



HHS Public Access

Author manuscript

IUBMB Life. Author manuscript; available in PMC 2017 March 31.

Published in final edited form as:

IUBMB Life. 2013 December ; 65(12): 991–998. doi:10.1002/iub.1229.

Regulation of Renal Function and Structure by the Signaling Na/K-ATPase

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Abstract

The Na/K-ATPase as an essential ion pump was discovered more than 50 years ago (1,2). The signaling function of Na/K-ATPase has been gradually appreciated over the last 20 years, first from the studies of regulatory effects of ouabain on cardiac cell growth. Several reviews on this topic have been written during the last few years (3–8). This article will focus on the molecular mechanism of Na/KATPase-mediated signal transduction and its potential regulatory role in renal physiology and diseases.

Introduction

The Na/K-ATPase

The Na/K-ATPase (or sodium pump) was discovered by Skou in 1957 (1). It is a member of the P-type ATPase family and the most important ion pump in animal physiology. By hydrolyzing ATP, Na/K-ATPase is capable of transporting sodium and potassium ions across the cell membrane against their concentration gradients. The Na/K-ATPase consists of two noncovalently linked α and β subunits. A γ subunit (a member of the FXYD proteins) is associated with the Na/K-ATPase in a tissue-specific manner and regulates the functionality of the enzyme (9,10). The α subunit (about 112 kDa) contains the ATP and other ligand binding sites, and couples ATP hydrolysis with ion movement; meanwhile the β subunit is essential for the assembly of a fully functional enzyme. Four α isoforms are found in human tissues and they are expressed in a tissue-specific manner. The $\alpha 1$ isoform is found in all cells. The $\alpha 2$ and $\alpha 3$ isoforms are mainly expressed in skeletal muscle, neuronal tissue, and cardiac myocytes. The $\alpha 4$ isoform is in testis and regulates sperm motility (11–19). Na/KATPase alpha subunits consist of three functional domains: The Actuator (A) domain is composed of the N-terminus and the second cytosolic domain (CD2)¹ connected to transmembrane helices M2 and M3 and which functions to regulate the sodium and potassium binding site. Next, the highly conserved phosphorylation (P) domain resides close

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Conflict of Interests The authors have no conflicts of interest to declare.

¹CD2: second cytosolic domain, P: phosphorylation, N: nucleotide binding, CTS: cardiotonic steroids, CHF: congestive heart failure, MBG: marinobufagenin, SH2: Src homology 2, SH3: Src homology 3, FRET: fluorescence resonance energy transfer, EGFR: EGF receptor, ADPKD: autosomal dominant polycystic kidney disease.

to the membrane and finally, the nucleotide binding (N) domain. The Na/K-ATPase resides in two main conformational states, the E1 sodium bound state and the E2 potassium bound state. During the transport cycle, the A domain rotates while the N domain closes up, which opens and closes the A, N and P domains in the E1 and E2 conformations, respectively (20–23). Studies during the last 20 years have indicated that the Na/K-ATPase possess an important cell-signaling role as well through its interactions with endogenous cardiotonic steroids (CTS) and signaling proteins such as Src kinase (3–8).

Molecular mechanism of Na/K-ATPase in signal transduction

Regulation of Cell Growth by Cardiotonic Steroids (CTS)

CTS include plant-derived digitalis drugs such as digoxin, and vertebrate-derived aglycones such as bufalin (3, 5). Digoxin has long been used to treat congestive heart failure (CHF) and/or atria fibrillation while bufalin and its derivatives represent the major active components of a traditional Chinese medicine called Chan'su, which is prescribed as a cardiotonic, diuretic and anodyne agent (3, 25, 26). Further, ouabain and marinobufagenin (MBG) have both been found in animal and human blood, and have been considered as endogenous steroids. Their synthesis and release appears to be regulated (3, 5).

It has been well established that CTS bind and fix Na/K-ATPase (either phosphorylated or nonphosphorylated) in an E2-like conformation, resulting in the inhibition of the pumping cycle (27, 28). Moreover, studies utilizing purified pig and shark Na/K-ATPase indicate that ouabain-bound Na/K-ATPase is less prone to thermal denaturation than non-ouabain bound pump (28), suggesting a more compact conformation that may make it less likely to interact with its partner proteins.

