Na/K ATPase: signaling versus pumping

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Na/K ATPase: Signaling Versus Pumping

Submitted by

Man Liang

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Na/K ATPase: Signaling Versus Pumping

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INTRODUCTION

Na/K-ATPase (or sodium pump) is an integral membrane protein found in most mammalian cells. It belongs to a family of evolutionarily ancient ATPases – P-type ATPase (Kaplan, 2002; Lingrel and Kuntzweiler, 1994). By keeping the electrochemical gradients across the plasma membrane, Na/K-ATPase plays a pivotal role in a variety of cellular functions. In recent years, more and more proteins such as lipid and protein kinases, membrane transporters, channels and cellular receptors have been identified to interact with Na/K-ATPase directly or indirectly. These findings have led to the proposal that Na/K-ATPase may also function as a signal transducer. Indeed, studies from many laboratories have now established that the binding of ouabain to this enzyme not only inhibits the ATPase activity, but also stimulates protein tyrosine kinases such as Src (Bertorello et al., 1991; Nelson and Veshnock, 1987; Tian et al., 2006; Yudowski et al., 2000). The activated Src in turn transactivates Epidermal Growth Factor Receptor (EGFR), resulting in the activation of multiple downstream signaling cascades such as the ERK1/2 and PLC-γ/PKC pathways (Haas et al., 2002; Yuan et al., 2005). Furthermore, it has been demonstrated that the signaling Na/K-ATPase resides with its partners in caveolae. This compartmentalized localization makes it possible for the Na/K-ATPase to interact and assemble with Src to form a functional receptor tyrosine kinase (Wang et al., 2004).

Since the activation of Src is essential for ouabain-induced changes in many cellular activities including the regulation of intracellular calcium concentration, gene expression, and cell growth (Aydemir-Koksoy et al., 2001; Liu et al., 2004;
Tian et al., 2001; Yuan et al., 2005), we have recently examined whether the Na/K-ATPase interacts directly with Src to form a functional signaling receptor complex (Tian et al., 2006). Using *in vitro* GST pull-down assays we have identified that the second and the third intracellular domains of the Na/K-ATPase α1 subunit interact with the Src SH2 and the kinase domains, respectively. Functionally, these interactions keep Src in an inactive state, and binding of ouabain to this inactive Na/K-ATPase/Src complex frees, and then activates the associated Src (Tian et al., 2006). These new findings suggest that the cellular Src-interacting Na/K-ATPase may play an important role in the regulation of basal Src activity and serve as a functional receptor for ouabain to stimulate protein tyrosine phosphorylation in live cells.

To test this hypothesis, we developed an siRNA-based assay to determine the effect of changes in the amount and properties of the Na/K-ATPase on both basal and ouabain-stimulated Src activity. This part of work is presented as the first manuscript in this dissertation. As expected, knock-down of the Na/K-ATPase α1 subunit by using siRNA-based approaches decreased the interaction of the Na/K-ATPase with Src, and resulted in an increase in basal Src activity and an inhibition of ouabain-induced signal transduction (Liang et al., 2006). Significantly, the enhanced basal Src activity also induced the increase of the phosphorylation of its direct effector focal adhesion kinase (FAK). Thus, the identification this pool of Src-interacting Na/K-ATPase has provided further physical evidence to support the capability of Na/K-ATPase in cell membrane to carry out non-canonical functions such as signal transduction and scaffolding in
addition to performing its canonical pumping function. The existence of different functional pools of Na/K-ATPase could be one of the regulatory formats utilized by cells to better conduct multiple functions concordantly.

To seek further evidence for the existence of the “non-pumping” pumps, we have characterized the ion-pumping properties of the Na/K-ATPase in the established α1 knockdown cells. The mismatch between the pumping activity and the amount of Na/K-ATPase molecule clearly demonstrate that over 65% of the plasma membrane Na/K-ATPases in LLC-PK1 cells are not involved in ion pumping. This “non-pumping” pool of Na/K-ATPase, like the pumping pump, binds ouabain. Depletion of either cholesterol or caveolin-1 is able to convert some of, if not all, “non-pumping” Na/K-ATPases into the pumping pump. Graded knock-down of the α1 subunit results in the loss of the non-pumping pool and the diminished ouabain induced activation of Src and/or ERK. These new findings indicate that, at least in some types of cells, there are a substantial amount of surface-expressed Na/K-ATPases which do not transport ions across the cell membrane, but function as ouabain-binding receptor to convert the extracellular ouabain signal into intracellular second messengers.

In order to gain some insights into the potential role of Na/K-ATPase in other signaling pathways such as EGFR mediated signal transmission, we also examined the EGF induced EGFR phosphorylation in the established Na/K-ATPase knock-down cells. The data presented in the third manuscript suggested that the knock-down of Na/K-ATPase can increase the EGF induced EGFR phosphorylation on several tyrosine residues and these potentiating effects can
be reversed by knocking in either wild type α1 subunit or its pumping null mutant. So far, the mechanisms underlying these processes are still unknown.
LITERATURE

THE IDENTIFICATION OF NA/K-ATPASE:

The story of the discovery of Na/K-ATPase started half a century ago. It was known as early as the 1920s that the ion composition within living cells is different from that in the surroundings. The cytosol of animal cells contains 20 times higher concentration of potassium ions (K\(^+\)) than that in the extracellular fluid. Conversely, the extracellular fluid contains a concentration of sodium ions (Na\(^+\)) as much as 10 times greater than intracellular. To explain how this ionic gradient across the membrane can be maintained, the concepts of active transport and hypothetic existence of an active “sodium pump” were developed in the 1940s and first half of the 1950s (Ussing, 1953). In 1953, Hodgkin and Keynes found that the transport of sodium out of the cell is coupled to a transport of potassium into the nerve cell (Hodgkin and Keynes, 1953). In 1954, Gardos discovered that ion pumping in red blood cell ghost was supported by ATP, which in turn became hydrolyzed (Gardos, 1954). However, the nature of the “sodium pump” started to come onto the stage until 1957, when J.C. Skou published his Nobel Award winning paper demonstrating that there is a link between transport of Na\(^+\) and K\(^+\) across the plasma membrane and a Na\(^+\) and K\(^+\)-activated ATPase activity in nerve tissues. He also found that ATPase actually promotes the directed transport of Na\(^+\) and K\(^+\) through the membranes (Skou, 1957). Since this initial identification of the Na/K-ATPase, a tremendous scientific effort has been devoted to elucidate the details of various aspects of Na/K-ATPase, including structural, functional and regulatory properties.
STRUCTURAL PROPERTIES OF NA/K-ATPASE:

Na/K-ATPase is a heteromeric, integral membrane protein found in most mammalian cells. It belongs to the P-type ATPase, a family of enzymes that is distinguished from other ATP-dependent ion pumps by involving the formation of a phospho-intermediate of the enzyme itself during the ion transporting cycle. The phosphorylation occurs on the aspartyl carboxyl group which is located in a highly conserved amino acid motif DKTGS/T (Moller et al., 1996) of the catalytic subunit (or α subunit). P-type ATPases have been identified in almost all organisms studied thus far. A variety of charged substrates such as K⁺, Na⁺, H⁺, Mg^{2+}, Ca^{2+}, Cu^{2+}, Cd^{2+} and phospholipids were transported through P-type ATPases. Different organisms contain various numbers of P-type ATPases, from very few in parasitic bacteria to 7 to 9 in free living bacteria to more than 30 in plants and animals. Unlike other P-type ATPase family members, which contain only one subunit, the Na/K-ATPase and its closely related cousin, the gastric H/K ATPase, have an additional regulatory β subunit.

α Subunit:

The α subunit is composed of approximately 1000 amino acids with a molecular mass of about 112 kDa. As the catalytic subunit, it spans the plasma membrane 10 times and keeps both the C and N terminals in the cytoplasm. Based on the structural similarity between the P-type ATPase family members and especially the high resolution structure of the Ca-ATPase of the
sarcoplasmic reticulum (SERCA) which is homologous to Na/K-ATPase, four distinct functional domains have been identified in the \( \alpha \) subunit (Sweedner and Donnet, 2001). The trans-membrane domain (T) is composed of a series of \( \alpha \) helices. The actuator (A) domain consists of the N-terminal tail of about 90 amino acids and the first cytoplasmic loop connecting the second and third transmembrane helices of about 120 residues. The highly conserved phosphorylation (P) domain and relatively isolated nucleotide binding (N) domain are located in the central loop formed by the forth and fifth transmembrane helices, which contains 430 amino acids. The P and N domains are responsible for ATP phosphorylation and binding, respectively. Since the distance between them is rather large, P and N domains must approach to each other before the ATP hydrolysis. This process might be induced by several conformational transitions. In addition to ATP, \( \alpha \) subunit also contains binding sites for Na\(^+\), K\(^+\), and cardiac glycoside such as ouabain, a highly specific ligand (or inhibitor) of the enzyme, and mediates the interaction of Na/K-ATPase with many other proteins (Kaplan, 2002; Lingrel and Kuntzweiler, 1994).

**Cation Binding Sites:**

Chemical modification and site-directed mutagenesis studies have shown that the amino acid with negatively charged and oxygen containing side chains as well as carbonyl groups of the polypeptide backbone in the forth, fifth, and sixth transmembrane helices of the of \( \alpha \) subunit are involved in Na\(^+\) and K\(^+\) binding. However, it is still unknown whether other domains or functional groups
are also involved in the gating system to facilitate the access of these ions to their binding sites (Lingrel and Kuntzweiler, 1994).

**Ouabain Binding Sites:**

Based on the random mutagenesis experiments, the inhibitory effects of ouabain on Na/K-ATPase can be affected by mutating different residues in the α subunit. The first extracellular loop connecting the first and second tranmembbrane domain, together with the regions encompassing from the forth to the eighth transmembrane helixes, influences the ouabain sensitivity and may represent the major binding sites for the cardiac glycoside class of drugs (Croyle et al., 1997; Price and Lingrel, 1988). By now, little direct information is available about the cardiac glycosides binding site on Na/K-ATPase.

