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The Plasmalemmal Na/K-ATPase: An Amplifier for Reactive Oxygen Species?

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Abstract

Although the plasmalemmal sodium potassium adenosine triphosphatase (Na/K-ATPase) is one of the most studied proteins in Biology or Medicine, a signaling function believed to be independent of its pumping function has only been noted in the past 20 years. This signaling function appears to require the generation of reactive oxygen species (ROS) which, in turn requires the activation of Src and the transactivation of the epithelial growth factor receptor (EGFR). Recent data suggest that the Na/K-ATPase may also serve as a receptor for some ROS and in this manner, serve to amplify oxidant signaling. The mechanisms involved and the implications for Physiology and Medicine are discussed in this review.

Keywords: Sodium pump; Cardiotonic steroids; ROS; Oxidant stress; Cell signalling; Renal salt handling

Introduction

Discovered only within the last fifty years, reactive oxygen species (ROS) are oxygen derived free radicals found in both intra- and extracellular space. Common forms of ROS include superoxide anion (O_2^-), hydroxyl radical (OH^-), and peroxide (O_2^{-2}), each classified as free radicals, due to the presence of an extra unpaired electron carried in their outer shell, giving each species paramagnetic tendencies. As metabolic by-products within normal physiological processes, such as the mitochondrial electron transport chain, ROS highly reactive nature arms them with the capability to damage surrounding cells--irrespective of cell type [1-3]. Consequently, if the magnitude of derived ROS exceeds the host's capacity to detoxify the reactive intermediates--via antioxidants--then the physiological system is said to be under oxidative stress. An unregulated imbalance between oxygen metabolism and ROS neutralization can threaten essential cellular macromolecules. Lipids are particularly vulnerable to oxidant damage, resulting in lipid peroxidation, threatening to disrupt the integrity of the cellular membrane [4]. Furthermore, cellular proteins may come under attack by way of carbonylation [5] or nitrosylation [6].

These disruptions at the cellular level can have very drastic systemic effects and, as mentioned above, prolonged oxidative stress has been implicated in the pathogenesis of several diseased states. Oxidative stress in the pancreas causes destruction of beta cells which, along with insulin resistance, is a staple effect of type II diabetes [7]. Pancreatic beta cells do not have a strong antioxidant response, making them easy targets for ROS. In the case of acute pancreatitis, ROS have been shown to induce edema and apoptosis in pancreatic cells [7]. Nearly all chronic liver diseases, both alcoholic and nonalcoholic alike, are heavily evidenced to be brought about by oxidative stress [8]. Furthermore, ROS directly attack both vasculature in the body, as well as cardiomyocytes in the heart. These attacks make cardiac events, such as myocardial infarction, far more likely, and even inevitable [3].

Systemic oxidative stress also appears to play a central role in the progression of uremic cardiomyopathy, which is defined as the development of cardiac disease secondary to renal disorders such as chronic kidney disease (CKD) and end-stage renal disease (ESRD); uremic cardiomyopathy is generally characterized by systolic and diastolic dysfunction, dilation of the left ventricle, and most notably, left ventricular hypertrophy [9]. The proposed mechanism in which cardiac damage ensues, appears to begin with the elevated systemic levels of cardiotonic steroids (CTS), specifically marinobufagenin (MBG). Under normal physiological conditions, MBG appears to act as a natriuretic hormone that allows sodium to be excreted when salt and water are accumulated [10]. This (or other) endogenous steroid hormone(s) binds to the plasmalemmal Na/K-ATPase (Na^+ pump) α1 subunit and triggers a conformational change in the functional receptor complex (described below) and activates a host of downstream intracellular signaling cascades, one of which results in the production of ROS [11]. The role of oxidative stress induced by this process may be a major contributing factor in the pathogenesis of fibrosis in the heart [12].

ROS as Signaling Molecules

Despite the longstanding stigmata that reactive oxygen species have a primarily malignant function within the body, research also suggests a means by which living organisms have actually adapted to the negative effects of ROS using free radicals to their benefit. More specific to the purpose of this review, free radicals have been shown to act as intermediate messengers within cell signaling pathways [13]. On a cellular level, ROS have a higher affinity for misfolded proteins than intact proteins; the interaction between ROS and specific amino acids within protein polypeptides perpetuates a molecular change signaling these macromolecules for proteolysis [14]. Some amino acids are particularly prone to oxidation by ROS (e.g, tryptophan, tyrosine, histidine, and cysteine) and modified in oxidative signaling [15-17]. Carbonylation of these amino acids lead to conformational modification of the parent protein [18]. These changes in conformation lead to changes in function as well as alter the protein's susceptibility to proteolysis [19,20]. Not only are misfolded proteins

degraded by this process but it can also be seen as a mechanism for reducing levels of oxidant stress [13].

