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Invited Review

Endogenous and exogenous cardiac glycosides: their roles in hypertension, salt metabolism, and cell growth

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Schoner W, Scheiner-Bobis G. Endogenous and exogenous cardiac glycosides: their roles in hypertension, salt metabolism, and cell growth. *Am J Physiol Cell Physiol* 293: C509–C536, 2007. First published May 9, 2007; doi:10.1152/ajpcell.00098.2007.—Cardiotonic steroids (CTS), long used to treat heart failure, are endogenously produced in mammals. Among them are the hydrophilic cardenolide ouabain and the more hydrophobic cardenolide digoxin, as well as the bufadienolides marinobufagenin and telecinobufagin. The physiological effects of endogenous ouabain on blood pressure and cardiac activity are consistent with the "Na^+-lag" hypothesis. This hypothesis assumes that, in cardiac and arterial myocytes, a CTS-induced local increase of Na^+ concentration due to inhibition of Na^+/K^+-ATPase leads to an increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) via a backward-running Na^+/Ca^{2+} exchanger. The increase in $[Ca^{2+}]_i$ then activates muscle contraction. The Na^+-lag hypothesis may best explain short-term and inotropic actions of CTS. Yet all data on the CTS-induced alteration of gene expression are consistent with another hypothesis, based on the Na^+/K^+-ATPase "signalosome," that describes the interaction of cardiac glycosides with the Na^+ pump as machinery activating various signaling pathways via intramembrane and cytosolic protein-protein interactions. These pathways, which may be activated simultaneously or selectively, elevate $[Ca^{2+}]_i$, activate Src and the ERK1/2 kinase pathways, and activate phosphoinositide 3-kinase and protein kinase B (Akt), NF-κB, and reactive oxygen species. A recent development indicates that new pharmaceuticals with antihypertensive and anticancer activities may be found among CTS and their derivatives: the antihypertensive rostafuroxin suppresses Na^+ resorption and the Src-epidermal growth factor receptor-ERK pathway in kidney tubule cells. It may be the parent compound of a new principle of antihypertensive therapy. Bufalin and oleandrin or the cardenolide analog UNBS-1450 block tumor cell proliferation and induce apoptosis at low concentrations in tumors with constitutive activation of NF-κB.

endogenous cardiotonic steroids; ouabain; marinobufagenin; rostafuroxin; bufalin; oleandrin; sodium pump; sodium/potassium-adenosinetriphosphatase; arterial hypertension; sodium metabolism; cell proliferation; cancer therapy

CHRONIC HEART FAILURE is a major public health problem that occurs with a greatly increased incidence in advanced age. A main reason for the development of this disease is the adaptation of the myocardium to arterial hypertension and, to a lesser extent, coronary artery disease. The incidence of heart failure correlates with the increase of heart rate (250). Cardiac, as well as arterial, myocytes respond to the constant exposure to increased concentrations of epinephrine and other hypertension-producing hormones by a process of molecular remodeling of the heart and arterial smooth muscle cells (315). This process of adaptation to a constant stress includes internalization of β-adrenergic receptors, uncoupling of the intracellular signaling pathway by altered expression of G proteins, lowered expression of K^+ channels and ryanodine receptors (RyRs), and increased expression of the Na^+/Ca^{2+} exchanger, events resulting in lowered intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Additionally, the expression of proteins of the contractile system is induced, resulting in increased muscle mass and reorganization of the myofilaments (29, 243, 315, 365). Eventually, a cardioinflammatory response due to hemodynamic overload leads to heart failure via increased myocardial cytokine production (interleukins and TNF-α) and apoptotic processes (95, 251, 291). Consequently, modern concepts of heart failure therapy focus on the protection against these stress-induced alterations (16, 29, 75).

Heart failure therapy with cardiac glycosides, however, is based on increasing cardiac output (40). It is mostly explained by the Na^+-lag hypothesis (37), which postulates that inhibition of myocardial Na^+/K^+-ATPase activity by cardiotonic steroids (CTS) leads to a local rise of intracellular Na^+ concentration ($[Na^+]_i$), which in turn increases $[Ca^{2+}]_i$ and, thereby, results in a positive inotropic action on the cardiac muscle. This therapeutic concept seems to contradict modern concepts of heart failure therapy that are based on avoiding a destructive rise of $[Ca^{2+}]_i$ that leads to further heart failure via altered protein expression and apoptosis. Yet one may not exclude that digoxin, the prominent cardiac glycoside used in

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therapy, also acts indirectly on the failing heart by depressing the activated neuroendocrine system and, hence, the adrenergic and renin-angiotensin system (109). Thus both therapeutic approaches may protect against stress. This apparently contradictory role of $[Ca^{2+}]_i$ in the two therapeutic approaches merits further investigation, especially since a recent clinical post hoc reanalysis of the beneficial effects of digitalis therapy (4, 5) contradicts the report of the Digitalis Investigation Group (360) and reestablishes digoxin as an important remedy in the treatment of heart failure (42). According to this study, treatment with low digoxin concentrations significantly reduces mortality and hospitalizations in patients with ambulatory chronic systolic and diastolic heart failure (4, 5).

The impressive benefit of digitalis therapy in the last century led to the postulate that an "endogenous digitalis" might exist (312, 354). Such endogenous inhibitors of the Na^+ pump might circulate in essential hypertension in higher concentrations in blood plasma and induce natriuresis and, thereby, decrease the fluid volume (35, 53, 126). Following this concept, Hamlyn et al. (131) were the first to demonstrate that an endogenous inhibitor of the Na^+ pump circulates in human blood plasma and that its concentration correlates with the blood pressure of the donors. This finding was soon confirmed (262). These observations paved the way for the identification of a number of endogenous CTS as a new type of steroid hormone belonging to the group of cardenolides and bufadienolides (Fig. 1). The Na^+ pump, which exists in all cells, acts as a hormone receptor for these substances. Consequently, endogenous CTS may control not only cardiac and kidney function, salt metabolism, and hypertension but, also, cell proliferation, cell half-life, and, more generally, cell function. Hence, this review discusses *1*) the chemical nature of endogenous cardiac glycosides, *2*) the mechanism of signal transduction via the Na^+ pump, *3*) the regulatory role of endogenous and exogenous cardiac glycosides in tissue proliferation and apoptosis, especially in cancer cells, *4*) the physiology and pathophysiology of endogenous cardiac glycosides in the circulatory system and its role in hypertension, and *5*) endogenous cardiac glycosides in diabetes mellitus.

CHEMICAL NATURE OF ENDOGENOUS CARDIAC GLYCOSIDES

It has long been known that certain vertebrates, such as amphibians, synthesize CTS with five- or six-member lactone rings (145, 308). Hence, it is not too astonishing that different CTS have been purified from mammalian fluids and tissues and structurally identified (Fig. 1).

Endogenous Ouabain

Endogenous ouabain has been isolated from human plasma (127, 247), bovine adrenal gland (333), bovine hypothalamus (177), and the supernatant of rat pheochromocytoma (PC-12) cells (193). Endogenous ouabain has been shown by ^{1}H-NMR (177, 333) and liquid chromatography-electrospray ionization-mass spectrometry (193) to be identical to the plant-derived

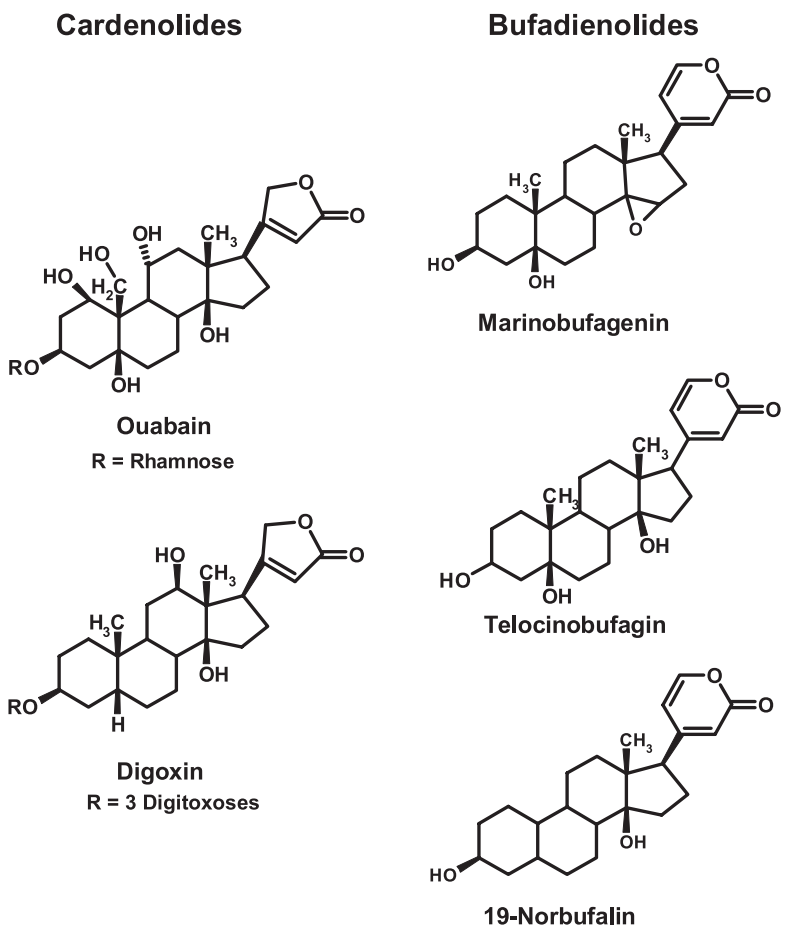


Fig. 1. Structures of the endogenous cardiac glycosides which were identified on the search for "endogenous digitalis" from mammalian tissues and fluids.

steroid. However, there is some speculation that an 11β-isomer of ouabain may circulate in human blood (128). Ouabain is able to form complexes under physiological conditions, for instance, with borate. This may explain some reports of an increased sensitivity of some preparations of endogenous ouabain compared with ouabain alone (12, 177, 178).

Adrenal gland as a source of ouabain. Orally and parenterally administered ouabain is selectively taken up by adrenal glands (187), but its intestinal uptake accounts for only 3–5% of the orally administered substance (122). Hence, it is essential to exclude the possibility that ouabain isolated from mammalian tissues does not simply represent resorbed ouabain from food. Conscious dogs release ouabain from their adrenal glands (38). Consistent with the assumption that the adrenal gland is a site of synthesis and/or storage of ouabain, adrenalectomy leads to a decline of ouabain levels in plasma (127, 246). Most likely, the zona glomerulosa and zona fasciculata of the adrenal cortex store and/or synthesize endogenous ouabain (203). The adrenal cortex contains more ouabain than the medulla (214), and plasma concentrations of ouabain have not been shown to be lower in medullectomized rats than in sham-operated controls (129). Overproduction of ouabain by adrenal tumors has been reported, and excision of these tumors lowered the elevated blood pressure (194, 233).

Biosynthesis of ouabain in adrenal and hypothalamic cells. De novo synthesis of ouabain and dihydroouabain has been demonstrated in tissue culture experiments (294, 301). The amount of ouabain secreted by bovine adrenocortical cells in vitro is up to 10-fold greater than the cellular content (69, 202, 294). The biosynthesis occurs in zona fasciculata cells. Pregnenolone and progesterone are precursors of endogenous ouabain (129, 193, 294). Inhibition of the conversion of pregnenolone to progesterone by trilostane, an inhibitor of 3β-hydroxysteroid dehydrogenase, inhibits ouabain synthesis (294). When [7-^{3}H]pregnenolone is added to primary cultures of rat adrenal cells, radioactivity is found in a fraction that has digitalis-like activity but does not contain ouabain (218). It is possible that the mechanism leading to 5-hydroxylation in ouabain (but not in digoxin) and the A/B conformation of the steroid backbone eliminates the ^{3}H atom in position 7. The sugar [^{14}C]rhamnose, which is part of the ouabain molecule, is synthesized in mammals (230), readily enters adrenocortical cells, and increases the biosynthesis of endogenous ouabain (294).

Since ouabain is synthesized by bovine adrenocortical cells in tissue culture, one may ask how its release is controlled hormonally. ACTH, α_1-adrenergic receptor agonists, and angiotensin II stimulate ouabain's release from bovine adrenocortical cells (203–205). Yet the release of ouabain from human CLR7050 cells (an adrenal cortex-derived cell line) is insensitive to ACTH and angiotensin II but sensitive to arginine vasopressin and phenylephrine (205). In bovine adrenocortical cells, angiotensin II acts via the angiotensin type 2 (AT_2) receptor, since the AT_2 receptor agonist CGP-42112 stimulates the release of ouabain and the AT_2 receptor antagonist PD-123319 inhibits it (203). The phenylephrine-dependent release of ouabain from human CRL7050 and bovine adrenocortical cells in culture is blocked by the α_1-adrenergic receptor antagonist doxazosin. This was interpreted to indicate that the sympathetic nervous system is involved in regulation of the release of this hormone to the bloodstream (204). In fact, evidence has been obtained in studies with salt-hypertensive rats that high Na^+ concentrations in plasma and cerebrospinal fluid may stimulate the release of endogenous ouabain via an Na^+ sensor (143) or epithelial Na^+ channels (ENaC) in the brain (376). This may then lead to a sympathetic hyperactivity and an elevation of brain angiotensin levels (87, 146).

When endogenous ouabain can be isolated from the hypothalamus (177), it may be synthesized there. A marked upregulation of genes coding for *P*-450 side-chain cleavage of cholesterol and Δ^5,3β-hydroxysteroid dehydrogenase/Δ^5,β_4-isomerase (catalyzing the synthesis of progesterone from pregnenolone) was seen in the hypothalamus of hypertensive compared with normotensive Milan rats, but not in adrenal cells. Knockdown of the latter enzyme decreased the production of endogenous ouabain in neural tissue (270).

