MINIREVIEW

Cardiotonic Steroids: Potential Endogenous Sodium Pump Ligands with Diverse Function[1]

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The highly conserved cardiotonic steroid (CS) binding site present on the ubiquitous membrane sodium pump, sodium, potassium-ATPase, appears to have been conserved by no force other than its capacity to bind CS: a family that includes plant-derived cardiac glycosides and putative endogenous vertebrate counterparts. Binding of ligand is inhibited by increased extracellular potassium. This implies functional coordination because inhibition of the sodium pump would be counterproductive when extracellular potassium is elevated. The interesting biology of the CS binding site continues to stimulate investigations into the identity of endogenous ligands, their role as pump regulators at the cellular level, and as mediators of body fluid balance and blood pressure regulation. In addition to inhibition of sodium and potassium transport, there is considerable recent evidence suggesting that the sodium pump may act as a cell signaling receptor activated by CS binding and responding by coordination of intracellular signaling pathways that can be dependent on and also independent of the reduction in transmembrane ion flux resulting directly from pump inhibition. This signaling may influence cell survival, growth, and differentiation. Recent insight into the biology of pump regulation by CS is reviewed. [Exp Biol Med Vol. 227(8):561–569, 2002]

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Cardiotonic steroids (CS) encompass a group of compounds that share the capacity to bind to the extracellular surface of the major cellular ion transport protein, the membrane-inserted sodium, potassium-ATPase (NKA, the sodium pump). Members of this group of compounds include plant-derived pharmaceuticals such as the digitalis steroid glycoside drugs as well as the more polar plant monoglycoside, ouabain, and also vertebrate-derived aglycone CS such as bufalin and marinobufagenin. The vertebrate CS are best known as amphibian venoms, however, there is growing evidence of their presence in mammals. The classic actions of CS relate to their ability to inhibit the membrane ion translocating activity of NKA and to increase intracellular sodium concentration (1). New evidence also suggests CS actions that occur in the absence of substantial erosion of the transmembrane sodium gradient and that operate through intracellular second messenger signaling pathways with ultimate effects on gene expression and cell growth and division.

Since this topic was last reviewed (2), there has been progress in a number of important areas. In particular, further clarification of the structure of ouabain-like material present in bovine brain has emerged; there has been accumulating evidence of adrenocortical release, biosynthesis, and regulation of CS production; and there is growing evidence of the ability of plant-derived glycosides to mediate effects on second messenger signaling with effects on pathways influencing cytoskeletal reorganization, cell growth, and cell division, as well as further evidence of involvement of endogenous CS in regulation of blood pressure and renal function. The present paper will survey these continuing areas of progress in the regulation of NKA function.

Is There an Endogenous Mammalian CS and if so, What is Its Structure?

This has been a major question in this field for a number of years and has been the subject of close scrutiny in previous reviews in this series. The proposal that ouabain itself is an endogenous mammalian compound is problematic for a number of reasons whose cogency remains largely

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unchanged (2, 3). Since our last appraisal of this topic, additional work has been reported examining the ouabain-like material present in bovine hypothalamus that was initially reported to be dissimilar from authentic ouabain in a number of key features. This recent work has confirmed its identity to plant ouabain and has explained a number of the anomalous features previously reported (4). However, the presence of this material in preparations from bovine hypothalamus leaves unanswered important questions concerning whether it is the product of bovine biosynthesis or a diet-derived material. The relationship between this hypothalamic ouabain and regulation of NKA in the periphery also remains unknown. Further evidence of an adrenocortical substance having numerous properties in common with the vertebrate CS marinobufagenin (MBG) have been presented, and additional work supporting the endogenous biosynthesis and release of this material is encouraging in establishing CS as endogenous mammalian endocrine hormones. However, this work also remains incomplete (5).

