MINIREVIEW

Regulation of Na,K-ATPase by Endogenous Ouabain-like Materials (43699)

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Abstract. **Sodium, potassium-ATPase (NKA) is an essential energy transduction mechanism in which the free energy released by hydrolysis of ATP is transferred to an electrochemical gradient across the cell membrane. The asymmetry of sodium in this gradient is coupled to membrane transport mechanisms which affect transmembrane movement of a range of solutes and electrolytes. Recent advances in the molecular biology of NKA have revealed important new aspects of structure-function relationships as well as illuminating the basis for variations in cardiac glycoside sensitivity of the enzyme. The search for endogenous mammalian counterparts of the cardiac glycosides, which regulate the activity of the enzyme by interacting from the extracellular surface at this receptor site, has moved ahead dramatically with evidence that ouabain is an endogenous product of the mammalian adrenal cortex. These advances, and problems raised by them, are explored in this review.** [P.S.E.B.M. 1994, Vol 205]

Sodium, potassium-ATPase (NKA) is the biochemical substrate of a fundamental, energy-dependent transport process: the maintenance of transmembrane gradients of sodium and potassium. This process is common to essentially all animal cells (1). It is the largest single means by which cell ATP is consumed. NKA conserves a portion of the energy released through ATP hydrolysis by charging an electrochemical gradient across the cell membrane. As the result of the charge and concentration differences of ions inside and outside the cell, a potential difference is developed across the cell membrane. The low permeability of the membrane to sodium and potassium serves to sustain this gradient and therefore the potential. Excitable cells have the capacity to rapidly alter this permeability. The resulting energy flow (depolarization) is a primary signal to other cellular processes. In addition, the NKA-generated electrochemical gradient powers a wide range of reactions affecting the transport of materials across the cell membrane via transport processes linked to the movement of sodium. These include glucose and amino acid cotransport; cotransport of sodium, potassium, and chloride; sodium-proton exchange; and sodium-calcium exchange.

Molecular Biology of the Sodium Pump

NKA is a heterodimer comprised of α (112 kDaltons) and glycosylated β (35 kDaltons—protein portion only) subunits. The alpha subunit contains the ATP catalytic domain and the ion transporting function which allows, in the normal operating mode, the outward movement of three sodium ions in exchange for two potassium ions. The charge different accounts for the electrogenic nature of the pump. The α subunit also appears to be the site at which cardiac glycoside (CG) drugs bind and inhibit the activity of the enzyme. The function of the β subunit is not entirely clear. It may regulate and/or affect assembly or membrane in-

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P.S.E.B.M. 1994, Vol 205: 202–212

0037-9727/94/2053-0202$10.50/0

sertion of the active complex. Possibly the β subunit is required to establish and/or maintain the necessary conformation for the α subunit to function. There are at least three isoforms each of the α and β subunits (1), and although the essential ion translocational function of the enzymes is the same regardless of isoforms, each isoform is presumably specialized in some as yet incompletely understood way. This specialization may be reflected in the tissue-specific expression of isoforms which has been well studied (1, 2).

Gene duplication has given rise to a family of ATPases including NKA, H, K-ATPases, and Ca-ATPases (2, 3). Within the NKA subfamily, remarkable conservation has taken place. The three α isoforms identified in mammals have three avian counterparts (4). Among the mammals at least, there is much greater sequence conservation across the species within isoforms than within a species across isoforms. Relatively high levels of conservation have occurred across wide phylogenetic distribution so that antibodies to a region close to the phosphorylation site of the rat α1 isoform are able to recognize NKA from human, pig, sheep, rat, chicken, toad, and fish samples, as well as from two invertebrates: fruit fly and brine shrimp (5).

The α isoforms from higher vertebrates have been studied by hydropathy analysis and appear to code for a protein which has eight transmembrane spanning domains (2). It is probable that at least part of the recognition site for CGs resides on the first extracellular loop. Alterations of amino acids in this location have been shown to affect the CG sensitivity of the enzyme which varies across isoforms and species. The native rat α-1 isoform is highly ouabain insensitive. Conversion of uncharged residues at Positions 111 and 122 in the sheep α-1 isoform with charged residues found in the rat α-1 isoform at these locations result in a 1000-fold decrease in ouabain affinity (6). Similar changes were found in mutations of the native α isoform expressed in *Xenopus* oocytes (7). Ouabain binding appears to be a feature which evolved early, since the NKA of the coelenterate *Hydra vulgaris* shows 50% inhibition at an ouabain concentration of 0.1 m*M* (8). This region in the brine shrimp, *Artemia*, is somewhat homologous to that in vertebrates. There is even greater homology in this region in the enzymes among *Drosophila* and several butterfly species (9–11). Reports of CG interactions with the enzyme from these species do not appear to have been published.

