Advances in understanding the role of cardiac glycosides in control of sodium transport in renal tubules

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Abstract

Cardiotonic steroids have been used for the past 200 years in the treatment of congestive heart failure. As specific inhibitors of membrane-bound Na^+/K^+ ATPase, they enhance cardiac contractility through increasing myocardial cell calcium concentration in response to the resulting increase in intracellular Na concentration. The half-minimal concentrations of cardiotonic steroids required to inhibit Na^+/K^+ ATPase range from nanomolar to micromolar concentrations. In contrast, the circulating levels of cardiotonic steroids under physiological conditions are in the low picomolar concentration range in healthy subjects, increasing to high picomolar levels under pathophysiological conditions including chronic kidney disease and heart failure. Little is known about the physiological function of low picomolar concentrations of cardiotonic steroids. Recent studies have indicated that physiological concentrations of cardiotonic steroids acutely stimulate the activity of Na^+/K^+ ATPase and activate an intracellular signaling pathway that regulates a variety of intracellular functions including cell growth and hypertrophy. The effects of circulating cardiotonic steroids on renal salt handling and total body sodium homeostasis are unknown. This review will focus on the role of low picomolar concentrations of cardiotonic steroids in renal Na^+/K^+ ATPase activity, cell signaling, and blood pressure regulation.

Key Words
- ion channels
- steroids
- renal
- physiology
- hypertension

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Introduction

Physiological concentrations of cardiotonic steroids play a role in sodium homeostasis. However, the mechanisms are unknown and a subject of current investigation in several laboratories. Cardiotonic steroids were discovered as specific inhibitors of the sodium pump, Na^+/K^+ ATPase. However, the circulating levels, at in picomolar concentrations, are insufficient to inhibit Na^+/K^+ ATPase. Endogenous cardiotonic steroids have been implicated in several forms of hypertension (Blaustein *et al.* 2009, Hamlyn & Manunta 2011), including hypertension associated with chronic kidney disease. The hypertensive effects of cardiotonic steroids on blood pressure have been attributed to their effects on vasculature and CNS centers that control sodium homeostasis and have been the subject of several recent excellent review articles (Tian & Xie 2008, Lingrel 2010, Blaustein *et al.* 2012). In vascular and cardiac cells, binding of cardiotonic steroids leads to a decrease in Na^+/K^+ ATPase activity and an increase in cytosolic Na^+ concentration ($[Na^+]$cyt). The increase in $[Na^+]$cyt results in reverse mode action of the Na^+/Ca^{2+}

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exchanger, which would cause an increase in cytosolic calcium that will in turn cause increased contraction and an increase in blood pressure (Askari *et al*. 1988, Blaustein *et al*. 2006). The positive ionotropic effects of digitalis and its derivatives on cardiac sarcolemma were described by Klaus & Lee (1969) and Lee & Klaus (1971). Akera and colleagues demonstrated that enhanced intracellular sodium transients caused by the positive ionotropic effects of digitalis and its derivatives would increase intracellular calcium, and hence, increase myocardial contractility (Akera 1977, Akera & Brody 1977, Brody & Akera 1977, Choi & Akera 1977, Weaver *et al*. 1977, Akera & Ng 1991). Studies from Askari's laboratory experimentally showed ionotropic effects of cardiotonic steroids on cardiac myocytes. They demonstrated that treatment of cardiac myocytes increases intracellular calcium and activates a signaling cascade that involves production of ROS and activation of the EGFR–Src kinase pathway leading to cardiac hypertrophy (Xie & Askari 2002, Mohammadi *et al*. 2003). This simplistic view was later on modified by the Blaustein hypothesis of the 'Plasmerosome'. He argued that in vascular smooth muscle, sarcoplasmic reticulum (SR) components called 'junctional SR' are close to the plasma membrane (PM) and this association forms a 'restricted cytoplasmic space' between the PM and SR. He called this complex of junctional SR, the adjacent PM, and the restricted cytoplasmic space the plasmerosome, which that contains the highly sensitive Na^+/K^+ ATPase α3 or α2 and the Na^+/Ca^{2+} exchanger. The plasmerosome has very high Na^+/K^+ and Ca^{2+} concentrations. The hypothesis suggests that very low levels of circulating endogenous cardiotonic steroids modify the ionic balance in the plasmerosome, causing changes in contraction and development of blood pressure (Blaustein *et al*. 1998). However, this hypothesis has been proven only in rodents, which express a less sensitive Na^+/K^+ ATPase α1-subunit unlike humans and other species that express a highly ouabain-sensitive α1. Therefore, it remains to be determined whether different Na^+/K^+ ATPase isoforms play such different roles in cardiotonic steroid-mediated blood pressure development in humans. This review highlights recent exciting studies investigating the effects of physiological and pathophysiological concentrations of cardiotonic steroids on renal salt reabsorption (Aizman *et al*. 2001, Abramowitz *et al*. 2003, Aizman & Aperia 2003, Contreras *et al*. 2006, Khundmiri *et al*. 2006, 2007, 2014, Aperia 2007, Nguyen *et al*. 2007, 2011, Holthouser *et al*. 2010, Jansson *et al*. 2012, Bignami *et al*. 2013, Blanco & Wallace 2013).