Endogenous Digoxin

There is much evidence that mammalian cells synthesize digoxin as well. A substance indistinguishable from digoxin was isolated from human urine and identified with fast atom bombardment-mass spectrometry, proton NMR, and several different HPLC systems (116). Deglycosylated and reduced forms of the immunoreactive digoxin-like factor were identified in bovine adrenal gland (118, 302). It was found in blood plasma, urine, adrenal gland, and breast cyst fluid (117, 334). Since digoxin is taken up from the gut at a much higher rate than ouabain, demonstration of digoxin's biosynthesis is a prerequisite to acceptance of the physiological role of this CTS. Qazzaz et al. (300) demonstrated that Y-1 murine adrenocortical tumor cells can use [1,2-^{14}C]acetate and [4-^{14}C]cholesterol as precursors for the synthesis of a [^{14}C]digoxin-like substance. Its synthesis from acetate was inhibited by the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor mevastatin. [7-^{3}H]pregnenolone seems to be a precursor for digoxin in bovine adrenal cells (218). Hence, the unsaturated lactone ring in digoxin is not formed from the isoprenoid side chain, a conclusion supported also by the finding that radioactivity from [26,27-^{3}H]25-cholesterol is not present in the CTS (218). Evidence for the existence of digitoxose sugars in mammals has been reported recently (303).

Endogenous Marinobufagenin and Other Bufadienolides

Marinobufagenin (3β,5β-dihydroxy-14,14-epoxybufadienolide; Fig. 1), originally discovered in amphibians, was isolated and identified from the urine of patients with myocardial infarction (18). Telocinobufagin, the reduced form of marinobufagenin, was identified, by high-resolution mass spectrometry and NMR, as a constituent of human plasma (192). Its plasma concentration is higher than that of marinobufagenin (192). The compound is synthesized from cholesterol in the adrenal cortex by a pathway that is independent of the cholesterol side-chain cleavage (65). 19-Norbufalin and its Thr-Gly-Ala tripeptide derivative were isolated from human cataractous lenses in an investigation of greater immunoreactivities against bufalin and ouabain in cataractous than in normal lenses (217).

Additional endogenous bufadienolides may circulate in mammalian blood. Sich et al. (343) demonstrated a correlation between the concentration of a proscillaridin A-immunoreactive substance with a polarity similar to that of ouabain with

systolic blood pressure. A bufalin-like immunoreactivity also correlated with systolic blood pressure (282).

MECHANISMS OF SIGNAL TRANSDUCTION OF CTS VIA THE SODIUM PUMP

CTS bind to Na^+/K^+-ATPase at a site formed in the extracellular part of the catalytic α-subunit by the H_1-H_2, H_3-H_4, and H_5-H_6 loops. Tentative three-dimensional structures of the cardiac glycoside binding site have been proposed (181, 304). Reversible interaction of CTS with this site may induce a conformational change of the Na^+/K^+-ATPase protein. In the actively pumping Na^+ pump, CTS are fixed by tight binding in the E_2 conformational state, a process leading to the enzyme's inactivation. Since the E_2-phosphoenzyme-CTS complex formed is almost irreversible (84), it is likely that, under cellular conditions, the ouabain-pump complex is internalized and degraded. K_d values of the CTS complexes vary with the form of the isozyme and the animal (33). Although K_d values of human α_1-, α_2-, and α_3-isoforms range from 10^{-8} to 10^{-9} mol/l (51, 265, 378), rodents exhibit an ouabain-insensitive α_1-isozyme (33). Since endogenous ouabain circulates in blood plasma of resting humans in the subnanomolar-to-nanomolar concentration range (132) and noninhibitory subnanomolar concentrations of ouabain stimulate the proliferation of smooth muscle (1, 17), endothelial (329), and kidney tubule cells in culture (66, 184), several mechanisms may transduce the docking of endogenous ouabain at the cell surface to the cell interior with or without inhibition of the Na^+ pump. Two different mechanisms of signal transduction have been proposed (see below).

Na^+-Lag Hypothesis and the PlasmERosome

The Na^+-lag hypothesis assumes that a partial inhibition of the Na^+ pump by cardiac glycosides leads to a transient increase of $[Na^+]_i$, which in turn increases $[Ca^{2+}]_i$ via the Na^+/Ca^{2+} exchange system (NCX1) running in a reverse mode (311). In cardiac and vascular smooth muscle cells, this exchanger is thought to contribute to Ca^{2+} extrusion from the cytosol in the relaxation process (36).

In smooth muscle cells, astrocytes, and hippocampal neurons of rodents, minimal inhibitory effects of CTS on the Na^+ pump seem to be amplified by a special arrangement of the α-isoforms in those cells (34, 37, 383). Immunochemical studies in rat arteries revealed that NCX1 and the ouabain-sensitive α_2- and α_3-isoforms (but not the rather insensitive α_1-isoform) of Na^+/K^+-ATPase reside in plasma membrane regions adjacent to the sarcoplasmic reticulum (SR) (113, 172, 173, 261, 411). The α_2-isoform of Na^+/K^+-ATPase is targeted and tethered to this site by Leu^{27} and Ala^{35} in its NH_2-terminal segment (346). This special arrangement may lead to a fortification of inhibitory effects of endogenous ouabain on α_2- and α_3-isozymes. In fact, enhanced Ca^{2+} transients evoked by the vasoconstrictors angiotensin II and phenylephrine have been recorded when the Na^+ pump was inhibited, and opening of store-operated Ca^{2+} channels led to coentry of Na^+ and Ca^{2+} (14). Since cytosolic Na^+ levels did not increase (15), a local transient rise of $[Na^+]_i$ may occur in a subplasmalemmal space known as the plasmERosome (14, 209). In addition, a local increase of $[Ca^{2+}]_i$ may occur via the NCX1. The transiently increased Ca^{2+} in the plasmERosome, as well as in the bulk cytoplasm, is thought to be taken up by the SR via Ca^{2+}-ATPase (SERCA). Because of the elevated Ca^{2+} content in the SR, more Ca^{2+} can be released when myocytes of vascular smooth muscle cells, astrocytes, or hippocampal neurons are stimulated by a rise of $[Ca^{2+}]_i$ in the plasmERosome. This occurs presumably via an activation of the inositol (1,4,5)-trisphosphate (IP_3) receptor (IP_3R) and leads to an augmented force of contraction (34, 37, 383) (Fig. 2). The specific subcellular localization of Na^+ pump isoforms and the NCX1 protein is consistent with this concept: mice with an ouabain-insensitive mutant α_2-isoform of the Na^+ pump failed to show cardiac inotropy upon ouabain treatment and did not develop ouabain-induced hypertension (71, 72). However, lowering of expression of the wild-type α_2-isoform increased blood pres-

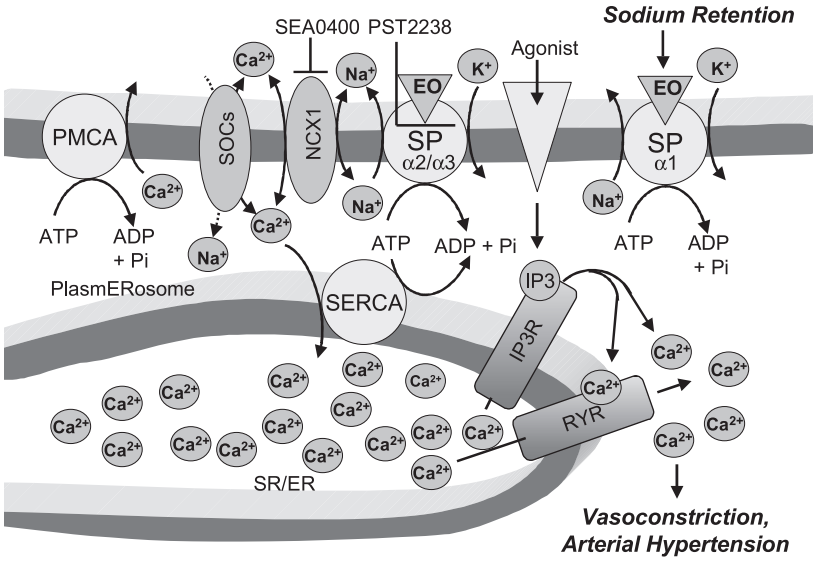


Fig. 2. Na^+-lag hypothesis of the action of endogenous ouabain (EO). The existence of a subplasmalemmal space, called the plasmERosome, is assumed. In this compartment, a transient local increase of intracellular Na^+ concentration ($[Na^+]_i$) caused by stimulation of store-operated channels (SOC) or by ouabain (O)-dependent inhibition of α_2- and α_3-subunits of the Na^+ pump (SP) may lead to a local rise of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). This signal is amplified by a greater amount of Ca^{2+} in the sarco/endoplasmic reticulum (SR/ER) from sarcoplasmic Ca^{2+}-ATPase (SERCA). Any agonist (angiotensin II or epinephrine)-stimulated Ca^{2+} release leads to an amplified Ca^{2+} release via the inositol trisphosphate receptor (IP_3R), which stimulates opening of the ryanodine Ca^{2+} channel (RyR) and, thus, results in a higher degree of vasoconstriction. In a noninhibited Na^+/K^+-ATPase, Ca^{2+} in the plasmERosome will be extruded by the Na^+/Ca^{2+} exchanger (NCX1) and the plasmalemmal Ca^{2+}-ATPase (PMCA). Inhibition of NCX1 by SEA-0400 will suppress the effect of endogenous ouabain, as does the ouabain antagonist PST-2238.

sure (411). The inhibition of Ca^{2+} entry via NCX1 by the inhibitor SEA-0400 lowers arterial blood pressure in salt-dependent hypertensive rat models. Heterozygous NCX1-deficient mice have low salt sensitivity. Transgenic mice with the NCX1.3 variant in their smooth muscle cells are salt hypersensitive (165, 166), but transgenic mice expressing the transgenic NCX1.1 are salt insensitive, and their blood pressure did not respond to SEA-0400 (166). These findings are consistent with Blaustein's plasmERosome hypothesis, which may explain, in part, the development of arterial hypertension at sustained elevated concentrations of endogenous ouabain. However, other mechanisms must exist as well, since, especially at subnanomolar ouabain concentrations, the Na^+ pump (23, 108, 329) and cell proliferation (1, 17, 329) are stimulated. Furthermore, there is no strict correlation between the hypertensive action of CTS and inhibition of the Na^+ pump (237).

In heart muscle cells, CTS may alter contractility in a different way. Consistent with the Na^+-lag hypothesis, a functional Na^+/Ca^{2+} exchanger seems to be necessary for an acute inotropic effect of cardiac glycosides (11, 36, 310). However, even though α_1-, α_2-, and α_3-isoforms of Na^+/K^+-ATPase were found (229), there is no evidence for a plasmERosome-like mechanism in heart muscle cells. Additionally, the Ca^{2+} release channel of the SR in the cardiac myocyte is the RyR (29, 260) and not, as in most other cell types, IP_3R type 2 (335). Moreover, and in contrast to a plasmERosome mechanism in the heart muscle, the α_1-isoform of Na^+/K^+-ATPase regulates cardiac contractility and functionally interacts and colocalizes with the Na^+/Ca^{2+} exchanger of the heart (73). The latter Na^+/K^+-ATPase isoform is significantly downregulated in human heart failure (267, 338, 340, 410). One should also bear in mind that, in humans, cardiac glycoside affinities of all α-isoforms of Na^+/K^+-ATPase are very similar (51, 265). Perhaps, in the heart muscle, no CTS-amplification mechanism is necessary to achieve inotropy. Consistent with such a conclusion is the observation that physical exercise of healthy men and dogs leads to a rise of endogenous ouabain within the therapeutic concentration range in blood (26).

Na^+/K^+-ATPase Signalosome

A large number of experiments support the hypothesis that Na^+ pump inhibition is not necessary for the inotropic effect of cardiac glycosides in the myocardium (276). Because of the pioneering work of Xie and colleagues (396, 397), we are now starting to understand how nano- and subnanomolar concentrations of endogenous and exogenous cardiac glycosides may lead to increased inotropy of cardiac muscle and to hypertension, proliferation, differentiation, and altered cell life span (37, 68, 108, 317, 327, 418) (Fig. 3, see Fig. 5). In contrast to the Na^+-lag hypothesis, the Na^+/K^+-ATPase signalosome uses all α-isoforms to transduce the information of ouabain's binding from the Na^+ pump to the cell interior and nucleus (7, 221, 223, 256, 329, 414). The signalosome is located in caveolar structures (56, 57, 98, 371, 397, 408) and may transfer signals to the cell interior, even when the pump is unable to work (216), and affect membrane recycling and trafficking, as well as cell-cell interactions (Fig. 3).