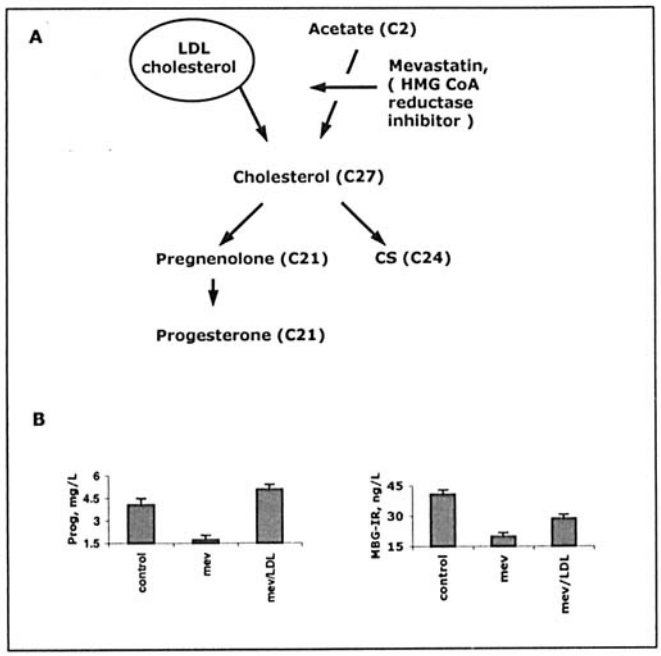

Figure 1. Role of cholesterol in CS biosynthesis. (A) Bufadienolide CS (MBG) is produced by the pathway of *de novo* cholesterologenesis that can be inhibited by the HMG-CoA reductase inhibitor mevastatin. Cholesterol complexed in the plasma LDL fraction was used for the production of bufadienolide CS material. (B) LDL-cholesterol (100 m*M*) reduced the inhibition effect of mevastatin on MBG and progesterone production.

Biosynthesis and Regulation of CS Production

Support for an adrenocortical origin of mammalian CS arises from studies that include purification and identification of sodium pump inhibitors from adrenocortical tissue (5–9) and studies showing secretion of CS from cultured adrenocortical cells in basal and stimulated conditions (5, 10–14). The hypothalamus has also been suggested as an endogenous source of mammalian CS (7, 15–17), but there is no data to address whether this material is released from the brain to affect peripheral NKA.

The mouse adrenocortical tumor cell line, Y-1, constitutes a model for studying the biochemical mechanisms of steroid hormone production in the adrenal gland. This cell line was used to investigate the biosynthetic pathway of the bufadienolide CS MBG in mammals (5). Although the chemistry of amphibian bufodienolides has been studied extensively for decades, the biosynthetic pathways are practically unknown. Using ^{14}C-cholesterol, Siperstein *et al.* (18) have shown that cholesterol is a precursor of cardiotonic bufadienolides in *Bufo marinus*. In Y-1 cells grown in the absence of exogenous cholesterol, MBG was produced by the pathway of *de novo* cholesterologenesis that can be inhibited by the HMG-conenzyme A (CoA) reductase inhibitor mevastatin (5). These adrenocortical cells may also use cholesterol complexed in the plasma low-density lipoprotein (LDL) fraction for the production of bufadienolide CS material (Fig. 1). MBG is produced in a pathway leading from acetate through mevalonate to cholesterol, which at least partially overlaps the pathway by which all steroid hormones are synthesized in the absence of exogenous cholesterol.

The first stage in the synthesis of steroid hormones from cholesterol is the removal of a C6 unit from the side chain of C27 cholesterol to form C21 pregnenolone. The sterol transfer protein, steroidogenic acute regulatory protein (StAR), provides the regulated flow of cholesterol to the inner mitochondrial membrane (19). Side chain cleavage occurs at this site by the action of a cytochrome P450 complex (P450scc). The side chain of cholesterol is hydrolyzed at C20 and then at C22, and the bond between these carbon atoms is then cleaved by desmolase, resulting in formation of pregnenolone, the progenitor of adrenocortical gluco- and mineralocorticoids and adrenal sex steroids. The role of P450scc in biosynthesis of CS differs between plants and animals. In plants, the biosynthesis of cardenolides can involve the formation of a C-21 steroidal precursor (20), and pregnenolone is a good precursor for plant CS glycosides. Synthesis of the lactone ring of plant cardenolides precedes through the condensation of acetyl CoA and a 20-keto-pregnane derivative (20), whereas the α-pyrone ring of plant bufadienolides (e.g., in squill, *Scilla maritima*) is formed by condensation of a pregnane derivative and one molecule of oxaloacetic acid (21). The animal bufadienolides are apparently biosynthesized by a different pathway. In amphibians, pregnenolone, the immediate product of side chain cleavage, is not incorporated into bufadienolides. Thus, pregnenolone was not a precursor to the production of MBG in *Bufo paracnemis* (22, 23) *Bufo arenarum* (24), and *Bufo marinus* (18).