Cardiotonic steroids: structure, synthesis, and hypertension

Cardiotonic steroids are a family of compounds that are referred to variously as cardiac glycosides, ouabain, digoxin, digitalis, bufadienolides, endogenous ouabain (EO), ouabain-like factor (OLF), and digoxin-like factor (DLF or DLIF) to name a few. For the remainder of the review, the term cardiotonic steroids will be used, unless referring to a specific member of the cardiotonic steroid family. The structural features important for the action of cardiotonic steroids are a cyclopentaphenanthrene nucleus with AB cis, BC trans, and CD cis, a C-14 hydroxyl group, and an unsaturated lactone ring in the β-configuration. The lactone rings are hydroxylated at positions 1, 5, 11, 14, and 19 for ouabain while digoxin is hydroxylated at position 14 of the lactone ring. In many plant-derived cardiotonic steroids, a sugar group is present at C3 (Fig. 1). Ouabain has a rhamnose sugar group while digoxin and digitoxin contain digitoxose. The sugar moiety does not appear to play a role in the effects of cardiotonic steroids on salt homeostasis (Goto *et al*. 1992, Blaustein & Hamlyn 2010). For example, similar to ouabain, ouabagenin binds with the same affinity to Na^+/K^+ ATPase (Yoda & Yoda 1981). Data from our laboratory indicate that treatment with picomolar concentrations of ouabagenin for 24 h increases Na^+/K^+ ATPase activity in human kidney proximal tubule cells similar to picomolar concentrations of ouabain (Fig. 2). Based on their origin, cardiotonic steroids are divided into plant-derived cardenolides (e.g., ouabain, digoxin, and digitalis) and animal-originated bufadienolides (e.g., marinobufagenin and telocinobufagin) (Bagrov & Shapiro 2008). Plant-derived cardiotonic steroids contain a five-membered lactone ring while the cardiotonic steroids of animal origin contain a six-membered lactone ring (Schoner & Scheiner-Bobis 2005). In humans, one cardiotonic steroid isolated from adrenal glands and plasma appears to be identical to plant-derived ouabain by NMR and mass spectroscopy (Nicholls *et al*. 2009) and is designated as EO.

Several investigators have demonstrated that cardiotonic steroids are synthesized and/or stored in the adrenal glands (Boulanger *et al*. 1993). Interestingly, both orally and parenterally administered cardiotonic steroids are taken up by the adrenal glands. The intestines only absorb 3–5% of orally administered cardiotonic steroids (Greeff & Fox 1984, Boulanger *et al*. 1993, Kitano *et al*. 1998). The mechanism of regulation of this process is unknown, as is the contribution of absorbed cardiotonic steroids to circulating plasma levels. Consistent with the hypothesis