Na^+/K^+-ATPase complexes with membrane and cytoskeletal proteins. Caveolae are known to be associated with molecules crucial for Ca^{2+} handling. They contain proteins such as neuronal nitric oxide (NO) synthase, NCX1, plasma membrane Ca^{2+}-ATPase, and L-type Ca^{2+} channels and interact with peripheral proteins of the SR (56). A complex of PLC tethered to the IP_3 receptor of the endoplasmic reticulum and the NH_2-terminal end of the α-subunit of Na^+/K^+-ATPase has been demonstrated (256, 408, 414). Binding motifs for caveolin, ankyrin, and phosphoinositide 3′-kinase (PI3K) exist on the α-subunit of Na^+/K^+-ATPase (397, 409). A defined site of ankyrin (415) tethers to the attenuator and nucleotide binding loop of the cytosolic side of Na^+/K^+-ATPase (61, 171, 195). Ankyrin also interacts with the Na^+/Ca^{2+} exchanger, voltage-gated Na_v channel, anion exchanger, H^+/K^+-ATPase, and cell adhesion molecules, as well as RyR and IP_3R (259). Interaction of the Na^+ pump with the cytoskeletal proteins ankyrin, fodrin, and, probably, adducin seems to be essential for the proper localization in polarized cells (263, 273), as well as for the

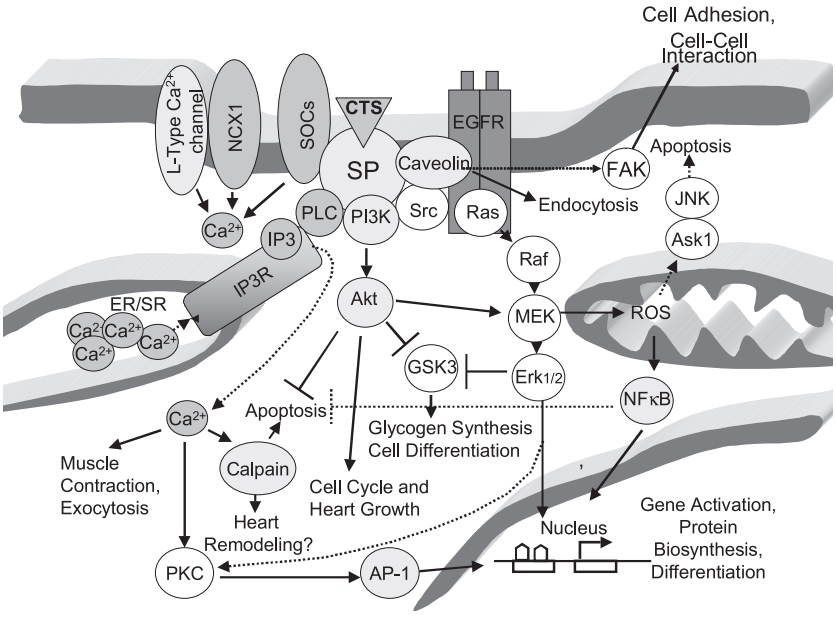


Fig. 3. Na^+/K^+-ATPase acting as a signalosome. Signaling reactions in various cells triggered by the interaction of ouabain with the Na^+ pump are shown. Endogenous or exogenous cardiotonic steroids (CTS) affect the processes in various signal transducing pathways. AP-1, activator protein-1; ASK-1, apoptosis signal-regulating kinase-1; FAK, focal adhesion kinase; GSK-3, glycogen synthase kinase-3; IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; MEK, mitogen-activated ERK-activating kinase (an MAPK kinase); NCX1, Na^+/Ca^{2+} exchanger; PI3K, phosphoinositide 3′-kinase; Raf, an MAPK kinase kinase kinase; Ras, an MAPK kinase kinase; ROS, reactive oxygen species; Src, sarcoma kinase; EGFR, epithelial growth factor receptor.

trafficking of Na^+/K^+-ATPase from the Golgi to the cell surface (62). It is not known, however, whether all possible interactions of Na^+/K^+-ATPase with membrane and cytoskeletal proteins occur at the same time or whether they are mutually exclusive. Since the cytoskeletal proteins ankyrin and adducin stimulate Na^+/K^+-ATPase activity (99), this ankyrin-Na^+/K^+-ATPase complex might stabilize a catalytically active Na^+ pump at the cell surface (393). In fact, a hypertensinogenic adducin variant leads to an increased number of Na^+ pumps in the basolateral membrane in transfected kidney tubule cells, probably because of a reduced endocytosis by an impaired phosphorylation-dephosphorylation cycle of adaptin-2 (AP2-μ2) (30). On the other hand, defects in ankyrin may lead to cardiac arrhythmias because of the loss of cellular targeting of Na^+/K^+-ATPase, Na^+/Ca^{2+} exchanger, and IP_3R (259). Internalization of the Na^+ pump is induced by ouabain and occurs via a caveolin- and clathrin-dependent mechanism (220, 221). It brings CTS tightly bound to the E_2 conformation of inactivated Na^+/K^+-ATPase (84) to the cell interior (78, 79). Intracellular CTS may then eventually activate cardiac RyRs (at EC_{50} ~0.2 nM ouabain) in cooperation with a 31.5-kDa protein to increase $[Ca^{2+}]_i$ (107, 325, 383). Cardiac glycosides have been found to alter the recycling of endocytosed membrane proteins and cargo (206, 317), as well as human ether-à-go-go-related gene cardiac K^+ channels (thereby prolonging the action potential) (381), and to stimulate exocytosis of endothelin-1 (ET-1) (329). This process is affected by $[Na^+]_i$ (163) and adrenergic hormones (208, 295). Thus, whether the activation of the Na^+ pump by subnanomolar concentrations of ouabain (23, 108, 329) is caused by an augmented incorporation of Na^+ pumps into the plasmalemma, an increased local rise of $[Na^+]_i$ activating the Na^+ pump, an altered interaction with cytoskeletal proteins, or a changed phosphorylation state of the NH_2-terminal sequence of the catalytic α-subunit that enhances Na^+ pump activity is an open question (361).

Ouabain-Na^+/K^+-ATPase complex in caveolae stimulates different pathways of signal transduction. Interaction of endogenous and exogenous cardiac glycosides with the cardiac glycoside receptor of Na^+/K^+-ATPase, even at nanomolar concentrations of the drug, may lead to conformational changes that are recognized by neighboring proteins (396). Both processes lead to the activation of various signal transduction pathways.

CALCIUM AS SECOND MESSENGER. Changes of $[Ca^{2+}]_i$ upon cardiac glycoside binding are well known (256, 327, 329, 363, 414). The rise of $[Ca^{2+}]_i$ caused by nanomolar concentrations of ouabain may result from a stimulation of T- and L-type currents (212), an increase of voltage-dependent Ca^{2+} influx (244), an activation of promiscuous Na^+ channels (327), or an activation of Ca^{2+} release channels of the SR (325). In cardiac myocytes, ouabain binding may lead to protein-protein interactions of the Na^+ pump with Na^+ channels, inducing the latter to become promiscuous in their ion selectivity and, thereby, allow Ca^{2+} influx from the extracellular fluid (327). This event may be connected to an activation of the α_2- and α_3-isoforms of the Na^+ pump (108). Clearly, the Na^+-lag hypothesis does not apply to such a condition (see above). Additional pathways can lead to a rise of $[Ca^{2+}]_i$. An ouabain-induced conformational change of the Na^+ pump may be transmitted via the α-subunit's NH_2-terminal end to the IP_3R of the endoplasmic reticulum/SR (256, 414). This complex also contains PLC-γ_1, the Src-dependent phosphorylation of which may increase IP_3 formation, which in turn enhances Ca^{2+} release from intracellular stores (257, 258, 408). Additionally, activation of PLC-γ_1 stimulates diacylglycerol formation and, thus, PKC activity (120). It may also affect, by phosphorylation, the activities of L-type voltage-dependent Ca^{2+} channels (82, 103, 269, 396), the Na^+/Ca^{2+} exchanger, and even the Na^+/K^+-ATPase itself (which may increase $[Ca^{2+}]_i$ further by Na^+ pump inhibition) (257, 363) (Figs. 2 and 3). Nanomolar concentrations of ouabain have been reported to induce Ca^{2+} oscillations in hippocampal astrocytes and kidney tubule cells, as well as endothelial cells, but toxic ouabain concentrations lead to constantly elevated $[Ca^{2+}]_i$ (7, 224, 329). $[Ca^{2+}]_i$ may, furthermore, increase as a result of ouabain exposure via the Na^+/K^+-ATPase-Src-epidermal growth factor receptor (EGFR) complex, which activates the Ras-MEK-ERK1/2 pathway. This pathway may lead to the activation and phosphorylation of Ca^{2+} channels and/or the Na^+/Ca^{2+} exchanger (257, 363) and, thereby, increase the heart's positive inotropy as well (257, 258) (Fig. 3). The frequency of Ca^{2+} oscillations seems to determine whether a cell undergoes proliferation or apoptosis (414). Low-frequency Ca^{2+} oscillations are known to improve the specificity and efficiency of gene expression (67). Ca^{2+} may stimulate protein biosynthesis via activation of PKC (160, 258, 292) and NF-κB (7, 256, 414) or, in the heart, by the loosening of cell-cell contacts and myofibril disassembly and, hence, promote cardiac remodeling via activation of calpain (105, 136, 207). Low concentrations of ouabain triggering low-frequency Ca^{2+} oscillations activate NF-κB and protect cells from apoptosis (414) (Fig. 3). Finally, activation of PKC and Ca^{2+}-calmodulin kinase may activate the expression of early-response genes, such as *c-fos* and *c-jun,* leading to formation of the transcription factor AP-1 (292). These events also may result in expression of late growth-related genes, such as skeletal α-actin, atrial natriuretic factor, myosin light chain 2, and transforming growth factor-β1, in the heart (160) and may differentially regulate the expression of the isoforms of the α- and β-subunits of Na^+/K^+-ATPase (159, 191, 377).

ACTIVATION OF PHOSPHATIDYLINOSITIDE 3′-KINASE AND AKT (PROTEIN KINASE B). The NH_2-terminal end of the catalytic α-subunit of Na^+/K^+-ATPase contains a binding motif for PI3K (397, 409). Its ouabain-induced conformational change was shown to activate cell proliferation in kidney proximal tubule cells via a Ca^{2+}-dependent phosphorylation of Akt (PKB) (184). In the heart, this conformational change may lead to cardiac hypertrophy (142) and metabolic alterations (83). Akt-1-knockout mice show a reduced heart size and body size and develop cardiac dilation and dysfunction. Activated Akt may induce hypertrophy and cardiac dysfunction over time in some models (142). Ouabain-induced activation of Akt was reported to show an antiapoptotic action (366). Hence, Akt plays an important role in ouabain-induced signal transduction.

CAVEOLIN-SUPPORTED PATHWAYS. In rat cardiac myocytes, 20–30% of cellular α_1- and α_2-isoforms of Na^+/K^+-ATPase are in caveolae, along with most of the cellular caveolin-3. Caveolin-3 and the α isoforms are located in peripheral sarcolemma and T tubules. Exposure of the contracting heart to ouabain led to a two- to threefold increase in activation/phosphorylation of ERK1/2 and a 50–60% increase in caveolar Src and the α_2-isoform of Na^+/K^+-ATPase, suggesting an

ouabain-induced recruitment of Src and α_2-isoforms to caveolae as a prerequisite for the manifestation of ouabain's inotropic response (223). Also, kidney tubule cells form an ouabain-induced Na^+/K^+-ATPase-EGFR-Src-caveolin-1 complex, leading to an increased tyrosine phosphorylation of caveolin-1 and the subsequent activation of the Ras-Raf-ERK cascade, with changes in $[Ca^{2+}]_i$ and gene activation, cell proliferation, and hypertrophy (98, 184). Abolition of ouabain-induced recruitment of Src and the depletion of cellular caveolin-1 blocked this cascade (371). Another pathway resulting in endocytosis and the formation of clathrin coats, as well as early and late endosomes, may be triggered by cardiac glycosides from this complex (221). The clathrin coat also contains AP-2, PI3K, and clathrin heavy chain (220, 221). Formation of a complex of PI3K with the proline-rich motif of the NH_2-terminal sequence of the Na^+ pump α-subunit and its phosphorylation at Ser^{11} and Ser^{18} by PKC [resulting in a critical conformational change (80)], as well as the binding of AP-2 to this NH_2-terminal domain, seems to regulate the trafficking of the Na^+ pump (46, 183, 409). Intracellular Na^+ modulates the phosphorylation of the α-subunit of Na^+/K^+-ATPase by PKC (163), which seems to counteract the internalization and activation of the Na^+ pump. This is also the case with the hypertension-linked mutation of adducin, which shows impaired trafficking in response to dopamine as a result of a higher phosphorylation state of the AP2-μ2 protein (81). A bufalin-induced stimulation of the formation of large vesicles adjacent to the nucleus (containing transferrin, low-density lipoprotein, and the Rab protein) was also seen in human NT2 (neuronal precursor) cells and interpreted to indicate stimulation of plasma membrane recycling (317). There have been reports that inhibition of endocytosis protects against the toxicity of cardiac glycosides (280) and that internalized hydrophobic cardiac glycosides may increase $[Ca^{2+}]_i$ and heart contractility via direct interaction with the RyR (281, 325).

SRC-EGFR-RAS-RAF-ERK CASCADE. Ouabain binding to the Na^+ pump stimulates Src kinase, which in turn phosphorylates the EGFR, leading to activation of the Ras-Raf-MEK-ERK pathway (Fig. 3). Fluorescence resonance energy transfer studies revealed a close proximity of Na^+/K^+-ATPase to Src at the plasma membrane (362). Binding of Src to Na^+/K^+-ATPase is independent of the Na^+ pump's catalytic activity (216). Binding of the SH2 domain of Src to cytosolic domains 2 and 3 of the Na^+/K^+-ATPase α-subunit inhibits Src activity if no CST is bound to the Na^+ pump (362). Apparently and as a consequence of an ouabain-induced conformational change of the Na^+ pump, Src is released from the Na^+/K^+-ATPase-Src complex and activated by phosphorylation at Tyr^{418} (124, 362). Consistent with an ouabain-induced release of Src from the Src-Na^+/K^+-ATPase complex, a knockout of the α_1-isoform of Na^+/K^+-ATPase in kidney epithelial LLC-PK1 cells led to an increased basal Src activity and also an increased tyrosine phosphorylation of focal adhesion kinase (216). As a consequence, dissociation of Src from the Src-Na^+/K^+-ATPase complex leads to an increased tyrosine phosphorylation of EGFR (which is not identical to epidermal growth factor-induced autophosphorylation at Tyr^{1173}) and to recruitment and phosphorylation of the adaptor protein Shc. This results in binding of the adaptor protein Grb2 to the Src-EGFR complex and, subsequently, activation of the p42/44 MAPK (123, 189). This signaling pathway seems to be activated with, as well as without, inhibition of the Na^+ pump. In cardiac A7r5 and kidney LLC-PK1 cells, inhibition of Na^+/K^+-ATPase by ouabain clearly correlated with ouabain-induced activation of Src and MAPK. Evidently, ouabain inhibition of α_1- and α_2-isoforms of Na^+/K^+-ATPase transactivates the EGFR and, subsequently, stimulates the Ras-MAPK cascade (124). Yet, in cultures of vascular and prostate smooth muscle cells as well as in renal epithelial cells, stimulation of proliferation and activation of MAPK and ERK1/2 by ouabain or marinobufagenin did not correlate with Na^+/K^+-ATPase inhibition (1, 17, 66, 112). This is important, because the Na^+/K^+-ATPase-Src complex is the only known receptor for ouabain and other CTS that stimulates the protein kinase cascades (216).

