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REVIEW ARTICLE

Na,K-ATPase as a target for endogenous cardiotonic steroids: What's the evidence?

Sergei N. Orlov [a,b,c], Artem M. Tverskoi [a,*], Svetlana V. Sidorenko [a,b], Larisa V. Smolyaninova [a,b], Olga D. Lopina [a], Nickolai O. Dulin [d], Elizaveta A. Klimanova [a,b]

[a] *MV Lomonosov Moscow State University, Moscow, 119234, Russia*
[b] *National Research Tomsk State University, Tomsk, 634050, Russia*
[c] *Siberian State Medical University, Tomsk, 634050, Russia*
[d] *University of Chicago, IL, 60637, USA*

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Abstract With an exception of few reports, the plasma concentration of ouabain and marinobufagenin, mostly studied cardiotonic steroids (CTS) assessed by immunoassay techniques, is less than 1 nM. During the last 3 decades, the implication of these endogenous CTS in the pathogenesis of hypertension and other volume-expanded disorders is widely disputed. The threshold for inhibition by CTS of human and rodent α1-Na,K-ATPase is ~1 and 1000 nM, respectively, that rules out the functioning of endogenous CTS (ECTS) as natriuretic hormones and regulators of cell adhesion, cell-to-cell communication, gene transcription and translation, which are mediated by dissipation of the transmembrane gradients of monovalent cations. In several types of cells ouabain and marinobufagenin at concentrations corresponding to its plasma level activate Na,K-ATPase, decrease the $[Na^+]_i/[K^+]_i$-ratio and increase cell proliferation. Possible physiological significance and mechanism of non-canonical Na_i^+/K_i^+-dependent and Na_i^+/K_i^+-independent cell responses to CTS are discussed.

* Corresponding author. Department of Biomembranes, Faculty of Biology, Lomonosov Moscow State University, 1-12 Leninskie Gory, 119234 Moscow, Russia.
E-mail address: garretam@yandex.ru (A.M. Tverskoi).
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Introduction

A half century ago, De Wardener and co-workers reported that in dogs natriuresis triggered by intravenous saline administration occurs even in the absence of significant changes in renal perfusion pressure and glomerular filtration rate. This observation suggesting an implication in renal salt handling new unknown system termed as a *Third Factor*.[1] Twenty years later, it was shown that natriuretic effect of the Third Factor might be at least partially explained by augmented production of atrial and brain natriuretic peptides (ANP and BNP) which inhibit in renal epithelial cells the basolateral Na,K-ATPase via their interaction with G protein-coupled receptors and activation of cGMP-mediated signaling.[2] At the same time, several research teams demonstrated that low molecular compounds distinct of ANP and BNP can also contribute to this phenomenon. Thus, Buckalew and co-workers found that serosal application of plasma ultrafiltrate from salt-loaded dogs to the frog skin lacking ANP and BNP receptors decreases the transepithelial potential difference and short current thus suggesting an inhibition of the basolateral Na^+ transport[3] (for historical details, see[4,5]).

The beneficial effect of digitalis in the therapy of heart failure, described more than 200 years ago, led to the identification of numerous plant-derived cardenolides, including ouabain, i.e. the most hydrophilic steroid used in an overwhelming number of *in vitro* studies. Other members of the cardiotonic steroid (CTS) superfamily, bufadienolides, were isolated from amphibians.[6] A long-lasting search for endogenous CTS (ECTS) resulted in the purification from mammalian species compounds identical to ouabain,[7–9] digoxin,[10] bufalin,[11] marinobufagenin (MBG),[12,13] telocinobufagin[14] and marinobufotoxin[15] (structure of some endogenous cardiotonic steroids are presented in Fig. 1) Data on association of cardiovascular, renal and neuronal diseases with increased ECTS level and preventive actions of passive immunization by anti-ECTS antibodies allowed researchers to propose an implication of ECTS in the pathogenesis of these and other volume-expanded disorders (for comprehensive review, see[16–21]).

Starting from early 90th the focus of investigations has been directed to the mechanisms by which ECTS may regulate various cellular functions. Since the seminal publication of Dr. J. Skou on the complete inhibition, by digitalis, of Mg^{2+}-dependent (Na^++K^+)-stimulated adenosine triphosphatase (NKA, EC 7.2.2.13),[22] this enzyme remains the only known target of CTS. As predicted, NKA inhibition by CTS sharply affects cellular functions that are directly linked to transmembrane gradients of monovalent cations, such as maintenance of electrical membrane potential (E_m), cell volume, intracellular concentration of Ca^{2+}, H^+, Cl^-, inorganic phosphate and low molecular weight organic compounds by Na^+- and K^+-coupled transporters. More recently, it was shown that side-by-side with dissipation of transmembrane gradients of monovalent cations ECTS trigger transcriptomic and proteomic changes and affect cell adhesion, proliferation and death. Several research teams proposed that these non-canonical cellular responses are mediated by Na_i^+,K_i^+-independent signaling and contribute to physiological and pathophysiological actions of ECTS.[16,17,20,23–25] In this review, we examine the potential role of ECTS in the triggering of canonical and non-canonical cellular responses by comparative analysis of their plasma content and dose-dependent actions of ECTS on NKA activity, intracellular content of monovalent cations, intracellular signaling pathways and cellular responses affecting cell proliferation and gene expression.

The content of circulating ECTS

Data on the content of ECTS in the extracellular fluids were mainly obtained by ELISA, RIA, DELFIA and other immunoassay approaches. Table 1 displays that with few exceptions the plasma concentration of immunoreactive ouabain and MBG in mammalian species is less than 1 nM. The huge variability of the plasma content of ouabain (from 0.05 to 1 nM) and MBG (from 0.2 to 0.6 nM) in healthy patients reported by different laboratories (Table 1) can be explained by numerous features of self-made reagents employed in these investigations. This comment becomes important because 3 research teams failed to detect any immunoreactive ouabain in the human plasma after its high-performance liquid chromatography separation.[26–28] More recently, the negative results were also obtained by newly developed ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). This sophisticated technique having a lower limit of quantification of 0.002 nM failed to detect ouabain in the plasma of healthy subjects as well as patients with heart failure.[29] Keeping these data in mind it might be proposed that immunoreactive ouabain-like substance(s) rather than authentic ouabain per se has been detected in part of the studies listed in Table 1.

Do ECTS inhibit Na,K-ATPase?

Na,K-ATPase is a complex of proteins integrated into plasma membrane, it is found in all types of animal cells. The enzyme is composed of α- and β-subunits. Larger α-subunit (~110 kDa) hydrolyses ATP, this results in the phosphorylation of Asp369 residue located in enzyme active site. After that the enzyme undergoes E_1-E_2 conformational change that, in turn, leads to enzyme dephosphorylation. As consequence of these events Na,K-ATPase provides electrogenic ion transport ($3Na^+$ vs $2K^+$) with a rate of 60—80 phosphorylation—dephosphorylation cycles per sec. Three other NKA α-subunit isoforms were found by screening cDNA libraries in addition to the ubiquitous α1-isoform. These isoforms are highly expressed in astrocytes, neuronal cells (α3 and α2), heart, skeletal muscle (α2), and testis (α4). In majority of tissues (possibly, with an exception of kidney epithelial cells) at least two different isoforms are expressed: the ubiquitous α1-NKA is supplied by another (usually regulated) isoform (for review, see[30,31]). The mechanism of CTS inhibiting effect on NKA was mostly investigated with ouabain originated from *Strophanthus gratus* and having much higher water solubility in comparison with other CTS. Lingrel with coauthors demonstrated that at least 10 amino acid residues in H1, H5 and H7 α-subunit transmembrane segments

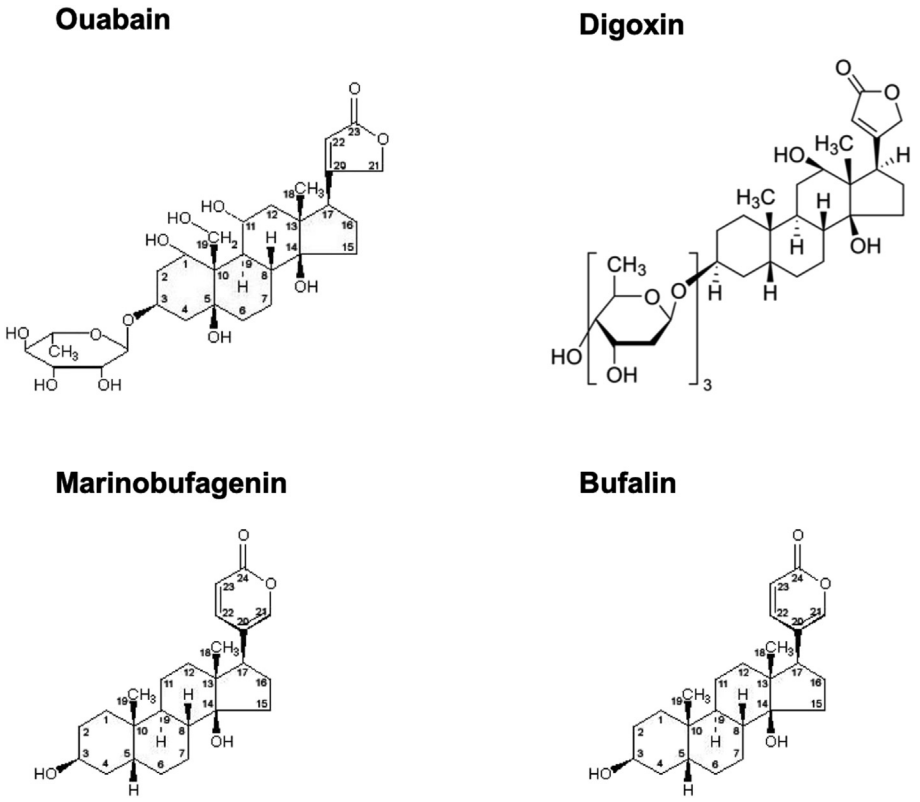

Figure 1 Structure of 4 cardiotonic steroids: cardenolides (ouabain and digoxin) having 5-membering lactone ring in 17-th position of steroid core; bufadienolides (marinobafagenin and bufalin) with 6-membering lactone ring in this position.