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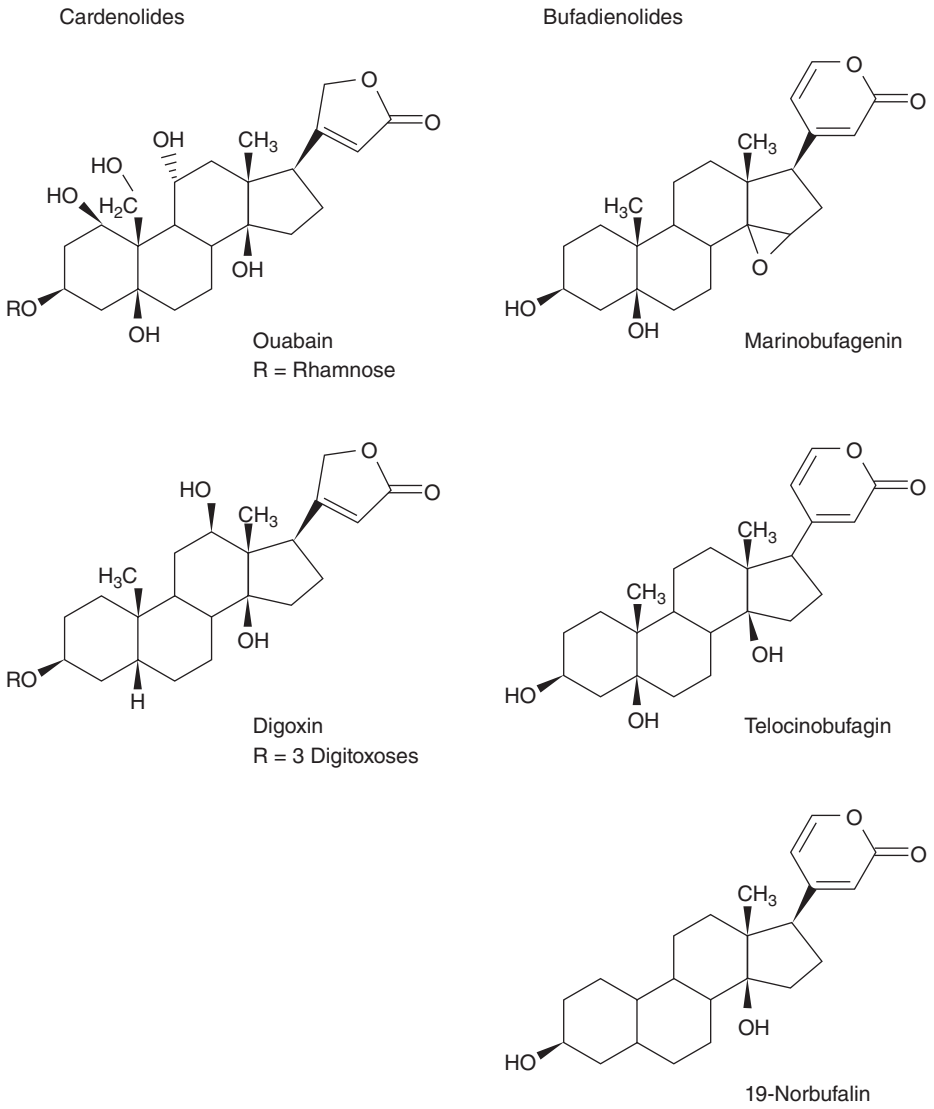

Figure 1
Structure of the common cardiotonic steroids.

that cardiotonic steroids are synthesized in the adrenal glands, adrenalectomy has been demonstrated to decrease circulating levels of cardiotonic steroids (Masugi *et al.* 1988, Hamlyn *et al.* 1991). In patients with adrenal tumors, the presence of high blood pressure appears to correlate with elevated circulating levels of cardiotonic steroids, as both blood pressure and circulating cardiotonic steroid concentrations were normalized after removal of the adenomas (Doris *et al.* 1989, Komiyama *et al.* 1999, Schoner & Scheiner-Bobis 2005). Synthesis of cardiotonic steroids in the adrenal glands, hypothalamus, and cells cultured from bovine adrenal glands and rat pheochromocytoma has been demonstrated (Hamlyn *et al.* 1991, Mathews *et al.* 1991, Schneider *et al.* 1998*a*,*b*, Kawamura *et al.* 1999, Komiyama *et al.* 2001). The synthesis of endogenous cardiotonic steroids is upregulated by ACTH, alpha adrenergic and dopaminergic stimulation, angiotensin II through stimulation of AT2 receptor, hypoxia, and physical exercise (el-Masri *et al.* 2002, Schoner 2002, Qazzaz *et al.* 2004, Dmitrieva *et al.* 2005, Schoner & Scheiner-Bobis 2005, 2007*a*, *b*, 2008, Loreaux *et al.* 2008, Lorenz *et al.* 2008, Manunta *et al.* 2010).

The role of nanomolar concentrations of cardiotonic steroids in the development of hypertension, regulation of blood pressure, salt homeostasis, and risk of cardiovascular disease (CVD) has been extensively studied and reviewed

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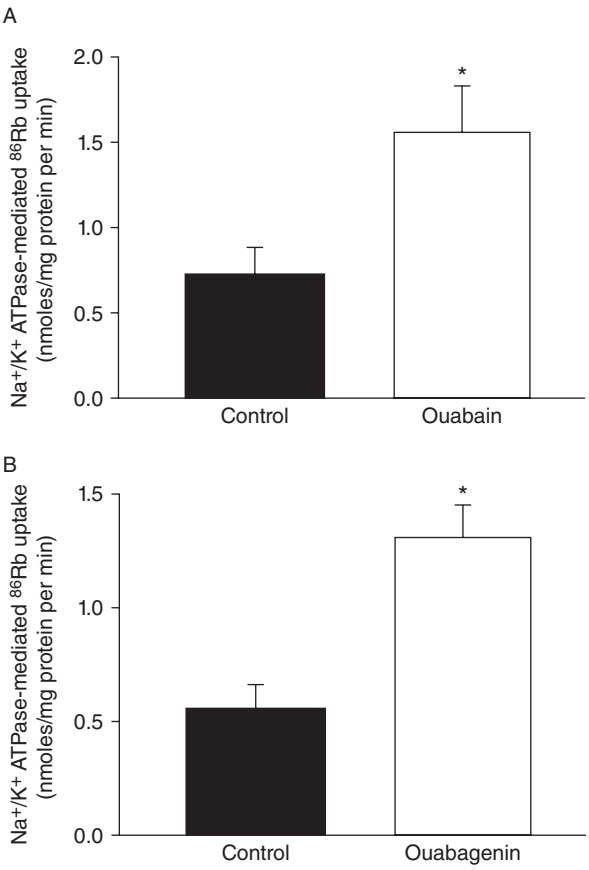