To study the role of P450scc in CS biosynthesis by adrenal cortex, two different approaches were used. Studies examining whether release of cardenolide-like CS by rat

adrenal tissue or Y-1 adrenocortical cells could be reduced by inhibiting the conversion of cholesterol to pregnenolone through the addition of aminoglutethimide (AG) to the incubation medium have been reported (25, 26). Release of pregnenolone was consistently blocked by AG, however, release of CS was not similarly affected. To exclude the possibility that CS could be accounted for by crossreaction with another known adrenal steroid, AG inhibition was accompanied with blockade of further pregnenolone metabolism by inhibition of 17 α-hydroxylase with SU-10603 and inhibition of 3 β-hydroxysteroid dehydrogenase with cyanoketone. Once again, pregnenolone release and metabolism were effectively inhibited, but no similar pattern of inhibition of CS release was observed. These observations support the principle that P450scc is not involved in mammalian adrenocortical CS biosynthesis pathway and pregnenolone is not a precursor of CS.

Genetically engineered adrenocortical cell lines have been useful to further elucidate the biosynthetic pathway of bufadienolide CS (5). Three clonal Y-1 lines have been used. The control line, Y-1/neo, is transfected with the selection vector (neomycin resistance) only. The second, Y-1/DAX, contains the same vector, but is also transfected to express the human DAX-1 transcription factor that downregulates the expression of a number of proteins involved in adrenal steroidogenesis (27, 28). Another transformed Y-1 line, Y-1/RIAB, expresses the dominant-negative form of the type I protein kinase A regulatory subunit. In both Y-1/DAX and Y-1/RIAB, StAR protein is undetectable at the level of both RNA and protein (28). Furthermore, expression of the cytochrome P450 complex responsible for side chain cleavage (P450scc) is reduced severalfold (29). The cAMP-dependent signaling pathway, which plays a major role in initiating steroidogenesis by activating side chain cleavage, remains functional in Y-1/DAX cells, but not in Y-1/RIAB (29). Both Y-1/DAX-1 and Y-1/RIAB cell lines showed markedly reduced production of progesterone compared with Y-1/neo, but the production of MBG by these transformed lines was at the same levels as in the control, Y-1/neo line. MBG-IR material produced by these cells lines was partly purified from conditioned medium and fractionated by several steps of HPLC and was found to elute in the same fractions as authentic MBG. Serial dilutions of HPLC-purified MBG-IR were compared with authentic MBG for their ability to compete for binding to the MBG antibody and very similar dilution curves were observed. These experiments indicate that MBG production is independent of StAR-mediated transfer of cholesterol to the inner mitochondrial membrane and that P450scc is not involved in MBG biosynthesis (Fig. 2).

Regulatory mechanisms controlling production of adrenocortical CS have been studied using the Y-1 adrenocortical cell line (5), primary adrenal cell cultures (12, 30), and *in vivo* techniques (8, 31). A series of studies performed using primary bovine adrenocortical cell cultures growing with serum present at various concentrations reported that

