified macrocylic derivative of inorganic carbon suboxide, which is a natriuretic, Na-K-ATPase inhibiting compound derived from plant tissue. We look forward with interest to the comparison between Kerek's 408 Da compound and the 408 Da compound discussed in this review.

The HMW compound of the present dissertation was previously referred to as "hypertension-associated protein" by Van de Voorde et al. (7). These authors claimed an approximate molecular weight of 15 kDa for the compound they isolated by chromatography after reduction of the disulfide bridges of the precursor 105 kDa protein molecule with beta-mercaptoethanol. In a prior study of plasma proteins in essential hypertension, utilizing SDS-PAGE to separate the plasma proteins, Nardi et al. (4) had earlier reported a 14 kDa protein present in such patients but not in patients with hypertension secondary to renovascular hypertension or renal parenchemal disease. Cloix et al. (6) had reported a 13 kDa protein in the plasma of hypertensive human beings and rats. Thus, we are left with four studies that purport to show either 12, 13, 14, or 15 kDa proteins in the plasma of human beings with essential hypertension but not in normal controls or possibly in renovascular hypertension or hypertension with CRF. What could this protein be? In the present study, we have referred to the 12 kDa protein as a "carrier protein" because the Na-K-ATPase inhibitor can be split off by heat and formic acid. But are there alternatives?

To explore this question in all of its ramifications, it is first necessary to review what has been learned about the "other" natriuretic system, namely the natriuretic peptides. Following the initial description of natriuretic peptides by deBold and associates in 1961 (30), it has been found that there are at least three natriuretic peptides released from the hypothalamus and cardiac tissue – atrial natriuretic factor (ANF), B-type natriuretic factor (BNF), and C-type natriuretic factor (CNF). All occur initially as pre-prohormones, which are degraded to prohormones and then finally to the active peptides (31).The molecular weight of the proANF, a circulating compound (32), has been described as 14 kDa (33). Is it possible that pro-ANF is identical to the hypertension-associated protein described by Van de Voorde et al. (7), Nardi et al. (4), Cloix et al. (6), and the present study? A suggestion that this may be the case comes from Melander et al. (34) who described in offspring of hypertensive human beings a strong correlation between salt sensitivity, as defined by the difference in sodium excretion while on a low salt diet and then on a high salt diet, and plasma proANP levels.

Initially, it was thought that EDLF, endogenous digitalis-like factor, or OLF, ouabain-like factor, as the Na-K-ATPase inhibitor became known, could be distinguished physiologically from ANF by its Na-K-ATPase inhibiting property as well as its tendency to increase, rather than decrease, vasoconstriction when applied to isolated blood vessels (10). However, it was recognized by Górny et al. (35) that ANF does inhibit Na-K-ATPase in the rat renal medulla, but not in the rat renal cortex, where the proximal tubule is located. In contrast, Chiou and Vesely (36) reported that kaliuretic peptide, a fraction split off from ANF prohormone, inhibits both renal cortical and medullary Na-K-ATPase. However, these experiments employed rat renal tissue rather than hog cerebral cortex for assay of Na-K-ATPase and the inhibition in the two studies quoted resulted from indirect inhibition of Na-K-ATPase through effects of second messengers, namely, dopamine in the first study (35) and prostaglandin E_2 in the second study (36). Thus, we may no longer be able to depend exclusively on the Na-K-ATPase assay to distinguish between ANF and EDLF. On the other hand, we can still depend on both the molecular weight and the direct vasoconstrictive (10) or vasodilatory (37) actions on isolated vascular smooth muscle preparations to distinguish between EDLF and ANF. The molecular weights for EDLF have been reported as varying between 360 and 620 Da (**Table 1**), while the molecular weights for ANF have been described as 3800 Da for rat ANF (38) and varying from 3000 Da (33) to 5499 Da (39) for human ANF.

Haupert (44) in 1988 first posed the question as to whether there is an interrelationship between natriuretic peptides and EDLF or OLF. That the interrelationship exists can no longer be in doubt. It has long been recognized that both ANF and EDLF are released from the hypothalamus (45, 46), and in fact from the AV3V region (47). Lesions produced in the AV3V region prevent the natriuresis following isotonic saline volume expansion in experimental animals. Furthermore blood drawn following expansion failed to show an anti-natriferic effect in the toad bladder in contrast to control animals, implying interference with release of the Na-K-ATPase inhibitor. The perfusate from incubation of fragments of rat brain inhibited the Na, K pump by a 77% reduction of ouabain-sensitive ^{86}Rb uptake into human erythrocytes. This did not occur when ANF was given intravenously before sacrifice of the test animals (48). ANF injected into lateral cerebral ventricles releases an Na-K-ATPase inhibitor measured as above in cultured aortic smooth muscle cells (49). Ouabain and digoxin, cardiotonic steroids resembling EDLF and OLF, increase ANF secretion by rat atrial cardiocyte superfusions (50). Liu et al. (51) also employed the perfused beating rabbit atria model to show that ouabain significantly increased ANF secretion in a dose-dependent manner, indicating that the interrelationship between Na-K-ATPase inhibitors and ANF can proceed in both directions.

Table 1 | Comparison of sources and molecular weights of various EDLFs.

Author	Source	Molecular weight (daltons)	Reference
Bricker et al.	Human uremic urine	360	(40)
McKinnon et al.	Human placenta	370	(41)
Kramer et al.	Na-loaded normal human urine	391	(38)
Cloix et al.	Normal human urine	431	(42)
Weiler et al.	Hypertensive human plasma	408	(10)
Kerek	Plant tissue	408	(29)
Tamura et al.	Pig urine	620	(43)

Finally, in an elegant experiment performed by Morgan et al. (52), using extracts from cultured rat hypothalamic cells separated on Sephadex G-25, a sodium transport inhibitor could be recovered from the post-salt fraction as indicated by three assays: (1) inhibition of transport in human erythrocytes, (2) displacement of ^{3}H ouabain from its binding site, and (3) direct inhibition of canine Na-K-ATPase. Could pro-ANF and EDLF be co-secreted by the hypothalamus in response to volume expansion or as an indicator of pre-disposition to essential hypertension? A suitable way to settle this question would be to perform immunoassays for pro-ANF and ANF on the 12 kDa protein of the present experiment. For this reason, I would again implore currently active investigators to separate the 12 kDa protein from human hypertensive plasma and test it for pro-ANF and ANF immunoreactivity.