Figure 2
Effect of ouabagenin on Na^+/K^+ ATPase activity in human kidney proximal tubule cells. Human kidney cells (HKC11) were treated with 10 pM ouabagenin or 10 pM ouabain for 24 h. Na^+/K^+ ATPase-mediated ^{86}Rb uptake was measured as ouabain-sensitive rubidium uptake. The results demonstrate that both ouabagenin and ouabain increase Na^+/K^+ ATPase activity in HKC11 cells. Each bar indicates values as mean ± S.E.M. from six individual experiments ($n=6$). *$P<0.05$ by the Student's *t*-test.

(Schoner & Scheiner-Bobis 2007*a*,*b*, Fedorova *et al.* 2010*a*,*b*, Lingrel 2010, Liu & Xie 2010). The average circulating level of cardiotonic steroids in normotensive controls is 253 pmol/l (30–600 pmol/l). The concentrations of cardiotonic steroids have been shown to increase to approximately 930 pmol/l (300–1700 pmol/l) in patients with hypertension, CVDs, cardiorenal syndrome, and chronic kidney disease (Mohmand *et al.* 2005, Kennedy *et al.* 2006, Stella *et al.* 2008), which is higher than in healthy subjects but still significantly lower than the levels achieved with pharmacological doses of digoxin and also lower than the levels required to inhibit Na^+/K^+ ATPase significantly. The correlation between levels of cardiotonic steroids and CVD indicates a potential pathophysiological role of cardiotonic steroids in the development of these disorders.

Na^+/K^+ ATPase structure and function

All cells maintain a cytoplasmic environment that is low in sodium and high in potassium. Studies in the early 1950s using red blood cells solved the mystery of how this ionic gradient is maintained (Glynn 1956). In 1957, Skou discovered Na^+/K^+ ATPase to be the enzyme responsible for the maintenance of these ion gradients for which he was honored with the Nobel Prize in 1997 (Skou 1957). Na^+/K^+ ATPase uses the energy from hydrolysis of ATP to transport three Na^+ out of the cell and two K^+ into the cell (Skou & Esmann 1992). Since its discovery, a tremendous scientific effort has been devoted to the understanding of the structure and function of this enzyme due to its physiological importance. The ionic gradients created by Na^+/K^+ ATPase create a membrane potential in the excitable cells of the nervous system and muscles. Na^+/K^+ ATPase also acts as an energy transducer by converting the chemical energy of ATP to create ionic concentration gradients. This gradient is utilized by cells to energize the transport of various ions and solutes such as glucose, amino acids, and neurotransmitters across the membrane against their chemical gradients, for absorption of nutrients from intestines and to regulate cell volume (Martin 2005). This gradient also plays an important role in the reabsorption of sodium and various vital solutes in kidney (Jorgensen & Skou 1969).

Na^+/K^+ ATPase is a heterotrimeric enzyme and it consists of an α-subunit, a β-subunit, and a FXYD (or γ-subunit) (Sweadner 1989, Lingrel & Kuntzweiler 1994, Malik *et al.* 1996, Kaplan 2002). The α-subunit, a 110 kDa protein, is the catalytic subunit that has binding sites for Na^+ and K^+ and ten transmembrane helices, three cytoplasmic domains, an actuator domain (A), the nucleotide-binding domain (N), and the phosphorylation domain (P). The catalytic subunit has recently been shown to have a canonical caveolin-binding domain (CBD) that regulates the association of the α-subunit with caveolin 1 (Cai *et al.* 2008). The β-subunit, a 36 kDa protein, has a small N-terminal cytoplasmic domain, one transmembrane helix, and a large highly glycosylated extracellular domain, and it is required for maturation and insertion of the Na^+/K^+ ATPase complex into the membrane (McDonough *et al.* 1990, Geering 1991, Lutsenko & Kaplan 1993, Beguin *et al.* 1998*a*,*b*, Hasler *et al.* 1998, Blanco 2005). The β-subunit also forms bridges between adjacent cells, which are critical for maintaining the integrity of tight junctions and apical and basolateral polarity in epithelial cells as well as preventing the translocation of proteins from apical to basolateral

