A Dissertation

entitled

Role of Different Isoforms of Na/K-ATPase in Signal Transduction

by

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biomedical Sciences

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Na/K-ATPase is an ion transporting pump which maintains the transmembrane ionic gradient by pumping three $\text{Na}^+$ out of the cell and two $\text{K}^+$ into the cell, at the expense of ATP.

Along with this pumping function $\alpha_1$ Na/K-ATPase is also involved in cell signaling. It regulates Src kinase function by associating through its second cytosolic domain (CD2) and N domain with SH2 (Src Homology 2) and Kinase domains of Src, respectively. The N domain-kinase domains interaction keeps the Src in an inactive form, preventing tyrosine 418 phosphorylation, which is known to be essential for the full activation of Src. When a ligand of Na/K-ATPase, such as ouabain, binds, this interaction is disrupted, causing activation of Src and downstream signaling.

For over a decade, significant amount of work has been done to confirm signaling role of $\alpha_1$ Na/K-ATPase. Whether this signaling capability also exists in other $\alpha$ Na/K-ATPase isoforms has not been assessed. In order to study the regulation of Src by other isoforms of $\alpha$ Na/K-ATPase, we have generated cell lines that express either $\alpha_2$ Na/K-ATPase or
α3 Na/K-ATPase. Studies with these cell lines along with the control α1 expressing cells show the following.

Cell expressing α2 Na/K-ATPase show disrupted Na/K-ATPase–Src interaction. Moreover ouabain fails to stimulate Src and ERK. Caveolin-1 expression in these cells cannot be rescued like that in α1 containing cells. Finally, these cells grow much slower than those of α1 Na/K-ATPase cells.

Cell expressing α3 Na/K-ATPase also show disrupted Na/K-ATPase–Src interaction. Like α2 expressing cells, ouabain fails to stimulate Src. But these cells can rescue Caveolin-1 expression normally as in α1 expressing cells. These cells also show ERK activation upon ouabain stimulation like α1, but in contrast to α1, the ERK activation is not regulated through Src, but through PI3K and PKC. Finally, these cell grow slightly slower than those of α1 Na/K-ATPase cells but faster than α1 knockdown cells and α2 containing cells.

We have derived a 20 amino acid peptide from the N domain of α1 Na/K-ATPase. This peptide called NaKtide binds and inhibits Src. Mutagenesis studies indicate the helical structure at the N terminus of NaKtide is essential for its Src interacting activity.

Because disruption of the helical structure by Ala to Pro mutation abolishes the effect of NaKtide on Src in vitro, we have generated stable cell lines expressing the mutant Na/K-ATPase-α1 having Ala to Pro mutation in the NaKtide region. Functional characterization of the mutant cell lines allowed us an identification of A420P mutant Na/K-ATPase-α1 that has normal pumping function but is devoid of the Src related signaling function.
I dedicate this work and all my accomplishments to my Parents for all their support and love along the journey.
Acknowledgements

Firstly I would like thank the person who guided me right from the first step into this magnificent world of research, my Advisor Dr. Zijian Xie.

I would also like to thank my committee members Dr. Andrew Beavis, Dr. Edwin Sanchez, Dr. Kevin Pan and Dr. Jiang Tian for their suggestions regarding my research and dissertation work.

I would like to thank my previous and current lab members, my friends and all my family members.

Last but not the least I am in debt to my parents, without whom I would not have been here.
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<tbody>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri phosphate</td>
</tr>
<tr>
<td>CD2</td>
<td>second cytosolic domain</td>
</tr>
<tr>
<td>CG</td>
<td>Cardiac Glycoside</td>
</tr>
<tr>
<td>CTS</td>
<td>Cardiotonic Steroids</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated Kinases</td>
</tr>
<tr>
<td>(K+)</td>
<td>potassium ions</td>
</tr>
<tr>
<td>Km/K0.5</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>(Na+)</td>
<td>sodium ions</td>
</tr>
<tr>
<td>Pi</td>
<td>free phosphate group</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
</tr>
<tr>
<td>SFK</td>
<td>Src Family Kinases</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology domain 2</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
</tbody>
</table>
List of Symbols

°C.................................Degree Celsius

<.................................Less than

>.................................More than

µM...............................Micro Molar

nM...............................Nano Molar

mM...............................Milli Molar

M.................................Molar
Chapter 1

General Introduction

1.1. Na/K-ATPase

Na/K-ATPase was discovered by Dr. Skou in 1957 [1]. It is a veteran membrane protein that not only maintains the ionic gradient across the plasma membrane by transporting three sodium ions (Na\(^+\)) out and two potassium (K\(^+\)) into cells, but also plays a role in signal transduction. Na/K-ATPase is the largest protein complex in the family of P-type cation pumps. At rest, it consumes 20–30% of ATP production to actively transport Na\(^+\) out of and K\(^+\) into the cell. Its pumping function helps in maintaining membrane potential, cell volume as well as transportation of other ions, nutrients, and neurotransmitters across the plasma membrane through secondary transport systems [2-4]. Its signaling function helps in regulation of signaling pathways controlling cell proliferation, cell cycle, fibrosis and cell adhesion [5-7]. Na/K-ATPase can bind to cardiotonic steroids (CTS) such as ouabain and digoxin which can modulate its pumping and signaling activities.[8, 9]
1.1.1 Structure

A functional Na/K-ATPase consists of α, β and γ subunits. The α subunit, a 112 kDa protein, is a catalytic subunit that contains binding sites for substrates such as ATP, ions and CTS. It has ten transmembrane domains and several cytoplasmic loops. Transmembrane domains contain ion binding sites and are necessary for transport [10]. Cytoplasmic region is responsible for ATP hydrolysis and is divided into three parts. The first part is Actuator or ‘A’ domain which acts as a piston during the pump cycling through E1 and E2 stages.

Figure 1-1- The figure showing a functional Na/K-ATPase, made up of α subunit consisting of ten transmembrane domains and cytosolic parts, β and γ subunit. (Figure reproduced with permission from Morth, J.P., et al., A structural overview of the plasma membrane Na+, K+-ATPase and H+-ATPase ion pumps. Nature Reviews Molecular Cell Biology, 2011. 12(1): p. 60-70 [11])
Second part is phosphorylation ‘P’ domain that contains the phosphorylation site - Asp-376 residue in a conserved DKTGT motif. The third part is the nucleotide binding domain or ‘N’ domain that also binds to many proteins including Src. The β subunit is essential for making a functional Na/K-ATPase and helps to assemble this functional enzyme onto the plasma membrane [12]. Although γ subunit that is a member of FXYD proteins was shown to be not required for normal Na/K-ATPase activity, there is increasing evidence that it may play a role in regulation of Na/K-ATPase [13-18].

1.1.2 Na/K-ATPase as a pump

The α subunit of Na/K-ATPase undergoes cycles of conformational changes between E1, E2 and many intermediate states that involve ATP binding, phosphorylation, and dephosphorylation leading to extrusion of three Na\(^{+}\) ions and uptake of two K\(^{+}\) ions. The mechanism is best explained by the Albers-Post scheme [19](figure 1.1.2-1). In the E1 conformation, which has high apparent affinity for Na\(^{+}\) and ATP, cation binding site faces intracellular region, enable three Na\(^{+}\) ions binding. This induces phosphorylation of the enzyme by ATP, which in turn leads to the formation of occluded [Na3]E1P–ADP state. After ADP is displaced, Na\(^{+}\) is occluded, the binding site of Na\(^{+}\) faces extracellular space and pump forms E2P conformation (E2+Pi). In the E2P conformation the enzyme has a high affinity for K\(^{+}\) allowing binding of two K\(^{+}\) on its outer surface. The K\(^{+}\) binding then dephosphorylates the enzyme and induces the release of Pi from the intracellular portion of the enzyme. After Pi is released, two K\(^{+}\) are occluded to form the [K2]E2 conformation. Triggered by ATP binding to its low affinity site on the [K2]E2 conformation, the conformation of the enzyme changes to [K2]E1 and releases K\(^{+}\) to intracellular region.
After the release of $K^+$, E1 conformation is established ready to undergo another cycle [20]. CTS such as ouabain, as specific ligands of Na/K-ATPase can bind to the enzyme and stabilize its E2P conformation to inhibit further conformational change.

![Na/K-ATPase reaction cycle](Figure reproduced with permission from Kaplan, J.H., Biochemistry of Na,K-ATPase. Annu Rev Biochem, 2002. 71: p. 511-35[20])

1.1.3 Na/K-ATPase as a signal transducer

Na/K-ATPase is now well known for being involved in signal transduction [21]. This signaling function of Na/K-ATPase was first implicated in the transcriptional regulation and hypertrophic growth in cardiac myocytes induced by ouabain treatment [22]. Na/K-ATPase can relay the signal of stimuli such as ouabain and activate several downstream pathways such as activation of Src kinase, tyrosine phosphorylation of epidermal growth factor receptors, production of ROS and activation of MAPK pathways [23].
The signaling events lead by Na/K-ATPase are shown to be due to direct and indirect association with proteins such as Src. Work in the last decade from our laboratory have shown the direct interaction of Na/K-ATPase and Src.

First indications that ouabain-induced protein tyrosine phosphorylation is Src dependent, came from studies in SYF cells. SYF cells are mouse embryo fibroblast cells deficient in Src, Yes and Fyn kinases. In these cells ouabain failed to induce tyrosine phosphorylation of proteins immunoprecipitated with α1 Na/K-ATPase [24]. Later Na/K-ATPase-Src
direct interaction was shown using immunoprecipitation [25], GST pull down assays, confocal imaging and FRET analysis [26]. Na/K-ATPase was found to interact with Src at two different sites. One interaction is between CD2 of Na/K-ATPase and SH2 of Src, and the other is between N domain of Na/K-ATPase and Kinase domain of Src. This interaction site on N domain was further mapped down to about 20 amino acids and a peptide – ‘NaKtide’ was generated as Na/K-ATPase mimetic which can bind to and regulate Src kinase activity. This peptide and cell permeable form ‘pNaKtide’ have shown to be ouabain antagonist and inhibitor of elevated Src activity in cancer cells [27, 28].

Na/K-ATPase in addition to Src may interact with other proteins such as caveolin-1, PI3K, PLC and IP3R, and forms a signaling transduction platform called ‘signalosome’ in caveolae [29-31]. Caveolae are plasma membrane invagination that are enriched in cholesterol, glycosphingolipids, and sphingomyelin. The termination of this signaling event induced by ouabain is regulated by the endocytosis of Na/K-ATPase in a caveolin-1 and clathrin-dependent manner [32, 33].

Overall the signaling function of Na/K-ATPase appears to be relevant in the regulation of membrane stability, cholesterol metabolism, hypertension, renal salt handling, cardiac ischemia-reperfusion injury and cancer cell growth [31, 34-37].

1.2. Carditonic Steroids (CTS)

Some of the Carditonic Steroids (CTS), compounds presenting a steroid nucleus with a lactone moiety, are endogenous inhibitors of the Na/K-ATP [8]. They include plant derived digitalis drugs such as digoxin and ouabain, and vertebrate-derived aglycone such as bufalin and marinobufagenin. Many laboratories including ours have demonstrated that
these steroids can activate protein kinases and regulate cell growth, gene expression, intracellular calcium, and the production of reactive oxygen species (ROS) [21, 22, 38-40], thus playing an important role in the control of renal and cardiovascular functions[41, 42]

1.3 Na/K-ATPase isoforms

The early indications of Na/K-ATPase having different isoforms came from studies of cardiotonic steroid sensitivity and different mobility in SDS-polyacrylamide gels of Na/K-ATPase. Marks and Seeds [43] found that the mouse brain Na-K-ATPase activities showed two distinct inhibition curves when treated with varying concentrations of ouabain, suggesting the presence of two separate isoforms. Sweadner[44] found a slower migratory Na/K-ATPase isoform (α+) in mice brain compared to renal Na-K-ATPase isoform (α1). As of now four distinct isoforms of α subunit have been identified [45-48]. These isoforms exhibit tissue specific expression. The α1 isoform is ubiquitous. Some cells, in addition to α1, express other isoforms. The α2 is mainly found in skeletal muscles and cardiac myocytes[12, 49], whereas α3 isoform is mainly expressed in neuronal tissue[50-52]. The expression of α4 isoform is restricted to testis[53].

