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THE NA/K–ATPASE-MEDIATED SIGNAL TRANSDUCTION AS A TARGET FOR NEW DRUG DEVELOPMENT

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1. ABSTRACT

The Na/K–ATPase, or Na^+ pump, is a member of the P-type ATPase superfamily. In addition to pumping ions, the Na/K–ATPase is a receptor that not only regulates the function of protein kinases, but also acts as a scaffold, capable of tethering different proteins into a signalplex. The signaling Na/K-ATPase resides in caveolae and forms a "binary receptor" with the tyrosine kinase Src. Endogenous cardiotonic steroids and digitalis drugs such as ouabain act as agonists and provoke this binary receptor, resulting in tyrosine phosphorylation of the proteins that are either associated with, or in close proximity to, the signaling Na/K-ATPase. Subsequently, this initiates protein kinase cascades including ERKs and PKC isozymes. It also increases mitochondrial production of reactive oxygen species (ROS) and regulates intracellular calcium concentration. Like other receptors, activation of the Na/K-ATPase/Src by ouabain induces the endocytosis of the plasma membrane Na/K-ATPase. Significantly, this newly appreciated signaling function of the Na/K-ATPase appears to play an important role in the pathogenesis of many cardiovascular diseases, therefore serving as an important target for development of novel therapeutic agents.

2. INTRODUCTION

J. Skou discovered the Na/K–ATPase or Na^+ pump in 1957 as an energy-transducing ion pump (1). Since then, extensive research has been conducted to characterize the pumping function and the regulation of the pump (2–4). The pump, composed of both catalytic alpha and regulatory beta subunits, also functions as a receptor for cardiotonic steroids (CTS) that consist of digitalis drugs and endogenous digitalis-like substances such as ouabain and marinobufagenin. The digitalis drugs increase intracellular Ca^{2+} and contractility in the myocardium, and thus have been used for the management of congestive heart failure for more than 200 years (5-7). Because CTS inhibit the Na/K–ATPase, the physiological and pharmacological functions of CTS were thought secondary to their effects on intracellular ion concentrations. However, this notion has been challenged by recent new findings, showing that the Na/K-ATPase also possesses a signaling function. Specifically, the signaling Na/K-ATPase interacts with protein kinases as well as other membrane and cytosolic proteins. Binding of CTS to the signaling Na/K–ATPase can activate protein kinases and generates secondary messages independent of changes in intracellular ion concentration (8–16). The purpose of this article is to provide a discussion of Na/K–ATPase-mediated signal

transduction and the potential utilization of this signaling function as a target for new drug development.

3. Na/K-ATPase STRUCTURE

Na/K-ATPase belongs to the family of P-type ATPases and consists of two noncovalently linked alpha and beta subunits (2-4,17). The alpha subunit (about 112 kDa) contains the ATP, digitalis, and other ligand binding sites, and is considered as the "catalytic subunit". The beta subunit is essential for the assembly of the functional enzyme. Several alpha and beta subunits have been identified and functionally characterized (2-4), and the isoforms are expressed in a tissue-specific manner. The $alpha_1$ isoform is found in all cells. The $alpha_2$ and $alpha_3$ isoforms are expressed in skeletal muscle, neuronal tissue, and cardiac myocytes. Finally, the $alpha_4$ isoform is found only in sperm. Both SERCA and the Na/K-ATPase belong to the type-II class of P-type ATPases. Recently, the crystal structure of SERCA1a has been determined at 2.6 Å resolution (18). Because the structure of the Na/K-ATPase has not been identified, we will use structure of SERCA as a template for further discussion of the Na/K-ATPase. In a recent review article, Sweadner and Donnet (19) revealed the structural similarities between these two enzymes and concluded that the Na/K-ATPase, like SERCA, contains four distinct functional domains. The A domain consists of the N-terminus and the first cytoplasmic loop connected to transmembrane helices M2 and M3. Although there is great sequence variation at the N-terminus between SERCA and the Na/K-ATPase, both enzymes appear to have the same two alpha-helix motifs. More importantly, based on the structure of SERCA, the A domain is highly exposed for the binding of other proteins. The enzyme also has the highly conserved phosphorylation (P) domain that is close to the membrane and a relatively isolated nucleotide-binding (N) domain that is found to interact with many cytoskeletal and signaling proteins (20-23).