Regulation of the Sodium Pump

Much has been learned about the regulation of sodium pump function, and several recent reviews have appeared (12–17). Briefly, regulation of rate of expression appears to be under the influence of thyroid hormone and corticosteroids. Increased intracellular sodium is also able to stimulate transcription of pump component genes, however, the mechanism by which increased intracellular sodium leads to this change is unknown. Likewise, there is still controversy as to the role of α or β subunit availability in controlling subunit dimer assembly and hence insertion. Under most circumstances there is coordinate expression of the two subunits, but important exceptions have been observed. In addition to its role in increasing transcription of pump genes, increased intracellular sodium elevates activity of existing pumps. Indeed, under normal conditions, this is likely to be the most important controller of the rate at which the sodium pump functions. Extracellular potassium is necessary for electrogenic pump function and can act as a noncompetitive antagonist of CG inhibition. ATP availability is likely to limit pump function only in anoxia. The existence of the highly conserved ouabain binding site has provided a basis to postulate and test the hypothesis that there also exist circulating agents which control sodium pump activity by binding to the extracellular ouabain receptor site. The focus of the remainder of this paper will be an analysis of evidence for an "ouabain-like factor" and an examination of recent evidence that mammals have the capacity to synthesize and secrete an endogenous substance presently indistinguishable from ouabain (18, 19).

The Endogenous Cardiac Glycoside

The possibility of a single agent which can explain several interrelated phenomena is a compelling subject for study. The idea of the existence of an endogenous inhibitor of the sodium pump holds forth several tantalizing attractions to lure the curious investigator. The first is the remarkable evolutionary conservation of the CG receptor site on NKA. As indicated above, the evolutionary origins of NKA are ancient. The ouabain binding region of the NKA α isoform present in the simple coelenterate, *Hydra*, is 50% homologous to the same region in mammals (8). The *Hydra* NKA shows inhibition by CGs. While this binding and modulatory role appears to have arisen early in evolution and to have been rigorously conserved, it is unclear whether primitive animals have the capacity to generate CG-like substances, or if they can, how production might be regulated and what functional role inhibition of the enzyme would play in these animals. An alternative explanation is that the ouabain binding phenomenon arose by chance and was subsequently enhanced and utilized to regulate enzyme activity by the appearance of endogenous CG-like substances. These hypotheses are generally unsatisfying in part because they are so relatively unexplored. They do not provide any argument against the possibility that the conservation of this region of the enzyme is under evolutionary pressure for some other reason and that the bind-

ing of CGs to this region is an epiphenomenon which has been exploited by some plants which produce CGs to protect themselves from consumption by herbivores (20).

Second among the attractions of the putative endogenous CG is the possibility that sodium excretion by the mammalian kidney may be subject to influence by agents which control the activity of NKA in this organ. Since most solute reabsorption by the kidney is linked either directly or indirectly to the reabsorption of sodium by the action of the sodium pump, regulation of the activity of this enzyme in the kidney would have important consequences for electrolyte homeostasis. De Wardener has been a pioneer of studies which sought to demonstrate the role of factors other than GFR and mineralocorticoids in the regulation of renal sodium excretion (21). The concept of a "third factor," which serves to increase sodium excretion without changes in GFR and in the presence of high levels of mineralocorticoids, was developed and reinforced by De Wardener's experiments. De Wardener proposed that the mechanism of this third factor is to inhibit the sodium pump (22). The discovery of atrial natriuretic hormone (23) revealed the existence of another means for enhancing sodium excretion, but it was quickly recognized that the mechanism of this agent does not involve the sodium pump (24). Meanwhile, findings that plasma from volume-expanded animals is able to alter the short-circuit current across amphibian skin reinforced the possibility that an additional natriuretic factor, which functions by modulating sodium pump activity (22, 25, 26), remained to be discovered.