**Protein-Protein interaction domains:**

Since both A and N domains are highly exposed, many specific motifs reside in these regions enable Na/K-ATPase to directly interact with various proteins (Xie and Cai, 2003). Two conserved caveolin-binding motifs (e.g, ΦxxΦxxxxΦ and ΦxΦxxxxΦ) in the α subunit of mammalian Na/K-ATPase, where Φ represents an aromatic amino acid residue, facilitate the interaction between α subunit and caveolin-1 protein. These two motifs are located at the border of cytoplasmic side of the first transmembrane helix and at the extracellular portion to the 10th transmembrane helix. A conserved proline rich (PR) motif at the N-terminus of α subunit was found to mediate the association of Na/K-ATPase with the SH3 domain of the Phosphatidylinositol-3'-kinase (PI-3K) (Yudowski et al., 2000). Some other functional domains also have been identified to be involved in
the interaction of the enzyme with phospholamban-like proteins, PKA, PKC, ankyrin, adducin, and cofillin (Beggah and Geering, 1997; Feschenko et al., 1997; Jordan et al., 1995; Lee et al., 2001). With respect to the topic of this dissertation, more detailed information regarding the functional domains involved in the interaction between α1 subunit and Src kinase will be discussed later. These functional domains and/or specific motifs constitute the structural basis for Na/K-ATPase to transduce ouabain signal through protein-protein interactions.

**α Isoforms:**

The classification of α subunit isoforms was based on the electrophoretic mobility of the polypeptide and the relative sensitivity toward the cardiac glycoside. At least four different and highly conserved α subunits have been identified so far. The expression of these α subunit isoforms is displayed in a tissue or developmental specific manner (Lingrel, 1992; Sweadner, 1991). The α1 isoform is universally expressed in virtually all kinds of cells and serves as the dominant form in mammal kidney. However, the liver is relatively low in α1 expression and expresses no other known α isoforms (Gick et al., 1993). The α2 subunit is found in fatty tissue, skeletal muscles, brain and cardiac myocyte. The α3 is mainly expressed in neuronal tissue and brain. The α4 is a testis specific isoform. A developmentally regulated transition in α2 and α3 expression was observed in myogenesis. The α3 mRNA was expressed primarily in fetal and neonatal heart tissue and then replaced by α2 in juvenile and adult tissue (Orlowski and Lingrel, 1988).
Different isoforms of the α subunit may have different affinities for Na⁺, K⁺ and cardiac glycoside. In HeLa cells expression system, rat α1 and α2 show similar affinity for Na⁺ (K₁/₂ = 17-19 mM) and K⁺ (K₁/₂ = 0.2 mM), whereas the α3 isoform shows considerably lower affinity for Na⁺ (K₁/₂ = 63 mM) and higher affinity for K⁺ (K₁/₂ = 0.09 mM). While the rat α1 is relatively resistant to ouabain binding (Kᵢ = 1-5 X10⁻⁵ M), the α2 and α3 isoforms are very sensitive (Kᵢ = 1-5 X10⁻⁷ M). However, in human there is no notable distinction in ouabain sensitivity among various α isoforms. (Munzer et al., 1994). Since more than one isoform can coexist in certain tissues, typically muscle, heart or brain, this specific expression pattern of the α isoforms in these tissues may provide the basis for the fine tuning of the pump activity responding to various physiological conditions.

β Subunit:

β subunit (~35 kDa) is composed of about 370 amino acids. It belongs to the type II membrane proteins featured by one transmembrane segment, a short N-terminal, cytoplasmic tail, and a large extracellular C-terminal ectodomain which interacts with the extracellular loop of α subunit connected by the seventh and eighth transmembrane helices. There are three consensus N-glycosylation sequences in the ectodomain and the apparent molecular mass of β is about 60 KD when these three sites are fully glycosylated. Mutations in N-linked glycosylation sites of β subunit can induce the decreased ability of β subunit to assemble with α subunit without affecting the apparent K⁺ and ouabain affinities
to the enzyme (Beggah et al., 1997). The presence of three consecutive disulfide (S-S) bonds in the ectodomain constitutes another signature feature of the β subunit family.

The primary functional role of the β subunit is to act as a specific chaperone assisting the structural and functional maturation of α subunits. The β subunits facilitate the translocation of α subunit from endoplasmic reticulum (ER) to plasma membrane as well as ensure the correct membrane insertion of the protein complex. In the absence of association with β subunit, α subunit undergoes rapid degradation. A role in ouabain and K+ binding to α subunit also has been proposed for β subunit. Co-expression of rat H/K ATPase β subunit in yeast cells with sheep Na/K-ATPase α1 subunit or rat α3 subunit led to the increase of the ouabain-binding affinity in yeast membranes (Eakle et al., 1992). The β subunit also contributes to the intrinsic transport properties of the α subunit. The reduction of S-S bridges in the ectodomain of the β subunit in the purified renal Na/K-ATPase leads to more than 50% decrease of the pumping activity (Lutsenko and Kaplan, 1993).

β Isoforms:

Three β isoforms have been characterized: β1 is the most widely distributed isoform, but it is absent from certain astrocytes and ear vestibular cells (Cameron et al., 1994; McGuirt and Schulte, 1994); β2 is found in excitable tissues such as muscle and nerve. It also functions as an adhesion molecule in glial cells (Gloor et al., 1990); β3 is specifically expressed in testis, adrenal gland
and brain. All β isoforms share a common basic structure and the transmembrane domain is the most highly conserved region among different isoforms. The α1 subunit, along with the β1 subunit, is found in nearly every tissue and α1-β1 complex is the predominant enzyme in kidney. The apparent K⁺ affinity of Na/K-ATPase can be regulated by β subunits in an isoform-specific manner (Blanco and Mercer, 1998). However, the differential physiologic importance of these β isoforms is still unknown.

**γ Subunit:**

In addition to α and β subunit, a third subunit γ (7-11kDa) has been identified in purified preparations of Na/K-ATPase (Reeves et al., 1980). This subunit belongs to type I membrane proteins and contains a signature FXYD motif in the N-terminus and several conserved glycine and serine residues in the trans-membrane domain. The γ subunits have been found in many species, like rat, human and *Xenopus laevis* in a tissue specific expression pattern (Beguin et al., 1997; Kim et al., 1997; Mercer et al., 1993). In order to be stably expressed and transported efficiently to the plasma membrane, γ subunit needs to be associated with the assembled α-β complex of Na/K-ATPase through interacting with the C-terminal domain of the α subunit. It has been suggested that γ subunit can help to stabilize certain conformation of the Na/K-ATPase by increasing the affinity of the enzyme to ATP (Therien et al., 2001) although γ subunit was not required for the catalytic function of Na/K-ATPase (Scheiner-Bobis and Farley,
Recent studies also have shown that γ subunit can modulate the K$^+$ activation of Na/K-ATPase expressed in *Xenopus* oocytes (Beguin et al., 1997).

Recently, additional three members of the FXYD family, including phospholemmann (PLM), corticosteroid hormone induced factor (CHIF) and FXYD7, have been found to interact with Na/K-ATPase and regulate the pump kinetics in different expression systems (Beguin et al., 2002; Crambert et al., 2002; Garty et al., 2002). The mechanistic details underlying these regulatory processes are still far from being understood fully.

**THE ION PUMPING FUNCTION OF NA/K-ATPASE:**

**The Catalytic Mechanism:**

Accompanying with the phosphorylation-dephosphorylation process, Na/K-ATPase binds, occludes, and transports ions by switching between two different cation dependent conformations, called E1 and E2. The E1 form has intracellular facing cation binding sites with high affinity for both Na$^+$ and ATP. Binding of the intracellular Na$^+$ catalyzes the phosphorylation of the enzyme by previous bound ATP and produces the E1P form. The following conformation transition from E1P to E2P exposes the Na$^+$ ions to the outside of the cell, where they are released. The E2P form of the enzyme shows high affinity for K$^+$ at the outer surface and binding K$^+$ ions can dephosphorylate the enzyme from E2P to E2 form. K$^+$ ions are then released from the enzyme at the inner surface and the enzyme returns from E2 to E1 and is ready to initiate a new reaction cycle. By
hydrolyzing one molecule of ATP, Na/K-ATPase pumps 3 Na$^+$ out and 2 K$^+$ ions in during one catalytic cycle.

**The Cellular Functions Associated with Ion Pumping:**

In a resting animal, greater than 20% of the cellular ATP production is consumed by the Na/K-ATPase to carry out the coupled extrusion of Na$^+$ and uptake of K$^+$ across the membrane. In tissues such as kidney and brain, which contain a large amount of Na/K-ATPase, as high as 60% of resting metabolism could be attributed to the ion pumping activity (Clausen et al., 1991; Rolfe and Brown, 1997). The electrical and chemical gradients generated through this process render the Na/K-ATPase to be the center of a constellation of fluxes of a wide variety of solutes which involved in different cellular functions, including metabolism, pH control, cell volume regulation, excitability and etc.

In neurons, by maintaining the resting membrane potential, Na/K-ATPase makes the action potential and neuronal transmission possible (Hodgkin and Huxley, 1952); In skeletal muscle cells, continuous stimulation of muscle fibers during exercise leads to the dissipation of the ionic gradient necessary for muscle contraction. Rapid activation of Na/K-ATPase activity under these conditions plays a very important role in delaying the muscular fatigue and reducing the toxic effect induced by high concentration of plasma K$^+$ (Clausen, 1996).

In addition, the ionic gradients maintained by Na/K-ATPase have provided the driving force for many Na$^+$ coupled transport systems, which regulates the uptake and extrusion of other ion species, glucose and amino acids or other
nutrients. In epithelia, the polarized distribution of Na/K-ATPase made the directional transport of solutes across the epithelial cell layer possible. The osmotic gradient created by the translocation of Na⁺ from one side of the epithelium to the other side drives the water absorption in intestine and the reabsorption in kidney (Caplan, 1997).

Recent studies also revealed a crucial role for Na/K-ATPase in maintaining the integrity and function of tight junctions, which are the multi-protein complexes located at the boundary of apical and basolateral plasma membrane domains of neighboring epithelial cells. Inhibition of Na/K-ATPase activity by ouabain or by K⁺ depletion prevents the formation of tight junction in Madin-Darby canine kidney (MDCK) cells (Rajasekaran and Rajasekaran, 2003; Rajasekaran et al., 2001a, b). No apparent effect on the formation of the cortical actin ring has been detected in epithelia cells after the pump inhibition. However, the pump activity seems to be involved into the organization of stress fibers (Rajasekaran et al., 2001a, 2003).

