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Regulation of Sodium Pump Endocytosis by Cardiotonic Steroids: Molecular Mechanisms and Physiological Implications

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Abstract

We have previously shown that ouabain and other cardiotonic steroids interact with the plasmalemmal Na/K-ATPase and cause a time and dose dependent endocytosis of the Na/K-ATPase. This endocytosis is demonstrable using fluorescence imaging as well as conventional biochemical and biophysical cell separation methods. In proximal tubule cells, this process appears to regulate the density of basolateral Na/K-ATPase expression directly as well as indirectly modulate transepithelial sodium transport.

Work with genetic manipulations, as well as pharmacological agents with cell culture models, have demonstrated that the cardiotonic steroid stimulated endocytosis of the plasmalemmal Na/K-ATPase requires caveolin and clathrin as well as the activation of c-Src, transactivation of the EGFR and activation of PI3K. Interestingly c-Src, EGFR and ERK1/2 all appear to be endocytosed along with the plasmalemmal Na/K-ATPase. These observations suggest a close analogy between a subset of plasmalemmal Na/K-ATPase and signaling companions with conventional receptor tyrosine kinases. While further studies are necessary to delineate the role of this endocytosis in the generation as well as the limit of signal transduction through the Na/K-ATPase signal cascade, we propose that it has an important role in the regulation of renal sodium handling as well as other important processes.

Keywords

Na/K-ATPase; Ouabain; NHE3; Kidney; Sodium; caveolin-1; c-Src; PI3K

1. Introduction

Endocytosis is the vesicle-mediated process used by all cells to internalize extracellular macromolecules and plasma membrane components, and is responsible for the transport of proteins between various compartments of the secretory and endocytic systems. It can be broadly divided into two categories based on the material internalized. Phagocytosis (or cell eating) refers to the internalization of large particles (>200 nm). Pinocytosis (or cell drinking) refers to the internalization of extracellular medium and may occur through four basic mechanisms: clathrin-dependent endocytosis, caveolae-mediated endocytosis, macropinocytosis, and dynamin- and clathrin-independent endocytosis [1]. Although there are several additional types of pinocytosis which have recently been described (e.g.,

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macropinocytosis and non-dynamin mediated pinocytosis [2-4]), clathrin and caveolin mediated endocytosis are the best described endocytosis pathways.

2. Mechanisms of endocytosis

Clathrin-coated vesicles are responsible for receptor-mediated endocytosis at the plasma membrane and sorting of proteins at the *trans*-Golgi network (TGN), and found associated with the cell membrane, the TGN, and on some endosomes [5-7]. At the plasma membrane, clathrin-mediated internalization is initiated by the self-assembly of clathrin lattice formation to provide an organizing structure. The formation of clathrin coated pits is triggered by the heterotetrameric adaptor complex AP-2, which recruits clathrin to the plasma membrane and binds to membrane receptors (with adaptor recognition signals). Clathrin polymerizes into a lattice that pulls the plasma membrane inside. Once the inward budding of the membrane is complete, interactions between AP-2, Eps15, dynamin, and other regulatory proteins, allow the pinching off the coated pits to form clathrin-coated vesicles [8-16]. AP-2 and other coat proteins interact with cell membrane proteins through endocytic motifs (tyrosine-based and dileucine codes), to provide a cargo selection function. After pinch off, the clathrin coat is removed in a process involving a number of proteins including auxilin, heat shock proteins (hrs 70), and synaptojanin. These uncoated vesicles are then fused with the peripheral early endosome in a process involving Rab5, which may be necessary for homotypic early endosome fusion [17,18]. PI3K also appears to play an important role in endocytosis regulation and early endosome fusion [19-21]. The cargo proteins either recycle back to the plasma membrane or transport to late endosome and lysosome for degradation.