as well as in H1—H2, H5—H6 and H7—H8 extracellular loops exert an influence on ouabain affinity of NKA.[31] Their important role in CTS binding was also approved by comparative analysis of NKA derived from different species. Ouabain affinity to rat and mouse NKA α1-subunit (CTS-resistant α1R-NKA) is 3 orders of magnitude lower in comparison with other mammalian species (CTS-sensitive α1S-NKA). It was shown that the replacement of neutral Gln111 and Asn122 in α1-subunit by charged Arg and Asp amino acids resulted in ~1000-fold decrease of ouabain affinity to it. The same amino acid replacement significantly decreased α2- and α3-NKA affinity for ouabain. Unlike ubiquitous α1-isozyme, the affinity for CTS of α2- α4-NKA in rodents and other mammalian species are about the same.[32]

In the early 1980s, M.P. Blaustein proposed that augmented ECTS production partially normalizes renal function by inhibiting the Na-pump located in basolateral membranes of renal epithelial cells but elevates total peripheral resistance via suppression of this enzyme in vascular smooth muscle cells.[33] It should be noted, however, that the treatment of congestive heart failure with commercially available cardenolides occurs in the absence of significant natriuresis.[34] The hypothesis on the implication of ECTS via NKA inhibition also contradicts to the comparative analysis of ECTS concentration detected in the extracellular fluids and their dose-dependent action on NKA activity (Fig. 2). Indeed, at concentrations less than 100 nM ouabain had no significant impact on NKA activity in rat renal epithelial cells and vascular smooth muscle cells isolated form rat aorta. In both cases, half-maximal modulation of intracellular cation content was observed at 500—1000 μM of ouabain[35] that was at least 3 orders of magnitude higher than the plasma content of immunoreactive ouabain and MBG. Data on the inhibitory action of ouabain and MBG on the activity of human α1S-NKA as well as CTS-sensitive α2-, α3-isozymes at concentrations less than 1 nM are limited to few publications (Table 2). To the best of our knowledge there is no report showing dissipation of transmembrane gradients of monovalent cations by ECTS at the range corresponding to their concentrations in plasma.

Comparative analysis of the dose-dependent actions of ouabain and MBG on NKA activity in membrane fractions enriched by vascular smooth muscle sarcolemma and perivascular nerve endings (neuronal plasmalemma) containing predominantly α1- and α3-isozymes respectively, demonstrated that affinity for ouabain and MBG of α1- and α3-isozymes are sharply different (IC_{50} for inhibition of α1-NKA by ouabain and MBG are 50 and 2 nM, respectively, vs 3 and 140 nM in the case of α3-NKA).[36] These data contradict to 5-fold elevation of the affinity for ouabain compared to MBG in Madin—Darby canine kidney cells abundant with α1-NKA[37] and about the same affinity demonstrated in α1-NKA purified from duck salt glands.[38] Possible mechanisms underlying this discrepancy should be examined further.

It is known that in some cell types (neurons, glia, vascular smooth muscle myocytes) α2- and α3-isozymes of NKA are located in microdomains of plasma membrane that

Table 1 Plasma levels of CTS in hypertension and states associated with sodium loading and/or plasma volume expansion and estimated by immunoassay technique.

CTS and groups under investigation	Values, nM	References
Ouabain		
Humans EH/PA/control	3.39 ± 0.57/4.09 ± 1.12/0.53 ± 0.10	110
Humans CHF/control	0.030—8.3/0.16—0.77	111
Humans CHF/control before HPLC	0.25—1.6/0.13—0.56	28
Humans CHF/control after HPLC	ND/ND	28
MHS/MNS	0.076 ± 0.029/0.027 ± 0.014	112
NaCl-loaded rats/control	1.43 ± 0.06/1.14 ± 0.05	113
ACTH-treated subjects/control	0.87 ± 0.25/0.64 ± 0.17	114
ACTH-treated rats/control	0.09 ± 0.01/0.10 ± 0.04	115
Humans: CRF/EH/PA/control	0.14 ± 0.02/0.13 ± 0.05/0.10 ± 0.03/0.09 ± 0.02	116
Humans: preeclampsia/control	0.70 ± 0.16/0.32 ± 0.07	117
3rd trimester of pregnancy/control	0.024 ± 0.004/0.009 ± 0.001	118
Mild EH/control	0.039 ± 0.024/0.029 ± 0.018	119
DS, high/low-NaCl diet	0.12 ± 0.02/0.10 ± 0.02	120,121
Rats, high/normal NaCl intake	0.28 ± 0.04/0.34 ± 0.06	122
Humans: volume expansion/control	0.21 ± 0.04/0.09 ± 0.02	121
Dogs: controls	0.138 ± 0.043	7
Humans: controls	0.037 ± 0.007	7
Humans: nephrectomy/control	0.20 ± 0.06/0.12 ± 0.06	123
Humans: mild hypertension/control	1.34 ± 0.91/0.38 ± 0.31	124
Humans: control	0.152 ± 0.067	125
Humans: low-renin EH/control	0.94 ± 0.22/0.37 ± 0.04	126
Marinobufagenin		
ACTH-treated rats/control	0.44 ± 0.06/0.21 ± 0.05	115
Patients with CRF/EH/PA/control	16.6 ± 5.3/1.7 ± 0.7/13.5 ± 12.9/0.26 ± 0.05	116
Patients with preeclampsia/control	2.63 ± 0.10/0.63 ± 0.07	117
DS, high/low-NaCl diet	1.24 ± 0.0.27/0.38 ± 0.04	120,121
Patients with CHF stage IV/stage I	1.90 ± 0.04/0.60 ± 0.14	127
Rats, high/normal-NaCl intake	1.14 ± 0.12/0.55 ± 0.06	122
Patients with AMI/control	1.9 ± 0.38/0.38 ± 0.01	13
Rats with volume expansion/control	0.49 ± 0.05/0.20 ± 0.06	121
Patients with nephrectomy/control	0.57 ± 0.04/0.36 ± 0.02	128
Patients: 24-hr low/high salt diet	0.16—0.30/0.18—0.37	108

Abbreviations: **AMI** — acute myocardial ischemia; **CHF** — congestive heart failure; **CRF** — chronic renal failure; **DS** — Dahl salt-sensitive rats; **EH** — essential hypertension; **HPLC** — high-performance liquid chromatography; **MHS** and **MNS** — Milan hypertensive and normotensive strains, respectively; **ND** — non detectable; **PA** - primary aldosteronism.

are in close proximity to the underlying endoplasmic reticulum. Keeping in mind this finding and the extremely low affinity of rodent α1R-NKA for CTS, Blaustein and coworkers proposed that ECTS evoked an increase of vascular tone via elevation of $[Na^+]_i$ in the space-limited plasma membrane-junctional endoplasmic reticulum compartment (plasmerosomes) abundant with ubiquitous isoform of the Na^+/Ca^{2+} exchanger (NCX1) and with α2-, α3-NKA.[39] A key role of NCX1 in the development of salt-sensitive hypertension was demonstrated by using a selective inhibitor of this carrier, compound SEA0400. Moreover, it was shown that DOCA-salt-induced increment of blood pressure was absent in NCX1-deficient *Slc8a1*$^{+/-}$ mice but was increased in transgenic mice expressing canine *Ncx1.3* driven by the smooth muscle-specific promoter.[40] To the best of our knowledge, the elevation of Na^+ concentration in cytoplasm or plasmerosome compartments by ECTS at concentrations detected in the extracellular fluids has not been demonstrated.

Do ECTS trigger non-canonical Na_i^+/K_i^+-mediated cellular responses?

In this section, we briefly summarized the data on non-canonical cellular responses triggered by CTS and mediated by elevation of the $[Na^+]_i/[K^+]_i$-ratio.

Gene expression

We observed that long-term inhibition of NKA in rat vascular smooth muscle cells (RVSMC) by ouabain results in sharp elevation of RNA synthesis[41] and appearance of hundreds of newly synthesized proteins.[42] Later on we employed Affymetrix technology for identification of cell-type specific and ubiquitous set of Na_i^+,K_i^+-sensitive transcripts by comparative analysis of the action of ouabain and K^+-free medium on transcriptomic changes in HUVEC, RVSMC and HeLa cell line.[43] In this study, we

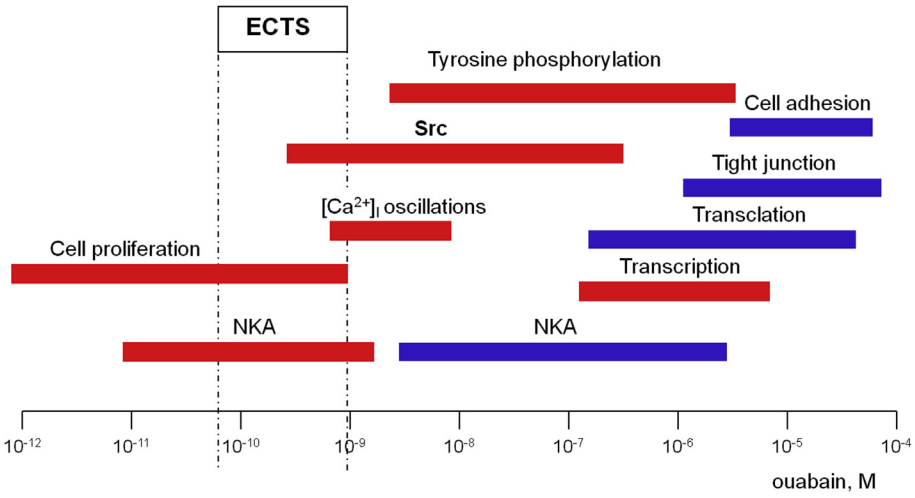

Figure 2 Plasma concentration of ECTS detected by immunoassay technique and dose-dependent cellular actions of ouabain in non-rodent mammalian cells. Activatory and inhibitory actions of ouabain are shown by red and blue, respectively. For original data, see text and Tables 1—3

detected changes in expression levels of hundreds of genes that were highly correlated between two treatments thus demonstrating a key role of Na_i^+/K_i^+-mediated mechanism of excitation-transcription coupling. Importantly, about 80 Na_i^+/K_i^+-sensitive transcripts were found in all types of cells. This set of ubiquitous Na_i^+/K_i^+-sensitive transcripts was highly abundant with early response genes (ERG) and other genes involved in transcription regulation.[43,44]