ROS appear to play an integral role in the propagation of various molecular signaling cascades in both physiologic and pathophysiologic processes. Kokusho and coworkers demonstrated the involvement of ROS derived from metabolically active cardiomyocytes, specifically H_2O_2, in blood flow regulation within coronary microvasculature during enhanced cardiac metabolism [21]. The overproduction of ROS during cardiac failure may be implicated in its pathogenesis. It is possible that enhanced ROS signaling produced by metabolically active cardiomyocytes leads to impaired regulation of coronary microvascular tone [22].

This review will focus on ROS as critical intermediates in the propagation of the CTS- activated Na/K-ATPase signal transduction cascade. We aim to outline the role of Na/K-ATPase as an amplifier for ROS, which are detrimental, not only in uremic cardiomyopathy, but in many systemic diseases.

Structure and Function of the Na/K-ATPase

The Na/K-ATPase was discovered in 1957 by Jens C. Skou during his work with crab nerve cells. Skou was awarded the Nobel Prize in 1997 for this discovery [23]. Na/K-ATPase is a member of the P-type family of membrane incorporated proteins. The sodium pump is present in all eukaryotic cells and its primary function is as an ion pump, in other words to maintain the concentration gradients for Na^+ and K^+ across the plasmalemmal membrane. The accepted functional mechanism (depicted in Figure 1) is known as the E1/E2 or Albers-Post theory.

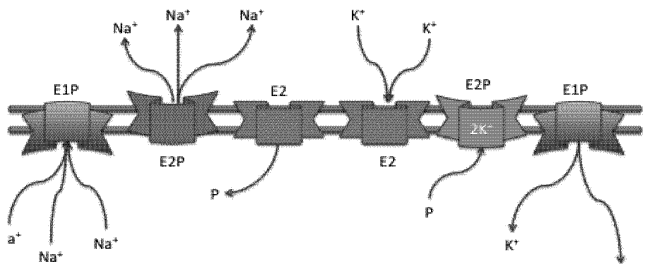

Figure 1: Schematic showing the Post-Albers model of the Na/K-ATPase. Recurring conformational changes of the pump diagramed, as 3 Na^+ ions are removed from the cytosol and transferred to the extracellular matrix (E1P and E2P conformations, respectively). The pump is then dephosphorylated (E2) whereby it gains a higher affinity for K^+ ions. Consequently, 2 K^+ ions from the extracellular space bind to Na/K-ATPase; the pump is then autophosphorylated (E2P), leading to another conformational shift and the 2 K^+ ions are released into the cytosol. The pump then returns to the E1P conformation, and the cycle continues.

During the E1 phase, the enzyme is phosphorylated (E1P), with the binding site having a high affinity for Na^+ and an open gate facing the cytoplasm. When $3Na^+$ bind to the enzyme, the Na/K- ATPase undergoes a conformational change, allowing the sodium ions to exit the gate into the extracellular matrix. When the sodium ions are released it causes the Na/K-ATPase to undergo another conformational change (E2P) which has a high affinity for (extracellular) K^+. The enzyme is then dephosphorylated (E2). When the conformation returns to E1, ATP again phosphorylates the pump, releasing $2K^+$ molecules into the cytoplasm, beginning the cycle again. This is known as the classical, or ionic, pathway.

The chemical gradient created by the Na/K-ATPase serves many secondary functions including: cytoplasmic pH and calcium regulation via Na^+/H^+ and Na^+/Ca^{2+} exchanger, respectively [24,25]. We refer to the regulation of cellular processes through inhibition (or stimulation) of the Na/K-ATPase pumping activity as the classical or ionic pathway of Na/K-ATPase signaling [26,27].