Evidence has accumulated demonstrating alternatives or adjuncts to clathrin mediated endocytosis. In recent years, caveolae has probably received the greatest attention since the discovery of the caveolin proteins (caveolin-1, -2, -3). Caveolae are believed to be specific membrane microdomains to regulate protein sorting and membrane dynamics in the endocytic pathway. Caveolae are 50- to 100-nm flask shaped, non-clathrin coated plasma membrane invaginations which are believed to play a central role in potocytosis and receptor-mediated transcytosis and endocytosis (reviewed in [22-27]). The underlying mechanism of invagination, budding, and vesicle trafficking differs significantly from the coated pit pathway. Caveolae-mediated transport may overlap with those events mediated by clathrin coated pits, but caveolae may also serve selective transport functions. Caveolins are 21-24 kDa membrane-associated scaffold proteins and are major structural components of caveolae. Three major groups of caveolins have been identified and named as caveolin-1, -2, and -3. The expression and distribution of caveolins are tissue-specific. While caveolin-3 is expressed highly in muscle cells, caveolin-1 is predominantly expressed in a wide variety of cells. Expression of caveolin-1 or caveolin-3 is sufficient and necessary to drive the caveolae formation. The primary sequence of highly conserved hydrophobic protein caveolin-1 contains several binding domains: a N-terminal membrane attachment domain (residues 82-101) containing a membrane-targeting sequence, a C-terminal membrane attachment domain (residues 135-150) containing a Golgi-targeting sequence, an oligomerization domain (residues 61-101), and a scaffolding domain (residues 82-101). Interaction between the oligomerization domains and the C-terminal domains results in formation of high molecular oligomers containing about 14 to 16 caveolins. This is important for the scaffolding function of caveolins. Caveolins stabilize caveolae and modulated signal transduction by attracting signaling molecules to caveolae and regulating their activity [28]. With the analysis of mice deficient in caveolins, the caveolins are believed to be essential in the conversion of lipid rafts (which are not invaginated and lack caveolin) into caveolae, although some cell signaling and even endocytosis appear to be possible in lipid rafts which are not caveolae [29-31]. The function of caveolae receives much interest because of the findings that caveolae are able to concentrate signaling molecules and form “preassembled signaling complexes” at the plasma membrane [32-34]. Functioning as

scaffolding proteins, caveolins attract and compartmentalize many signaling molecules that play pivotal roles in intracellular signal transduction into caveolae and caveolae-related microdomain, including G-protein-coupled receptors, receptor tyrosine kinases, H-Ras, Src family tyrosine kinases, protein kinase C, and endothelial nitric oxide synthase (eNOS), etc. In these signaling molecules, their enzymatically active catalytic domains contain an aromatic amino acid-based caveolin-binding motif (CBM, $\Phi\text{XXXX}\Phi\text{XX}\Phi$ and $\Phi\text{X}\Phi\text{XXXX}\Phi$, where Φ represents an aromatic amino acid [35]), which could interact with the scaffolding domain (residues 82-101) in caveolin-1.

In some cases, clathrin-dependent and caveolae/lipid rafts-dependent endocytic pathways could co-exist and function differently. The interleukin 2 receptor (IL-2R) and glycosyl phosphatidylinositol (GPI)-anchored proteins were shown to enter cells in a rafts-dependent and clathrin/Eps15-independent pathway, but might be further internalized from endosomes to lysosomes for degradation in a clathrin-dependent pathway [36,37]. Interestingly, the fate of endocytosed receptors can be depending on the endocytosis pathway. The TGF- β receptors can be internalized in parallel either by a clathrin- or caveolae/lipid rafts-dependent pathway [38]. Endocytosis via the clathrin-dependent pathway accumulated the TGF- β receptors in EEA-1 (early endosome antigen-1)-positive endosome and stimulated TGF- β -Smad2 signaling from these early endosomes, whereas endocytosis via the caveolae/lipid rafts-dependent pathway stimulated TGF- β -Smad7 signaling and accelerated receptor degradation.

3. Endocytosis and signal transduction

Endocytosis of cell surface receptors is an important regulatory event in signal transduction. The classic concept of receptor downregulation by endocytosis has been established over the past several decades. In general, receptor-mediated endocytosis results in internalization of the receptor and ultimate destruction of the receptor in lysosomes. In this situation, the endocytosis is part of a negative feedback loop meant to attenuate or minimize the signal associated with receptor activation by limiting the number of receptors available to signal through. Ligand-mediated endocytosis may attenuate the signaling of an activated surface receptor and/or translocate the activated surface receptor to appropriate compartments to interact with downstream effectors. While receptor-mediated endocytosis has been traditionally considered as an effective mechanism to attenuate ligand-activated responses, it is becoming clear that signaling continues on the endocytic pathway, especially from endosomes [39-41]. Endocytosis plays an important role in the activation and propagation of signaling pathways [42,43], and signal transduction can also regulate endocytosis [44,45]. Many signaling molecules and membrane receptors are regulated by dynamically associated with clathrin and caveolin, such as Src-family kinases, Ras, PKC, ERK, insulin receptor, EGFR, and some entire signaling modules like PDGFR-Ras-ERK [28,34,46]. Clathrin-coated pit may also represent a specialized microdomain, like caveolae, where proteins are assembled into active signaling complexes. Interaction with the components of the clathrin-coated pit machinery may facilitate some signaling functions of transmembrane receptors [47,48]. Both caveolin and clathrin heavy chain are substrates of Src kinases [44,49,50], and there is evidence that caveolin or the integrity of caveolae/lipid rafts may be important in regulating clathrin-dependent endocytosis through their interactions with clathrin [50-53].