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Spherical oligo-silicic acid SOSA disclosed as possible endogenous digitalis-like factor

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The Na^+/K^+-ATPase is a membrane ion-transporter protein, specifically inhibited by digitalis glycosides used in cardiac therapy. The existence in mammals of some endogenous digitalis-like factors (EDLFs) as presumed ATPase ligands is generally accepted. But the chemical structure of these factors remained elusive because no weighable amounts of pure EDLFs have been isolated. Recent high-resolution crystal structure data of Na^+/K^+-ATPase have located the hydrophobic binding pocket of the steroid glycoside ouabain. It remained uncertain if the EDLF are targeting this steroid-receptor or another specific binding site(s). Our recently disclosed spherical oligo-silicic acids (SOSA) fulfill the main criteria to be identified with the presumed EDL factors. SOSA was found as a very potent inhibitor of the Na^+/K^+-ATPase, Ca^{2+}-ATPase, H^+/K^+-ATPase, and of K-dp-ATPase, with IC_{50} values between 0.2 and 0.5 μg/mL. These findings are even more astonishing while so far, neither monosilicic acid nor its poly-condensed forms have been remarked biologically active. With the diameter ϕ between 1 and 3 nm, SOSA still belong to molecular species definitely smaller than silica nano-particles with $\phi > 5$ nm. In SOSA molecules, almost all Si-OH bonds are displayed on the external shell, which facilitates the binding to hydrophilic ATPase domains. SOSA is stable for long term in solution but is sensitive to freeze-drying, which could explain the failure of countless attempts to isolate pure EDLF. There is a strong resemblance between SOSA and vanadates, the previously known general inhibitors of P-type ATPases. SOSA may be generated endogenously by spherical oligomerization of the ubiquitously present monosilicic acid in animal fluids. The structure of SOSA is sensitive to the concentration of Na^+, K^+, Ca^{2+}, Mg^{2+}, and other ions suggesting a presumably archaic mechanism for the regulation of the ATPase pumps.

Keywords: oligo silicic-acid, ATPase regulation, digitalis-like factor, ouabain

INTRODUCTION

Investigating the staircase effect on frog ventricular muscle, it was revealed in the early 1950s that human serum contains a factor, which improves the contractile power of the heart, similar to plant-derived digitalis glycosides (1). Besides the serum, this digitalis-like activity was reproduced by a total extract of adrenal but, individual cortisol steroids were, with one exception, inactive. Referring to their finding Szent-Györgyi concluded (2) in more general terms that "such digitalis-like factors are ubiquitously distributed in mammals" and "digitalis glycosides from plants are actually not drugs but only substitutes of the body's own digitalis-like factors." This kind of relationship between exogenous drug-substances, which fits accidentally into the receptor of a body's own factor has been verified by the discovery of endorphins, the endogenous counterparts of the plant-derived morphine (3). Why this broadly accepted rationale was not consequently applied in the differentiation between endogenous digitalis-like factors (EDLFs) and exogenous digitalis glycosides is a subject of the present review.

A further milestone finding of the 1950s was that digitalis glycosides inhibit the transport of Na^+ and K^+ ions across the erythrocyte membrane (4) and above all the seminal discovery of the membrane transport protein of the Na^+ and K^+-ions by Skou (5). This Na^+/K^+-ATPase (adenosine-triphosphatase) abbreviated NKA or sodium pump is present in the membrane of all eukaryotic cells. Every pumping cycle of the NKA moves 3 Na^+ ions outward and 2 K^+ ions inward, powered by the energy of a phosphate bond from ATP.

At the beginning of the 1960s, the existence of a circulating natriuretic factor was postulated by De Wardener et al. (6) showing that the blood, transfused from a saline-loaded, hypertensive dog, produces natriuresis in the recipient normotensive animal. It was further observed that the plasma ultra-filtrate of saline-loaded dogs inhibits the sodium transport in toad bladder and the volume expansion was accompanied by increasing concentrations of a sodium pump-inhibitory factor (7). Ascertaining that both effects were caused by the same factor the name natriuretic hormone was proposed. The natriuretic fraction extracted from the plasma of volume-expanded dogs inhibited the ouabain-insensitive NKA from rat kidney (8).

After the identity of the natriuretic hormone with the sodium pump-inhibitory factor was confirmed, the name EDLF came into use (9). The initial idea that EDLF could be a natriuretic peptide

was rejected after disclosure of the atrial natriuretic peptide (ANP), which has, contrary to EDLF no NKA-inhibitory activity (10). Though the structure of EDLF remained obscure, some of its particular characteristics were established as for instance: its non-peptide nature or its specific interaction with different ATPase isoforms. But the failure of all attempts to obtain weighable amounts of pure EDLF impeded for more than five decades the disclosure of its chemical structure.

Na^+/K^+-ATPase controls a broad spectrum of essential cellular functions such as ion homeostasis, membrane potential, pH, temperature, and water osmosis, thereby regulating important physiological processes, e.g., muscle contraction, nervous signal transmission, renal sodium retention, and vascular tone. Study data in animal models and clinical observations in human beings suggest that cardiac insufficiency, essential hypertension, and other diseases may be caused by or connected to malfunction or dysregulation of the sodium pump.

The extensive research work related to the structure, characterization, mechanism of action, and physiological implications of the Na^+/K^+-ATPase was comprehensively reviewed by Gadsby et al. (11), Glynn (12), Kaplan (13), and Jørgensen et al. (14). Similarly, extensive reviews on the whole P-type-ATPase field have been published by Møller et al. (15), Kühlbrandt (16) and by the original contributions of Axelsen and Palmgren (17, 18) with special focus on evolutionary aspects of the P-type ATPases.

ATPase RECEPTOR SITE

P-type ATPase is the generic designation of several ATP-driven transmembrane ion pumps found in bacteria, archaea, and eukaryotes. The prefix P refers to the ability of these proteins for phosphorylation and de-phosphorylation of their catalytic aspartate residue (15). By the binding and removal of the phosphate group, ATPases interconvert between two conformations, denoted by E1 and E2, each with different affinity to the nucleotide ATP (adenosine triphosphate) and the transported ions (16). A common feature of P-type ATPases is their inhibition by vanadate ions at micro- and sub-micro-molar concentrations.

From about 200 members of the P-type ATPase family, the most prominent pumps are the cell membrane Na^+/K^+-ATPase (NKA); the Ca^{2+}-ATPase (SERCA) from sarcoplasmic reticulum (SR), the gastric H^+/K^+-ATPase, and the bacterial K-dp-ATPase. Based on 80–90% similarities of the amino acid (AA) sequences in the conserved regions, it is assumed that P-type ATPases evolved from a common ancestor, probably 3500 million years ago (17, 18).

A seminal breakthrough for the detailed structural understanding of the transmembrane ion pumping was achieved by the first high-resolution (2.6Å) crystal structure of the SERCA Ca^{2+}-ATPase protein from SR solved by Toyoshima et al. (19). This study established the detailed 3D structure and accomplished the functional characterization of the cytoplasmic subunits designated P (phosphorylation), N (nucleotide binding), and A (actuator) domains. It was found that the ion-binding sites are surrounded by the M4–M6 and M8 transmembrane helices where M4 and M6 provide the efficient geometry for the coordination of the Ca^{2+} ions. The over 50 Å distance between the membrane site of the Ca^{2+} ion translocation and the cytoplasmic phosphorylation site is remarkably long.