**Cardiac Glycosides And Cardiac Contractility:**

Cardiac glycosides, or cardiotonic steroids, are widely used to increase the contractile strength of the heart. They consist of a group of chemicals that specifically bind to Na/K-ATPase, such as the plant-derived digitalis drug digoxin or ouabain and the vertebrate-derived aglyconebufalin (Dmitrieva and Doris, 2002). Some cardiac glycosides are also endogenous steroids released by hypothalamus and adrenal gland (Schoner, 2002). The traditional explanation for
the positive inotropic effects of these drugs is that partial inhibition of sodium pump activity by ouabain in cardiac myocytes results in a modest increase in intracellular Na\(^+\) concentration, which, in turn, leads to a significant increase in intracellular Ca\(^{2+}\) concentration by reducing the extrusion of Ca\(^{2+}\) from the cell via Na/Ca exchanger and the subsequent Ca\(^{2+}\) release from the sarcoplasmic reticulum. The elevation of the intracellular Ca\(^{2+}\) concentration results in the increase in force of contraction of cardiac muscle (Schwartz et al., 1988). The excess intracellular Ca\(^{2+}\) is then taken up back into the sarcoplasmic reticulum (SR) through the sarco-endoplasmic reticulum Ca-ATPase. The increased intracellular storage of Ca\(^{2+}\) can further strengthen the subsequent contractions.

Several lines of evidence also imply that the mechanisms underlying the enhanced cardiac contractility by cardiotonic drugs are more complex than what we thought. McGarry found that 0.1nM digoxin appear to activate the sarcoplasmic reticulum Ca-release channels, which may serve as the potential direct target for cardiac glycosides in cardiac inotropy in addition to Na/K-ATPase (McGarry and Williams, 1993). In response to cardiac glycoside, the slip mode conductance of the Na\(^+\) channel may contribute significantly to local and global cardiac Ca\(^{2+}\) signaling independent of both Na/K-ATPase and Na/Ca exchanger (Santana et al., 1998). Furthermore, by serving as a hormone receptor, Na/K-ATPase itself can deliver message from the ouabain-liganded enzyme to the cytoplasm or nucleus via protein-protein interactions. Multiple growth related signaling pathways are activated through this process (Haas et al., 2000, 2002; Kometiani et al., 1998). Significantly, some of the ouabain-activated signaling
pathways (e.g., Src and ERKs, extra-cellular signal regulated kinase) are essential in the regulation of intracellular Ca^{2+} (Tian et al., 2001) and the following increase in contractility. Both the ion pumping function and the signal transduction function of Na/K-ATPase are involved in the classic digitalis effects on the heart.

**THE SIGNAL TRANSDUCTION FUNCTION OF NA/K-ATPASE:**

It has been known for a long time that Na/K-ATPase is capable of controlling expression of its own genes (Boardman et al., 1974). The chronic inhibition of Na/K-ATPase by ouabain leads to the increased functional pump expression in the plasma membrane. This adaptive up-regulation has been generally ascribed to the intracellular ion concentration changes occurred following the pump inhibition. However, several later observations on lymphocytes clearly indicated that the growth-related effects of ouabain in cells could be dissociated from the changes of intracellular ion concentrations induced by ouabain (Brodie et al., 1995; Szamel and Resch, 1981). To explore the possible role of Na/K-ATPase in the heart hypertrophy and understand how the cardiac Na/K-ATPase and its clinically used specific inhibitors, the digitalis drugs, are involved in this non-proliferative growth process, our laboratory and others have focused on mapping the signaling pathways coupled with Na/K-ATPase and then clarifying the mechanisms involved in interactions of Na/K-ATPase with its signaling partners.
In cardiac myocytes and many other cells, the signaling pathways that are rapidly elicited by the binding of ouabain to Na/K-ATPase include: (1) activation of Src tyrosine kinase followed by Src-induced transactivation of EGFR and recruitment of adaptor protein Grb2 and Ras (Haas et al., 2002). This recruitment subsequently leads to the activation of Ras, which is independent of ouabain-induced rise in Ca\(^{2+}\) or Na\(^+\) concentration. (2) Activated Ras leads to two branched pathways, one is increased mitochondrial reactive oxygen species (ROS) generation and the other is activation of the Ras/Raf/MEK/ERK cascade (Haas et al., 2002; Liu et al., 2000). Ouabain induced activation of PKC is also necessary for ERK activation. These events can result in an increase in both systolic and diastolic Ca\(^{2+}\) in cardiac myocytes (Tian et al., 2001) and Ca\(^{2+}\) oscillation in renal epithelial cells (Aizman et al., 2001). Most recent studies also demonstrated the role of ouabain-bound Na/K-ATPase in tethering PLC-\(\gamma\) and IP3 receptors to regulate Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) store (Yuan et al., 2005). (3) related downstream events include the expression of some early response proto-oncogene and activation of transcription factors AP-1 (activator protein 1) and NF-kB, which eventually modulates cell growth and gene expression in targeted cells (Kometiani et al., 1998). Notably, most of these signaling events activated by ouabain are independent of changes in intracellular ion concentrations, but depend on the direct interactions of the Na/K-ATPase with its signaling partners (Liu et al., 2000).

Interestingly, in both cardiac myocytes and renal epithelial cells, the above ouabain-activated events originated from the caveolar Na/K-ATPase. Disruption
of caveolae structure by depletion of either cholesterol or caveolin-1 inhibited ouabain-induced signal transduction (Liu et al., 2003; Wang et al., 2004; Yuan et al., 2005).

THE REGULATION OF NA/K-ATPASE:

The regulation of Na/K-ATPase is a complex process occurring at many different levels. Several factors and multiple underlying mechanisms have been identified in regulating Na/K-ATPase activity. For example, the Na/K-ATPase activity can be regulated by changing the substrate concentrations; by reorganizing the subunit composition or other associated components of Na/K-ATPase; by varying the Na/K-ATPase localization and/or its expression level; by a series of signaling events featured by phosphorylation, de-phosphorylation and protein-protein interactions. A variety of hormones have been shown to execute both acute (short term) and chronic (long term) regulation on Na/K-ATPase through these mechanisms.

Substrate Regulation:

Na/K-ATPase is activated by intracellular Na\(^+\), ATP and extracellular K\(^+\). So, the concentrations of these substrates serve as the most direct and simplest determinants of the enzyme activity. The concentration of intracellular Na\(^+\) required for half-maximal activation of the enzyme (K\(_{1/2}\)) ranges from below 10mM in red blood cells and tight epithelia, such as urinary bladder or choroid plexus, to almost 40 mM in leaky epithelia, such as kidney proximal tubule (Eaton,
1981; Saito and Wright, 1982; Soltoff and Mandel, 1984a). These \( K_{1/2} \) values are usually higher than the cytosolic \( Na^+ \) concentrations in the corresponding cell type during resting state. A small change in \( Na^+ \) will be sufficient to elicit dramatic increase or decrease of the enzyme activity. External \( Na^+ \) has only a minimal regulatory effect on pumping activity even though it may slow the turnover of the sodium pump by inhibiting the dephosphorylation reaction (Blostein, 1979).

In contrast, the \( K_{1/2} \) of Na/K-ATPase for ATP is between 0.22 mM to 0.8 mM in a variety species. Since the cytosolic ATP concentration in most cells (> 1mM) is saturating for Na/K-ATPase, a modest shift in ATP affinity should not lead to dramatic effects and it is almost impossible to detect the ATP-dependent enzyme activity. However, in certain circumstances, changes in ATP level may elicit signals that could in turn regulate the Na/K-ATPase activity. Under the anoxic (or hypoxia) conditions, even with the demonstrated saturation of ATP, a linear and non-saturating dependence of the Na/K-ATPase activity on the ATP concentration could still be observed in kidney proximal tubule (Brezis and Rosen, 1995; Soltoff and Mandel, 1984b). Aw and Jones also found that in heptocytes, under conditions where the cellular ATP concentration was lowered to 40% of control, \( ^{86}Rb^+ \) uptake was nearly zero (Aw and Jones, 1985).

The ATP concentration appears to affect the affinity of Na/K-ATPase for \( K^+ \) (Hastings and Skou, 1980). However, the relative high affinity of Na/K-ATPase for extracellular \( K^+ \) precluded the significant role of \( K^+ \) in the regulation of enzyme activity.
**Regulation By Membrane Lipids:**

Aside from the isoform specific differences in the relative affinity for different enzyme substrates, the membrane milieu, where the Na/K-ATPase resides, also contribute to the regulation of sodium pump kinetics in a species and tissue specific manner. The membrane phospholipid or fatty acid composition, as well as cholesterol content vary from one type of cell to another. Most of the studies so far have suggested that the regulatory effect of membrane lipids on Na/K-ATPase activity is mainly related to the essential role of lipids in keeping the membrane integrity, fluidity and thickness and thus to promote the optimal Na/K-ATPase activity.

Recent functional reconstitution experiments involving lipid crossovers between species clearly demonstrated the role of membrane lipid composition as one of the major determinants of the enzyme activity (Cornelius, 1991). The removal of 80-90% of membrane phospholipids from cardiac membrane with detergents led to the 95-100% inhibition of the Na/K-ATPase activity (Hegyvary et al., 1980), whereas the presence of the phospholipid vesicle composed of phosphatidylserine or phosphatidylglycerol is able to reactivate the delipidized preparation of Na/K-ATPase (Kimelberg and Papahadjopoulos, 1972).

Free fatty acids presented in the membrane have been shown to inhibit the Na/K-ATPase activity by altering the Na$^+$ affinity and other enzyme kinetic properties (Gerbi et al., 1993, 1994). In heart, the inhibitory effects of free fatty acids on Na/K-ATPase have been observed both in vivo and in vitro (Karli et al., 1978; Lamers and Hulsmann, 1977). Furthermore, higher concentrations of
Na/K-ATPase have been detected in liver and kidney obtained from rats chronically fed on essential fatty acid-deficient diets, whereas the affinity of the binding sites for ouabain remained unchanged (Lin et al., 1979).

Probably partially due to the relationship between cholesterol and cardiovascular disease, the effects of cholesterol on sodium pump activity have been studied most extensively (Grundy, 1988). It has been reported that rabbit lens fiber cells, which contain higher membrane cholesterol content than lens epithelial cells, showed less Na/K-ATPase activity even with higher expression of Na/K-ATPase $\alpha_1\beta_1$ polypeptide than that in the lens epithelial cells (Dean et al., 1996). It is well accepted that cholesterol can reduce the fluidity of phospholipids fatty acyl chains, decrease the molecular motion or conformational freedom of Na/K-ATPase and thus result in the enzyme activity inhibition (Papahadjopoulos et al., 1973). Claret et al. also have suggested that at least some of the effects induced by cholesterol depletion on enzyme activity are due to the alteration of the apparent affinity of Na/K-ATPase for internal Na$^+$ (Claret et al., 1978). However, in some other systems, the stimulatory effects of cholesterol on Na/K-ATPase activity also have been noticed. One of the possible mechanisms may be the direct interaction of cholesterol, the essential positive effecter, with the Na/K-ATPase, although no firm evidence has been provided to support this hypothesis (Giraud et al., 1981; Yeagle, 1983).