In the trafficking of receptor tyrosine kinase (RTK) receptors, the receptor trafficking could regulate receptor signaling in different ways, depending on the specific cell and tissue type. Endocytic (endosomal) receptors could control the magnitude of the response (the same signaling cascades as surface-localized receptors) or initiate distinct signaling cascades (from those generated at the cell surface), and the signaling from endosomes could be qualitatively different from that generated at the cell surface (reviewed in [54]). In polarized epithelial cells,

distribution of RTKs' substrates could affect cellular responses[55], and the endosomal signaling appears to be dependent on both the receptor and the cell type.

4. Na/K-ATPase: an ion pump and a signal transducer

The Na/K-ATPase was first discovered as the molecular machine for the ATP-dependent and -coupled transport of Na^+ and K^+ across the plasma membranes of all eukaryotic cells [56, 57]. Na/K-ATPase is a heterodimeric membrane protein that belongs to the type II class of P-type ATPases and consists of two noncovalently linked α and β subunits [57-59]. The α subunit is considered as the "catalytic subunit" containing the binding sites for ATP, ouabain, and other ligands. The β subunit is essential for the assembly of the functional enzyme and membrane delivery. The isoform expression of α and β subunits is tissue-specific. Besides the α and β subunits, other small, single-membrane-spanning polypeptides (like γ subunit and other polypeptides sharing a FXDY signature motif with the γ subunit) have been found to be able to interact with the Na/K-ATPase [60,61], which may influence the transport and enzymatic activities of the Na/K-ATPase. With the availability of the crystal structure of the SERCA1a (skeletal-muscle sarcoplasmic-reticulum/endoplasmic-reticulum Ca^{2+} -ATPase), another type II P-type ATPase [62], it has been concluded that the Na/K-ATPase has four distinct functional domains[63], in which both A and N domains are highly exposed and capable of protein binding. As a classic active ion transporter of Na^+ and K^+ , the central role of the Na/K-ATPase is to maintain intracellular Na^+ and K^+ balance as well as to keep an inwardly directed Na^+ gradient in the expense of adenosine triphosphate (ATP). This Na^+ gradient, in turn, is the driving force to keep other passive co-/counter-transporters active to maintain intracellular ion homeostasis and nutrients uptake (reviewed in [57]).

In addition to ion pumping, the Na/K-ATPase was also shown to interact with other proteins and regulate gene expression and cell growth [60,64-66]. Low concentrations of ouabain augment cell proliferation, DNA synthesis, and activation of several signaling pathways without significant inhibition of Na/K-ATPase activity and elevation of bulk intracellular sodium concentration ($[\text{Na}^+]_i$) [67-70]. Recent studies [71-82] have clearly demonstrated that the Na/K-ATPase also functions as a receptor and signal transducer, converting extracellular ouabain signal into the activation of various protein kinase cascades without changes in intracellular Na^+ and K^+ concentrations. Although the Na/K-ATPase itself lacks tyrosine kinase activity, the Na/K-ATPase and Src assemble into a functional receptor complex in caveolae microdomain capable of initiating a tyrosine kinase cascade in response to the binding of cardiotonic steroids. The formation of a functional CTS receptor complex in caveolae microdomain appears to involve the binding of the Na/K-ATPase $\alpha 1$ subunit (via clathrin-binding motif) to caveolin-1 (via scaffolding domain) as well as c-Src (via SH3/SH2 and kinase domains) to the Na/K-ATPase $\alpha 1$ subunit (via A domain in CD2 and N/P domain in CD3) (Figure 1. The schematic illustrations are based on [23,63,83,84]). Under basal condition, the Na/K-ATPase directly and constitutively interacts with Src to form a functional receptor for CTS. This interaction occurs via multiple domains, and the binding of the Src kinase domain to the N domain of Na/K-ATPase $\alpha 1$ subunit keeps the complex in an inactive state. Binding of ouabain to the Na/K-ATPase/Src receptor complex changes the interaction and frees the Src kinase domain from the Na/K-ATPase, resulting in the activation of Src and subsequent tyrosine phosphorylation of multiple proteins [79,85]. Specifically, the activated Src transactivates EGF receptor, leading to the subsequent activation of PI3K, Ras/Raf/ERKs, PLC/PKC, and ERK1/2 in several different cells including smooth muscle cells and kidney proximal tubular cells. Activation of Src and PI3K by ouabain binding to Na/K-ATPase-Src complex induces endocytosis of this receptor complex, as seen in other receptors. Furthermore, the activated Src by ouabain also increases mitochondrial production of ROS and regulates intracellular Ca^{2+} concentration/oscillation, two well-established second messengers.