In the next high-resolution (3.1 Å) crystal structure of the SERCA pump, the Toyoshima group applied the sesquiterpene lactone thapsigargin, to stabilize the Ca-free E2-(TG) state (20). The comparison of both crystallized forms Ca^{2+}E1 and E2-(TG) revealed further details of the ion transport mechanism. In a following contribution (21), the structure of the Ca^{2+}-ATPase was solved in the E1 state fixed by the ATP-analog AMPPCP. In the same year, the structure at 2.3 Å resolution of SERCA with phosphate analogs such as $[MgF_4]^{2-}$ has been resolved (22). The studies of the Ca-pump fixed with ATP- or phosphate analogs have completed the structural insight into almost all important states of the pump turnover.

Resolving the crystal structure of the SERCA pump with the phosphate mimic $[BeF_3]^-$, Olesen et al. (23) provided support for the presence of an open ion pathway in the pump in which the transmembrane domains form a funnel-shaped geometry. As described later, the crystal structure data confirmed that P-type ATPases share the same architecture regardless of the size, charge, and number of ions that they transport. It seems that the differences are largely confined to the ion-binding pocket (23, 24).

Na^+/K^+-ATPase is sensitive to inhibition by digitalis glycosides (e.g., digoxin, ouabain), isolated from medicinal plants used over centuries to treat congestive heart failure. The term "digitalis" designates the entire group of cardiac glycosides and aglycones without regard of their structure and origin (25). Responsible for the cardiac activity is the aglycone, i.e., the steroid nucleus, which results after the sugar moiety from position 3 is removed. Steroid aglycones with five-membered lactone ring in position 17 are named cardenolides while those with six-membered lactone ring bufadienolides. Both terms, cardiotonic (CTS) or cardiac steroids (CSs), are in use but the adjective "tonic" is less rigorous as it refers to a species-dependent physiological response; thus, the designation CS should be preferred over CTS.

Since Na^+/K^+-ATPase is the target of digitalis drugs in heart failure patients, it was of prior importance to establish structure–activity relationships. A general correlation between binding affinity and NKA-inhibitory potency of cardiac glycosides was revealed (26) but some notable exceptions were also identified. By replacement of the five-membered lactone ring in cardenolides with the 2-pyrone ring of bufadienolides, the binding affinity declines but the inhibitory potency increased. Furthermore, while the removal of ouabain's rhamnose moiety had little effect on inhibitory potency, it caused a decline in ligand binding affinity.

In a recent study, 30 different cardiac glycosides were investigated for their interaction with the α1, α2, and α3 isoforms of the human NKA expressed in *Pichia pastoris* (27). The study revealed significant isoform selectivity by digoxin glycosides but ouabain was found moderately α2 selective. The observed influence of the sugar moiety on the selectivity was surprising since according to pharmacological data this part influences rather the bioavailability and metabolism of the digitalis drugs.

Biophysical methods provided further insight into the charge transfer processes during the pump cycle. The binding of ouabain is associated with movement of electrical charges; thus, it can be followed by the charge-sensitive fluorescence indicator RH421. These data revealed that the binding of ouabain or generally of

a cardiac glycoside to the Na^+/K^+-ATPase protein will stabilize under physiological conditions the E2P·2Na^+ stage (28).

Na^+/K^+-ATPase is expressed in all animal cells and shows highly conserved AA sequences in the main α subunit (~1000 AA residues) responsible for the catalytic function, similar to that found in SERCA. This catalytic subunit of NKA has 10 transmembrane TM helices numbered M1–M10 from the amino-terminal. Experiments with punctual mutations of AA residues evidenced CS binding sequences in the extracellular loops L1/2, L5/6, and L7/L8. The cytosolic loop L2/3 contains the nucleotide (ATP) binding N site and the phosphorylation site P, while the NH_2 terminal and the L2/3 loop are responsible for the de-phosphorylation step. NKA contains further the heavily glycosylated ß-subunit with ca. ~300 AA and the tissue specific auxiliary γ subunits FXYD of ca. 70–180 residues The multiple regulatory potential of the NKA is explained by the existence of tissue-specific assemblies of different structural subunits or isoforms (29).

In comparison with more than 20 X-ray structures for Ca^{2+}-ATPase, for NKA only 5 crystal structures at better than 5 Å resolution have been published. Some difficulties come from the source of NKA protein, which is limited to rabbit kidney and shark rectal gland, expressing selectively the α1 isoform. The lack of very high-affinity inhibitors able to fix the enzyme in one particular conformation posed further limitations to NKA crystallization (29).

The crystal structure of the pig-renal Na^+/K^+-ATPase with 2 Rubidium ions as K ion congeners was solved at 3.6 Å resolution by the PUMPKIN group in Denmark (30). The structure shows that the conformation of the: α unit (in Rb/K occlusion) closely matches the conformation of the Ca^{2+} ion bound state of SERCA.

The crystal structure of the Na^+/K^+-ATPase from shark enzyme was resolved (31) at 2.4 Å resolution in the: E2·2K~Pi state in which the pump has a high affinity to K^+ ions. The coordination of K^+ ions in the transmembrane sites and the critical role of the β subunit in binding of the K^+ ions were remarked. Despite identical coordinating residues, small differences with the Ca^{2+}-ATPase pump were noted.

Solving with 2.8 Å resolution, the crystal structure of Na^+/K^+-ATPase, co-crystallized with ouabain in the E2·2K^+~Pi state, the authors concluded (32) that ouabain is deeply inserted into the transmembrane domain, with lactone ring near to the K^+ binding site with partial unwinding of the M4E helix (see **Figure 1A** below). This unwinding should explain why ouabain binding is so slow. The data suggest reconsideration of previous data that CSs bind to the extracellular surface of the ATPase α-subunit. Since ouabain interacts with transmembrane segments M3, M4, and M6 involved in ion transport this steroid can influence or block these processes.

Based on the crystal structure at 4.6 Å resolution of the pig kidney Na^+/K^+-ATPase with ouabain bound in the E2P state, it was suggested that the high-affinity binding of ouabain stabilizes the phosphorylated state (33). The steroid binds to a site formed between αM1 and αM6 domains, plugging the ion pathway from the extracellular side. A high-affinity interaction is formed between the steroid and the αM1–2 part from domain α, which is rotated following the phosphorylation. Solving the crystal structure at 3.4 Å resolution of the phosphorylated pig kidney NKA in complex with ouabain has revealed that the steroid binds with intensive hydrogen bonding network to the αM1, αM2, and αM6 transmembrane segments (34). It was concluded that the binding pocket in the [Mg^{2+}] E2P state allows deep ouabain binding with possible long-range interactions with Mg^{2+} and K^+ ions.