In red blood cell, the progressive cholesterol depletion can induce a biphasic response in the pumping activity of Na/K-ATPase. The stimulatory effects were induced by moderate depletion and the inhibitory effects were
associated with larger cholesterol depletion. The cross-over between these two responses occurred at 25-35% depletion (Lucio et al., 1991). The biphasic regulation by membrane lipid also has been observed in the studies of Na/K-ATPase activity in rabbit renal medulla membrane vesicles, as well as in the glucose transport in human erythrocytes and 3T3 mouse fibroblasts (Yeagle et al., 1988; Yuli et al., 1981). Despite the fact that under physiological conditions, cholesterol reduces the cation translocation rate and increases the selectivity of the pump for internal Na\(^+\), the existence of the specific lipid domains on the plasma membrane such as lipid rafts and caveolae could also contribute to this biphasic effect of lipid depletion on the Na/K-ATPase activity. The modest cholesterol depletion probably only released the regional restricting effects on Na/K-ATPase located in the lipid enriched area, whereas the larger depletion may cause the instability of the whole membrane and thus the collapse of the molecular working of the enzyme.

For a long time, people have found that many clinical syndromes are associated with the alterations in plasma lipid composition and sodium pump activity. For example, the elevated erythrocyte cholesterol content in renal failure has been reported to correlate with and account in part for the lowered Na/K-ATPase activity in this syndrome (Fervenza et al., 1989). However, the physiological or pathological importance of the regulation of Na/K-ATPase by membrane lipid remains unclear.
**Regulation by Phosphorylation:**

It is well established that Na/K-ATPase activity can be regulated by tyrosine kinases and serine/threonine kinases, including cAMP-dependent protein kinase (PKA), protein kinase C (PKC) and cGMP-dependent protein kinase (PKG) in a tissue and species-specific manner. Through direct phosphorylation of pump catalytic subunit and other indirect mechanisms, these kinases can affect Na/K-ATPase activity at different levels.

The PKA has been shown to phosphorylate the Na/K-ATPase at Ser-943 and result in the inhibition of enzyme activity in both *in vitro* and *in vivo* studies (Fisone et al., 1994). In rat renal proximal tubules, both an increase and decrease of the pump activity upon PKA activation have been observed (Carranza et al., 1996; Hussain et al., 1997). Besides direct phosphorylation of the enzyme, several other mechanisms have been suggested to be involved in the PKA regulatory effects on pump activity. In renal tubule cells, the activation of a protein phosphatase 1 (PP1) inhibitor by PKA also was involved in the regulation of Na/K-ATPase activity (Aperia et al., 1991). In other systems, by altering the function of Na\(^+\) transporters, PKA is able to induce the intracellular Na\(^+\) changes and thus lead to the alteration of Na/K-ATPase activity (Hughes et al., 1988). There is also evidence to support the notion that the number of the plasma membrane sodium pump is also under the control of PKA. Taub et al. have observed the increased membrane pump expression upon PKA activation in MDCK cells (Taub et al., 1992).
A similar role for PKC in inhibiting or stimulating Na/K-ATPase activity has been documented both in vivo and in vitro in rat hepatocyte, human lymphocytes, opossum kidney cells and many other tissues (Lynch et al., 1986; Middleton et al., 1993). The direct phosphorylation of the enzyme by PKC not only induces the conformational change of Na/K-ATPase, which affects the catalytic turnover rate of the enzyme or the apparent affinity of the enzyme for Na\(^+\) and K\(^+\), but also leads to the endocytosis of pumps as observed by Chibalin et al. in isolated proximal tubule cells and a cell line derived from opossum kidney (Chibalin et al., 1998).

Cheng et al. have suggested that the inhibitory or stimulatory effect of PKA/PKC on sodium pump may be determined by the intracellular Ca\(^{2+}\) concentration, even though the mechanism under which Ca\(^{2+}\) modulates the activity of Na/K-ATPase remains to be elucidated (Cheng et al., 1999).

Similarly, PKG also have been found to either inhibit the Na/K-ATPase activity in brain or skeletal muscle, or activate the enzyme activity in duck salt gland, or exert both of the opposite effects on kidney Na/K-ATPase (Li and Sperelakis, 1993; Pontiggia et al., 1998; Scavone et al., 1995; Stewart and Sen, 1981; Syren, 1997). So far, the precise mechanism of PKG regulation is still unknown.

**Regulation Through Protein-protein Interactions:**

In addition to the protein kinases mentioned above, Na/K-ATPase is also directly or indirectly associated with various membrane or peripheral membrane
proteins including: lipid kinases, cytoskeleton proteins, membrane transporters as well as cellular receptors.

**PI-3K:**

Phosphatidylinositol-3'-kinase (PI-3K) is an important lipid kinase comprised a 110 kD catalytic subunit (p110) and an 85 kD regulatory subunit (p85). p85 subunit contains SH2 and SH3 domains which mediate the interaction of PI3K with Na/K-ATPase and other cellular proteins. This interaction is enhanced by the phosphorylation of Na/K-ATPase by PKC on Ser-18 which in turn leads to the increase of PI3K activity. The activation of PI3K is essential to the dopamine-induced decrease of Na/K-ATPase activity in kidney proximal tubules by facilitating the internalization of enzyme through clathrin-dependent endocytic pathway (Yudowski et al., 2000). Conversely, in vascular smooth muscle cells (VSMC), low K\(^+\) induced up-regulation of membrane expression of Na/K-ATPase can be blocked by PI3K inhibitor, which implies a potential role of PI3K in translocation of sodium pump from intracellular stores to the cell membrane (Aydemir-Koksoy and Allen, 2001).

**Cytoskeleton:**

Cytoskeleton is lattice like network of proteins underlying the cell membrane. This dynamic cytoplasmic filamentous structure exists in virtually all types of cells. The association of Na/K-ATPase with cytoskeleton proteins, including ankyrin, fordrin (an actin-binding protein analogous to erythrocyte spectrin), actin, adducin and etc, plays an essential role in transporting and positioning Na/K-ATPase to appropriate membrane compartments (Kashgarian
et al., 1988; Koob et al., 1990; Nelson and Veshnock, 1987). In nonpolarized cells, Na/K-ATPase is randomly distributed over the plasma membrane, while in polarized cells such as kidney proximal tubules and intestinal epithelium, Na/K-ATPase is usually found along the basolateral membrane due to the association with ankyrin and fordrin (spectrin) (Amerongen et al., 1989; Morrow et al., 1989). In retinal pigment epithelial cells, Na/K-ATPase also can be re-localized to apical membrane domain through the interaction with a different set of cytoskeleton complexes (Huotari et al., 1995; Nelson et al., 1990).

The direct interaction between Na/K-ATPase and ankyrin has been observed in different cell types. Two specific ankyrin binding domains in Na/K-ATPase α1 subunit have been identified (Devarajan et al., 1994). Through the association with ankyrin, Na/K-ATPase can be attached to the subcortical cytoskeleton proteins including fordrin (spectrin) and actin. The spectrin-ankyrin complex is required for the transport of Na/K-ATPase from the endoplasmic reticulum to the Golgi apparatus in MDCK cells (Devarajan et al., 1997).

Recently, a role for cytoskeleton proteins in regulating Na/K-ATPase activity also has been suggested. Addcuin, an spectrin binding protein that interacts with Na/K-ATPase directly, can affect the apparent ATP binding affinity and stimulate Na/K-ATPase activity (Ferrandi et al., 1999). Similarly, by altering the rates of conformational transitions, ankyrin may exert a stimulatory effect on pump catalysis (Ferrandi et al., 1999; Mays et al., 1995). Actin has been shown to activate the enzyme activity through a PKA mediated pathway (Cantiello, 1995, 1997). The association of Na/K-ATPase with cofilin, a pH sensitive actin
depolymerization protein, results in the enhanced pump activity measured by \(^{86}\text{Rb}^+\) uptake, although the molecular mechanism underlying such regulation is still unknown (Lee et al., 2001).

**Hormone Regulation:**

The regulation of Na/K-ATPase in various tissues is under the precise control of a number of circulating hormones through either a short-term or a long-term action. Through a variety of intracellular signaling pathways, catecholamine, peptide hormone and some of the steroid hormones can exert acute effects on pump activity in response to sudden perturbation of the environment. The short term regulation usually affects the enzyme kinetic properties including the pump turnover rate and the affinity to substrates. It also modulates the Na/K-ATPase trafficking between the reserved intracellular pool and the different functional pools of pumps in the plasma membrane, which eventually determines the availability of the enzyme. Long-term regulation of pump capacity by steroid or thyroid hormones is mainly due to their effects on gene transcription as well as the stability of the corresponding mRNAs and proteins. Major hormonal controls over pump activity are summarized as follows:

**Thyroid Hormones:**

The regulation of Na/K-ATPase activity by thyroid hormones is isoform specific and varies among tissues (Horowitz et al., 1990). It has been well accepted that thyroid hormone stimulates Na/K-ATPase activity by increasing the expression of the sodium pump instead of by changing the catalytic properties of
the enzyme. Upon the thyroid treatment, the elevation of both mRNA and protein level of Na/K-ATPase have been observed in skeletal muscle and kidney (Azuma et al., 1993; McDonough et al., 1988).

**Aldosterone:**

Aldosterone is a steroid hormone synthesized and released by the adrenal cortex. Both rapid and sustained increases in membrane pump numbers upon aldosterone treatment have been observed.

The rapid stimulation of pump activity by aldosterone is a Na$^+$-dependent process. The increased intracellular Na$^+$ concentration induced by the Na$^+$ entry through Na$^+$ channel or Na/H exchanger upon aldosterone treatment can’t only accelerate the single membrane pump turnover rate, but also recruit previously inactive or reserved pump units into the membrane, which may be facilitated by the microtubular network.

