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Signaling Mechanisms that Link Salt Retention to Hypertension: Endogenous Ouabain, the Na^+ Pump, the Na^+/Ca^{2+} Exchanger and TRPC Proteins

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Abstract

Salt retention as a result of chronic, excessive dietary salt intake, is widely accepted as one of the most common causes of hypertension. In a small minority of cases, enhanced Na^+ reabsorption by the kidney can be traced to specific genetic defects of salt transport, or pathological conditions of the kidney, adrenal cortex, or pituitary. Far more frequently, however, the salt retention may be the result of minor renal injury or small genetic variation in renal salt transport mechanisms. How the salt retention actually leads to the increase in peripheral vascular resistance (the hallmark of hypertension) and the elevation of blood pressure remain an enigma. Here we review the evidence that endogenous ouabain (an adrenocortical hormone), arterial smooth muscle α2 Na^+ pumps, type-1 Na/Ca exchangers, and receptor- and store-operated Ca^{2+} channels play key roles in the pathway that links salt to hypertension. We discuss cardenolide structure-function relationships in an effort to understand why prolonged administration of ouabain, but not digoxin, induces hypertension, and why digoxin is actually anti-hypertensive. Finally, we summarize recent observations which indicate that ouabain upregulates arterial myocyte Ca^{2+} signaling mechanisms that promote vasoconstriction, while simultaneously downregulating endothelial vasodilator mechanisms. In sum, the reports reviewed here provide novel insight into the molecular mechanisms by which salt retention leads to hypertension.

Keywords

Salt-dependent hypertension; Calcium; Sodium Pump; Sodium/Calcium Exchanger; Receptor-operated Channel

1. Introduction

Hypertension, or chronic high blood pressure (BP) is a major contributor to ischemic heart disease, cerebrovascular disease, heart failure and renal failure, and is estimated to cause more than 7 million premature deaths per year worldwide [1]. Appropriate treatment, and even prevention, of hypertension depends upon better understanding of the underlying causes and

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mechanisms of the elevated BP. Despite extensive research during the past few decades, some critical questions about the pathogenesis of elevated BP remain unanswered. Here, we focus on recent findings that provide novel insight into the linkage between salt retention and hypertension.

2. Kidneys, salt retention and hypertension

The kidneys, which play the primary role in salt and water balance, have long been at the center of hypertension research. The kidney glomeruli of a 70 kg man filter ~25,000 mEq of Na^+ and 180 liters of water per day, and then reabsorb nearly 99.5% of this filtered load by a variety of Na^+ transport mechanisms [2]. It is not surprising, therefore, that defects in any mechanisms that alter renal Na^+ transport may contribute to the net gain or loss of salt (and water).

The association of hypertension with renal parenchymal diseases [3,4], monogenic diseases of renal salt transport [5-7] and renal transplant studies [8-10], as well as Guyton's seminal work on the “over-riding dominance of the kidneys” in controlling BP [11-13], all point to the critical role of the kidneys in hypertension. Likewise, epidemiological studies as well as acute and chronic dietary studies [14,15], volume expansion studies [13,16-18], the efficacy of diuretic therapy [19], and monogenic diseases of renal salt transport [5,6,20], all indicate that NaCl retention and a tendency toward plasma volume expansion [21] play a fundamental role in the chronic elevation of BP. Conversely, genetic defects that reduce salt retention, such as those associated with Bartter's and Gittelman's syndromes, tend to lower BP and protect against the development of hypertension [22]. Nevertheless, the specific mechanism(s) responsible for salt retention in most forms of human essential hypertension (EH) is(are) unresolved. Perhaps subtle renal damage [23], which increases with age, including that which may result from obesity [24,25], causes the salt retention. Importantly, any of several genetic variants (single nucleotide polymorphisms, SNPs), such as those in G-protein coupled receptor kinase type 4 [26-28], alpha-adducin [29,30], or serine/threonine kinase (*STK39*) [31] genes may favor salt retention by the kidneys and, therefore, predispose the bearers of these genes to salt-dependent hypertension. It is apparent, however, that in virtually all of these situations, extracellular fluid (ECF) neither progressively increases nor decreases. Instead, homeostatic physiological (feedback) mechanisms come into play to protect against large ECF volume changes [21]. As we shall see, some of these mechanisms may alter BP to defend against changes in plasma (and ECF) volume.

3. Vascular tone

A related, unresolved issue in hypertension, and our main focus, is the specific mechanism(s) or “signaling pathway” by which salt retention actually elevates BP [32]. To explore this issue, we begin with some basic hemodynamic principles: Mean arterial BP is a function of cardiac output (CO), heart rate (HR), stroke volume (SV) and total peripheral vascular resistance (TPR) [33]. At constant CO, mean BP ≈ CO × TPR. CO, which is equal to HR × SV, is, in turn, directly related to the ECF volume and the volume of venous return to the heart. TPR is regulated dynamically by vasoconstriction/dilation in small “resistance” arteries by three groups of mechanisms: baroreflexes and other neuro-humoral mechanisms, endothelial mechanisms, and myogenic mechanisms [33]. The local (myocyte and endothelial) factors that maintain tonic arterial constriction, or ‘tone’, can be studied in isolated, cannulated small arteries. These arteries develop spontaneous ‘myogenic’ tone (MT) when the lumen is pressurized [34,35]. Indeed, the level of tone in isolated arteries “is often comparable to that observed in the same vessels *in vivo*” [34,35], and may even be used to predict BP changes [36].

Hypertension has often been associated with structural changes that decrease the lumen-to-wall thickness ratio and increase wall stiffness [37-39] due to vascular remodeling [40,41]. It is not clear, however, whether this vascular remodeling is usually the cause or the effect of the hypertension. Recently, we reported that, in some hypertension models, most of the increase in TPR can be attributed to functional, and not structural, alterations in small resistance arteries [42]. Here, we will explore the basis of the dynamic, reversible arterial functional changes, the augmented tone and contractile responses that are observed in hypertension [43,44]. To understand the generation of vascular tone, it is prudent to examine the fundamental mechanisms that influence arterial myocyte contraction. We will start with the mechanisms that regulate myocyte Ca^{2+} because contraction is activated by a rise in the cytosolic Ca^{2+} concentration, $[Ca^{2+}]_{CYT}$ [45].