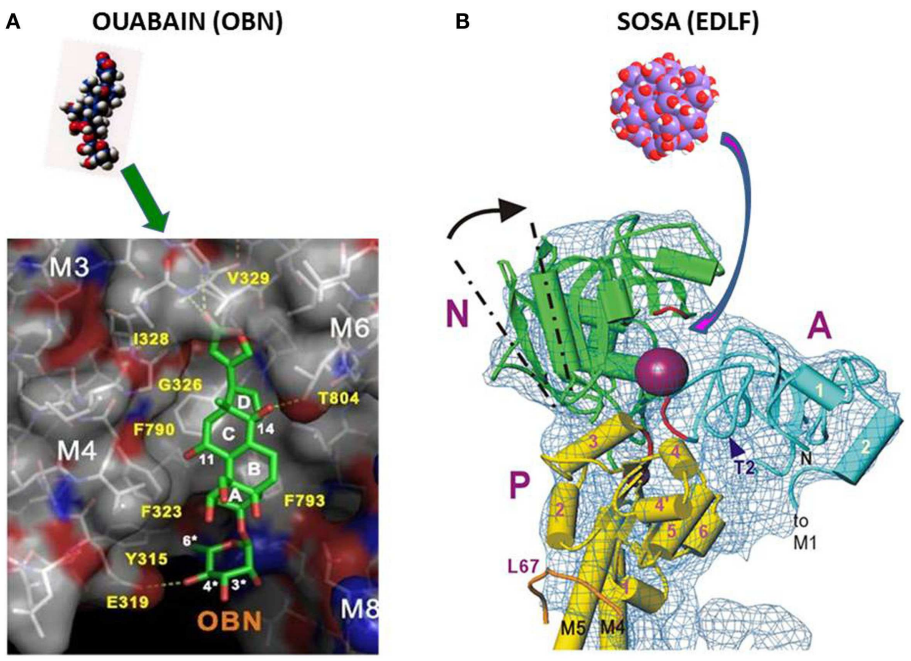

FIGURE 1 | (A) Binding of ouabain to the hydrophobic site in NKA (32). **(B)** Binding of SOSA to the hydrophilic receptor site of decavanadate in SERCA (19).

Applying fluorescein-labeled ouabain and a lanthanide binding tag in the NKA derived from *Xenopus oocytes* the data of spectroscopic measurements (35) suggested two different type binding sites for ouabain: one external, low-affinity site on the extracellular end of the ion pathway as previously assumed; the second high-affinity site is slightly deeper toward the intracellular end of the ion pathway, as indicated by recent X-ray diffraction studies.

In conclusion, the high-resolution crystal structure data have identified the location of ouabain in the NKA together with the protein domains involved in the interaction with this CS. The ouabain-binding site described in Ref. (32) has a flat hydrophobic surface suitable for the interaction with the steroid frame as shown by **Figure 1A**.

For some hydrophilic ATPase ligands such as the decavanadate or the here disclosed spherical oligo-silicic acid (SOSA) (see below), it is improbable to target the hydrophobic receptor site of CSs and thus to compete directly with ouabain or digoxin. A more suitable target for hydrophilic inhibitors in P-type ATPases is the phosphorylation site in the cytoplasmic region between the N, A, and P domains (**Figure 1B**), as it was suggested for the decavanadate ion in SERCA (19).

The high-structural analogy between the hydrophilic polyanionic decavanadate and the here disclosed SOSA suggests that SOSA should target ATPase pumps at the phosphorylation site in a similar manner as decavanadate. This assumption is illustrated by **Figure 1B**) with the high-resolution structure of the cytoplasmic domain of SERCA with the decavanadate ion bound to the hydrophilic phosphorylation site (19).

DILEMMA: EDLF OR CARDIAC STEROIDS

The physiological role of the CS receptors and the existence of endogenous non-steroidal ligands of NKA have been investigated by the group of Lingrel (36, 37). The experiments were conducted among others on some ouabain-resistant and ouabain-sensitive isoforms (cc2, cc3, and cc4) genetically engineered in mice and rat. The biological function and significance of the CS binding site was evidenced with rigorous distinction between cardiotonic steroids (CTS) and the yet undisclosed endogenous ATPase ligands. Reviewing the whole set of his experimental results and their significance for the physiological role of the NKA receptors, Lingrel concluded three main points: (1) the ouabain-binding site of the Na/K-ATPase plays a physiological role, (2) an endogenous ligand for the Na^+/K^+-ATPase must exist, and finally, (3) whether the endogenous ligand acts through a change in intracellular Na^+ or through a signaling mechanism is unknown (37).

Despite the convincing arguments of Lingrel, the relation between EDL factors and CSs remained a dilemma, i.e., "a problem offering two possibilities, neither of which is acceptable." In fact, there are some experimentally identified but physically not isolated (probably labile) EDL factors assumed as ATPase ligands that differ markedly from CSs except for the inhibition of the NKA. Lacking weighable amounts of EDL factors, the studies have been performed only with the commercially available CSs and, therefore, could neither demonstrate nor exclude the identity of EDLF with CS.

This unsolved dilemma has marked the extensive research work focused to disclose the structure and properties of the putative endogenous ligands of the ATPases as thoroughly reviewed by Goto et al. (38), Hollenberg and Graves (39), Buckalew (40), Schoner and Scheiner-Bobis (41), Nesher et al. (42), and Bagrov et al. (43).

Numerous attempts to isolate pure EDL factors from various biological sources (organs, glands, plasma, or urine) were listed by these surveys (38–42). Despite applying extraction procedures on several kilogram amounts of starting material and efficient separation techniques, the final yields after multiple purification steps were invariably small: trace, sub-microgram amounts of EDLF, definitely insufficient for structural studies.

The low chemical stability of the EDL factors should also have been considered as possible explanation for the dramatically vanishing EDLF amounts along the purification processes. But this instability was not investigated in detail. Once a labile sodium pump inhibitor was signalized in peritoneal dialyzate (44) with even significantly higher inhibitory potency than ouabain, but the experiment was not reproduced. Of historical interest is the mention published 60 years ago by Szent-Györgyi (2) that, "the cardio-active serum factor if lyophilized and stored, loses its activity, as it also loses it on repeated freezing and thawing."

Because of these persistent failures to isolate EDL factors, the search for endogenous ATPase ligands and assessment of their putative biological role became increasingly discrepant. Lacking measurable amounts of the pure EDLF on one side and the growing body of evidences that toxic CSs of herbal or amphibian origin are unable to function as endogenous ATPase ligands in mammals became an unsolvable problem. Further controversies were caused by the sugar moieties of the cardiac glycosides comprising desoxy-sugars (e.g., rhamnose, digitoxose) which were never identified in mammals, making it unlikely that such desoxyglycosides can genuinely exist and act as endogenous ligands in these animals.

Estimated from their evolutionary history CSs identified in flowering plants could not be older than 100 million years and steroids from amphibians must be "younger" than 400 million years. It is very unlikely that such compounds have existed as regulatory ligands of the ATPase pumps 3500 million years ago in the prokaryote membrane.