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membranes and vice versa (Rajasekaran & Rajasekaran 2003, 2009, Barwe *et al*. 2005, Tokhtaeva *et al*. 2009, 2011). Members of the FXYD protein family are tissue-specific and regulate the activity of the pump (Sweadner *et al*. 2003, Geering 2008). To date, four isoforms of the α-subunit (α1–4), three isoforms of the β-subunit (β1–3), and at least seven isoforms of FXYD have been identified (Forbush *et al*. 1978, Sweadner & Rael 2000, Blanco 2005) adding to the diversity of Na^+/K^+ ATPase function. The α1-subunit is ubiquitously expressed and is the only subunit expressed in the kidneys, while the other isoforms are tissue-specific. The α2-subunit is expressed in skeletal muscle, smooth muscle, heart, brain, lung, and adipocytes (Donnet & Sweadner 2003, Blanco 2005, Bystriansky & Kaplan 2007, Lingrel 2010). The α3-subunit is expressed in neurons, ovaries, and adult human heart (Sweadner 1989, Kaplan 2002, Lingrel 2010), while the α4-subunit is expressed in sperm (Shamraj & Lingrel 1994, Woo *et al*. 1999, 2000, Wagoner *et al*. 2005, Sanchez *et al*. 2006, Jimenez *et al*. 2010, 2011*a*,*b*, 2012). The Na^+/K^+ ATPase α1-subunit in rodents is the least sensitive to cardiotonic steroids, whereas the α2-, α3-, and α4-subunits are highly sensitive to cardiotonic steroids. However, all α-subunits have a similar sensitivity to cardiotonic steroids in humans, pigs, and other species. Recent studies using high-resolution structure analysis provide in-depth understanding of the ion-transporting function and allosteric modulations of Na^+/K^+ ATPase in the Na^+ and K^+ transporting states (Morth *et al*. 2007, 2009, 2011, Pedersen *et al*. 2007, Schack *et al*. 2008, Ogawa *et al*. 2009, Shinoda *et al*. 2009, Toustrup-Jensen *et al*. 2009, Kanai *et al*. 2013, Laursen *et al*. 2013).

Na^+/K^+ ATPase is the main target of cardiotonic steroids such as ouabain and digoxin. Extensive research has primarily been directed at the ion-transporting properties of therapeutic doses of cardiotonic steroids, and the resulting ionotropic effect due to inhibition of Na^+/K^+ ATPase and also the resulting increase in intracellular calcium concentrations in vasculature and heart muscle. In addition to its transporting function, Na^+/K^+ ATPase plays an important role in cell signaling (Xie & Askari 2002). The elegant work from the laboratories of Xie and Askari has elucidated this important role of Na^+/K^+ ATPase that is probably involved in diverse cellular functions and pathophysiological states such as cell growth, hypertrophy, ischemia, development and postnatal maturation of kidneys, and as yet undefined processes (Kometiani *et al*. 1998, 2005, Xie *et al*. 1999, Liu *et al*. 2000, 2007*a*,*b*, Aizman *et al*. 2001, Mohammadi *et al*. 2001, Aizman & Aperia 2003, Andersson *et al*. 2004, Li *et al*. 2006, 2009, 2010, Liang *et al*. 2006, Tian *et al*. 2006, 2010, Aperia 2007, 2012, Nguyen *et al*. 2007, Kennedy *et al*. 2008, Tian & Xie 2008, Khodus *et al*. 2011, Brashear *et al*. 2012, Blanco & Wallace 2013, Burlaka *et al*. 2013, Fontana *et al*. 2013, Wu *et al*. 2013). The laboratory of Zijian Xie made an important discovery by demonstrating that a population of non-ion-transporting and high-affinity Na^+/K^+ ATPase resides in caveolae, forming a signaling complex with Src kinase (Pierre & Xie 2006). When cardiotonic steroids at low picomolar to nanomolar concentrations bind to Na^+/K^+ ATPase in the caveolae, this activates Src, which in turn results in transactivation of EGFR and activation of the PI3K–Akt pathway, the Ras–Raf–ERK MAP kinase pathway, IP3R activation, and calcium oscillations, resulting in regulation of early-response genes associated with cell growth, motility, and metabolic pathways (Peng *et al*. 1996, Kometiani *et al*. 1998, Contreras *et al*. 1999, 2004, 2006, Aizman *et al*. 2001, Abramowitz *et al*. 2003, Dmitrieva & Doris 2003, Dong *et al*. 2004, Khundmiri *et al*. 2006, 2007, Liu & Askari 2006, Liu *et al*. 2007*a*,*b*, 2011, Pierre *et al*. 2008, Quintas *et al*. 2010, Morrill *et al*. 2012, Bai *et al*. 2013, Wu *et al*. 2013, Rincon-Heredia *et al*. 2014).