Since the 1970s, many studies have documented a role of CTS in the regulation of cell growth (29,30). Recent studies have revealed some important pathways relevant to this regulation. Specifically, it has been demonstrated that CTS activate multiple protein/lipid kinases and stimulate either differentiation/apoptosis or hypertrophic/proliferative growth in a cell type-specific manner (30–53). Moreover, it appears that the signaling role that CTS play could occur at nano- and subnanomolar concentrations that would not affect the overall cellular Na/K-ATPase pumping activity. Therefore, it becomes evident that the Na/K-ATPase may have CTS-regulated non-pumping functions that are important to cell growth.

Interaction between the Na/K-ATPase and Src

Src family kinases are 52–62 kDa membrane associated non-receptor tyrosine kinases (54,55). They are present in a wide variety of cell types including kidney epithelial cells, and play a vital role in the regulation of signal transduction pathways. For example, activation of Src has been implicated in the generation of reactive oxygen species, the development of tissue fibrosis, and the growth and metastasis of cancer. Src contains a kinase domain, a N terminus that regulates the kinase association with the plasma membrane, and a C-terminal regulatory domain. The Src homology 2 (SH2) and Src homology 3 (SH3) domains represent the most crucial domains of Src regulation. The two most important phosphorylation sites are Y529 and Y418. Phosphorylation of Y529 keeps Src in an inactive

state by facilitating the interaction between pY529 and the SH2 domain. Due to this, the SH3 domain then becomes bound to the kinase domain linker, preventing the formation of a salt bridge in the kinase domain between E310 and K295. Autophosphorylation of the second site, Y418, leads to an open conformation of Src, which stimulates Src activity. Detection of phosphorylation in this site is a marker for overall Src activity.

Many laboratories have investigated the role of Src in Na/K-ATPase-mediated signal transduction (37, 46, 56–85). Moreover, the following data indicate that the Na/K-ATPase and Src most likely interact directly to form a functional receptor complex (46, 58–64, 68–71). First, the Na/K-ATPase and Src are co-enriched in caveolar fractions in different cell lines and in tissues. Second, immunofluorescence imaging analyses show a co-localization of these two proteins in the plasma membrane. Third, both proteins could be co-immunoprecipitated by either anti-Na/K-ATPase or anti-Src antibodies. Fourth, fluorescence resonance energy transfer (FRET) analysis indicates that both proteins are in close proximity, providing further support of a direct interaction in live cells. Finally, *in vitro* GST pull-down assays demonstrate direct interactions between the $\alpha 1$ subunit of Na/K-ATPase and Src. Based on these assays, binding of the $\alpha 1$ and Src involves at least two contacting sites: one being the CD2 of $\alpha 1$ and Src SH2, and the other consisting of the N domain of $\alpha 1$ and the Src kinase domain (64). This N domain/kinase domain interaction is responsible for keeping Src in an inactive state (64). Further mapping of this interaction leads to the identification of 20 amino acid peptide (NaKtide) that binds and inhibits Src in an ATP-independent manner (86).

It should be noted that both Na/K-ATPase and Src are highly expressed in the plasma membrane, especially in epithelial cells. Moreover, it has been estimated that the pool of Na/K-ATPase is much larger than that of Src in renal epithelial cells. Therefore, it is likely that the Na/K-ATPase represents an important negative regulator of cellular Src signaling through its interaction with the SH2 and kinase domain of Src. Indeed, both *in vitro* and *in vivo* studies have revealed a Src-interacting pool of Na/KATPase (62,67,83). There is evidence that a reduction in cellular expression of Na/K-ATPase is sufficient to increase Src activity. Moreover, restoration of Na/K-ATPase expression, even by a nonfunctional mutant pump, is capable of reducing cellular Src activity in cell cultures (62). Although it remains to be further studied, the potential clinical relevance of these findings is self-evident. It is known that the expression of Na/K-ATPase is reduced under many clinical conditions such as chronic renal insufficiency, heart failure and in certain types of cancer. It is also known that Src activity is elevated and plays an important role in the pathogenesis of these diseases. Thus, this newly appreciated Na/K-ATPase/Src interaction may serve as a potential target for therapeutic intervention. To this end, recent studies have demonstrated the effectiveness of the Na/K-ATPase-derived pNaKtide in blocking Src-mediated signal transduction, and growth of human prostate cancer cells and tumor xenografts (87).