A third attraction drawing interest in the existence of an endogenous sodium pump inhibitor were observations from studies in volume-expanded models of hypertension. Earliest evidence of a circulating hypertensive factor can be traced to cross-circulation experiments between hypertensive and normotensive dogs (27) and, later, rabbits (28). Pressure was found to rise in the normotensive cross-circulation partner, indicating a circulating hypertensive substance. In a parabiosis experiment with rats, Dahl *et al.* showed that their salt-resistant rats became hypertensive when in a parabiotic arrangement with a salt-sensitive animal on high sodium intake (29). Suggestions of a change in NKA function in volume-expanded hypertension arose from observations of potassium vasodilatation. Dogs with volume-expanded hypertension were shown to response less than controls to potassium vasodilatation (30). When it was recognized that potassium antagonizes ouabain inhibition of the sodium pump, the suggestion that such inhibition was present in volume-expanded hypertension arose and was further explored. Ouabain-like activity was found in the plasma in several models of volume-expanded hypertension (31). What was needed was a means to connect inhibition of the sodium pump to the etiology of volume-expanded hypertension. Blaustein provided this by suggesting that the accumulation of intracellular sodium which results from inhibition of the sodium pump leads to activation of outward sodium movement by the sodium:calcium exchanger. As a consequence, calcium enters the cell and accumulates, increasing the available pool of calcium for contraction of vascular smooth muscle (32, 33). Thus, a mechanism which may function to increase sodium excretion becomes hypertensinogenic when its effects are generalized to extrarenal tissues including vascular smooth muscle.

A final compelling piece of the puzzle emerged with the advent of radioimmunoassay techniques for therapeutic monitoring of individuals treated with CGs. Numerous reports emerged indicating the presence of immunoreactivity in the plasma of individuals who had never been treated with CGs. Endogenous digoxin-like immunoreactivity (DLIF) has been detected in the circulation of normal human subjects (34), in patients with impaired renal function (35), in normal (36) and hypertensive pregnancy (37, 38), and in newborn humans (39, 40). Evidence has been generated to suggest that the DLIF measured can not be attributed to known steroids or fatty acids (41). This material may interact with a circulating binding protein (34). A recent study has isolated and partially identified DLIF from bovine adrenal and indicates that this material is structurally very similar to digoxin but different from ouabain (42).

Gruber *et al.* reported that digoxin antiserum cross-reactive material was present in dogs and was increased in volume-expansion (43). Plasma digoxin immunoreactivity was reported to be elevated in rats consuming increased sodium chloride (44), in spontaneously hypertensive rats (45) and in hypertensive nonhuman primates (46). Treatment of hypertensive animals with crude but not Fab fragments from anti-digoxin antibodies has lowered blood pressure (47, 48), though the possibility that this may have been attributable to activation of vasodilatory agents from immune reactions cannot be completely excluded. Some inconsistent observations of CG immunoreactivity have emerged. Great care must be taken to exclude possible cross-reactivities with known endogenous materials which may contain sufficient structural similarity to permit some recognition by CG antibodies. Since CGs have a steroid nucleus, cross-reactivity with endogenous steroids is an obvious pitfall which requires careful and complete evaluation of antibody recognition properties.

This intertwined network of observations has propelled the search to identify the endogenous digitalis. The results of this search have raised many expectations and generated many disappointments. This top-

ic's importance arises from its relevance to electrolyte homeostasis at both cellular and organismal levels and from the useful role that such an agent might play in providing the long sought link between increased dietary sodium intake and hypertension in human populations. Furthermore, the identification of an endogenous regulator of NKA holds out the possibility of new treatments for congestive heart disease as well as hypertension, and even while these are developing, the ability to monitor levels of endogenous NKA inhibitors may be of great utility while administering CGs to patients.

Many efforts to identify the endogenous CG material have not been successful. Several reasons may account for this. First, since CGs appear quite widely in the plant kingdom, it is possible that omnivores and herbivores have a continued low level of dietary intake of such materials. Second, CG drugs are widely prescribed, so random screening of a population may result in some subjects whose tissues and body fluids contain CGs of therapeutic origin. Third, inhibition of the ouabain-sensitive forms of NKA occurs in the range of low nanomolar concentrations in humans, thus, if the endogenous ligand has a similar or greater affinity, circulating levels would be very low. Fourth, there is disagreement as to the material's tissue of origin—the adrenal (49–56) and hypothalamus (57–63) being the front runners. Finally, there is no single assay to identify this material. Rather, it is necessary to select from a range of assays which allow the properties of an NKA inhibitor to be manifest. These include antibody recognition, inhibition of labeled ligand binding to NKA, inhibition of the ATP hydrolytic activity of preparations of NKA of varying purity and inhibition of the ion translocating action of the enzyme in various circulating blood cells or vascular tissue preparations. Presumably, an authentic endogenous NKA inhibitor would be active in all these assays, show specificity for ATP hydrolysis by NKA rather than other ATPases, require the same ionic conditions for activity, show the same competition of action with extracellular potassium, and even have the inotropic effects characteristic of digitalis.