4. Ca^{2+} homeostasis and arterial constriction

Arterial constriction/dilation and, thus, BP are under neural control, and are also regulated by various endocrine and paracrine substances. Especially noteworthy is the role of the endothelium, which normally tends to restrict excessive vasoconstriction by secretion of nitric oxide (NO) and other vasodilatory factors [46]. In small “resistance arteries”, MT induced by intra-vascular pressure [34,35], plays a key role in controlling BP.

At the cellular level, contraction depends directly on $[Ca^{2+}]_{CYT}$ and the activation of myosin light chain kinase by Ca^{2+}-calmodulin, as well as on modulation of the contractile apparatus' sensitivity to Ca^{2+} (e.g., by Rho/Rho kinase) [47,48]. Myocyte $[Ca^{2+}]_{CYT}$ is regulated by various Ca^{2+} entry, exit and storage systems [45]. Ca^{2+} enters myocytes from the ECF through voltage-gated, receptor-operated, store-operated and stretch-activated channels (VGCs, ROCs, SOCs and SACs, respectively; see Fig. 1). Most of the myocyte Ca^{2+} is sequestered in the sarcoplasmic reticulum (SR) by the sarco-/endoplasmic reticulum Ca^{2+} pump (SERCA). Myocytes can be activated by various hormones and neurotransmitters. For example, stimulation of the sympathetic nerves that innervate the arteries releases norepinephrine (NE), ATP and neuropeptide Y, all of which contribute to activation of the myocytes [49]. Myocytes can also be activated by increased intra-lumenal pressure and wall tension; this opens cation-selective SACs, which depolarize the myocytes, thereby opening Ca^{2+}-selective VGCs. Neurotransmitter release, as well as NO release by the endothelium, are also activated by a rise in $[Ca^{2+}]_{CYT}$ in the respective cell types, but the neurotransmitters promote myocyte contraction, while NO antagonizes contraction. Nevertheless, Ca^{2+} homeostasis in neurons and endothelial cells utilizes many of the same mechanisms that operate in arterial myocytes.

Vasoconstrictors such as NE bind to agonist receptors (ARs), which are G-protein coupled receptors (GPCRs) located in the myocyte PM (Fig. 1). This induces the phospholipase C-mediated synthesis of inositol trisphosphate (IP_3) and diacylglycerol (DAG). The IP_3 interacts with its receptors/channels (IP_3Rs) on the SR membrane, thereby releasing Ca^{2+} into the cytosol to activate contraction. Ca^{2+} (and Na^+) [50,51] can also enter the cytosol from the ECF through cation-selective ROCs (opened by DAG) and SOCs (opened by SR Ca^{2+} depletion). The Ca^{2+} can be re-sequestered in the SR by SERCA, or it can be extruded from the myocytes by ATP-driven PM Ca^{2+} pumps (PMCA). Importantly, Ca^{2+} can also either exit or enter the cells via the Na^+/Ca^{2+} exchanger (NCX) which is driven by the Na^+ electrochemical gradient across the PM under the control of the Na^+ pumps [52]. NCX uniquely links Na^+ metabolism to Ca^{2+} regulation and, thus, to arterial myocyte constriction. These mechanisms provide critical insight into question of how salt retention elevates BP.

6. Whole body autoregulation

A seminal advance in elucidating the pathophysiology of hypertension was the introduction of the concept of long-term[1] "whole body autoregulation" of blood flow [53] and its experimental verification [16,18,54,55]. These studies showed that salt retention and consequent plasma volume expansion initially elevates BP because of an increase in CO. With sustained volume expansion, even for just a few days, however, the CO declines and TPR increases to maintain the elevated BP. Thus, relatively normal CO and elevated TPR are routinely observed in established hypertension [11]. Nevertheless, in experimentally-induced hypertension, for example with mineralocorticoids [55] or renal artery clipping [56], a transient initial state of increased CO can often be detected. Failure to observe this high CO stage could be the result of compensatory mechanisms ("autoregulation") that turn on very shortly after the volume starts to expand. In most humans with (essential) hypertension, the salt retention and (tendency toward) volume expansion likely occur gradually, over days to years. In that case, the mechanisms that tend to lower plasma volume and CO, including the rise in TPR and pressure natriuresis [12,54,57], likely operate simultaneously to prevent an overt increase in CO. This corresponds to a condition of "virtual hypervolemia," however, because blood volume is still inappropriately high relative to the BP [21]. Importantly, the effects of volume expansion on TPR and BP are rapidly reversed if the stimulus (e.g., the volume load or the mineralocorticoid and salt) is withdrawn [54,55,57,58]. This implies that the (initial) rise in TPR must be functional and not structural, and it must almost certainly be hormonal because this "autoregulation" involves *all* of the vasculature, veins as well as arteries, and pulmonary as well as systemic vessels [59].

Despite the elapse of forty years since the demonstration of long-term autoregulation, efforts to elucidate the specific underlying mechanisms have been surprisingly scant. In the mid-1970's, we [60] and others [61] raised the possibility that an endogenous Na^+ pump inhibitor, i.e., a ouabain-like compound with vasotonic action, might be secreted in response to salt retention and plasma volume expansion. In other words, this substance might be a missing hormonal link between salt retention, and the increased TPR and hypertension. Strict conservation of the high affinity ouabain-binding site amino acid sequence throughout mammalian evolution implies that there must be an endogenous ligand that interacts with this site. We suggested that partial Na^+ pump inhibition by the endogenous inhibitor should promote the net gain of Ca^{2+} via the myocyte NCX, and thereby augment Ca^{2+} signaling and vasoconstriction [59,60]. The central roles of these three molecular entities, the endogenous Na^+ pump inhibitor, Na^+ pumps and NCX, is described below. New evidence that certain TRPC proteins, components of Ca^{2+}- and Na^+- permeable ROCs and SOCs [50,51], also make key contributions to the altered Ca^{2+} signaling [62], is discussed as well.

7. Endogenous ouabain and its receptor

The aforementioned ideas fueled the search for the postulated endogenous Na^+ pump inhibitor, a ligand for the pump's cardiotonic steroid (CTS) binding site, that might mediate the vascular response. In 1991, we purified endogenous ouabain (EO) from human plasma; the endogenous substance was identified as ouabain by mass spectroscopy [63]. It is now possible to quantitate EO by liquid chromatography-tandem mass spectroscopy (LC-MS-MS) methods [64] starting from small (1 ml) samples of human or animal plasma [65,66]. The idea that EO might be the 11β isomer of ouabain [67,68] has been excluded because the 11-epimers of ouabain are chromatographically different [69].