Interestingly, recent data from our laboratories suggest that the Na/K-ATPase involved in signaling may actually not be pumping at all [86].

5. Endocytosis of the Na/K-ATPase in the regulation of renal sodium excretion

The regulation of renal tubule epithelial cell sodium transport by endocytosis of the Na/K-ATPase has been extensively studied. Most of this work has been done in the context of G protein receptor mediated signal transduction induced by dopamine. Dopamine stimulates the Na/K-ATPase trafficking and alters renal tubular epithelial sodium handling by decreasing plasmalemmal Na/K-ATPase content [87-90]. Endocytosis of the Na/K-ATPase in response to dopamine is triggered by the phosphorylation of Ser¹⁸ of rat α 1 subunit and activation of PI3K. The binding and activation of PI3K facilitates the binding of the α 1 subunit with adaptor protein AP-2, providing the inclusion of the Na/K-ATPase into clathrin-coated pits [91]. However, Ser¹⁸ is found only in rat α 1 subunit and is not present in pig and dog α 1 subunit [92]. Depending on the type of renal tubular epithelium, internalization of the sodium pump may be mediated through PKC or PKA dependent mechanisms [93-95]. Tyr⁵³⁷ on the α 1 subunit is essential for AP-2 binding and clathrin-dependent endocytosis of the Na/K-ATPase in OK cells expressing the rodent α 1 isoform [89] whereas Ser¹⁸ phosphorylation (also on α 1) is essential for dopamine induced endocytosis in primary culture of rat proximal tubules cells [88,90]. Other than dopamine, Rho small GTPase has also been shown to translocate the Na/K-ATPase in renal epithelial cells [96]. Although the binding of radioactive-labeled ouabain or digoxin to the Na/K-ATPase has been utilized as a way to follow the trafficking of the pump through the different cell compartments [97,98], our data are the first to suggest that ligand modulated internalization of the Na/K-ATPase as a mechanism by which sodium transport by proximal tubular epithelium is altered in a physiologically meaningful manner [99-103].

6. Ouabain-induced endocytosis of the Na/K-ATPase in LLC-PK1 cells

In LLC-PK1 cells, acute treatment with low concentrations of ouabain or MBG (≤ 100 nM) do not cause detectable inhibition of the Na/K-ATPase activity, but chronic treatment with ouabain or MBG causes significant decreases in Na/K-ATPase activity and transepithelial Na⁺ flux without changing in intracellular Na⁺ concentration [99-101]. Since simply inhibition of the Na/K-ATPase with low extracellular potassium does not produce these effects, we reasoned that other regulatory mechanism(s) might be involved, and ouabain-induced redistribution of the Na/K-ATPase was the most likely candidate. Ouabain-induced redistribution of the Na/K-ATPase was first introduced by Cook and Lamb in their early studies in HeLa cells [97,98], which demonstrated that [³H]ouabain-Na/K-ATPase complex translocated from the plasmalemmal membrane to intracellular (lysosomal) compartments. In their studies, [³H]ouabain accompanied the Na/K-ATPase. To determine if ouabain could stimulate redistribution of the Na/K-ATPase in renal proximal tubule, we investigated subcellular distribution of the Na/K-ATPase and functional studies in response to ouabain.

To ensure that the ouabain concentrations used were not toxic to cells, LLC-PK1 cells were exposed to different concentrations of ouabain for different times. These control experiments showed that low concentrations of ouabain (up to 100 nM, comparing to IC₅₀ $\approx$ 1 μ M for ouabain-sensitive ⁸⁶Rb uptake) has no effect on cell morphology and viability, examined by LDH release (12 h treatment), Trypan blue exclusion (12 h treatment), and cellular ATP level (30 min treatment). 12/26/2007 of ouabain ($\geq 1 \mu$ M, 12 h) caused changes in cell morphology and viability. By using ratiometric imaging method, real-time [Na⁺]_i measurements did not show any significant [Na⁺]_i change in response to ouabain (100 nM, up to 30 minutes). However, [Na⁺]_i was significantly increased within 5 min in response to higher concentration ouabain

(10 μ M), consistent with previous observations that high concentration of ouabain was required to alter intracellular Na^+ [104]. Increasing extracellular K^+ from 5 to 10 mM also showed no significant effect on cell viability. Furthermore, the effects of ouabain on LLC-PK1 cells are fully reversible [100] in terms of the Na/K-ATPase activity and cell surface expression.