Data on the time- and dose-dependent actions of ouabain and MBG on intracellular Na^+ and K^+ content and gene expression in human endothelial cells strongly suggest that both ECTS affect excitation-transcription coupling via NKA inhibition and $[Na^+]_i$ elevation.[45] This study demonstrated that 4-fold elevation of c-Fos mRNA occurs within 30 min after the addition of ouabain. At that moment $[Na^+]_i$ was increased by 5-fold whereas $[K^+]_i$ was declined by less than 15%.[46]

After Lubin and Ennis[47] pioneer observation, a number of laboratories have shown K^+ requirement for protein synthesis thus assuming the bimodal effect of CTS on gene expression via activation and inhibition of transcription and translation, respectively (for review, see[48,49]). In reticulocytes $[Na^+]_i$ increase reduces the efficiency of K_i^+-dependent regulation of protein synthesis probably through the competition for one binding site within hypothetical K_i^+-sensor.[50] As another hypothesis it might be suggested that elevation of $[Na^+]_i$ reduces the elongation factors transcription. Moreover, we discovered that 6-hr incubation of HUVEC in the presence of ouabain resulted in 3-fold decrease of mRNA encoding eukaryotic translation initiation factor 5 (eIF5).[51] This factor plays common role in protein synthesis by inducing mRNA translation and GTP hydrolysis.[52,53] The molecular origin of intracellular Na^+ and K^+ sensors involved in activation of gene transcription and translation remains unknown.

Tight junctions and cell adhesion

Gupta and co-workers have shown that discrepancies in dose-dependent decrease the attachment of human and monkey cells possessing CTS-sensitive α1S-NKA by ouabain, vs mouse and hamster cells, having CTS-resistant α1R-NKA, positively correlate with discrepancies in dose-dependent suppression of ^{86}Rb influx.[54] At large doses, ouabain severely decreased the adhesion of COS-7 [55] and human retinal pigment epithelial cells[56] and blocked tight junctions in Madin—Darby canine kidney (MDCK) cells,[57] RVSMC[58,59] and HeLa cells.[58] Importantly, tight junction and adhesion breakdown in cells with α1R- and α1S-NKA was observed at concentration of ouabain ~1000 and 1 μM, respectively,[55,57—60] i.e. in the range of these enzymes' complete inhibition. It should be noted that these ouabain effects were revoked in the medium without Na^+ and were imitated by NKA inhibition in K^+-depleted medium.[56,57,60,61] Viewed collectively, these data assume that Na^+ and K^+ transmembrane gradient maintenance is an obligatory factor for the establishment the adhesion and cell-to-cell communications. The relative contribution the gain of Na_i^+ and loss of K_i^+ in this phenomenon remains a matter of speculations.[62]

Do ECTS activate Na,K-ATPase?

Numerous research team reported that low doses of CTS activate rather than inhibit Na,K-ATPase. Thus, it was demonstrated that Na-pump providing ion current in single cardiac myocytes from dog, human hearts, and guinea pig hearts[63] is augmented with 10 nM and less ouabain concentration. At this low ouabain doses Na_i^+ concentration in guinea pig atria[64,65] was decreased. At 0.1 nM ouabain an activation of ^{86}Rb uptake was seen in human

Table 2 Thresh-old and half-maximal concentration (IC50) of ouabain (OU) and marinobufagenin (MBG) triggered Na,K-ATPase and $K^+/^{86}Rb$ uptake inhibition.

Cell type	Tested CTS	Thresh-old, nM	Half-maximal effect, nM	References
Na,K-ATPase				
c-kidney	OU	1	20	129
r-kidney	*OU*	*5×10^3*	*10^5*	130
m-kidney	OU	10^5	10^6	131
r-sarcolemma from aorta	*OU*	*10*	*50*	36
r-kidney medulla	*OU*	*10^4*	*$>10^5$*	132
r-intravascular nerve ending	*OU*	*1*	*2.6*	36
h-rings from mesenteric artery	OU	1	14	12
r-sarcolemma from left ventricle	*OU*	*10*	*2.5×10^3*	133
h-erythrocytes	MBG	1	70	134
r-sarcolemma from aorta	*MBG*	*1*	*2.1*	36
r-intravascular nerve ending	*MBG*	*100*	*140*	36
r-sarcolemma from left ventricle	*MBG*	*1*	*5.5×10^3*	133
r-kidney medulla	*MBG*	*0.5*	*10*	132
r-kidney medulla	*MBG*	*0.1*	*50*	120
h-sarcolemma from mesenteric artery	MBG	1	50—60	117,135
h-rings from mesenteric artery	MBG	1	50	136
h-sarcolemma from pulmonary artery	MBG	0.1	50	137
Sf-9 cells transfected with r-$\alpha 1\beta 1$	*OU*		*43,000*	138
Sf-9 cells transfected with r-$\alpha 2\beta 1$	*OU*		*170*	138
Sf-9 cells transfected with r-$\alpha 1\beta 1$	*OU*		*31*	138
$K^+/^{86}Rb$ uptake				
h-erythrocytes	OU	1—2	50	139,140
r-erythrocytes	*OU*	*$>10^3$*	*2×10^5*	141
h-leukocytes	OU	0.1	0.56	142
c-renal epithelial cells	OU	>3	50	37
r-fetal brain	*OU*	*<10*	*20*	143
r-renal epithelial cells	*OU*	*10^3*	*5×10^4*	79
r-renal epithelial cells	*OU*	*10^4*	*3×10^5*	129
gp-carotid artery	OU	<1	10	144
c-renal epithelial cells	MBG	30	80	37

Abbreviations: **c** — canine; **gp** — guinea pig; **h** — human; **m** - mouse; **r** — rat.
Data obtained in rodents appear in *italics*.

erythrocytes,[66] whereas at 10 nM and 10 pM ouabain augmented ^{86}Rb uptake was found in opossum and human kidney proximal tubule cells, respectively.[67,68] Stimulation of Na-pump and increment of NKA activity by ouabain and MBG were also documented in hippocampal slice cultures and human mesenteric arteries[12,69] as well as microsomal fractions from mammalian kidney and duck salt glands.[51,70] We observed that prolonged incubation of HUVEC with ouabain (1 and 3 nM) decreased $[Na^+]_i$ and increased $[K^+]_i$ resulting in $[Na^+]_i/[K^+]_i$-ratio attenuation by 30—50%. It should be noted that low doses of ouabain increased the rate of ^{86}Rb influx suggesting that elevation the $[Na^+]_i/[K^+]_i$-ratio is caused by NKA activation.[51] Considered the data on the plasma content of ECTS obtained by immunoassay technique (Table 1, Fig. 2) it might be assumed that their actions *in vivo* documented using anti-CTS antibodies[16,17,25] are at least partially mediated by NKA activation.

The mechanism of bimodal actions of CTS on NKA activity (activation and inhibition at low and high concentration, respectively) remains poorly understood.[71] Keeping in mind data on NKA functioning within the plasma membrane as dimer ($\alpha\beta$) and tetramer ($(\alpha\beta)2$)[72] we proposed that binding of CTS at low doses with $\alpha\beta$ activates enzyme whereas the occupation of $(\alpha\beta)_2$ dimer binding sites with higher CTS concentrations suppresses the enzyme activity.[73] This suggestion is now investigated in our laboratory.

Do ECTS trigger cell proliferation?

Proliferation of cultured human and canine VSMC,[74,75] proximal tubule cells from opossum kidney,[67] HUVEC[75,76] and human polycystic kidney cells[77] having α1S-NKA increased by 20—30% after the addition of ouabain at concentrations less than 1 nM, that is in the range corresponding to its plasma concentrations (Table 1). At doses lower than 1 nM ouabain also increased growth of rat proximal tubule cells,[75] rat astrocytes[78] and rat VSMC[75] expressing α1R-NKA. As noted above at these concentrations ouabain did not inhibit NKA[67,74,76,77,79] thus indicating that CTS proliferation action is mediated by Na_i^+,K_i^+-independent signaling induced by $[Na^+]_i/[K^+]_i$-ratio elevation.

Table 3 Major signaling pathways triggered by ouabain.

Cellular response	Type of cells/ouabain concentration, nM	References
$[Ca^{2+}]_i$ oscillations & elevation	*r-PTC, 10—100*	100
	h-EC, 1—10	76
	r-PTC, >5 × 10⁴	100,145
	r-CM, 10⁵	87,146
ERK phosphorylation & activation	*r-PTC - 0.1—10***	79
	h-PSMC, 0.1***	147
	c-VSMC, 1***	74
	h-EC, 1—10	76
	h-HeLa, 10^6	148
	r-CM, 10⁵	149
	p-LLC-PK1, $>10^2$	88
	r-A7r5, 10⁴	88
	gp-heart, 10³	150
	r-heart, 5 × 10⁴	150
	h-breast cancer cells, 10^2	151
	h-SkMC, 10^2	152
	c-VSMC, 1	74
Src activation	**p**-LLC-PK1, 10^3	88
	r-A7r5, 10⁶	88
	r-CM, 10⁵	87
	h-breast cancer cells, 10^2	151
	h-SkMC, 10^2	152
Protein tyrosine phosphorylation	*r-CM, 10⁵*	87
	h-HeLa cells, 10^3	87
	c-REC, 10^4	153
	c-VSMC, 1	74
Akt phosphorylation	**o**-kidney PTC, 10*	67

Abbreviations: **c** — canine, **f** — fish, **gp** — guinea pig, **h** — human, **m** — mouse, **mn** — monkey; **o** — opossum; **p** — pig, **r** — rat; CGC — cerebellar granule cells; CM — cardiomyocytes; CytD — cytochalasin D, EC — endothelial cells, EGFR — epidermal growth factor receptor, PKC — protein kinase C; PLC — phospholipase C; PSMC - prostate smooth muscle cells, PTC — proximal tubule cells, SkMC — skeletal muscle cells; VSMC — vascular smooth muscle cells.
Data obtained in rodents appear in *italics*.
* $P < 0.05$; ** $P < 0.01$; *** $P \leq 0.001$.