Endogenous cardiotonic steroids in cardiac and renal diseases

As early as the 1960s, it became evident that activation of the renin–angiotensin–aldosterone system alone cannot explain the physiological and pathophysiological responses to acute or chronic blood volume regulation in salt-sensitive hypertension (Schrier & De Wardener 1971*a*,*b*, Schrier & Berl 1975). de Wardener *et al*. (1961) demonstrated that saline infusion-induced natriuresis is maintained even under normal renal perfusion pressure and glomerular filtration rate. This observation led to the postulation of 'the third factor' theory, which suggests that volume expansion is associated with an increase in a circulating factor that inhibits Na^+/K^+ ATPase activity (de Wardener *et al*. 1961, de Wardener & MacGregor 2002, de Wardener 2003, de Wardener *et al*. 2004). Bricker and colleagues, based on their studies in uremic human subjects and experimental rat models of chronic kidney disease, postulated that the circulating Na^+/K^+ ATPase inhibitory factor is involved in the progression of renal failure and pathogenesis of uremic syndrome (Bricker *et al*. 1968, 1970, 1972, 1993, Slatopolsky *et al*. 1968, 1970, Bricker & Klahr 1970, Bourgoignie *et al*. 1971, Bricker 1972). The identification and characterization of the circulating Na^+/K^+ ATPase antagonist remained elusive

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for a long time. After painstaking research spanning three decades, two groups independently identified the circulating factor(s) as substances similar to plant-derived ouabain and digoxin that are synthesized in the mammalian adrenal glands (Hamlyn *et al.* 1982, 1987, 1989, 1991, 1996, 1998, Craver & Valdes 1983, Graves *et al.* 1983, 1984, Valdes 1985*a*,*b*,*c*, Valdes *et al.* 1985, 1988, Skogen *et al.* 1987, Lackner *et al.* 1988, Siegfried & Valdes 1988, Shaikh *et al.* 1991, Qazzaz & Valdes 1996, Qazzaz *et al.* 1996*a*,*b*, 2000, Ferrandi *et al.* 1997, Jortani & Valdes 1997, Perrin *et al.* 1997, Grider *et al.* 1999, Manunta *et al.* 2001*a*,*b*, Pierdomenico *et al.* 2001, el-Masri *et al.* 2002, El-Mallakh *et al.* 2007). Bagrov and colleagues identified the presence of endogenous bufadienolides, marinobufagenin, and telocinobufagin, in plasma of rats with renal failure, diabetes, and preeclampsia, and in plasma from patients with congestive heart failure and renal failure (Bagrov *et al.* 1996, 2005, 2009, Fedorova *et al.* 1998, 2005*a*,*b*, 2010*a*,*b*, 2012, Gonick *et al.* 1998, Lopatin *et al.* 1999, Averina *et al.* 2006, Kennedy *et al.* 2006, Zvartau *et al.* 2006, Anderson *et al.* 2008, Bagrov & Shapiro 2008, Tian *et al.* 2010, Kolmakova *et al.* 2011). Recent studies have indicated that endogenous cardiotonic steroids also play a significant role in the pathogenesis of polycystic kidney disease (Nguyen *et al.* 2007, 2011, Blanco & Wallace 2013, Jansson *et al.* 2013).

Effects of low nanomolar to picomolar concentrations of ouabain on Na^+/K^+ ATPase activity

A significant advance in the understanding of the role of Na^+/K^+ ATPase and cardiotonic steroids in human physiology has been the recognition of the dose-dependent effects of cardiotonic steroids on Na^+/K^+ ATPase activities. In a recent study, Manunta *et al.* (2006) have demonstrated that, in normotensive control subjects, plasma levels of cardiotonic steroids were 0.43 ± 0.08 nmol/l and urinary cardiotonic steroid excretion was 1.04 ± 0.13 nmol/day. In patients with high salt loading for 3 days, the plasma levels of endogenous cardiotonic steroids increased to 5.8 ± 2.2 nmol/l, and the urinary cardiotonic steroid excretion increased to 1.69 ± 0.27 nmol/day. Similar concentrations were reported by Stella *et al.* (2008) in end-stage renal disease (ESRD) patients. They demonstrated that increased endogenous cardiotonic steroid levels are independently associated with left ventricular hypertrophy (LVH) and eccentric non-dilated hypertrophy (EH) in ESRD patients. They concluded that LVH and EH in this patient cohort were associated with increased cardiac glycoside levels stimulated by chronic volume overload. One possible explanation is that sodium retention due to renal failure would increase volume load, and increase synthesis and/or