While low concentrations of ouabain have no significant acute effect on Na/K-ATPase activity, chronic treatments with low concentrations of ouabain or MBG significantly inhibited Na/K-ATPase activity (measured by ouabain-sensitive $^{86}\text{Rb}^+$ uptake and enzymatic activity) in a dose- and time-dependent manner. For example, exposure of LLC-PK1 cells to 25 nM ouabain for 12 h caused about 50% inhibition of ouabain-sensitive $^{86}\text{Rb}^+$ uptake. Interestingly, these chronic treatments also caused a concomitant decreases in transepithelial Na^+ transport (measured by transepithelial $^{22}\text{Na}^+$ flux from apical to basolateral aspect) in LLC-PK1 monolayers [99-102]. This inhibition of the Na^+ transport was also in a dose- and time-dependent manner, similar to that shown in Na/K-ATPase activity inhibition.

By using cell surface biotinylation, [^3H]ouabain binding pulse-chase assay, and subcellular fractionation technique, we found that ouabain translocated the Na/K-ATPase $\alpha 1$ and β subunits from the cell surface to clathrin-coated pits as well as early and late endosomes, leading to profound removal of cell surface Na/K-ATPase and inhibition of Na/K-ATPase enzymatic activity. Ouabain-induced decreases in surface Na/K-ATPase were well correlated with ouabain-induced inhibition of ouabain-sensitive $^{86}\text{Rb}^+$ uptake. As noted above, these effects of ouabain were fully reversible and were not due to change in cell viability. [^3H]ouabain binding pulse-chase assay (combined with immunoprecipitation assay) showed that the [^3H]ouabain-Na/K-ATPase complex was endocytosed together, in agreement with the early observations in HeLa cells [97,98]. Ouabain-induced endocytosis of the Na/K-ATPase is dose- and time-dependent and in a clathrin-dependent pathway. Immunofluorescence staining showed internalization of the Na/K-ATPase $\alpha 1$ subunit as well as co-localization of Na/K-ATPase $\alpha 1$ subunit and clathrin both before and after exposure to ouabain. Pharmacological blockage of the clathrin-dependent endocytic pathway (by chlorpromazine [105,106] and intracellular potassium depletion with hypotonic shock [107]) significantly reduced ouabain-induced endocytosis.

Ouabain-stimulated endocytosis of the Na/K-ATPase was initiated by the ouabain-activated Na/K-ATPase signaling, required activation of c-Src and PI3K [100,101]. Ouabain treatment enhanced protein-protein interactions among the Na/K-ATPase $\alpha 1$ subunit, clathrin heavy chain, adaptor protein AP-2 α subunit (AP-2 α), and PI3K p85 α subunit. Inhibition of c-Src (by PP2) or PI3K (by wortmannin or LY294002) significantly attenuated ouabain-induced endocytosis and protein-protein interaction. The central role of activated Src in these processes was further confirmed by the observation that ouabain-induced endocytosis of the Na/K-ATPase was abolished in the c-Src-deficient SYF cells and was rescued in SYF+c-Src cells in which c-Src is re-introduced into the SYF cells. The c-Src-deficient SYF cells are derived from mouse embryos harboring functional null mutations in the alleles of the Src family kinases Src, Yes, and Fyn. The SYF+c-Src cells are the stable transfectant of the SYF cells that expressing c-Src [108]. Inhibition of c-Src also abolished ouabain-induced activation of PI3K, suggesting that PI3K is a down-stream effector of c-Src in ouabain-activated signaling. Moreover, some signaling molecules (such as EGFR, c-Src, and ERK1/2) were also accumulated in endocytic compartments (clathrin-coated pits, early and late endosomes) with endocytosed ouabain-Na/K-ATPase complexes.