Nevertheless, it should be noted that the lack of ouabain effect on NKA documented in above-cited studies might be explained by the variety of incubation times used in these measurements. Indeed, cells were placed in the medium with ouabain for more than 24 h in order to estimate proliferation, whereas to assess ^{86}Rb influx rate and NKA activity[67,74,76,77,79,80] 15—30 min of incubation were used. This is important because of long—time interaction of NKA with CTS at their low concentrations documented in human lymphocytes[81] and HUVEC.[51] Thus, in HUVEC indeed, half-maximal elevation of $[Na^+]_i$ by 100 nM ouabain was detected in 6 h, whereas in 24 and 48 h the same increment was detected at ouabain concentrations of 30- and 10 nM, respectively.[51] We observed that 48—72 h exposure of HUVEC to low nanomolar to picomolar concentrations of ouabain increased cell growth of by 20—40%.[51] Importantly, prolonged exposure to 1 and 3 nM ouabain increased $[K^+]_i$ and decreased $[Na^+]_i$ resulting in attenuation of the $[Na^+]_i/[K^+]_i$-ratio by 30—50%. At these concentrations, ouabain increased the rate of ^{86}Rb influx suggesting that side-by-side with Na_i^+,K_i^+-independent signaling augmented cell proliferation might be caused by NKA activation and elevation the $[Na^+]_i/[K^+]_i$-ratio.[51] Data on the inhibitory actions of CTS at concentrations 3—4 order of magnitude higher than their plasma level on cell proliferation, oncosis and apoptosis is out of scope of our review and considered elsewhere[73,82—84]

Do ECTS evoke Na_i^+/K_i^+-independent signals?

Xie and Askari were probably the first to propose that side-by-side with monovalent ions transmembrane gradient dissipation CTS affect cellular function by triggering Na_i^+,K_i^+-independent signals.[85] Table 3 displays the early data on dose-dependent ouabain effects on intracellular signaling intermediates. Recent studies considering the comparative contribution of Na_i^+,K_i^+-mediated and -independent signaling are briefly described below.

Src-kinase

First evidence supporting membrane-associated non-receptor tyrosine kinase Src activation came from experiments that demonstrated time- and dose-dependent tyrosine phosphorylation in cells treated by CTS.[86] Hence, exposure of cardiac myocytes, HeLa, L929, and A7r5cells to ouabain led to fast activation of Src, its epidermal growth factor receptor (EGFR) and several proteins tyrosine phosphorylation that was removed by Src kinase inhibitors PP2 and herbymicin A.[87,88] It was demonstrated that ouabain activates ERK MAPK and Src in transfected pig renal epithelial cells (PY-17) having α1-but not α2-NKA.[89] Detailed mapping of α1-NKA nucleotide binding domain reveled 20-amino acid peptide (Ser-415 to Gln-434, NaK-tide). This chemically synthesized NaKtide inhibited Scr (IC_{50} = 70 nM). Positively charged analogs of NaKtide entered into LLC-PK1 cells and suppressed ouabain-induced Src and ERK MAPK activation.[90] Using FRET technology it was shown that ouabain induces Src kinase domain dissociation from α1-NKA nucleotide binding domain that leads to Src tyrosine phosphorylation and activation[91] in LLC-PK1 cells. Lately, however, Gable et al. re-examined this effect and reported that Src-418 phosphorylation as the measure of Src activation is increased in cell-free systems not only by ouabain but also by two other NKA inhibitors (oligomycin and vanadate). They concluded that decrease of Src phosphorylation is primary result of ATP-sparing effect and cannot serve as an evidence of NKA and Src interaction triggered by CTS binding.[92] Further investigations were carried by Yu et al. using native and mutant forms of α2-NKA.[93] Native α2-isoform is known to lack putative Src-binding sites and fail to carry on Src-dependent signaling. Authors introduced key amino acid residues of the two Src-interacting domains that are on α1-but not α2-sequence into the α2-polypeptide and generate stable cell lines expressing this mutant. Comparison Src-signaling properties of cells expressing this mutant demonstrated that in contrast to wild-type α2, the mutant cells gained α1-like signaling function.

It has been proposed that signaling cascades triggered by NKA interaction with Src does not depend on the change of

intracellular Na^+, K^+ and Ca^{2+} concentrations.[85,86] Indeed, initial publications reported about increased EGFR and several other proteins tyrosine phosphorylation at ouabain concentrations that have no considerably influence on intracellular Na^+ content and ^{86}Rb influx.[74,79,94] It should be noted, however, that cells loaded with fluorescent dye possessing low Na^+/K^+-selectivity was employed in this study. Using Na^+/K^+-selective isotope technique we found that MAPK phosphorylation in HUVEC occurs at ouabain concentrations leading to elevation of the $[Na^+]_i/[K^+]_i$-ratio.[51] This observation is consisting with other reports showing Src-mediated signaling at CTS concentrations that inhibit NKA[88,91,95–98] and data on the complete dissociation of Src from α1S-NKA in the presence of 1 μM ouabain, i.e. at concentration resulted in the full-scale Na^+-pump inhibition.[91] Importantly, the augmented tyrosine phosphorylation was mimicked by NKA inhibition in K^+-depleted medium.[87] Viewed collectively, these data strongly suggest that in CTS-treated cells raised $[Na^+]_i/[K^+]_i$-ratio contributes to Src-mediated signaling triggering/progression.

PI3K-Akt

Liu et al.[99] reported about activation of serine/threonine-specific protein kinase B also known as Akt in the presence of 50 μM ouabain that was abolished by phosphatidylinositol 3-kinase (PI3K) inhibitors in cultured neonatal rat cardiac myocytes. They also detected that ouabain induces phosphatidylinositol 3,4,5-triphosphate (PIP3) content increase and leads to co-immunoprecipitation of Na,K-ATPase α-subunit and p85 subunit PI3K (class IA). To reveal Src role in PI3K/Akt signal inducing, Wu and co-workers studied mouse fibroblasts without Src (SYF cells) and control Src^{+++} cells. They discovered that ouabain triggers PIP3 accumulation, PI3KIA and Akt activation, and p85 subunit of PI3KIA and NKA co-immunoprecipitation in both cell types that was insensitive to Src inhibitor PP2. These data allowed to suggest that Akt activation is triggered by interaction of NKA α-subunit proline-rich domain with SH3 domain of PI3KIA p85 subunit induced by CTS.[97] Unfortunately, the role of elevated $[Na^+]_i/[K^+]_i$-ratio connected with NKA inhibition in PI3K/Akt-mediated signaling has not been studied yet.

Ca_i^{2+}-oscillations

Aperia and co-workers reported that in rat proximal tubule cells partial NKA inhibition by 50–250 μM ouabain was accompanied by increased amplitude of low-frequency $[Ca^{2+}]_i$ oscillation which were abolished by L-type Ca^{2+} channel blocker nifedipine.[100] It is well-documented that $[Ca^{2+}]_i$ oscillations activate transcription factors NF-kB and CREB.[101,102] In fact, ouabain-induced $[Ca^{2+}]_i$ oscillations blockade that eliminated NF-kB and CREB activation was provided by their enter into the nucleus and phosphorylation, respectively.[80,100] $[Ca^{2+}]_i$ oscillations in human COS-7 cells were found in the presence of 100 nM ouabain that induces 10% ^{86}Rb influx inhibition.[103] Similar oscillations were also observed in the presence of 100 nM MBG and digoxin.[104]

Importantly, unlike modest ouabain concentrations, complete suppression of NKA by 2 mM of ouabain did not produce $[Ca^{2+}]_i$ oscillations but resulted in continuous $[Ca^{2+}]_i$ increase. It was also demonstrated that $[K^+]_o$ decrease from 4.0 to 0.5 mM led to the same $[Na^+]_i$ increase as 250 μM of ouabain. Inversely to ouabain $[K^+]_o$ decrease abolished $[Ca^{2+}]_i$ oscillation rather than enhanced them. Based on these observations, authors suggested that $[Ca^{2+}]_i$ oscillations found in ouabain-treated cells are not primary result of NKA inhibition.[100] Additional investigations should be accomplished to reveal the role of Na_i^+,K_i^+-independent signaling and dissipation of monovalent cations transmembrane gradient in $[Ca^{2+}]_i$ oscillations produced by interaction of NKA and $InsP_3$ receptor interaction.