4. MOLECULAR MECHANISM OF Na/K–ATPase-MEDIATED SIGNAL TRANSDUCTION

Na/K-ATPase serves as a receptor for CTS that include plant-derived digitalis drugs such as digoxin and ouabain, and vertebrate-derived aglycones such as bufalin and marinobufagenin. Although CTS have been considered only as drugs since their discovery, recent studies have identified both ouabain and marinobufagenin as endogenous steroids whose production and secretion are regulated by multiple pathological or physiological stimuli including angiotensin II and epinephrine (24-27). Early studies have demonstrated that the Na/K-ATPase, like many membrane transporters (28,29), interacts with multiple soluble and membrane proteins (21-23) and that CTS regulates gene expression and cell growth (30,31). Recent work on the Na/K–ATPase has made the connection between the Na/K–ATPase-mediated protein interaction and CTS-evoked changes in cellular function, showing that the Na/K-ATPase can transmit extracellular CTS signal via mechanisms independent of changes in intracellular Na^+ and K^+ concentrations (32).

4.1. Na/K-ATPase as a pseudo-receptor tyrosine kinase

We and others have shown that the binding of ouabain to the Na/K-ATPase stimulates tyrosine phosphorylation of multiple proteins in the absence of changes in intracellular ion concentration and that increases in tyrosine phosphorylation play a pivotal role in ouabain-induced changes in gene expression and cells growth (10-16). Because the Na/K-ATPase has no intrinsic tyrosine kinase activity, we speculated that the pump might function as a cytokine receptor, employing non-receptor tyrosine kinases such as Src to form a pseudo-receptor tyrosine kinase (33). Indeed, we and others demonstrated that ouabain activated Src kinase in primary cultures of rat cardiac myocytes, canine smooth muscle cells as well as several cell lines including the pig kidney LLC-PK1 cells (11,12). Furthermore, inhibition of Src or knockout of Src family kinases abolished ouabain-activated signaling pathways including the Ras/Raf/ERK cascade and mitochondrial production of ROS (14,15). It also prevents ouabain-induced endocytosis of the Na/K-ATPase (34). Mechanistically, the ouabain-activated Na/K-ATPase/Src complex recruited and assembled multiple proteins into various signalplexes. For example, we found that the ouabain-activated Na/K-ATPase/Src complex transactivated EGF receptor, which in turn recruited Shc and Grb to the complex, resulting in the activation of the Ras/Raf/ERK cascade (14,15). In addition, the ouabain-activated Na/K-ATPase/Src complex was able to recruit and activate PI3-kinase, which in turn assembled endocytotic cargo for removal of the activated Na/K-ATPase signalplex from the plasma membrane (34). Apparently, this process not only terminates/targets the signaling complexes to intracellular compartments, but also reduces overall pumping capacity of the cells due to the loss of plasma membrane Na/K-ATPase (35).