Ras activation leads to further activation of three different branches of the signal transduction cascades. One of the pathways communicates with the mitochondria: the activation of MAPK and increase in $[Ca^{2+}]_i$ result in opening of mitochondrial ATP-sensitive K^+ channels (364) and generation of mitochondrial reactive oxygen species (ROS) (363, 398). Both processes cooperate to increase cardiac muscular contraction (258, 363, 364). ROS subsequently activate NF-κB (256, 398) and slow $[Ca^{2+}]_i$ oscillations at nanomolar ouabain concentrations (7). NF-κB is involved as a transcription factor in processes related to growth, differentiation, and inflammatory responses (7) and inhibits apoptosis (111, 213, 366, 414) (Fig. 3). The second pathway, the Ras-Raf-MEK-ERK1/2 cascade (159, 189, 398), leads to gene activation when ERK1/2 is activated in cooperation with PLC in the presence of Ca^{2+} (257) and ROS (222). Interestingly, ERK1/2 controls surface expression of Na^+/K^+-ATPase in kidney tubule and muscle cells (10, 253). JNK might be a substrate of ERK (190), as well as the JNK kinase SEK1 (215). A third pathway of ouabain-dependent Ras activation in muscle cells results in activation of p90 ribosomal S6 kinase and inactivation of glycogen synthase kinase (GSK-3α/β) via activation of ERK1/2 and phosphorylation; this process stimulates glycogen synthesis (196) (Fig. 3). Since GSK-3 is a master switch regulating cell-fate specificity and tumorigenesis (185), inhibition or suppression of GSK-3 may also affect transcription factors and increase cardiac hypertrophy (133).

Ouabain, Na^+ pump, and cell-cell interaction. A number of bidirectional interactions between the cytoskeleton and the Na^+/K^+-ATPase signaling pathway have been reported (31, 48–50, 342). The c-Src, $p190^{Rho\text{-}GAP}$, and ERK1/2 signaling pathways are known to regulate cell adhesion through specific attachment molecules and to modify the interaction with the extracellular matrix (50). Stimulation of a complex signaling cascade by CTS may promote cell-cell communication by means of gap junctions in epithelial cells by specifically enhancing connexin32 expression (206). The opposite may occur, however, on blockade of the Na^+ pump: prolonged ouabain blockade of Na^+/K^+-ATPase in Madin-Darby canine kidney cells leads, via an increase in $[Ca^{2+}]_i$, to a detachment of cells from each other, an increase in MAPK activity, and a redistribution of molecules involved in cell attachment (occludin, ZO-1, desmoplakin, cytokeratin, α-actinin, vinculin, and actin). Inhibition of protein tyrosine kinases and MAPK kinase, respectively, blocks this detachment. The content of $p190^{Rho\text{-}GAP}$, a GTPase-activating protein of the Rho small G protein subfamily, is increased by ouabain (48), suggesting that the Rho/Rac and MAPK pathways are involved (50). However,

in the MA104 rhesus monkey epithelial cell line, in which ouabain binding to Na^+/K^+-ATPase is of high affinity (K_m $\sim 4 \times 10^{-8}$ M), blockade of the Na^+ pump failed to modify phosphorylation, as well as the pattern of distribution of associated molecules (50). Furthermore, other inhibitors of the Na^+ pump were also without effect (48). This was interpreted to mean that, in MA104 cells, the signaling sequence is faulty and, in normal cells, inhibition of the Na^+ pump affects their retrieval (perhaps in plasmalemma turnover) and, eventually, leads to a loss of cell-cell contact by β-β subunit interaction (110, 268, 332, 342) (Fig. 3).

EFFECT OF ENDOGENOUS AND EXOGENOUS CARDIAC GLYCOSIDES ON CELL PROLIFERATION AND DEATH OF NORMAL AND CANCER CELLS

It is evident from the literature cited above that CTS may influence cell proliferation, differentiation, and, eventually, cell death via the Na^+/K^+-ATPase signalosome pathways (Fig. 3). It is unclear whether endogenous cardiac glycosides may affect tumor growth. In a preliminary study, Weidemann (388) found lowered plasma concentrations of endogenous digitalis immunoreactivity in ~74% of patients with breast cancer but markedly increased concentrations in 10.8% of these patients. Since tumor cells may show abnormal Na^+/K^+-ATPase activity, which is explained by the "Na^+/K^+-leakage theory" (176, 388), clinical studies on the therapeutic effects of CTS seem reasonable. In fact, a recent clinical study by Stenkvist (349) reported that cardiac glycosides (digoxin and digitoxin) may have therapeutic effects on cancer: a group of 175 patients with breast cancer, 32 of which were on digitalis therapy when the disease was diagnosed, were studied over >22 yr. The death rate was significantly lower in patients treated with digitalis glycosides than in those not treated with digitalis (6% vs. 34%). In vitro studies show that a number of different cancer cell types are blocked in the G_2/M phase of the cell cycle (140, 249). Yet, in another careful study of 9,271 patients, Haux et al. (138) were unable to confirm Stenkvist's observation. However, fewer cases of leukemia were diagnosed in a group of patients treated for cardiac failure with digitoxin than in a control group of patients, who, after cancer diagnosis, required digitoxin treatment. There might be a cancer-protective trend for higher concentrations of digitoxin for lymphoma/leukemia and kidney/urinary organ cancers (138). Interestingly, studies with immune-compromised mice seem to indicate that failure of the adrenal glands to release endogenous digitalis-like immunoreactivity may facilitate the establishment of tumors (389). Bufalin derived from the toad skin has been used for several hundred years in traditional Chinese medicine to treat malignant diseases such as hepatocellular and hematologic cancers (169, 170, 404). Oleandrin, another hydrophobic cardenolide, is in phase I trials in the United States as an anticancer remedy for refractory human cancers (275). Since the therapeutic window for cardiac glycosides is very narrow (188), there is a need for cardiac glycoside derivatives without cardiotonic action, but with cytotoxic action, to overcome this problem. Fortunately, such compounds have been detected recently (Fig. 4, see Fig. 7) (54, 255, 368). One of these compounds, UNBS-1450, entered the candidate drug development stage of phase I clinical trials in Belgium in 2006 (255).

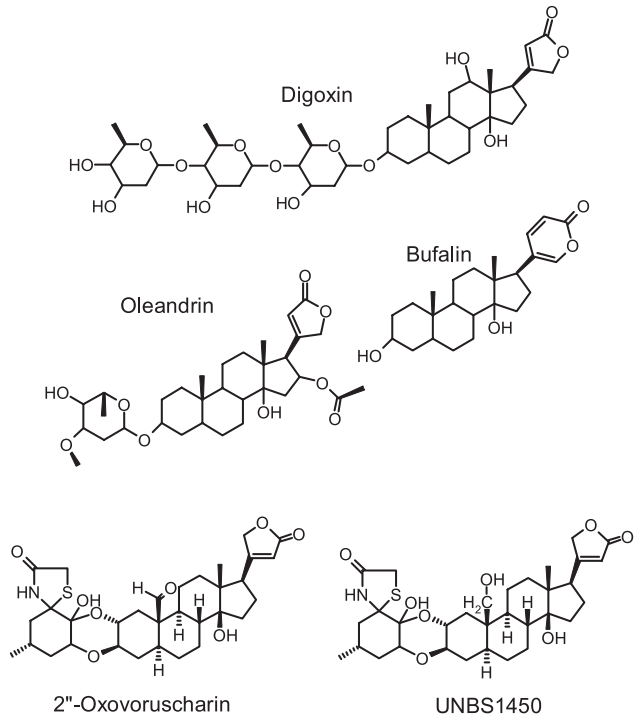


Fig. 4. Cardiac glycosides and their derivatives with anticancer action.

In Vitro Studies on Cytotoxicity of Cardiac Glycosides on Tumor Cells

In vitro observations that leukemia cells undergo apoptosis in the presence of bufalin and oleandrin, but normal leukocytes do not, are consistent with the hypothesis of a therapeutic effect of CTS on tumor growth (169, 179, 245, 348). An endogenous bufalin-like factor in human blood may induce differentiation of leukemia cells (277). Hence, studies on the effects of various CTS on normal and tumor cell growth are published more frequently. A survey of the data reveals that exogenous CTS in the nanomolar concentration range and, certainly, endogenous CTS as well may stimulate cell proliferation and differentiation (1, 17, 47, 98, 112, 184, 306, 309) and protect normal cells and some cancer cells from apoptosis (213, 366), but there are also reports that hydrophobic CTS at rather low concentrations may induce apoptosis or death of cancer cells (47, 190, 219, 272, 278, 306, 405, 406) (Table 1).

The variation in the effects of CTS on cell growth is certainly due to differences in the gene expression of these cells. Among the most common perturbations in malignant, transformed cells is the overexpression and/or mutation of growth factor tyrosine kinase receptors, leading to an increased phosphorylation of the enzyme ERK (74, 137) and constitutive NF-κB activation, which enable malignant cells to escape apoptosis (271). Also, the question of cell attachment is of considerable relevance for cardiac glycoside sensitivity of apoptosis (48, 50). Hence, tumor cells and normal cells may vary considerably in their sensitivities to cardiac glycosides.

Tumor cells are more sensitive to hydrophobic CTS, such as bufalin and oleandrin (Fig. 4), which may induce differentiation (279, 412) and/or apoptosis in a number of human myeloid leukemia cells, including HL-60, U937, and K562 cells (231, 384–386) (Table 1). Lower (nanomolar) concentrations of

Table 1. *Effects of cardiotonic steroids on various cell types*

Protection Against Apoptosis		Induction of Apoptosis	
Cardiac Glycoside	Cell Type	Cardiac Glycoside	Cell Type
Ouabain (10–100 nM)	Endothelial cells (285, 366)	Bufalin (0.03–30 nM)	Ovarian endometriotic cyst stromal cells (272)
Ouabain (1–10 μM)	Renal proximal tubule cells (213)	Bufalin (>10 nM)	Human monocytic leukemia THP-1 (198, 278), HL-60, MLl, U937 (169, 384-386) cells
Ouabain (0.05–1 mM)	Vascular smooth muscle cells (284, 357, 358)	Bufalin, cinobufagin (0.1–10 μM)	Prostate cancer LNCaP, DU145, PC3 cells (407)
Bufalin (10 nM)	Human monocytic leukemia THP-1 (198), erythroleukemia K562 (277, 413), promyelocytic HL-60 (413), monoblastic U937 (413), myeloblastic ML1 (413) cells	Oleandrin (1.7–170 nM)	PC-3 human prostatic (249, 345), DU145 (345) BRO human melanoma (275), U937 lymphoma (231, 385, 386), HeLa epithelial (231), CaCOV3 ovarian cancer (231), Jurkat T (231) cells
Digoxin, digoxigenin (<10 nM)	HeLa cells (306)	Digoxin, digoxigenin (>10 nM)	HeLa epithelial cells (306)
		Digitoxin (2–33 nM), digoxin (2–30 nM)	Human cancer TH-10 (renal), MCF-7 (breast), UACC-62 (melanoma), K-562 leukemia (226, 227), prostate cancer LNCaP, PC-3, TSU-pr1, DU-145 (140, 406) renal adenocarcinoma (TK-10) (226) cells
		Ouabain (high concentrations)	Human prostatic smooth muscle (47), estrogen receptor-negative human breast cancer MDA-MB-435s (190) cells

cardiac glycosides may stimulate differentiation (54, 169, 180, 264, 341, 359, 384, 412) or proliferation (306), but at >10 nM they induce cell death (306). In the picomolar-to-nanomolar concentration range, bufalin strongly induces differentiation of human promyelocytic HL-60, monoblastic U937, and myeloblastic ML1 cells, whereas other CTS, such as cinobufagin, ouabain, and digitoxigenin, had no effect or only weak effects (54, 169, 180, 264, 341, 359, 384, 412). The murine leukemia cell line Ml-T22 was insensitive to bufadienolides (279).

Higher concentrations of bufalin and oleandrin, however, induce apoptosis in leukemic and other tumor cells (169, 198, 232, 249, 272, 275, 278, 345, 384–386, 406) (Table 1). Induction of apoptosis by bufalin in human tumor cells is associated with an increase in $[Na^+]_i$, i.e., an inhibition of the Na^+ pump (179). The apparent bufalin specificity of the apoptotic effect in K562 human erythroleukemia cells is due to the higher affinity of Na^+/K^+-ATPase for bufalin, but other cardiac glycosides at higher concentrations may induce apoptosis as well (279). Human leukemic cell lines such as Jurkat (T cell leukemia) and Daudi (B cell leukemia) cells are among the most susceptible to induction of apoptosis by digitoxin treatment in vitro (139). Digitoxin may inhibit proliferation and promote apoptosis at concentrations commonly found in patients on digitalis therapy (140, 226).

Cytotoxic effects of cardiac glycosides in vitro have also been reported for a number of additional tumor cells, including mammary tumor (190), lung cancer (254), prostate cancer (140, 219, 249, 405), renal cancer (226), malignant melanoma cells (226, 413), and human skin squamous cell carcinoma (9).

Mechanisms of the Anticancer Effect of Cardiac Glycosides

We are far from understanding how cells, on interaction with CTS, can undergo responses such as proliferation, differentiation, cell cycle arrest, and apoptosis, which are so opposed in nature. Some intracellular signals for differentiation and apoptosis are activated simultaneously (197, 198, 385, 386). It might be the orchestration of various intracellular signals and the duration of activation that decide the path that is finally taken. A rough scheme incorporating most of the information is shown in Fig. 5.