[1]This long-term, or day-to-day, whole body autoregulation, can be distinguished temporally and, therefore, almost certainly mechanistically, from the minute-to-minute autoregulation that maintains constant blood flow in, for example, the brain or kidney vasculature.

Rat adrenal cortex is highly enriched with EO, and human and cow adrenals also contain very high levels [63]. Bilateral adrenalectomy greatly reduces EO in rat plasma; conversely, treatment of uni-nephrectomized rats with DOCA (deoxycorticosterone acetate) + salt greatly increases plasma EO and elevates BP [63]. These findings indicate that EO is an adrenocortical hormone. Other reports, however, suggest that EO may also be synthesized in, and secreted by, the hypothalamus [70,71].

Numerous human and intact animal studies, as well as adrenocortical cell culture studies, indicate that EO is synthesized in the adrenal cortex, and that its synthesis and secretion is stimulated by adrenocorticotropic hormone (ACTH) [63,72-83]. In humans [79] and animals [72,75], ACTH-induced hypertension is associated with elevation of EO. Indeed, preliminary reports indicate that certain rare adrenocortical tumors, which are associated with severe hypertension, may produce prodigious amounts of EO [84,85]. In ACTH-induced hypertension [75,86], as well as in DOCA-salt hypertension [87] and reduced renal mass hypertension [88], BP is lowered by Digibind (digoxin-selective Fab fragments), which also binds ouabain with high affinity [89].

About 50% of humans with untreated essential hypertension and a majority of patients with adrenocortical adenomas and hypertension have significantly elevated plasma EO; moreover, BP correlates directly with plasma EO [90-93]. Even in normal human subjects, a high salt diet raises plasma EO [66], and a 10 min infusion of low dose ouabain increases vascular resistance and elevates BP for >60 min [94-96].

Critical support for the idea that EO might play a key role in the pathogenesis of hypertension was the demonstration that prolonged administration of ouabain to normal rats induces hypertension [97]. This observation has been replicated in many laboratories [98-100].

Plasma EO levels are elevated in several rodent salt-sensitive hypertension models [63,88, 101-103], and chronic administration of low dose ouabain to normal rodents usually induces hypertension in 1-3 weeks (Fig. 2) [90,97-99]. Also, sub-pressor doses of ouabain and DOCA act synergistically to induce hypertension [104]. Ouabain-induced BP elevation in rodents is counteracted by the ouabain antagonist, Rostafuroxin (PST-2238) [105,106], and hypertension induced by ACTH or DOCA+salt is antagonized by Digibind [75,86,87].

The aforementioned findings are strong evidence that circulating EO has a key role in the pathogenesis of salt-sensitive hypertension. Other studies suggest, however, that brain, not plasma, EO [70], or even marinobufagenin [70,107], may be important.

Interestingly, low-dose ouabain increases TPR in dogs, but doesn't raise BP, presumably because heart rate and CO are markedly reduced [108]. Ouabain also doesn't induce hypertension in sheep [109] or in mineralocorticoid-resistant [110] Long-Evans rats [111]. Such exceptions not only show that the genetic background is important, but may provide novel information to help clarify the relationship between EO and hypertension.

Na^+ pumps are widely accepted as *the* CTS receptor, but this greatly oversimplifies the situation. Na^+ pumps are αβ heterodimers. The catalytic subunit, α, contains the Na^+, K^+, ATP and ouabain binding sites, and is phosphorylated during each pump cycle. β is essential for pump function; it stabilizes the α subunit conformation and chaperones the αβ complex to the PM [112-114]. The 4 mammalian α subunit isoforms (α1-α4) are products of different genes, but have nearly 90% sequence identity; they have different expression patterns and different kinetics, and are differently regulated [112,115-121]. Many (most) cell types express Na^+ pumps with an α1 subunit *and* Na^+ pumps with a second α isoform [112,119,122]. Astroglia [123-125], endothelial cells [126], and all types of muscles [42,112,127-129] express Na^+ pumps with an α2 subunit as well as pumps with an α1; most neurons express α1 and α3

[112,123,125,129]. Renal epithelia express predominantly (>90-95%) Na^+ pumps with α1, which mediate the final step in net transepithelial Na^+ reabsorption [120,130].

The Na^+ pump α subunit CTS binding site has been highly conserved during the evolution of higher animals. Nevertheless, not all α subunit isoforms, nor the isoforms in all species, have the same high affinity for CTS. For example, rodent α1 Na^+ pumps have unusually low affinity for CTS [112,131]. Thus, it is important to understand better both the CTS and their interactions with their Na^+ pump α2 subunit binding sites.

8. The myriad uses and roles of cardiotonic steroids

Recorded use of CTS dates back more than 1500 years. CTS have been employed not only as diuretics and cardiotonics, but as emetics, as abortion agents, and as poisons. For more than two centuries following William Withering's classic clinical study [132], *Digitalis* glycosides were the drugs of choice for the treatment of congestive heart failure and certain cardiac arrhythmias.

Recently, the novel roles of CTS and the Na^+ pump in cancer therapeutics, and mood/behavioral [133] and neurological disorders [134] have been discussed. For example, one striking observation is that mortality from breast cancer was markedly reduced in patients on digitalis therapy [135]; this has prompted greatly renewed interest in CTS and their possible role in cancer therapy [136-140]. In addition, ideas about the action of EO as a natriuretic agent [60] have been revived [141,142]. Furthermore, many observations now indicate that EO also is a growth hormone: EO may participate in a variety of kinase-mediated and other signaling pathways, independent of its effects on Na^+ pump-mediated Na^+ transport [143-150]. This might contribute, for example, to the target organ damage that often occurs in hypertension.

9. Cardiotonic steroid structure-activity relationships: hypertensinogenic and anti-hypertensinogenic cardenolides

Cardiotonic steroids have been widely used clinically to treat heart failure and cardiac arrhythmias. It has long been accepted that the cardiotonic effect of CTS results from their ability to inhibit Na pumps (Na,K-ATPase) [151] and thereby promote Ca^{2+} entry via NCX [152,153]. The CTS include two structurally distinct groups – the cardenolides, in which the steroid is attached to a five member singly unsaturated lactone ring (Fig. 3, Table 1), and the bufadienolides, in which the lactone has six members and is doubly unsaturated. When one or more sugars are attached to the CTS at carbon 3, they are termed ‘cardiac glycosides’; common examples include ouabain and digoxin. With the exception of the bufanolide, proscillaridin, the steroid nucleus (aglycone) in the common bufadienolides is usually not glycosylated, but it may be conjugated with suberyl arginine or various other congeners.