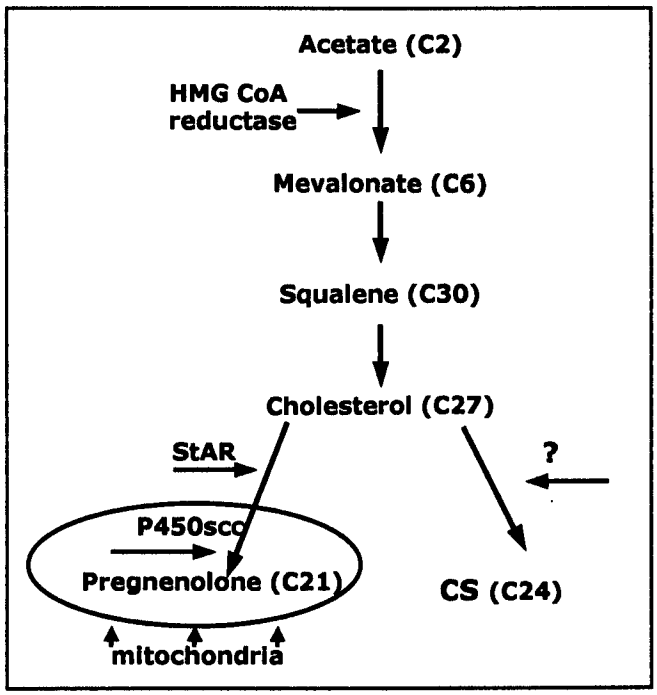

Figure 2. Scheme of CS biosynthetic pathway. CS MBG is produced in a pathway leading from acetate through mevalonate to cholesterol, which partially overlaps the pathway by which all steroid hormones are synthesized in the absence of exogenous cholesterol. However, MBG production is independent of StAR-mediated transfer of cholesterol to the inner mitochondrial membrane, and P450scc is not involved in MBG biosynthesis.

angiotensin II stimulated release of aldosterone, cortisol, and a CS that had similar polarity to ouabain and was recognized by a ouabain antibody (12, 30). Stimulation of steroid secretion by angiotensin II was dose dependent for all three steroids: half-maximally effective concentrations of angiotensin II were 0.31–0.38 n*M*/l for secretions of aldosterone and cortisol and 2.3 n*M*/l for ouabain-like CS. The nonselective mammalian antagonist of angiotensin II, [Sar^1, Ile^8]-AII, blocked stimulation of secretion of all three steroids without affecting basal output. Using specific agonists and antagonists, the angiotensin effects on mineralo- and glucocorticoid were shown to be mediated by AT1 receptors, whereas ouabain-like material was regulated by AT2 receptors. The second messenger mechanisms involved in the ouabain-like CS secretion were studied (13). Treatment of adrenocortical cells with cAMP and phorbol ester both stimulated aldosterone secretion, but had no effect on the secretion of CS. Incubation with the nonspecific phosphatase inhibitor sodium orthovanadate blocked angiotensin II-stimulated secretion of CS. cGMP stimulation maximally activated secretion of CS.

Recently, Chen *et al.* (32) showed that CS digoxin and digitoxin, though not ouabain, decreased progesterone release by rat adrenal granulosa cells via a mechanism involving inhibition of a post-cAMP pathway and cytochrome P450scc and StAR protein functions. The reduction of P450scc and StAR function was in part due to the decrease of P450scc and StAR protein expression. This observation

may point out coordination in the regulation of pregnenolone-derived steroids and CS. Interactions occur between ouabain, angiotensin-II, atrial natriuretic peptide, and potassium in the regulation of aldosterone secretion, and an inhibitory effect of ouabain on aldosterone production and angiotensin action in the human adrenal has been observed (33–35). However, other studies have reported stimulation (36, 37) or transient stimulation followed by inhibition (38, 39) of aldosterone secretion in response to ouabain. The varying doses of ouabain used, the possibility of signaling pathways independent of effects of NKA inhibition of membrane ion gradients (see below), and the species-dependent resistance of NKA to ouabain inhibition complicate interpretation of these findings.

Additional difficulties in performing and interpreting experiments to study CS regulation may arise from accumulation of sodium pump inhibitor in cell culture medium in response to stimulation. Because adrenocortical cells contain NKA binding sites for CS, the resulting inhibition of NKA may result in feedback regulation of CS synthesis. Using an approach that reduces this problem, Hinson and co-workers (8) investigated the effect of ACTH on release of ouabain-like CS from the intact isolated rat adrenal, perfused *in situ*. Basal corticosterone level was generally higher than CS, whereas aldosterone was generally lower. In three of the five perfusions, the addition of ACTH was followed by rapid increases in both CS and corticosterone secretion rates within 10 min of stimulation. Stimulated levels of CS were 2- to 4-fold and of corticosterone were 3- to 7-fold those found in basal samples. CS was found to co-elute with plant ouabain under isocratic HPLC analysis, whereas perfusion medium itself contained no detectable CS immunoreactivity.