Differentiation vs. apoptosis. Regulation of the cell cycle is an important target of intracellular signaling. At low nanomolar concentrations, the hydrophobic cardiac glycoside bufalin arrests the cell cycle of ovarian endometrial cyst stromal cells in the G_0/G_1 phase (272) and of human leukemia ML 1 cells and several prostate cancer cells in the G_2/M phase (140, 170) (Fig. 5). In ovarian endometrial cyst stromal cells, bufalin (10^{-9} M) downregulates the expression of cyclin A, which is important for entering the S phase (DNA replication). The arrest of the cell cycle in the G_1/S phase is further supported by the upregulation of p21 (a suppressing cofactor of G_1S-Cdk). In human leukemia ML 1 cells, bufalin (10^{-9} M) leads to changes in cyclin-dependent protein kinase (Cdk-2) and the Cdk inhibitor protein CKII, which controls transition from the G_2 to the M phase. Additionally, activities of PKC, PKA, and DNA topoisomerases I and II are inhibited (32, 170). Cell cycle arrest and activation of the non-cell cycle-dependent Cdk-5 and *p25* formation (via *p35* cleavage) by digoxin may induce apoptosis (219) (Fig. 5).

The mechanism by which a normal cell or a tumor cell enters the pathway of differentiation and proliferation or apoptosis seems to be essentially the same for normal and malignant cells. Nanomolar concentrations of CTS leading to low-frequency $[Ca^{2+}]_i$ oscillations and activation of NF-κB protect cells from apoptosis (Fig. 3), whereas higher concentrations lead to a sustained increase of $[Ca^{2+}]_i$ and apoptosis (7). Protection from apoptosis by cardiac glycosides is achieved by activation of Akt (184, 417), which inactivates the killer protein Bad by phosphorylation (58, 184) (Fig. 5). Bufalin-induced differentiation is associated with activation of the conventional PKC subfamily (dependent on Ca^{2+} and diacylglycerol) (197). However, at higher concentrations (100 nM) of bufalin (which certainly lead to an inhibition of Na^+/K^+-ATPase and a rise of $[Ca^{2+}]_i$), THP-1 cells undergo apoptosis via the activation of novel PKC, which is activated by diacylglycerol but does not require Ca^{2+} (198) (Fig. 6). An essential

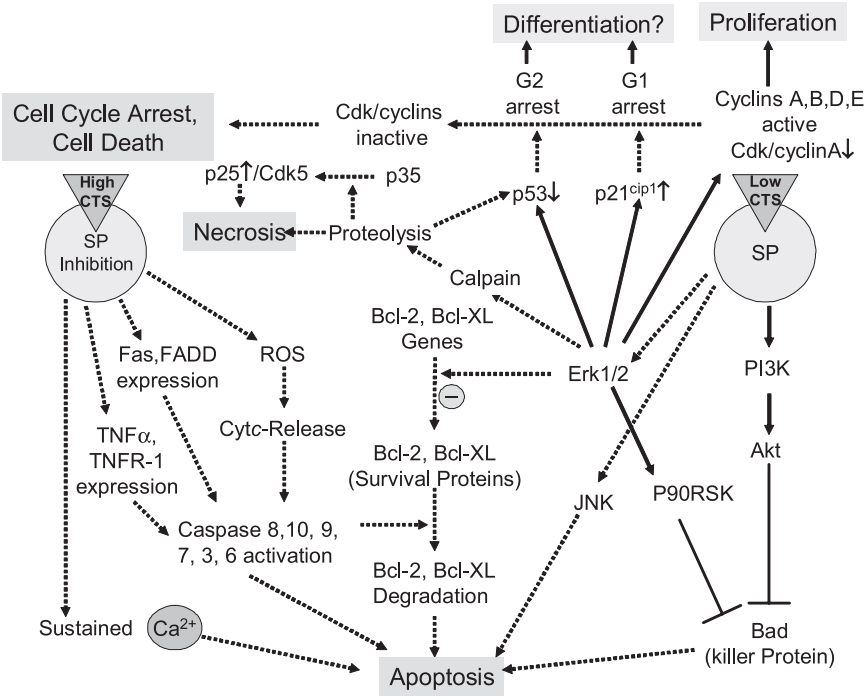


Fig. 5. Effects of CTS on cell proliferation and apoptosis. Effects favoring proliferation/differentiation are indicated by solid arrows and those favoring apoptosis/cell death with dashed arrows. CDK, cyclin-dependent kinase; TNFR-1, TNF receptor type 1; Cyt *c*, cytochrome *c*; RSK, ribosomal S6 kinase.

role of PKC in inducing apoptosis is also evident from the strong resistance of cells defective in conventional PKC-β and novel PKC-δ isoforms to the bufalin-induced DNA ladder formation (198). Treatment of human leukemia THP-1 cells with bufalin sequentially induces c-*fos* and expression of inflammatory cytokine IL-1β and TNF-α genes before the appearance of mature phenotypes of monocytic cells (197). Induction of differentiation of human monocytic leukemia THP-1 cells by bufalin into macrophage-like cells is characterized by loss of proliferation, cell adherence, increased ability to reduce nitro blue tetrazolium, and increased expression of IL-1β. During this process, c-*myb* and c-*myc* expression is downregulated and c-*fos* and Egr-1 transcripts are induced. Furthermore, bufalin fails to induce c-*fos* expression and downregulate c-*myb* transcripts in low-Na^+ medium. These findings indicate the importance of $[Na^+]_i$ handling in triggering the change in protooncogene expression and differentiation (200, 278, 283), inasmuch as it is the "Na^+ cycle" for cell proliferation (324).

It is puzzling that the ERK cascade seems to control bufalin-induced cell differentiation and apoptosis simultaneously (197, 385, 386). Experiments in PC-12 cells suggest that transient activation of ERK promotes proliferation, whereas sustained ERK activation promotes differentiation (350). Possibly, distinct preceding signals (perhaps $[Ca^{2+}]_i$) modulate the ERK cascade. It has been suggested that scaffold or adaptor proteins may participate in such a regulation (391, 399). Additionally, differences in the subcellular localization and substrate specificity of PKC isozymes (316), as well in the ERK cascade, seem to be important. ERK activation has been shown to play a critical role in the bufalin-induced differentiation of human monocytic leukemia THP-1 cells. p38 MAPKs and their downstream mediators may modulate ERK activity and, eventually, cell differentiation (197).

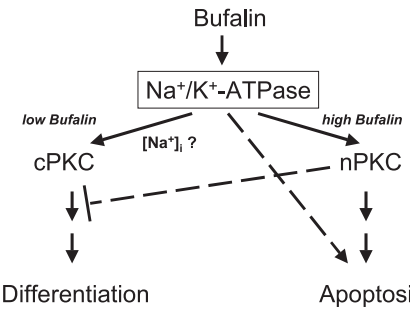


Fig. 6. Mechanism for discrimination of bufalin in human monocytic THP-1 cells between differentiation and apoptosis. nPKC and cPKC, novel and conventional PKC. [Modified from Kurosawa et al. (198).]

Sustained increase in $[Ca^{2+}]_i$ by cardiac glycosides proves to be an important factor in cell death in cardiac glycoside-triggered apoptosis of tumor cells (219, 226, 249, 279, 392, 405, 406). The bufalin-induced apoptosis in endometrial cells in the G_0/G_1 cell cycle is connected to the downregulation of cyclin A, Bcl-2, and Bcl-xL expression and the simultaneous upregulation of p21 and Bax expression and caspase-9 activation (272) (Fig. 5). In the estrogen receptor-negative human breast cancer cell line MDA-MB-435s, ouabain lowers cell proliferation by cell cycle arrest and activation of ERK1/2, leading to an increased expression of the cell cycle inhibitor $p21^{CIP1}$ but a decreased expression of the tumor suppressor protein p53 and activation of JNK (190). Oleandrin, another potent carcinostatic cardiac glycoside (Fig. 4), suppresses the activation of NF-κB, AP-1, and the associated JNK in lymphoma cell lines (231, 348). It also activates the expression of Fas, which is considered to be responsible for apoptosis. Such a Fas induction is not seen in primary cells (348). Persistent activation of the MAPK pathway by bufalin (386) and altered expression of c-*myc* and *bcl-2* genes are involved in apoptosis (179, 180, 245, 272, 385, 386, 392), as well as in the activation of Rac1, p21-activated kinase, and JNK pathways (180, 231).

Overexpression of the survival protein Bcl-2 has an antiapoptotic effect (392). Oleandrin stimulates the formation of ROS in melanoma cells, which leads to a release of cytochrome *c* from mitochondria. This release in turn activates caspases, leading to apoptosis (275). Additionally, activation of apoptosis signal-regulating kinase 1 (ASK-1) may occur (Fig. 3), resulting in activation of JNK and apoptosis, as shown with palytoxin, a very potent ligand at the cardiac glycoside binding site of Na^+/K^+-ATPase (387). In the prostate cell lines PC-3, LNCaP, and DU145, the CTS digoxin, ouabain, and bufalin may induce apoptosis specifically via activation of caspase-3, -8, and -9 (249, 406), early cytochrome *c* release from mitochondria, and ROS generation without damaging other cells (161, 249, 405, 406). This is also true for the cytotoxic effects of bufadienolides and their derivatives on malignant T lymphoblasts that occur via the classical caspase-dependent pathway with damage to mitochondria and internucleosomal DNA fragmentation (54). In endometriotic stromal cells, bufalin also supports apoptosis by activation of caspase-9 (272) (Fig. 5). The Ca^{2+}-activated protease calpain is known to be activated by ouabain in myocardial cells (136). This activation of protease activity by phosphorylation through ERK (104, 105) may occur also in tumor cells. Activated calpain may release cytochrome *c* from mitochondria and inactivate antiapoptotic proteins such as Bcl-2 and Bcl-xL by proteolysis, thereby promoting cell death (134, 307) (Fig. 5).

Search for More Sensitive Antitumor Derivatives of Cardiac Glycosides

The cardiotoxic effects of cardiac glycosides represent a major obstacle for their use in cancer therapy. Hence, Daniel et al. (54) searched for bufadienolide derivatives that induce apoptosis without affecting the heart (Fig. 7). Such compounds contain the configuration of the specific bufadienolide steroid backbone with A/B and C/D *cis* configurations. Additionally, a cardenolide derivative from the root bark of *Calotropis procera*, 2″-oxovoruscharin, and its derivative UNBS-1450 (Fig. 4) are potent drugs with antitumor activity at nanomolar concentrations in a panel of 57 human cancer cell lines (368). UNBS-1450 had the maximally tolerated dose (i.e., the highest tolerated daily administered intraperitoneal dose for 28 days) of 80 mg/kg in mice, which is 24 times higher than that of ouabain (5 mg/kg) and 12 times higher than that of the parent compound oxovoruscharin (368). In non-small lung cancers (NSCLC), which are associated with a very dismal prognosis, chronic in vivo intraperitoneal and oral UNBS-1450 treatment of immunodeficient mice with metastases into the brain and liver had very significant therapeutic effects (255). Human A549 NSCLC cells showed highly activated cytoprotective NF-κB signaling pathways. UNBS-1450 affected the expression and activation status of different constituents of the NF-κB pathways in A549 tumor cells. The modifications induced by UNBS-1450 led to a decrease in the DNA binding capacity of the p65 subunit and the NF-κB transcriptional activity (255). Furthermore, UNBS-1450 leads to the induction of nonapoptotic cell death and decreases heat shock protein 70 at the mRNA and protein levels and downregulates nuclear factor of activated T cells/tonicity-responsive enhancer-binding protein (a factor responsible for the transcriptional control of heat shock protein 70). It also induces an increase in permeability of lysosomal membranes of NSCLC cells (254). Since UNBS-1450 showed effective in vivo antitumor activity in nude mice carrying subcutaneous xenografts of human NCl-H727 and A549 cells, it is entering phase I clinical trials in Belgium (254, 255).

PHYSIOLOGY AND PATHOPHYSIOLOGY OF ENDOGENOUS CARDIAC GLYCOSIDES IN THE CIRCULATORY SYSTEM

Because of the existence of manifold CTS-induced intracellular signaling pathways in a great number of cells, it is likely that the various endogenous cardiac glycosides act as a new class of steroid hormones. In fact, the quite substantial information on the role of the different endogenous CTS in cardiac and kidney function and the regulation of salt and mineral metabolism strengthens the concept that endogenous cardiac glycosides play an essential role in the physiology and pathophysiology of blood pressure regulation and development of arterial hypertension. It is probable that the various endogenous CTS with different functional and tissue specificity cooperate in the physiological tasks. Differences in the specificity of endogenous ouabain and marinobufagenin for kidney and heart function, as well as for the circulatory system, have been described (235). On a molecular level, such differences may be caused by variations in the affinities of various Na^+/K^+-ATPase isoforms for specific CTS, by altered interaction times of the cardiac glycoside receptor site with hydrophobic vs. hydrophilic CTS, by a slightly different conformation of the α-subunit upon CTS binding due to an induced-fit mechanism,

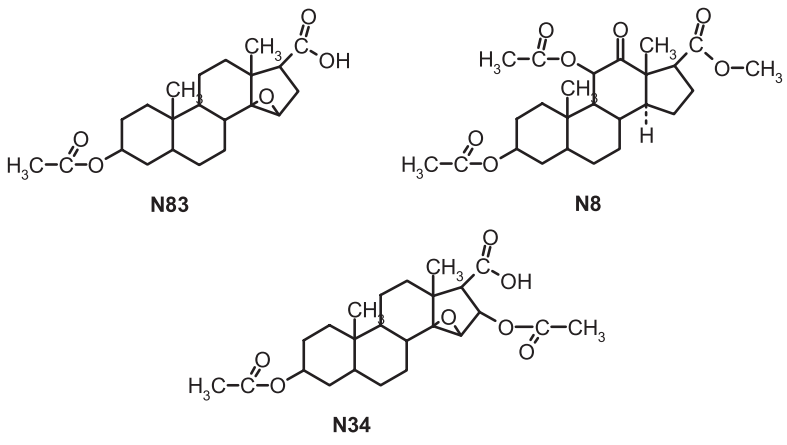


Fig. 7. Bufadienolide derivatives with tumor-specific cytotoxicity but without cardiac activity (54). N8, 3β,11α-diacetoxy-12-keto-5β,14α-aetianic acid methyl ester; N34, 3β,16β-diacetoxy-14β,15β-epoxyaetianic acid methyl ester; N83, 3β-acetoxy-14β,15β-epoxyaetianic acid.

and, certainly, also by variations of the gene expression pattern of the various intracellular signaling proteins and other proteins in the target cells.