Cardenolides and bufadienolides are synthesized in certain plants, some amphibians and insects, and possibly all higher animals. Crude as well as highly enriched extracts from plants and the parotid secretions of the toad have been used in homeopathic remedies to treat heart failure and some cancers, and as general tonics for metabolism and immune function, especially in China (e.g., *Chan Su*) and Japan (*Senso*). The advent of modern pharmacology, coupled with the desire to use purer preparations in therapy, led to extensive studies on the *Digitalis* and *Strophanthus* glycosides and their aglycones, and the more prominent entities in toad secretions, including bufalin and resibufagenin.

Most research on CTS, and on various natural and synthetic analogs, has focused on the positive inotropic response (enhanced contraction) of heart preparations, and on the inhibition of isolated kidney enzyme (Na,K-ATPase). Thus, the bulk of knowledge about the structure-

activity relationships is relevant to the heart, or to (renal) Na^+ pumps with an α1 catalytic subunit. Overall, the inotropic response appears to be correlated with the ability to bind and inhibit the Na^+ pump. Introduction of various substitutions in the steroid nucleus and lactone ring indicate that the configuration of the steroid is crucial for these effects.

The classic adrenocortical, ovarian and testicular steroids lack the cis-trans-cis fusion of the AB, BC, and CD rings found in the CTS (Fig. 3 and Table 1), and do not bind to, or inhibit, the Na^+ pump. Certain steroids with trans-trans-cis ring fusions are cardiotonic [154,155], while those with cis-trans-trans ring fusions (e.g., common bile salts, 14α-digitoxigenin and 14α-artebufogenin) are either inactive or very weak [156,157]. Addition of one or more sugars to the cardenolide steroid nucleus increases the potency, while inversion of the lactone configuration at C17 from β to α [158], or saturation (e.g., dihydroouabain and dihydrodigoxin) of the lactone ring, reduces the cardiotonic activity 10-30 fold. These fundamental relationships, obtained with cardiac preparations, have been widely assumed to be valid in other physiological systems.

Both ouabain and digoxin, when administered acutely *in vivo*, and often in high doses, induce vasoconstriction [95,159-164]. Nanomolar ouabain, however, augments myogenic constriction in rodent isolated arteries [36,42,65,165,166]. The first experimental evidence of a previously-unrecognized cardenolide structure-activity relationship was the observation that the prolonged administration of digoxin, also an Na,K-ATPase inhibitor [167], does *not* raise BP in normal rats, whereas ouabain does (Fig. 2) [168]. This result has been confirmed by several investigators [99,100,169,170]. Moreover, while digoxin itself does not raise BP [171], digoxin and a related CTS, digitoxin, are very effective in lowering the elevated BP in rats with ouabain-induced hypertension (Fig. 2) [99,100]. Importantly, digoxin also is known to lower BP in hypertensive humans [172]. These remarkable observations can only be explained by structural differences between the *Strophanthus* (e.g., ouabain) and *Digitalis* steroids, even though they are ostensibly similar Na^+ pump inhibitors. The sugar(s) is(are) not crucial for these effects: the aglycone of ouabain, ouabagenin (Table 1), is also pro-hypertensive [173], while Rostafuroxin, a derivative of digitoxigen [174], is anti-hypertensive in humans and rats [105,106]. Thus, differences in the steroid moieties of digoxin/digitoxin and ouabain account for their disparate effects on long term BP. Excluding the common oxygen at C3, ouabain is hydroxylated in positions 1,5,11,14 and 19, while digoxin is hydroxylated in positions 12 and 14 (Table 1). The major structural difference between the two steroids therefore lies in the extensive hydroxylation of ouabain in the A and B rings (and well away from the lactone ring) and the 12 hydroxyl group in digoxin. Like digoxin and ouabain, Rostafuroxin has a steroid nucleus that is cis-trans-cis fused and has a 14β hydroxyl group. However, it lacks the ouabain hydroxyls at positions 1,5,11 and 19 and the lactone has been replaced with a doubly unsaturated furane [174].

In sum, the key structural components that underlie the long term pressor activity of the cardenolides appear to include a steroid nucleus whose rings are fused in a cis-trans-cis configuration with oxygenation of the AB ring at C5. The depressor activity of the cardenolides appears to be linked with the cis-trans-cis steroid configuration, deoxygenation of the AB ring at C5 and substituents at C17 that augment potency as Na^+ pump inhibitors including unsaturated 5- and 6-member lactone rings. Many of the naturally occurring cardenolides are mixtures of structural features at opposite ends of the steroid nucleus that confer prolonged pressor and depressor activity *in vivo*. Synthetic analogs with either improved pressor or depressor activity, the latter exemplified by Rostafuroxin, may be of clinical relevance. Clearly, the physiology and pharmacology of these agents is still full of surprises.

9. The “PLasmERosome”: a structural basis for ouabain's action

The roles of the different α subunit isoforms were clarified by the discovery that, in the various cell types, Na^+ pumps with the α2 or α3 subunit were confined to PM microdomains situated adjacent to “junctional” sarco-/endoplasmic reticulum (jS/ER) (Fig. 1) [117,118]. Na/Ca exchangers are confined to the same PM microdomains (Fig. 1) [118], as are various TRPC proteins [175] that are components of ROCs and SOCs [176-178]. In contrast, Na^+ pumps with an α1 subunit are more ubiquitously distributed in the PM, but are apparently excluded from these PM microdomains [122,179,180]. The functional as well as structural interrelationship of these transport proteins is supported by the remarkable observation that α2 (but not α1) Na^+ pumps, NCX1, and TRPC6 and -1, are all upregulated by prolonged ouabain administration, both *in vivo* and *in vitro* [176].

The PM microdomains are separated by only 12-20 nm from the jS/ER [181], and these structures form a functional unit, the “PLasmERosome” [182,183]. The volume of cytosol in the junctional space (J) between the PM and jS/ER of a single PLasmERosome (Fig. 1) is on the order of only 10^{-19} to 10^{-18} liters, and diffusion of Na^+ and Ca^{2+} between this space and bulk cytosol is restricted. Thus, standing Na^+ and Ca^{2+} concentration gradients between these compartments and bulk cytosol can be maintained [51,127,131,179,184,185].