After the successful evolution of the archaic ATPase 3.5 billion years ago, it was no more evolutionary pressure to improve this perfectly working ion-pumping mechanism or to change its endogenous regulatory ligand. The adaptation on the growing complexity of multicellular organisms was accomplished by diversification of the subunits or by the combination of substructures, without essential changes of the basic pumping mechanism.

Finally, it should be remarked that the search for identification of the EDL factors had considered almost exclusively organic candidates (38). This was not fully justified as the essential chemical reaction of the pumping cycle is the binding (and release) of a simple inorganic phosphate group. The lack of specific 3D structures in solution makes simple inorganic salts rather unable to fit a receptor site and to work as endogenous ligands contrary to organic substances. However, some inorganic poly-oxo-acids derived from metalloids such as Be, Al, Si, Ge, As, V, Cr, or Mn are able to form 3D structures in solution to fit into a receptor site. Actually the predominant part of these poly-oxo-acids and their

salts are toxic for living organisms, which reduces the number of candidate endogenous ligands.

Several criteria of the putative EDLFs have been formulated previously, among others by Goto et al. (38). Completing the earlier list with a few criteria, we consider that the endogenous ATPase ligands should have characteristics as:

a. inhibitor of the Na^+/K^+-ATPase,
b. inhibitor of other P-type ATPases,
c. ubiquitously distributed,
d. bioavailable and eliminable,
e. non-toxic for animals,
f. was present in a very early stage of the evolution,
g. sensitive to drying and freeze-drying,
h. specific 3D structure in solution.

These properties can also explain the difficulties met earlier in the isolation and characterization of pure EDLFs. Further efforts are needed to disclose the detailed mechanism of action at cellular and physiological level of the actual EDL factors.

ATPase INHIBITORY VANADATES

The inhibition of the Na^+/K^+-ATPase by vanadate was discovered in year 1977 with the accidental observation (45, 46) that the reagent grade ATP of Sigma was contaminated with an ATPase inhibitory substance, identified as sodium vanadate (Na_3VO_4). The similar (isoelectronic) structure of the vanadate and phosphate ions was considered as a probable mechanism of the inhibition by vanadate in competition to the phosphate binding site.

Investigating the interaction of vanadates (47) with fluorescein-labeled SERCA, it was observed that vanadate impeded the high-affinity Ca^{2+} binding to the enzyme at 4°C. Vanadate inhibits the phosphorylation reaction by inorganic phosphate but had no effect on the phosphorylation by ATP. It was suggested that vanadate binds to the low-affinity ATP binding site of the ATPases, which is exposed only in the E2 conformation of the enzyme.

Interactions between SERCA and vanadate ions in solution have been investigated by ^{51}V-NMR spectra indicating that mono- and oligo-vanadates are bound to SR membrane influencing the structure of Ca^{2+}-ATPase (48). Actually, the mono and oligo-vanadate species form some complex equilibria impeding the establishment of rigorous structure–activity correlations.

In the presence of Ca^{2+}, it was observed that tetra- and deca-vanadate $[V_{10}O_{28}]^{6-}$ binds to the SERCA pump, whereas monomeric vanadate binds to the SR only when ATP is present. There are further arguments that decavanadate clearly differs from mono- or small-vanadate oligomers in preventing the accumulation of Ca^{2+} ions by SR vesicles, which is coupled to ATP hydrolysis (49).

Biological studies with vanadium often disregarded the formation of decameric vanadate species known to manifest high-affinity interaction with many proteins such as myosin and the SR calcium pump (50). Vanadium is accumulated in mitochondria in particular when decavanadate is administered. These findings point out the contributions of decavanadate to *in vivo* effects induced by vanadium in biological systems.

An increasing volume of data suggests the putative biological importance of decavanadate, a vanadate oligomer that eventually occurs in the cytoplasm more often than expected (51). Specific interactions of decavanadate have been clearly demonstrated for Ca^{2+}-ATPase, myosin, and actin, considered as major proteins in muscle contraction and its regulation. Based on crystal structure data, the binding of the SERCA inhibitory decavanadate was localized (19, 52) to the ATP binding site between the cytoplasmic domains A, N, and P of the thapsigargin-inhibited enzyme in the absence of Ca^{2+} as shown by **Figure 1B** of the present paper.

Vanadate compounds show a significant antidiabetic efficacy. Sodium vanadate was applied in diabetes therapy 22 years before the first use of insulin to treat diabetes in human beings (53, 54). Besides its insulin-mimetic action, vanadate inhibits the glucose-6-phosphatase (G6P) enzyme, with a key role in glucose metabolism.

The proposal of Kramer et al. (55) to consider vanadium diascorbate with a molar mass of 403 Da as a candidate EDLF is worth mentioning. However, this hypothesis has not been confirmed since vanadate ions are not ubiquitously distributed in mammals and are toxic in particular by accumulation in some organs.

ATPase INHIBITORY MCS-FACTORS

Our way to disclose the structure of the assumed EDLFs is a typical example of serendipity. At the end of 1990s, we investigated at the Max-Planck Institute for Biochemistry in Munich an herbal product isolated from the roots of *Helleborus species*. The plant product contained, besides other components, the cardiac glycoside hellebrin with strong NKA-inhibitory potency. The chemical stability of hellebrin was monitored by measuring the inhibition of Na^+/K^+-ATPase, starting therewith a very intensive and prolific collaboration with Hans-Jürgen Apell and Robert Stimac from the University of Konstanz (GER), which led finally to the disclosure of the novel ATPase-inhibitory factor.

The alkaline treatment destroyed hellebrin and annihilated the NKA-inhibitory effect but, as the alkaline boiling was accidentally prolonged for several hours, a very potent novel NKA inhibitor was generated (56). By the HPLC on RP-18 column, the pure inhibitory compound eluted closely after the injection peak, or delayed if it was attached to some lipophilic components. Similar characteristics have been reported by the HPLC analysis of the earlier EDLF preparations from biological samples (38–43).

The main component of the plant material subjected to alkaline boiling was a resin-like compound, similar to the polymeric carbon suboxide (57). Therefore, it was thought that the obtained potent ATPase inhibitor could be a low molecular weight decomposition product of this polymer. For the structure of the de-polymerization product, we assumed a repeatedly condensed 4-pyrone frame that forms a supplementary cage-type macrocycle with formula $(C_3O_2)_n$ where (n = 4, 6, or 8). We named the inhibitor MCS, macrocyclic carbon suboxide (56). Tentatively, this MCS was suggested as probable EDLF and natriuretic factor (58).

MCS factors showed a rigorously reproducible potent inhibition of the Na^+/K^+-ATPase, Ca^{2+}-ATPase, H^+/K^+-ATPase, and K-dp-ATPase with IC_{50} values in the 0.2–0.5 μg/mL range. The mechanism of Na^+/K^+-ATPase inhibition by the MCS factor was investigated with the fluorescent styryl dye RH421, a dye known to

reflect changes of local electric fields in the membrane dielectric. It was found that the binding of the MCS to the Na^+/K^+-ATPase is not competitive with ouabain (59, 60). MCS factors interact with the Na^+/K^+-ATPase in the E1 conformation of the ion pump and induce a structural rearrangement that causes a change of the equilibrium dissociation constant for one of the first 2 intracellular cation binding sites. The MCS-inhibited state was found to have bound one cation (H^+, Na^+, or K^+) in one of two non Na^+ specific binding sites, and the other Na^+ ion was bound at high Na^+ concentrations to the highly Na^+-selective ion-binding site (60).