The Identification of Ouabain, or its Stereoisomer, as an Endogenous Mammalian Cardiac Glycoside

Material meeting these criteria had never been identified until the recent series of reports from Hamlyn *et al.* concluding with the identification of ouabain, or a stereoisomer of ouabain, as an endogenous product of the mammalian adrenal (18, 19, 64–67). Hamlyn's approach has been to collect large volumes of plasma from volume-expanded subjects. The pooled materials were then dialyzed and the dialysate absorbed on Amberlite XAD-2. After the absorbed material was eluted with methanol, the resulting dried solids were redissolved in water and subjected to an initial HPLC fractionation over a reversed phase column. NKA inhibitory activity was screened by examining 86Rb uptake into washed human red cells. The most active peak was subject to further purification by affinity extraction with purified lamb kidney NKA. After collection of the material bound to the enzyme, two further HPLC steps led to the purification of a single homogenous peak of activity. The material in this peak was subject to mass spectroscopy which revealed a spectrum identical to that of authentic plant ouabain. An ELISA assay was developed for ouabain and was subsequently used to confirm the presence of ouabain immunoreactivity in solid phase extracts of human, dog, and rat plasma (18). Ouabain immunoreactivity was also present in the adrenal and was added to serum-free medium by cultured bovine adrenal cells. HPLC purification of rat adrenal extracts yielded a material which contained ouabain immunoreactivity which coeluted with authentic ouabain. Adrenalectomy lead to a reduction in the levels of ouabain immunoreactivity in plasma extracts. This latter evidence is important since it supports the endogenous synthesis of ouabain. Furthermore, work in our own laboratory has shown that the adrenal cortex is a likely source of an endogenous NKA inhibitor and that cultured adrenocortical cells have the capacity to release material, which meets several NKA inhibitor criteria, into medium (this material, however, was shown not to be ouabain) (50, 51, 68). Indeed, the adrenal cortex is a steroidogenic organ involved in fluid and electrolyte homeostasis, so the finding of ouabain in this tissue was in harmony with the steroid nature of ouabain as well as its possible function in fluid balance. Further evidence of an arterio-venous gradient of ouabain immunoreactivity across the adrenal has provided additional support that ouabain is an endogenous NKA inhibitor and that the adrenal is its site of origin (49).

Efforts to examine the hypertensinogenic properties of ouabain have progressed. This is an important undertaking when it is considered that CGs are generally not hypertensive when given acutely and reports of hypertension during chronic clinical treatment are rare. Indeed, it seems counterintuitive that an agent which is natriuretic should also be hypertensive, and therefore it has been important to assess whether ouabain could elevate blood pressure. Yuan *et al.* have recently presented evidence of ouabain-induced hypertension in the rat (69). Gottlieb *et al.* have also studied the presence of ouabain immunoreactivity in extracts of the plasma of heart failure (70). A small elevation in plasma ouabain immunoreactivity was observed. However, the extremely wide range of plasma ouabain levels in both controls and patients was sur-

prising and much greater than had been previously reported. Similarly, the mean level of this control population was twice what had been reported from a previous normal population.

The identification of a new hormone, especially one so long sought after and with a function of interest to so broad a constituency, can be expected to be followed by a deafening clamor of reaction, generally of concurrence, occasionally of dissent. The response to the identification of ouabain has been remarkable for the paucity of either. Perhaps this is attributable to the lag time necessary for other laboratories to develop assays and perform and publish studies. In addition, an expected commercialization of the ouabain ELISA has still not materialized at the time of writing. To this point, however, limited independent support for the conclusion that ouabain is present in mammalian body fluids and may be synthesized by mammals has emerged. Tamura *et al.* have identified a material in bovine adrenals which they have not been able to distinguish from ouabain (71). It shares identical HPLC elution characteristics and the same cross-reactivity curve with antiouabain antiserum as authentic ouabain, though they have not completed chemical identification of this material. Ferrandi *et al.* have studied a ouabain-like factor from Milan hypertensive and normotensive rats (72). A material which shared the same HPLC elution time as ouabain was found in the hypothalamus of both strains of rat and in the adrenals of the hypertensive, but not normotensive, animals. Like authentic ouabain, this material was a reversible inhibitor of NKA, inhibition was reduced by potassium, there was high-affinity binding to NKA, and there was no effect on Ca^{++}-ATPase. However, unlike ouabain, this material was able to more effectively inhibit the ouabain-resistant rat isoform of NKA, and NKA from younger animals seemed to have an increased resistance to this material than NKA from older animals.