4.2. Na/K-ATPase signals from caveolae

Because the Na/K–ATPase has to interact with Src, EGFR, and other proteins to transmit the ouabain signal, we proposed that the signaling Na/K-ATPase might be preassembled with its partners in membrane microdomains such as caveolae. Caveolae are membrane microdomains that were first identified as flask-shaped vesicular invaginations of plasma membrane enriched in cholesterol, glycosphingolipids, and sphingomyelin (36,37). Caveolins are 21-24 kDa membrane-associated scaffolding proteins that serve as a protein marker of caveolae (36). Caveolins directly interact with many signaling proteins such as Src family kinases via the scaffolding domain's binding to the caveolin-binding motifs of the target proteins. Based on the observation that the Na/K-ATPase alpha subunit contains two conserved caveolin-binding motifs, we and others have recently tested the role of caveolin and caveolae in the Na/K-ATPase-mediated signal transduction. In many different cells including cardiac myocytes, smooth muscle and renal epithelial cells, the Na/K-ATPase is co-localized with caveolin-1 and concentrated in caveolae (16,38,39). GST pull-down assay showed that Na/K-ATPase bound to the N-terminus of caveolin-1. Significantly, ouabain regulated this interaction in a time- and dose-dependent manner and stimulated tyrosine phosphorylation of caveolin-1 in a Src-

dependent manner in LLC-PK1 cells. When added to the isolated membrane fractions, ouabain increased tyrosine phosphorylation of proteins from the isolated caveolae, but not other membrane fractions. Inhibition of Src or removal of ATP prevented ouabain-induced tyrosine phosphorylation of the membrane proteins. Interestingly, ouabain also induced the formation of a Na/K-ATPase/Src/caveolin complex in the isolated caveolae preparations as it did in live cells (16). Clearly, the caveolar Na/K-ATPase possessed an ion-pumping independent signaling function when it interacts with its partners. Consistent with this notion, depletion of either cholesterol by methyl β-cyclodextrin or caveolin-1 by siRNA re-distributed the Na/K-ATPase and Src from the caveolae to other compartments, and abolished ouabain-induced recruitment of Src to the Na/K-ATPase signaling complex and the subsequent activation of ERKs. These findings suggest that both caveolin and cholesterol are important for the signaling Na/K-ATPase to interact with its partners.

4.3. CTS as agonists of the signaling Na/K-ATPase

Because CTS bind specifically to the Na/K-ATPase with high affinity, and consequently inhibit the pumping function of the enzyme, it is generally accepted that the Na/K-ATPase serves as a receptor for CTS. However, if the ion pumping activity of the Na/K-ATPase is considered as the receptor function, CTS have to be termed as inverse agonists because they stabilize the receptor in its inactive state and inhibit the basal activity of the receptor. The newly appreciated signaling function of the Na/K-ATPase, on the other hand, will make CTS true agonists of the receptor. As discussed in the above paragraphs, the binding of CTS to the signaling Na/K-ATPase will convert the Na/K-ATPase/Src complex from an inactive conformation to an active conformation, resulting in tyrosine phosphorylation and subsequent assembly of down-stream signaling complexes. This classification is important for understanding the physiological function of endogenous CTS. It is well accepted that under normal physiological conditions, the endogenous CTS circulate at sub-nano molar concentrations. At such low concentration, they can only bind to less than 1% of cell surface Na/K-ATPase. Because most of mammalian cells contain about 1 million pump molecules per cell (40-42), inhibition of such small amount of the surface pump cannot cause significant change in intracellular Na^+ and K^+ concentration. Thus, it is highly inefficient for CTS to function as inverse agonists to provoke sufficient signals against the large pumping capacity of the cells. On the other hand, activation of 1% of the signaling Na/K-ATPase/Src complexes by CTS shall produce at least a few thousand active molecules per cell. Based on the findings of EGF signaling in HeLa cells (43), this shall be sufficient to generate strong signals via kinase cascades, especially if the signaling event occurs in caveolae or other membrane microdomains (16).