It is also important to note that reactive nitrogen species (RNS), such as peroxynitrite, play an important regulatory role of Na/K-ATPase function. Multiple experiments have shown that peroxynitrite, a product of nitric oxide (NO) and superoxide (O_2^-), causes inhibition of the sodium pump by modifying susceptible amino acids (namely tyrosine and cysteine). Findings from Reifenberger et al. suggest that peroxynitrite mediated nitrosylation of the cysteine thiol group, specifically, is responsible for Na pump inhibition as the antioxidant glutathione was unable to reverse pump inhibition [28,29]. To highlight the importance of peroxynitrite as a signaling molecule, Zhang and colleagues demonstrated its involvement in angiotensin II-mediated (ANGII) pump activation. The researchers originally determined that ANGII stimulates the ionic pathway of the sodium pump in a concentration dependent manner; at picomolar concentrations, ANGII increases Na/K-ATPase activity, but when concentrations rise into the nanomolar range this effect is lost [30]. The increased stimulation of the Na/K-ATPase leads to Ca^{2+} - mediated activation of NO-synthase and eventual peroxynitrite (and other RNS) generation; this causes inhibition of the Na pump, explaining the attenuation of ANGII stimulation. This was proven further by the addition of RNS scavengers, which unmasked the stimulating effect of ANGII on Na/K-ATPase [31].

Structure, isoforms, and distribution

The main structure of Na/K-ATPase (depicted in Figure 2) consists a heterodimer involving an α and β subunit.

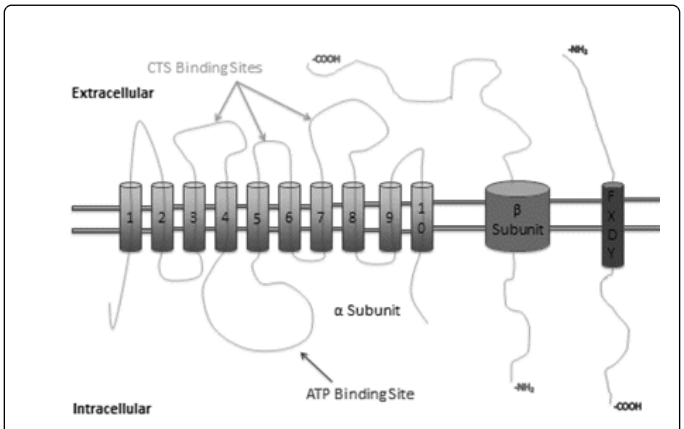

Figure 2: Schematic showing subunits of the Na/K-ATPase. The three essential subunits include the α subunit (comprised of ten transmembrane domains), the β subunit, and the FXYD protein (γ subunit).

We have highlighted three separate locations on the extracellular domain of the subunit depicting CTS binding sites, as well as the ATP

binding site on the intracellular domain (responsible for the pump's catalytic activity). The β subunit is responsible for inserting the enzyme into the cell membrane, and provides structural stability. FXYD helps to regulate the catalytic activity of the pump.

The α subunit is responsible for the pump's catalytic activity, and is comprised of ten transmembrane domains. It is the α subunit that harbors extracellular binding sites for Na^+ and cardiotonic steroids and intracellular binding sites for K^+ and ATP. The α subunit of the Na/K-ATPase is divided into three regions (A, N, and P depicted in Figure 3) with ATP binding to the N domain [32].