Endogenous Ouabain, a Blood Pressure-Modulating Hormone?

Ouabain and hemodynamic effects. Endogenous ouabain is structurally identical to plant-derived ouabain (127, 177, 193, 247, 333), and they have identical cardiotonic and vasotonic actions in guinea pig left atria and aortic rings (39). Plasma concentration of endogenous ouabain is mostly determined after a specific cleanup process by cross-reaction with ouabain-specific antibodies (26, 96, 135). Endogenous ouabain increases in blood plasma in stress situations demanding acutely increased blood supply of organs in humans and dogs running on a treadmill (26) or in swimming rats (119). Ouabain rises rapidly and concomitantly with epinephrine (119) when physical exercise starts and declines quickly upon rest. It is likely that the substantial increases in endogenous ouabain induce an inotropic effect on heart function. Pretreatment of dogs with the β-blocker atenolol, as well as with the angiotensin-converting enzyme (ACE) inhibitor benazepril, abolished the exercise-dependent rise in endogenous ouabain levels, indicating that the release of ouabain in dogs is controlled by epinephrine and angiotensin II (26, 146). Also, ACTH raises the plasma concentration of endogenous ouabain in humans and rats (347, 402). Since plasma concentration of endogenous ouabain correlates with systolic and mean arterial blood pressure, the behavior of endogenous ouabain is similar to that of a blood pressure-modulating factor (235, 242, 297, 379). Most likely, the increased concentration of endogenous ouabain in the cord blood of newborns (64) is the result of stress during delivery. Intra-arterial injection of ouabain (8 μg/100 ml tissue) into the forearm of normotensive subjects and mildly hypertensive patients increased vasoconstriction 30% and 52%, respectively (162). On the other hand, a single intravenous injection of ouabain into healthy human volunteers did not produce hypertension, nor did it affect renal blood flow, glomerular filtration rate, hourly urine volume, or Na^+ and K^+ excretion; it did lead, however, to a significant reduction in heart rate and plasma angiotensin II levels and a rise in plasma epinephrine levels. Hence, ouabain is neither an acute pressor nor a natriuretic substance in a healthy individual (296). No enhancing effect of nanomolar ouabain on vasoconstrictor agents was seen in anesthetized normotensive Wistar rats (321), but an effect was noted in isolated rat tail arteries after a longer (1-h) treatment (320). Yet, in spontaneous hypertensive rats and those in which NO synthase was blocked, low doses of ouabain increased arterial blood pressure by increasing the vascular tone (321). Apparently, the innervations, as well as the gene expression pattern, of cells and organs contributing to the regulation of contraction are important factors determining how ouabain affects hemodynamics. Intravenous application of ouabain produced an excitatory effect on baroreceptor nerve activity that was greater in hypertensive rats (2). Regulation of baroreceptor nerve activity may also explain the decrease in heart rate in humans after a single ouabain injection (296). Exposure of rats to nanomolar ouabain concentrations for a longer period of time leads to arterial hypertension (238, 290, 407) and smooth muscle proliferation (1, 17). In patients with essential hypertension, circulating endogenous ouabain concentrations correlate directly with the heart's relative wall thickness and the total peripheral resistance but inversely with the left ventricular end-diastolic index, stroke index, and cardiac index (297). Since concentrations of endogenous ouabain above the plasma level are present in hearts of Wistar rats, which are even higher in myocardial ischemia, this CTS may also be released locally into the bloodstream during exercise (76). In congestive heart failure, elevated plasma ouabain concentrations were measured (19, 24, 121), but, in contrast to the reports on patients without cardiac failure (235, 242, 297, 379), endogenous ouabain correlated inversely with mean arterial pressure (121). In their prognostic study of the plasma concentration of endogenous ouabain and the aggravation of heart failure in optimally treated patients with idiopathic dilated cardiomyopathy, Pitzalis et al. (298) found a much more rapid progression of heart failure in patients with ouabain levels >233 pmol/l than in those with lower ouabain levels. Interestingly, endogenous ouabain concentrations were significantly higher in patients receiving digitalis therapy. This suggests that endogenous ouabain may contribute to digoxin toxicity (234). Ouabain has been found to induce IL-1β, IL-6, and TNF expression in human peripheral blood mononuclear cells (248). Production of TNF-α by cardiac myocytes is known to be stimulated during hemodynamic overload (95, 251, 291). Hence, it is feasible that constantly increased blood plasma levels of endogenous ouabain may lead to an increased biosynthesis of TNF-α in cardiac myocytes and, subsequently, to myocardial contractile dysfunction and apoptosis (Fig. 5). ROS, which are released in response to ouabain (161, 364, 398), are known to stimulate apoptotic cell death of brain cells via ASK-1 and MAPK signaling (339, 351), as does palytoxin, another inhibitor of the Na^+ pump, in tumor cells (387) (Fig. 3). A similar mechanism may lead to cardiac failure.

Ouabain and Na^+ metabolism. The interrelationship between endogenous ouabain and salt in the homeostatic regulation of blood pressure is rather complex (235). Exposure of fish to increasing salinity of their surrounding water raises plasma ouabain levels, and cortisol concentrations rise in parallel with the increase in plasma osmolality (175). Elevated circulating levels of Na^+ pump inhibitors have been described in experimental low-renin forms of hypertension and essential hypertension (131, 228, 262, 289, 299). Increased concentrations of endogenous ouabain have been reported under a number of conditions, such as Na^+ imbalance, chronic renal failure, hyperaldosteronism, and preeclampsia (34, 129, 236, 401). Hence, one may ask whether changes in the plasma Na^+ concentration may directly and immediately affect the release of endogenous ouabain into circulating blood. However, this does not seem to be the case. Acute intravenous salt volume expansion (2 liters of saline in 4 h) in low-renin hypertension did not increase endogenous ouabain in salt-sensitive or salt-resistant patients (240) but did increase the levels of atrial natriuretic peptide (ANP) (25). However, when healthy humans were exposed for several days to a high-Na^+ diet, a parallel increase in urine Na^+ excretion and a rise in endogenous ouabain in plasma and urine were seen beginning on *day 3*, whereas plasma renin activity and aldosterone levels were suppressed. This salt-evoked increase in plasma endogenous ouabain was greater in older individuals (236). In a careful study of >100 patients with essential hypertension, there was

no evidence that plasma volume expansion or an increase in plasma NaCl concentration is a trigger for the release of endogenous ouabain. On the contrary, interventions that specifically promoted the loss of body Na^+ increased the plasma concentration of endogenous ouabain (240). Hence, the existing data do not support the concept that endogenous ouabain is a natriuretic hormone but, rather, suggest that it is involved in the adaptation of humans to Na^+ depletion (240). Consistent with the latter concept is another careful study in the general population (379 subjects) revealing that endogenous ouabain concentration correlates positively with urinary K^+ excretion. Endogenous ouabain was not dependent on urinary Na^+ excretion, nor was it dependent on serum Na^+ or K^+ concentration (379). A statistically significant interaction of plasma ouabain with urinary Na^+ excretion in relation to systolic and diastolic blood pressure was observed (379). It was suggested that endogenous ouabain is released in response to K^+, either inhibiting the pressure effect of an excessive salt intake or counteracting the depressor action of Na^+ depletion (379). Furthermore, a positive association between hematocrit and plasma ouabain (379) points to the possibility that an increase in blood viscosity may raise the blood pressure (and the release of endogenous ouabain). This would then require an increased force of contraction of the heart, which would benefit from the release of endogenous ouabain (see above). Unfortunately, a detailed study of this aspect has not been performed. Another possibility is that Na^+/K^+-ATPase acts as a sensor of extracellular K^+ concentration. A decrease of plasma K^+ concentration makes Na^+/K^+-ATPase, as the cellular signal transducer in the periphery and brain, much more sensitive to endogenous ouabain (8). One may not exclude that cells in the hypothalamus become more sensitive to ouabain and, thereby, activate the sympathetic pathway and, hence, the release of endogenous ouabain from the adrenal cortex (Fig. 8).

Long-term effects of ouabain induce arterial hypertension. In addition to its rapid effects, ouabain has long-term consequences on protein synthesis (330, 396) and affects other hormonal systems. Ouabain has been reported to affect hormones involved in the control of the circulatory system, including catecholamine release and synthesis from atrial and chromaffin tissue cells (125, 286), acetylcholine release (114, 328), secretion of the atrial natriuretic hormone (331), ET-1 (55, 63, 274), and NO (68, 85, 164, 287, 319, 329, 395), and alteration of aldosterone synthesis (13, 356, 400) and the renin-angiotensin system (146, 394).

Long-term exposure of rats to small (nanomolar) doses of ouabain or other cardenolides leads to hypertension via central and peripheral mechanisms (44, 98, 241, 290, 319, 323, 344, 369, 407). This means that long-term exposure to ouabain may produce hypertension, even in adrenalectomized animals, which also leads to a rise of plasma aldosterone (241). In contrast to ouabain, however, digoxin does not induce hypertension but, rather, reverses ouabain-induced hypertension in rats (186, 238). The hypertensinogenic action of CTS in rats is unrelated to their ability to inhibit Na^+/K^+-ATPase activity (237). Hence, signal-transducing mechanism(s) other than that proposed by the Na^+-lag mechanism (37) (Fig. 2) must exist for endogenous cardiac glycosides (1, 112, 397) (Fig. 3). Nanomolar ouabain concentrations induce the proliferation of proximal tubule kidney cells in association with Akt phosphorylation (184) and of arterial smooth muscle cells in vitro along with EGFR phosphorylation and ERK1/2 activation (1, 17).

Approximately 50% of Caucasians with uncomplicated essential hypertension and hyperaldosteronism exhibit elevated concentrations of endogenous ouabain. The hypertension is unrelated to plasma renin activity and is not affected by dopaminergic (DA_2) receptor blockade and stimulation (318). Hypertensive patients show a reduced heart rate and greater left

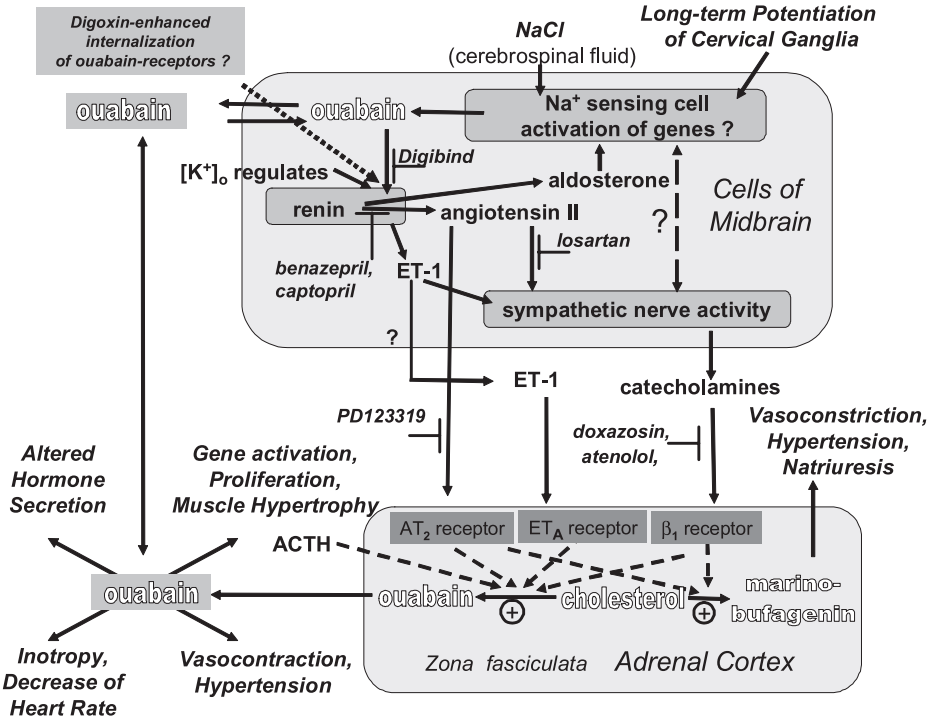


Fig. 8. Overview of hormonal control of ouabain release from cells of the midbrain and adrenal cortex and interrelationship of ouabain and hormones regulating blood pressure and salt/water metabolism. Upon binding to a cellular surface via the Na^+ pump, ouabain acts as a signal transducer (396, 397) or inhibitor (37). A decrease of extracellular K^+ concentration ($[K^+]_o$) may increase affinity of Na^+/K^+-ATPase as a receptor for cardiac glycosides and, hence, lead to arterial hypertension (8) as a result of activation of the hypothalamic renin-angiotensin system (146). Sympathoinhibitory (109) and antihypertensive effects of digoxin (151, 238, 375) are interpreted to be the result of an internalization of the Na^+ pump cardiac glycoside receptors in special areas of the brain (149). ACTH-induced stimulation of ouabain secretion has been demonstrated (347, 402). Angiotensin II receptor in the brain is an AT_1 receptor (147). Endothelin-1 (ET-1) acts via the ET_A receptor in ouabain-induced hypertension (63, 77).

ventricular mass and stroke volume of the heart (242, 297). Arterial hypertension may be related to a mutated α-adducin $Gly^{460}Trp$ allele (379), a ubiquitously expressed tetrameric cytoskeletal protein with a defect that may lead to a lowering of endocytosis of Na^+ pumps and, hence, an increase in the number of basolateral Na^+ pumps in kidney epithelial cells. This results in an increased tubular Na^+ resorption (30), which then may stimulate the secretion of endogenous ouabain in humans as well as in rats (52, 97) and, consequently, lead to an increase in blood pressure (201). If this concept applies to inherited hypertension in humans, then normotensive subjects with familial factors favoring the development of hypertension might show elevated plasma concentrations of endogenous ouabain. In fact, this has been reported recently (239).