The proposal with cage-form condensed macrocyclic carbon suboxide structure was apparently supported by the mass ion peaks (m/z) containing multiples of the 68.03 Da unit, the molar mass of the C_3O_2.

The main m/z peaks in ESI-MS spectra (**Figure 2**) have been assessed as small multiples of the carbon suboxide unit (68 Da) with 1 Na^+ ion according to the formula $[(C_3O_2)_n \cdot Na]^+$ thus, m/z = 159 Da correspond to $n = 2$; 295 Da ($n = 4$); 431 Da ($n = 6$), and 567 Da ($n = 8$). The small MH^+ peaks at 275, 409.2, and 544.2 Da were also perceptible in the mass spectrum (56).

Interestingly, a molar mass ion at M = 408 Da ($MH^+ = 409$; $MNa^+ = 431$ Da) was identified in some earlier EDLF preparations from biological sources, e.g., human plasma (61), placenta (62), or bound to a hypertension-associated plasma protein (63). It can be speculated about the possible identity of these factors and our MCS product but only the same molar mass ion value is not sufficient to prove or disprove this identity. The fine structure of the mass spectra may also differ due to the different ionization techniques, i.e., FAB used by Weiler et al. (63) and ESI-MS applied by us (56).

Although the ATPase-inhibitory effect of the MCS factors on several P-type ATPases and the mechanism of action on NKA were rigorously reproducible, the structure with head-to-tail condensed pyran-4-one rings supplementary bond in a cage-like macro-cycle could not be confirmed by synthesis despite huge experimental efforts with Frank Freudenmann and Luis Moroder at the MPI for biochemistry in Munich.

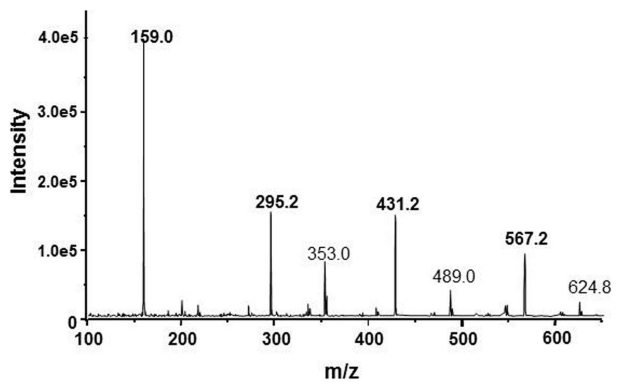

FIGURE 2 | ESI-mass spectrum of the NKA-inhibitory MCS factor corresponding to some mass ion fragments resulted by decomposition of SOSA.

Likewise not confirmed were the specific ^{13}C-NMR signals and the UV-absorbance peaks expected for the macrocyclic condensed pyran-4-one structure. The assessment of the mass spectrum as multiples of a 68 Da unit was correct but the attribution of this mass to C_3O_2 was erroneous. These disagreements required the revision of the proposed macrocyclic cage-structure.

SPHERICAL OLIGO-SILICIC ACID (SOSA)

The decisive hint to disclose the actual chemical structure of our NKA-inhibitory factor came from revision of the blind probe of the described alkaline preparation. Surprisingly, the several hours boiling of the NaOH solution alone, without any other reagent yielded a similarly potent ATPase inhibitor as that obtained by alkaline boiling of the plant polymer.

The mystery was explained by identifying small amounts of sodium silicate in the solution leached from the glass flask by its prolonged alkaline heating in oil bath at 120°C. Applying the proper neutralization-activation procedure (56), this silicate was transformed to the highly active ATPase-inhibitory factor identified as spherical oligomers of silicic acid. The generation of biologically active oligomeric condensation products from the inactive monosilicic acid was totally unexpected and thus very surprising.

We considered that this finding could have implications in clearing of some controversial disputes within the following research areas:

Regarding the biological role of silicon, it was generally agreed that Si provides structural support in plants and is beneficial of bones and elasticity of cartilages in animals. But neither a Si containing biologically active substance, nor a protein, which needs Si has been found in animals. Identifying the SOSA as biologically active water-soluble Si compound (64), a decisive argument has been provided in support of the assumed biological role (essentiality) of this element.

The assumed existence of EDLFs and their putative identity with cardiac glycosides was the subject of debates for several decades. The proposed identity of EDLF (65) with the SOSA was a novel approach suggesting the reconsideration of the divergent opinions.

The structure of the ATPase-inhibitory SOSA is formed by successive condensation of a few (oligos in Greek) molecules of monosilicic acid H_4SiO_4 according to the equation (Eq. 1) where "n" should be in the range of 16–200.

$$\begin{array}{ccc} n H_4SiO_4 & \rightarrow & [SiO_x(OH)_{4-2x}]_n \\ \text{mono-silicic acid} & & \text{oligo-silicic acid} \\ x = 1.5 & \rightarrow & [SiO_{1.5}(OH)]_n \\ & & \text{SOSA} \end{array} \quad (1)$$

The value $x = 1.5$ in the general formula $[SiO_x(OH)_{4-2x}]_n$ is congruent with a particular symmetry of the multi-cyclic silicic acid oligomers corresponding to polyhedral symmetry, i.e, prismatic hexamer ($n = 6$), cubic octamer ($n = 8$), and prismatic decamer ($n = 10$) structure, known as silsesquioxanes with general formula $[SiO_{1.5}OH]_n$ and shown by **Figure 3**.

The spherical form of the oligo-silicic acid SOSA is accomplished for $x = 1.5$ in the general formula $[SiO_x(OH)_{4-2x}]_n$ and

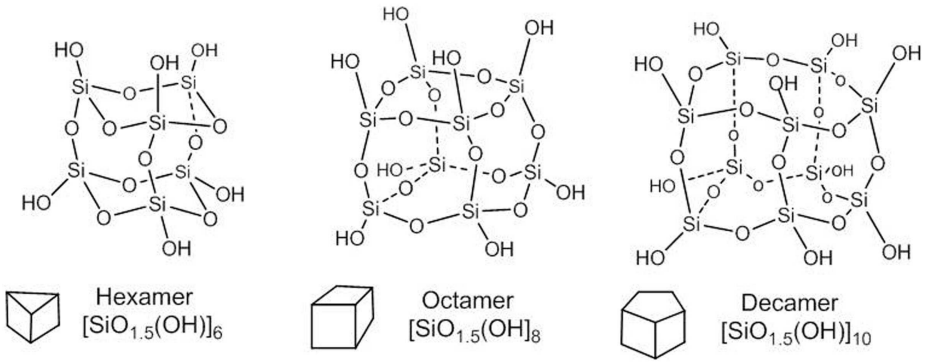

FIGURE 3 | Structure of polyhedral silsesquioxanes.

is assumed as the natural continuation of the polyhedral series with formula $[SiO_{1.5}(OH)]_n$ for values of $n > 20$. There is an interesting formal resemblance with the series of Platonic bodies (tetrahedron, cube,. . .sphere) from geometry.