Caveolae microdomain is pivotal in assembly of ouabain-Na/K-ATPase-Src signaling complex, and caveolin-1 has been described as indispensable for ouabain-induced signal transduction from caveolar Na/K-ATPase. To determine the function of caveolae and/or lipid rafts, their functional structures were disrupted and re-established by cholesterol depletion

(using methyl-beta-cyclodextrin, M β -CD) and repletion. As expected, disrupting caveolae and/or lipid rafts by cholesterol depletion prevents ouabain-induced endocytosis, and cholesterol repletion restored the effects of ouabain. Furthermore, ouabain-induced endocytosis of the Na/K-ATPase also requires caveolin-1, the major structural component of caveolae microdomain where receptor Na/K-ATPase-Src complex was assembled. When using LLC-PK1 cells stably transfected with an empty vector (P-11, as control) or a vector expressing caveolin-1-specific siRNA (C2-9, as caveolin-1 knockdown cells), ouabain treatment induced endocytosis of the Na/K-ATPase in P-11 cells similarly to the effect seen in the wild-type LLC-PK1 cells, but not in the caveolin-1 knockdown C2-9 cells. While depletion of caveolin-1 significantly reduced the protein-protein interaction among $\alpha 1$ subunit, clathrin heavy chain, AP-2 α , and PI3K p85 α , depletion of caveolin-1 also significantly reduced the ouabain-induced accumulation of Na/K-ATPase $\alpha 1$ subunit, EGFR, Src, and ERK1/2 in clathrin-coated pits, as well as in early and late endosomes [101].

While LLC-PK1 cells were pretreated with a conventional “Na⁺-clamping” method [109] (20 μ M monensin, or 10 μ M monensin plus 5 μ M gramicidins) to equilibrate intracellular [Na⁺] with extracellular concentrations, ouabain is still able to induce accumulation of Na/K-ATPase and NHE3 in early endosome (unpublished data). These observations further suggest that ouabain-induced inhibition of transepithelial Na⁺ transport is originated from ouabain-activated signaling and independent of intracellular sodium concentration.

In short, our data have demonstrated that chronic stimulation of the Na/K-ATPase/Src complex by ouabain can stimulate endocytosis of the Na/K-ATPase-Src complex and compartmentalize signaling molecules, which in turn results in a significant removal of the Na/K-ATPase from plasma membrane and concomitant inhibition of pumping activity. This process requires ouabain-activated caveolar Na/K-ATPase signaling pathways, but is independent of intracellular sodium concentration (Figure 2).

7. Ouabain regulates sodium handling in renal proximal tubule

Sodium reabsorption in the proximal tubule involves the coupling of apical sodium entry mainly through the NHE3 (sodium/hydrogen exchanger, isoform 3) and basolateral sodium extrusion primarily through the Na/K-ATPase. Accumulated evidence supports the notion that endogenous cardiotonic steroids may cause a physiologically meaningful regulation of transepithelial sodium transport in the proximal tubule. Since low concentrations of ouabain decrease basolateral sodium extrusion by depletion of the cell surface Na/K-ATPase and have no effect on intracellular sodium concentration, it is logical to propose that apical sodium entry via NHE3 must be down-regulated simultaneously. Recently, we have demonstrated that low concentrations of ouabain caused a biphasic effect on NHE3 regulation, trafficking regulation in short-term and transcriptional regulation in long-term ([102] and unpublished data). Most interestingly, these effects of ouabain on NHE3 regulation are dependent on ouabain-activated Na/K-ATPase-Src signaling.

In the renal proximal tubules, NHE3 is expressed in the apical membrane [110-112], mediating Na⁺, HCO₃⁻, and fluid reabsorption. NHE3 null mice have shown reduced Na⁺ and HCO₃⁻ reabsorption in the proximal tubule with urinary Na⁺ and HCO₃⁻ wasting [113]. *In vivo*, salt loading not only depresses NHE3 expression, but also induced Na/K-ATPase endocytosis in an MBG dependent manner [103,114]. NHE3 activity is regulated at various levels, including phosphorylation [111,115,116], trafficking [117,118], and transcriptional regulation [119]. In LLC-PK1 cells, exposure to low concentrations of ouabain (≤ 100 nM, up to 12 h) had no effect on intracellular Na⁺ concentration ([Na⁺]_i), but caused a significant inhibition of NHE3 activity (measured by both H⁺-driven ²²Na⁺ uptake and Na⁺-stimulated pH recovery rate). The