Similar to α subunit, β subunit has three distinct isoforms. The ubiquitous β1 isoform is expressed in most of tissues. The β2 isoform is found primarily in neurons while the β3 is in testis, retina, liver, and lung [54].

1.3.1 Enzymatic Properties of Na-K-ATPase Isoforms.

The amino acid sequence comparison of all four isoforms of α shows that α2 and α3 have greater similarities to α1 compared to α4 as shown in table 1. Amino terminus of
these sequences show the most variability whereas middle cytoplasmic region shows most similarities[54].

Table 1-1. Polypeptide sequence identity of different α isoforms compared to α1

<table>
<thead>
<tr>
<th>α1</th>
<th>α2</th>
<th>α3</th>
<th>α4</th>
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<tbody>
<tr>
<td>100%</td>
<td>~92%</td>
<td>&gt;96%</td>
<td>78%</td>
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The most significant difference among rodent α isoforms is their sensitivity to CTS, ATP and Na\(^+\) and K\(^+\) ions. Many studies have been done to study the kinetic properties of isoforms. Although some differences in substrate affinities have been reported, a number of inconsistencies are observed (table 1.3.1-2), which may be due to fact that the isoforms either expressed in non-mammalian cells where Na/K-ATPase is not expressed or in mammalian cells that contains large number of endogenous α1 Na/K-ATP.

1.3.2 Differences in protein interactions

Along with structural and functional differences, the α isoforms are also shown to bind to other proteins with different preferences. For example α1 and α2 Na/K-ATPase are shown to interact with Na\(_x\) channels but not α3 Na/K-ATPase [55]. The α3 Na/K-ATPase is found to interact with dopamine D1 receptor in the spines of striatal neurons either directly or indirectly though scaffolding protein PSD95 which is found only in neurons [56].
Table 1-2 Comparison of $K_{0.5}$ values of $\alpha$ isoforms reported in literature.

<table>
<thead>
<tr>
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<th>$\alpha$2</th>
<th>$\alpha$3</th>
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<tr>
<td></td>
<td>Reference</td>
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<tr>
<td></td>
<td>cell system</td>
<td>Ouabain</td>
<td>ATP</td>
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<td>Swedner et al., JBC, 1985</td>
<td>cell system</td>
<td>Kidney</td>
<td>Ouabain</td>
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<td></td>
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<tr>
<td>Lingrel et al., JBC, 1991</td>
<td>cell system</td>
<td>HeLa cells</td>
<td>Ouabain</td>
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<td>Blasco et al., Frontiers in \textit{Bioscience}, 2005</td>
<td>cell system</td>
<td>Sf-9 cells</td>
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<td>Gilles Cambert et al., JBC, 2000</td>
<td>cell system</td>
<td>Xenopus laevis Oocytes</td>
<td>Ouabain</td>
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<td>Alisha McDonough et al., \textit{Am J Physiol Cell Physiol}, 2001.</td>
<td>cell system</td>
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<td>Therien et al., JBC, 1996</td>
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<td>Kidney</td>
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1.3.3. Na/K-ATPase α2

The α2 isoform is found in glial cells, myocytes and adipocytes. The α2 isoform appears to possess the unique ability to regulate intracellular Ca\(^{2+}\) levels in myocytes and co-resides with the Na\(^+/\)Ca\(^{2+}\)- exchanger\([57, 58]\) and plays a more prominent role in calcium release in cardiac and smooth muscle myocytes than that of α1\([59]\) \([9]\). Recently our lab has created a mammalian cell line that expresses α2 (LX-α2-4) without detectable levels of α1\([60]\). Signaling studies using these cells imply that α2 does not regulate Src like α1. Ouabain fails to activate Src and ERK in these cells (figure 1.3.3-1).

![Figure 1-4. Effects of ouabain on α2 cells.](image)

Na/K-ATPase α1 containing AAC-19 α2 containing LX-α2-4 and Cells were treated with 100 μM ouabain for indicated time. Cell lysates were collected and subjected to Western blot analysis for Src pY418 and pERK (Figure adapted from\([60]\))
1.4 Rationale and Aims of the study

Different α isoforms are expressed in a tissue specific manner. While α1 has both pumping and signaling function, it remains to be tested whether α3 isoform exhibits α1-like activity. In order to study this it is necessary to develop a mammalian cell line that expresses α3 in the absence of detectable α1.

Although a lot of work has been done by many laboratories to establish the signaling role of Na/K-ATPase by its direct interaction to Src, questions have continually been raised whether the signaling effect is due to ionic changes by the pumping function of Na/K-ATPase and whether these signaling events are due to direct interaction with Src. In this work we identified a mutant α1 Na/K-ATPase that pumps but fails to signal.
Reference


Chapter 2

Characterization of pumping and signaling functions of α3 Na/K-ATPase in mammalian cells.

Unpublished manuscript

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Running title – Na/K-ATPase α3 in signal transduction
Abstract

We and others have demonstrated that $\alpha_1$ Na/K-ATPase has both pumping and signaling functions. Both $\alpha_1$ and $\alpha_3$ Na/K-ATPase are expressed in neurons and mutations of $\alpha_3$ are linked to several neurological disorders. Here we generate a stable mammalian cell line that express essentially only $\alpha_3$ Na/K-ATPase. The data shows following key findings. First, the expressed $\alpha_3$ Na/K-ATPase could form a normal pumping enzyme, and rescue expression of Na/K-ATPase $\beta_1$ and Caveolin-1. Second, Na/K-ATPase $\alpha_3$ could not regulate Src and finally, while ouabain failed to activate Src, it stimulated ERK/Akt via Src-independent pathways involving PI3K and PKC.

Introduction

Na/K-ATPase was discovered by Dr. Skou and is a ubiquitously expressed membrane protein in most mammalian cells. It transports three Na$^+$ in and two K$^+$ out of the cell at the expense of ATP. This maintains ionic homeostasis as well as provides transmembrane Na$^+$ gradients for the Na$^+$-dependent transport of nutrients [1]. Over the last decade we and others have shown that the $\alpha_1$ Na/K-ATPase also has important signal transduction function, which is dependent on its interaction with membrane, structural and cytosolic proteins [2]. We have reported that the $\alpha_1$ Na/K-ATPase interacts with Src kinase and this complex acts as a functional receptor for cardiotonic steroids to activate protein kinase cascades [3-5], which play an important role in renal salt handling and remodeling of the heart and the kidney under pathological conditions [6]. The $\alpha_1$ Na/K-ATPase interacts with Src kinase at two sites to form a functional signal transducing receptor-protein complex.
One of the interaction is between the second cytoplasmic domain (CD2) of α1 Na/K-ATPase subunit and Src SH2 domain, which is constitutive. Tyrosine 260 in the CD2 domain is important for binding of Src SH2 domain to Na/K-ATPase α1 [7]. The other non-constitutive interaction is between the nucleotide (N) domain of α1 subunit and Src kinase domain. The latter interaction keeps Src in an inactive state. Binding of cardiotonic steroids such as ouabain to α1 Na/K-ATPase disrupts the latter interaction, resulting in an activation of the pump-associated Src [5]. The α1 Na/K-ATPase also interacts with caveolin-1, in cholesterol rich invagination of the plasma membrane called caveolae [8-10]. In caveolae, Na/K-ATPase α1 interacts directly or indirectly with many other proteins such as phosphoinositide 3-kinase (PI3K), EGFR and PLC.[8, 11]

Recently we have found that α1 Na/K-ATPase-Src interaction requires Tyr 260. Sequence comparison indicate that Tyr 260 is specific to α1 Na/K-ATPase, suggesting that the Src-interaction related signaling of Na/K-ATPase may be isoform specific. Which is consistent with the fact that α2 Na/K-ATPase has no detectable signaling function[12].

The α3 Na/K-ATPase has gained importance in research due to its essential role in nervous system. The α3 Na/K-ATPase has been predicted to be necessary for restoring basal membrane potential after depolarization and has been shown to play a role in long term memory [13]. Mutations in α3, have been linked to neurological disorders such as rapid onset dystonia parkinsonism (RDP)[14] and alternating hemiplegia of childhood (AHC)[15, 16]. Although some studies have shown that these mutations change sodium and ouabain affinity for α3 resulting in the disorders, exact neuropatho-physiological mechanisms remains unknown. It has been demonstrated that the expression and activity of Na/K-ATPase are altered in aging and Alzheimer’s diseases [17, 18]. The activity
changes in pumping activity are predicted to be due to both the isoforms α1 and α3, and individual participation of these isoforms has not been resolved.

In this paper, we address the issue of whether α3 Na/K-ATPase possesses signaling abilities similar to that of α1 Na/K-ATPase. The newly generated LM-α3-1 cells express α3 Na/K-ATPase with undetectable amount of α1 Na/K-ATPase. When these cells were treated with ouabain they displayed signaling patterns different from that of AAC-19 cells which express only α1 Na/K-ATPase. These data, taken together with other published and unpublished data from our laboratory, indicate that the signaling functions of Na/K-ATPase is likely to be isoform specific.

Methods and Materials

Materials: All the peptides of more than 95% purity (checked by RP-HPLC) were purchased from HD Biosciences (China) Co. Ltd. The polyclonal anti-Src[pY418] antibody, cell culture media, fetal bovine serum, trypsin, Lipofectamine 2000 were purchased from Invitrogen (Grand Island, NY). QuikChange mutagenesis kit was obtained from Stratagene (La Jolla, CA). Anti-Na/K-ATPase α1 polyclonal, anti-Na/K-ATPase β1 (clone C464.8) antibody and recombinant human Src were obtained from Upstate (Lake Placid, NY). The monoclonal anti-α1 antibody (α6F) was from the Developmental Studies Hybridoma Bank at the University of Iowa. Anti-c-Src (B-12) monoclonal antibody, the anti-Cav1 polyclonal antibody, and all the secondary horseradish peroxidase-conjugated antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti Na/K-ATPase α3 monoclonal antibody was purchased from Thermo-Fisher. Radioactive $^{86}\text{Rb}^+$ was from PerkinElmer Life Science Products (Boston, MA). Protease inhibitor
cocktail was purchased from Sigma (St. Louis, MO). The specific inhibitors GO6983 and PD153035 were ordered from Santa cruz, LY 29400 from cell signaling and PP2 from Calbiochem.

**Methods**

*Expression vectors, gene transfer, and selection* - Rat wild-type α3 cDNA was received from Dr. Blanco and was subcloned into the HindIII and XbaI sites of the eukaryotic expression vector CMV5 (received from Dr. Kevin Pan, University of Toledo), with the aid of a HindIII and XbaI adaptor.

A variant of the rat wild-type α3 isoform, designated α3*, was constructed by site-directed mutagenesis (Q108R and N119D) by using QuikChange II XL Site-Directed Mutagenesis Kit from Agilent Technologies to obtain an isoform resistant to micromolar concentrations of ouabain as described in our prior publications [4]. The sequence of the α3* mutant cDNA was confirmed by DNA sequencing.

*Generation of α3 stable cell lines* – This was done as previously described [4]. Briefly, PY-17 cells were cultured on 6 well plates and transfected with the α3* containing CMV5 vector. The transfected cells were selected with 3µM ouabain for one week and the survived ouabain resistant colonies were collected and diluted into 96 well plates to isolate single colony. Once the colony was expanded into stable cell line, the expression of mutant rat α3 was verified by α3 -specific antibody from Thermo-Fisher. Clone 1 was selected as α3 expressing cell-line and was names as LM- α3-1.
Src kinase assay - The activity of NaKtide and other peptides was measured using in vitro Src kinase assay as described [2]. Briefly, purified Src (4.5 U) was incubated with different concentrations of peptides in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) for 15 min at 37°C. Afterward 2.0 mM ATP/Mg²⁺ was added to induce phosphorylation. The reaction was continued for 15 min at 37°C and was stopped by addition of SDS sample buffer. Afterward, Western blot analyses of Src pY418 was conducted [2].

Cell Culture—Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 1 µg/ml puromycin. When cells reached 90-100% confluence, they were serum-starved overnight and used for experiments unless otherwise noted.