Aldosterone can also induce the synthesis of new α and β subunits. This long term effects of aldosterone on Na/K-ATPase have been found in a variety of tissues originated from different species such as the kidney and heart from rat and the colon from rabbit. Even though the effects of aldosterone on Na/K-ATPase in most tissues are mainly through the regulation of α1 and β1 expression, the specific induction of α3 isoform has also been observed in rat brain hippocampus (Farman et al., 1994).

**Insulin:**

In addition to regulating carbohydrate metabolism, insulin plays a pivotal role in Na$^+$ and K$^+$ homeostasis and has multiple effects on the Na/K-ATPase
activity. The increased uptake of K\(^+\) by various tissues induced by insulin is mainly due to the stimulation of Na/K-ATPase activity.

The short term effects of insulin on Na/K-ATPase are mainly through the following mechanisms: (1) inducing the rapid translocation of the sodium pumps from the intracellular stores to the cell surface (Omatsu-Kanbe and Kitasato, 1990); (2) stimulating Na/K/2Cl co-transporter to increase the cytoplasmic Na\(^+\) concentration and activate the latent pumps already present in the membrane (Grinstein and Erlij, 1974); (3) phosphorylating Na/K-ATPase \(\alpha_1\) subunit at Tyr-10 to regulate pump activity (Feraille et al., 1999); and (4) reducing the dissociation constant of Na\(^+\) binding and altering the Na/K-ATPase sensitivity for Na\(^+\) (Feraille et al., 1994; Feraille et al., 1995).

Long-term exposure of cells to insulin may induce the changes in gene expression in a tissue and isoform specific manner (Tirupattur et al., 1993).

**Measurements of Pump Activity and Availability:**

Several approaches have been developed to measure the pump activity and quantify the pump numbers. The affinity of the Na/K-ATPase to its substrates can be determined by measuring the kinetic parameters of ion transport. The pump activity can be obtained through the ouabain sensitive \(^{86}\text{Rb}^+\) uptake assay in intact cells or tissues or the ouabain sensitive ATP hydrolysis assay in either permeabilized cell preparations or isolated membranes (Esmann, 1988; Norby, 1988). The numbers of the active sodium pumps available at the cell membrane can be assayed by calculating how much \(^3\text{H-ouabain}\) bind to the \(\alpha\) subunits in
isolated membrane or intact cells. Detailed methods to measure the pump activity and availability have been described in the first and second manuscripts enclosed in this dissertation.

**SRC FAMILY KINASE:**

Src family kinases are 52-62 kDa membrane-associated non-receptor tyrosine kinases and play important roles in a variety of biological functions such as cell proliferation, migration, differentiation and survival. The transforming protein v-Src of the oncogenic retrovirus, Rous Sarcoma Virus (RSV) is the prototype member of Src family, which originated from its cellular progenitor c-Src. So far, nine other proteins that contain similar overall structure to c-Src have been identified, such as Yes, Fyn, Yrk, Blk, Lyn, Lck, Hck and Fgr. Among these family members, Src, Fyn and Yes are ubiquitously expressed, whereas Blk, Fgr, Hck, Lck and Lyn are found primarily in hematopoietic cells (Bolen and Brugge, 1997). Src expression level appears to be much higher in platelets and neurons than in other types of cells (Brown and Cooper, 1996).

**c-Src Structure and Mechanisms of Regulation:**

c-Src is a 60 kD protein that is composed of seven distinct functional domains: (1) the N-terminal membrane association domain, also termed as Src homology (SH) 4 domain. The SH4 domain is composed of 15 amino acids and contains signals for lipid modification of Src kinase (Buser et al., 1994); (2) the unique domain, which is important for each family members to specifically
interact with a unique set of signaling proteins; (3) the SH3 domain, which contains 50 amino acids and plays an essential role in the recruitment of substrates, in the Src localization and in its catalytic activity; (4) the SH2 domain, which has an globular structure of about 100 amino acids and plays a similar role as the SH3 domain. The intra-molecular interactions of the SH2 or SH3 domains with the specific amino acids in the catalytic domain and other regions stabilize the inactive conformation of the kinases. Structural studies also suggested that SH2 and SH3 domains may act together to regulate the interaction if Src with its partner proteins (Xu et al., 1997); (5) SH2- kinase linker domain, which can intra-molecularly interact with the SH3 domain and regulate the enzyme activity; (6) the catalytic (or SH1) domain, which is a well conserved region among all tyrosine kinases and responsible for the catalytic activity of Src. It contains a tyrosine residue (Tyr-419 in human c-Src) that becomes phosphorylated upon Src activation; (7) the negative regulatory domain at the c-terminus of the Src protein. When the tyrosine residue (Tyr-530 in human c-Src) in this region is phosphorylated, usually by the cytoplasmic tyrosine kinase Csk, it is capable of binding the SH2 domain and inhibiting the Src kinase activity. Tyr-530 and several surrounding amino acids are found deleted in v-Src and some active c-Src mutants (Reynolds et al., 1987). Binding of a tyrosine phosphorylated cellular proteins to the SH2 domain can destabilize the Tyr-530/SH2 interaction and release the structural constraints, which eventually lead to the kinase activation.

Taken together, the presence of these modular domains makes the regulation of Src kinase by a diverse group of proteins possible. There are
multiple ways to activate Src family kinases, such as by modification of certain residues or disruption of the auto-inhibitive intra-molecular interactions within different domains by high affinity ligands. Recent studies from our laboratory have shown that the SH3 and SH2 domains of Src constitutively bind to the second cytosolic domain (CD2) of Na/K-ATPase α1 subunit, whereas the Src kinase domain interacts with the third cytosolic domain (CD3) of α1. The conformational changes of Na/K-ATPase caused by ouabain binding can release the Src kinase domain from the α1 CD3 and result in the Src activation, while still preserving the attachment of Src to Na/K-ATPase through the SH2/SH3-CD2 interaction (Tian et al., 2006).

**FOCAL ADHESION KINASE (FAK)**

Focal adhesion kinase (pp125FAK or FAK) is a cytoplasmic tyrosine kinase enriched in focal adhesions (the dense cellular structures linking the extracellular matrix proteins to actin filament bundles via integrin receptors). The FAK homologues have been identified in a variety of species, including human, rodent, chicken, Xenopus with an over 90% amino acids sequence identity. In adult tissues, FAK is primarily found in brain, lung, heart and testis. Several FAK isoforms have been identified in rat brain and chicken fibroblast (Burgaya and Girault, 1996).

**Structural Properties of FAK:**

The FAK contains three major functional domains: (1) a central kinase domain, (2) a c-terminal non-catalytic domain termed FRNK (Fak-Related Non-
Kinase) which is rich in protein-protein interaction sites (Schlaepfer et al., 1999), and (3) an N-terminal domain which exhibits sequence similarity to a structurally conserved FERM domains found in many proteins such as talin and erythrocyte band 4.1ezrin-radixin-moesin (ERM) family proteins (Girault et al., 1999) (Figure 1).

The focal adhesion targeting (FAT) domain is located at the end of FRNK encompassing residues from 920 to 1053 and is essential for the sub-cellular localization of FAK (Hildebrand et al., 1993). Binding of the cytoskeleton protein paxillin and talin to FAT domain is one of the critical steps in recruiting FAK to the focal adhesions (Brown et al., 1996). The direct interaction between the FAT domain and the cytoplasmic tail of integrin β1 subunits can further stabilize the association of FAK with integrins in focal contacts (Chen et al., 2001).

The FERM domain of FAK is critical for the interaction between FAK and activated EGFR. Although FAK is targeted in the EGF- stimulated cell migration, the kinase activity of FAK appears to be dispensable in this process, indicating that FAK may act as an adaptor to recruit molecules that promote adhesion turnover instead of a solely protein kinase (Sieg et al., 2000). Through binding to the PH domain of Etk, a member of Btk family of tyrosine kinases, the FERM domain also is involved in the regulation of extracellular matrix proteins exerted by Etk/BMX (Schaller et al., 1995). A role for the FERM domain in regulating the catalytic activity and subcellular localization also has been suggested recently (Dunty and Schaller, 2002).
Figure 1. FAK Structure and Domain Organization (Modified from Parsons et al., 2000).

In the c-terminal FAT domain, two proline-rich motifs (PRM) are responsible for the interaction between FAK and SH3-domain containing proteins, such as Graf (GTPase regulator associated with FAK), CAP (c-Cbl-associated protein) and multi-functional adaptor protein p130Cas (Crk-associated substrate). By binding to Graf, FAK can regulate actin remodeling and the formation of stress fibers or focal adhesions via the increase of the GTPase activity of the GTP-binding proteins RhoA and Cdc42 (Hildebrand et al., 1996; Ribon et al., 1998). The FAK mutants which have defects in p130Cas binding exhibit compromised downstream signal transduction and then cause the ensuing instability of the adhesion complex.

**Activation of Fak:**

The FAK is usually maintained in an inactive state due to the intra-molar interactions of the N-terminal with its c-terminal or kinase domain. Disrupting this
auto-inhibitory conformation of FAK by a simple N-terminal deletion can result in the FAK activation (Schlaepfer and Hunter, 1996). Upon the engagement of integrins with the ECM, FAK undergoes a conformational change which leads to the unmasking of the active sites on FAK and allows the catalytic domain to adopt an active conformation. A transient dimerization then can occur and followed by the auto-phosphorylation on Tyr-397. The phosphorylated residue Tyr-397 serves as a consensus binding site for SH2-domain containing proteins including Src family kinases, the adaptor protein Shc and Grb7, the p85 subunit of PI-3K, and phospholipase Cγ (Chen and Guan, 1994; Han and Guan, 1999; Schaller et al., 1994; Xing et al., 1994; Zhang et al., 1999). Binding of Src to FAK through Tyr-397 elicits a series of intermolecular trans-phosphorylation or intramolecular cis-phosphorylation of FAK on additional tyrosine residues, such as Tyr-407, Tyr576/577 and Tyr-925, which further increases FAK kinase activity. The Src specific phosphorylation of FAK on Tyr-925 creates a high affinity binding site for adaptor protein Grb2/SOS complex, which then leads to the activation of downstream targets such as the ERK2/MAP kinase.