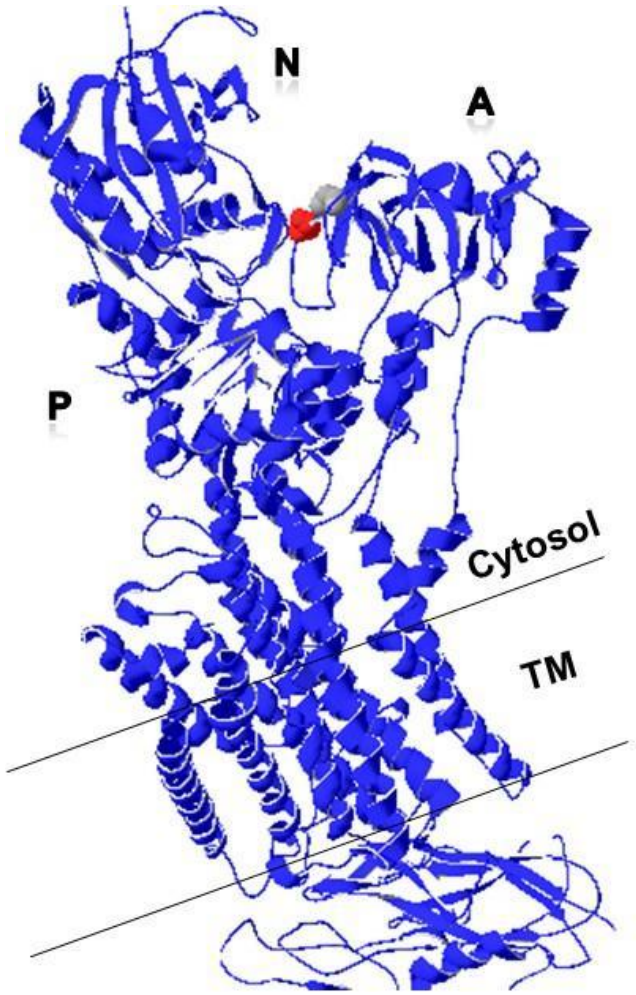

Figure 3: Crystalline structure of the Na/K-ATPase highlighting the cytosolic A, N, and P domains. Two amino acids (proline 222 in red and threonine 224 in grey) in the pig form of the α1 subunit of the Na/K-ATPase) are noted. Reprinted with permission from the Journal of Biological Chemistry.

The A Domain serves a purpose in the stability during conformational shift between the E1 and E2 states [33] and may be important in the regulation of Src kinase activity [34] which we will discuss in some detail. The β subunit, on the other hand appears to be involved in cellular trafficking as well as modulate the pumping function of the Na/K-ATPase. There is also a third component of Na/K-ATPase, consisting of seven single transmembrane proteins, each of them tissue specific; they are called FXYD after their amino acid sequence motif. FXYD2 is also known as the γ subunit. These proteins also help modulate pump distribution and function [26,27].

There are also different isoforms among the α and β subunits: α1, α2, α3, and α4 and β1, β2, and β3, respectively. Each isoform, along with the seven separate FXYD proteins, are regulated by single genes corresponding to their specific isoform [35]. The α1 isoform is found in all tissues throughout the body and is the primary isoform in the kidney, as is β1 [36]. The α1/β1 conformation of the pump is the most populous of the different configurations. The α2 isoform is also fairly widespread, being found in adult cardiac, vascular smooth and skeletal muscle as well as adipose, cartilage, and bone tissues. The α3 isoform has a more specific role, being found in excitable tissues such as nerves and Purkinje fibers [36]. The α4 isoform has only been encountered in the testis, and is the most tissue specific of the α isoforms [37]. α1, 2, and 3 isoforms have about 87% homology whereas the α4 subunit differs a bit more. The β2 and β3 isoforms can be found in the brain, cartilage, and erythrocytes. β2 can also be found in cardiac tissue, with β3 also expressed in the lungs [38].

Endogenous Cardiotonic Steroids

The use of the digitalis purpurea plant, "Foxglove", has been incorporated into herbal remedies over thousands of years. Serious examination of digitalis began with the writings of William Withering [39]. Substances with similar pharmacological effects can also be found in Eastern medicine. The dried skin of toads has been used in the treatment of cardiac dysfunction dating back at least one thousand years [40]. Nearly a century after Withering's examination, the idea of an endogenous compound that mimicked digitalis was set forth by Ringer in 1885. With the discovery of the sodium pump in the 1950's, the exact effect of digitalis as a pump inhibitor was postulated. Based on the work of several workers, a role for endogenous digitalis in sodium homeostasis was proposed [41,42] with Bricker's "trade off hypothesis" further suggesting a pathophysiological role in progressive renal failure and the uremic syndrome for these endogenous digitalis like factor (EDLF) or cardiotonic steroids (CTS) [43] as we now refer to them [44].

There has been a fair amount of controversy regarding endogenous CTS as the measurement of these substances has been plagued by inconsistency. Ouabain, a cardenolide CTS similar in structure to digoxin, has received perhaps the most attention, but the bufadienelides, marinobufagenin (MBG), telecinobufagenin (TCB), and bufalin, have also been implicated in Biology and Medicine [45,46]. Both types of CTS have a base structure of a steroid ring system, and are differentiated by either having a 5 member unsaturated lactone ring (cardenolides) or a 6 member unsaturated lactone ring (bufadienolides) [27]. Although the immunological measurements of these compounds have been employed in a number of detailed studies, more recent mass spectroscopy data has cast doubt upon what precisely is being measured. In particular, endogenous ouabain which had been postulated to play an important role in blood pressure homeostasis as well as renal salt handling, is simply not detectable as such in human plasma by high sensitivity mass spectroscopy [47]. Whether there is something chemically "close" to ouabain that functions as an ouabain like chemical (OLC) still requires careful study. Mass spectroscopy has identified various bufadienolides (e.g, telecinobufalin and marinobufagenin) in human plasma and urine [48], but their exact measurements in normal and pathological states are still controversial [49]. Cardiotonic steroids also play a role