Immunoblot Analysis—After treatment, cells were washed by ice-cold PBS, and lysed in ice-cold radioimmunoprecipitation (RIPA) buffer (0.25% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4 and 1% protease inhibitor cocktail). After the cell lysates were centrifuged at 14,000x g for 15 min, the supernatants were collected and measured for the protein content. The same amount protein of each sample was loaded, separated by SDS-PAGE, then transferred to an Optitran membrane and probed by indicated antibody. ImageJ 1.46 was used for analyzing the data.

Ouabain sensitive Na/K-ATPase activity – Cells were collected and homogenized in ice-cold buffer A (150 mM sucrose, 5 mM HEPES, 4 mM EGTA, 0.8 mM dithiothreitol) and briefly sonicated. After centrifugation (800 g, 10 min), the post nuclear fraction was further centrifuged (100,000Xg, 45 min) in order to get crude membrane. The crude membrane
pellet was resuspended in buffer A and the protein content was determined. The aliquots of protein were treated with alamethicin (0.1 mg/mg of protein) for 10 min at room temperature and then added to the buffer containing 50 mM Tris (pH 7.4), 1 mM EGTA, 1 mM MgCl₂, 25 mM KCl, 100 mM NaCl, 5 mM NaN₃. After 15 min preincubation at 37°C, ATP/Mg was added to a final concentration of 2 mM to start the reaction. The reaction continued for 45 min and was stopped by adding 8% ice-cold trichloroacetic acid. Phosphate generated during the ATP hydrolysis was measured by BIOMOL GREEN Reagent (Enzo Life Science). Ouabain-sensitive Na/K-ATPase activities were calculated as the difference between the presence and absence of 1 mM ouabain.

Cell counting assay — 20,000 cells/well were seeded in 12-well plate and cultured in 10% FBS DMEM medium. At indicated time, 3 wells from each cell line were trypsinized and the number of viable cells was counted as described [10].

Ouabain-sensitive ⁸⁶Rb⁺ Uptake — Ouabain-sensitive ⁸⁶Rb⁺ was measured to estimate the transport function of Na/K-ATPase as previously described [19]. Briefly, cells were cultured in 12-well plates and serum starved overnight when the cells were over 90% confluence. Cells were washed and incubated in DMEM with or without 1.0 mM ouabain for 10 min at 37 °C. ⁸⁶Rb⁺ (1 μCi/well) was then added to the cells for 10 min and the reaction was stopped by washing with ice-cold 0.1 M MgCl₂. Trichloroacetic acid soluble ⁸⁶Rb⁺ was counted in a Beckman scintillation counter. All counts were normalized to protein amount. In certain experiments, different concentrations of ouabain were added.
Data Analysis — Data presented are mean± S.E. of at least three independent experiments, and statistical analysis was performed using the Student’s t test and significance was accepted at P < 0.05.

Results:

Generation of stable cell lines expressing α3 Na/K-ATPase: To evaluate the role of α3, we wished to generate mammalian cells that express α3 in the absence of α1. To reach this goal, we have developed a knock down and rescue protocol[4]. In brief, we used a α1 Na/K-ATPase knockdown PY-17 cells and transfected these cells with α3 Na/K-ATPase expressing plasmid. PY-17 cells are derived from pig kidney LLC-PK1 cells and the expression of endogenous α1 Na/K-ATPase was reduced to about 8% of LLC-PK1 cells. These cells are previously confirmed for the absence of other isoforms of Na/K-ATPase [4]. Wild-type rat-α1 rescued PY-17 cells (AAC-19) we made previously were used as a control [4]. Like wild type rat α1 [4], ouabain selection of transfected PY-17 cells resulted in numerous clones that expressed α3 Na/K-ATPase (Supplementary fig.1). Western blot analyses using Na/K-ATPase α3-specific antibody showed that every viable clone expressed rat α3. Based on the western blot analysis, we selected clone-1 that expressed highest amount of α3. When α1 was measured in AAC-19, LM- α3-1 and PY-17 cells, we detected α1 in AAC-19 cells (Fig 1A). PY-17 cells showed a faint band upon higher exposure. When these cells were probed with α3 specific antibody, only LM- α3-1 showed the presence of Na/K-ATPase α3 (Fig. 1B). The selected clone was named LM- α3-1 and was used in the following experiments.
To further assess the level of endogenous pig α1 expression in LM- α3-1, we conducted
3H-ouabain binding analyses (Fig. 1C). Parental LLC-PK1 cells and PY-17 cells contain
pig α1 which has high affinity for ouabain, whereas the expressed α3 is ouabain resistant.
This difference in ouabain binding between endogenous α1 and the expressed α3 made it
possible for us to use 3H-ouabain binding to estimate the maximal level of endogenous α1
in the LM- α3-1 cells. The 3H-ouabain binding for endogenous pig α1 in PY-17 was set as
100% and LM- α3-1 displayed less 3H-ouabain binding as compared to that of PY-17 cells,
which is consistent with the western blot data (Fig. 1A)

To form a functional enzyme in the plasma membrane, the α subunit needs to associate
with the Na/K-ATPase β subunit, hence we tested whether α3 can form a functional Na/K-
ATPase in association with β1. As shown in Fig.1D, α3 was able to rescue the expression
of glycosylated β1 as that of AAC-19. PY-17 cells were used as a negative control [20].
These findings indicate that the expressed α3 is fully capable of assembling with the β1
subunit into functional Na/K-ATPase.

**Characterization of the pumping function of α3 Na/K-ATPase:** In order to assess the
pumping function, we measured ouabain-sensitive ATPase activity and 86Rb+ uptake in
AAC-19 and LM- α3-1 cells. Na/K-ATPase activity in the membrane preparations made
from AAC-19 and LM- α3-1 cells was comparable (Fig. 2A). In contrast, LM- α3-1 cells
showed much lower ouabain -sensitive 86Rb+ uptake activity than that of AAC-19 cells
(Fig. 2B).
**Kinetic properties of α3 Na/K-ATPase:** To further verify whether the expressed α3 possess the normal pumping function, we measured kinetic properties of α3 Na/K-ATPase by measuring the $K_{0.5}$ values of Na$^+$ and K$^+$. In accordance with most of the previous studies, Na/K-ATPase α3 showed lower Na$^+$ affinity than that of α1 Na/K-ATPase (Fig.3A). The K$^+$ affinity was comparable between α1 and α3 Na/K-ATPase (Fig.3B).

**Regulation of α3 Na/K-ATPase by intracellular Na$^+$ in LM-α3-1 cells:** Since Na/K-ATPase α3 was confirmed to have lower Na$^+$ affinity than α1, we predicted that LM-α3-1 cells should show higher increase in ouabain sensitive $^{86}$Rb$^+$ uptake than that of AAC-19 cells when monensin is added to the medium. Monensin is a Na$^+$ ionophore, capable of clamping intracellular Na$^+$ to that of extracellular Na$^+$. Because the $K_{0.5}$ of α1 Na/K-ATPase for Na$^+$ is about 10mM, monensin produced about two fold increase (Fig.4) in ouabain sensitive $^{86}$Rb$^+$ uptake in accordance with the reported intracellular Na concentration in LLC-PK1 cells. On the other hand, we detected about three fold increase in ouabain sensitive $^{86}$Rb$^+$ uptake activity in LM-α3-1 cells. These data demonstrated that α3 Na/K-ATPase is much more sensitive to change in intracellular Na$^+$, which is in agreement with the essential role of α3 in maintaining ionic balance in neurons.

**Caveolin expression in LM-α3-1 cells:** The α1 Na/K-ATPase associates with caveolin-1 in caveolae and knockdown of α1 Na/K-ATPase increases endocytosis and degradation of caveolin-1 in PY-17 cells [21]. Caveolae also play a critical role in cell signal transduction [22, 23]. Hence we checked the expression of caveolin-1 in LM- α3-1 cells. As seen in Fig. 5, caveolin-1 expression in LM- α3-1 cells was rescued as that in AAC-19 cells.
Basal phosphorylation levels of protein kinases. – To assess whether α3 Na/K-ATPase can regulate Src, like the α1 Na/K-ATPase, we first determined the basal Src activity in these cells. We have previously reported that knockdown of Na/K-ATPase increased basal Src activity in PY-17 cells and that rescuing the knockdown cells with rat α1 reduced the elevated basal Src activity in AAC-19 cells [4]. LM- α3-1 cells showed increased basal levels of phosphorylation of Src at pY418 in comparison to that of AAC-19. This suggested that α3 Na/K-ATPase may not be able to regulate Src activity as that of α1 Na/K-ATPase. (Fig. 6A)

In addition to the phosphorylated Src, we also checked basal activity of ERK and Akt. Similar to Src, ERK also showed increased levels of phosphorylation (Fig. 6B). There was no statistically significant increase in Akt activity (Fig. 6C).

Effect of Na/K-ATPase peptides on Src by In Vitro kinase assay: Since AAC-19 cells and LM- α3-1 cells showed differences in basal levels of phosphorylation of protein kinases, to understand this difference between α1 and α3, we compared NaKtide sequences of α1 and α3 Na/K-ATPase (Fig. 7). We have previously demonstrated that the regulation of Src by the α1 Na/K-ATPase depends on the interaction between the NaKtide sequence of α1 N domain and Src kinase domain. As depicted in Fig. 7, the corresponding NaKtide sequence from human α3 subunit show about 20% difference from that of α1 subunit. In accordance, in vitro kinase assays indicated that α3 NaKtide is much less effective in inhibiting Src than α1 NaKtide.
**Effect of ouabain on ATPase activity:** In order to determine appropriate dose of ouabain for signaling studies, we measured ATPase activity as a function of ouabain concentration. As shown in table 1, ouabain at 10 and 100 µM produced about the same degree of inhibition of α1 and α3 Na/K-ATPase. Therefore, we selected 10µM and 100 µM of ouabain for signaling studies.

**Ouabain-induced signaling transduction:** Binding of ouabain causes conformational changes in α1 Na/K-ATPase resulting in the activation of Src, and consequently the transactivation of EGFR and the stimulation of Ras/Raf/ERK cascade [11]. This activation is blocked in α1 knockdown PY-17 cells and restored when PY-17 cells were rescued by the expression of rat α1 Na/K-ATPase (AAC-19 cells). Consistently we found that 10 and 100 µM ouabain stimulated phosphorylation of Src within minutes. Unlike AAC-19 cells, ouabain failed to stimulate Src even at 100µM in LM-α3-1 cells (Fig. 8A).

To our surprise, when LM-α3-1 cells were exposed to different concentrations of ouabain, and assayed for ERK activation, we found that ouabain was able to activate ERK in LM-α3-1 cells as in AAC-19 cells (Fig. 8B). These findings suggest that α3 may wire the signaling partners differently from that of α1 using Src-independent signaling process. This is consistent with the *in vitro* peptide data(Fig.7) as well as the identical role of Y260 in the formation of receptor α1/Src complex[7].

**Ouabain stimulation of Akt in LM-α3-1 cells:** Ouabain also regulated PI3K/Akt pathway in many types of cells including LLC-PK1 cells [10]. To test whether α3 regulates
PI3K/Akt pathway we stimulated the LM-α3-1 cells with 10 and 100 μM of ouabain. As shown in Fig.9 ouabain could stimulate Akt phosphorylation in LM-α3-1 cells as in AAC-19 cells.

**Mechanism of Ouabain-induced activation ERK:** Since ouabain was shown to stimulate ERK but not Src in LM-α3-1 cells, we wanted to assess the cause of ERK activation, using pathway specific inhibitors.

Firstly we confirmed whether ERK activation is independent of Src by using PP2, a Src inhibitor. As shown in Fig.10, ouabain could stimulate ERK even in the presence of 5μM PP2. These findings are consistent with the fact that EGFR inhibitor PD153035 failed to block ouabain-induced activation of ERK in LM-α3-1 cells. In addition to Src and EGFR, ERK could be activated via PI3K and PKC pathways [24, 25]. To test them out we pre-treated LM-α3-1 cells with PI3K and PKC inhibitors and then with ouabain. As depicted in Fig.10 these inhibitors were effective in blocking ouabain induced ERK activation.