Actually, the cage-type condensed polyhedral silsesquioxanes have all Si-OH groups at the external vertices with nearly axial orientation. A similarly external distribution of the Si-OH groups is accomplished by the here disclosed nearly spherical oligomers of the silicic acid. SOSA molecules as next term in the series of polyhedral structures with the same general formula $[SiO_{1.5}(OH)]_n$ have the same ratio between the Si: O: H atom = 1: 2.5: 1.

But, there are significant differences between the chemical structure and properties of the polyhedral and of the spherical silica. The predominant difference is that polyhedral silsesquioxanes inhibit neither Na^+/K^+-ATPase nor other ATPases conversely to the strong ATPase-inhibitory SOSA. Structurally, all polyhedral silsesquioxanes (**Figure 3**) comprise only Q3 type Si atoms, i.e., each one is involved in three (Si)-O-Si bonds and one (Si)-OH bond.

The spherical shape of the oligo-condensed silicic acid with $x = 1.5$ is accomplished by some preferred "n" values. **Figure 4** shows the SOSA molecule with $n = 36$, as ball and stick model. This SOSA molecule with formula $[Si_{36}O_{54}\ (OH)_{36}]$ comprises in its internal shells $4 + 8 = 12$ Si atoms of type Q^4 (without Si-OH bonds). In the external shell, there are 12 Si atoms of type Q3 (with one Si-OH bond) and 12 Si atoms of type Q^2 (with two Si-OH bonds). It is observed that the 36 external Si-OH bonds are displayed on the external surface strongly facilitating the hydrophilic interactions with proteins.

Applying the gel-permeation chromatography (GPC) method, molar mass values in the range of 1.2–6.0 kDa have been obtained of the spherical condensed silicic acid oligomers. For the SOSA molecule illustrated on **Figure 4** there resulted $M = 3.2$ kDa, corresponding to molecular diameter $\phi = 2.2$ nm further confirmed by dynamic light scattering (DLS).

The mass ion peaks of SOSA obtained by ESI-MS technique were practically the same as those identified in the spectrum of our MCS factor (**Figure 2**). Actually these m/z peaks correspond to small ionic fragments resulted by the split of the large SOSA molecules in the ionization chamber. The main mass ion peaks

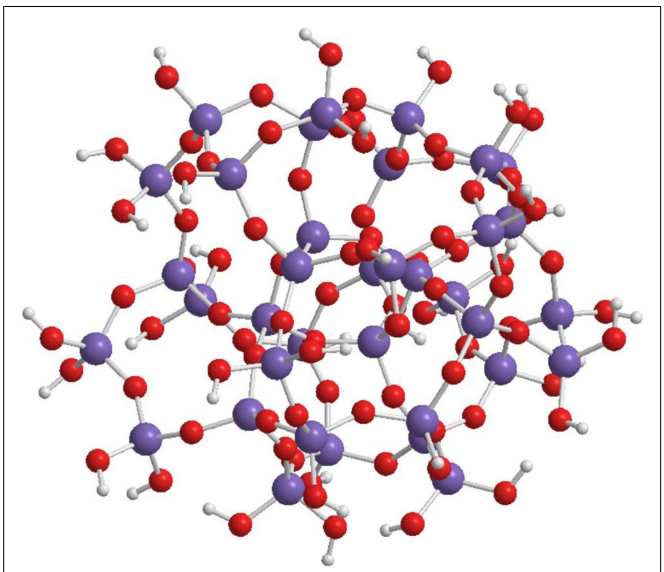

FIGURE 4 | Structure of SOSA with formula: $[Si_{36}O_{54}\ (OH)_{36}]$ with 36 Si-OH bonds on the external surface of the spherical molecule.

correspond to the formula $[(Si_2O_5)_q \cdot Na]^+$ with m/z = 159 Da for $q = 1$; 295 Da for $q = 2$; 431 Da for $q = 3$, and 567 Da for $q = 4$. Similar mass spectra have been observed by decomposition of condensed silsesquioxanes (66, 67).

In fact, the erroneous assessment of the mass ion peaks as derived from carbon suboxide was caused by the accidental identity of the molar mass of C_3O_2 M = 68.03 Da and the mass of the 1/2 Si_2O_5 (di-silicate) ion with M = 68.08 Da, resulted by the decomposition of SOSA. In conclusion, the mass ion peaks on **Figure 2** correspond rather to the formula $[(Si_2O_5)_q \cdot Na]^+$ of SOSA and not to the formula $[(C_3O_2)_n \cdot Na]^+$ as initially suggested.

SOSA AS POSSIBLE ENDOGENOUS FACTOR

With the confirmed potent inhibition of several P-type ATPases, SOSA fulfills the main condition as a candidate ATPase ligand. Its classification as an endogenous factor requires further

that SOSA should be produced within the organism, tissue, or cell. This condition is satisfied with the presumed biosynthetic pathway of SOSA by spherical oligomerization of the monosilicic acid (H_4SiO_4) ubiquitously distributed in plants and animals (68, 69).

A human body contains approximately 1 g of Si in various combinations with oxygen (generic name: silica). Almost all silica in the body is bound to biomolecules and tissues and only a minor part circulates as dissolved silicic acid in blood plasma and urine (70). The plasma level of silicic acid is 0.7 mg/L corresponding to 0.2 mg/L silicon. Infants have two- to three-fold higher Si plasma level, but in contrast aged persons and pregnant women have significantly lower values of Si. For human beings, the daily ingested amount of Si is estimated to be 30–50 mg and the same amount is eliminated in urine. After meals, the plasma concentration of Si increases 30–50%, and returns after a few hours to the initial level (71). The regulatory mechanisms of Silicon homeostasis require elucidation.

The catalytic action of a biomolecule to accomplish the spherical oligomerization of silicic acid yielding SOSA is presumed. Proteins catalyzing high-grade polymerization of silicic acid have been found (67) in algae (silaffin) or sponges (silicatein). Biomolecules favoring the spherical oligomerization of silicic acid have not yet been identified.

The mechanism of ATPase regulation by the probable endogenous ligand SOSA is not fully understood. According to the unusual physical–chemical properties of SOSA and to its specific interactions with the Na^+, K^+, H^+, Mg^{2+}, or Ca^{2+} ions and with the ATPase protein, some challenging proposals for the regulation mechanism may be formulated.