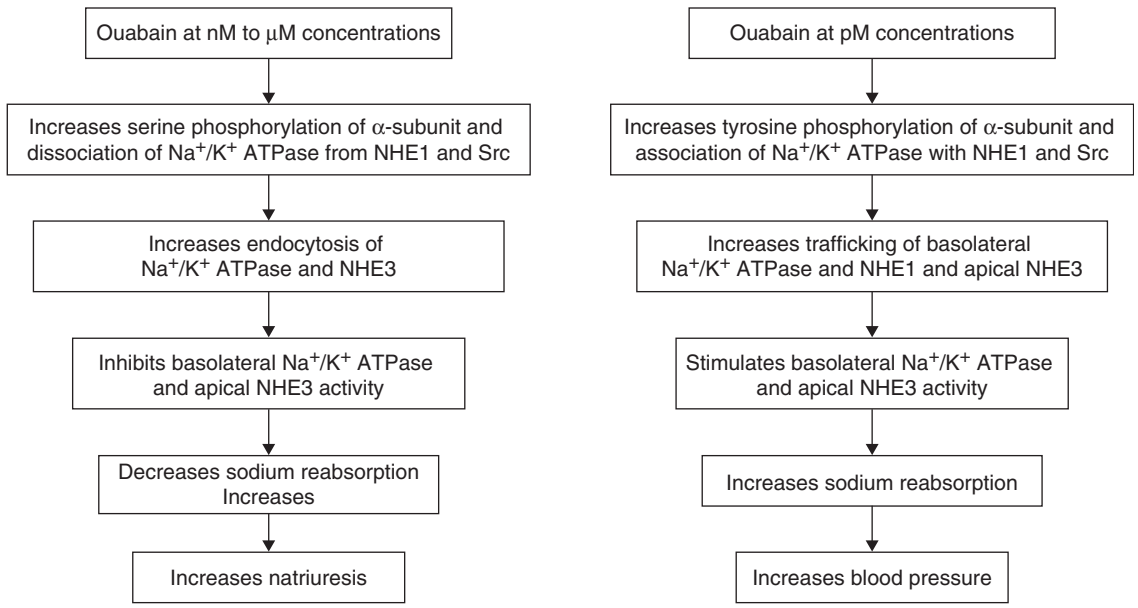

Figure 3
A comparison of the effects of high and low concentrations of ouabain on renal proximal tubular sodium reabsorption.

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decrease clearance of cardiotonic steroids. The increased circulating levels of endogenous cardiotonic steroids would result in increased cardiac contractility and peripheral vascular resistance (Schoner & Scheiner-Bobis 2007*a*,*b*). Ferrari *et al*. (2006) proposed a dual mechanism for the hypertensogenic action of endogenous cardiotonic steroids, where they suggest that at, low concentrations, cardiotonic steroids inhibit the Na^+/K^+ ATPase α2-subunit in vessels and increase the activity of renal Na^+/K^+ ATPase α1-subunits, resulting in an increase in the intracellular calcium concentration in the vascular cells and volume expansion due to increased sodium reabsorption in the kidney. Both these factors together would contribute to the increase in blood pressure. In fact, Brown *et al*. (1962) described stimulation of the sodium pump by cardiotonic steroids at concentrations that cause positive ionotropic effects. Subsequently, Lee & Klaus (1971) and Cohen *et al*. (1976) described similar effects of digitalis and its derivatives on the sodium pump. We (Khundmiri *et al*. 2006, 2007) and others (Bluschke *et al*. 1976, Gao *et al*. 2002, Ferrari 2010) have demonstrated that low concentrations of cardiotonic steroids increase Na^+/K^+ ATPase-mediated ion transport in renal proximal tubule cells. As early as 1914, Douglas Cow discovered microcirculation between kidneys and adrenal glands (Cow 1914), which would indicate that EO synthesized in the adrenal glands could directly affect the sodium reabsorption in the kidneys without reaching the systemic circulation. The increased sodium reabsorption would lead to an increase in the volume followed by vascular changes to increase blood pressure. Recent studies from our laboratory have demonstrated that treatment with low-dose ouabain, concentrations similar to those present in ESRD patients, increases blood pressure in rats and increases Na^+/K^+ ATPase activity in basolateral membranes prepared from kidney cortex. The increase in activity was dependent upon the presence of NHE1 (SLC9A1) expression and was inhibited by specific inhibitors of NHE1 (Holthouser *et al*. 2010). We also demonstrated that low-dose cardiotonic steroids increase the association between Na^+/K^+ ATPase and NHE1 and that this association is critical for activation of the Src–EGFR signaling cascade. Mutation analysis of the Na^+/K^+ ATPase and *NHE1* demonstrated that the N-terminal CBD of the Na^+/K^+ ATPase α-subunit and the scaffolding domain of NHE1 are the critical components for this association. Interestingly, total internal reflection fluorescence microscopy (TIRFM) showed that deletion of the N-terminal CBD of Na^+/K^+ ATPase resulted in the failure of trafficking of Na^+/K^+ ATPase α-subunit and NHE1 to the basolateral membranes. Mutations in the scaffold domain or the activity domain of *NHE1* prevented the induction of the increase in Na^+/K^+ ATPase activity by ouabain (Khundmiri *et al*. 2014). These data indicate that an association between Na^+/K^+ ATPase and NHE1 triggered by low-dose ouabain is essential for Src activation and the increase in sodium reabsorption in the proximal tubular cells (Fig. 3). Traditionally, the β-subunit of Na^+/K^+ ATPase has been demonstrated to facilitate the trafficking of the αβ heteroenzyme to the PM. Our recent observation that mutations affecting the CBD prevented the membrane trafficking of the Na^+/K^+ ATPase in the presence of endogenous β-subunit indicates an exciting possibility that the CBD may also play a role in the association of the α- and β-subunit. Further experiments are required to confirm this possibility.