**Expression of α3 is sufficient to rescue the cell growth defect:** The expression of α1 Na/K-ATPase is important for cell proliferation. Knockdown of α1 Na/K-ATPase inhibited cell growth in PY-17 cells. Restoration of α1 expression rescued the growth defect. To assess the role of α3, we measured cell proliferation in LM-α3-1 cells. AAC-19 and PY-17 cells were used as a positive and as a negative control, respectively. As shown in Fig.11 the growth rate of LM-α3-1 cells was between AAC-19 and PY-17 cells.
Discussion

We report here the generation of stable cell-lines expressing α3 Na/K-ATPase in the absence of detectable amount of α1. Functional characterization of these cells indicate that α3 Na/K-ATPase has a signaling function in response to ouabain stimulation. However this signaling is different from that of α1 Na/K-ATPase. Moreover the expressed α3 exhibits distinct kinetic properties from that of α1 allowing unique regulation of pumping activity by changing in intracellular Na⁺. These and other important issues are further discussed.

Generation of stable cell lines expressing α3:

Because α2/α3 isoforms express together with α1 isoform, it has been difficult to assess their functions in their native state. Several expression systems have been developed over the years and produced important findings regarding different isoforms[26-32]. However each system has its own limitations which result in inconsistent findings for different kinetic properties of different isoforms of Na/K-ATPase, and make it difficult to interpret the findings.

For example, insect cells (Sf9)- based expression system is advantageous since these cells do not express endogenous Na/K-ATPase. However, because they do not express normal Na/K-ATPase, the cellular environment and its post translational modifications are different from mammalian cells. It has been also demonstrated that most Na/K-ATPase expressed in Sf9 is inactive[33].

Although yeast has been used successfully to express functional Na/K-ATPase, post-translational modification and expression system is also different since yeast do not express
Na/K-ATPase. It has also been difficult to measure $K^+$ transport activity and substrate dependent pumping in these two cell systems.

Mammalian cell systems (e.g. HeLa and OK cells) are very useful since they provide native environment for Na/K-ATPase. However, there is a large amount (up to 50%) of endogenous Na/K-ATPase[34]. Therefore, interpretation of the data obtained from these cells is complex, especially when ouabain induced signal transduction is assessed.

To develop mammalian cell expression system which can allow both pumping and signaling to be studied, we have tried to express different isoforms of Na/K-ATPase or their mutants in mammalian cells in the absence of detectable levels of endogenous Na/K-ATPase.

PY-17 cells provide such a system. It contains very low levels of endogenous pig $\alpha_1$ and do not express either $\alpha_2$ or $\alpha_3$ Na/K-ATPase[4]. When exogenous $\alpha$ subunits are expressed in these cells, endogenous Na/K-ATPase expression is further reduced, which has been confirmed with western blotting and ouabain binding. The speculations made about Na/K-ATPase $\alpha_3$ being functional only at higher intracellular concentration of $Na^+$ can now be confirmed by using our LM-$\alpha_3$-1 cell line.

Although LM-$\alpha_3$-1 cells present a unique cell system to study Na/K-ATPase $\alpha_3$, they have some limitations. Since we used epithelial cells to generate these cells, it might not represent native working environment for Na/K-ATPase $\alpha_3$ because it is normally expressed in neurons.
**Na/K-ATPase and signal transduction:**

Most of our initial studies on the Na/K-ATPase and signal transduction were performed in LLC-PK1 cell that express only α1 isoform. These studies indicate that ouabain could activate many protein and lipid kinase cascades. The activation of Src and subsequent transactivation of EGFR are essential for ouabain–induced signal transduction. Such ouabain-induced transduction has been reported in cell expressing both α1 and α2/α3 isoforms. These studies raise the question whether other isoforms of Na/K-ATPase also possesses α1-like signal transduction function.

Pierre *et al.* have tried to assess it in Sf-9 insect cells[32]. Their studies indicate that ouabain could activate ERK in Sf-9 cells expressing α1, α3 and α4 Na/K-ATPase. Because Sf-9 cells do not normally express Na/K-ATPase and Src. It became necessary to develop a mammalian cell system to re-examine the issue. As shown in Fig.7 unlike α1 NaKtide, NaKtide derived from α3 did not produce an effective inhibition of Src in vitro kinase assay. Consistently, expression of α3 was ineffective in reducing the elevated Src activity in PY-17 cells due to the down regulation of α1 Na/K-ATPase. Moreover, as predicted, ouabain failed to activate Src in LM-α3-1 cells. However, as in Sf-9 cells [32] ouabain was able to activate ERK. Mechanistic studies indicate that ouabain–induced ERK activation was independent of Src/EGFR, but dependent on PI3K and PKC. Boldyrev *et al.*, using intact neuronal suspension prepared from cerebellum of rat pups have shown that ouabain-sensitive (α2 and α3) and ouabain-resistant (α1) Na/K-ATPase are involved in cell signaling by different modes. They found that α2 and α3 regulate PKC and PIP3 kinase whereas the α1 regulate tyrosine kinases like Src and ERK[35].
All together, the newly developed α 3-expressing cells allowed us to illustrate a distinct cell signaling mechanism of ouabain, separate from the one mediated by α1 Na/K-ATPase. The ouabain inducible α1 Na/K-ATPase-Src receptor-complex is in a way similar to G protein coupled receptors. While Na/K-ATPase provides a ligand binding site (i.e. G protein coupled receptors), Src acts as a signal transducer (i.e. G protein). The data presented here suggest that α3 Na/K-ATPase may associate with a transducer other than Src, possibly PI3K or PKC (as shown in Fig.12). In contrast to both α1 and α3, α2 Na/K-ATPase has no apparent signaling role. Of course, more studies are needed to not only determine the signal transduction of α3 Na/K-ATPase, but also to verify the lacking of signaling of α2 Na/K-ATPase or other unknown signaling functions.

**Na/K-ATPase and cell growth**

Na/K-ATPase is required for normal cell growth. This has been confirmed by many studies. In rodents, knocking out α1 Na/K-ATPase is shown to be embryonic lethal [36]. The α1 Na/K-ATPase knockdown cells (PY-17) show slower cell growth rate [4]. This defect is rescued when α1 Na/K-ATPase is knocked back in (AAC-19 cells). When the PY-17 cells are rescued with α3 instead of α1 Na/K-ATPase, the cell growth rate is rescued but not equal to that of α1 Na/K-ATPase. When PY-17 cells are rescued with α2 Na/K-ATPase, the cell growth defect cannot be rescued [12]. This is consistent with the apparent signaling functions of α1, α2 and α3 Na/K-ATPase. The α1 Na/K-ATPase which is fully capable of signaling function can restore normal cell growth, α2, incapable of cell signaling, cannot restore the normal cell growth. The α3 Na/K-ATPase which possess partial signaling function, can restore the cell growth more efficiently than α2 but not as much as α1. These
observations suggest that for a cell to grow normally, not only pumping function of Na/K-ATPase is required but also the signaling function.

**The role α3 Na/K-ATPase in physiology and pathology:**

The α3 Na/K-ATPase is present mainly in neuronal tissues. Due to low Na\(^+\) affinity of α3 Na/K-ATPase, it can play important role in neuronal action potential[13]. Mutations in α3 give rise to many neurological diseases like rapid onset dystonia Parkinsonism (RDP)[14], cognitive deficits[37, 38], mood disturbances[39, 40] and alternating hemiplegia of childhood (AHC)[15]. It will be of interest to assess whether signaling function of α3 Na/K-ATPase is responsible for the observed neuronal disorder related to α3 mutations.

The data provide strong evidence that the α3 Na/K-ATPase possesses signaling functions, but different from those of α1 Na/K-ATPase. The analysis of \(^{86}\text{Rb}^+\) studies indicate that under normal conditions α3 may not be as efficient pump as that of α1, but can function more efficiently when intracellular Na\(^+\) is increased as membrane depolarizes. So far this speculation was made based on the kinetic studies, but we, for the first time can prove this using our newly generated cell system. This ability of α3 Na/K-ATPase is essential in neuronal cells during action potential when there is increased intracellular Na\(^+\) and α1 Na/K-ATPase alone may not be able to restore the basal membrane potential. This may explain diverse localization of Na/K-ATPase isoforms among different cells and tissues. Different pathways taken by different isoforms may provide versatility to the cells and may provide regulation of cell/tissue specific signaling events.
Uncertainties and Implications:

As we have discussed, although this study has its own limitations, it opens lot more paths directing to many different research areas in α3 Na/K-ATPase field. We present a cell system to study the characteristics of α3. Moreover our lab has generated cells expressing α1, α2 or α3 derived form a common parental cells and without presence detectable of other isoforms of Na/K-ATPase. Changes in Na\(^+\) affinity and pumping ability of mutated α3 Na/K-ATPase is implicated to be the root cause of severe neurological disorders such as Alzheimer’s disease, Alternating hemiplegia of childhood (AHC), rapid-onset dystonia-parkinsonism (RDP). But signaling changes due to these mutations has not been evaluated as yet. Our newly generated cell-lines can certainly provide tools to compare characteristics and functionality of α3 Na/K-ATPase.
Reference


39. Kirshenbaum, G.S., et al., Decreased neuronal Na+,K+-ATPase activity in Atp1a3 heterozygous mice increases susceptibility to depression-like

Table.

Table 1: Effect of ouabain on ATPase activity.

<table>
<thead>
<tr>
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<th>AAC-19*</th>
<th>LM-α3-1</th>
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<tbody>
<tr>
<td>10μM</td>
<td>100μM</td>
<td>10μM</td>
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<tr>
<td>~8%</td>
<td>~49%</td>
<td>7.1±5.2%</td>
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*The ouabain inhibition was inferred from ref [4]

Figure legends.

Figure 1. Generation of stable cell lines expressing α3 Na/K-ATPase.

Total cell lysates from AAC-19, LM-α3-1 and PY-17 were separated by SDS-PAGE and analyzed by Western blot for the expression of the (A) α1 Na/K-ATPase and (B) α3 Na/K-ATPase and (C) β1 Na/K-ATPase. A representative Western blot is shown.

(D) ^3^H ouabain binding: Cells were treated for 2μM ouabain for 30 min and then assayed for ouabain binding as described under methods. Quantification of data are shown as mean±S.E and were calculated from at least three separate experiments. * P<0.05, ** P<0.001 compared to control.

Figure 2. Characterization of the pumping function of α3 Na/K-ATPase.

(A) Ouabain sensitive ATPase activity was measured from the membrane preparations of AAC-19 and LM-α3-1. (B) Ouabain sensitive ^86^Rb^+^ uptake was measured as described under methods. Values are normalized per protein amount.
Data are shown as mean±S.E and were calculated from at least three separate experiments. * P<0.05 compared to AAC-19.

**Figure 3. Kinetic properties of α3 Na/K-ATPase.**

Crude membranes were prepared from AAC-19 and LM-α3-1 cells and ATPase activity was measured under varying concentration of (A)Sodium Chloride (Na⁺) and (B)Potassium Chloride (K⁺).

Data are shown as mean±S.E and were calculated from at least three separate experiments.

**Figure 4. Na⁺ regulation of α3 Na/K-ATPase.**

Ouabain sensitive ⁸⁶Rb⁺ uptake was measured along with monensin as described under methods. Values are normalized per protein amount.

Quantification of is are shown as mean±S.E and they were collected from at least three separate experiments. *P<0.05 compared to basal, #P<0.05 compared to fold change in AAC-19.

**Figure 5. Caveolin expression in LM-α3-1.**

Total cell lysates from AAC-19, LM-α3-1 and PY-17 cells were separated by SDS-PAGE and analyzed by Western blot for the expression of Caveolin-1.

A representative Western blot is shown. Quantification of respective protein is shown as mean±S.E and they were calculated from at least three separate experiments. * P<0.05 compared to AAC-19.
Figure 6. Basal phosphorylation levels of protein kinases.

Total cell lysates collected from AAC-19 and LM-α3-1 cells were separated by SDS-PAGE and analyzed by Western blot for (A) Src pY418 (B) pERK (C) pAkt.

