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The physiological and clinical importance of sodium potassium ATPase in cardiovascular diseases

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The Na/K-ATPase has been extensively studied, but it is only recently that its role as a scaffolding and signaling protein has been identified. It has been identified that cardiotonic steroids (CTS) such as digitalis mediate signal transduction through the Na/K-ATPase in a process found to result in the generation of reactive oxygen species (ROS). As these ROS also appear capable of initiating this signal cascade, a feed forward amplification process has been postulated and subsequently implicated in some disease pathways including uremic cardiomyopathy.

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Introduction

The Na/K-ATPase enzyme (EC 3.6.3.9.), or 'sodium pump' was first identified by Skou on the crab nerve in 1957 [1]. Besides its transportation of ions, in the late 1990s, Dr. Zijian Xie and our research group discovered a scaffolding and signaling function for the Na/K-ATPase, where the Na/K-ATPase/Src complex acts as a unique receptor for cardiotonic steroids (CTS). This signaling pathway appears to be involved a number of clinical disorders including cardiovascular diseases and hypertension, salt balance, renal diseases, diabetes and other metabolic diseases, as well as neurological disorders [2,3]. Moreover, the alteration of signaling receptor function is also seen in hypertension, cardiac hypertrophy, ischemia/reperfusion injury, cancer, and tissue fibrosis. Therefore, this enzyme assumes an increasing importance for researchers and clinicians [4].

Structure and pumping function of the Na/K-ATPase

Structurally, Na/K-ATPase primarily consists of three subunits denoted by α, β, and γ, with only the α and β subunits necessary for ion pumping. The catalytic α subunit contains binding sites for the cations (Na^+ and K^+), ATP and cardiotonic steroids (CTS) such as ouabain [5]. The β subunit is also essential for pump function, and it appears to stabilize the α subunit conformation as well as chaperone the delivery of the α/β complex to the plasma membrane. In some tissues, a third subunit, γ subunit (FXYD protein) may help to regulate sodium pump activity [6,7]. The Na/K-ATPase α subunit with 11 transmembrane domains has 4 isoforms. The α1 isoform is found in all cells, α2 and α3 isoforms are mainly expressed in skeletal muscle, neuronal tissue, and cardiac myocytes. The α4 isoform is in testis and regulates sperm motility.

The structure–function relationships in Na/K-ATPase were extensively studied in the later portions of the 20th century and has received new attention due to the recently recognized Na/K-ATPase scaffolding and signaling functions which we will discuss further [8•,9]. The alteration between two major conformational states is responsible for pumping of ions. In E1 (Na^+-form), the cation-binding sites have high affinity for Na^+ and face the cytoplasm. In E2 (K^+-form), the cation-binding sites have high affinity for K and face the extracellular side [9,10]. Functionally, the Na/K-ATPase α subunit has 3 cytoplasmic A (actuator), N (nucleotide binding) and P (phosphorylation) domains. During the ion pumping cycle, the relative positions of these domains change as they also do in response to binding CTS [10].

Third factor

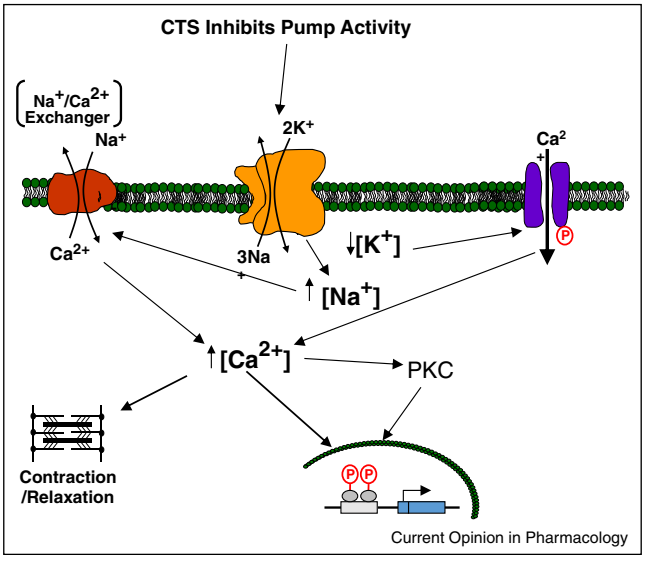
It was clear to renal physiologists and nephrologists that changes in glomerular filtration rate (factor 1) and mineralocorticoids (factor 2) could not explain natriuretic responses to acute or chronic expansion of blood volume [11]. This point was first demonstrated in 1961 in a classic paper by de Wardener and colleagues [12]. Natriuresis induced by saline infusion occurred even if renal perfusion pressure and glomerular filtration rate and aldosterone concentrations were prevented from changing. This so called 'third factor,' which we now understand is (are) CTS, was proposed by Bricker and colleagues to be an inhibitor of the Na/K-ATPase and, as such, produced natriuresis by inhibiting Na reabsorption in the kidney [13]. However, doubt as to the validity of circulating Na/K-ATPase inhibitors developed during the 1980s and 1990s because of inconsistencies in the reported results. In particular, prevailing CTS assays were based on cross-reactivity of CTS with antibodies to digoxin. The most