Differences in Na^+ pump α subunit isoform kinetics are the key to PLasmERosome function. Rodent α1 has an unusually low affinity for ouabain ($K_{Ouabain}$ > 100 μM, vs < 0.05 μM in humans) [112,131]; thus, nanomolar ouabain inhibits only the α2 Na^+ pumps in rodent arterial myocytes. Even in humans, however, where α1 Na^+ pumps have high affinity for ouabain, partial inhibition of Na^+ pumps by nanomolar ouabain will elevate $[Na^+]$ in the junctional space much more than in bulk cytosol. The reason is that the affinity of α2 Na^+ pumps for Na^+ is much lower ($K_{Na} \approx 22$ mM) than is the affinity of α1 Na^+ pumps ($K_{Na} \approx 12$ mM) [121].

The broad distribution of α1 Na^+ pumps implies that they control, primarily, $[Na^+]$ in bulk cytosol. In contrast, pumps with an α2 (in smooth muscle, for example) or α3 catalytic subunit regulate *local* $[Na^+]$ in the junctional space. Thus, these α2/α3 Na^+ pumps control the local Na^+ electrochemical gradient that influences Ca^{2+} transport by the adjacent NCX. The ROCs and SOCs located here (Fig. 1) are cation-selective channels that admit Na^+ as well as Ca^{2+} [50]. This organizational arrangement (Fig. 1) links Na^+ metabolism to cell Ca^{2+}. Thus, the transporters in the PLasmERosome regulate not only $[Ca^{2+}]$ in the junctional space, but S/ER Ca^{2+} stores and global Ca^{2+} signaling in the cells as well [182,183]. Therefore, modulation of α2 Na^+ pumps in arterial myocyte PLasmERosomes by EO can influence arterial tone and BP. In the ensuing discussion we summarize data from recent experiments in which genetic engineering and pharmacological manipulation of mouse Na^+ pumps and NCX have been used to examine the roles of these transporters in the long-term control of BP.

10. How does ouabain (EO) elevate blood pressure? The downstream effector mechanisms

α2 Na^+ Pumps

The fact that chronic administration of exogenous ouabain induces hypertension in rodents has already been mentioned. The questions we now address are: How does ouabain (or EO) elevate BP? Is it due to inhibition of smooth muscle α2 Na^+ pumps, as implied by the preceding discussion?

We have reported that acute application of nanomolar ouabain to isolated, pressurized rodent arteries with myogenic tone augments the tone. The EC_{50} is on the order of 1 nM ouabain in intact arteries, and even lower in arteries without endothelium [36,42].

If circulating ouabain (or EO) elevates BP by inhibiting arterial smooth muscle (ASM) α2 Na^+ pumps, reduced expression of α2 Na^+ pumps should have a similar effect. Indeed, mice with a null mutation in one α2 Na^+ pump allele ($\alpha2^{+/-}$) [128] express ~50% of the normal complement of α2 in arteries [36,127] and have elevated BP (Fig. 3) [36,165]. Mice with a null mutant α1 allele ($\alpha1^{+/-}$) express half the normal complement of α1 Na^+ pumps, but have normal BP (Fig. 4) [36,165]. Moreover, mesenteric small arteries from the $\alpha2^{+/-}$, but not $\alpha1^{+/-}$ mice, exhibit augmented myogenic reactivity and myogenic tone (MT).[36] The $\alpha2^{+/-}$ mice are also "salt-sensitive": a high salt diet increases BP much more in these mice than in their wild type littermates (Fig. 4).

The $\alpha2^{+/-}$ mice are "global" single allele null mutants, but it is important to determine if the effects are the result of reduced α2 Na^+ pump activity/expression in ASM. Recently, we found that expression of a short N-terminal segment of the α2 Na^+ pump was dominant negative (DN) for expression of full-length α2 pumps [180]. Therefore, we generated mice ($\alpha2^{SM/DN}$) that expressed the N-terminal segment with a smooth muscle (SM)-specific myosin heavy chain promoter [186]. These mice, with greatly reduced α2 Na^+ pump expression in artery smooth muscle, have elevated BP (Fig. 4). Conversely, mice that overexpress α2, but not those overexpress α1, Na^+ pumps in smooth muscle, have, on average, significantly reduced BP compared to wild type (WT) mice (Fig. 4) [187].

The roles of ouabain/EO and α2 Na^+ pumps in elevating BP was also examined in two other ways. We tested Rostafuroxin, which antagonizes the inhibitory action of ouabain on Na,K-ATPase [188]. In isolated arteries, Rostafuroxin counteracts the augmentation of MT by nanomolar ouabain, but not the (ouabain-independent) augmenting effect of reduced α2 expression on MT [36]. Rostafuroxin also lowers BP in ouabain-induced hypertension [105, 106] and in nearly 50% of humans with essential hypertension [105].

Alternatively, mice that expressed mutant, ouabain-resistant α2 pumps ($\alpha2^{R/R}$) [75,115,189] are resistant to ACTH-induced hypertension [75,115] as well as to ouabain-induced hypertension [189]. These results demonstrate that ACTH-induced and ouabain-induced hypertension depend a high-affinity cardiotonic steroid binding site on the α2 Na^+ pump. The hypertension also depends upon a water-soluble ligand that binds to this site because the plasma level of this ligand (presumably EO) is increased by ACTH and, like ouabain [89], bind to Digibind with high affinity [189].

The studies on mice with genetically altered α2 Na^+ pumps reveal that arterial myocyte α2 Na^+ pumps mediate the effects of EO and play a role in the long-term regulation of BP. Genetically or pharmacologically reduced α2 activity elevates BP, whereas increased α2 activity lowers BP. It is not yet clear, however, how to reconcile these results with the evidence that isoouabain, with a saturated lactone ring tethered to C14 of steroid ring D, is hypertensinogenic, but a poor inhibitor of α1 Na^+ pumps [173]. One possibility is that CTS structure-function relations may be different for α1 and α2 Na^+ pumps.

NCX Type-1

The next question is: By what specific mechanism does the altered α2 Na^+ pump activity influence BP? The answer appears to lie in Na/Ca exchange, which *directly* links Na^+ to Ca^{2+} metabolism and is a distal regulator of cytosolic Ca^{2+}. There are two classes of Na/Ca exchangers, those that co-transport K^+ with Ca^{2+} (NCKX), and those that do not (NCX) [190]. Although NCKX has been found in ASM [191]. the dominant exchanger in arterial myocytes is NCX. There are three mammalian NCX isoforms (NCX1-NCX3), each the product of a different gene [192]. NCX1, which is expressed in ASM, has multiple splice variants; NCX1.3 is the dominant variant in ASM [193].