in nitrosylation. Ouabain has been shown to activate NO-Synthase, leading to the production of nitric oxide, and ultimately peroxynitrite [28]. Discussion as to the probable function, biosynthesis and elimination of the cardenolide and bufadienolide CTS can be found in several references [26,27,50].

CTS and Na/K-ATPase Signaling Pathway

Only within the last two decades, has an abundance of experimental evidence surfaced in support of a second type of signaling pathway involving the plasmalemmal Na/K-ATPase. This novel pathway is independent from the classical pathway that is imperative to the establishment and maintenance of transcellular ionic gradients. Discovery of the signaling pathway has illuminated the Na^+ pump's ability to function as a receptor complex that, upon activation, has the capacity to transduce a variety of downstream cytosolic signaling cascades [11,51]. Opposed to the classical pathway in which ligands such as ouabain play an antagonistic role to the "pumping" function of the Na/K-ATPase, the "signaling" pathway suggests that ouabain binding actually disinhibits Src by inducing a conformational change in the α1 subunit removing a portion of the N domain from covering the Src kinase domain and allowing it to become active. This essentially makes the Na/K-ATPase-Src complex function together as a receptor-tyrosine kinase with CTS serving as receptor-ligands [34]. In this postulated pathway, it is only the "non pumping" α1 Na/K-ATPase residing in caveolae or lipid rafts which is associated with Src and thus participating in this signal cascade [52]. The intermediate molecules generated amongst the activation of this cascade (including, but not limited to: PLC, PKC, MEK, ERK, ROS, PI3K, Akt) provide a host of potential targets for pathway-dependent modulation, and may have therapeutic implications in various disease states, such as uremic cardiomyopathy [27,44,49].

Evidence supporting caveolar localization of Na/K-ATPase functional receptor complex as well as the critical involvement of these cholesterol-rich microdomains in the ouabain- mediated signaling pathway was further illuminated by Liu, et al. and Wang et al. [53,54]. Cell-free in vitro GST pull down assay was used in order to demonstrate the direct interaction between specific domains present on the subunit of the Na/K-ATPase (CD2 and CD3) and the Src protein (SH2 and SH1 kinase domain), respectively [34]. In the absence of stimuli, the interaction within the functional receptor complex, specifically the α1 N-domain/Src kinase interaction, maintains Src in its inactive state. It is therefore proposed that the extracellular binding of ouabain to the Na+ pump's α subunit alters the conformation so that the N domain is withdrawn from Src, allowing its kinase domain to be functional, in turn leading to the activation of protein tyrosine phosphorylation [55]. After Src activation, further ouabain-mediated signal transduction is seen in the recruitment and transactivation of EGFR, which continues the potentiation and propagation of multiple downstream protein kinase signaling cascades [34,56]. This cascade involves the generation of ROS, probably through the subsequent activation of Ras, Raf and Rac [11,57,58].

Recently, Weigand, et al. [59] and Gable, et al. [60] have called into question the existence of direct molecular contact between Src and the Na+ pump, along with the mechanistic role of Src in the digitalis-induced cell signaling pathway. The latter studies suggest that Src activation may be dependent upon the ATP-sparing effects of Na+ pump antagonists, rather than the previously hypothesized functional receptor complex. At the time of this review, it is unclear how pNaKtide developed based on the direct molecular-contact hypothesis (and discussed below) would have its observed effects in the ATP-sparing model proposed by Gable and coworkers as pNaKtide does not appear to have appreciable inhibition or stimulation of Na/K-ATPase enzymatic activity [55,61].