In short, recent studies from our laboratory as well as others have demonstrated that mammalian cell Na/K-ATPase interacts with various membrane and cytosolic proteins, thus serving as a classic signaling receptor. Binding of an agonist (e.g., ouabain) to the signaling Na/K-ATPase activates Src, which results in tyrosine phosphorylation of down-stream proteins and subsequent assembly of different signaling cascades. This signaling function of the Na/K-ATPase, not its pumping function, depends on the ability of the enzyme to interact with its partners. Furthermore, the cell-specific signaling may arise from the cell-specific or compartment-specific expression of the partners of the signaling Na/K-ATPase. However, it is important to note that the ion pumping function of the enzyme may also contribute/interfere with the above mentioned signaling pathways or act independently to change cellular function if the Na/K-ATPase fails to balance intracellular ion concentration. This can occur when the Na/K-ATPase is significantly inhibited by CTS and other factors. This also transpires when the plasma membrane enzyme is reduced due to either increased endocytosis (chronic over-stimulation by CTS) or decreased delivery of the enzyme (inhibition of the expression of Na/K-ATPase).

4.4. Regulation of cardiac function by the signaling Na/K-ATPase

4.4.1. CTS and inotropy

The inotropic effects of digitalis on the heart are well documented (4-7). Although it is accepted by the field that these effects involve the pumping function of the Na/K-ATPase and Na^+-Ca^{2+}-exchanger (NCX) (4-7), early work did suggest that the activation of protein kinases and Ca^{2+} channels also participated (44,45). Using cultured cardiac myocytes as a model, we found that the activation of protein tyrosine kinases by ouabain preceded increases in $[Ca^{2+}]_i$ (11), which has led us to test whether the signal transducing function of Na-K–ATPase contributes to inotropic effects of ouabain on myocytes. These studies have revealed the following. First, the cardiac signaling Na/K-ATPase resided with Src, EGFR, and ERKs in caveolae (38). Second, ouabain activated the Na/K-ATPase/Src complex in cultured cardiac myocytes as well as isolated hearts at the concentrations that induced positive inotropy (11,46,47). Subsequently, the activated Src transactivated EGFR, resulting in the activation of Ras/ERK cascade. It is important to note that the above signaling events occur not only in ouabain-insensitive rodent hearts, but also the ouabain-sensitive guinea pig heart (47). Third, ouabain caused a significant increase in the production of reactive oxygen species (ROS) in a Src and Ras-dependent manner in cardiac myocytes (48). Inhibition of mitochondrial site III or blocking mitochondrial K_{ATP} channel by 5-HD significantly reduced ouabain-induced ROS production, indicating that ouabain stimulated mitochondrial ROS production via pathways that open the K_{ATP} channel in a Src and Ras-dependent manner (10). Third, inhibition of either Src or Ras also abolishes ouabain-induced increases in both $[Ca^{2+}]_i$ and contractility. Interestingly, although activation of ERKs was required for ouabain-induced increases in $[Ca^{2+}]_i$, both ERKs and ROS contribute to ouabain regulation of cardiac contraction (46). Fourth, the effects of ouabain on $[Ca^{2+}]_i$, at least in part, are due to the activation of L-type Ca^{2+} channels. This activation is most likely evoked by ERK–mediated phosphorylation of the channels as demonstrated

in neuronal cells (49). Finally, ouabain regulation of cardiac contractility also involves opening of mitochondrial K_{ATP} and resultant increases in ROS production (46). Thus, ouabain regulates cardiac contractility via activation of at least two major pathways. Activation of ERKs and inhibition of the ion pumping function of the Na/K–ATPase by ouabain increased $[Ca^{2+}]_i$ whereas opening of mitoK_{ATP} stimulated the production of ROS. Both $[Ca^{2+}]_i$ and ROS, in turn, worked in concert and raised contractility in cardiac myocytes. This notion is supported by a recent report showing that ouabain was able to stimulate contraction in myocytes that were incubated in Na^+-free medium (50). These observations need to be replicated in the isolated heart and whole animals; nevertheless, it is important to note the significance of these studies. First, these findings suggest that stimulation of the signaling function of Na/K–ATPase alone may be sufficient to increase cardiac contractility. Because the signaling function can be separated from the pumping function of the enzyme, these data point to the possibility of developing a new class of agonists that only activate the signaling function of the Na/K-ATPase. Second, because the opening of the mitoK_{ATP} channel participates in ischemic preconditioning (51), it will be of great interest to test whether digitalis drugs, especially the future agonists that only affect the signaling function of the Na/K-ATPase, can protect the heart from ischemic injury. Finally, it is important to note that these new findings are not in conflict with what has been learned about the role of intracellular Na^+ or Na^+/Ca^{2+} exchanger in regulation of intracellular calcium (52-54). As a matter of fact, both mechanisms could operate in concert or in a loop in regulation of intracellular calcium because increases in intracellular calcium can affect many signaling events activated by ouabain and activation of the signaling function of the Na/K-ATPase may regulate the coupling between different membrane transporters (e.g. Na/K-ATPase and Na^+/Ca^{2+} exchanger).

