REVIEW ARTICLE

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

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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⁺]/[K⁺]-ratio and increase cell proliferation. Possible physiological significance and mechanism of non-canonical Na⁺/K⁺-dependent and Na⁺/K⁺-independent cell responses to CTS are discussed.

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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. 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. 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⁺ transport² (for historical details, see³-⁵).

The beneficial effect of digitals 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 cardioactive 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 telocinobufagin¹⁴ and marinobufutoxin¹⁵ (structure of some endogenous cardioactive 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, see¹⁶-²¹).

Starting from early 90th the focus of investigations has been directed to the mechanisms by which ECTS may 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 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 E₁-E₂ 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, see³⁰,³¹). The mechanism of CTS inhibiting effect on NKA was mostly investigated with ouabain originating 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
as well as in H1–H2, H5–H6 and H7–H8 extracellular loops exert an influence on ouabain affinity of NKA. 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.

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. However, that the treatment of congestive heart failure with commercially available cardenolides occurs in the absence of significant natriuresis. 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 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 (IC50 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). 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 and about the same affinity demonstrated in α1-NKA purified from duck salt glands. 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
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 co-workers proposed that ECTS evoked an increase of vascular tone via elevation of \([\text{Na}^+]_i\) in the space-limited plasma membrane-junctional endoplasmic reticulum (plasmerosomes) abundant with ubiquitous isoform of the \(\text{Na}^+/\text{Ca}^{2+}\) exchanger (NCX1) and with \(\alpha2^{-}\), \(\alpha3^{-}\)-NKA. 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. To the best of our knowledge, the elevation of \(\text{Na}^+\) concentration in cytoplasm or plasmerosome compartments by ECTS at concentrations detected in the extracellular fluids has not been demonstrated.

### Do ECTS trigger non-canonical \(\text{Na}^+_i/\text{K}^+_i\)-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 \([\text{Na}^+]_i/[\text{K}^+]_i\)-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 and appearance of hundreds of newly synthesized proteins. Later on we employed Affymetrix technology for identification of cell-type specific and ubiquitous set of \(\text{Na}^+/\text{K}^+\)-sensitive transcripts by comparative analysis of the action of ouabain and \(\text{K}^+\)-free medium on transcriptomic changes in HUVEC, RVSMC and HeLa cell line. In this study, we

| 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     |            |
| 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 ± 0.02/0.27/0.38 ± 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.
detected changes in expression levels of hundreds of genes that were highly correlated between two treatments thus demonstrating a key role of Na\(^+\)/K\(^+\)-mediated mechanism of excitation-transcription coupling. Importantly, about 80 Na\(^+\)/K\(^+\)-sensitive transcripts were found in all types of cells. This set of ubiquitous Na\(^+\)/K\(^+\)-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 \(\alpha\)1S-NKA by ouabain, vs mouse and hamster cells, having CTS-resistant \(\alpha\)1R-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, RVSMC\(^{58,59}\) and HeLa cells.\(^{58}\) Importantly, tight junction and adhesion breakdown in cells with \(\alpha\)1R- and \(\alpha\)1S-NKA was observed at concentration of ouabain \(\sim1000\) and 1 \(\mu\)M, 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\(^+\) and loss of K\(^+\) 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\(^{55}\) is augmented with 10 nM and less ouabain concentration. At this low ouabain doses Na\(^+\) concentration in guinea pig atria\(^{64,65}\) was decreased. At 0.1 nM ouabain an activation of \(^{86}\)Rb uptake was seen in human
erythrocytes, whereas at 10 nM and 10 pM ouabain augmented \(^{86}\)Rb uptake was found in opossum and human kidney proximal tubule cells, respectively. Stimulation of Na-pump and increment of NKA activity by ouabain and MBG were also documented in hippocampal slice cultures and human mesenteric arteries, as well as microsomal fractions from mammalian kidney and duck salt glands. 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. 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 are at least partially mediated by NKA activation.

Do ECTS trigger cell proliferation?

Proliferation of cultured human and canine VSMC, proximal tubule cells from opossum kidney, HUVEC, and human polycystic kidney cells 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, rat astrocytes and rat VSMC expressing \(\alpha_1R\)-NKA. As noted above at these concentrations ouabain did not inhibit NKA, thus indicating that CTS proliferation action is mediated by Nai\(^+\), K\(^+\)-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 | c-PTC, 10−100 | 100 |
| | h-EC, 1−10 | 76 |
| | r-PTC, >5 × 10\(^4\) | 100,145 |
| | r-CM, 10\(^3\) | 87,146 |
| ERK phosphorylation & activation | p-LCC-PK1, >10\(^2\) | 88 |
| | r-A7r5, 10\(^3\) | 88 |
| | gp-heart, 10\(^2\) | 150 |
| | r-heart, 5 × 10\(^4\) | 150 |
| | h-breast cancer cells, 10\(^2\) | 151 |
| | h-SKMC, 10\(^2\) | 152 |
| | c-VSMC, 1 | 74 |
| Src activation | p-LCC-PK1, 10\(^3\) | 88 |
| | r-A7r5, 10\(^3\) | 88 |
| | r-CM, 10\(^3\) | 87 |
| | h-breast cancer cells, 10\(^2\) | 151 |
| | h-SKMC, 10\(^2\) | 152 |
| Protein tyrosine phosphorylation | c-PTC, 10\(^3\) | 87 |
| | c-REC, 10\(^3\) | 153 |
| | c-VSMC, 1 | 74 |
| Akt phosphorylation | o-kidney PTC, 10\(^6\) | 67 |

Abbreviations: c – canine, f – fish, gp – guinea pig, h – human, m – mouse, mm – monkey; p – pig, r – rat; CGC – cerebellar granule cells; CM – cardiomyocytes; CytD – cytoskeletal 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.