A representative Western blot is shown, and the quantitative data are mean ± S.E. from at least three independent experiments. * P<0.05 compared to AAC-19

Figure 7. Effect of Na/K-ATPase peptides on Src by In-Vitro kinase assay

Recombinant Src (4.5 units) was incubated with different concentrations of NaKtide and α3 NaKtide for 15 minutes and then assayed for pY418 Src.

The quantitative data are shown as mean ± S.E. from at least three independent experiments. * p<0.05 compared to control.

Figure 8. Effect of Ouabain on Src and ERK in cells expressing α1 or α3 Na/K-ATPase.

Cells were serum-starved overnight and harvested after ouabain treatment of 10 minutes. Total cell lysates collected from AAC-19 and LM-α3-1 cells were separated by SDS-PAGE and analyzed by Western blot for (A) Src pY418 and c-Src (B)pERK and ERK.

A representative Western blot is shown, and the quantitative data are mean ± S.E. from at least three independent experiments. * P<0.05 compared to control.
**Figure 9. Ouabain stimulation of Akt in LM-α3-1 cells.**

Cells were serum-starved overnight and harvested after ouabain treatment of 10 minutes. Total cell lysates collected from AAC-19 and LM-α3-1 cells were separated by SDS-PAGE and analyzed by Western blot for pAkt and Akt.

A representative Western blot is shown, and the quantitative data are mean ± S.E. from at least three independent experiments. * P<0.05 compared to control.

**Figure 10. Effect of inhibitors on ouabain stimulated ERK activation.**

LM-α3-1 cells were pretreated with following inhibitors: (A) 5µM SFK inhibitor PP2 for 30 min. (B) 1µM PKC inhibitor GO6983(GO) for 60min. (C)50µM PI3K inhibitor LY 29400(LY) for 60min.(D)2µM EGFR inhibitor PD153035(PD) for 60min. The cells were then treated with10 µM of ouabain for 10 minutes. Cell lysates were collected, separated by SDS-PAGE and analyzed by Western blot for pERK and ERK.

A representative Western blot is shown, and the quantitative data are mean ± S.E. from at least three independent experiments. * P<0.05 compared to control.

**Figure 11. Effects of α3 expression on cell growth.**

Cells were plated in 12-well plates (20,000 cells/well), cultured different times in DMEM + 10% FBS as mentioned then collected and counted as described under Methods.

Data shown are mean ± S.E. from at least three independent experiments. **p<0.01 compared to AAC-19.
Figure 12. Possible signaling transduction mechanisms by different isoforms of Na/K-ATPase.

The α Na/K-ATPase isoforms functions as a receptor for ligands such as ouabain. Src acts as a transducer with α1 Na/K-ATPase and activate downstream signaling. The α2 Na/K-ATPase fails to interact with Src and do not show any downstream signaling. The α3 Na/K-ATPase forms a signaling complex with an unconfirmed transducer.

Supplementary Figure 1. The expression level of α3 Na/K-ATPase of different clones in the α3 rescued cells.

Cell lines were generated by transfection of rat α3 Na/K-ATPase containing expression vectors into PY-17 cells. Total cell lysates from different cell lines were separated by SDS-PAGE and analyzed by Western blot for the rat α3 expression. Quantification of respective protein shown as mean±S.E and were calculated from at least three separate experiments.
FIGURE 1

A. α1
α-tubulin
AAC-19 LM-α3-1 PY-17

Ratio of density of α1 and Tubulin (% of AAC-19)

0 20 40 60 80 100 120

AAC-19 LM-α3-1 PY-17

**  **

B. α3
α-tubulin
AAC-19 LM-α3-1 PY-17

Ratio of density of α3 and Tubulin (% of LM-α3-1)

0 20 40 60 80 100 120

AAC-19 LM-α3-1 PY-17

**  **

C. β1
α-tubulin
AAC-19 LM-α3-1 PY-17

Basal Levels of [3H]Tubulin (% of AAC-19)

0 20 40 60 80 100 120

AAC-19 LM-α3-1 PY-17

**

D. %ouabain Binding (% of PY-17)

0 20 40 60 80 100 120

PY-17 LM-α3-1

*
FIGURE 2

A. 

B. 

FIGURE 3

A. 

B. 

<table>
<thead>
<tr>
<th>$K_{0.5}$ values</th>
<th>Na$^+$</th>
<th>K$^+$</th>
</tr>
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<tbody>
<tr>
<td>AAC-19</td>
<td>10.72±.7mM</td>
<td>1.76±0.26mM</td>
</tr>
<tr>
<td>LM-α3-1</td>
<td>27.7±2.5mM</td>
<td>1.84±0.3mM</td>
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FIGURE 4

Fold change in the activity compared to basal $^{86}$Rb uptake

<table>
<thead>
<tr>
<th></th>
<th>AAC-19</th>
<th>LM-α3-1</th>
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<tr>
<td>Basal</td>
<td>1.82</td>
<td>2.78</td>
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FIGURE 5

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<th>Cav-1</th>
<th>α-tubulin</th>
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<tr>
<td>AAC-19</td>
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<tr>
<td>LM-α3-1</td>
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<td></td>
</tr>
<tr>
<td>PY-17</td>
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<td></td>
</tr>
</tbody>
</table>

Ratio of density of Caveolin and Tubulin (% of AAC-19)
FIGURE 6

A. pSrc
   C-Src
   AAC-19   LM-α3-1

B. pERK
   ERK
   AAC-19   LM-α3-1

C. pAkt
   Akt
   AAC-19   LM-α3-1

FIGURE 7

<table>
<thead>
<tr>
<th>NaKtide</th>
<th>415-SATWFSRSLGCLNARVFQ-434</th>
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<tbody>
<tr>
<td>α3 NaKtide</td>
<td>405-SHTWVALSHIAGLCNARVFK-424</td>
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Relative Src pY418 (% of control)

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<th>Concentration</th>
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<th>α3 NaKtide</th>
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<td>0.1 μM</td>
<td><img src="image" alt="Graph" /></td>
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<td>1.0 μM</td>
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<tr>
<td>10 μM</td>
<td><img src="image" alt="Graph" /></td>
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</tbody>
</table>

* **P < 0.05**
**FIGURE 12**

Supplementary Figure 1

**α3**

α-tubulin

Clone 1  Clone 4  Clone 11  Clone 15  Clone 16

Integrated density (normalized with tubulin)

Clone 1  Clone 4  Clone 11  Clone 15  Clone 16
Chapter 3

Identification of a Mutant α1 Na/K-ATPase That Pumps but is Defective in Signal Transduction

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Running title – Na/K-ATPase in signal transduction

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Abstract

Background: It has not been possible to study the pumping and signaling functions of Na/K-ATPase independently in live cells.

Results: Both cell-free and cell-based assays indicate that the A420P mutation abolishes the Src-regulatory function of Na/K-ATPase.

Conclusion: A420P mutant has normal pumping but not signaling function.

Significance: Identification of Src regulation-null mutants is crucial for addressing physiological role of Na/K-ATPase.

Introduction

The α1 Na/K-ATPase possesses both pumping and signaling functions. However, it has not been possible to study these functions independently in live cells. We have further identified a 20 amino acid peptide (Ser-415 to Gln-434) (NaKtide) from the nucleotide binding domain of α1 Na/K-ATPase that binds and inhibits Src in vitro. The N-terminus of NaKtide adapts a helical structure. In vitro kinase assays showed that replacement of residues that contain bulky side chain in the helical structure of NaKtide by alanine abolished the inhibitory effect of the peptide on Src. Similarly, disruption of helical structure by proline replacement, either single or in combination, reduced the inhibitory potency of NaKtide on Src. To identify mutant α1 that retains normal pumping function but is defective in Src regulation, we transfected Na/K-ATPase α1 knockdown cells with expression vectors of wild type or mutant rat α1 carrying Ala to Pro mutations in the region
of NaKtide helical structure, and generated several stable cell lines. We found that expression of A416P or A420P or A425P mutant fully restored the α1 content and consequently the pumping capacity of cells. However, in contrast to A416P, either A420P or A425P mutant was incapable of interacting and regulating cellular Src. Consequently, expression of these two mutants caused significant inhibition of ouabain-activated signal transduction and cell growth. Thus we have identified α1 mutant that has normal pumping function but is defective in signal transduction.

Na/K-ATPase is a ubiquitously expressed, integral membrane protein transporting Na\(^+\) and K\(^+\) across the plasma membrane by hydrolyzing ATP [1]. This pumping function is essential for eukaryotic cells to maintain ionic homeostasis as well as to provide transmembrane Na\(^+\) gradients for the Na\(^+\)-dependent transport of nutrients. Recent studies from different laboratories have revealed that the α1 Na/K-ATPase also has important signal transduction functions that are could act as a functional receptor for cardiotonic steroids to activate protein kinase cascades [2-4], which play an important role in renal salt handling and remodeling of the heart and the kidney under pathological conditions [5]. Using in vitro binding assays we have identified two pairs of domain interactions that seem to be essential for the formation of this functional receptor. One is between the second cytoplasmic domain (CD2) of Na/K-ATPase α1 subunit and Src homology 2 (SH2) domain and the other between the nucleotide binding domain of α1 subunit and Src kinase domain. The latter interaction keeps Src in an inactive state. Binding of cardiotonic steroids such as ouabain to the Na/K-ATPase disrupts the latter interaction, resulting in an activation of the pump-associated Src [4]. Besides Src, the α1 Na/K-ATPase interacts with many other
partners including phosphoinositide 3-kinase and caveolin-1, and is involved in the 
regulation of PI3K/Akt pathway and the formation of caveolae [6-8].

To further probe the Src-regulatory function of Na/K-ATPase, we have recently mapped 
the structural determinant of nucleotide binding domain of α1 subunit that is involved in 
the interaction with the Src kinase domain, which led to the identification of NaKtide, a 20 
amino acid peptide located in the N-terminus of nucleotide binding domain [9]. We have 
further engineered a cell-permeable NaKtide (pNaKtide). This peptide is a potent Src 
inhibitor in the test tube and acts as a receptor antagonist by blocking the formation of 
functional Na/K-ATPase/Src complex when applied to cultured cells [9]. Moreover, 
pNaKtide was effective in inducing tumor regression and inhibiting tumor growth in vivo[10]. To understand the molecular basis of NaKtide-mediated Src regulation, we made 
several mutants of NaKtide and tested their effects on Src. These in vitro studies indicate 
that the N-terminal helical structure of NaKtide appears to be important for its interaction 
with Src. To further test this hypothesis, we made several α1 mutants and generated stable 
cell lines expressing these mutants. Functional studies of these stable cell lines demonstrate 
that A420P mutant α1 has normal pumping function, but has lost its capacity of Src 
regulation.

Experimental Procedures

Materials:

All the peptides of more than 95% purity (checked by reverse phase HPLC) were 
purchased from HD Biosciences (China) Co. Ltd. The polyclonal anti-Src (Tyr(P)418) 
antibody, cell culture media, fetal bovine serum, trypsin, Lipofectamine 2000 were
purchased from Invitrogen (Grand Island, NY). QuikChange mutagenesis kit was obtained from Stratagene (La Jolla, CA). Image-iT FX signal enhancer, antifade kit, Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 546-conjugated anti-rabbit IgG antibodies were from Molecular Probes (Eugene, OR). Anti-Na/K-ATPase α1 polyclonal, anti-Na/K-ATPase β1 (clone C464.8) antibody and recombinant human Src were obtained from Upstate (Lake Placid, NY). The monoclonal anti-α1 antibody (α6F) was from the Developmental Studies Hybridoma Bank at the University of Iowa. Anti-c-Src (B-12) monoclonal antibody, the anti-Cav1 polyclonal antibody, and all the secondary horseradish peroxidase-conjugated antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Polyclonal rat α1-specific antibody (anti-NASE) was provided by Dr. Thomas Pressley (Texas Tech University, Lubbock, TX). Radioactive $^{86}\text{Rb}^+$ was from PerkinElmer Life Science Products (Boston, MA). Protease inhibitor cocktail was purchased from Sigma (St. Louis, MO).