Recent work has added to a growing body of evidence that adrenocortical production of CS is subject to regulation by pathways including those already shown to influence adrenal steroidogenesis. Indeed, adrenocortical steroids with opposing effects on NKA function (i.e., aldosterone and CS) may have mutually opposing influences on their production. Much remains to be learned about the adrenocortical biosynthesis of CS, and considerable effort will be required to elucidate the precise sequence of events in mammals CS biosynthesis, the subcellular locations and identities of the critical enzymes, and the points at which regulatory signals impinge to control production and release.

CS, Sodium Excretion, and Blood Pressure

In a recent summary, Aperia (40) concluded that the renal tubular NKA comprises a final site for regulation of renal sodium transport by many factors, including angiotensin II, norepinephrine, dopamine, and atrial natriuretic peptide. A shift in the balance between these forces may lead to salt retention and hypertension. An endogenous CS sodium pump inhibitor must also fit into this scheme. Its unique attribute is that inhibition of NKA in the kidney may facilitate renal sodium excretion while the broader endocrine effects of an endogenous CS NKA inhibitor on sodium pumps in the vascular system may contribute to increased vascular contraction and elevated blood pressure. This puts CS in the position of being able to transduce defective renal sodium excretion into a signal generating elevated blood pressure. This activates the pressure-natriuresis mechanism that is the final determinant of long-term blood pressure control (41).

Evidence that ouabain is able to influence renal sodium excretion is controversial. Manunta and co-workers (42) studied plasma levels of ouabain-like factor during acute and chronic changes in sodium balance in 138 patients with essential hypertension who underwent an acute volume expansion/contraction maneuver (2 days) and 20 patients who entered a blind randomized crossover design involving chronically controlled sodium intake and depletion (170 to 70 m*M*/day; 2 weeks each period). In both studies, plasma levels of ouabain-like factor were higher during sodium depletion. No significant change in plasma ouabain-like factor was observed after a 2-hr saline infusion or controlled sodium. When patients were divided into salt-sensitive or salt-resistant groups, no differences in plasma ouabain-like factor were observed in the two groups at baseline; however, circulating ouabain-like factor was increased by sodium depletion in both groups. These new results suggest that ouabain-like factor is involved in the adaptation of humans to sodium depletion and argue against the hypothesis that ouabain-like factor is a natriuretic hormone.

Yates and co-workers proposed (43) that endogenously produced ouabain has a role in the regulation of sodium excretion in certain physiological situations. They have shown that renal arterial infusion of aldosterone (3 μg/hr) reveals a natriuretic response to plant ouabain that was absent in animals that did not receive aldosterone. Two and 6 days after commencement of aldosterone infusion, ouabain was infused into the renal artery for 60 min. Ouabain infusion after 40 hr of aldosterone treatment increased sodium excretion more than 15-fold in the 2nd hr after cessation of ouabain infusion. Ouabain infusion after 6 days of aldosterone treatment increased sodium excretion to a similar high level. Aldosterone treatment produced a significant increase in mean arterial pressure after 6 days, but not 2 days. At this time point, the natriuresis occurring in response to intrarenal ouabain infusion was accompanied by a significant lowering of blood pressure. These results suggest that the relevance of endogenous digitalis-like factor in the physiological control of sodium homeostasis may be increased in conditions of chronic renal sodium retention and volume expansion.

Ouabain-like activity in the third ventricle region of the hypothalamus may also be involved in the sympathoexcitatory and pressor responses to a high-sodium diet in Dahl salt-sensitive (DS) (44, 45) and spontaneously hypertensive (SHR) (46, 47) rats. Introduction of ouabain into the cerebral ventricles of rats increases blood pressure and is accompanied by a rise in sympathetic nervous activity. These

responses can be prevented by the intracranial administration of Fab fragments of digoxin antibody (Digibind), which presumably act by binding ouabain through a crossreaction (15, 48–50). The mechanisms regulating the biosynthesis, release, and inactivation of brain "ouabain" remain unknown.