important inconsistency was that digitalis did not appear to be natriuretic in normal subjects, something one would expect in a candidate natriuretic substance [14]. Also, atrial (and brain) natriuretic peptide(s) were discovered and found to be natriuretic [15–19]. Undoubtedly, these points, detracted focus from the study of CTS. However, enthusiasm was renewed in the recent past for the following reasons. First, several CTS have been isolated from experimental animals and humans and chemically characterized. Specifically, marinobufagenin (MBG) as well as telecinobufagin (TCB) have been isolated from plasma and urine [20]. Ouabain has also been identified although there is still some debate as to whether this is ouabain or something distinct which also reacts to anti-ouabain antibodies [21•,22]. The concentrations of ouabain (or ouabain like compound) and MBG appear to be in the range of 2–30 $\times$ 10^{-10} M in humans, depending on whether disease is present [2,3,23]. Plasma levels of TCB and bufalin are less well defined at present.

Ionic model for Na/K-ATPase signaling

The concept proposed by Bricker and others was that third factor or cardiotonic steroids acted like digitalis and inhibited the enzymatic, ion pumping function of the Na/K-ATPase. The effects on sodium transport by the kidney seemed obvious in that clearly a failure to pump sodium out of epithelial cells would decrease net sodium reabsorption and effect natriuresis. Coupling the Na/K-ATPase to the Na/Ca exchanger, cardiac and smooth muscle relevance of these substances also seemed very clear in that subtle increases in cytosolic sodium would have amplified effects on cytosolic calcium, effecting changes in contractility in the heart as well as contractile tone in smooth muscle cells. The concept is illustrated in Figure 1.

Figure 1



A schematic illustrating the proposed ionic consequences of Na/K-ATPase inhibition. Note that such inhibition would predict increases in cytosolic sodium (Na^+) which, through Na/Ca exchange could increase cytosolic calcium (Ca^{2+}) concentration ([Ca^{2+}]. Decreases in cytosolic potassium (K^+) would change the membrane potential, favoring more Ca^{2+} entry, further increasing cytosolic [Ca^{2+}]. This would potentially activate phosphokinase C (PKC) and other Ca^{2+} dependent proteins, which in turn, would have a number of downstream effects.

Although we will explore an alternate hypothesis for the much of the remainder of this review, it must be said that this ionic model may explain some of the effects of digitalis and other cardiotonic steroids previously characterized as third factor. However, for reasons we will discuss below, certain discrepancies provoked scientists in our group, in particular Dr. Zijian Xie, to consider and elaborate a very different mechanism by which these cardiotonic steroids signal through the Na/K-ATPase.