Conclusion and unresolved issues

Scheme illustrating data considered in this review are presented on Fig. 3. Results we examined demonstrate that with exception of few reports the plasma concentration of ouabain and MBG, i.e. two mostly studied CTS assessed by immunoassay techniques, is less than 1 nM. The threshold for inhibition by CTS of human and rodent α1-NKA, i.e. the only isoform detected in renal epithelial cells, is ~1 and 1000 nM, respectively, that rules out the functioning of ECTS as natriuretic hormones (at least in rodents). As predicted, at concentrations <1 nM CTS have no impact on non-canonical cellular responses, including cell adhesion, cell-to-cell communication via tight junction, gene transcription and translation, which are mediated by dissipation of the transmembrane gradients of monovalent cations (for review see[73]). It should be noted, however, that local ECTS concentration might be essentially higher than that detected in plasma. In addition, NKA sensitivity to CTS is augmented by diverse stimuli, increasing its content in the E_2~P state, including attenuation of $[K^+]_o$ and elevation of $[Na^+]_i$. Importantly, baseline $[K^+]_o$ in CSF and tubular fluid delivered to distal nephrons is decreased by ~2-fold compared to plasma.[105,106] In neurons, short periods of synaptic activity produce increases of $[Na^+]_i$, from ~10 to 30 and 100 mM in apical dendrites and dendritic spines, respectively.[107]

At concentrations less than 1 nM ouabain increases by 20–30% proliferation of several cell types (cultured human and canine VSMC,[74,75] proximal tubule cells from opossum kidney,[67] HUVEC[75,76] and human polycystic kidney cells[77]) having α1S-NKA. Because many authors reported that ouabain within this range (0.1–1 nM) activates NKA by about 25% (for review see[73]) we may suggest that cell proliferation is due to NKA activation and elevation the $[Na^+]_i/[K^+]_i$-ratio.

So, more experiments should be performed to investigate ECTS role in the triggering of Na_i^+/K_i^+-mediated cellular responses. What is $[Na^+]_i$- and $[K^+]_i$-sensors molecular origin participating in regulation of gene transcription, translation and other non-canonical cellular responses triggered by NKA inhibition and elevation of the $[Na^+]_i/[K^+]_i$-ratio? What is the mechanism of NKA activation by low doses of CTS? Does this mechanism contribute to proliferative effects and activation of several signaling pathways documented in cells subjected to chronic exposure to low

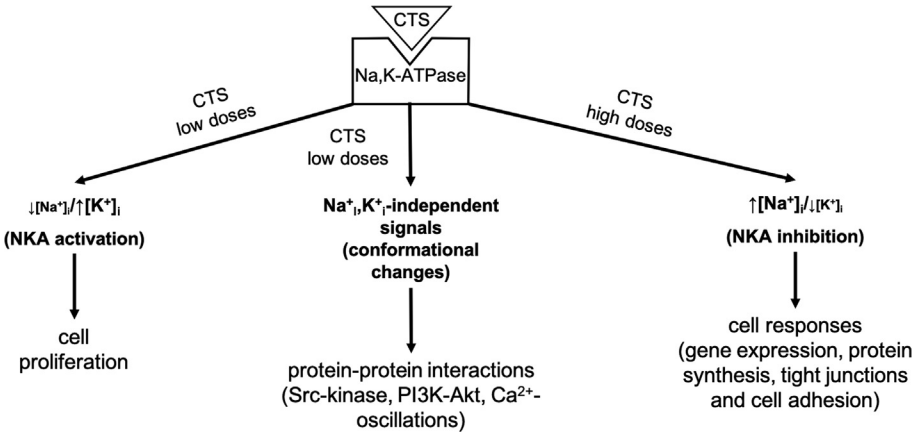

Figure 3 Effects of CTS binding to Na,K-ATPase: role of $[Na^+]_i/[K^+]_i$ -ratio changes (increase and decrease) that are produced by Na,K-ATPase inhibition and activation, respectively, and triggering cell signaling due to Na,K-ATPase conformational changes and protein—protein interaction.

doses of CTS? Do these actions provide a link between the augmented content of ECTS and pathogenesis of volume-expanded disorders proposed by several research teams?[16—21,53,71,108,109] We address these questions to forthcoming studies.

Conflict of Interests

The authors declare no conflict of interests.

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In memorium: Sergei N. Orlov (1947—2019) Paper "Na^+,K^+-ATPase as a target for endogenous cardiotonic steroids: what's the evidence?" was the last one written by our dear friend and colleague professor Sergei N. Orlov. He passed away on 13 October 2019. Sergei N. Orlov was born on 6 December 1947 in small town Kashira (Russia) that is located on the pictorial bank of the Oka. He graduated from middle school here and in 1966 was accepted at Lomonosov Moscow State University, Faculty of Biology. He graduated from the university in 1971 with outstanding academic achievement and entered a PhD program at Moscow State University in the specialty "biophysics". His PhD thesis was related to the study of free radical oxidation of higher fatty acids in phospholipids of biological membranes. Since 1975 he stated to work in Central scientific research laboratory of Ministry of Health under the guidance of chief forensic pathologist professor Yu.V. Posnov. S.N. Orlov and Yu.V. Postnov collected a group of young scientists and started to study the peculiarities of transport of monovalent cations through the cell membranes of hypertensive animals and patients. In 1983 they established discovery: "Phenomenon of propagated disturbances of cation transport through plasma membrane in essential hypertension". In 1993 Sergei Orlov was awarded by International Society on Hypertension (Pfizer Award) and became a recipient of professorship in Montreal University. Since 1993 up to 2013 he conducted his research in Research Centre of Montreal University. Main problem that was interested professor S.N. Orlov during this time was effect of monovalent cations fluxes on physiological state of different animal cells and especially on gene expression. In 2013 professor S.N. Orlov returned to Moscow State University where he continued research studies up to the fall of 2019. S.N. Orlov is an author of about 350 scientific papers, 7 books and 6 patents. He was a member of editorial teams of 9 scientific journals. Last years of life S.N. Orlov devoted to the search of sensors of monovalent cations, which were considered him as second messengers. Being seriously ill Sergei continued to work until last day of his life. His grave is on the local cemetery of his lovely town Kashira.

References

1. de Wardener HE, Mills IH, Clapham WF, Hayter CJ. Studies on the efferent mechanism of the sodium diuresis which follows the administration of intravenous saline in the dog. *Clin Sci*. 1961;21:249—258.
2. de Bold AJ, Borenstein HB, Veress AT, Sonnenberg H. A rapid and potent natriuretic response to intravenous injection of atrial myocardial extracts in rats. *Life Sci*. 1981;28(1):89—94.
3. Buckalew VM, Martinez FJ, Green WE. The effect of dialysates and ultrafiltrates of plasma of saline-loaded dogs on toad bladder sodium transport. *J Clin Invest*. 1970;49(5):926—935.