**Methods:**

Src kinase assay - The activity of NaKtide and its mutant peptides was measured using *in vitro* Src kinase assay as described [9]. Briefly, purified Src (4.5 U) was incubated with different concentrations of peptides in PBS (137 mM NaCl, 2.7 mM KCl, 10mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, pH 7.4) for 15 min at 37°C. Afterward 2.0 mM ATP/Mg$^{2+}$ was added to induce phosphorylation. The reaction was continued for 15 min at 37°C and was stopped by addition of SDS sample buffer. Afterward, Western blot analyses of Src (Tyr(P)418) was conducted [9].

Circular Dichorism (CD) - These studies were done as previously described [11]. Briefly, CD measurements were performed at room temperature (20°C) using Jasco J-715 and
quartz flow cell with a 1mm path length. Peptides was dissolved in PBS (pH 7.4) at a concentration of ~ 0.17 mg/ml. Spectra were collected at 50nm/min, using a band width of 1nm, average over 10 scans, and the baseline (PBS only) was subtracted from each spectrum. Percent helicity was then calculated by equation: % helicity = [θ]_222 / [θ]_max * 100 (where [θ]_222 mean residue ellipticity and [θ]_max is maximum mean ellipticity)

Computer Modeling - Computer modeling was done using PyMol (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.)

Site-directed Mutagenesis - QuikChange mutagenesis kit was used. We used the vector pRc/CMV-α1 AAC m1 as a template as described in our prior publications [3]. According to the GenBank™ rat α1 sequence (NM_01254), we created A416P, A420P, A425P and A420/425P mutant vector. These four mutants were verified by DNA sequencing.

Generation of A to P mutant stable cell lines – This was done as previously described [3]. Briefly, PY-17 cells were cultured on 6 well plates and transfected with the 4 different mutant vectors. The tranfected cells were selected with 3µM ouabain for one week and the survived ouabain resistant colonies were collected and diluted into 96 well plates to isolate single colony. Once the colony was expanded into stable cell line, the expression of mutant rat α1 was verified by rat α vC1-specific antibody.

Cell Culture—Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 1 µg/ml puromycin . When cells reached 90-100% confluence, they were serum-starved overnight and used for experiments unless otherwise noted.

Immunoblot Analysis—After treatment, cells were washed by ice-cold PBS, and lysed in ice-cold radioimmunoprecipitation (RIPA) buffer (0.25% sodium deoxycholate, 1%
Nonidet P-40, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride, 1mM sodium orthovanadate, 1mM NaF, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4 and 1% protease inhibitor cocktail). After the cell lysates were centrifuged at 14,000x g for 15 min, the supernatants were collected and measured for the protein content. The same amount protein of each sample was loaded, separated by SDS-PAGE, then transferred to an Optitran membrane and probed by indicated antibody. ImageJ 1.46 was used for analyzing the data.

Confocal Fluorescence Microscopy – Immunostaining of α1 and pY418 was done was previously described.[10]. Briefly, cells were cultured on cover-slips in 6 well plates. When cells reached 90-100% confluence, they were serum-starved overnight. After indicated treatment, cells were washed twice with ice-cold PBS, fixed with pre-chilled (-20°C) methanol for 15 min, and then blocked with 100 µl Image-iT FX signal enhancer for pY418Src and PBS containing 1% FBS for α1 at room temperature for 30 min. The blocked cells were incubated with primary antibody overnight at 4°C, washed and incubated with Alexa Fluor conjugated secondary antibody for 2 hrs at room temperature. The stained cells on cover-slips were washed, mounted, and then visualized using a Leica DMIRE2 microscope (Wetzlar, Germany).

[^3]H]Ouabain Binding—To measure the surface expression of the endogenous pig Na/K-ATPase,[^3]H]ouabain binding assay was performed as described (8). Briefly, cells were cultured in 12-well plates until confluent and serum-starved overnight. Afterward, the cells were incubated in K⁺-free Krebs solution (142.4 mM NaCl, 2.8 mM CaCl₂, 0.6 mM NaH₂PO₄, 1.2 mM MgSO₄, 10 mM glucose, 15 mM Tris (pH 7.4)) for 15 min and then exposed to 200 nM[^3]H]ouabain for 30 min at 37 °C. After incubation, the cells were washed 3 times with ice-cold K⁺ free Krebs solution, solubilized in 0.1 M NaOH, 0.2%
SDS, and counted in a scintillation counter for \(^{3}H\)ouabain. Nonspecific binding was measured in the presence of 1 mM unlabeled ouabain and subtracted from total binding. All counts were normalized to protein amount.

Ouabain sensitive Na/K-ATPase activity – Cells were transiently transfected as described [12]. After 24 h of culture, the transfected cells were collected and homogenized in ice-cold buffer A (150 mM sucrose, 5 mM HEPES, 4 mM EGTA, 0.8 mM dithiothreitol) and briefly sonicated. After centrifugation (800 X g, 10 min), the post nuclear fraction was further centrifuged (45,000 X g, 45 min) in order to get crude membrane. The crude membrane pellet was resuspended in buffer A and the protein content was determined. The aliquots of protein were treated with alamethicin (0.1 mg/mg of protein) for 10 min at room temperature and then added to the buffer containing 50 mM Tris (pH 7.4), 1 mM EGTA, 1 mM MgCl\(_2\), 25 mM KCl, 100 mM NaCl, 5 mM NaN\(_3\). After 5 min preincubation at 37°C, ATP/Mg was added to a final concentration of 2 mM to start the reaction. The reaction continued for 15 min and was stopped by adding 8% ice-cold trichloroacetic acid. Phosphate generated during the ATP hydrolysis was measured by BIOMOL GREEN Reagent (Enzo Life Science). Ouabain-sensitive Na/K-ATPase activities were calculated as the difference between the presence and absence of 1 mM ouabain. In certain experiments, indicated vanadate amount was added in the reaction mixture.

Cell counting assay — 20,000 cells/well were seeded in 12-well plate and cultured in 10% FBS DMEM medium. At indicated time, 3 wells from each cell line were trypsinized and the number of viable cells was counted as described [8].

Cell Surface Biotinylation of Na/K-ATPase — Biotinylation of cell surface protein of these mutant cell lines was undertaken by the protocol previously described [13]. Briefly, the full
confluent cells grown on 60-mm dishes were washed 3 times with ice-cold PBS followed by incubation with 2 ml of NHS-SS-biotin (1.5 mg/ml) in biotinylation buffer (10 mM triethanolamine, pH 9.0, 150 mM NaCl) for 25 min at 4°C with very gentle horizontal motion to ensure mixing. The un-reacted biotin was quenched and the biotinylated cells were lysed with ice cold lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 1% protease inhibitor cocktail, pH 7.5). Equal amounts of protein (250 µg) were incubated with streptavidin-agarose beads (150 µl) overnight at 4°C. The beads-bound biotinylated protein and total cell lysate (25 µg) was subjected to western blot analysis.

Ouabain-sensitive $^{86}$Rb$^+$ Uptake — Ouabain-sensitive $^{86}$Rb$^+$ was measured to estimate the transport function of Na/K-ATPase as previously described [14]. Briefly, cells were cultured in 12-well plates and serum starved overnight when the cells were over 90% confluence. Cells were washed and incubated in DMEM with or without 1.0 mM ouabain for 10 min at 37 ºC. $^{86}$Rb$^+$ (1 µCi/well) was then added to the cells for 10 min and the reaction was stopped by washing with ice-cold 0.1 M MgCl$_2$. Trichloroacetic acid soluble $^{86}$Rb$^+$ was counted in a Beckman scintillation counter. All counts were normalized to protein amount. In certain experiments, different concentrations of ouabain were added.

Data Analysis — Data presented are mean± S.E. of at least three independent experiments, and statistical analysis was performed using the Student’s t test and significance was accepted at $p < 0.05$.

Results

In vitro mutagenesis analyses of NaKtide: We have shown that NaKtide binds and inhibits Src [9]. Its mode of action is similar to that of purified α1 Na/K-ATPase in which
it inhibits Tyr-418 phosphorylation without affecting the phosphorylation of Tyr-529 [4].
The deduced structure from crystals of either E2P (2ZXE)[15] or high ouabain affinity
(3N23)[16] form indicate that NaKtide may adapt a helical structure in the N-terminus
(Thr-417 to Leu-427) followed by C-terminal loop tail (Cys-428-Gln-434) (Figure 1A)
To test the importance of this helical structure in NaKtide-mediated Src inhibition, we
conducted the following in vitro mutagenesis studies.

Based on the crystal structure (Figure 1A), the side chains of Trp-418, Leu-419 and
Arg-423 are solvent exposed. If the helical structure is involved in the interaction with Src,
these side chains may provide the contacting site and be important for Src inhibitory effect
of the peptide. Indeed, replacement of these three residues with alanine significantly
attenuated the inhibitory effect of peptide on Src (Figure 1B). To further test the
importance of helical structure, we replaced Ala-420 with proline. It is known that proline
replacement bends the backbone of helical structure [17]. Ala-420 resides in the middle of
the helical structure. Therefore, if the helical structure is important, A420P mutation would
alter the structure and attenuate the inhibitory effect of NaKtide on Src. When the dose-
dependent effect of A420P mutant peptide was assessed, we found that the mutant peptide
was about ten times less effective than that of NaKtide (Figure 1B and C)[9]. To further
verify the importance of helical structure, we also determined the effect of A425P and
A420P/A425P mutant peptides on Src. As depicted in Figure 1D, while A425P had a
reduced effect on Src as A420P peptide, A420P/A425P double mutant exhibited no
inhibition of Src.

To assess whether the helix is sufficient for the inhibition of Src, we synthesized a
C-terminally truncated peptide P3A (Ser-415 to Arg-430), removing the last five amino
acid residues. As depicted in Figure 2A, although this peptide showed inhibitory effect on Src, both efficacy and potency were reduced. To be sure that truncation of last five amino acid residues did not reduce the helicity of the peptide, we measured the Circular Dichorism (CD) spectra of NaKtide and peptide P3A. As shown in Figure 2B and Table 1, the helicity in PBS was actually increased when the C-terminal five amino acid residues were removed. In short, the above in vitro mutagenesis analyses indicate that the helical structure as well as the C-terminal tail is important for the interaction and inhibition of Src by NaKtide.

**Generation of stable cell lines expressing A to P mutants:** To verify the importance of helical structure in α1 Na/K-ATPase-mediated Src regulation, we constructed the following A to P mutants based on a rat α1 cDNA expressing vector we described in our previous publications [3]: A416P, A420P, A425P and a double mutant (A420/425P). Based on the crystal structure, A416 resides out of helical structure. Thus, A416P mutant was selected as a control. To reduce the interference from endogenous α1 Na/K-ATPase, we rescued α1 knockdown PY-17 cells with these mutants. PY-17 cells were derived from pig LLC-PK1 cells and the expression of endogenous α1 Na/K-ATPase was reduced > 90% by the expression of α1-specific siRNA [3]. Note that these cells do not express other isoforms of Na/K-ATPase [3]. Wild-type rat-α1 rescued PY-17 cells (AAC-19) we made previously were used as a control [3]. Like wild type rat α1 [3], ouabain selection of transfected PY-17 cells resulted in numerous clones for A416P, A420P and A425P.

Interestingly, cells transfected with the double mutation (A420/425P) cDNA did not produce any viable clone in the presence of ouabain. To explore whether the expressed
A420/425P double mutant could function as a pump, we conducted a transient transfection assay, made crude membrane preparations and measured ouabain-sensitive ATPase activity. Like wild type rat α1, transient transfection of PY-17 cells with A420/425P double mutant produced a comparable increase in ouabain-sensitive ATPase activity as the wild type rat α1(Table 2). Thus, it is unlikely that the failure of generating viable clones from A420/425P mutant α1 is because of defects in the pumping capacity of this mutant.