Na/K-ATPase signaling and ROS generation

Recent studies have implicated the involvement of ROS functioning as a secondary messenger in CTS-activation of hypertrophic pathways. Conclusions drawn from Elkareh et al. [62] study helped elucidate the molecular basis of MBG effects on promoting collagen synthesis in rat cardiac fibroblasts; the team suggested that the effects were mediated through CTS activation of the Na/K-ATPase-Src-EGFR-ROS cascade, promoting preferential protein synthesis and gene expression. Similar to Elkareh et al. [62], El-Okdi et al. [63] tested MBG and other CTS effects on promoting collagen production in human dermal fibroblasts; western blot analysis revealed dose-dependent increases in procollagen expression in response to MBG. Furthermore, it appears that while CTS-mediated ROS generation may be implicated in regulating cell growth, it has been hypothesized that ROS may also be involved in generating a signal amplification loop by acting directly on the Na/K-ATPase, thus perpetuating the cascade [64,65].

Regulation of feed forward signaling mechanism

The binding of ouabain to the Na+ pump in renal proximal tubule cells (RPT) and subsequent activation of the Na/K-ATPase/c-Src signaling cascade appears to be implicated in the increased natriuresis and diuresis seen in conditions involving diminished renal function and/or high sodium intake. This protective mechanism of salt excretion, appears to aid in counterbalancing BP elevation often seen under these conditions. It has been observed that downstream effects of CTS-mediated activation of RPT Na/K-ATPase signaling pathway leads to basolateral Na+ pump and apical Na+/H+ exchanger (NHE3) redistribution; as a result of decreased transporters, due to receptor-mediated endocytosis, less Na+ is capable of reabsorption and consequently increased natriuresis and diuresis follows [10,61,66-67]. Evidence that first suggested ouabain could trigger the internalization of the Na/K-ATPase and itself was supplied by Cook et al. in 1982. Their experiment followed [3H] ouabain into lysosomal pockets in HeLa cells [68].

Interestingly, oxidation of the Na/K-ATPase appears to occur on the α1 subunit in the form of reversible carbonylation of 2 amino acids residing in the A domain. Specifically, Yan and coworkers demonstrated that both proline 222 and threonine 224 on the porcine α1 subunit undergo reversible carbonylation with ouabain or H_2O_2 stimulation. The effects of low doses of H_2O_2 (induced by addition of glucose oxidase to the extracellular media) on Src and ERK activation were identical to that seen with signaling doses of ouabain [69]. This suggests a feed forward oxidation process where ROS could directly stimulate their own production through Na/K-ATPase signaling, a process which might then be terminated by endocytosis of the entire signaling complex [49].

Manipulations of Na/K-ATPase in Experimental Renal Failure

As discussed earlier, Bricker specifically hypothesized that endogenous digitalis like substances whose concentrations were increased with renal failure in an effort to maintain sodium

homeostasis might create some of the tissue injury seen with renal failure [43]. In a study conducted by Ferrandi and coworkers in 2004, the researchers injected rats with specified doses of ouabain over the course of 18 weeks. At 6 weeks, half of the rats began receiving oral doses of PST 2238, an antagonist of ouabain which blocks its interaction with the Na/K-ATPase (ionic or signaling), [70,71]. The results of the experiment showed the injections of ouabain caused the expected results of hypertension and hypertrophy formation. The mice treated with PST 2238, however, saw these symptoms attenuated. Kennedy and colleagues measured circulating levels of MBG in renal failure and found substantial elevations. They found that administration of MBG with minipumps to mimic this elevation produced a similar phenotypical pattern of cardiomyopathy and that immunization against MBG (discussed below) ameliorated the cardiomyopathy associated with experimental renal failure [12]. More recently, Tian and coworkers examined whether the administration of spironolactone might attenuate the cardiomyopathy associated with renal failure, and by what mechanisms this protection might occur. In addition to being a mineralocorticoid antagonist, spironolactone (and its major metabolite, canrenone) is (are) also known to interact with Na/K-ATPase and prevent CTS binding [72]. The administration of spironolactone attenuated hypertrophy in the cardiac tissue. In vitro studies conducted alongside animal studies suggested that the mechanism of action of spironolactone (and canrenone) were to antagonize CTS mediated signal transduction through the Na/K-ATPase [73].

As mentioned above, antibodies to MBG have also been used to attenuate the effects of oxidative stress induced by the signaling pathway of Na/K-ATPase in experimental renal failure. This was seen with active immunization [12] as well as passive immunization with either Digibind™, a fragment of a bovine antibody against digitalis, or 3E9, a specific monoclonal antibody developed to marinofugenin [74]. In both these settings, attenuation of uremic cardiomyopathy occurred in concert with amelioration of oxidant stress and Na/K-ATPase signaling.