Studies on primary cultured rat arterial myocytes indicated that inhibition of Na^+ pumps by nanomolar ouabain augments Ca^{2+} signaling without elevating bulk cytosolic Na^+ [182]. Even knockout of α2 Na^+ pumps in cultured cells (astrocytes) had only minimal effect of bulk cytosolic Na^+, but a large effect on Ca^{2+} signaling [194]. These results are consistent with a functional linkage between α2 (but not α1) Na^+ pumps and NCX1, and *local* reduction of the trans-PM Na^+ gradient when α2 activity is reduced, as implied by the PLasmERosome model (Fig. 1). Also, recent pharmacological and genetic engineering studies reveal that NCX1 influences not only arterial myocyte Ca^{2+} metabolism, but long-term vascular tone and BP as well.

Mice in which NCX1 is overexpressed in smooth muscle ($NCX1^{SM/Tg}$) have elevated BP that is markedly increased by a high salt diet (i.e., the mice are "salt-sensitive") (Fig. 4) [166]. The elevated BP in the NCX1 overexpressors on high dietary salt is abolished by SEA0400, a selective NCX1 inhibitor [195], but not if the overexpressed NCX1 contains a G833C mutation [166], which specifically antagonizes the action of SEA0400 [196].

To perform the converse experiment, mice with floxed NCX1 ($NCX1^{flx/flx}$) [197] were crossed with mice containing a Cre recombinase gene under the control of the smooth muscle myosin heavy chain promoter [186] to generate smooth muscle-specific NCX1 knockout ($NCX1^{SM-/-}$) mice. These $NCX1^{SM-/-}$ mice have abnormally low blood pressure (Fig. 4), and isolated, pressurized small arteries from these mice have abnormally low MT[198]. Indeed, SEA0400 also lowers BP by about 5-10 mm Hg in WT mice [166] and reduces MT by about 10% in isolated arteries from these mice [36,166]. Thus, NCX1 activity apparently makes a small, but direct, contribution to normal MT and BP. SEA0400 also attenuated the increased MT in arteries from $\alpha2^{+/-}$ mice [36], indicating that NCX1 mediates effects that are distal to those of the α2 Na^+ pumps. The BP and MT data from $\alpha2^{+/-}$ and $NCX1^{SM-/-}$ mice support the view that MT in isolated arteries is an *in vitro* reflection of BP [34] and, most likely, TPR.

The mice with genetically engineered NCX1 demonstrate that this exchanger contributes to long-term BP regulation: increased NCX1 expression increases BP, while knockout of NCX1 reduces BP. This view is also supported by the effects of NCX blockers in several rodent models of salt-dependent or ACTH-induced hypertension. In DOCA+salt hypertensive rats, spontaneously hypertensive rats (SHR) on a high salt diet, and Dahl salt-sensitive rats on high salt, SEA0400 markedly reduced BP [166]. Also, KB-R7943, a less potent blocker, prevents ACTH from elevating BP in mice.[75] Moreover, although a null mutation in one NCX1 allele has negligible effect on BP ($NCX^{+/-}$ in Fig. 4) or MT[198], it does prevent the induction of hypertension by DOCA+salt [166]. Importantly, SEA0400 does not lower BP in several *salt-independent* rat hypertension models: SHR on a normal salt diet, stroke prone-SHR, and the renin-dependent two-kidney/one-clip rat [166]. The implication is that NCX1 contributes to the pathogenesis of salt-dependent hypertension, but not to salt-independent hypertension. As detailed elsewhere [65], these findings reveal that NCX1, along with SACs and L-type VGCs, contribute to the elevated $[Ca^{2+}]_{CYT}$ that generates and maintains MT and, thus, influences TPR and BP.

11. The central role of Ca^{2+} signaling

At the outset, we noted that arterial myocyte contraction depends, ultimately, upon the availability of cytosolic Ca^{2+}, and the sensitivity of the contractile apparatus to that Ca^{2+}. NCX1, under the control of the Na^+ gradient generated by the adjacent α2 Na^+ pumps, helps regulate myocyte Ca^{2+} homeostasis (Fig. 1). For example, nanomolar ouabain-induced increases in MT are associated with increases in myocyte $[Ca^{2+}]$ [36]; conversely, reduction of MT by SEA0400 is associated with reduced myocyte $[Ca^{2+}]$ [166]. Thus, α2 Na^+ pumps and NCX1 are relatively distal mechanisms in the final common path that links salt to

vasoconstriction and hypertension. Indeed, *all upstream vasoconstrictor and vasodilator mechanisms (neural and humoral) must, inevitably, be influenced by the activity of these two transporters* [165].

As an alternative, it was recently suggested that activation of Rho/Rho kinase via the G_{12}-G_{13}-mediated G protein-coupled receptor pathway, which modulates the Ca^{2+} sensitivity of the contractile apparatus in ASM, is selective for salt-dependent hypertension [199]. Interference with the G_{12}-G_{13} pathway, however, whether at the agonist receptor level [200], or at the level of Rho kinase [201], also lowers BP in salt-independent models such as the stroke-prone spontaneously hypertensive rat (SPSHR) [200] and the NO synthase-inhibited rat [201]. In our view, the findings of Wirth and colleagues [199] better fit the view that, once salt-sensitive NCX1-mediated Ca^{2+} entry has occurred [65], the G_{12}-G_{13} pathway becomes a critical determinant of the increases in vascular tone and BP. The G_{12}-G_{13} pathway is, therefore, downstream, and distinct from the key salt-sensitive steps in Na^+-dependent hypertension.

12. Acute versus chronic effects of ouabain on the vasculature and blood pressure

Much of the preceding discussion concerns, primarily, the acute actions of ouabain on the vasculature. Nanomolar ouabain increases the myogenic reactivity of normal rodent arteries with a time course of seconds to minutes, and with an apparent EC_{50} (concentration for half-maximal effect) of 0.6-1.3 nM [36,42]. A comparable effect is observed in arteries isolated from rats with ouabain-induced hypertension [42]. Nevertheless, *in vivo* ouabain administration (~15-30 μg/kg/day), whether by injection, subcutaneous pellet, or osmotic minipump, elevates BP very slowly. BP usually rises with a delay, and takes about 14-21 days to plateau (Fig. 2) [97,99]. A likely explanation for this slow rise, despite the increased myogenic reactivity, is that normal feedback mechanisms defend the BP and counteract the elevation. Important examples include the baroreceptor reflex and local endothelium-initiated vasodilator mechanisms [33]. With maintained administration, however, the BP slowly begins to rise (Fig. 2) [97,99] as the chronic effects of ouabain become manifest and feedback controls are down-regulated or reset.