Despite the evidence of a direct interaction between the Na/K-ATPase and Src, it is important to point out other potential mechanisms of regulation. Biochemical assays using purified Na/K-ATPase demonstrate that ouabain and Na/K-ATPase may exert regulation over Src activity through the changing energetic state of the cell, in addition to a direct interaction with Src (88). Moreover, others have reported that ouabain was incapable of regulating the

activity of Src that was co-purified with the Na/KATPase from pig kidney (134). Although these studies took place in a cell-free system, it is clear that more studies are required to further clarify the role of Na/K-ATPase in Src regulation.

The Na/K-ATPase/Src complex as a potential receptor mechanism of CTS-induced protein tyrosine phosphorylation

Studies utilizing Src inhibitors as well as Src knock out and knock in cells provided the first clue that the Na/K-ATPase/Src complex may function as a receptor mechanism of CTS-induced protein tyrosine phosphorylation (58–69). Subsequently, studies using recombinant Src kinase and other functional domains indicate that the binding of the kinase domain to the Na/K-ATPase is inhibited by ouabain, suggesting that the Na/K-ATPase/Src interaction may depend on the conformational state of Na/K-ATPase. Indeed, it has been demonstrated that chemical modifiers that stabilize the Na/K-ATPase in either E1- or E2-like conformation have opposite effects on Src activity (89). Moreover, expression of E1/E2 conformation transition-defective mutants is sufficient to alter cellular Src activity and inhibits ouabain-induced activation of protein kinases in live cells (90). Finally, mutation of the Src binding site at the N domain of Na/K-ATPase increases intracellular Src activity and inhibits ouabain-induced signal transduction (91). These new findings have the following two important implications. First, they reveal that the Na/K-ATPase/Src complex could serve as a receptor mechanism and that cardiotonic steroids such as ouabain are agonists of this newly appreciated receptor. Second, it suggests that ligands other than cardiotonic steroids may also use this receptor mechanism to transduce signals as long as they are capable of altering the conformational states of Na/K-ATPase. To this end, it has been demonstrated that changes in intracellular Na⁺ or extracellular K⁺ can affect cellular kinase activity through the expression of Na/K-ATPase (89).

In short, recent studies suggest that the Na/K-ATPase/Src complex mimics the actions of a receptor tyrosine kinase. Ouabain and other CTS serve as the ligand of this receptor complex, leading to the generation and amplification of signaling cascades through the recruitment and assembly of a cell-specific Na/K-ATPase signalosome (Fig. 1). For example, in renal epithelial cells, CTS activation of the Na/K-ATPase/Src receptor complex has been shown to trans-activate the EGF receptor (EGFR) in a Src-dependent manner (37,59,68,71,72). The activation of EGFR in turn results in the assembly of several signaling platforms through recruitment of Shc, PLC- γ and PI3K to the receptor complex. This leads to the stimulation of the Ras/Raf/MEK/ERK1/2 and the activation of PLC- γ /PKC cascades, resulting in the production of the second messengers, DAG and IP3 (66,75,76). The termination of this signaling process has also been investigated and the data suggest the endocytosis of Na/K-ATPase signalosome as being important. Moreover, it appears that the endocytosis requires the activation of Src, EGFR, PI3K and PKCs (92–94). It is of interest to note that this endocytosis seems to involve both clathrin and caveolin-1. Because caveolin-1 is important for ouabain to activate Src and EGFR, it seems to be likely that the ouabain-induced endocytosis of Na/K-ATPase is mediated through the clathrin pathway (93).