Problems in the Validation of Ouabain as a Relevant Endogenous Sodium Pump Regulator

In light of the surprising finding that ouabain is an endogenous mammalian material, and the fact that this finding has yet to be fully confirmed independently two years after its publication and even the supportive independent data that has emerged is not wholly concurrent, it seems worthwhile to make a fresh appraisal of the evidence. Without doubt, strong evidence has been presented which supports the conclusion that ouabain is an endogenous mammalian substance. However, since this evidence derives principally from a single source, it seems wise to cautiously appraise issues raised by such a conclusion to test the strength of the original conclusion.

Issue 1. NKA Isoforms. If ouabain is to play a role in regulating renal excretion of sodium, it is necessary that the circulating levels of ouabain reach sufficient levels to modulate NKA function in the kidney. The predominant NKA isoform expressed in the kidney is the α-1 isoform, which is least sensitive to ouabain. The K_d of human heart NKA (probably reflecting predominantly the α-1 isoform) by ouabain is approximately 2.5 n*M* (73). So far, mean plasma ouabain levels in the range 0.14–1.1 n*M* have been reported. Thus, it seems possible that this isoform is subject to limited regulation by circulating ouabain. There is evidence of a second ouabain isoform in the kidney. This was first indicated by observations of increased ouabain sensitivity in lower portions of isolated nephrons (74). Results from studies of gene expression in the kidney have been contradictory, revealing consistent α-1 expression, while reporting varying observations of presence or absence and amount of α-2 and α-3 isoforms (2). However, more recent, sensitive and quantitative studies of this issue more convincingly demonstrate the presence of the α-2 isoform at lower levels than α-1 (75, 76). Since the ouabain sensitivity of this isoform is probably greater than the α-1 isoform, it is conceivable that α-2, and perhaps α-3, isoforms are expressed and regulated in lower nephron regions to permit important effects on the control of sodium reabsorption by the action of ouabain on the lower nephron.

Issue 2. Second Messengers. Kelly and Smith have drawn attention to the fact that the membrane receptors to which hormones bind are coupled to second messenger systems by which signaling is transferred intracellularly and amplified (20). The binding of ouabain to NKA activates no second messenger or amplification system and is therefore highly uncharacteristic of a receptor-ligand interaction. This view is, of course, parsimonious. It is conceivable that the ouabain receptor on NKA represents the conservation of a primitive hormone-receptor system. Because important changes in cell function can occur as a result of changes in intracellular ionic composition secondary to NKA inhibition, it could be argued that no second messenger system is required.

Issue 3. Steroid Nucleus Configuration. The biosynthesis of ouabain in mammalian tissues requires the capacity for 14 β-hydroxylation of the steroid nucleus. This modification places the C and D rings of the steroid nucleus in the *cis* configuration (Fig. 1). This configuration appears to be critical for receptor recognition. Until the identification of ouabain as an endogenous mammalian steroid, there had been no previous reports of the identification of endogenous steroids with this modification in mammals. This is not, however, an issue which has received focused investigation. Some lower vertebrates clearly have this capacity, since synthesis of cardiotonic steroids (bufotoxins) hydroxylated in the 14 β position has been demonstrated in toads of the *Bufonidae* family

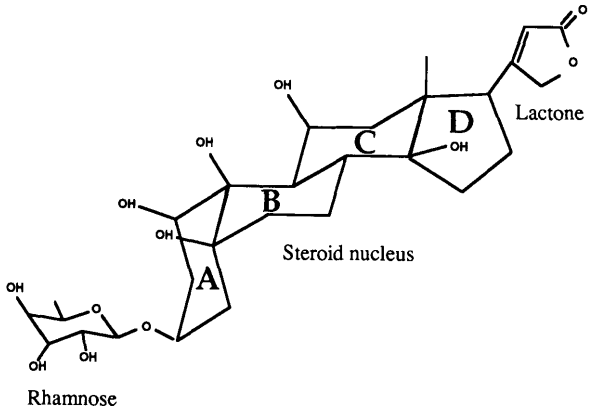

Figure 1. Structure of ouabain showing lactone group at C17, steroid nucleus with A/B and C/D rings in *cis* conformation, and rhamnose residue.

which synthesize these materials as cutaneous venoms (77).