**FAK and Cancer:**

Studies have shown that elevated FAK expression and activity correlate with the increasing tumorigenic potential such as cell motility, invasiveness and proliferation in a number of human cancer cell lines, whereas FAK deficient cells exhibit an increased number of prominent focal adhesions and decreased rate in cell spreading and in chemotactic and haptotactic migration (Ilic et al., 1995;
Owen et al., 1999). v-Src transformation can induce the phosphorylation of FAK on Tyr-925 in the absence of integrin stimulation in NIH3T3 cells and the stable association of v-Src, Grb2 and Sos with FAK was independent of the cell adhesion to fibronectin (Schlaepfer et al., 1994). The tyrosine phosphorylation of FAK modulated by v-Src transformation is critical for the disassembly of cell-cell junctions that is associated with the epithelial-mesenchymal transition (Avizienyte et al., 2002; Brunton et al., 2005; Guan and Shalloway, 1992). In the mouse FAK<sup>+/−</sup> skin carcinogenesis model, it has been shown that FAK is involved in both of the tumor formation and progression (McLean et al., 2001, 2004). However, further studies in additional mouse models are still needed to determine the physiological function of FAK in different types of tumor.

CAVEOLAE AND CAVEOLIN:

Caveolae are 50-100 nm diameter flask-shaped vesicular invaginations in plasma membrane found in many types of mammalian cells including endothelial cells, smooth muscle cells, lung epithelial cells, adipocytes and nervous system (Schlegel and Lisanti, 2001). They are enriched in cholesterol, sphingolipids but poor in phospholipids relative to the bulk membrane. It was discovered that caveolae also contain a variety of signal transduction molecules including G protein-coupled receptors, G proteins and adenylyl cyclase, the endothelial isoform of nitric oxide synthase, multiple components of the tyrosine kinase-mitogen-activated protein kinase pathway, and numerous lipid signaling molecules (Shaul and Anderson, 1998). Caveolae-mediated modulation and
integration of signaling events at the cell surface play an essential role in cholesterol transport, potocytosis, tumor suppression and many other cell signaling events.

The major structural constituents and biochemical markers for caveolae are the caveolins, a family of integral membrane proteins (21-24 kDa) that are expressed in a tissue specific manner. Three groups of caveolins have been identified and are designated as caveolin-1, -2 and -3 (Okamoto et al., 1998). While caveolin-3 expression is muscle specific, caveolin-1 is ubiquitously expressed in endothelial and epithelial cells, adipocytes, fibroblasts, and smooth muscle cells. Caveolin-2 is usually localized along with caveolin-1. The primary sequence of caveolin-1 contains: (1) one central hydrophobic domain (residues 102-134) that anchors the protein to the plasma membrane and leaves both the N- and C- terminals in the cytosol; (2) one oligomerization domain (residue 61-101) that facilitates the formation of high molecular caveolin oligomers which contains up to 14~16 individual molecules. These oligomers bind directly to cholesterol and require cholesterol for the insertion into the membrane; (3) one scaffolding domain (residues 81-101), which is a short cytoplasmic, membrane-proximal domain mediating the direct interaction between caveolin-1 and multiple caveolae-associated signaling proteins, such as EGFR, Src and Raf through their caveolin-binding motif (Couet et al., 1997; Schlegel and Lisanti, 2001). Through forming a scaffold onto which many signaling molecules are assembled, caveolins are able to regulate the activation state of these caveolae-associated
signaling proteins, and eventually achieve a variety of signaling effects concordantly.

**EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR):**

**Structural Properties of EGFR:**

The EGFR is an intrinsic membrane protein of 1186 amino acids and belongs to a family of receptor tyrosine kinases which is composed of four members: EGFR (ErB1), ErB2, ErB3, and ErB4 (Ullrich et al., 1984). The EGFR contains a large highly glycosylated extracellular domain, a single transmembrane domain and an intracellular domain. Two cysteine-rich domains located in the extracellular domain comprise the ligand binding domain. In the intracellular domain, three sub-domains have been identified: (1) the juxtamembrane domain, which serves as the regulatory site for PKC induced feedback attenuation; (2) the tyrosine kinase domain contiguous to the juxtamembrane domain; and (3) the carboxyl-terminal tail, which contains multiple autophosphorylation sites and mediates the interaction of EGFR with a variety of SH2 domain or PTB (phospho-tyrosine binding) domain containing proteins (Wells, 1999).

**Biological Functions:**

The binding of specific polypeptide ligands, including EGF, transforming growth factor alpha (TGFα), betacellulin, epiregulin or amphiregulin to the monomeric EGFR, triggers the dimerization of receptor tyrosine kinase followed
by the activation of its intrinsic kinase, and then the phosphorylation of multiple tyrosine residues in the cytoplasmic region. These phosphorylated residues provide the binding sites for EGFR to interact with a variety of the signaling proteins. Through a rich network of cellular signaling pathways, EGFR is actively involved in many fundamental cellular processes such as proliferation, migration, differentiation and survival (Holbro and Hynes, 2004; Ullrich and Schlessinger, 1990; Yarden and Sliwkowski, 2001).

Upon ligand binding and activation, EGFR undergoes an immediate signal attenuation process symbolized by receptor internalization and degradation (Yarden and Sliwkowski, 2001). The endocytosis of EGFR is facilitated by a saturable endocytic system. By clustering over the clathrin-coated regions of the cell membrane, the ligand/EGFR complex is internalized through the invagination of endocytic vesicles. Along with the intracellular trafficking, these vesicles gradually decrease their internal pH, accumulate different hydrolytic enzymes and mature from early endosomes to late endosomes (Gill, 2002). Usually, the ligands dissociate from their corresponding receptors in the early endosomes and the receptors in late endosome either recycle back to plasma membrane or enter into lysosomes for degradation. Studies also have shown that the protein tyrosine phosphatase PTP1B, which resides in endoplasmic reticulum (ER) may remove phosphate groups from tyrosine residues in the cytoplasmic domain of the EGFR before it goes to lysosomes for degradation (Haj et al., 2002).
**EGFR and Related Diseases:**

The EGFR presents on almost all epithelial and stromal cells as well as on selected glial and smooth muscle cells. In polarized epithelial cells, EGFR is largely restricted to the basolateral sides (Bishop and Wen, 1994; Gesualdo et al., 1996; Mori et al., 1987; Playford et al., 1996; Scheving et al., 1989). Mislocalization of EGFR as well as Na/K-ATPase has been observed in polycystic kidney disease (PKD) and the abnormally distributed EGFR has been suggested to play a major role in the progression of PKD (Avner et al., 1992; Du and Wilson, 1995; Wilson, 2004; Wilson et al., 1991). The relationship between the aberrant signaling through the over-expressed or mislocalized EGFR and tumorigenesis in breast, brain, bladder, lung, head & neck, cervix, ovary also has been established. A series of EGFR tyrosine kinase inhibitors have already shown great potentials in the clinical therapies (Dancey and Freidlin, 2003; Sweeney et al., 2000).

**EGFR and Caveolae:**

It has been observed that EGFR is greatly enriched in specialized membrane domains known as caveolae or lipid rafts (Mineo et al., 1996; Wang et al., 2004). Depletion of cholesterol with methyl-β-cyclodextrin can increase the cell surface EGF binding by 40%, which is independent of the receptor externalization from intracellular pool (Pike and Casey, 2002). Upon ligand binding, EGFR rapidly dissociates from caveolae and migrates to the bulk membrane. This process appears to be regulated by Src kinase and occurs
independently of the clathrin-mediated endocytosis (Mineo et al., 1999). The sequestration of EGFR in the caveolae plays an important role in the EGF signaling. A prolonged or reduced residence of EGFR in this domain may contribute to abnormal cell behavior.

**RNA INTERFERENCE:**

The RNA interference (RNAi) is a cellular surveillance mechanism which was first discovered in 1998 by Andrew Fire and Craig Mello in the nematode worm *Caenorhabditis elegans* (Fire et al., 1998). As an evolutionally highly conserved process, RNA interference refers to the post-transcriptional gene silencing (PTGS) induced by the direct introduction of double stranded RNA (dsRNA) into a cell. Double stranded RNA triggers the degradation of a homologous mRNA and leads to the loss-of-function phenotypes. It is now established as an important biological strategy for gene silencing in a wide variety of organisms, including mammals. (Elbashir et al., 2001; McManus and Sharp, 2002)

The working mechanism of RNAi includes three major steps: (1) the delivery of double-stranded RNA (dsRNA) into the cells. This can be accomplished by either direct injection or transfection of chemically synthesized dsRNA or through invitro transcription from dsRNA expression vectors; (2) the dsRNAs inside the cells then are processed into the 21–23-nucleotide small interference RNA (siRNA) by a processing enzyme DICER; and (3) siRNAs incorporate into a nuclease complex, called the RNA-induced silencing complex
(RISC) and direct the RISC to a homologous cellular mRNA by base pairing. Then, the targeting mRNAs undergo degradation through exonucleolytic or endonucleolytic cleavages (Tuschl, 2001).
Functional Characterization of Src-Interacting Na/K-ATPase

Using RNA Interference Assay*

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Running Title: Regulation of Src by Na/K-ATPase

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We have shown that the Na/K-ATPase and Src form a signaling receptor complex. Here, we determined how alterations in the amount and properties of the Na/K-ATPase affect basal Src activity and ouabain-induced signal transduction. Several α1 subunit knock-down cell lines were generated by transfecting LLC-PK1 cells with a vector expressing α1-specific siRNA. While the α1 knock-down resulted in significant decreases in Na/K-ATPase activity, it increased the basal Src activity and tyrosine phosphorylation of FAK, a Src effector. Concomitantly, it also abolished ouabain-induced activation of Src and ERK1/2. When the knock-down cells were rescued by a rat α1, both Na/K-ATPase activity and the basal Src activity were restored. In addition, ouabain was able to stimulate Src and ERK1/2 in the rescued cells at a much higher concentration, consistent with the established differences in ouabain sensitivity between pig and rat α1. Finally, both fluorescence resonance energy transfer analysis and co-immunoprecipitation assay indicated that the pumping-null rat α1 (D371E) mutant could also bind Src. Expression of this mutant restored the basal Src activity and FAK tyrosine phosphorylation. Taken together, the new findings suggest that LLC-PK1 cells contain a pool of Src-interacting Na/K-ATPase that not only regulates Src activity, but also serves as a receptor for ouabain to activate protein kinases.
INTRODUCTION

Na/K-ATPase was discovered by Skou as the molecular machinery of the cellular sodium pump (1). It belongs to a family of evolutionarily ancient ATPases that couple the hydrolysis of ATP to membrane ion translocation (2,3). A major difference between the Na/K-ATPase and other ATPases is its ability to bind cardiotonic steroids such as ouabain. Studies from many laboratories have now established that the binding of ouabain to this enzyme not only inhibits the ATPase activity, but also stimulates protein tyrosine kinases such as Src (4,5). The activated Src in turn transactivates EGFR\(^1\), resulting in the assembly and activation of multiple signaling cascades such as the ERK1/2 and PLC-\(\gamma\)/PKC pathways (5,6).