inhibitory effects of ouabain on transepithelial $^{22}\text{Na}^+$ transport only occurred when ouabain was added to the basolateral, but not the apical aspect of LLC-PK1 monolayer cultured on the Transwell® membrane support, which clearly suggesting that these effects were mediated by the basolateral Na/K-ATPase. We found that acutely (0.5-4 h), ouabain significantly reduced NHE3 content in the apical membrane in LLC-PK1 monolayers as well as stimulated accumulation of NHE3 in early endosomes. Chronically (12 to 24 h), low concentrations of ouabain significantly reduced NHE3 protein and mRNA expression, as well as NHE3 promoter activity in LLC-PK1 cells [102]. Ouabain-induced down-regulation of NHE3 mRNA is highly correlated with those observed in NHE3 protein expression. In ouabain-induced down-regulation of NHE3 promoter activity, ouabain-response elements are mapped to a region between -450 and -1194 nt in NHE3 promoter, where decreased binding of Sp1 to its cognate *cis*-element are demonstrated both *in vitro* and *in vivo*.

Ouabain-activated signaling function and endocytosis of the Na/K-ATPase might be involved in the mechanism ultimately resulting in NHE3 regulations. Inhibition of Src and PI3K or disruption of caveolae structure, which has been shown to block ouabain-induced endocytosis of Na/K-ATPase, was sufficient to block ouabain-induced NHE3 regulation, indicating that activation of the signaling function of the Na/K-ATPase by ouabain is required. Again, disruption of caveolae and/or lipid rafts by cholesterol depletion prevented the ouabain-induced accumulation of NHE3 in early endosomes, while cholesterol repletion restored the endosomal accumulation of NHE3 in response to ouabain. Consistently, depletion of caveolin-1 by siRNA was equally effective in abolishing the effects of ouabain on NHE3 promoter activity as well as mRNA expression. Moreover, blocking ouabain-induced endocytosis of Na/K-ATPase by PI3K inhibitor (wortmannin, 100 nM) or a blocking peptide (TPRPTTPE, which resembling the proline-rich domain in Na/K-ATPase $\alpha 1$ N-terminal for binding of PI3K [91]) was also sufficient to abolish the effects of ouabain on NHE3 endocytosis, suggesting a role of the endocytosed Na/K-ATPase signaling complex in regulation of NHE3 trafficking. In short, other than inducing endocytosis of the Na/K-ATPase, ouabain-activated signaling also regulates apical NHE3 expression and trafficking, leading to the inhibition of apical sodium entry.

8. Ouabain-Na/K-ATPase in endocytic pathway: signaling termination or propagation?

Binding of ouabain to the receptor Na/K-ATPase signaling complex activates the Na/K-ATPase-bound c-Src, leading to endocytosis of the signaling complex. In early endosomes, functioning as a ion pump, native or endocytosed Na/K-ATPase may regulate endosomal pH by generation of a interior-positive membrane potential [120,121]. This regulation kept a mild pH (about 6.0) environment in early endosomes, which is sufficient to dissociate most ligand-receptor complexes and facilitate receptor recycling back to cell surface or degradation in lysosomes. It appears that ouabain-Na/K-ATPase complex was intact along endocytic pathway, since our [3H]ouabain binding pulse-chase and immunoprecipitation data suggested that [3H]ouabain came along with endocytosed Na/K-ATPase. This is in agreement with early observation in HeLa cells, which indicated that [3H]ouabain might still bind to endocytosed Na/K-ATPase in lysosomes [97,98]. Considering that lysosomes are typically the most acidic organelle in mammalian cells (about pH 4.7-4.8)[122], the mild pH environment in early and late endosomes may not be sufficient to completely dissociate ouabain-Na/K-ATPase complex. These lead to the possibility that ouabain-Na/K-ATPase complex might execute its signaling function in endosomes, even though it is unknown whether Src still could bind to this complex and be activated as seen in plasma membrane. In OKP cells, acidosis activates c-Src and MEK/ERK/c-fos cascade [123]. Strikingly, recycling endosomes in MDCK cells are enriched in the major structural components of caveolae, like caveolin-1, cholesterol, and sphingomyelin [124]. Caveolin-1 is also present in the early endosome in LLC-PK1 cells (unpublished data).

If these major caveolae structural components could form a caveolae or caveolae-like microdomain, it may provide a 'docking station' for endocytosed ouabain-Na/K-ATPase-Src receptor complex, like the caveolae microdomain in plasmalemmal membrane.