Issue 4. Rhamnose, the Sugar Residue of Ouabain. Rhamnose belongs to a family of pentoses present in bacteria and plants, but hardly known in mammals. It is used clinically as an index of the permeability of the gut mucosal barrier across which it passes via the transcellular route (78, 79). There is only limited evidence for its normal presence in mammals, which makes it useful as a probe of intestinal permeability. However, rhamnose has been purified by simple chromatographic methods and identified in rabbit skin (80). Rhamnose has also been reported to be present in human urine (81). Furthermore, it is reported to be a constituent of brain mucopolysaccharides (82). Whether rhamnose is synthesized by mammals is unclear, and there are only limited studies of its intermediary metabolism in mammals (83). However, even if the source of mammalian rhamnose is dietary, this does not exclude the incorporation of rhamnose into products of mammalian metabolism, perhaps including ouabain. The bufotoxins, however, are aglycones (77).

Issue 5. The Lactone Ring. Plant CGs, such as ouabain and digitalis, contain an unsaturated cyclical group (lactone) at the C17 of the steroid nucleus. In amphibian venoms, there is a similar (α-pyrone) group. It is uncertain whether mammals possess the ability to perform this modification of cholesterol and whether cholesterol side chain cleavage is necessary prior to such modification. Evidence from amphibians reveals that cholesterol is a likely precursor of bufotoxins (84), though there is no evidence that side chain cleavage of cholesterol is part of the biosynthetic pathway. The identification of ouabain in mammals has not yet lead to studies to define the biosynthetic pathway. In the absence of this information, a strong piece of confirmatory evidence for the mammalian biosynthesis of ouabain is lacking.

Issue 6. Is Extraction and Radioimmunoassay Sufficient? Subsequent to the initial identification of ouabain in human plasma, an extraction technique was developed to remove ouabain from plasma prior to immunoassay of the extract (65). Presumably, this was to limit interference in the assay by "nonspecific" materials of unknown identity. Single step solid phase extraction is a useful method of sample enrichment. However, selectivity of this method is not high. Since polarity is the principal determinant of extraction and elution in this system, all material with similar polarity can be recovered from plasma into an extract which is then assayed. While less likely than in assays employing whole plasma, it remains possible that ouabain immunoreactivity in the extracts could be accounted for by material which is not ouabain. As Kelly and Smith have suggested, routine HPLC fractionation of samples to increase the stringency of purification prior to assay is highly desirable at this early stage in the evaluation of ouabain as a candidate mammalian substance (20). Indeed, solid phase extraction of adrenal tissue homogenates has already provided evidence of ouabain immunoreactive material which does not coelute with authentic ouabain in HPLC (67). However, Harris *et al.* reported that in plasma the dilution curves of solid phase extracts of human plasma were similar to those produced by ouabain and that the addition of ouabain to plasma also yielded a parallel dilution curve (65).

Issue 7. An Extracellular Steroid Receptor. Cardiac glycosides are steroids, generally more polar than known mammalian steroids, and therefore generally less accessible to the cytoplasm. Indeed, ouabain is an extremely polar cardiotonic steroid with eight hydroxyl groups per molecule. Steroid hormones are known to function by binding to intracellular receptors, which then allow alterations in gene expression to occur as the pathway of hormone action. The idea of the presence of a membrane receptor (NKA) for an endogenous steroid is highly novel and poses some new questions. For example, are endogenous cardiotonic steroids the only steroids which act in this way? Can cardiotonic steroids also act intracellularly, and are there intracellular binding sites available which allow them to function by modification of gene expression? The acceptance of ouabain as an endogenous mammalian substance requires a revision of the tenets of steroid hormone function. More evidence must be developed before such revision is warranted.

Issue 8. Volume Status and Plasma Ouabain Levels. A significant appeal of the concept of an endogenous CG is the ability to link natriuresis via inhibition of the sodium pump to a consistent set of observations of increased plasma NKA inhibitory activity in volume-expanded states. The fact that such an agent may be hypertensive as a consequence of its

inhibition of NKA in vascular smooth muscle cells only reinforces the appeal by explaining how hypertension is generated in volume-expanded models of the disease. Since chronic ouabain treatment has now been shown to produce hypertension, there is even more impetus to believe that plasma ouabain levels should rise in volume-expansion (69). The available evidence, though limited, is less reassuring. Acute volume-expansion in dogs has been shown to have no effect on plasma ouabain levels (49). Perhaps this is because the normal stimulus linking volume status to ouabain secretion is slower to develop or be expressed than the time course of acute expansion. However, studies in patients with chronic heart failure failed to reveal a relationship between atrial pressure and plasma ouabain (70). Certainly, it is premature to conclude that ouabain is not responsible for the increase in plasma NKA inhibition in volume-expansion on the basis of these two studies, however, the available evidence does not support such a role. What then does drive the production of ouabain in mammals?