The role of CS-like factors in renal sodium handling in Dahl model of hypertension has been studied by Bagrov and co-workers (51, 52). The genetically determined hypertension that develops in DS on a high NaCl intake is believed to be due several factors. One of them is mutation of α-1 subunit of NKA (53). Due to this mutation, the DS sodium pump exhibits an abnormal Na/K coupling ratio (2:2 vs 3:2) that results in the impairment of pressure-natriuresis and the inability of DS to fully accommodate the excess intake of NaCl. Experiments were performed in which MBG and ouabain sensitivity of renal NKA (measured as the ability of various doses of ouabain and MBG to inhibit NKA ATP hydrolysis by renal medullary microsomal preparations) in Wistar, DS, and Dahl salt-resistant (DR) rats was studied (51). MBG exhibited a greater overall potency of inhibition than ouabain. There was a greater NKA inhibitory effect of MBG in the membranes from Wistar rats. In DS, the effects of MBG were less than in Wistar rats, but greater than in DR. The authors concluded that the higher sensitivity of renal NKA to MBG in DS comprises an adaptive mechanism targeted toward compensation of impaired properties of the sodium pump. Inhibition of renal NKA by MBG occurs over 6 or 7 log orders of magnitude, yet appears to occur through a single substrate, the α1 subunit of NKA. Additional work will be required to explain the unusual inhibition kinetics observed.

The effects of acute sodium loading on tissue and plasma levels and on urinary excretion of MBG and ouabain-like compound (OLC) were studied (51). MBG and OLC were crude preparations, defined as the material present in C18 sample preperation cartridge extracts crossreacting with MBG and ouabain antisera, respectively. Following acute NaCl loading, adrenal and plasma OLC exhibited 40% increases within 1 hr, and then decreased to baseline levels within 8 hr. These responses did not differ between DS and DR. Plasma and tissue levels of MBG doubled in both strains within 1 hr of NaCl loading, but in contrast to OLC, they remained elevated for the duration of the experiment. The total 8-hr urinary MBG excretion in DS was 4-fold greater than in DR, whereas OLC excretion in DS was only 30% greater than in DR. A weaker natriuretic response observed in DS than in DR, in spite of a greater renal excretion of MBG, suggests a blunted renal response of DS to MBG, perhaps related to the mutant renal α-1 sodium pump in DS. Recently Oda *et al.* (54) showed that material crossreactive with another vertebrate CS, bufalin, was increased in serum of DS rats during a period of high-salt diet, whereas the serum levels of healthy volunteers were significantly correlated with their systolic blood pressure.

Endogenous Sodium Pump Inhibitor in Preeclampsia

Preeclampsia is an example of a rapidly developing, volume-dependent and sodium-sensitive hypertension. In several aspects of its pathogenesis (decreased endothelial nitric oxide production, elevated levels of endothelin-1 and CS, and relatively low plasma levels of angiotensin II), preeclampsia resembles hypertension occurring in DS on a high NaCl intake. Clinical and experimental studies demonstrate that pregnancy is associated with dysregulation of NKA function (55). Reductions in α NKA isoform number or alterations in NKA distribution in preeclampsia may contribute the elevated cell sodium reported (55), which may in turn increase pressor sensitivity or smooth muscle tone directly and thereby contribute to hypertension in preeclampsia. Several studies have found elevated plasma of levels of digoxin-like immunoreactivity in pregnant subjects (56–59). Digibind, a commercially available digoxin antibody Fab fragment used to treat digitalis poisoning, lowered the blood pressure in 26 patients with preeclampsia (60). Lopatin and co-workers (61) have reported the levels of two CS immunoreactivities in preeclamptic plasma. Substantial increases in plasma ouabain-like and MBG-like immunoreactivity in preeclampsia were observed. Taken together with the reported vasoconstrictor properties of authentic MBG (61, 62) and NKA inhibitory activity of human MBG immunoreactive factor purified from preeclamptic plasma (61), this evidence suggests that plasma concentrations of MBG in preeclampsia are enough to substantially inhibit the sodium pump in cardiovascular tissues.

CS: A Hormone Involved in Signal Transduction?

New functions of NKA have begun to emerge that go well beyond its established role in maintaining the normal gradients of $[Na^+]$ and $[K^+]$ across the cell membrane. Several recent studies suggest that NKA may also act as a signal transducer and transcription activator involved in cell growth and differentiation. Research in this area has recently shown that concentrations of CS, causing only partial NKA inhibition, activate multiple interrelated signal pathways.