From our observations, it is conceivable to propose that endocytosed ouabain-Na/K-ATPase complex is still functional as a signaling complex to propagate its signaling, even though it is not clear whether this endosomal signaling is the same as the original one or not. This is supported by the following evidences. Ouabain-induced both transcriptional and trafficking regulation of NHE3 are initiated by the activation of Na/K-ATPase signal function and independent of intracellular sodium. Moreover, ouabain-induced trafficking and transcriptional regulation of NHE3 was prevented when ouabain-induced Na/K-ATPase signaling/endocytosis was blocked. In short, we propose that ouabain-activated receptor Na/K-ATPase signaling complex has biphasic effect. First, it decreases in basolateral sodium extrusion. Ouabain binding to Na/K-ATPase activates Src and PI3K, leading to endocytosis of the Na/K-ATPase and decreases in cell surface Na/K-ATPase. Second, it decreases in apical sodium entry. Endocytosed ouabain-Na/K-ATPase initiates signaling from endosome, leading to down-regulation of NHE3 via trafficking and transcriptional regulation. Ultimately, these processes inhibit renal sodium reabsorption and stimulate renal sodium excretion, resulting in the physiological control of sodium homeostasis.

9. Physiological implications

Renal adaptation to both volume expansion and hypertension involves a complicated interplay among different hormonal and cellular regulatory mechanisms. Since the finding of proposed endogenous cardiotonic steroids, accumulated evidences have indicated that these steroids might be related to a number of health conditions such as sodium imbalance, chronic renal failure, hypertension, and congestive heart failure. It is known that CTS are elevated during volume expansion. The cardiotonic steroid binding site of the Na-K-ATPase is believed to be the molecular target of the hypertensive effects of these compounds, and appears to play an important role in blood pressure regulation [125,126]. Our recent *in vivo* studies have demonstrated that elevated endogenous CTS and endocytosis of the Na/K-ATPase play an important role in renal adaptation to volume expansion. Based on our observations, it is conceivable that the Na/K-ATPase mediated cellular signaling mechanism could play an important role in renal adaptation to volume expansion and hypertension, in which the endocytosed CTS-Na/K-ATPase complex might serve as a coupling point for controlling apical Na⁺ entry and basolateral Na⁺ extrusion. Physiologically, elevated endogenous or exogenous CTS concentrations may provide a meaningful adaptation to volume expansion and hypertension by controlling sodium handling in renal proximal tubule, which resonates with the early theories proposing one or more endogenous natriuretic compounds introduced by Dahl [127], deWardener [128] and Blaustein [129].

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Abbreviations

CTS	cardiotonic steroid(s)
EGFR	epidermal growth factor receptor
ERK1/2	extracellular-signal-regulated kinase 1 and 2
LLC-PK1 cells	an epithelial cell line derived from the porcine renal proximal tubules
MBG	marinobufagenin
Na/K-ATPase	sodium potassium adenosine triphosphatase
NHE3	Na/H exchanger isoform 3
PI3K	phosphoinositide-3-kinase

Figure 1.

Schematic illustration of Na/K-ATPase α 1 subunit, caveolin-1 and c-Src, A: In the Na/K-ATPase α 1 subunit, caveolin-binding motif (CBM) is located in the cytosolic side of N-terminus, proximally to TM1 (transmembrane domain 1). CD2 is the first cytosolic loop between TM2 and TM3, bearing activation domain (AD). CD3 is the second cytosolic loop between TM4 and TM5, bearing nucleotide-binding domain (ND) and phosphorylation domain (PD) B: In caveolin, C-terminal and N-terminal membrane attachment domains (C-MAD and N-MAD) are important for membrane attachment. The scaffolding domain (SD) is overlapped with N-MAD and oligomerization domain (OD). The transmembrane domain (TMD) is for membrane insertion. C: c-Src is a cytosolic kinase, containing SH3, SH2, and kinase (N-lobe and C-lobe) domains. C-terminal tail Y527 is the negative regulatory phosphorylation site, and Y416 (bearing in the A-loop helix between kinase domain N-lobe and C-lobe) is the major autophosphorylation site.

Figure 2.

Schematic illustration ouabain-induced endocytosis of the Na/K-ATPase and NHE3. Binding of ouabain to the Na/K-ATPase α 1 activates c-Src and consequent signal cascades. This process also recruits PI3K P85 α subunit, AP-2, and clathrin to the α 1 subunit as well as promotes the formation of clathrin-coated pits. In response to ouabain, the Na/K-ATPase is internalized into clathrin-coated vesicles (CCV), early endosomes (EE), and late endosomes (LE). Some important signaling molecules (EGFR, c-Src, and ERK1/2) are also accumulated in CCV, EE, and LE. Interestingly, ouabain-activated signaling also induces endocytosis or downregulation of NHE3. The ultimately effect is reduced transepithelial sodium reabsorption.