4.4.2. CTS and hypertrophic growth

Cardiac hypertrophy represents an independent risk factor for development of congestive heart failure. There has been a long-standing interest in whether CTS regulate cardiac growth. Based on clinical observations, Christian maintained that **"**in patients with heart disease but not heart failure, digitalis retarded cardiac enlargement and delayed the appearance of symptoms of cardiac insufficiency" (55). This observation prompted investigators to use various animal models to study the effects of CTS on cardiac hypertrophy. These early studies produced rather inconsistent results, showing that digitalis induced or amplified cardiac hypertrophy, or that digitalis had no effect on cardiac growth or prevented overload-induced cardiac hypertrophy (56-58). Because overload-induced hypertrophy of the heart in an intact animal is due to a complex interplay of changes in a multitude of mechanic and neurohumoral stimuli, treatment of intact animals with digitalis adds not only another growth stimulus that acts on myocardium directly, but also a drug that, through its well-established neural and vascular effects, can exacerbate existing pathophysiological stimuli. Furthermore, the different ouabain sensitivities of the experimental animals add another layer of complexity to these studies. To directly address the effect of CTS on cardiac growth, several years ago we determined whether ouabain stimulated hypertrophic growth in cultured neonatal rat cardiac myocytes (59-61). These *in vitro* studies demonstrated that ouabain increased protein synthesis, cell size and contractile protein contents. In addition, ouabain behaved similarly as other hypertrophic stimuli, inducing expression of several fetal genes and suppressing the expression of the alpha3 isoform of the Na/K-ATPase in the cultured myocytes. Interestingly, ouabain also activated the expression of TGF-beta1, suggesting that it may activate a cardiac fibrosis pathway. Mechanistically, ouabain activated several pathways that are well characterized in hypertrophied heart (48,62). Specifically, it activated the Ras/ERK pathways and increased intracellular calcium and mitochondrial production of ROS. Remarkably, the addition of anti-oxidants such as N-acetyl cysteine and tea polyphenol compounds prevented ouabain from the induction of hypertrophic growth in cardiac myocytes (48). It is important to note that CTS other than ouabain also stimulate hypertrophic growth and that marinobufagenin appears to be more potent than ouabain in inducing this effect in rat cardiac myocytes (72). Several recent *in vivo* studies support our *in vitro* findings. First, a recent study on endogenous ouabain and left-ventricular mass demonstrated a correlation between plasma ouabain concentration and increases in left-ventricular mass in human subjects (63); this correlation exists even when the blood pressure is accounted for. In addition, rats infused with nM concentrations of ouabain were found to develop cardiac hypertrophy (64). Clearly, activation of the signaling function of the Na/K-ATPase by CTS can result in hypertrophic growth in cardiac myocytes. However, it remains to be determined as to which endogenous CTS (ouabain vs marinobufagenin) plays a major role in the activation of signaling functions of the Na/K-ATPase under different disease states (see Bagrov's review for detail).