Development of pNaKtide

Perhaps the most direct test of the Na/K-ATPase-Src signaling pathway has been the development and deployment of pNaKtide. Li et al. identified a 20 amino acid sequence within the N domain of the Na/K-ATPase α1 subunit that binds and inhibits Src. Li and coworkers took this sequence and engineered a cell permeable version of the protein with a TAT leading sequence (now 33 AA long) which they called pNaKtide [54]. With pNaKtide inhibiting Src independently of Na/K-ATPase activity, pNaKtide appears to have possible efficacy against some types of cancer as multiple types of cancer may have decreased Na/K-ATPase expression and, as a consequence, constitutive activation of Src [75,76]. Administration of pNaKtide, however, greatly attenuates the overactivity of Src in prostate cancer cells, inhibiting cancer cell growth as well as stimulating apoptosis and reducing tumor size [61].

The Na/K-ATPase as an ROS Amplifier

We have seen that the "excessive" signaling through the Na/K-ATPase may produce systemic oxidant stress and a characteristic injury phenotype in several settings, most notably experimental renal failure [49]. However, the observation of specific oxidation sites on the α1 subunit [69] strongly suggests that this feed forward system might be involved in other oxidant stress states as well as those induced by increased circulating concentrations of CTS (Figure 4). Based on the data that we've summarized, we would suggest that any pathway which involves ROS capable of carbonylation (e.g. O_2-, H_2O_2) may become amplified by the Na/K- ATPase-Src-ROS signal cascade. If this is the case, the Na/K-ATPase may prove to be a useful therapeutic target in a variety of disease states associated with excessive oxidation.

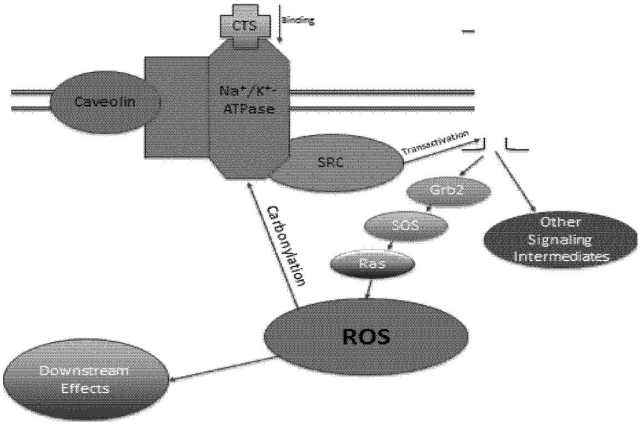

Figure 4: Schematic of Na/K-ATPase signaling and feed forward amplification of ROS. CTS binds to the caveolin-embedded Na/K-ATPase. This causes activation of the signaling pathway of the pump, and causes Src activation. Activation of Src initiates transactivation of the closely associated EGFR, which propagates a host of signaling pathways; one of which leads to a cascade of intermediates (Grb2, SOS, Ras) that lead to the eventual generation of ROS. From ROS generation two potential effects are highlighted: (1) pump carbonylation, which leads to receptor endocytosis and (2) a number of downstream effects.

Conclusion

The evidence that we have compiled in this review attempts to shed light upon the importance of the CTS-mediated activation of the Na/K-ATPase signaling pathway, independent from the classical (ionic) pathway, and the downstream generation of ROS. Understanding the mechanism that underlies the amplification of ROS is beneficial for potential therapeutic intervention, as oxidative stress is implicated in a variety of disease states. Consequently, there are many experimental manipulations that have been shown to selectively target the inhibition of ROS production from pump signaling, at levels that interfere with both CTS binding to the receptor complex and at levels that interfere with downstream cascade intermediates. At this point in time, this research has not yet bridged into the realm of translational medicine. The numerous cellular and animal studies that are, and have been, underway help elucidate new molecular targets for potential pharmacologic intervention in the future. Taken together, there is still much to be learned about the specific interactions between cascade intermediates as well as their roles in the potential amplification of ROS Therefore, future experimental manipulations of the signaling cascade will be needed to more comprehensively understand the exact mechanistic actions of ROS and its role as a signaling intermediate.

Acknowledgements

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