It is important to note that signaling proteins other than Src may also interact with the Na/K-ATPase in a conformation-dependent manner. For example, it has been shown that the Na/K-

ATPase interacts with IP3 receptor (IP3R) directly (95,96). Ankyrin-B, an intracellular scaffolding protein, is thought to be essential in linking the IP3R with Na/K-ATPase as it has been shown to bind to the C-terminus of the IP3R as well as to the $\alpha 1$ subunit of Na/K-ATPase. This direct interaction between the IP3R and Na/K-ATPase allows ouabain to function in regulating low frequency calcium oscillations. Similarly, the Na/K-ATPase may directly interact and regulate PI3K through the N-terminal polyproline domain (97–100). However, it is equally important to point out that cross-talk and redundancy do exist among these different interactions. For example, both IP3R and PI3K are enriched with the Na/K-ATPase/Src receptor complex and caveolin-1 (75,76). Functionally, inhibition of Src blocked ouabain-induced calcium oscillations (7) and attenuated ouabain-induced activation of PI3K (98). Moreover, we have demonstrated recently that while expression of an E2 mutant of Na/K-ATPase blocked ouabain-induced activation of Src and ERK, it also produced a partial inhibition of ouabain-induced stimulation of PI3K/Akt pathway in LLC-PK1 cells (90).

The signaling Na/K-ATPase and the Kidney Structure and Function

CTS and renal salt handling

Guyton suggested that the kidney is the most important organ in the regulation of Na^+ handling and thus blood pressure (101). This concept has now been well accepted (102). The Na/K-ATPase, and in particular, the $\alpha 1$ isoform, reside on the basolateral membrane of renal epithelial cells, and therefore plays a crucial role in renal salt handling (2–5). Because CTS inhibit the ionic pumping function of Na/K-ATPase, endogenous CTS have been considered as the natriuretic hormones that Dahl and others suggested many years ago (103–105). Hamlyn and others have provided supporting evidence as they found a correlation between levels of serum CTS and blood pressure in patients suffering from hypertension (106,107). Furthermore, the application of anti-digoxin antibodies was able to lower blood pressure (108). However, the most convincing evidence that CTS and Na/K-ATPase are involved in renal salt handling comes from Lingrel's laboratory. By utilizing transgenic mice, they were able to demonstrate greater natriuretic response in response to Na^+ loading, in mice expressing a mutated form of Na/K-ATPase $\alpha 1$ isoform that is highly sensitive to ouabain, than in wild type mice (109). Finally, studies have shown that elevated levels of endogenous CTS are found in patients suffering from chronic renal disease as well as congestive heart failure (107,110–112).

Because CTS can also activate the Na/K-ATPase/Src receptor complex, it has been suggested that CTS may be able to regulate renal salt handling via pathways separate from its role in inhibiting the pumping function of Na/K-ATPase. There is evidence that CTS induce the endocytosis of Na/K-ATPase through the activation of Src/PLC/PKC and PI3K cascades in renal epithelial cells (92–94). Concomitantly, CTS also reduced surface expression of NHE3 through both short-term (i.e., endocytosis/exocytosis-mediated) and long-term (i.e., gene expression-related) mechanisms (113–116). The NHE3 belongs to a family of electroneutral mammalian Na^+/H^+ exchangers (117). In the renal proximal tubules, NHE3 resides in the apical membrane, and acts as a major Na^+ transporter, responsible for Na^+ , HCO_3^- , and fluid reabsorption (117). As expected, up-regulation of NHE3 in the proximal tubular cells is associated with the development of hypertension (118). Thus, this

coordinated regulation of basolateral Na/K-ATPase and apical NHE3 by the CTS may represent an important mechanism of salt handling by the kidney. Indeed, application of CTS to the basolateral but not apical side of renal epithelial cell monolayer in culture reduces the transcellular movement of Na⁺ (113,114). Ex vivo studies provide additional evidence of the importance of CTS and Na/K-ATPase-mediated signal transduction in the regulation of tubular Na⁺ movement (84). Finally, studies with Dahl salt-sensitive rats have revealed the inability of CTS in the activation of Na/K-ATPase-mediated signal transduction in renal proximal tubular cells, resulting in decreased ability of renal salt handling and consequently an increase in blood pressure in response to salt loading (119).