4. Buckalew VM. Endogenous digitalis-like factors: an overview of the history. *Front Endocrinol*. 2015;6, e49.
5. Hamlyn JM. Natriuretic hormones, endogenous ouabain, and related sodium transport inhibitors. *Front Endocrinol*. 2014;5, e199.
6. Krenn L, Kopp B. Bufadienolides from animal and plant sources. *Phytochemistry*. 1998;48(1):1—29.
7. Hamlyn JM, Blaustein MP, Bova S, et al. Identification and characterization of a ouabain-like compound from human plasma. *Proc Natl Acad Sci U S A*. 1991;88(14):6259—6263.
8. Kawamura A, Guo J, Itagaki Y, et al. On the structure of endogenous ouabain. *Proc Natl Acad Sci USA*. 1999;96(12): 6654—6659.
9. Schneider R, Wray V, Nimtz M, et al. Bovine adrenals contain, in addition to ouabain, a second inhibitor of the sodium pump. *J Biol Chem*. 1998;273(2):784—792.
10. Goto A, Ishiguro T, Yamada K, et al. Isolation of an urinary digitalis-like factor indistinguishable from digoxin. *Biochem Biophys Res Commun*. 1990;173(3):1093—1101.
11. Lichtstein D, Gati I, Samuelov S, et al. Identification of digitalis-like compounds in human cataractous lenses. *Eur J Biochem*. 1993;216(1):261—268.
12. Bagrov AY, Fedorova OV. Effects of two putative endogenous digitalis-like factors, marinobufagenin and ouabain, on the Na^+,K^+-pump in human mesenteric arteries. *J Hypertens*. 1998;16(12 Pt 2):1953—1958.
13. Bagrov AY, Fedorova OV, Dmitrieva RI, et al. Characterization of a urinary bufodielnolide Na,K-ATPase inhibitor in patients after acute myocardial infarction. *Hypertension*. 1998;31(5): 1097—1103.
14. Komiyama Y, Dong XH, Hishimura N, et al. A novel endogenous digitalis, telocinobufagin, exhibits elevated plasma levels in patients with terminal renal failure. *Clin Biochem*. 2005; 38(1):36—45.
15. Yoshika M, Komiyama Y, Konishi M, et al. Novel digitalis-like factor, marinobufotoxin, isolated from cultured Y-1 cells, and its hypertensive effect in rats. *Hypertension*. 2007;49(1): 209—214.
16. Schoner W, Scheiner-Bobis G. Endogenous and exogenous cardiac glycosides: their role in hypertension, salt metabolism, and cell growth. *Am J Physiol Cell Physiol*. 2007; 293(2):C509—C536.
17. Bagrov AY, Shapiro JI, Fedorova OV. Endogenous cardiotonic steroids: physiology, pharmacology, and novel therapeutic targets. *Pharmacol Rev*. 2009;61(1):9—38.
18. Leenen FHH. The central role of the brain aldosterone-"ouabain" pathway in salt-sensitive hypertension. *Biochim Biophys Acta*. 2010;1802(12):1132—1139.
19. Hodes A, Lichtstein D. Natriuretic hormones in brain function. *Front Endocrinol*. 2014;5:201.
20. Khalaf FK, Dube P, Mohamed A, et al. Cardiotonic steroids and the sodium trade balance: new insights into trade-off mechanisms mediated by the Na^+,K^+-ATPase. *Int J Mol Sci*. 2018; 19(9), e2576.
21. Blaustein MP, Chen L, Hamlyn JM, et al. Pivotal role od a2 Na+ pumps and their high affinity ouabain binding site in cardiovascular health and disease. *J Physiol*. 2016;594(21): 6079—6103.
22. Skou JC. Further investigation on a Mg^{2+} + Na^+-activated adenosinetriphosphatase possibly related to the active transport of Na^+ and K^+ across the nerve cell membrane. *Biochim Biophys Acta*. 1960;42:6—23.
23. Dvela M, Rosen H, Feldmann T, Nesher M, Lichtstein D. Diverse biological responses of different cardiotonic steroids. *Pathophysiology*. 2007;14(3—4):159—166.
24. Xie Z, Xie J. The Na/K-ATPase-mediated signal transduction as a target for new drug development. *Front Biosci*. 2005;10: 3100—3109.
25. Hamlyn JM, Blaustein MP. Endogenous ouabain: recent advances and controversies. *Hypertension*. 2016;68(3):526—532.
26. Doris PA, Jenkins LA, Stocco DM. Is ouabain an authentic endogenous mammalian substance derived from the adrenal? *Hypertension*. 1994;23(5):632—638.
27. Gomez-Sanchez EP, Poecking MF, Sellers D, Gomez-Sanches CE. Is the circulation ouabain-like compound ouabain? *Am J Hypertens*. 1994;7(7 Pt 1):647—650.
28. Lewis LK, Yandle TG, Lewis JG, et al. Ouabain is not detectable in human plasma. *Hypertension*. 2019;24(5):549—555.
29. Baecher S, Kroiss M, Fassnacht M, Vogeser M. No endogenous ouabain is detectable in human plasma by ultra-sensitive UPLC-MS/MS. *Clin Chim Acta*. 2014;431:87—92.
30. Clausen M, Hilbers F, Poulsen H. The structure and function of the Na,K-ATPase isoforms in health and disease. *Front Physiol*. 2017;8, e371.
31. Lingrel JB, Croyle ML, Woo AL, Argьello JM. Ligand binding sites of Na,K-ATPase. *Acta Physiol Scand*. 1998;163(suppl. 643):69—77.
32. Lingrel JB. The physiological significance of the cardiotonic steroid/ouabain-binding site of the Na,K-ATPase. *Annu Rev Physiol*. 2010;72:395—412.
33. Blaustein M. Sodium transport in hypertension. Where are we going? *Hypertension*. 1984;6(4):445—453.
34. Hauptman PJ, Garg R, Kelly RA. Cardiac glycosides in the next millennium. *Prog Cardiovasc Dis*. 1999;41(4):247—254.
35. Orlov SN, Taurin S, Hamet P. The α1-Na/K pump does not mediate the involvement of ouabain in the development of hypertension in rats. *Blood Pres*. 2002;11(1):56—62.
36. Fedorova OV, Bagrov AY. Inhibition of Na/K ATPase from rat aorta by two Na/K pump inhibitors, ouabain and marinobufagenin: evidence of interaction with different alpha-subunit isoform. *Am J Hypertens*. 1997;10(8):929—935.
37. Akimova OA, Bagrov AY, Lopina OD, et al. Cardiotonic steroids differentially affect intracellular Na^+ and $[Na^+]_i/[K^+]_i$-independent signaling in C7-MDCK cells. *J Biol Chem*. 2005;280(1): 832—839.
38. Klimanova EA, Petrushenko IY, Mitkevich VA, et al. Binding of ouabain and marinobufagenin leads to different structural changes in Na,K-ATPase and depends on the enzyme conformation. *FEBS Lett*. 2015;589(19 Pt B):2668—2674.
39. Blaustein MP, Zhang J, Chen L, Hamilton BP. How does salt retention raise blood pressure? *Am J Physiol Regul Integr Comp Physiol*. 2006;290(3):R514—R523.
40. Iwamoto T, Kita S, Zhang J, et al. Salt-sensitive hypertension is triggered by Ca^{2+} entry via Na^+/Ca^{2+} exchanger type-1 in vascular smooth muscle cells. *Nat Med*. 2004;10(11): 1193—1199.
41. Orlov SN, Taurin S, Tremblay J, Hamet P. Inhibition of Na^+,K^+ pump affects nucleic acid synthesis and smooth muscle cell proliferation via elevation of the $[Na^+]_i/[K^+]_i$ ratio: possible implication in vascular remodeling. *J Hypertens*. 2001;19(9): 1559—1565.
42. Taurin S, Seyrantepe V, Orlov SN, et al. Proteome analysis and functional expression identify mortalin as an anti-apoptotic gene induced by elevation of $[Na+]_i/[K+]_i$ ratio in cultured vascular smooth muscle cells. *Circ Res*. 2002;91(10):915—922.
43. Koltsova SV, Trushina Y, Haloui M, et al. Ubiquitous $[Na+]_i/[K+]_i$-sensitive transcriptome in mammalian cells: evidence for Ca2+i-independent excitation-transcription coupling. *PLoS One*. 2012;7, e38032.
44. Klimanova EA, Sidorenko SV, Smolyaninova LV, et al. Ubiquitous and cell type-specific transcriptomic changes triggered by dissipation of monovalent cation gradients in rodent cells: physiological and pathophysiological implications. *Curr Top Membr*. 2019;83:107—150.
45. Klimanova EA, Tverskoi AM, Koltsova SV, et al. Time- and dose-dependent actions of cardiotonic steroids on

transcriptome and intracellular content of Na^+ and K^+: a comparative analysis. *Sci Rep*. 2017;7, e45403.
46. Taurin S, Dulin NO, Pchejetski D, et al. c-Fos expression in ouabain-treated vascular smooth muscle cells from rat aorta: evidence for an intracellular-sodium-mediated, calcium-independent mechanism. *J Physiol*. 2002;543(Pt 3):835—847.
47. Lubin M, Ennis HL. On the role of intracellular potassium in protein synthesis. *Biochim Biophys Acta*. 1964;80:614—631.
48. Orlov SN, Hamet P. Intracellular monovalent ions as second messengers. *J Membr Biol*. 2006;210(3):161—172.
49. Orlov SN, Hamet P. Salt and gene expression: evidence for Na_i^+,K_i^+-mediated signaling pathways. *Pflueg Arch Eur J Physiol*. 2015;467(3):489—498.
50. Cahn F, Lubin M. Inhibition of elongation steps of protein synthesis at reduced potassium concentrations in reticulocytes and reticulocyte lysate. *J Biol Chem*. 1978; 253(21):7798—7803.
51. Tverskoi AM, Sidorenko SV, Klimanova EA, et al. Effects of ouabain on proliferation of human endothelial cells correlate with Na^+,K^+-ATPase activity and intracellular ratio of Na^+ and K^+. *Biochemistry (Mosc)*. 2016;81(8):876—883.
52. Das S, Maitra U. Mutational analysis of mammalian translation initiation factor 5 (eIF5): role of interaction between β subunit of eIF2 and eIF5 in eIF5 function in vitro and in vivo. *Mol Cell Biol*. 2000;20(11):3942—3950.
53. Jennings MD, Pavitt GD. eIF5 is a dual function GAP and GDI for eukaryotic translational control. *Small GTPases*. 2010; 1(2):118—123.
54. Gupta RS, Chora A, Stetsko DK. Cellular basis for the species differences in sensitivity to cardiac glycosides (digitalis). *J Cell Physiol*. 1986;127(2):197—206.
55. Belusa R, Aizman O, Andersson RM, Aperia A. Changes in Na^+-K^+-ATPase activity influence cell attachment to fibronectin. *Am J Physiol Cell Physiol*. 2001;282(2):C302—C309.
56. Rajasekaran SA, Hu J, Gopal J, et al. Na,K-ATPase inhibition alters tight junction structure and permeability in human retinal pigment epithelial cells. *Am J Physiol Cell Physiol*. 2003;284(6):C1497—C1507.
57. Rajasekaran SA, Palmer LG, Moon SY, et al. Na,K-ATPase activity is required for formation of tight junctions, desmosomes, and induction of polarity in epithelial cells. *Mol Biol Cell*. 2001;12(12):3717—3732.
58. Martin PE, Hill NS, Kristensen B, Errington RJ, Griffith TM. Ouabain exerts biphasic effects on connexin functionality and expression in vascular smooth muscle cells. *Br J Pharmacol*. 2003;140(7):1261—1271.
59. Matchkov VV, Gustafsson H, Rahman A, et al. Interaction between Na^+/K^+-pump and $Na^+/Ca2^+$-exchanger modulates intercellular communication. *Circ Res*. 2007;100(7):1026—1035.
60. Violette MI, Madan P, Watson AJ. Na^+/K^+-ATPase regulates tight junction formation and function during mouse preimplantation development. *Dev Biol*. 2006;289(2):406—419.
61. Lee J-M, Grabb MC, Zipfel GJ, Choi DW. Brain tissue responses to ischemia. *J Clin Invest*. 2000;106(6):723—731.
62. Rajasekaran AK, Rajasekaran SA. Role of Na-K-ATPase in the assembly of tight junctions. *Am J Physiol Ren Physiol*. 2003; 285(3):F388—F396.
63. Gao J, Wymore RS, Wang Y, et al. Isoform-specific stimulation of cardiac Na/K pumps by nanomolar concentrations of glycosides. *J Gen Physiol*. 2002;119(4):297—312.
64. Li J, Zelenin S, Aperia A, Aizman O. Low doses of ouabain protect from serum deprivation-triggered apoptosis and stimulate kidney cell proliferation via activation of NF-kappaB. *J Am Soc Nephrol*. 2006;17(7):1848—1857.
65. Ghysel-Burton J, Godfraind T. Stimulation and inhibition of the sodium pump by cardiotonic steroids in relation to their binding sites and ionotropic effect. *Br J Pharmacol*. 1979; 66(2):175—184.
66. Balzan S, D'Urso G, Nicolini G, Forini F, Pellegrino M, Montali U. Erythrocyte sodium pump stimulation by ouabain and an endogenous ouabain-like factor. *Cell Biochem Funct*. 2007;25(3):297—303.
67. Khundmiri SJ, Metzler MA, Ameen M, Amin V, Rane MJ, Delamere NA. Ouabain induces cell proliferation through calcium dependent phosphorylation of Akt (protein kinase B) in opossum kidney proximal tubule cells. *Am J Physiol Cell Physiol*. 2006;291(6):C1247—C1257.
68. Khundmiri SJ, Salyer SA, Farmer B, et al. Structural determinants for te ouabain-stimulated increase in Na-K ATPase activity. *Biochim Biophys Acta*. 2014;1843(6):1089—1102.
69. Oselkin M, Tian D, Bergold PJ. Low-dose cardiotonic steroids increase sodium-potassium ATPase activity that protects hippocampal slice cultures from experimental ischemia. *Neurosci Lett*. 2010;473(2):67—71.
70. Holthouser KA, Mandal A, Merchant ML, et al. Ouabain stimulates Na-K-ATPase through a sodium/hydrogen exchanger-1 (NHE1)-dependent mechanism in human kidney proximal tubule cells. *Am J Physiol Ren Physiol*. 2010; 299(1):F77—F90.
71. Khundmiri SJ. Advances in understanding the role of cardiac glycosides in control of sodium transport in renal tubules. *J Endocrinol*. 2014;222(1):R11—R24.
72. Askari A. Na^+,K^+-ATPase: on the number of the ATP sites of the functional unit. *J Bioenerg Biomembr*. 1987;19(4):359—374.
73. Orlov SN, Klimanova EA, Tverskoi AM, Vladychenskaya EA, Smolyaninova LV, Lopina OD. Na_i^+,K_i^+-dependent and -independent signaling triggered by cardiotonic steroids: facts and artifacts. *Molecules*. 2017;22(4), e635.
74. Aydemir-Koksoy A, Abramowitz J, Allen JC. Ouabain-induced signaling and vascular smooth muscle cell proliferation. *J Biol Chem*. 2001;276(49):46605—46611.
75. Abramowitz J, Dai C, Hirschi KK, et al. Ouabain- and marinobufagenin-induced proliferation of human umbilical vein smooth muscle cells and a rat vascular smooth muscle cell line, A7r5. *Circulation*. 2003;108(24):1049—1054.
76. Saunders R, Scheiner-Bobis G. Ouabain stimulates endothelin release and expression in human endothelial cells without inhibiting the sodium pump. *Eur J Biochem*. 2004;271(5): 1054—1062.
77. Nguyen AN, Wallace DP, Blanco G. Ouabain binds with high affinity to the Na^+,K^+-ATPase in human polycystic kidney cells and induces extracellular signal-regulated kinase activation and cell proliferation. *J Am Soc Nephrol*. 2007;18(1): 46—57.
78. Murata Y, Matsuda T, Tamada K, et al. Ouabain-induced cell proliferation in cultured rat astrocytes. *Jpn J Pharmacol*. 1996;72(4):347—353.
79. Dmitrieva RI, Doris PA. Ouabain is a potent promoter of growth and activator of ERK1/2 in ouabain-resistant rat renal epithelial cells. *J Biol Chem*. 2004;278(30):28160—28166.
80. Desfrere L, Karlsson M, Hiyoshi H, et al. Na,K-ATPase signal transduction triggers CREB activation and dendritic growth. *Proc Natl Acad Sci USA*. 2009;106(7):2212—2217.
81. Segel GB, Lichtman MA. The apparent discrepancy of ouabain inhibition of cation transport and lymphocyte proliferation is explained by time-dependency of ouabain binding. *J Cell Physiol*. 1980;104(1):21—26.
82. Orlov SN, Hamet P. Apoptosis vs oncosis: role of cell volume and intracellular monovalent cations. *Adv Exp Med Biol*. 2004; 559:219—233.
83. Akimova OA, Platonova AA, Koltsova SV, et al. Cell death triggered by cardiotonic steroids: role of cell volume perturbations and α1-Na^+,K^+-ATPase subunit. *Siberian Med Bull*. 2013;12:24.
84. Akimova OA, Tverskoi AM, Smolyaninova LV, et al. Critical role of the a1-Na^+,K^+-ATPase subunit in insensitivity of rodent