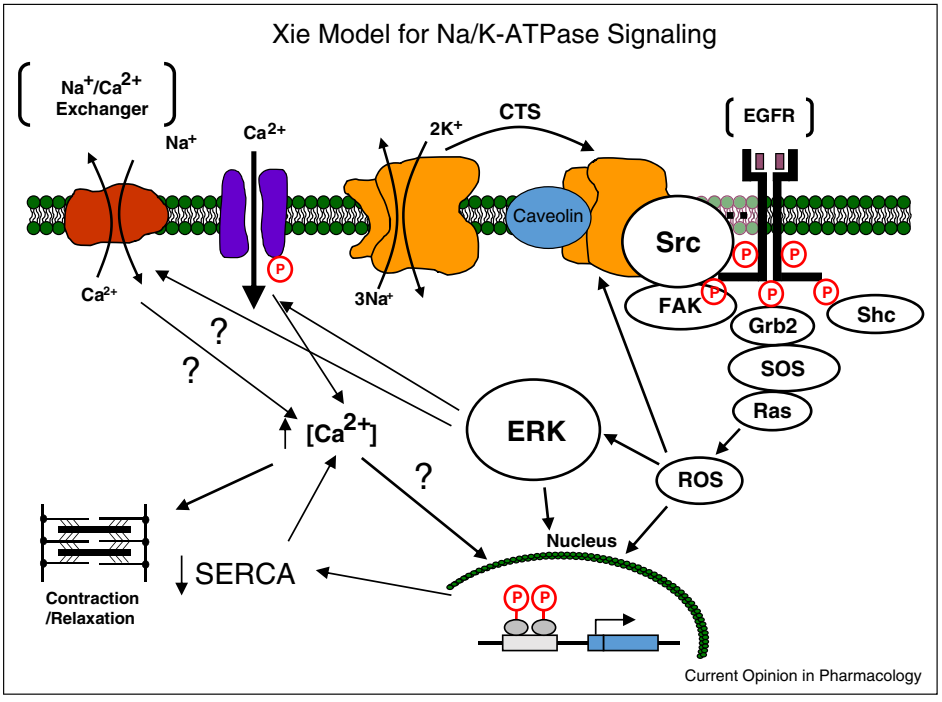
Xie model of Na/K-ATPase signaling

The Xie model for the Na/K-ATPase signaling function was derived from difficulties explaining signaling with the ionic model along with experimental observations regarding reactive oxygen species (ROS) and tyrosine kinase activities being critical to such signaling. This model proposed that the caveolar Na/K-ATPase alpha1 subunit serves as a negative regulator of Src, and that during conformational changes in alpha1 induced by CTS or oxidation, Src is allowed to become active and trigger a signal cascade which involves the generation of reactive oxygen species (ROS). This model is shown schematically in Figure 2, and in our admittedly biased opinion constitutes an important advance in our understanding of sodium pump signaling.

Problems with ionic model

Although the ionic model makes some predictions consistent with the observed pharmacology of Na/K-ATPase inhibitors, in general the concentrations of such inhibitors exceed by orders of magnitude those concentrations achieved in physiologically relevant models and clinical therapy. Although there is still some debate about this, it is generally accepted that endogenous cardiotonic steroids circulate at concentrations ranging from 10^{-10} to 10^{-8} M concentrations and pharmacologic treatment with digitalis compounds achieves concentrations of approximately 1–2 $\times$ 10^{-9} M [2]. In mice and rats used for many experiments, the IC50 for the α1 subunit approximates 10^{-5} M meaning that no detectable inhibition is seen with these lower concentrations. Even in humans which have a much more sensitive α1 subunit, the IC50 is approximately 10^{-7} M suggesting that these compounds might have relatively little effect. More importantly, changes in bulk cytosolic sodium are essentially impossible to detect with existing methods. Although these methods are not ideal and cannot identify sodium concentration changes in cytosolic subregions, it is still concerning. Perhaps of greater concern, sodium ionophores

Figure 2



A schematic illustrating the involvement of cardiotonic steroid (CTS)-induced Na/K-ATPase signal cascade initiated by the Na/K-ATPase mediated activation of Src tyrosine kinase and subsequent downstream targets eventually leading to the development of reactive oxygen species (ROS). Specifically, we postulate that in the microdomain of caveolae, the Na/K-ATPase functions as a scaffolding protein, interacting with CTS and changing conformation so as to active Src. Src then transactivates the EGFR which leads to a signal cascade involving FAK, Shc, Grb2 and SOS resulting in the generation of ROS which in turn activates additional Na/K-ATPase molecules as well as causes downstream activation of ERK as well as effects on the nuclear transcription [68]. ERK activation has effects on both L-type channels and possibly the Na/Ca exchanger with net effect to increase cytosolic Ca^{2+} in some tissues [35]. Nuclear effects in myocardial tissue include downregulation of SERCA transcription and translation [69]. *Abbreviations*: Epidermal growth factor receptor (EGFR); focal adhesion kinase (FAK); Src homology-2 domain containing protein (Shc); growth factor receptor-bound protein-2 (Grb2); son of sevenless protein (SOS); extracellular-signal-regulated kinase (ERK); sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA).

which can produce measurable changes in bulk cytosolic sodium do not appear to produce physiological changes similar to those produced by cardiotonic steroids [2,3].