Issue 9. Pathway of Ouabain Clearance. Ouabain is known to be cleared from plasma predominantly by the kidney in man (85). Thus, a relationship might be expected between plasma ouabain and renal function; defective renal function (reduced GFR) might be expected to produce increased plasma ouabain levels. In their report of plasma ouabain immunoreactivity in heart failure patients, Gottlieb *et al.* were able to find no relationship with GFR in these subjects (70). This might be attributable to other heterogeneous derangements of fluid regulation across this patient population, of course, but this remains a troubling observation in need of further exploration.

Issue 10. Are the Adrenals the Source of Ouabain? Several enigmas are posed by the proposal that the adrenal is the source of ouabain. The first of these stems from the fact that ouabain is an extremely polar steroid. This is in contrast to the much less polar cardiotonic steroids elaborated by amphibians (77). Adrenal steroids are released to the circulation by diffusion across the cell membrane. Ouabain's polarity excludes this as a possible mechanism of secretion from the adrenal. Thus, to accommodate the hypothesis that ouabain is an adrenal product, a whole new mechanism of secretion by the adrenal cortex must be established. While such a mechanism has yet to be evaluated, validity of the general hypothesis that ouabain is an endocrine product of the adrenal cortex presently rests on speculation.

Studies in our own laboratory have investigated whether the release of CG-like material from the adrenal occurs as the result of an extension of the known steroidogenic pathways in this gland. All adrenal steroid production is presumed to result from the conversion of cholesterol to pregnenolone as the result of side chain cleavage. Our work has shown that the release of CG-like material from cultured adrenocortical cells is not inhibited by blockade of cholesterol side chain cleavage and further metabolism of pregnenolone (50, 51). This, then, implies that either cholesterol is not a precursor or that side chain cleavage is not part of the biosynthetic pathway. Production of cardiotonic steroids by amphibians, however, does employ cholesterol as a precursor (84). While both these possibilities exist, they again involve fundamental revision of yet another aspect of steroid hormone endocrinology.

There also remains the possibility that the adrenal production of ouabain might affect adrenal function itself. This could be either adaptive or maladaptive. The presence of high local concentrations of ouabain in the adrenal gland would presumably put the organ at risk to toxic effects from these high local concentrations. Undoubtedly, this risk is mitigated somewhat by the high ratio of blood flow to tissue volume in this gland. However, preliminary studies from our own laboratory indicate high levels of ouabain binding sites in the cell membranes of cultured bovine adrenocortical cells. These cells share the capacity for internalization of bound ouabain previously demonstrated and most thoroughly characterized in HeLa cells grown in culture (86). Our work indicates that the kinetics of release of internalized labeled ouabain from adrenocortical cells is indistinguishable from that reported from HeLa cells. Furthermore, the labeled ouabain present in the cytoplasm and released after internalization does not appear to undergo any modification, based on HPLC elution of labeled ouabain recovered from the cytoplasmic fraction (Doris and Trimmer, unpublished findings). These experiments suggest that the adrenal cortex is likely to be highly sensitive to locally produced ouabain, that there is no evidence that ouabain that enters the cytoplasm can gain access to a secretory pathway (leading to enhanced exocytosis), and that there is no further metabolic pathway into which cytoplasmic ouabain can enter. One study (87), but not another (69), suggests a preferential uptake of injected ouabain into the adrenal gland of intact rats. In the former study, after cessation of radiolabeled ouabain administration, plasma ouabain levels were sustained apparently as the result of slow release of ouabain from the pool sequestered in the adrenals. Once again, if our view of ouabain as an adrenal product is to be supported and sustained, these are considerations which must be explored and resolved.

Issue 11. Matrix Effects. Early reports of plasma levels of ouabain measured by ELISA of C18 extracts of plasma indicated plasma levels of 0.138 ± 43 n*M* in normal human subjects (18). A subsequent review from the same group reports plasma levels in normal subjects at 0.694 ± 0.11, 0.74 ± 0.09 and 1.10 ± 0.31

n*M* (88). This range of values and the fact that they appear to be rising progressively is troubling. Unidentified sample "matrix effects" are speculated as being the source of this variation; however, this does not appear to include protein binding of ouabain which has a very low affinity for plasma proteins at "physiological" concentrations (88). It is essential that these unidentified matrix effects be identified and eliminated. Without removing this confounding factor, all measurements of ouabain in C18 extracts of plasma may be under- or overestimates.