Western blot analyses using rat α1-specific antibody showed that every viable clone tested expressed rat α1 mutant and that the expression level of mutant α1 varied among different clones (Supplementary Figure 1). After this initial Western blot screening, we picked clone 4 from A416P transfected (named as LL-A416P-4, abbreviated as A416P), clone 20 from A420P transfected (named as LL-A420P-20, abbreviated as A420P) and clone 9 from A425P transfected (named as LL-A425P-9, abbreviated as A425P) cells. As depicted in Figure 3A, the expression of mutant α1 in these cell lines was comparable to that of wild type rat α1 in AAC-19 cells. Because rat α1 specific antibody was used, no detectable signal of rat α1 was observed in the PY-17 cell lysates, derived from pig LLC-PK1 cells (Figure 3A). However, when the blot was analyzed by a generic α1-specific monoclonal antibody, a weak signal was detected in samples from PY-17 cells as we previously reported [3]. Moreover, the overall α1 expression level in the rescued cell lines was comparable to each other. This is further verified by immunostaining of these cell lines. Strong and comparable signals were detected in the plasma membrane area in all of the rescued cell lines but the parental PY-17 cells (Figure 3B). Finally, biotinylation analysis indicate that the ratio of biotinylated surface α1 to total α1 in the cell lysates was similar among AAC-19, A416P mutant, A420P and A425P mutant cells (Figure 3C).
**Na/K-ATPase activity in mutant-rescued cells** – To characterize the pumping function of mutant Na/K-ATPase, we first checked the expression of β1 subunit. Knockdown of α1 subunit reduced the expression and glycosylation of β1 subunit [13]. As shown in Figure 3D, all three α1 mutants were able to rescue the expression and glycosylation of β1 as did wild-type α1. These findings indicate that the expressed mutant α1 is fully capable of assembling with the β1 subunit into functional Na/K-ATPase, which is consistent with the findings depicted in Figure 3B and C.

To assess the pumping capability of these mutants, we measured ouabain-sensitive \(^{86}\)Rb\(^+\) uptake in different cell lines (Figure 4A). No difference in pump activity was detected among different cell lines, indicating that the Ala to Pro mutation made in this particular area of nucleotide binding domain of α1 subunit did not cause apparent defect in pumping function of the Na/K-ATPase. This notion is further supported by the fact that the expressed mutants showed the similar sensitivity to vanadate (Figure 4B) and ouabain (Figure 4C).

To further confirm that these mutations do not alter the Na/K-ATPase pumping properties, we compared the kinetic properties of A416P and A420P mutants. The crude membranes were prepared from A416P and A420P cells, and ATPase activity was measured in the presence of different concentrations of Na\(^+\) and K\(^+\). As depicted in Fig. 4, D and E, the Km values of Na\(^+\) and K\(^+\) were comparable between A416P and A420P mutants. These values are similar to those reported in the literature for α1 Na/K-ATPase (17).

To assess the level of endogenous pig α1 expression in the mutant-rescued PY-17 cells, we conducted \(^{3}H\) ouabain binding analyses. Because ouabain dissociates from rat α1 much faster than that from the endogenous pig α1, this binding assay allows us to assess
the surface expression of endogenous α1 in the presence of highly expressed rat α1 in the rescued cells. The parental PY-17 cells were used as control. As depicted in Figure 4D, expression of endogenous α1 in either AAC-19 cells or mutant-rescued cells was further reduced compared to that in PY-17 cells.

Taken together, the above findings indicate that expression of mutants restored total cellular α1 Na/K-ATPase and consequently the pumping capacity in A416P, A420P and A425P mutant cells to the level comparable to that in AAC-19 cells. Moreover, the level of endogenous α1 in the rescued cells was lower than that of PY-17 cells, amounts to less than 10% of total α1 Na/K-ATPase.

**The expressed mutants have different effects on caveolin-1 expression:** We showed that knockdown of α1 Na/K-ATPase increased endocytosis and degradation of caveolin-1 in PY-17 cells. As reported [18], this defect could be rescued by the expression of rat α1 as shown in Figure 5. When caveolin-1 was measured in A to P mutant-rescued cells, we found that expression of A416P and A420P, but not A425P, mutant was sufficient to restore the expression of caveolin-1 (Figure 5).

**Regulation of Src by mutant α1** – To assess whether the Src-regulatory function of α1 Na/K-ATPase was altered in the mutant-rescued cells, we first determined the basal Src activity in these cells. We previously reported that knockdown of Na/K-ATPase increased basal Src activity in PY-17 cells and that rescuing the knockdown cells with rat α1 reduced the elevated basal Src activity in AAC-19 cells [3]. This is indeed the case as shown in Figure 6A by Western blot analysis of active Src as indicated by Y418 phosphorylation.
Interestingly, only the expression of A416P, but not A420P and A425P, mutant showed the same effect as wild type α1. The basal Src activity in both A420P and A425P cells were as high as that in PY-17 cells. To complement the Western blot analyses, we also immunostained these cultured cells with a monoclonal anti-Y418 antibody. As shown in Figure 6B, in contrast to A416P cells, both A420P and A425P showed much higher positive staining of active Src. These data suggest that bending the helical structure of intact α1 subunit, like in NaKtide (Figure 1), may reduce the capability of α1 Na/K-ATPase to interact and regulate Src. Taken together, the above experiments indicate that A416P mutant works like wild type α1 whereas expression of A420P mutant restores the expression of caveolin-1 but fails to inhibit Src. The expression of A425P mutant, on the other hand, could not restore either Src regulation or caveolin-1 expression.

**Expression of A420P mutant inhibits ouabain-induced activation of Src pathway.** We have shown that ouabain is a specific agonist of α1 Na/K-ATPase/Src complex [4]. Binding of ouabain to the α1 Na/K-ATPase stimulated Src pathway within minutes and increased the expression of α1 Na/K-ATPase in hours [2, 4]. Indeed, these changes in response to ouabain (10 to 100 μM) were observed in the control AAC-19 cells and A416P cells (Figure 7A and B). If A420P mutant could not interact with Src as evidenced by the findings in Figure 1 and Figure 6, this mutant would not be able to constitute a functional receptor for cardiotonic steroids such as ouabain to activate Src. This was the case as shown in Figure 7A and B. Ouabain was able to stimulate Src in A4, but not B20 cells. Furthermore, we reported that ouabain could increase the expression of α1 in LLC-PK1 and rat α1-rescued AAC-19 cells [8]. It failed to do so in A420P cells. To seek further evidence that the helical structure is important for the formation of a functional Na/K-
ATPase/Src complex, we repeated these studies in A425P cells. As shown in Figure 7A and B, A425P mutant, like A420P mutant, lost its ability to allow ouabain to stimulate Src pathway. It is important to note that AAC-19 cells express ouabain-insensitive rat α1 Na/K-ATPase. Therefore, μM instead of nM concentration of ouabain was used in these experiments [3].

**Expression of A420P and A425P mutants inhibits cell proliferation:** Src-mediated pathways are known to play an important role in cell proliferation. We have shown that alteration in Na/K-ATPase-mediated Src regulation affects cell growth[19]. However, these previous studies were done in cell lines where both pumping and signaling functions of Na/K-ATPase were altered. To further demonstrate a role of α1 Na/K-ATPase-mediated Src regulation in control of cell growth, we cultured control and mutant cells in full medium and counted the number of cells at different time points. As shown in Figure 8, A416P grew similarly as AAC-19 cells. However, cell growth was significantly reduced in A420P and A425P cells.

**Discussion**

We report here the identification of α1 mutants that retain normal pumping activity but fail to interact and regulate Src and Src-mediated signaling pathways in cell cultures. These findings reaffirm the importance of helical structure of NaKtide in binding and regulating Src. Moreover, we suggest that the newly identified α1 mutants would allow us to fully assess the importance of Src-regulatory function of α1 Na/K-ATPase as well as the CTS-induced signaling in animal physiology, pathology and pharmacology.
Identification of A420P mutant α1 as a Src regulation-null pump: Based on the crystal structure [20], the NaKtide sequence could adapt a helical structure at the N-terminus followed by a C-terminal loop (Figure 1). Kinase activity assays indicate that replacement of either Ala-420 or Ala-425 by proline was sufficient to reduce the peptide-induced inhibition of Src (Figure 1). Moreover, alanine replacement of residues that contain bulky side chain in the helical structure (i.e., Trp-418, Leu-419 and Arg-423) also resulted in a loss of inhibitory effect of the peptide on the Src. These new findings suggest an important role of the helical structure and amino acid residue Trp-418, Leu-419 and Arg-423 in the binding and regulation of Src. Because pNaKtide acts as an effective antagonist of receptor Na/K-ATPase/Src complex in vitro and in vivo [9, 10], the new findings provide important structural information for directing the development of second generation of pNaKtide derivatives.

PY-17 cells were derived from LLC-PK1 cells stably transfected with plasmids expressing α1-specific siRNA [3]. The expression of α1-specific siRNA produced more than 90% down-regulation of endogenous α1 and consequently abolished the signaling function of Na/K-ATPase. As such, these cells have been used by us to study the signaling function of α1 Na/K-ATPase and the structure/function relationships of the pump in signal transduction after the cells were rescued by either wild-type rat α1 or α1 mutants [3]. The intrinsic limitation of these early studies is the fact that we could not separate the pumping function of Na/K-ATPase from its signaling function. For instance, knockdown of α1 Na/K-ATPase abolished ouabain-induced activation of protein kinases in PY-17 cells. However, we could not rule out the influence of reduced transport activity across the cell
membrane and/or the consequent changes in intracellular ion concentrations on protein kinase activity [13]. Furthermore, we could not attribute the effects of either endogenous or exogenous ouabain on cellular functions solely to the changes in protein kinase activity since ouabain also inhibits the pumping function of Na/K-ATPase. Thus, it would be more desirable to have a mutant Na/K-ATPase that can pump but not signal. We believe that A420P mutant α1 is such a mutant pump. First of all, expression of A420P not only rescued cellular α1, but also restored the expression and glycosylation of β1 subunit, indicating that the mutant α1 is fully capable of assembling with the β1 subunit into functional pump in the plasma membrane. This is consistent with the findings presented in the Figure 3, showing that the mutant α1 was expressed in the plasma membrane as revealed by both immunostaining and biotinylation. Functionally, A420P mutant exhibited comparable pumping activity to that of wild type rat α1 as measured by ouabain-sensitive $^{86}$Rb$^+$ uptake. Moreover, the expressed mutant showed the same ouabain-sensitivity as that of wild-type α1. This is in sharp contrast to many reported mutants of α1 Na/K-ATPase in the literature [21], for example, the identified mutants defective in E1/E2 transitions exhibit reduced pump activity and altered ouabain sensitivity [22]. Our recent rescuing studies of I279A and F286A in PY-17 cells confirm these early findings[19]. Second, unlike the wild type α1 or A416P mutant, expression of A420P mutant failed to restore the basal Src activity in the rescued cells. Most importantly, although ouabain was able to inhibit the pumping function, it failed to stimulate Src and Src effectors in the rescued A420P cells (Figure 6). Third, it is of interest to compare A420P and A425P mutants. While both of these mutants showed defect in Src regulation, expression of A420P, but not A425P, was able to, like wild type α1, rescue the expression of caveolin-1. This is important because caveolae play
a critical role in cell signal transduction in general [23, 24], and use of A425P in the study would further complicate the signaling pathways.

Src is known to play an important role in regulation of cell growth [25]. The α1 Na/K-ATPase exists in two major conformations, namely E1 and E2. We have found that expression of α 1 Na/K-ATPase mutants that are defective in either E1 or E2 conformational transition alters the dynamic nature of Src regulation, resulting in cell growth inhibition[19]. These early findings suggest that the α1 Na/K-ATPase be a key regulator of cellular Src activity and consequently cell growth. However, because E1 and E2 mutants are also defective in pumping[19], it is conceivable that changes in cellular pumping activity could at least be part of altered Src regulation and cell growth. To this end, the new findings as depicted in Figure 8 are very important, because they clearly demonstrate that alteration in the Src-regulatory capacity of α1 Na/K-ATPase is sufficient to inhibit cell growth. Therefore, we believe that the α1 Na/K-ATPase provides cells a critical ability of dynamic Src regulation.