Evidence for tyrosine kinase signaling

In the late 1990s, Dr. Xie and colleagues observed that in neonatal cardiac myocytes, ouabain caused increases in reactive oxygen species (ROS) measured with CMDCF [24]. These increases in ROS could be demonstrated even when cytosolic calcium was maintained low by removal of extracellular calcium [25]. It was further noted that some of the downstream effects of ouabain, specifically those on gene expression, calcium cycling and contractility could be blocked by N-Acetyl Cysteine (NAC) or vitamin E [24–26]. It was further noted that Ras activation appeared to be necessary to see increases in ROS [25]. Other studies determined that interactions between the Na/K-ATPase and Src appeared to initiate the signal cascade [27,28]. The α1 subunit of the Na/K-ATPase binds Src and appears to maintain it in an inactive state. However, binding a CTS appears to alter the Na/K-ATPase structure allowing Src became activated which, in turn, trans-activates the EGFR, and begins the signal cascade which causes increases in ROS [27–30]. The Na/K-ATPase-Src complex appears to function similar to a receptor tyrosine kinase. Downstream activation of PLC, PI(3)K and PKC has also been established [31–35].

Some of our studies have shed light on the molecular basis of the Na/K-ATPase α1 subunit-Src interaction. It appears that there is a critical binding of the tyrosine kinase domain of Src by a portion of the N domain of the α1 subunit. Under basal conditions, this binding inhibits the tyrosine kinase function of Src. We speculate that conformational changes induced in the Na/K-ATPase by cardiotonic steroids and/or the specific oxidation of some amino-acids (vida infra) result in the internalization of this epitope and the disinhibition of the tyrosine kinase function of Src with attendant downstream signaling. This is illustrated in Figure 2. From this speculation, we have developed a peptide based on this epitope in combination with a tat leader sequence that allows for

cellular penetration which we refer to as pNaKtide. Although pNaKtide has no effect on Na/K-ATPase enzymatic or pumping function, it prevents a portion of Src activation which is usually regulated by the plamalemmal Na/K-ATPase [36,37].

Oxidant stress in chronic renal failure

Our group and others first proposed that oxidant stress contributed to the progression of chronic renal failure in the mid 1980s [38]. The concept which we proposed was that oxygen consumption by the chronic renal failure kidney could not be explained by the amount of tubular sodium transport performed in the setting of a reduced glomerular filtration rate. Patients with chronic renal failure consistently demonstrate elevations in circulating levels of oxidized proteins and byproducts of lipid peroxidation. This oxidant stress has been implicated in the pathogenesis of uremic cardiovascular disease on several levels [39].

A number of studies utilizing echocardiography have demonstrated that both left ventricular hypertrophy (LVH) and diastolic dysfunction (as assessed by left ventricular, atrial and pulmonary venous doppler flow studies) are extremely common in end stage renal disease (ESRD) patients treated with hemodialysis(HD) [40] as well as patients incident to ESRD [41–43]. In general, most studies have demonstrated that LVH predicts diastolic dysfunction with some accuracy. Systolic dysfunction while not uncommon is much less often demonstrable than diastolic dysfunction and LVH [44]. In experimental chronic renal failure, we have observed that left ventricular hypertrophy develops quite early and that impaired myocyte relaxation accompanies the cardiac enlargement. This impaired myocyte relaxation appears to be associated with a marked downregulation of SERCA2a mRNA, protein and activity. SERCA2a is the dominant isoform of the sarcoplasmic reticulum calcium ATPase and is responsible for the rapid reduction in cytosolic calcium following systole. We have found excellent correlations between the reduction in SERCA2a expression and SERCA enzymatic activity as well as calcium renormalization following electrical stimulation. We have also found marked abnormalities in cardiac myocyte calcium concentrations during both systole and diastole [26,45]. It is unclear at present whether the abnormalities in SERCA2a expression explain all of the changes in calcium cycling or active relaxation. Regarding passive relaxation, it is very clear that uremic cardiomyopathy is associated with profound cardiac fibrosis, both clinically and in both rats and mice with experimental renal failure [46•]. Our group has developed extensive evidence that blockade of Na/K-ATPase signaling results in amelioration of oxidant stress, downregulation of SERCA2a and cardiac fibrosis in these experimental uremic cardiomyopathy models [47–50].