Chronic infusion of ouabain (just double the nanomolar concentration of endogenous ouabain) induces hypertension, as well as hypertrophic growth and the transcriptional regulation of several early- and late-response genes (7, 17, 160, 330). Na^+/K^+-ATPase, the molecular receptor of ouabain, exists in four different isoforms of the catalytic subunit. Although the α_1-subunit is generally considered the workhorse of Na^+ transport, the physiological role of the α_2- and α_3-isoforms is less clear. In the rat, but not in humans, the α_1-subunit is ouabain insensitive, whereas the α_2- and α_3-isoforms are much more sensitive (51, 265, 378). Hearts from mice with reduced α_2-isoform expression exhibit hypercontractility (168), whereas those from mice with reduced expression of α_1-isoforms show a reduced force of heart and muscle contraction (141). The reduced contractility may be due to compensatory effects and changes in other genes (70). There is much evidence that the α_2- and α_3-isoforms are important for the development of inotropic and hypertensive responses [see Blaustein's plasmERosome hypothesis (37)] (Fig. 2). This theory is also supported by experiments with transgenic mice: when the cardiac glycoside receptor site of the α_2-isoform was converted to an ouabain-resistant form, the mice became resistant to ACTH- and ouabain-induced arterial hypertension (70, 72). However, when transgenic mice were constructed with an ouabain-sensitive α_1-subunit and an ouabain-insensitive α_2-subunit, the α_1-subunit substituted for the α_2-subunit in the wild-type animal (70). Both catalytic isoforms are coupled to the Na^+/Ca^{2+} exchanger (73). In other words, any of the catalytic α-subunits could act in humans as cardiac glycoside receptors in the induction of hypertension (70).

A prohypertrophic effect of low ouabain concentrations was demonstrated in cultured rat cardiac myocytes, in renal tubule cells, and after chronic infusion in conscious rats (98, 124). Therefore, it is likely that constantly elevated plasma concentrations of ouabain in humans may lead to arterial hypertension and remodeling of the heart. In the right atrium of hypertensive patients, this remodeling process results in a pronounced increase in expression of the α_3-isoform and a fivefold increase in expression of the α_2-isoform of Na^+/K^+-ATPase as well as an increase in expression of the Na^+/Ca^{2+} exchanger and Ca^{2+}-ATPase of the plasma membrane (167). Endomyocardial biopsies from human patients with heart failure showed a ~40% fall of total Na^+/K^+-ATPase, which corresponded with the decrease of heart function (337, 340, 410). There are regional differences between the right atrium and the left ventricle (267). Development of heart failure in humans is associated with a downregulation of the α_1-isoform of Na^+/K^+-ATPase in the left ventricular myocardium (338), a reduced sensitivity to marinobufagenin, an upregulation of the α_3-isoform (410), and an enhanced sensitivity to ouabain (336, 340). A similar change of the expression pattern of α-subunits of Na^+/K^+-ATPase was seen in ouabain-induced hypertension in rats (377). Chronic exposure of rat cardiac myocytes to ouabain also results in an increase in the protein expression of the Na^+/Ca^{2+} exchanger (266, 370). Apparently, constantly elevated levels of ouabain remodel the heart and may, eventually, produce heart failure (340). Nanomolar concentrations of ouabain activate calpain, a Ca^{2+}-activated cysteine protease in human-derived myoblasts, an event suggested to be involved in the remodeling of hearts in uremia (136). Ouabain and digoxin affect cell proliferation and expression of Na^+/K^+-ATPase isoforms in a different way in vitro (396) as well as in vivo (377). Hence, termination of raised endogenous cardiac steroids might prevent arterial hypertension and cardiac remodeling and, consequently, heart failure. Interestingly, immunization of rats against ouabain lowers arterial blood pressure and plasma aldosterone concentrations (129, 400), as does infusion of the commercially available Fab fragment of an anti-digoxin antibody (Digibind) that cross-reacts with ouabain in humans and rats (3, 115).

Ouabain, endothelium, and vascular smooth muscle cells. In various arteries, vascular endothelium may modify the acute vascular action of ouabain in different ways (319, 320, 322, 323, 326, 344). The long-term effect of ouabain on the induction of hypertension is dependent on the presence of the endothelium (72). Perfusion pressure was not affected by perfusion of rat tail arteries with 1 nM ouabain for 1 h in normotensive animals but was increased in salt-hypertensive animals. Presence of the endothelium was mandatory, indicating an increased endothelial synthesis and release of angiotensin II (344). Yet, in normotensive rats, a 10-fold-higher concentration of ouabain also produced arterial contraction (320). Ouabain-induced hypertension in rats increases expression of genes of prepro-ET-1 and the ET_A receptor in the aorta without affecting the ET_B receptor (394).

In human umbilical endothelial cells in vitro, incubation with nanomolar ouabain concentrations increased release and expression of ET-1, proliferation of cells, and Na^+/K^+-ATPase activity. Such nanomolar ouabain concentrations led to oscillations of $[Ca^{2+}]_i$ and stimulation of MAPK phosphorylation (329). They also increased NO production in rat aortic endothelial cells (68). Nanomolar concentrations of ouabain stimulated NO release by an increased translocation of endothelial NO synthase and an activation of PI3K. These events were followed by phosphorylation of Akt and activation of endothelial NO synthase by phosphorylation. An activation of NO release due to ET-1 binding to the ET_B receptor could be excluded (85). Ouabain also stimulated vasodilatation by increasing release of an endothelial hyperpolarizing factor that, presumably, opens a Ca^{2+}-dependent K^+ channel (319). At concentrations that are inhibitory to the Na^+ pump, ouabain activated the expression of the vascular cell adhesion molecule (VCAM-1) in murine microvascular cells and potentiated the effect of interferon-γ on this process. Moreover, ouabain provided a complementary signal for TNF or interferon-γ by stimulating inducible NO synthase expression. This was accompanied by an activation of the transcription factor NF-κB (28).

In vascular smooth muscle cells, exposure to nanomolar concentrations of ouabain stimulated proliferation (1, 17, 112) and increased collagen content (41). In rat vascular smooth muscle cells, 10 nM ouabain also stimulated the formation of NO by a $[Ca^{2+}]_i$-dependent mechanism (418). Nanomolar ouabain increased expression of endothelial NO synthase and neuronal NO synthase in the aorta (319), but not endothelial NO synthase in mesenteric, supermesenteric, and caudal arteries (319, 322).

Ouabain, a neurosteroid mediating sympathetic hyperactivity in salt-sensitive hypertension. Hypertension has been associated with increased sympathetic tone, which also may be due to activation of the central regulatory system involving renin-angiotensin (146) and endothelin (55, 63). Ouabain was isolated from the hypothalamus (177) and is present in the pituitary gland and medullary neurons (334). When Dahl salt-hypertensive or spontaneously hypertensive rats were exposed to a high-NaCl diet, an increase of the Na^+ concentration of the cerebrospinal fluid preceded the increases of arterial blood pressure and heart rate by several days (156). This was accompanied by an increase of the sympathoexcitatory response and of endogenous ouabain in the brain and cerebrospinal fluid (158, 210). Moreover, dietary Na^+ raised the concentration of endogenous ouabain in the hypothalamus and pituitary gland, even in adrenalectomized rats, to the same extent as in sham-operated control animals (211). Apparently, the central nervous system may represent the major source of central and peripheral endogenous ouabain (211), although a contribution of other tissues, such as the heart (77), cannot be excluded. In conscious rats, acute intracerebroventricular injection of ouabain raises sympathetic activity, blood pressure, and heart rate (149, 152). Such effects can be prevented by the simultaneous administration of Fab fragments of Digibind, which cross-react with ouabain with high affinity (149, 156, 355). The effects of increased Na^+ concentration in the cerebrospinal fluid and of intracerebroventricular injection of ouabain on blood pressure and heart rate were attenuated in transgenic rats deficient in brain angiotensinogen (148). In normal rats, sympathetic hyperactivity and hypertension induced by chronic ouabain and hypertonic saline treatment are prevented by the angiotensin type 1 (AT_1) receptor (148) and blockade of the α_2-adrenergic receptor. Hence, locally produced angiotensin II seems to play an important role in the sympathoexcitatory effects of ouabain and Na^+ (211), which is evident from the increased vascular resistance and the decreased blood flow of kidneys, skeletal muscle, skin, stomach, spleen, testes, and intestine (55, 63). This concept is supported by other studies as well (43, 44, 149, 152–155). Differences in Na^+ responsiveness of various Dahl rats may be due to genetically caused variations in the sympathoexcitatory-and-pressor response sequence (158) (Fig. 8). The highly polar ouabain molecule probably enters the hypothalamic region via fenestrated epithelia adjacent to the circumventricular organ and, by enhancing the sympathetic nerve activity, evokes sustained elevations of blood pressure (6, 130, 150, 155, 407). Chronic ouabain infusion suppressed plasma angiotensin I and II concentrations and did not alter angiotensin I in the heart and kidneys but, rather, led to an increase of angiotensin II content in the hypothalamus (44). This was associated with decreases in the amount of central AT_1 receptors and the density of ACE, supporting the involvement of the brain renin-angiotensin system in the central hypertensive mechanism of the action of ouabain (44).

A role of central ET-1 in ouabain-induced hypertension has also been suggested (55, 63). Ouabain increased the central synthesis of ET-1 in the brain, whereas ET_A receptor mRNA was decreased. Microinjections of ET_A, but not ET_B, receptor antagonists into the intraperiaqueductal gray area led to a significant reduction of ouabain-induced hypertension. One may wonder whether an adaptation of the endocrine system in the brain to a high-salt diet may alter the ouabain response. Indeed, a high-salt diet over a period of weeks attenuated the response to exogenous intracerebroventricular ouabain. Such a diet doubled the hypothalamic content of endogenous ouabain (149). The attenuating effect of a high-salt adaptation might lead to an internalization of ouabain receptors in the hypothalamus. Whether such a mechanism may also explain why prolonged treatment of rats with digoxin lowers blood pressure in ouabain-hypertensive animals remains unknown (151, 186, 238).

The baroreflex control also seems to be desensitized by a rise of endogenous ouabain in the brain. This may in turn facilitate the development of arterial hypertension (152). Digoxin seems to counteract this process (151). Long-term potentiation of the isolated superior cervical ganglia is also tightly linked to ouabain-dependent hypertension (6). It is interesting that central sympathectomy acutely decreased the concentrations of endogenous ouabain in the hypothalamus and plasma, but peripheral sympathectomy did not alter plasma concentrations of endogenous ouabain (403). Apparently, cells in the brain nuclei seem to be interlinked by adrenergic compounds, ouabain, and angiotensin II (Fig. 8).

Apparently, even small increases of Na^+ in the cerebrospinal fluid are sensed by benzamil-sensitive Na^+ channels (373, 374), the Na^+ sensor Na_x (143), or the aldosterone-inducible ENaC (147). The enhanced Na^+ entry into relevant brain areas may increase ouabain release in the brain and, subsequently, sympathetic outflow and blood pressure (146, 373). In humans, several days are needed for a rise of Na^+ intake to lead to an increase in blood pressure and alterations in plasma and cerebrospinal concentrations of endogenous ouabain (236). It may be that an Na^+-dependent induction of genes is necessary before changes in blood pressure and other alterations become visible. In fact, in Dahl salt-sensitive rats, a high-salt intake increased the expression and activity of ACE in the hypothalamus and pons (but did not increase local angiotensin II) (416). Chronic blockade of endogenous ouabain in the brain by intraventricular infusion of an ouabain antibody lowered the NaCl-dependent rise of ACE mRNA (416). Elevated Na^+ in the cerebrospinal fluid for 1 wk increased the density of AT_1 receptors within specific brain nuclei (147, 380) and hypothalamic aldosterone (147). Chronic intracerebroventricular infusion of aldosterone into Dahl salt-sensitive rats in turn led to sympathetic hyperactivity, hypertension, and an increase of endogenous ouabain in the hypothalamus, but not in plasma and adrenal glands. It attenuated excitatory responses to intracerebroventricularly applied ouabain (157). Spironolactone, an aldosterone antagonist, attenuated the effects of aldosterone infusion (147). In other words, intracerebroventricular infusion of aldosterone into Dahl S rats mimicked the responses of high-salt intake, possibly via increased uptake of Na^+ due to the increased expression of ENaC and Na^+/K^+-ATPase (157).

A recent report shows a marked attenuation of sympathetic hyperactivity and left ventricular dysfunction in transgenic rats deficient in brain angiotensinogen 8 wk after experimental myocardial infarction. This demonstrates that the brain renin-angiotensin system has a substantial impact on the development of left ventricular dysfunction of the heart (372) and that therapy with AT_1 receptor blockers such as losartan should be beneficial (394).

Endogenous Digoxin, a Hormone Opposing Endogenous Ouabain?