The receptor Na/K-ATPase and tissue fibrosis

The first suggestion that chronic activation of receptor Na/K-ATPase by CTS could lead to tissue fibrosis came from studies of cardiac fibrosis in 5/6 nephrectomized rats (77). Subsequent studies showed that infusion of CTS was sufficient to increase tissue fibrosis and that neutralization of the increase in endogenous CTS by either passive or active immunization against MBG or by administration of a non-specific ouabain inhibitor spironolactone was effective in preventing or reversing fibrosis in 5/6 nephrectomized rats (78–80, 120, 121). Moreover, these studies demonstrated that CTS were potent stimulators of collagen production in dermal and cardiac fibroblasts as well as renal epithelial cells. These stimulatory effects were initiated by the activation of receptor Na/K-ATPase/Src complex and involved the increased production of ROS, activation of PKC and the down-regulation of transcriptional factor Fli-1 (80).

More recently, an interaction between the Na/K-ATPase and scavenger receptor CD36 in the formation of proinflammatory signaling loop has been demonstrated in the renal proximal tubular cells (122). In addition, the data suggest that ligands generated in hyperlipidemia could work in concert with endogenous CTS to activate the receptor Na/K-ATPase and CD36 signaling loop, which promotes the development of chronic inflammation, ROS stress and renal fibrosis underlying the renal dysfunction common to proatherogenic hyperlipidemic states. In short, we suggest that while the activation of receptor Na/K-ATPase/Src complex by endogenous CTS is required for the kidney to get rid of excess salt, the trade-off could be the development of tissue fibrosis especially under the stress conditions such as chronic renal insufficiency, high salt intake and hyperlipidemic states where the receptor function has been compromised (78, 119, 122).

CTS in relation to other renal conditions

Blanco's group has provided evidence that the CTS-activated Na/K-ATPase/Src receptor complex may play a role in the development of Autosomal Dominant Polycystic Kidney Disease (ADPKD) by stimulating cell proliferation (8,67,123,124). ADPKD occurs in approximately 1 in 500 to 1000 births and is marked by the presence of fluid filled cysts that form within the kidney (125). ADPKD is considered one of the most prevalent causes of end stage renal disease and approximately half of all patients with ADPKD develop end-stage renal disease between the ages of 50 and 60. The cysts begin to form in utero and eventually, lead to the expansion of the kidney. In addition to the stimulation of human ADPKD cell proliferation, ouabain, working in concert with the increase in cellular cAMP, promotes the

formation and growth of cysts (124). Interestingly, Blanco and others have demonstrated an increased sensitivity of human ADPKD cells to ouabain (68). This increase in ouabain sensitivity could be an important link between endogenous ouabain and the pathogenesis of cysts. Mechanistically, it appears that the increase in ouabain sensitivity is due to the interaction between the Na/K-ATPase and polycystin-1 (126). As discussed below, ouabain appears to also have anti-apoptotic effects in renal epithelial cells. This could provide further stimulation of ADPKD cell proliferation. In short, the new findings suggest a potential role of Na/K-ATPase signaling in the pathogenesis of ADPKD.

Cereijido and his colleagues have long used MDCK cells as a model to study the role of CTS-activated signal transduction in epithelial biology. They and others have demonstrated that ouabain exerts a complex regulation of the expression, trafficking and degradation of proteins that are important for the formation of tight junctions in MDCK cells (127–130, 133). These regulations are mediated by the activation of Src-dependent signaling pathways. While ouabain at lower, more physiologically relevant doses, promotes the formation of tight junction, it causes the disassembly of tight junction at higher doses. This disassembly pathway may be dependent on Src and ERK1/2 signaling pathways (133). Moreover, recent studies have shown that ouabain also regulates the assembly and growth of cilia in MDCK cells (130). In view of the role of cilia in the pathogenesis of ADPKD, the new findings also call for more mechanistic investigations of the Na/K-ATPase-mediated signal transduction in the development of ADPKD and in cilia biogenesis.