Because several laboratories have demonstrated that the activation of Src is essential for ouabain-induced changes in many cellular activities including the regulation of intracellular calcium, gene expression, and cell growth(6-9), we have recently examined whether the Na/K-ATPase interacts directly with Src to form a functional signaling receptor (10). Using in vitro GST pull-down assays we have identified that the second and the third intracellular domains of the Na/K-ATPase \(\alpha1\) subunit interact with the Src SH2 and the kinase domains, respectively. Functionally, these interactions keep Src in an inactive state, and binding of ouabain to this inactive Na/K-ATPase/Src complex frees, and then activates the associated Src (10). These new findings suggest that the cellular Src-interacting Na/K-ATPase may play an important role in regulation of the basal Src activity and serve as a functional receptor for ouabain to stimulate
protein tyrosine phosphorylation in live cells. To test this hypothesis we have developed an siRNA-based assay that allows us to determine the effect of changes in the amount and properties of the Na/K-ATPase on both basal and ouabain-stimulated Src activity.

EXPERIMENTAL PROCEDURES

Materials: Chemicals of the highest purity were purchased from Sigma (St. Louis, MO). The GeneSuppressor vector was purchased from BioCarta (San Diego, CA); Cell culture media, fetal bovine serum, trypsin, Lipofectamine 2000 and restriction enzymes were purchased from Invitrogen (Burlington, ON); EYFP expression vector (pEYFP) and ECFP expression vector (pECFP) were obtained from Clontech (Palo Alto, CA); QuickChange Mutagenesis kit was purchased from Stratagene (La Jolla, CA); Optitran nitrocellulose membrane was from Schleicher & Schuell (Keene, NH); Enhanced chemiluminescence (ECL) super signal kit was purchased from Pierce (Rockford, IL); Image-iT FX signal enhancer, Antifade kit, Alexa Fluor 488-conjugated anti-mouse/rabbit IgG and Alexa Fluor 546-conjugated anti-mouse/rabbit IgG antibodies were obtained from Molecular Probes (Eugene, OR); Anti-Src (clone GD11) monoclonal antibody, anti-Na/K-ATPase α1 polyclonal and monoclonal (clone C464.6) antibodies, anti-Phosphotyrosine (clone 4G10) antibody, protein G-agarose were from Upstate (Lake Placid, NY); the polyclonal anti-pY^{418}-Src and anti-pY^{529}-Src antibodies were from Biosource (Camarille, CA). The polyclonal anti-FAK and anti-pY^{925}-FAK antibodies were from Cell Signaling (Danvers, MA). The monoclonal anti-α1
antibody (α6F) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Anti-c-Src (B-12) monoclonal antibody, anti-c-Src (SRC 2) polyclonal antibody, anti-ERK (C-16) polyclonal antibody, anti-pERK (E-4) monoclonal antibody and all the secondary horseradish peroxidase-conjugated antibodies were from Santa Cruz (Santa Cruz, CA). Polyclonal rat α1-specific antibody (anti-NASE) was provided by Dr. Thomas Pressley (Texas Tech University, Texas).

**Cell culture:** LLC-PK1 cells and HEK 293T cells were obtained from American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium containing 10% FBS, 100-units/ml penicillin, and 100 µg/ml streptomycin in 5% CO₂ humidified incubator as previously described (10,11).

**Construction of the siRNA expression vectors:** siRNAs were constructed using the GeneSuppressor Construction Kit as previously described (11). Briefly, four pairs of oligonucleotides (A1~A4) were synthesized using the human α1 cDNA (accession no. NM_000701) as template (see Table I for details) and the inserts were prepared by annealing two complementary oligonucleotides. The annealed inserts were then cloned into pSuppressorᵀᴹ-U6 vector digested with Sall and Xbal. Positive clones were confirmed by nucleotide sequencing.

**Site-directed mutagensis:** Rat-α1 expression vector pRc/CMV-α1AAC was provided by Dr. Pressley (12). To make the expression of rat α1 insensitive to A4 siRNA, the α1 siRNA-targeted sequence was silently mutated from ⁡₂⁵₃⁰ggtcgtcgtatctt (accession no. NM_012504) to ²⁵₃⁰ggcaggctaatatc using
QuickChange Mutagenesis kit. The SspI (aat/att) restriction site was generated to facilitate the clone screening. The positive mutant (pRc/CMV-α1AACm1 or AAC in short) was verified by DNA sequencing and then used in this study. The pumping-null mutant (D371E) was generated by mutating the $^{1126}$ga$\text{caag}$ to $^{1126}$gagaag using pRc/CMV-α1AACm1 as the template (13).

**Generation of stable α1 subunit knock-down and knock-in cell lines:** HEK 293T cells were transiently transfected with different siRNA expression vectors along with pEYFP using Lipofectamine 2000 as we previously described (11). After 48 h, cells were first examined for the expression of EYFP for assessing the transfection efficiency, and then collected for analysis of endogenous α1 content by Western blot. To generate stable cell lines, one batch of LLC-PK1 cells was transfected with the A4 siRNA expression vector (pSuppressor-A4 siRNA) (Table I) and a puromycin selection marker (pBade-puro). Another batch of cells was co-transfected with pEYFP together with the pSuppressor-A4 siRNA and pBade-puro, so that the co-expressed EYFP could be used as a marker to pick clones. Empty vector (pSuppressor) or A1 siRNA transfected cells were co-selected and used as a control. The cells were selected with puromycin (1µg/ml) 24 h post transfection. Puromycin-resistant colonies were cloned and expanded. To rescue the Na/K-ATPase knock-down cells, cells were transfected with the pRc/CMV-α1AACm1. Selection was initiated with 3µM ouabain because untransfected cells were very sensitive to ouabain. After about one week, ouabain-resistant colonies were isolated and expanded into stable cell lines in the absence of ouabain. G418 was not used because these cells are resistant to it, requiring
more than 3 mg/ml to kill the un-transfected cells. The knockdown cells were also sensitive to blasticidin (15µg/ml) and we have recently used this agent for other selections.

**Immunoprecipitation and Immunoblot analysis:** Cells were washed with PBS and solubilized in modified and ice-cold RIPA buffer, and subjected to immunoprecipitation or Western blot analysis as previously described (11). Protein signal was detected using the ECL kit and quantified using Bio-Rad GS-670 imaging densitometer.

**Na/K-ATPase Activity assay:** Na/K-ATPase enzymatic activity was determined as we previously described (14). Briefly, cells were collected from the cultures in Tris-EGTA buffer (pH7.2) and briefly sonicated. The cell lysates were then treated with alamethicin at a concentration of 0.1 mg/mg protein for 30 min at room temperature. ATPase activity was measured by the determination of the initial release of $^{32}$P from [γ-$^{32}$P]ATP and the reaction was carried out in a reaction mixture (1 ml) containing 100 mM NaCl, 25 mM KCl, 3 mM MgCl$_2$, 1 mM EGTA, 2 mM ATP, 5 mM NaN$_3$, and 50 mM Tris-HCl (pH 7.4). Na/K-ATPase activity was calculated as the difference between the activities measured in the absence of ouabain and in the presence of 1 mM ouabain. To determine the ouabain concentration curve, the alamethicin-treated cell lysates were preincubated with different concentrations of ouabain for 15 min before ATP was added to start the reaction.
**Confocal fluorescence microscopy:** Cells cultured on cover slips were washed twice with PBS and fixed for 15 min with methanol pre-chilled at -20 °C. The fixed cells were then rinsed with PBS for 3 times and blocked with 200 µl Image-iT FX signal enhancer for 30 min at room temperature. The cells were washed again and incubated with the primary antibodies in PBS containing 1% BSA for 1 h at room temperature. After 3 washes with PBS, the cells were incubated with corresponding Alexa Fluor conjugated secondary antibodies. Image visualization was performed by Leica DMIRE2 confocal microscope (Leica, Mannheim, Germany). Leica confocal software was used for data analysis.

**FRET Analysis by Acceptor Photobleaching:** ECFP was fused to the C-terminus of Src, and EYFP was fused to the N-terminus of rat Na/K-ATPase α1 subunit or its mutant. FRET analysis was performed in cells co-transfected with Src-ECFP and EYFP-rat α1 expression vectors using acceptor photobleaching protocol as we described (10). Briefly, after 24 h culture, cells on a glass cover slip were fixed with methanol pre-chilled at -20 °C for 15 min, and washed twice with PBS solution. The EYFP-rat α1 was photobleached by applying high intensity of 515 nm laser, and the emission of ECFP excited by 456 nm laser was recorded before (D$_{pre}$) and after (D$_{post}$) EYFP photobleaching. The FRET efficiency was then calculated by the ratio of (D$_{post}$ - D$_{pre}$) / D$_{pre}$. Cells transfected with either Src-ECFP and EYFP or EYFP-α1 and ECFP expression vectors were used as a control and no detectable FRET was observed in these control cells.