Although it is evident that digoxin is synthesized in the adrenal gland (300), despite an impressive amount of literature on the action of this drug, there is not much information as to why the physiological effects of this CTS apparently differ from those of ouabain. In critically ill patients, digitalis-like immunoreactive substances, but not endogenous ouabain, were related to left ventricular function (27). The most striking difference is that digoxin acts as an antagonist of ouabain-induced hypertension (151, 238, 241, 369, 407). Additionally, the digoxin-induced arterial baroreflex opposes the sympathetic excitatory pressor responses to ouabain in the periphery and the brain (109, 151, 238) and no longer activates the chemoreflex in patients with chronic heart failure (288). The reason for this on a cellular level is unknown (Fig. 2). The plasma concentration of endogenous digoxin (determined by cross-reactivity with antibodies) is increased in renal failure and hypertensive pregnancy, during prolonged, strenuous exercise, and in newborn infants (117, 334, 367). Hence, whether the reported rise in plasma digoxin levels represents counteractive effects against the stress-induced rise in endogenous ouabain is an open question. One may also ask whether digoxin, when used as a treatment for heart failure, may act preferably via the suppression of the sympathetic excitatory pressor responses that had been exerted via release of ouabain from the adrenal gland and brain (109, 151, 372) as a result of an activation of the chemoreflex in patients with chronic heart failure (288, 372), rather than by a direct inotropic response on heart muscle cells. It is unclear how these two substances, which are specific inhibitors of the Na^+ pump, can produce opposite physiological effects. Perhaps the higher hydrophobicity of digoxin than ouabain would lead to a different tissue distribution. However, other reasons may exist as well.

Endogenous Marinobufagenin, a Natriuretic Hormone?

Endogenous marinobufagenin differs in its action from ouabain as follows (235). *1*) It exhibits a greater affinity for the ouabain-resistant α_1-subunit of Na^+/K^+-ATPase (88, 90, 92), which is the main isoform of rodent kidney tubule cells (33). *2*) The acute and chronic NaCl load of rats and dogs is accompanied by a sustained increase in the level of endogenous marinobufagenin (21, 89, 91, 92, 94, 293). This increase is preceded by a transient increase in endogenous ouabain levels in the brain (as well as in blood plasma and urine) (92, 94) that stimulates, at least in NaCl-loaded Dahl salt-sensitive rats, peripheral release of marinobufagenin via an AT_1 receptor pathway and, probably, via sympathetic activation (87) (Fig. 2). Possibly, the increased blood plasma marinobufagenin concentration (determined immunologically) promotes natriuresis and compensates for the genetically impaired pressor natriuretic mechanism (21, 90). The bufadienolide, which acts in a manner similar to ouabain as a vasoconstrictor (18), is elevated in volume expansion and preeclampsia in humans (225) and in a rat model (144). Interestingly, marinobufagenin also impairs first-trimester cytotrophoblast differentiation (199). Similar to ouabain, marinobufagenin is increased upon voluntary hypoventilation of human volunteers (20), which increases arterial blood pressure. *3*) In patients with chronic heart failure, plasma levels of marinobufagenin exhibit a strong correlation with α-ANP, which in turn correlates with changes in left ventricular function (106). It is of high interest that prepro-ANP and human α-ANP potentiate marinobufagenin-induced Na^+/K^+-ATPase inhibition in the kidney and that the effect is reversed in the aortic sarcolemma (86). A detailed study of this phenomenon revealed that the effects are caused by a different tissue distribution of the PKG isoforms PKG1 and PKG2. In kidney tubule cells, PKG2 leads to a prepro-ANP- and ANP-increased phosphorylation and inhibition of the α_1-subunit of Na^+/K^+-ATPase, whereas in aortic plasmalemma, PKG1 leads to a prepro-ANP-decreased phosphorylation and inhibition of the α_1-subunit of Na^+/K^+-ATPase. Hence, the concurrent production of a vasorelaxant, ANP, and a vasoconstrictor, marinobufagenin, potentiate each other's natriuretic effects, but ANP may offset the deleterious vasoconstrictor effect of marinobufagenin (86). Moreover, patients with chronic renal failure developing a "uremic" cardiomyopathy characterized by diastolic dysfunction, cardiac hypertrophy, and systemic oxidant stress show increased circulating concentrations of marinobufagenin and telocinobufagin (192). Experimentally, with constant administration of marinobufagenin to rats, the effects of a "uremic cardiomyopathy" could be mimicked: in rats treated with marinobufagenin for 4 wk, steroids rose to levels comparable to those observed at 4 wk of partial nephrectomy. This caused increases in conscious blood pressure, cardiac weight, and the time constant for left ventricular relaxation similar to those for a partial nephrectomy. Decreases in the expression of the cardiac SR ATPase, cardiac fibrosis, and systemic oxidant stress were observed under both conditions. Immunization of the animals against marinobufagenin attenuated the cardiac hypertrophy, impairment of diastolic function, cardiac fibrosis, and systemic oxidant stress seen with partial nephrectomy without a significant effect on conscious blood pressure. Hence, increased concentrations of marinobufagenin seem to be important for stimulated natriuresis in cooperation with ANP (86), as well as for the remodeling of the heart leading to cardiac disease in patients with renal failure (182). Coordinated shifts of Na^+/K^+-ATPase isoforms and their endogenous ligands were observed during cardiac hypertrophy and failure: the α_1-isoform decreased and the α_3-isoform increased (93). A causative therapy of this type of heart failure would be lowering of the plasma levels of marinobufagenin. In fact, administration of antibodies against marinobufagenin, but not anti-ouabain, lowered blood pressure in rats with NaCl-induced hypertension (94), arterial hypertension in chronic renal failure (293), and pregnant rats with NaCl-induced hypertension (91).

Agonists and Antagonists of Cardiac Glycoside Action

Interaction of a number of CTS with the cardiac glycoside receptor site of the Na^+ pump by induced fit results in a plethora of diverse signaling pathways starting from the Na^+/K^+-ATPase signalosome (Fig. 3). Hence, it makes sense to look for steroid derivatives acting as antagonists (60, 101) or agonists (368) of cardiac steroid action.

Rostafuroxin, an antihypertensinogenic agent. Rostafuroxin (or PST-2238), a compound resembling CTS, acts as an antihypertensive agent when given orally at microgram per kilogram body weight doses (101, 102, 305) (Fig. 9). At 10^{-12}–10^{-14} M in vitro, rostafuroxin inhibited the stimulatory effect of 10^{-12}–10^{-8} M ouabain on Na^+/K^+-ATPase activity after 5 days of incubation of normal rat kidney NRK-52E (epithelium-like) cells. This new prototype of an antihypertensive drug also has effects in Milan hypertensive rats, where a genetic alteration of adducin genes is associated with hypertension and an upregulation of renal Na^+/K^+-ATPase. Hence, PST-2238 might be useful for the treatment of human essential hypertension (100, 101).

Anti-digoxin. Compound 16 [4-(3′α,15′β-dihydroxy-5-estran-17′β-yl)furan-2-methyl alcohol], which resembles cardiac glycosides (Fig. 9), is claimed not to bind to the CTS receptor on Na^+/K^+-ATPase or to increase the force of heart muscle contraction but, rather, to inhibit the digoxin-induced increase in the force of contraction and arrhythmias in guinea pig papillary muscle and human atrial appendages. The steroid also inhibited digoxin-induced alteration in endocytosed membrane traffic, indicating a novel mechanism of action (60).

Inotropic agents with lower toxicity. PST-2744 (Fig. 9), a 5α,14α-androstane derivative, represents a new class of Na^+/K^+-ATPase inhibitors with inotropic activity comparable with that of digitalis but with greater safety. The more favorable inotropy-to-toxicity ratio appears to be associated with a direct stimulation of SERCA and/or a lack of enhancement of Ca^{2+} leak in the presence of digoxin (59, 252, 313, 314).

ENDOGENOUS CARDIAC GLYCOSIDES IN DIABETES MELLITUS

Plasma concentrations of endogenous ouabain in humans correlate with urinary K^+ excretion (379). K^+ uptake into muscle cells is activated by insulin by stimulation of the Na^+ pump via signaling pathways involving PI3K and PKC (352, 353). Hence, one may ask whether endogenous cardiac glycosides may control the body's K^+ metabolism via insulin secretion. In fact, toxic ouabain concentrations have been reported to suppress the glucose-induced ATP production and insulin release in pancreatic islets by generating ROS (174). Whether ouabain in a more physiological concentration range may act similarly is unknown. Insulin resistance is associated with high concentrations of endogenous ouabain-like immunoreactivity in patients with non-insulin-dependent (type 2) diabetes mellitus (382). However, in Wistar rats with streptozotocin-induced type 1 and 2 diabetes, neither plasma levels of endogenous ouabain nor renal excretion of ouabain increased. Yet the plasma levels and renal excretion of marinobufagenin increased. Na^+/K^+-ATPase activity of erythrocytes was inhibited more in rats with type 1 than in those with type 2 diabetes (22). In rat soleus muscle, insulin seems to increase Na^+ pump activity (390) via an increased plasmalemmal surface exposure of the α_2-subunit of Na^+/K^+-ATPase due to the PKC- and tyrosine kinase-dependent phosphorylation of the Na^+ pump (45). In human skeletal muscle cells, ouabain decreased surface abundance of the α_2-subunit, whereas α_1-subunit abundance was unchanged. Ouabain and marinobufagenin in the nanomolar concentration range increased glycogen synthesis in a way

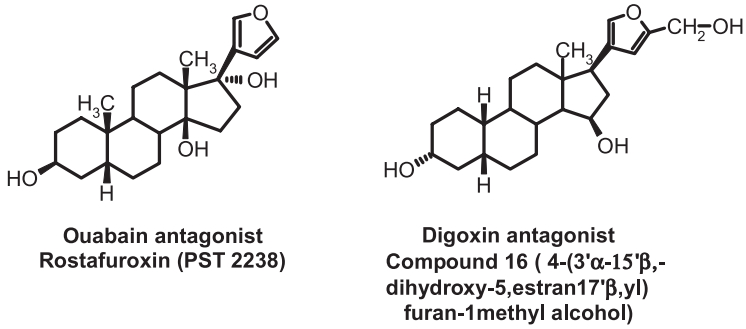
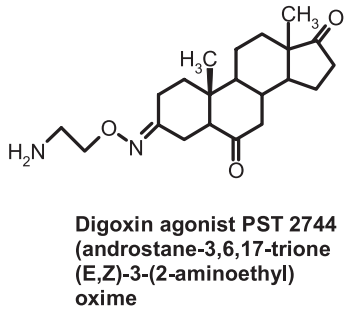


Fig. 9. Agonists and antagonists of CTS.



that was additive with insulin by activation of Src-, ERK1/2-, p90 ribosomal S6 kinase-, and GSK-3-dependent signaling (196) (Fig. 3). Hence, insulin and endogenous cardiac glycosides seem to control the number of ouabain receptor sites (α_2-subunits) on the surface of muscle cell membranes in an opposing way but to stimulate cell proliferation additively.

GENERAL CONCLUSIONS

It is evident that CTS, which are considered to be synthesized exclusively in plants and amphibians, are present in mammals. Endogenous CTS control blood pressure, salt metabolism, and, probably, cardiac function, and they also act as growth factors affecting the proliferation and differentiation of heart and smooth muscle cells. The hydrophilic endogenous ouabain differs in its cellular and physiological spectrum of action from the more hydrophobic endogenous digoxin and marinobufagenin. A short-term increase in the concentration of endogenous ouabain in the blood increases cardiac inotropy and smooth muscle contraction. On the other hand, long-term elevation in the nanomolar concentration range results in a sustained rise of blood pressure and remodeling of cardiac and smooth muscle cells. Secretion of CTS is controlled by hormones of the renin-angiotensin system, epinephrine, and ET-1. Hence, behavior of endogenous cardiac glycosides is similar to that of a new class of steroid hormones with secretion that is controlled by the hypothalamus and midbrain.

One single mechanism of the interaction of cardiac glycosides with the Na^+ pump cannot explain the complexity of cellular responses resulting in cardiac inotropy, arterial hypertension, and remodeling of the circulatory system in association with tissue proliferation, as well as apoptotic processes. The well-known Na^+-lag hypothesis, which assumes that inhibition of the α_2-isozyme of Na^+/K^+-ATPase leads to an increase in Ca^{2+} concentration, may explain short-term and inotropic actions of CTS. However, long-term effects leading to activation of genes and resulting in activation of cell proliferation, as well as apoptotic processes, are better described by the Na^+/K^+-ATPase signalosome hypothesis, which involves specific activation of signal transduction machinery. Depending on the gene expression pattern of the target cell, endogenous and exogenous cardiac glycosides may induce different physiological responses affecting not only the physiological action of cells of the circulatory system and an organism's salt metabolism but, also, the growth of cancer cells.

FUTURE PERSPECTIVES

Na^+/K^+-ATPase seems to respond to CTS by an induced-fit mechanism. Hence, the intramolecular signaling from the exterior to the interior cytosolic surface of Na^+/K^+-ATPase and proteins within the signalosome complex may differ depending on the nature of the steroid. A preference of the activation for specific pathways may thereby be induced. Such a model would explain why hypertension is induced in rats by long-term application of ouabain, but not digoxin. It would also explain why rostafuroxin (Fig. 9) can act as an antihypertensive antagonist of endogenous ouabain, lowering p42/44 MAPK phosphorylation in kidneys via the Src-EGFR-ERK pathway (101). Since rostafuroxin does not affect blood pressure in normotensive patients, it is not only an important representative of a new class of antihypertensive drugs but, also, a good example of a probably soon-to-be-increasing number of drugs affecting intracellular signaling pathways starting from the Na^+ pump. For example, it is feasible that rostafuroxin suppresses proliferation of tumor cells constitutively overexpressing the ERK1/2 and NF-κB pathways; such CTS with antitumor activity have recently been described (Figs. 4 and 7). Oleandrin shows cytostatic activity in leukemia tumor cells but does not affect the growth of normal cells. Another very effective substance is the cardenolide analog UNBS-1450, which acts in tumor cells with constitutive activation of the NF-κB pathway (255). Analogs of CTS with a greater therapeutic inotropic spectrum than digoxin or antidigoxin action (Fig. 9) or with antitumor activity without cardiotonic activity (Fig. 7) have been described. In other words, the long-lasting search for endogenous cardiac glycosides and for analogs with a greater therapeutic spectrum and antihypertensive and cancerostatic activities was finally successful. It is likely that, in the near future, much more information and a wider variety of substances will be available for treatment of heart failure, arterial hypertension, and cancer.

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