Na/K-ATPase is a receptor for natriuretic hormones

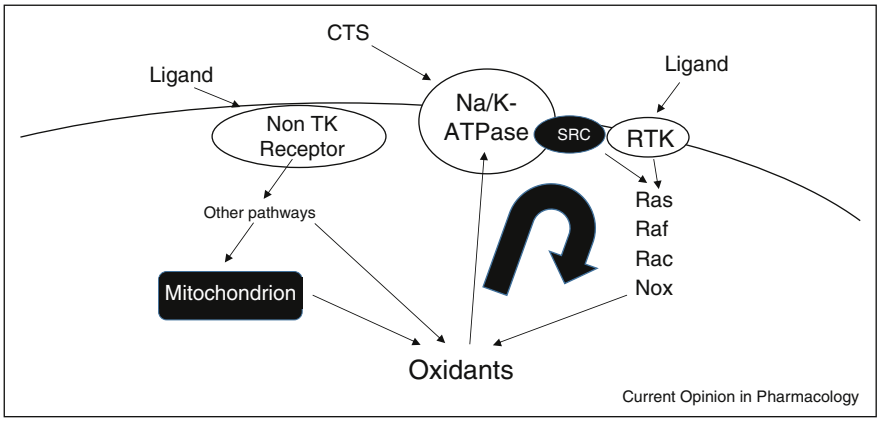
Our group has identified that concentrations of CTS below that necessary to significantly inhibit the enzymatic function of the Na/K-ATPase directly induce endocytosis of the Na/K-ATPase in cell lines approximating proximal tubules, decreasing plasmalemmal Na/K-ATPase expression and function [51–53]. We have also found that this ligand-induced endocytosis appears to play a role in sodium homeostasis in intact animals [54]. We will discuss some of these mechanistic data below.

In renal proximal tubules, binding of CTS to Na/K-ATPase stimulates Na/K-ATPase-Src signaling pathway and reactive oxygen species (ROS) generation, which induces the redistribution of basalateral α1 subunit of Na/K-ATPase and apical sodium proton exchanger3 (NHE3). NHE3 (SLC9A3) is responsible for two-third of filtered sodium and fluid reabsorption as well as maintenance and regulation of intravascular volume and blood pressure [55–57]. Therefore, the downregulation of NHE3 will decrease transepithelial sodium transportation from apical membrane into basalateral membrane, leading to a net increase in urinary sodium excretion [25,52,58••,59–62].

We have begun to address the molecular basis of this regulation [63]. We have demonstrated that ouabain increases the carbonylation of α1 Na/K-ATPase in cultured porcine renal proximal tubular LLC-PK1 cells. A GO-glucose system was used to mimic overall ROS stress since GO induces a low and sustained generation of H_2O_2 in the presence of glucose in culture medium [24,25,64]. Like ouabain, the GO-generated ROS were able to activate Src/ERK and cause redistribution of the Na/K. Interestingly, the increase in intracellular ROS generated by the addition of glucose oxidase (GO) to the culture medium resulted in a nearly identical pattern of protein carbonylation to that seen with ouabain as well as what appears to be specific carbonylation of the α1 Na/K-ATPase. We have further identified that Pro222 and Thr224 residues of α1 Na/K-ATPase are specifically carbonylated in response to either exposure to ouabain or GO-generated ROS. Finally, using well-established animal models we have demonstrated that high salt intake is capable of eliciting this oxidative modification of the Na/K-ATPase as well as the associated signaling and salt handling events in the renal PT of Sprague Dawley rats and Dahl-salt resistant (R) but not Dahl salt-sensitive (S) rats [65]. Overall, our studies show that ROS are involved in CTS-mediated sodium handling through Na/K-ATPase signaling in a feed-forward mechanism [58••,66]. This is illustrated in Figure 3.

The implications of this feed-forward amplification are profound. One might think of the circulating CTS as the rheostat on an amplifier which promiscuously allows

Figure 3



Schematic showing the possible promiscuous consequences of feed forward oxidant amplification of the Na/K-ATPase. Here oxidants can be formed by receptor tyrosine kinases (RTK) or non-tyrosine kinase receptors (Non TK Receptor). Activation of the Na/K-ATPase signal cascade or RTK would activate the Ras-Raf-Rac-Nox pathway. As this leads to further generation of oxidants, a feed-forward pathway is thus established. We speculate that the endocytosis of the Na/K-ATPase (not shown on this schematic) which we discuss in the text terminates this feed-forward signal amplification.

oxidant signals from a variety of signal cascades to be amplified. This is clearly something which may be relevant beyond the circumstances discussed in this review. In fact, we have recently observed that the Na/K-ATPase amplification of such oxidant signals is essential for the development of an obesity phenotype, and the application of pNaKtide, a peptide we designed to bind the tyrosine kinase domain of Src based on the region of the Na/K-ATPase α1 subunit N domain which normally does so, dramatically attenuates this process [67••].

Conclusions

The recently discovered oxidant amplification function of the Na/K-ATPase appears relevant to a number of physiological and pathological processes including renal sodium handling and progressive cardiac fibrosis. Exploitation of this understanding may allow for modulation of such processes and the potential development of new clinical therapies.

Conflict of interest statement

Neither Dr. Yan or Dr. Shapiro have conflicts to report.

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