ASSUMED REGULATORY MECHANISM OF SOSA

Transmembrane ion pumping may be physically influenced by SOSA located in a receptor cavity along the ion-transport pathway of the ATPase molecule. The SOSA molecule should behave like a multi-anionic gel with selective binding or permeability effects on cationic species depending on their concentration, charge and size.

The interaction of SOSA with the ATPase protein is assumed on the cytoplasmic site of the ATPases in the E1 conformation as revealed by charge transfer investigations with the dye RH421 (60). According to these data, SOSA should inhibit the X1E1 state, manifesting complex interactions with different X ions (Na^+, K^+, or H^+) and with Na^+ ions in the (Na)NaE1 state. Structural details of the very complex binding of the sodium ions to the NKA in the state preceding phosphorylation have been disclosed by high-resolution crystal structure (72).

The ion-sensitive nature of our ATPase inhibitory factors MCS (disclosed as SOSA) was remarked in Ref. (60) assuming that the concentration of the Na^+ and of other ions may cause significant structural rearrangements. The *in vitro* ion-sensitive structure of SOSA is also supported by DLS measurements. Tentatively, it could be assumed that the SOSA structure at $[Na^+] < 5$mM inhibits the sodium pump but the structure at $[Na^+] \geq 5$ mM should activate it. A possible activation mechanism could be by favoring the differentiate binding of Na^+ ions to the E1 conformation and promoting the phosphorylation step. Accepting this hypothesis, the generally found cytosolic level of $[Na^+] = 5.0$ mM in the eukaryotic cells could be a consequence of the structural change of the archaic ligand SOSA, which happens accidentally at this Na^+ ion concentration.

The here proposed probably archaic regulatory mechanism of the NKA pump by ion-dependent structural changes of the endogenous ligand could also work for other ATPases. The ion concentration threshold for the activation of other ATPases should depend upon the concentration of the ions to be pumped and their interaction with SOSA. This regulatory mechanism, assuming the ion-sensitive structural variation of the archaic ligand SOSA, can explain the astonishing manifoldness of the same well-conserved ion-pumping mechanism in the hitherto identified more than 200 different P-type ATPase pumps.

CONCLUSIONS AND OUTLOOK

With almost all Si-OH bonds disposed on the external surface of SOSA, this substance should bind preferentially to hydrophilic domains of the target proteins. In P-type ATPases, the well-conserved phosphorylation site between the cytoplasmic domains P and N provides an adequate binding site for SOSA molecules similar to decavanadate (**Figure 1**).

Although SOSA inhibits NKA at sub-micro-molar concentration, its direct competition with ouabain for the hydrophobic steroid binding site in NKA is less probable. The non-competitive binding mechanism of our hydrophilic ATPase inhibitor and ouabain was confirmed by the fluorescence dye measurements of Stimac et al. (59, 60). An apparent competition may appear if the NKA conformation required for SOSA binding and that required for ouabain binding are different (C. Toyoshima, personal communication).

The ion-concentration dependent structural changes of SOSA suggest a probable archaic regulation mechanism of the sodium pump and of other ATPases where the pump ligand is sensing the nature and molarity of the ions to be pumped. It is a challenging idea that the transmembrane ion pumping with fundamental importance for many essential life processes should be regulated by the cation-sensitive structure of an inorganic acid.

The identified chemical properties and enzymatic activities of SOSA are congruent with the predicted characteristics of the EDLFs of the P-type ATPases. **Table 1** shows a synoptic presentation of the assumed characteristics of the putative EDLF factors in comparison with that of candidate substances: ouabain, marinobufagenin, vanadate, and the here disclosed SOSA.

Summarizing the characteristics of the SOSA, it may be concluded that these match the predicted criteria of the endogenous ligands of the NKA and probably of further P-type ATPases:

a. SOSA inhibits with similar potencies ($IC_{50} \sim 0.2$–$0.5\,\mu g/mL$) the ouabain-sensitive Na^+/K^+-ATPase from rabbit medulla and the ouabain-insensitive enzyme from rat.
b. SOSA inhibits Ca^{2+}-ATPase from SR, H^+/K^+-ATPase from gastric membrane and of K-dp-ATPase from *Escherichia coli* with IC_{50} values in the range of 0.2–0.5 μg/mL.
c. There are no sensitive methods to differentiate between mono and oligo-silicic acids in cells. But the assay of the NKA-inhibitory factors EDLF in urine and plasma suggest the probable presence of SOSA in these biological fluids.

Table 1 | Comparison of the endogenous ATPase ligand candidates.

		EDLF	Ouabain	Marino-bufagin	Vanadate	SOSA
Characteristics						
C-1	Chemical nature	ND	Cardiac glycoside	Bufadienolide steroid	Inorganic, poly-oxo-acid	Inorganic poly-oxo-acid
C-2	Molar mass (kDa)	0.4–5.0	0.58	0.4	0.12–1.5	1.4–6.0
C-3	Stability by drying	Low	Stable	Stable	Limited	Low
C-4	Structural stability	ND	Stable	Stable	pH sensitive equilibra	Cation and pH sensitive
C-5	Nature to ATPase binding site	ND	Hydrophobic	Hydrophobic	Hydrophilic	Hydrophilic
C-6	Distribution	Ubiquitous	Only in a few plant species	Predominantly in amphibians	Limited	Ubiquitous
C-7	Mammalian occurrence	Yes	No	No	Only in traces	Ubiquitous
C-8	Evolutionary age million years Myr	>3500 Myr	<60 Myr	<360 Myr	>3500 Myr	>3500 Myr
Biochemistry						
B-1	Toxicity	Low	High	High	Moderate	Low
B-2	Biosynthesis	Predicted	Only in a few plant species	Predominantly in amphibians	Improbable	From monosilicic-acid
B-3	Na,K-ATPase	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor
B-4	SERCA	Inhibitor	No	No	Inhibitor	Inhibitor
B-5	H/K-ATPase	Inhibitor	No	No	Inhibitor	Inhibitor
B-6	K-db-ATPase	Inhibitor	No	No	Inhibitor	Inhibitor

ND: not determined.

d. Monosilicic acid is present in almost all cells; thus, its adequate transformation into SOSA can occur *in situ*, catalyzed and/or controlled by proteins.
e. Preliminary data revealed a reduced toxicity by per-oral and a moderate toxicity by intravenous or intramuscular administration of SOSA. Renal elimination is assumed.
f. There are no reasons to doubt the presence of silicic acid and of SOSA in the early history of the evolution.
g. SOSA is stable in solution for several years but it loses its activity by freeze-drying probably through the forced intermolecular condensation of water molecules.

It is planned to obtain further structural details of the interaction of SOSA with ATPase proteins with possible implication for the regulation of the pump. One of the very intriguing questions is to investigate the influence of the concentration of Na^+, K^+, Mg^{2+},Ca^{2+}, and other ions on the structure of SOSA. The further great challenge for ongoing research is to establish the role of SOSA in ATPase related cellular and physiological processes and to explore its possible health-care applications in some connected pathologies (73, 74).

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