cells to cytotoxic action of ouabain. *Apoptosis*. 2015;20(9):1200—1210.
85. Xie Z, Askari A. Na^+/K^+-ATPase as a signal transducer. *Eur J Biochem*. 2002;269:2434—2439.
86. Liu J, Xie Z. The sodium pump and cardiotonic steroids-induced signal transduction protein kinases and calcium-signaling microdomains in regulation of transporter trafficking. *Biochim Biophys Acta*. 2010;1802(12):1237—1245.
87. Haas M, Askari A, Xie Z. Involvement of Src and epidermal growth factor receptor in the signal transducing function of Na^+/K^+-ATPase. *J Biol Chem*. 2000;275(36):27832—27837.
88. Haas M, Wang H, Tian J, Xie Z. Src-mediated inter-receptor cross-talk between the Na^+,K^+-ATPase and epidermal growth factor receptor relays the signal from ouabain to mitogen-activated protein kinases. *J Biol Chem*. 2002;277(21):18694—18702.
89. Xie J, Ye Q, Cui X, et al. Expression of rat Na-K-ATPase a2 enables ion pumping but not ouabain-induced signaling in a1-deficient porcine renal epithelial cells. *Am J Physiol Cell Physiol*. 2015;309(6):C373—C382.
90. Li Z, Cai T, Tian J, et al. NaKtide, a Na/K-ATPase-derived peptide Src inhibitor, antagonizes ouabain-activated signal transduction in cultured cells. *J Biol Chem*. 2009;284(31):21066—21076.
91. Tian J, Cai T, Yuan Z, et al. Binding of Src to Na^+,K^+-ATPase forms a functional signaling complex. *Mol Biol Cell*. 2006;17:317—326.
92. Gable ME, Abdallah SL, Najjar SM, Liu L, Askari A. Digitalis-induced cell signaling by the sodium pump: on the relation of Src and Na^+,K^+-ATPase. *Biochem Biophys Res Commun*. 2014;446(4):1151—1154.
93. Yu H, Cui X, Zhang J, et al. Heterogeneity of signal transduction by Na-K-ATPase α-isoforms: role of SRC interaction. *Am J Physiol Cell Physiol*. 2018;314(2):C202—C210.
94. Liu J, Tian J, Haas M, Shapiro JI, Askari A, Xie Z. Ouabain interaction with cardiac Na^+/K^+-ATPase initiates signal cascade independent of changes in intracellular Na^+ and Ca^{2+} concentrations. *J Biol Chem*. 2000;275(36):27838—27844.
95. Tian J, Gong X, Xie Z. Signal-transducing function of Na^+,K^+-ATPase is essential for ouabain's effect on $[Ca^{2+}]i$ in rat cardiac myocytes. *Am J Physiol*. 2001;281(5):H1899—H1907.
96. Kulikov A, Eva A, Kirch U, Boldyrev A, Scheiner-Bobis G. Ouabain activates signaling pathways associated with cell death in human neuroblastoma. *Biochim Biophys Acta*. 2007;1768(7):1691—1702.
97. Wu J, Akkuratov EE, Bai Y, Gaskill CM, Askari A, Liu L. Cell signaling associated with Na^+/K^+-ATPase; activation of phosphatidylinositide 3-kinase IA/Akt by ouabain is independent of Src. *Biochem*. 2013;52(50):9059—9067.
98. Liu L, Abramowitz J, Askari A, Allen JC. Role of caveolae in ouabain-induced proliferation of cultured vascular smooth muscle cells of the synthetic phenotype. *Am J Physiol Heart Circ Physiol*. 2004;287(5):H2173—H2182.
99. Liu L, Zhao X, Pierre SV, Askari A. Association of PI3K-Akt signaling pathway with digitalis-induced hypertrophy of cardiac myocytes. *Am J Physiol Cell Physiol*. 2007;293(5):C1489—C1497.
100. Aizman O, Uhlen P, Lal M, Brismar H, Aperia A. Ouabain, a steroid hormone that signals with slow calcium oscillations. *Proc Natl Acad Sci USA*. 2001;98(23):13420—13424.
101. Dolmetsch RE, Xu K, Lewis RS. Calcium oscillations increase the efficiency and specificity of gene expression. *Nature*. 1998;392(6679):933—936.
102. Lonze BE. Function and regulation of CREB family transcription factors in the nervous system. *Neuron*. 2002;35(4):605—623.
103. Zhang S, Maimersjo S, Li J, et al. Distinct role of the N-terminal tail of the Na,K-ATPase catalytic subunit as a signal transducer. *J Biol Chem*. 2006;281(31):21954—21962.
104. Fontana JM, Burlaka I, Khodus G, Brismar H, Aperia A. Calcium oscillations triggered by cardiotonic steroids. *FEBS J*. 2013;280(21):5450—5455.
105. Somjen GG. *Ions in the Brain: Normal Functions, Seizures, and Stroke*. New York: Oxford University Press; 2004.
106. Vander AJ. In: *Renal Physiology*. 5 ed. New York: McGraw-Hill, Inc.; 1991.
107. Rose CR, Konnerth A. NMDA-receptor-mediated Na^+ signals in spines and dendrites. *J Neurosci*. 2001;21(12):4207—4214.
108. Fedorova OV, Lakatta EG, Bagrov AY, Melander O. Plasma level of the endogenous sodium pump ligand marinobufagenin is related to the the salt-sensitivity. *J Hypertens*. 2015;33(3):533—541.
109. Schoner W, Scheiner-Bobis G. Role of endogenous cardiotonic steroids in sodium homeostasis. *Nephrol Dial Transplant*. 2008;23(9):2723—2729.
110. Rossi G, Manunta P, Hamlyn JM, et al. Immunoreactive endogenous ouabain in primary aldosteronism and essential hypertension: relationship with plasma renin, aldosterone and blood pressure levels. *J Hypertens*. 1995;13(10):1181—1191.
111. Gottlieb SS, Rogowski AS, Weinberg M, Krichten CM, Hamilton BP, Hamlyn JM. Elevated concentration of endogenous ouabain in patients with congestive heart failure. *Circulation*. 1992;86(2):420—425.
112. van Horck FP, Ahmadian MR, Haeussler LC, Moolenaar WH, Kranenburg O. Characterization of p190RhoCEF, a Rho-specific guanine nucleotide exchange factor that interacts with microtubule. *J Biol Chem*. 2001;276(7):4948—4956.
113. Butt AN, Semra YK, Ho CS, Swaminathan R. Effect of high salt intake on plasma and tissue concentration of endogenous ouabain-like substances in the rat. *Life Sci*. 1997;61(24):2367—2373.
114. Butt AN, Semra YK, Lane SJ, Lee T, Swaminathan R. Endogenous ouabain secretion in man is not regulated by ACTH. *J Steroid Biochem Mol Biol*. 1998;66(3):151—157.
115. Fedorova OV, Anderson DE, Bagrov AY. Plasma marinobufagenin-like and ouabain-like immunoreactivity in adrenocorticotropin-treated rats. *Am J Hypertens*. 1998;11(7):796—802.
116. Gonick HC, Ding Y, Vaziri ND, Bagrov AY, Fedorova OV. Simultaneous measurement of marinobufagenin, ouabain, and hypertension-associated protein in various disease state. *Clin Exp Hypertens*. 1998;20(5—6):617—627.
117. Lopatin DA, Ailmazian EK, Dmitrieva RI, et al. Circulating bufodienolide and cardenolide sodium pump inhibitors in preeclampsia. *J Hypertens*. 1999;17(8):1179—1187.
118. Vakkuri O, Arnason SS, Pouta A, Vuolteenaho O, Leppaluoto J. Radioimmunoassay of plasma ouabain in healthy and pregnant individuals. *J Endocrinol*. 2000;165(3):669—677.
119. Balzan S, Neglia D, Ghione S, et al. Increased circulating level of ouabain-like factor in patients with asymptomatic left ventricular dysfunction. *Eur J Heart Fail*. 2001;3(2):165—171.
120. Fedorova OV, Kolodkin NI, Agalakova NI, Lakatta EG, Bagrov AY. Marinobufagenin, an endogenous α-1 sodium pump ligand, in hypertensive Dahl salt-sensitive rats. *Hypertension*. 2001;37(2 Pt 2):462—466.
121. Fedorova OV, Talan MI, Agalakova NI, Lakatta EG, Bagrov AY. Endogenous ligand of α1 sodium pump, marinobufagenin, is a novel mediator of sodium chloride-dependent hypertension. *Circulation*. 2002;105(9):1122—1127.
122. Fedorova OV, Kolodkin NI, Agalakova NI, et al. Antibody to marinobufagenin lowers blood pressure in pregnant rats on high NaCl intake. *J Hypertens*. 2005;23(4):835—842.