NKA establishes the plasma membrane potential, whereas pump inhibition leads to depolarization and subsequent activation of voltage-gated Ca^{2+} channels that initiate Ca^{2+} influx and Ca^{2+}-induced Ca^{2+} release (CICR). Recently, Aizman and colleagues (63) have reported studies in renal epithelial cells showing that the plant CS ouabain, in doses causing only partial NKA inhibition, acts as a biological inducer of regular, low-frequency intracellular calcium ($[Ca^{2+}]_i$) oscillations that elicit activation of transcriptional factor, NF-κB. Because low K^+-mediated NKA inhibition did not bring about $[Ca^{2+}]_i$ oscillations, it seems that ouabain-induced $[Ca^{2+}]_i$ oscillations are not a primary result of pump inhibition. Accordingly, these observations suggest

a more direct, ligand-specific effect on NKA-dependent Ca^{2+} signaling. The results implicate inositol triphosphate receptor (IP3) in ouabain-induced Ca^{2+} signaling, but the mechanism by which NKA and IP3 receptor communicate is unclear. Because of the close spatial relation between IP3 receptor in endoplasmic reticulum and NKA in plasma membrane, one scenario proposed involves their physical interaction through the cytoskeleton. Another possibility is that ouabain binding to NKA stimulates a signal cascade that leads to activation of phospholipase C and subsequent generation of IP3. Such a scenario has been suggested to occur in ouabain-treated cardiac myocytes (64).

Kometiani and co-workers (65) have studied signal transduction pathways linking NKA to four growth-related genes in cardiac myocytes. They have shown that the signal pathways initiated by the partial inhibition of cardiac NKA by ouabain are totally dependent on extracellular Ca^{2+} and all are prevented by a Ca^{2+}/calmodulin antagonist or a protein kinase C (PKC) inhibitor, which provides evidence of role of Ca^{2+}/calmodulin and PKC in these ouabain-initiated pathways (65). Interestingly, signal pathways linked to sarcolemmal NKA shared early elements involving Ca^{2+} and PKC, but then diverged into multiple branches involving Ras, p42/44 mitogen-activated protein kinases (MAPKs), or both. Evidence of a Ca^{2+}-dependent activation of PKC by ouabain in neonatal rat cardiac myocytes and its role in epidermal growth factor receptor (EGFR) regulation and activation of the Ras/ERK1/2 cascade has also been generated (64). Ouabain-induced proliferation of vascular smooth muscle cells via a signaling cascade involving Src, EGFR, and MAPK42/44 was also demonstrated (66).

Ouabain stimulates at least two important signal pathways downstream from Ras: one leads to the activation of MAPKs (64); the other increases production of reactive oxygen species (ROS) (67). Both may play a role in regulation of cell growth and gene expression in neonatal cardiac myocytes (68). Several of the early signaling events may be independent of ouabain-induced changes in intracellular ion concentration (67). Stimulation of ROS generation in cardiac myocytes may result from signals generated at the plasma membrane that follow from induction by ouabain binding of transient interactions between NKA and neighboring proteins leading to generation of ROS. The question again arises whether the ouabain-induced increase in $[Ca^{2+}]_i$ in cardiac myocytes can result from a signal transduction function of NKA that does not depend on altered NKA ion transport. Early studies (69) had suggested that the effects of ouabain on $[Ca^{2+}]_i$ may involve not only the Na/Ca exchanger, but other mechanisms regulated by ouabain-activated protein kinases stimulated through an ion transport-independent signal transduction function of NKA. Tian (70) has demonstrated a relationship between this NKA signal transduction function and the effect of ouabain on $[Ca^{2+}]_i$ and has provided evidence that inhibition of ouabain activation of the Ras/MAPK cascade, but not ROS generation, is necessary for ouabain-induced $[Ca^{2+}]_i$ increases in rat cardiac myocytes.