Ca^{2+} signaling is altered by prolonged ouabain treatment in both arterial smooth muscle and endothelium, but in different directions. Arterial smooth muscle from rats with ouabain-induced hypertension exhibits up-regulation of the protein components of the "Ca^{2+} signaling pathway" that includes the α2 Na^+ pumps, NCX1, and TRPC6 and TRPC1 (the latter are components of ROCs and SOCs, respectively; see Fig. 2) [176]. The consequently enhanced Ca^{2+} signaling further augments myogenic reactivity and vasoconstrictor-evoked responses [42].

Acute administration of low dose ouabain also promotes Ca^{2+} signaling in the endothelium, and thereby augments vasodilator mechanisms such as the response to acetylcholine [126]. Importantly, however, these endothelial mechanisms are impaired in arteries from rats with ouabain-induced hypertension [126]. In other words, at the local (vascular) level, prolonged exposure to ouabain enhances the vasoconstrictor mechanisms in the arterial smooth muscle while, simultaneously, downregulating the endothelial feedback mechanisms that normally help prevent the BP from rising. The net effect, of course, is the development of hypertension.

These findings may have much broader relevance to the pathogenesis of hypertension. In many forms of human and animal hypertension, including the DOCA-salt model and the Dahl salt-sensitive model (both of which are associated with high EO levels), endothelial vasodilator

mechanisms [202-205] and baroreflexes [206-208] are impaired, while vasoconstrictor responses are augmented [44,203,205].

13. Coda

In this review, we have explored some of the critical steps that link salt retention to the elevation of BP. Recent results, especially those from chemical analyses of human and rodent plasma samples, and from genetic engineering and pharmacological studies in rodents and rodent arteries, are summarized above. These studies give new insight into some of the molecular events that help regulate cytosolic Ca^{2+} and vascular tone. The data provide compelling evidence that EO, smooth muscle α2 Na^+ pumps, NCX1, and TRPC channel proteins, are key molecular links in the pathway that leads from salt retention to hypertension.

These findings provide a framework, but the story is far from complete. A key area where knowledge is lacking is at the early steps between plasma volume expansion and the release of EO. The astonishing difference between the actions of ouabain and digoxin on BP demonstrate that cardenolide structure-activity relationships need to be better understood. Even the central role of the kidneys is still not completely resolved: For example, the renal and extra-renal arteries make apparently independent (and equal) contributions to the long-term regulation of BP [34,209], but how the distal mechanisms, discussed above, affect the renal and extra-renal vasculature and renal function, and thereby contribute to BP control, is still unexplored. The progress outlined here should help identify new directions for hypertension research to resolve these issues.

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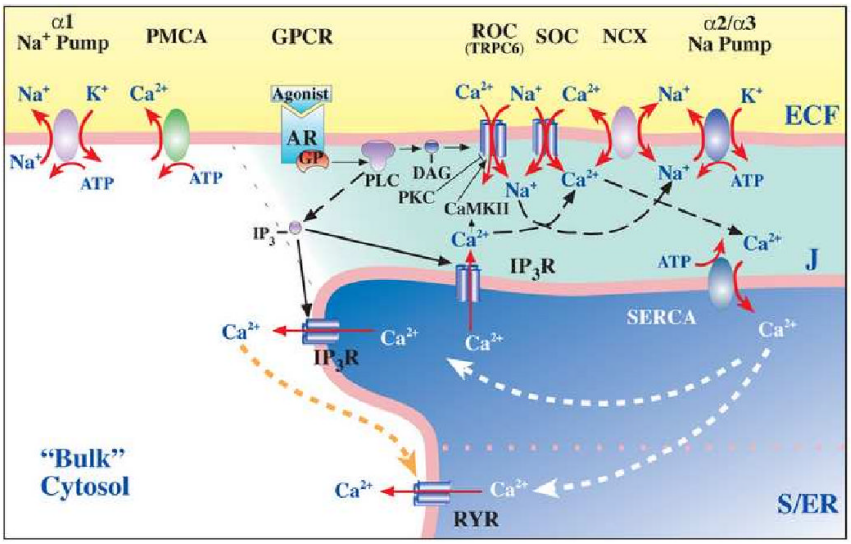

Fig. 1.

Model of the plasma membrane-junctional sarco-/endoplasmic reticulum (PM-jS/ER) region, the PLasmERosome, showing the location of key transport proteins involved in local control of jS/ER Ca^{2+} stores and Ca^{2+} signaling. The PLasmERosome consists of a PM microdomain, the adjacent jS/ER (with SERCA, IP_3R and ryanodine receptors, RYR), and the intervening 'diffusion-restricted' junctional space (J). The PM microdomain contains agonist receptors, ARs (GPCRs), ROCs and SOCs (composed of various transient receptor potential channel proteins or TRPCs), α2 (in smooth muscle) or α3 Na^+ pumps, and NCX. Activation of GPCRs and release of G-proteins (GPs) stimulates phospholipase C (PLC) to produce IP_3 and diacylglycerol (DAG). DAG may activate ROCs directly. Na^+ may enter locally, through ROCs, SOCs or, perhaps, SACs (not shown) to promote Ca^{2+} entry via NCX. Shading indicates relative Na^+ and/or Ca^{2+} concentrations. Other regions of the PM contain α1 Na^+ pumps and PMCA. Other abbreviations are defined in the text. Reprinted with permission [184].