A major observation in all studies where Na^+/K^+ ATPase activity was shown to be stimulated by low concentrations of cardiotonic steroids was that the effect was acute. The mechanisms of the chronic effects of cardiotonic steroids at low concentrations are not known. Recent work at our laboratory has indicated that the increase in Na^+/K^+ ATPase activity was accompanied by an increase in secretion of angiotensin II in human proximal tubule cells (Khundmiri SJ, unpublished observations). These results indicate that the chronic

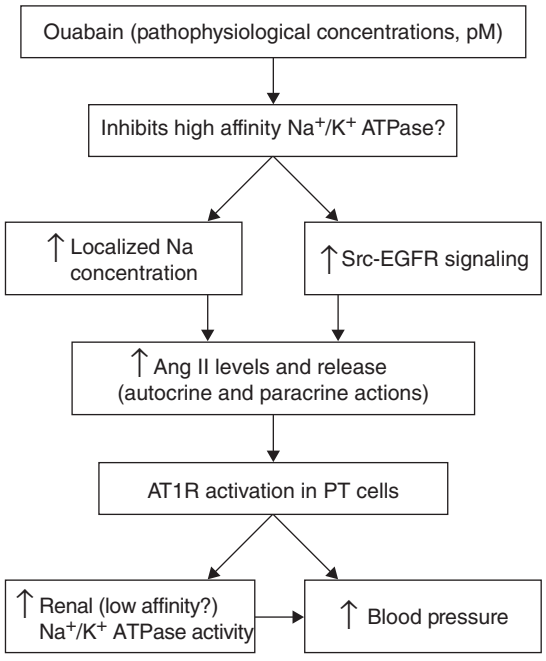

Figure 4
A hypothetical model for the effects of pathophysiological (pM) concentrations of ouabain (endogenous cardiotonic steroids) on blood pressure.

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effects of cardiotonic steroids may be mediated through angiotensin II. Based on our preliminary unpublished data, we propose a hypothesis that pathophysiological concentrations of endogenous cardiotonic steroids such as ouabain would inhibit high-affinity Na^+/K^+ ATPase residing in caveoli, resulting in a localized increase in Na^+ concentration near the caveolar membranes. The increase in localized $[Na^+]i$ and Src and EGFR activation promotes synthesis and release of angiotensin II from the proximal tubule cells. The released angiotensin II acts in both autocrine and paracrine manners to activate proximal tubular AT1R. Activation of AT1R will then increase sodium reabsorption by increasing the activities of NHE3 and Na^+/K^+ ATPase resulting in an increase in blood pressure (Fig. 4). In turn, angiotensin II may have effects on cardiotonic steroid release, activity, or intracellular signaling. Thus, these studies indicate a connection between the renin–angiotensin–aldosterone system, ACTH, and cardiotonic steroids that would result in a sustained increase in blood pressure in patients with chronic kidney disease and cardiorenal syndrome. Another possible mechanism would be a decrease in secretion of hormones that inhibit Na^+/K^+ ATPase activity (PTH) or loss of their effects on inhibition of sodium transport in the kidney (dopamine) due to pathophysiological concentrations of cardiotonic steroids (Brown *et al*. 1983, 1987, Zhang & Yuan 2010, Zhang *et al*. 2010, Apel *et al*. 2013). Further studies will unravel these pathways.

Conclusions

Understanding of the role of cardiotonic steroids in the pathophysiology of cardiac and renal disorders has progressed exponentially in the last two decades. The discovery of the role of Na^+/K^+ ATPase in signal transduction, a function distinct from its ion transport properties, demonstrates that Na^+/K^+ ATPase is a multifaceted protein involved in the regulation of several physiological processes over and above its classical role in the maintenance of the intracellular ionic milieu. Understanding the roles of low physiological and pathophysiological concentrations of cardiotonic steroids in the regulation of Na^+/K^+ ATPase, intracellular signaling, and hormonal signaling is essential to delineating the functions of these substances in the regulation of sodium homeostasis in normal and pathophysiological states. These studies will open up development of new therapies for the control of blood pressure and renal disorders linked with aberrant salt homeostasis.

Declaration of interest
The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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