4.4.3. Endogenous CTS and down-regulation of the Na/K-ATPase in the diseased heart

Recently, Lingrel's laboratory has provided solid evidence that the Na/K-ATPase plays an important role in regulation of cardiac function (54). They found that the knockout one copy of the alpha1 isoform gene resulted in 50% reduction of the alpha1 protein in the mouse heart. Consequently, it caused a significant decrease in myocardial contractility. Interestingly, lowering of extracellular Ca^{2+} exacerbated the decrease in contractility in the hearts of these alpha1+/- mice, indicating that the dominant alpha1 isoform of Na/K-ATPase is essential for normal functions of the Ca^{2+} handling machinery of the heart. These findings are significant because many prior studies have shown that both Na/K-ATPase proteins and enzymatic activity are significantly reduced in failing human hearts (65,66). Reduction in the Na/K-ATPase activity was also seen in several animal models of hypertensive and other forms of cardiomyopathies (67-72). Interestingly, circulating endogenous cardiotonic steroids are elevated significantly in both CHF patients and animals who suffered from hypertension, renal failure and heart failure (24). Notably, a recent human study has indicated

that plasma marinobufagenin level can serve, like brain natriuretic peptide or atrial natriuretic peptide, as a marker of CHF severity (73). However, while brain natriuretic peptide reduces cardiac stress by removal of Na+ and water from the circulation, increases in plasma marinobufagenin due to diminished cardiac function could act as norepinephrine and cause more stress on the diseased heart by stimulating hypertrophic growth and increasing contraction of cardiac myocytes (59-61). Because the sustained increases in CTS also correlate inversely with the surface contents of the Na/K-ATPase in failed cardiac myocytes, we suggest that the sustained increase in CTS is a mal-adaptive response that could result in the reduction of surface Na/K-ATPase by stimulating endocytosis of the signaling Na/K-ATPase and subsequent transcriptional down-regulation of the enzyme in the heart due to the activation of hypertrophic pathways (34,48,59). We emphasize this occurs as a mal-adaptive response because we would expect that physiological changes in CTS could have a different effect on the Na/K-ATPase. This notion is supported by many animal studies. Pressure-overload, for example, reduced Na/K-ATPase activity by more than 60% in the hypertrophied rat heart (69,70). Since total cellular alpha1 contents were not significantly reduced, the surface α1 isoform is most likely removed by endocytosis as we saw in the cultured cells as well as in intact renal tubules (34). On the other hand, it is well established that pressure overload also down-regulates the expression of the alpha2 isoform transcriptionally (70). Since Na/K-ATPase is coupled with other transporters and plays a key role in regulation of intracellular ion concentrations, significant decreases in ion transporting capacity would certainly impair both contractile and conducting functions of the heart by altering the intracellular calcium, especially under stress conditions (54). This scenario is reminiscent of the changes in the adrenergic receptors seen in the hypertrophied hearts (74). The new paradigm for clinic management of CHF patients calls for unloading the stressed heart (e.g., using beta-blockers) (75). This practice is derived from the understanding that further stress of the diseased heart will eventually kill the heart. Because digitalis drugs, especially when used at high doses, stimulate cardiac contraction and hypertrophic growth, they could, at least in principle, exacerbate the heart chronically although they improve the cardiac performance acutely.

5. TARGETING THE SIGNALING NA/K-ATPASE FOR NEW DRUG DEVELOPMENT

As discussed in the above sections, while the signaling Na/K-ATPase plays an important role for maintaining cardiac function, over-stimulating the same group of receptors may contribute to the development of cardiac hypertrophy and dysfunction of cardiac contractile and conducting functions chronically. Thus, development of agents that can specifically antagonize or stimulate the signaling function without affecting the pumping function of the Na/K-ATPase will not only provide researchers with new tools for studying the signaling Na/K-ATPase, but also offer novel approaches to various cardiac diseases.