As discussed above, Aperia's group has reported the interaction between the Na/K-ATPase and IP3R in primary cultures of rat proximal tubular cells and COS-7 cells derived from embryonic monkey kidney. Moreover, they have demonstrated that ouabain stimulates this interaction, resulting in slow calcium oscillations (7, 95,96). We have subsequently confirmed the interaction between the Na/K-ATPase and IP3R, and provided evidence that this regulation involves the activation of receptor Na/K-ATPase/Src complex in LLC-PK1 cells (75,76). The importance of Src activation has been recently confirmed by Aperia's laboratory in COS-7 cells (7). Interestingly, several studies have indicated that CTS-induced calcium oscillations have anti-apoptotic effects in renal epithelial cells (131, 132). Functionally, the anti-apoptotic effects of CTS have been found to be protective in the developmental programming of kidney exposed to malnutrition and in kidneys exposed to shiga toxin.

A Look Ahead

The appreciation of the Na/K-ATPase in physiology and diseases has only grown in recent years as the mechanisms by which the Na/K-ATPase/Src receptor complex functions as a receptor have been elucidated. There is ample evidence for a role of this receptor complex in the formation of tight junction, kidney development and in the maintenance of renal function. Furthermore, a chronic stimulation of the receptor appears to be involved in renal fibrosis, inflammation and the pathogenesis of ADPKD. It has become clear that further studies still need to be conducted in order to better understand the intricacies and complexities of Na/K-ATPase-mediated signal transduction in the kidney. Moreover, there is a clear need to develop, and test novel specific receptor agonists and antagonists in animal

models of renal diseases. Such work is crucial in the pursuit of new therapeutic strategies to remedy renal diseases related to CTS and the Na/K-ATPase/Src receptor complex.

Acknowledgments

This work was supported by National Institutes of Health under grants HL-109015 from NHLBI.

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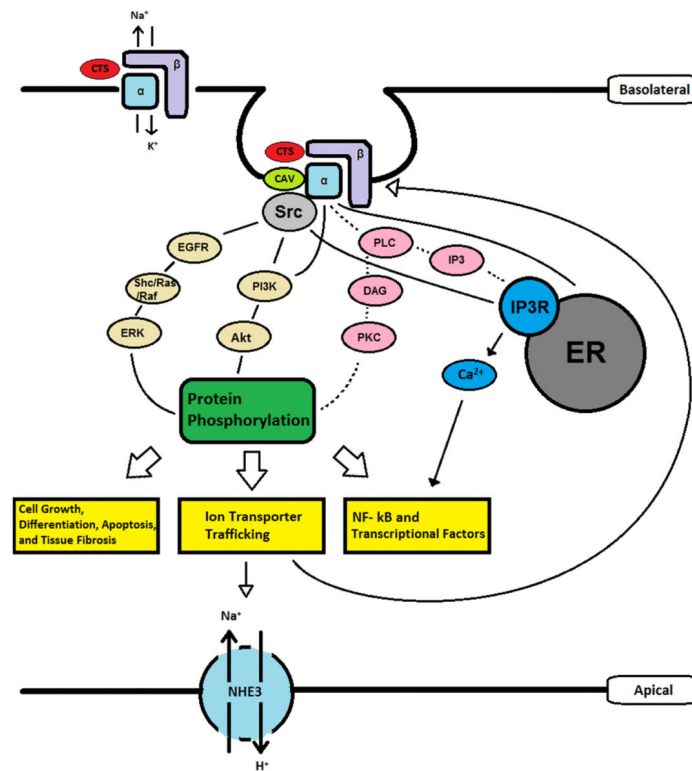


Figure 1.

Schematic presentation of Na/K-ATPase-Mediated Signal Transduction in Renal Epithelial Cells. Src, proto-oncogene tyrosine-protein kinase Src (sarcoma); CTS, cardiotonic steroids; Cav, caveolin-1; EGF, epidermal growth factor; EGFR, EGF receptor; PLC, phospholipase C; DAG, diacyl glycerol; IP3, Inositol trisphosphate; IP3R, IP3 receptor; PI3K, Phosphoinositide 3-kinase; Akt, protein kinase B; ERK, extracellular signal-regulated kinase.