Issue 12. The Immunological Profile. Another aspect of the evidence concerning the role of ouabain as an endogenous inhibitor of the sodium pump that must be appraised is whether ouabain is the only physiologically regulated endogenous inhibitor of the sodium pump, and if it is not, what role does it play and how is this role integrated with that of other NKA regulatory mechanisms. Some insight into the nature and scope of this question arises from studies reported by Naomi *et al.* who have analyzed the immunological profile of digoxin-like immunoreactivity in human plasma using seven separate antibodies and assay systems (89). Further, they compared the profile of digoxin-immunoreactivity produced by pure ouabain, bufalin, progesterone, and 17-OH progesterone with that obtained in plasma using the various assay systems. Their results indicate that the ability of each assay to recognize digitalis-like material in plasma was very different from the ability to recognize ouabain, suggesting that ouabain is not likely to account for much of the digoxin-like immunoreactivity in plasma. In contrast, the immunological profile of bufalin was much more similar to the profile obtained by the seven assays for digoxin-like immunoreactivity in plasma. There is an indication here, which warrants closer investigation, that ouabain may have only a limited role in accomplishing alterations in the circulating levels of NKA inhibitors.

Issue 13. The NKA Sink and Regulation of NKA Activity by Ouabain. Recent experiments have shown that chronic, but not acute, administration of ouabain produces hypertension in the rat (69). Chronic administration at doses sufficient to raise blood pressure in the rat does not cause elevated plasma levels of ouabain. However, ouabain does accumulate in various tissues, including the kidneys. Thus, there is reason to question whether the elevation of blood pressure produced by ouabain is mediated through an effect of circulating ouabain on blood vessels and direct effects on vascular smooth muscle contractility. This problem is amplified by the presence of highly ouabain-resistant α-1 isoform in vascular smooth muscle (90). Therefore, effects on blood pressure may occur indirectly, arising from outside the relative confines of the vascular smooth muscle compartment.

NKA is essentially ubiquitous to all animal cell membranes. Its localization and density on the cell membrane varies, but typically there are from 500 to >10,000 pump sites per cell. This means that there is an enormous sink for ouabain. If evidence from HeLa cells can be correctly generalized, a large fraction of the ouabain interacting with receptor sites in tissues is internalized, may be inactive in this location, and may be retained for a prolonged period only to be released again, apparently intact (86), from whence it may be able to repeat the process of binding, inhibition, dissociation or internalization, and release. How then, does the adrenal, which normally elaborates only relative small quantities of ouabain (49), issue sufficient signal to target modification of sodium pump activity in any physiologically meaningful way? For example, the dose (n*M*/kg/day) required to produce hypertension in the rat (69) is approximately 500 times the basal adrenal secretion in the dog (49), and this dose must be sustained for several weeks to produce detectable hypertension. If there is an answer to this dilemma it must certainly begin by attributing physiological regulation by ouabain to a very long time course in which moment by moment, hour by hour, perhaps even day by day alteration of ouabain release are not relevant. Ouabain's effect to assist in body sodium balance may be limited to excursions of altered sodium balance of prolonged and persistent duration, and its effect on blood pressure may require compromised renal function to be revealed (69).

Issue 14. Sodium Pump Dynamics. To this point in our discussion, we have referred to ouabain as if it were a static component in the system, unresponsive to forces introduced when it inhibits the sodium pump's activity. This is certainly far from accurate. The first consequence of sodium pump inhibition is an increase in intracellular sodium which is a powerful stimulus to increased pump activity. Thus, the pump itself responds to overcome inhibition with a time course which makes it difficult to detect altered intracellular sodium concentrations (91). By mechanisms which are presently not understood, prolonged increase in intracellular sodium also leads to increased NKA gene expression as part of a process which ensures stability of the intracellular ionic milieu (14, 92, 93). Thus, all actions of ouabain on the pump are opposed by cellular homeostatic responses. Is endogenous ouabain production sufficient to overcome these responses? This is a question which must be addressed before the observation of endogenous ouabain production can be extended to an understanding of how such material is relevant to fluid balance and hypertension.

Conclusion

There is strong evidence that ouabain may be an endogenous mammalian material. This evidence pres-

ently awaits thorough independent confirmation. Such confirmation will serve only as the starting point to address a wide range of questions concerning ouabain and its potential role as the mediator of responses to altered fluid balance and as a regulator of cardiovascular function in health and disease.

The author is grateful to Elizabeth Roth, Richard Beech, Denise Durham, and Tu Nguyen for technical assistance in parts of the work described in this paper.

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