5.1. Development of antagonistic agents

Improvements in ejection fraction, exercise tolerance, and mortality have been observed in CHF patients treated chronically with beta-antagonists such as metoprolol and carvedilol (74,75). These drugs prevent the over-stimulation of the receptor and the resultant toxic effect on myocyte. Eventually, these drugs reduce cardiac remodeling and bring a partial recovery of CHF-associated derangements in gene expression and cardiac function including those constituting the signaling and Ca^{2+}-handling machinery down-stream from the receptor. Since plasma membrane Na/K-ATPase is down-regulated in failing human hearts, administration of drugs that prevent over-stimulation of the signaling Na/K-ATPase might work as beta-adrenergic antagonists. This notion appears to be supported by a recent report that analyzes the clinic data from The Digitalis Investigation Group Trial (76). Although digoxin treatment did not change the overall mortality in CHF patients, there is a strong association of high serum digoxin concentration with increases in mortality in CHF patients. The data shows that patients with serum digoxin at 1.2 ng/ml or higher had an 11.8% increase in mortality rate compared with patients receiving placebo. It is important to note that in most of CHF patients' serum ouabain and marinobufagenin levels have already elevated. In severe CHF patients, the amount of marinobufagenin equals about 1.2 ng/ml of digoxin in term of its ability to bind and inhibit the Na/K-ATPase (73). Thus, it is conceivable that treatment of severe CHF patients with digoxin or other digitalis could over-stimulate the signaling function and concomitantly over-inhibit the pumping function of cardiac Na/K-ATPase, which may act as a beta1-adrenergic receptor agonist. This could improve cardiac function initially, but would eventually worsen the heart and increase the mortality. Therefore, we speculate that employment of a drug that functionally antagonizes the effects of CTS on the heart may offer a better means for reducing mortality rate in the severe CHF patients. Needless to say, many issues remain to be experimentally tested. For example, it will be of interest to determine the levels of both digitalis drug and endogenous CTS, and correlate the values to clinic outcomes of these patients. Nevertheless, it is important to discuss the potential means of developing drugs that can antagonize the effects of CTS on the heart.

Literature review indicates that there are several potential means to prevent over-stimulation of the CTS receptor. The first approach is to develop a pure CTS antagonist that binds to the receptor and antagonizes CTS from activation of the signaling Na/K-ATPase. The newly developed PST 2238 (17b-(3-furyl)-5b-androstan-3b,14b,17a-triol) appears to fit in this class (64). PST 2238 displaces ouabain from its binding to the purified Na/K-ATPase *in vitro*. In animal studies it antagonized ouabain-induced hypertension. More significantly, a recent study demonstrated that PST 2238 prevented ouabain from induction of cardiac hypertrophy in rat and that this effect appeared to be independent of its effect on blood pressure because lowering blood pressure by calcium blocker did not prevent the effects of ouabain on cardiac growth. At the molecular level, administration of PST 2238 prevented ouabain-induced formation of the active Na/K-ATPase/Src complex. Although this compound was developed for the therapy of hypertension, these new findings warrant further

study on its effectiveness in reversing/suppressing the cardiac remodeling in other animal models and, eventually, its effectiveness on CHF shall be tested. The second approach is to develop anti-Na/K-ATPase or anti-CTS antibodies. There is strong evidence that antibodies to various receptors can function as effective antagonists (77-79). For example, anti-EGFR antibodies have been proven to be effective in blocking the activation of the receptor and some of these antibodies are in clinic trial for therapy of certain cancers (78). It will be of interest to test whether antibodies raised against the extracellular domains of the Na/K-ATPase can block ouabain-induced signal transduction in both *in vitro* and *in vivo* kinase assays. Alternatively, anti-CTS antibodies could also be utilized to block the effect of CTS on the signaling Na/K-ATPase. For instance, anti-digoxin antibodies have been successively used to treat digoxin or digitoxin intoxication in CHF patients treated with these drugs. The same antibodies appear to also be effective clinically in preeclampsia. These antibodies effectively compete with the Na/K-ATPase for the binding of CTS. Significantly, a recent study by Bagrov has demonstrated that administration of anti-marinobufagenin antibodies was effective in antagonizing the hypertensive effect of high salts in rat (80). The third approach is to target the distal pathways from the receptor. For example, we reported several years ago that addition of antioxidants prevented ouabain from induction of hypertrophic growth in cultured neonatal cardiac myocytes (48). Interestingly, supplementation of tea pigments and tea polyphenols such as EGCG in drinking water was also found to be effective in reduction of cardiac hypertrophy in 5/6 nephrectomized rats (71,72).