\( * P < 0.05; ** P < 0.01; *** P < 0.001.\)

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\(^{57,74,76,79,80}\) 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 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\(^+\)/K\(^+\)-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, oncrosis and apoptosis is out of scope of our review and considered elsewhere\(^{23,82–84}\).

**Do ECTS evoke Na\(^+\)/K\(^+\)-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\(^+\)/K\(^+\)-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\(^+\)/K\(^+\)-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 A7r5 cells 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 herbimycin A.\(^{87,88}\) It was demonstrated that ouabain activates ERK MAPK and Src in transfected pig renal epithelial cells (PY-17) having z1-but not z2-NKA.\(^{89}\) Detailed mapping of z1-NKA nucleotide binding domain revealed 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 z1-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 z2-NKA.\(^{93}\) Native z2-isofrom 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 z1-but not z2-sequence into the z2-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 z2, the mutant cells gained z1-like signaling function.

It has been proposed that signaling cascades triggered by NKA interaction with Src do not depend on the change of
intracellular Na⁺, K⁺ and Ca²⁺ concentrations. 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 Rb influx. 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⁺]/[K⁺]-ratio. This observation is consistent with other reports showing Src-mediated signaling at CTS concentrations that inhibit NKA and [K⁺]-selectivity. In LA, [K⁺]-selectivity was employed in this study. Using Na⁺/K⁺-selectivity protein kinase B also known as Akt in the presence of 100 nM MBG and [Ca²⁺]i oscillations were also observed in the presence of 100 nM MBG and digoxin. Importantly, unlike modest ouabain concentrations, complete suppression of NKA by 2 mM of ouabain did not produce [Ca²⁺]i oscillations but resulted in continuous [Ca²⁺]i increase. It was also demonstrated that [K⁺]o decreased from 4.0 to 0.5 mM led to the same [Na⁺]o increase as 250 μM of ouabain. Inversely to ouabain ([K⁺]o), decrease abolished [Ca²⁺]i oscillation rather than enhanced them. Based on these observations, authors suggested that [Ca²⁺]i oscillations found in ouabain-treated cells are not primary result of NKA inhibition. Additional investigations should be accomplished to reveal the role of Na⁺/K⁺-independent signaling and dissipation of monovalent cations transmembrane gradient in [Ca²⁺]i oscillations produced by interaction of NKA and InsP₃ receptor interaction.

PI3K-Akt

Liu et al. 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 and Rb influx. Importantly, the augmented tyrosine phosphorylation was mimicked by NKA inhibition in K⁺-depleted medium. Viewed collectively, these data strongly suggest that in CTS-treated cells raised [Na⁺]/[K⁺]-ratio contributes to Src-mediated signaling triggering/progression.

Ca²⁺-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 [Ca²⁺]i oscillation which were abolished by L-type Ca²⁺ channel blocker nifedipine. It is well-documented that [Ca²⁺]i oscillations activate transcription factors NF-kB and CREB. In fact, ouabain-induced [Ca²⁺]i oscillations blockade that eliminated NF-kB and CREB activation was provided by their enter into the nucleus and phosphorylation, respectively. [Ca²⁺]i oscillations in human COS-7 cells were found in the presence of 100 nM ouabain that induces 10% Rb influx inhibition. Similar oscillations were also observed in the presence of 100 nM MBG and digoxin.

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 see73). 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 E₂ ~P state, including attenuation of [K⁺]o, and elevation of [Na⁺]o. Importantly, baseline [K⁺]o in CSF and tubular fluid delivered to distal nephrons is decreased by ~2-fold compared to plasma. In neurons, short periods of synaptic activity produce increases of [Na⁺]o, from ~10 to 30 and 100 mM in apical dendrites and dendritic spines, respectively.

At concentrations less than 1 nM ouabain increases by 20–30% proliferation of several cell types (cultured human and canine VSMC, proximal tubule cells from opossum kidney, and HUVEC proximal tubule cells from opossum kidney, and human polycystic kidney cells) having α1S-NKA. Because many authors reported that ouabain within this range (0.1–1 nM) activates NKA by about 25% (for review see73) we may suggest that cell proliferation is due to NKA activation and elevation the [Na⁺]/[K⁺]-ratio.

So, more experiments should be performed to investigate ECTS role in the triggering of Na⁺/K⁺-mediated cellular responses. What is [Na⁺]- and [K⁺]-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⁺]/[K⁺]-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
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\textsuperscript{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\textsuperscript{+},K\textsuperscript{+}-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.

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