123. Harwood S, Mullen AM, McMahon AC, Dawnay A. Plasma OLS is elevated in mild experimental uremia but is not associated with hypertension. *Am J Hypertens*. 2001;14(11 Pt 1):1112—1115.
124. Berendes E, Cullen P, van Aken H, et al. Endogenous glycosides in critically ill patients. *Crit Care Med*. 2003;31(5):1331—1337.
125. Wang JG, Staessen JA, Messangio E, et al. Salt, endogenous ouabain and blood pressure interactions in general population. *J Hypertens*. 2003;21(8):1475—1481.
126. Balzan S, Nicolini G, Iervasi A, Di Cecco P, Fommei E. Endogenous ouabain and acute salt loading in low-renin hypertension. *Am J Hypertens*. 2005;18(7):906—909.
127. Fridman AI, Matveev SA, Agalakova NI, Fedorova OV, Lakatta EG, Bagrov AY. Marinobufagenin, an endogenous ligand of α-1 Na/K-ATPase, is a marker of congestive heart failure severity. *J Hypertens*. 2002;20(6):1189—1194.
128. Kennedy DJ, Vetteth S, Periyasamy SM, et al. Central role for the cardiotonic steroid marinobufagenin in the pathogenesis of experimental uremic cardiomyopathy. *Hypertension*. 2006;47(3):488—495.
129. Ferrari P, Torielli L, Ferrandi M, et al. PST2238: a new antihypertensive compound that antagonizes the long-term pressor effect of ouabain. *J Pharmacol Exp Therapeut*. 1998;285(1):83—94.
130. Lee K, Jung J, Kim M, Guidotti G. Interaction of the α subunit of Na,K-ATPase with cofilin. *Biochem J*. 2001;353(Pt 2):377—385.
131. Dostanic I, Schultz JEJ, Lorenz JN, Lingrel JB. The α1 isoform of Na,K-ATPase regulates contractility and functionally interacts and co-localizes with the Na/Ca-exchanger in heart. *J Biol Chem*. 2004;279(52):54053—54061.
132. Fedorova OV, Lakatta EG, Bagrov AY. Differential effects of acute NaCl loading on endogenous ouabain-like and marinobufagenin-like ligands of the sodium pump in Dahl hypertensive rats. *Circulation*. 2000;102:3009—3014.
133. Fedorova OV, Talan MI, Agalakova NI, Lakatta EG, Bagrov AY. Coordinated shifts in Na/K-ATPase isoforms and their endogenous ligands during cardiac hypertrophy and failure in NaCl-sensitive hypertension. *J Hypertens*. 2004;22(2):1—9.
134. Dmitrieva RI, Georgiev IY, Shpen VM, Bagrov AY. Bufadienolide nature of an endogenous inhibitor of sodium-potassium adenosine triphosphatase in humans. *J Evol Biochem Physiol*. 1997;33(3):355—363.
135. Fedorova OV, Dorofeeva NA, Lopatin DA, Lakatta EG, Bagrov AY. Phorbol diacetate potentiates Na^+,K^+-ATPase inhibition by a putative endogenous ligand, marinobufagenin. *Hypertension*. 2002;39(2):298—302.
136. Sceinin M, Koulu M, Laurikainen E, Allonen H. Hypokalemia and other non-bronchial effects of inhaled fenoterol and salbutamol: a placebo controlled dose-response study in healthy volunteers. *Br J Clin Pharmacol*. 1987;24(5):645—653.
137. Bagrov AY, Dmitrieva RI, Fedorova OV, Kazakov GP, Roukoyatkina NI, Shpen VM. Endogenous marinobufagenin-like immunoreactive substance: a possible endogenous Na,K-ATPase inhibitor with vasoconstrictor activity. *Am J Hypertens*. 1996;9(10 Pt 1):982—990.
138. Blanco G, Sanchez G, Mercer RW. Comparison of the enzymatic properties of the Na,K-ATPase $\alpha3/\beta1$ and $\alpha3/\beta2$ isozymes. *Biochem*. 1995;34(31):9897—9903.
139. Brownlee AA, Johnsosn P, Mills IH. Actions of bufalin and cinobufotalin, two bufadienolides respectively more active and less active than ouabain, on ouabain binding and 86Rb uptake by human erythrocytes. *Clin Sci*. 1990;78(2):169—174.
140. Flier JS. Ouabain-like activity in toad skin and its implication for endogenous regulation of ion transport. *Nature*. 1978;274(5668):285—286.
141. Senn N, Lelievre LG, Braquet P, Garay R. High sensitivity of the Na^+,K^+-pump of human red blood cells to genins of cardiac glycosides. *Br J Pharmacol*. 1988;93(4):803—810.
142. Moller B, Vaag A, Johansen T. Ouabain inhibition of the sodium-potassium pump: estimation of ED_{50} in different types of human leucocytes in vitro. *Br J Clin Pharmacol*. 1990;29(1):93—100.
143. Tao QF, Hollenberg NK, Price DA, Graves SV. Sodium pump isoform specificity for digitalis-like factor isolated from human peritoneal dialysate. *Hypertension*. 1996;29(3):815—821.
144. Rodriguez-Manas L, Pareja A, Sanchez-Ferrer CF, Casado MA, Salacies MJ. Endothelial role in ouabain-induced contraction of Guinea pig carotid arteries. *Hypertension*. 1992;20(5):674—681.
145. Miakawa-Naito A, Uhlйn P, Lal M, et al. Cell signaling microdomain with Na,K-ATPase and inositol 1,4,5-triphosphate receptor generates calcium oscillations. *J Biol Chem*. 2003;278(50):50355—50361.
146. Peng M, Huang L, Xie Z, Huang W-H, Askari A. Partial inhibition of Na^+/K^+-ATPase by ouabain induces the Ca^{2+}-dependent expression of early-response genes in cardiac myocytes. *J Biol Chem*. 1996;271(17):10372—10378.
147. Chueh SC, Guh JH, Chen J, Lai MK, Teng CM. Dual effect of ouabain on the regulation of proliferation and apoptosis in human prostatic smooth muscle cells. *J Urol*. 2001;166(1):347—353.
148. Li S, Wattenberg EV. Differential activation of mitogen-activated protein kinases by palytoxin and ouabain, two ligands for the Na^+,K^+-ATPase. *Toxicol Appl Pharmacol*. 1998;151(2):377—384.
149. Kometiani P, Li J, Gnudi L, Kahn BB, Askari A, Xie Z. Multiple signal transduction pathways link Na^+/K^+-ATPase to growth-related genes in cardiac myocytes: the roles of ras and mitogen-activated protein kinases. *J Biol Chem*. 1998;273(24):15249—15256.
150. Mohammadi K, Liu L, Tian J, Kometiani P, Askari A. Positive ionotropic effect of ouabain on isolated heart is accompanied by activation of signal pathways that link Na^+/K^+-ATPase to ERK1/2. *J Cardiovasc Pharmacol*. 2003;41(4):609—614.
151. Kometiani P, Liu L, Askari A. Digitalis-induced signaling by Na^+/K^+-ATPase in human breast cancer cells. *Mol Pharmacol*. 2005;67(3):929—936.
152. Kotova O, Al-Khalili L, Hooke C, Fedorova OV, Bagrov AY, Chibalin AV. Cardiotonic steroids stimulate glycogen synthesis in human skeletal muscle cells via a Src- and ERK1/2-dependent mechanism. *J Biol Chem*. 2006;281(29):20085—20094.
153. Valente RC, Capella LS, Monteiro RQ, Rumjanek VM, Lopes AG, Capella MAM. Mechanisms of ouabain toxicity. *Faseb J*. 2003;17(12):1700—1702.