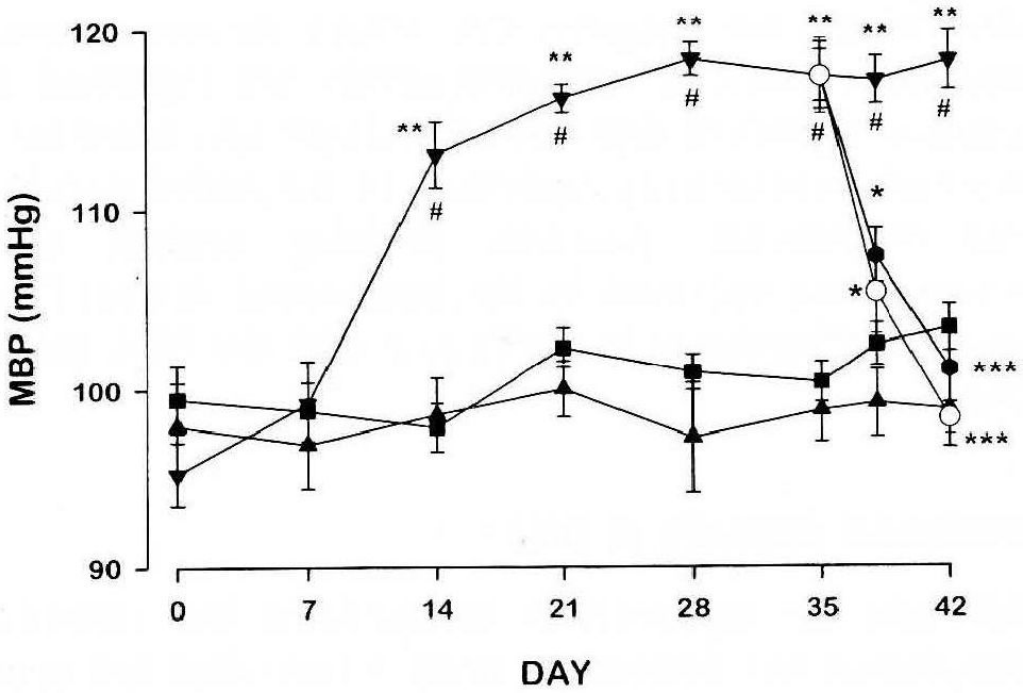

Fig. 2.

Oubain, but not digoxin, induces hypertension; digoxin and digitoxin reverse ouabain-induced hypertension. Rats were infused with vehicle (■), ouabain, 15 μg/kg/day (▼), or digoxin, 30 μg/kg/day (▲), for 42 days. From days 35 to 42, three groups of 8 ouabain-infused rats received a secondary infusion of digoxin, 30 μg/kg/day (●), digitoxin, 30 μg/kg/day (○), or vehicle (▼). Mean blood pressures (MBP) were obtained by tail cuff at weekly intervals or as indicated. **P*<0.05 vs ouabain; ****P*<0.001 vs ouabain; #*P*<0.005 vs vehicle; ***P*<0.001 vs digoxin. Reprinted with permission [99].

Effects of Ouabain, Digoxin and Digitoxin on BP

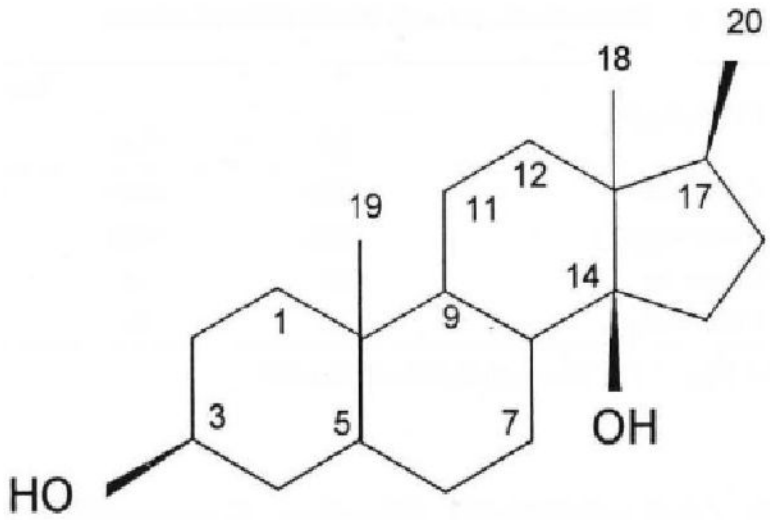

Fig. 3.

Prototypical cardenolide steroid skeleton. The primary feature is a steroid skeleton with the rings fused in a cis-trans-cis arrangement. The cardenolides discussed here have a 14βOH, an unsaturated lactone ring attached via C17 in the β configuration, and a methyl group at C18. When present, sugars are attached via the steroid 3βOH group. See Table 1 for the list of substituents in ouabain, ouabagenin, digoxin, digitoxin and Rostafuroxin. Reprinted with permission [99].

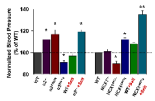

Fig. 4.

Relative blood pressures of mice with genetically-engineered α2 Na^+ pumps and NCX1. The data from several sources, are normalized to the BPs of the respective control wild type (WT) mice. Mice with a null mutation in one α2 Na^+ pump allele ($\alpha2^{+/-}$) [36] or smooth muscle-specific α2 knockdown ($\alpha2^{SM/DN}$) (Song, Chen, Zhang, Lee, Kotlikoff and Blaustein, unpublished), or increased smooth muscle-specific NCX1 overexpression ($NCX1^{SM/Tg}$) [166], had significantly elevated BP. A high salt diet augmented the elevated BP in $\alpha2^{+/-}$ mice (4% NaCl × 2 weeks) and $NCX1^{SM/Tg}$ mice (8% NaCl + 1% NaCl in tap water × 4 weeks). Smooth muscle-specific overexpression of α2 Na^+ pumps ($\alpha2^{SM/Tg}$)[187] or knockdown of NCX1 ($NCX1^{SM-/-}$) [198] significantly reduced BP. * = $P < 0.05$, ** = $P < 0.01$ vs WT or the respective genotypes on a normal (0.5%) salt diet. Reprinted with permission [65].

Table 1

Some relevant cardenolides and their substituents.

Compound	Steroid substituent[1]						Sugar at C3
	1β	5β	11α	12β	17α	19	
Ouabain	OH	OH	OH	H	H	CH_2OH	Rhamnose
Ouabagenin	OH	OH	OH	H	H	CH_2OH	None
Digoxin	H	H	H	OH	H	CH_3	Digitoxose (3)
Digitoxin	H	H	H	H	H	CH_3	Digitoxose (3)
Digitoxigenin	H	H	H	H	H	CH_3	None
Rostafuroxin	H	H	H	H	OH	CH_3	None

[1]The common cardenolides have a 5-member singly-unsaturated lactone ring in the 17β position, but Rostafuroxan has a doubly-unsaturated furane substutent at this position.

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