5.2. Development of agonistic agents

The Na/K-ATPase exhibits dual functions. Binding of CTS to the Na/K-ATPase not only activates the receptor-mediated signal transduction, but also inhibits the pumping function of the enzyme. Because the amount of plasma membrane Na/K-ATPase is significantly reduced in the diseased cardiac myocytes, additional inhibition of the pumping function by CTS drugs will certainly exacerbate the derangement of intracellular ion regulation. This notion is supported by the clinic observation that CHF patients treated with digoxin or digitoxin are prone to develop ventricular tachycardia due to increases in intracellular Na^+ and the resultant calcium overload. *In vitro* studies using cultured cardiac myocytes as a model also revealed that severe inhibition (about 40%) of the surface Na/K-ATPase resulted in a significant rise in intracellular Na^+ and the resultant Ca^{2+} overload due to the inhibition of Na^+/Ca^{2+} exchanger (11). Thus, it is desirable to develop agents that only provoke the receptor function of the Na/K-ATPase. In principle, the same approaches discussed in the above paragraph can be used. Because the activation of Src appears to be the earliest event of the signaling process evoked by the binding of ouabain to the Na/K-ATPase, we have recently attempted to establish an *in vitro* assay for studying the effects of various chemicals on the Na/K-ATPase/Src interaction. Interestingly, preliminary studies indicated that unlike ouabain, several known Na/K-ATPase inhibitors failed to stimulate the Na/K-ATPase/Src complex in membrane preparations. These findings not only support the notion that inhibition of the ATPase can be dissociated from the activation of Src, but also demonstrate the feasibility of using this assay to screen for the agonist of the signaling Na/K-ATPase.

There are many reports in the literature showing that anti-receptor antibodies can also function as agonists and directly activate the receptor (77, 79). For instance, early studies of insulin receptors had led to the identification of anti-insulin receptor antibodies in the serum of diabetic patients who were resistant to insulin therapy (77). Many of these antibodies blocked receptor activation in response to insulin. Similarly, recent studies also revealed that auto-antibodies to the beta1-adrenergic receptor not only stimulate the beta1-receptor, but also act as classic beta-agonist such as isoproterenol, causing cardiomyopathy (78). Interestingly, Xu's laboratory recently reported that antibodies raised against the Na/K-ATPase appeared to function as an agonist for the signaling Na/K-ATPase (81). The antibody appears to have no effect on enzymatic activity, but block the binding of ouabain to the Na/K-ATPase. Interestingly, when applied to cardiac myocytes, it acted as ouabain and increased intracellular calcium, thus contractility. Needless to say, it remains to be tested whether this antibody can activate the signaling function of the Na/K-ATPase in cardiac myocytes.

6. CONCLUSION

The work of the past few years has demonstrated that Na/K–ATPase is an important signaling receptor. This has changed our view on how CTS work under both physiological and pathological conditions. Recent work from many laboratories has begun to define the Na/K-ATPase signalosome and to map the functional domains that are involved in the organization of the individual signaling module. The new information will certainly re-shape the focus of the field and direct us to establish new molecular targets for the development of specific agonists and antagonists of the signaling Na/K-ATPase during the next few years. Clearly, development of these chemicals will not only provide us with new tools for studying the signaling Na/K-ATPase, but also give physicians novel therapeutics for treatment of cardiovascular diseases as well as other illnesses.

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