**Uncertainties and Implications:** Recent studies have identified both ouabain and MBG as endogenous steroids whose production and secretion are regulated by stimuli including angiotensin II and adrenocorticotropic hormone [26, 27]. Although their pathophysiological significance has been a subject of debate for many years [28], several gene replacement studies from Lingrel’s and co-workers [29, 30] have unequivocally demonstrated an important role of endogenous CTS in regulation of renal Na\(^+\) excretion and blood pressure. In addition, we and others showed that CTS not only induced hypertension in rats, but also caused significant cardiovascular remodeling independent of
their effect on blood pressure [5, 30-32]. Moreover, CTS may play an important role in embryonic development [33]. However, question remains whether the signaling function of α1 Na/K-ATPase is involved in the aforementioned physiological and pathological processes. To this end, we know that Src pathway is activated by high salt intake and by infusion of physiologically relevant amount of CTS [5]. Thus, α1 gene replacement using the newly identified A420P mutant would allow us to conduct experiments similar to those carried out by Lingrel and co-workers [29, 30] to assess specifically the Src-regulatory function of Na/K-ATPase in renal salt handling, organ remodeling and embryonic development.

Although our new findings suggest that the helical structure of NaKtide be important for regulating Src and the formation of receptor Na/K-ATPase/Src complex, more studies are required to reveal the detailed structural determinants. For example, it remains to be determined whether mutation of either Trp-418 or Leu-419 or Arg-423 is sufficient for abolishing the inhibitory effect of NaKtide on Src. It will also be of interest to test whether alanine replacement of these residues could produce another mutant α1 that retains the full pumping capacity but no Src regulatory function. Finally, we do not know why expression of the double mutant A420/425P α1 failed to produce viable clones in the presence of ouabain. Transient transfection assays indicate that the expressed mutant apparently had a normal ATPase activity as other mutants. However, it is possible that the double mutation alters ouabain sensitivity or makes the mutant pump interact with key growth-related pathways other than Src.

In short, this work, taken together with our prior reports, reveals that the α1 Na/K-ATPase is an important Src regulator. Mutations that affect the interaction between the
NaKtide region of α1 subunit and Src appears to have specific effect on the α1 Na/K-ATPase-mediated Src regulation without compromising the normal pumping function.
Reference


FIGURE LEGENDS

Figure 1. Structure-function analysis of mutant NaKtide.

*Panel A*, shown is the structure of NaKtide deduced from the crystal structure of Na/K-ATPase (PDB ID 2ZXE). The structure shows that side chains of Trp-418, Leu-419, and Arg-423 are exposed in the solvent. The *green color* represents the non-helical part, whereas the *red color* indicates the helical part of the peptide. The structure was generated by PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.

*Panel B–D*, shown is dose-dependent inhibition of Src Tyr-418 phosphorylation by NaKtide mutants. Recombinant Src (4.5 units) was incubated with different concentrations of peptides for 15 min and then assayed for Src Tyr(P)-418. Mutated residues are represented in *bold* and *underlined*. Combined data from at least four independent experiments are shown. Curve fit analysis was performed with GraphPad Prism 5. *, *p* < 0.05.

Figure 2. Effect of truncation of C terminus of NaKtide on Src inhibition.

*Panel A*, recombinant Src (4.5 units) was incubated with different concentrations of the peptide P3A for 15 min and then assayed for Tyr(P)-418 Src as described under “Experimental Procedures.” Combined data from at least four independent experiments are shown. Curve fit analysis was performed with GraphPad Prism 5. *Panel B*, CD spectra of NaKtide and P3A dissolved in PBS (pH 7.4) were measured as described under “Experimental Procedures.”
Figure 3. Expression of Na/K-ATPase.

*Panel A*, total cell lysates from different cell lines were separated by SDS-PAGE and analyzed by Western blot for the expression of the rat α1 Na/K-ATPase using polyclonal rat α1-specific antibody (anti-NASE) and the total α1 using monoclonal anti-α1 antibody (α6F). A representative Western blot is shown, and quantitative data (mean ± S.E.) of total α1 subunit were calculated from at least three separate experiments. **, *p*<0.01 versus AAC-19 cells. *Panel B*, cells were immunostained with anti-α1 primary and Alexa Fluor-conjugated secondary antibody as described under “Experimental Procedures.” Representative image of three separate experiments is shown for each cell line. *Panel C*, cells were biotinylated and processed as described under “Experimental Procedures.” An aliquot of 25 µg of cell lysate (T) and biotinylated membrane protein (M) from 250µg of total cell lysates was subjected to SDS-PAGE and probed with α6F antibody. Representative Western blots are shown, and quantitative data are calculated based on at least three independent experiments as relative ratio of membrane α1 to total α1. Values are the mean±S.E. *Panel D*, total cell lysates were analyzed by Western blot using anti-Na/K-ATPase α1 antibody. A representative Western blot of at least three independent experiments is shown.

Figure 4. Pumping activity in mutant-rescued cells.

*Panel A*, ouabain-sensitive $^{86}$Rb$^+$ uptake was measured as described under “Experimental Procedures.” Values are normalized to per protein amount and then calculated as % of AAC-19 cells (mean ± S.E.). *Panel B*, vanadate sensitivity analysis is shown. Crude
membrane fractions were prepared from AAC-19, A416P, A420P, and A425P cells and Na/K-ATPase activity was assayed as described under “Experimental Procedures” in the presence of different concentrations of vanadate. The data points are shown as the percentage of the Na/K-ATPase activity in the absence of vanadate (mean ± S.E.). Panel C, ouabain dose-response curves are shown. AAC-19, A416P, A420P, and A425P cells were cultured in 12-well plates and serum-starved overnight. After reaching 100% confluency, cells were pretreated with different concentrations of ouabain as indicated for 10 min and assayed for \(^{86}\text{Rb}^+\) uptake. Data are calculated from at least three repeats and shown as % of respective control (mean ± S.E.). Curve fit analysis was performed by GraphPad Prism 5. Panel D, measurement of \(\text{Na}^+\) \(K_m\) is shown. Crude membrane preparations were made from A416P and A420P cells and measured for the Na/K-ATPase activity as a function of \(\text{Na}^+\) concentration as described under “Experimental Procedures.” Choline chloride was used to substitute NaCl so that ionic strength was kept constant. The combined data from at least three repeats were shown, and \(K_m\) values (mean ± S.E.) were calculated using GraphPad Prism 5. Panel E, measurement of \(\text{K}^+\) \(K_m\) is shown. Crude membrane preparations were made from A416P and A420P cells and measured for the Na/K-ATPase activity as a function of \(\text{K}^+\) concentration as described under “Experimental Procedures.” Choline chloride was used to substitute KCl so that ionic strength was kept constant. The combined data from at least three repeats were shown, and \(K_m\) values (mean ± S.E.) were calculated using GraphPad Prism 5. Panel F, \([\text{H}^3]\text{ouabain binding was performed as described under “Experimental Procedures.” Cells were incubated with 200 nM ouabain for 30 min and then assayed for ouabain binding. \(\ast, p < 0.05; \ast\ast, p < 0.01\) versus PY-17 cells.\)
Figure 5. Caveolin-1 expression level in the mutant cells.

Total cell lysates from AAC-19, A416P, A420P, and A425P cells were separated by SDS-PAGE and analyzed by Western blot for caveolin-1. A representative Western blot is shown, and quantitative data (mean ± S.E.) were calculated from at least three separate experiments. * p< 0.05 versus control AAC-19 cells.

Figure 6. Regulation of Src by mutant α1.

Total cell lysates from AAC-19, A420P, and A425P cells were separated by SDS-PAGE and analyzed by Western blot for Tyr(P)-418 (pY418) Src and total Src. Quantitative data are the mean ±S.E. from at least three independent experiments. *, p < 0.05 versus control AAC-19 cells.

Figure 7. Expression of mutant α1 inhibits ouabain-induced signal transduction.

Panel A, confluent cells were treated with different concentrations of ouabain for 10 min and then harvested, and cell lysates were analyzed by Western blot for Src Tyr(P)-418 (pTyr 418). Panel B, confluent cells were treated with different concentrations of ouabain for 24 h, then harvested and analyzed for Na/K-ATPase α1 expression by Western blot. A representative Western blot is shown, and quantitative data are presented as the mean ±S.E. of at least three independent experiments. * <0.05 versus 0mM ouabain
**Figure 8. Effects of mutant α1 expression on cell growth.**

AAC-19, A416P, A420P, and A425P cells were plated in 12-well plates (20,000 cells/well), cultured for different times, and then collected and counted. The values are the mean±S.E. from four independent experiments. **, p<0.01 versus AAC-19 cells.

**Supplementary Figure 1. The expression level of rat α1 Na/K-ATPase of different clones in the rescued mutant cells.**

Cell lines were generated by transfection of rat α1 Na/K-ATPase mutant expression vectors into PY-17 cells. Total cell lysates from different cell lines were separated by SDS-PAGE and analyzed by Western blot for the rat α1 expression.
**Tables**

**Table 1.**
Calculated helicity of peptides in PBS.

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**Table 2**
Ouabain-sensitive ATPase activity.

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<td>Ouabain sensitive ATPase Activity (%), n≥3</td>
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Figures

Figure 1

A

B

C

D

89
Figure 2

A

P3A: SATWLALSRIAGLCNR

Src p(Y418)
(% of control)

Peptide Log[Conc] M

0 20 40 60 80 100 120

B

[θ], deg cm² dmol⁻¹ a.a.⁻¹

wavelength, (nm)

NaKtide
P3A
Figure 3

A

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<th>A416P</th>
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<td>α-tubulin</td>
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Total α1 level (% of AAC-19 cells)

0 20 40 60 80 100 120

AAC-19  PY-17  A416P  A420P  A425P

B

AAC-19  PY-17

A416P  A420P  A425P

C

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<td>α1</td>
<td>T</td>
<td>M</td>
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\[\text{ratio (membrane/total)}\]

D

AAC-19  PY-17  A416P  A420P  A425P

β1
Figure 5
Figure 6

![Diagram showing protein expression levels for pY418 and e-Src in different cell lines.

AAC-19  PY-17  A416P  A420P  A425P

pY418

e-Src

Bar graph showing Src pY418 levels (% of AAC-19 cells) for different cell lines: AAC-19 (white), PY-17 (striped), A416P (light gray), A420P (gray), A425P (black). Bars with asterisks indicate statistically significant differences.]
Figure 7

A

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B

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<table>
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Figure 8

Supplement Figure 1
Chapter 4

Summary, Discussion and Future Directions

4.1 Summary

This work presents two major findings. First, Na/K-ATPase regulates Src and other signaling mechanisms in an isoform specific manner. The α1 Na/K-ATPase interacts with Src to form a functional receptor for CTS to activate Src, ERK and other protein kinase cascades.

In contrast α2 Na/K-ATPase does not bind to and regulate Src. Ouabain fails to stimulate Src and ERK in cells that only express α2. α3 Na/K-ATPase on the other hand does not bind to Src to form a functional receptor complex however, ouabain stimulates ERK and Akt pathways in cell expressing only α3. The activation of ERK by ouabain in LM-α3-1 cells appears to be regulated by pathways related to PI3K and PKC.

Second we have for the first time, identified a mutant Na/K-ATPase that pumps but is defective in signal transduction via Src. This newly identified α1 mutant could serve as tool to fully assess the importance of Src-regulatory function of α1 Na/K-ATPase as well as the CTS-induced signaling in animal physiology, pathology and pharmacology.
4.2 Discussion and Future Directions

As these new findings provide long due answers, they also raise many new questions and fail to answer some old ones.

The exact molecular mechanism of Na/K-ATPase and Src interaction remains elusive. Although mutagenesis studies reveal some indications of molecular requirement for the interaction, the exact molecular bindings cannot be answered based on peptide or in-vitro studies. It is possible that individual peptides may bind to Src differently from the Na/K-ATPase molecule due to spatial restrictions. Ideally, a crystal structure and other molecular interaction studies are needed to reveal the Na/K-ATPase-Src interaction.

The newly generated cell systems LX-\(\alpha2\)-4 and LM-\(\alpha3\)-1 provide simple and basic cell systems to study the individual \(\alpha\) isoforms but in these cells, these isoforms may not behave in the same manner as they do in their native tissues. It will be more relevant and interesting to see functions of \(\alpha2\) in cardiac cells and \(\alpha3\) in neuronal cells. Still, LX-\(\alpha2\)-4 and LM-\(\alpha3\)-1 are cell line that can be used to study the role for these isoforms and more importantly, allow us comparative analysis with that of \(\alpha1\) expressing AAC-19, since all the three cell lines are derived from the same parental PY-17 cells.
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Chapter 2


Chapter 3