To date, there remains an incomplete picture of the signal transduction pathway activated by the ouabain-liganded NKA complex, in particular, the subsequent steps initiated after NKA-ligand interaction must be elucidated to determine if they depend solely on changes in calcium signaling attributable to altered ion flux through NKA or whether interaction of liganded NKA with other signaling molecules occurs. In summary, the findings in this field show that partial inhibition of NKA activates multiple signaling pathways that regulate muscle cell growth, proliferation, and differentiation and that there is evidence that some of this signaling derives from an action of ouabain-liganded NKA acting as a signal transduction molecule and is not directly dependent on alter ion transport by NKA. Additional studies on this novel role for NKA as a plasma membrane-bound steroid receptor involved in signal transduction are needed.

CS, Cancer, and Apoptosis

In the 1960s, *in vitro* inhibition of malignant cells by cardiac glycosides was reported and since then, several anti-cancer effects of digitalis have been observed (71, 72). In 1979, observations of the morphology of breast cancer cells from patients on digitalis suggested more benign characteristics than cancer cells from control patients not on CS therapy (73, 74). The 5-year recurrence after mastectomy among patients not taking digitalis was 9.6 times more than in patients taking digitalis (75). A long-term follow-up (22.3 years) of 175 breast cancer patients, of which 32 were on digitalis treatment, have again indicated a lower death rate (6%) among the patients on digitalis compared with patients not on digitalis (34%) (76).

Recently, digitalis and other CS in nontoxic concentrations have been shown to induce apoptosis in different malignant cell lines *in vitro* (72, 77, 78). The cardiac glycosides oleandrin, ouabain, and digoxin induce apoptosis in androgen-independent human prostate cancer cell lines *in vitro* (78). Single-cell imaging of intracellular Ca^{2+} fluxes demonstrated that the proapoptotic effects of the cardiac glycosides were linked to their abilities to induce sustained $[Ca^{2+}]_i$ increases in the cells. A vertebrate CS, bufalin, induced typical apoptosis in human leukemia U937 cells (79). Bufalin was also shown to have growth inhibitory and differentiation-inducing activities on leukemia cells (80–82), malignant melanoma cells (80), and human skin squamous-cell carcinoma cells *in vitro* (83). The mechanism of the anti-cancer effects of CS is not well established. The role of sustained $[Ca^{2+}]_i$ increases in the cells (78), persistent activation of the MAPK pathway (79), altered of expression of c-myc and bcl-2 genes involved in apoptosis (84–88), and activation of Rac1, p21-activated kinase, and c-Jun NH2-terminal kinase pathway (85, 89) have all been considered as potentially involved. The increase in cytosolic Ca^{2+} caused by cardiac glycosides might, in part, explain the

anti-tumor effects of CS in cancer patients (78). Ca^{2+} has a key role in the apoptotic process. Intracellular or extracellular Ca^{2+} chelators, Ca^{2+} channel blockers, and calmodulin antagonists can all delay or abolish apoptosis in several model systems.

It has been suggested that the activation of the ERK cascade is an event necessary for bufalin-mediated apoptosis (79, 90). ERK activation has also been shown to play a critical role in the induction of differentiation of human monocytic leukemia THP-1 cells by bufalin. P38 MAPKs and their downstream mediators may modulate ERK activity and eventually cell differentiation (91). Bufalin-mediated cell differentiation and apoptosis responses are interlinked with distinct PKC isoenzymes directing cell differentiation and apoptosis, respectively. These pathways might be coupled to determine the fate of the cell in response to CS (92). The recent work showing that ouabain can function as a physiological inducer of regular, low-frequency intracellular calcium $[Ca^{2+}]_i$ oscillations that elicit activation of the transcription factor, NF-κB, known to direct transcription for a large number of genes involved in regulation of cell growth, transcription factors, cytokines, and cell surface receptors (63) may explain some of the actions of CS on cell division and growth.

Summary

Recent work has added insight into the chemical identity of possible endogenous CS. The range of biological function in which endogenous CS may participate continues to extend from the whole animal level down to subcellular signaling pathways. Important issues remain to be resolved. These include whether functions of CS are specific to the isoforms of the heterodimeric (multiple α and β subunits) or heterotrimeric (α and β subunits integrating the γ subunit) physical states of the sodium pump; the identity, source, biosynthetic pathway, and regulation of CS; the role of endocrine, paracrine, and autocrine functions in CS biology; and refinement of insight into the cellular signaling pathways activated by CS and the implications of these pathways for cellular biology.

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