**Data Analysis:** Data are given as Mean ± SEM. Statistical analysis was performed using the Student’s *t*-test, and significance was accepted at p<0.05.
RESULTS

**Manipulation of the cellular Na/K-ATPase content by siRNA-based assays:**

As shown in Table I, a total of four pairs of the α1-specific siRNAs was selected based on the strategy we previously used (11). Transient transfection assay in HEK 293T cells showed that expression of A4 siRNA resulted in over 40% decreases in the expression of the human α1 subunit while others gave 0 (A1 siRNA) to 20% (A2 and A3 siRNA) reduction. Because the transfection efficiency was about 50% as indicated by the co-expressed EYFP, we reasoned that A4 siRNA is effective in silencing the expression of endogenous Na/K-ATPase. Therefore, LLC-PK1 cells were transfected with A4 siRNA expression vector (pSuppressor-A4 siRNA) and a puromycin selection marker (pBade-puro) either with or without pEYFP as described in the Experimental Procedure. After two rounds of selection, we collected 20 stable transfectants. Western blot analysis using a monoclonal (α6F) antibody showed that the expression of the α1 subunit in these clones was significantly reduced in comparison to the control P-11 cells that were transfected with empty vector (pSuppressor) and selected. In contrast, cell clones (e.g. A1) obtained from the LLC-PK1 cells that were transfected with A1 siRNA expressed 1 at the level comparable to that in P-11 cells (see Table II). Therefore, we expanded and further characterized three A4 siRNA-expressing clones. As shown in Fig.1A, expression of the α1 subunit was significantly reduced in A4-11, TCN23-19, and PY-17 cells. Of these cell lines, the PY-17 cells, which were cloned by using the co-expressed EYFP as a marker,
expressed the lowest level of the Na/K-ATPase. Table II shows the quantitative data on the relative amount of the α1 in these and other cell lines we generated. Because control Western blot using purified Na/K-ATPase prepared from pig kidney showed that it was only possible to perform reasonable quantitative assay comparing two samples with less than six-fold differences in the amount of α1 (data not shown), we measured the relative amount of 1 in these cells by comparing A4-11 with the control P-11, and then TCN23-19 and PY-17 with A4-11. To confirm the above Western blot data, we also probed the blots with a different anti-Na/K-ATPase α1 monoclonal antibody (clone C464.6) and an anti-Na/K-ATPase α1 polyclonal antibody, showing essentially the same as in Fig. 1A. In addition, when co-cultured P-11 and PY-17 cells were immunostained using anti-Na/K-ATPase α1 antibody (clone C464.6), we found that the green PY-17 cells exhibited no detectable expression of the α1 while the plasma membrane of control P-11 cells was clearly labeled by the antibody (Fig. 1B). To be sure that knock-down of the α1 subunit does not induce the expression of other isoforms, we analyzed the cell lysates for both α2 and α3 and found no detectable signals in the above cell lines. Finally, when ouabain-sensitive ATPase activity was measured in the cell lysates, a significant (80%) reduction was noted in the PY-17 cells in comparison to the control P-11 cells (Table III).

Because PY-17 cells have very low endogenous Na/K-ATPase, these cells will be very useful for studying the structure-function properties of the Na/K-ATPase if we can rescue the cells by knocking in an exogenous α1. To test this possibility, we first made silent mutations of the rat α1 cDNA to change the
siRNA-targeted sequence. We then transfected PY-17 cells with the mutated rat α1 expression vector (pRc/CMV-α1AACm1) and generated several stable transfectants. Further analysis of the clone AAC-19 showed that these cells, unlike both P-11 and PY-17, expressed rat α1 (Fig. 2A). When the same blots were analyzed for total α1 using the monoclonal anti-α1 antibody (α6F), we found that AAC-19 cells expressed comparable amount of α1 as control P-11 cells (Fig. 2A). This result was further confirmed by immunostaining of the co-cultured P-11 and AAC-19 cells using anti-α1 (clone C464.6) antibody. As depicted in Fig. 2B, the green AAC-19 and control P-11 cells exhibited the similar levels of the Na/K-ATPase in the plasma membrane. Control experiments also demonstrated that the rat α1 was stably expressed in this cell line for at least 20 passages in the absence of ouabain. Functionally, knock-in of the rat α1 into PY-17 cells was able to restore ouabain-sensitive ATPase activity (Table III). Most importantly, it shifted the dose-response curve of ouabain on the ATPase activity and made the rescued cells less ouabain-sensitive as rat cell lines (5) that express only the α1 isoform (Fig. 3). It is important to note that PY-17 cells were as sensitive to ouabain as the control P-11 cells, and that 10 M ouabain caused a complete inhibition of the Na/K-ATPase.

**Regulation of basal Src activity by the Na/K-ATPase:** Our in vitro studies have shown that the Na/K-ATPase directly binds and keeps Src in an inactive state. If this mode of regulation operates in live cells, we would expect that reduction of intracellular Na/K-ATPase will decrease the interaction, resulting in
an increase in basal Src activity. To test it out, we measured the phosphorylation of Src (pY\textsuperscript{418}-Src), indicative of Src activation (4,10), in the cell lysates from the above cell lines. As depicted in Fig. 4A, the expression of total Src was not altered by knock-down of the endogenous Na/K-ATPase. However, the levels of active Src were significantly increased in A4-11, TCN23-19, and PY-17 cells. Interestingly, the increase in Src activity appeared to be inversely correlated with the amounts of Na/K-ATPase expressed in these cells (Fig. 4B). These findings were further confirmed by immunostaining the cells with anti-pY\textsuperscript{418}-Src antibody, showing that TCN23-19 cells contained much more active Src than that of P-11 cells (Fig. 4C). It is important to note that there was no difference in the amount of active Src between two control cell lines P-11 and A1 cells (data not shown).

To test whether the increase in Src activity due to the decreased expression of the Na/K-ATPase is reversible upon repletion of the Na/K-ATPase, we determined the total Src and the active Src in AAC-19 cells. As depicted in Fig. 2, AAC-19 cells were derived from the rat α1-transfected PY-17 cells, and expressed a comparable amount of the Na/K-ATPase as in control P-11 cells. While knock-in of the rat α1 did not change the total Src in AAC-19 cells, it did reduce the level of the active Src to that seen in control P-11 cells (Fig. 5A and B). As illustrated in Table III, the Na/K-ATPase activity was reduced 80% in PY-17 cells. When intracellular Na\textsuperscript{+} was measured after the cells were incubated in \textsuperscript{22}Na\textsuperscript{+} (0.5µCi/ml) medium for 60 min to fully equilibrate exchangeable intracellular Na\textsuperscript{+} with \textsuperscript{22}Na\textsuperscript{+} (15), we found that the steady state intracellular Na\textsuperscript{+} in PY-17 cells was about twice as much as in P-11 cells\textsuperscript{2}. To be sure that changes in Src
activity observed in AAC-19 cells is not due to the restoration of the functional Na/K-ATPase and subsequent decreases in intracellular Na\(^+\), we tested whether knock-in of a pumping-null mutant of the rat \(\alpha_1\) is sufficient for the observed interaction between the Na/K-ATPase and Src. PY-17 cells were transiently transfected with either silently mutated wt rat \(\alpha_1\) (pRc/CMV-\(\alpha_1\)AACm1) or the rat \(\alpha_1\) pumping-null mutant (D371E). As shown in Fig. 5C, expression of either rat \(\alpha_1\) or the mutant reduced active Src in PY-17 cells. To further confirm these findings, we also transiently transfected TCN23-19 cells with the EYFP-fused rat \(\alpha_1\) mutant expression vector (pEYFP-D371E), and immunostained for active Src. As depicted in Fig. 5D, the cells expressing the rat \(\alpha_1\) mutant had much less active Src in comparison to the un-transfected TCN23-19 cells. These data suggest that the pumping-null Na/K-ATPase mutant is still able to interact and regulate Src. To seek additional support for this notion, we also performed FRET analysis in TCN23-19 cells transiently transfected with EYFP-rat \(\alpha_1\) mutant (D371E) and Src-ECFP expression vectors. As depicted in Fig. 6A, the pumping-null mutant was targeted to the plasma membrane. When FRET was measured in these transfected cells by acceptor photobleaching protocol, an energy transfer from Src-ECFP to EYFP-D371E was clearly demonstrated (Fig. 6B). The FRET efficiency measured from a total of twenty cells in three separate experiments ranged from 10.4 to 15.6 (13.2 \(\pm\) 1.4). These data indicate that the pumping-null Na/K-ATPase, acts like the wt \(\alpha_1\) (10), can interact with Src to form a signaling complex. This conclusion is further supported by the co-
immunoprecipitation assay showing that the rat α1 mutant could be co-
precipitated by anti-Src antibody (Fig. 6C).

FAK is a known Src effector that plays an important role in regulation of cell
migration and proliferation (16-18). Activation of Src stimulates phosphorylation
of FAK Y\textsubscript{925}, which subsequently can lead to the activation of ERK1/2 (19,20).
To examine whether an increase in basal Src activity can result in the activation
of Src effectors, we measured tyrosine phosphorylation of FAK in α1 knock-down
cells. As depicted in Fig.7A, cell lysates were immunoprecipitated by an anti-
phosphotyrosine antibody, and the immunoprecipitates were probed by anti-FAK
antibody. The data clearly showed that the α1 knock-down was capable of
increasing the amounts of tyrosine-phosphorylated FAK. Specifically, when cell
lysates were probed for pY\textsubscript{925} FAK, we found a significant increase in pY\textsubscript{925} FAK
in both A4-11 and PY-17 cells (Fig. 7B). Interestingly, when total ERK1/2 and p-
ERK1/2 were measured, we found a modest increase in the amount of active
ERK1/2 in PY-17 cells (Fig. 7C). This is in accordance with the known function
of pY\textsubscript{925} FAK (19,20). Significantly, this increase in pY\textsubscript{925} was sensitive to Src
inhibitor PP2 (Fig. 7D). It is important to note that the FAK phosphorylation
correlated well to the levels of active Src in the PP2-treated knock-down cells.
Taken together, these data indicate that the increased Src activity due to the 1
knock-down can stimulate tyrosine phosphorylation of Src effectors. This notion
is further supported by the observation that expression of the pumping-null
mutant (D371E) not only restored the basal Src activity, but also reduced FAK
Y\textsubscript{925} phosphorylation in PY17 cells (Fig. 7E).
Knock-down of the Na/K-ATPase abolishes ouabain-induced activation of Src and ERK1/2: Because the Na/K-ATPase/Src complex serves as a functional receptor for ouabain to induce Src activation and subsequent stimulation of ERK1/2 (5,10), the above findings prompted us to test if knock-down of the Na/K-ATPase affects ouabain-activated signal transduction. As shown in Fig. 8A, while ouabain activated Src in P-11 cells as we reported previously (4,5), this effect of ouabain was essentially abolished in PY-17 cells whereas a significant reduction was observed in A4-11 cells. To be sure that this inhibition is not due to non-specific defects in receptor signal transduction, we also measured the effect of EGF on Src. We found that EGF was able to stimulate Src pY418 in both P-11 and PY-17 cells (2.5 ± 0.3 fold increase in P-11 vs 1.7 ± 0.2 fold increase in PY17, n=3). Consistent with the findings on Src, we also failed to detect any ouabain-induced change in ERK1/2 phosphorylation in PY-17 cells (Fig. 8B). In contrast, EGF was able to stimulate ERK1/2 in PY17 cells (data not shown). These data support the notion that the Na/K-ATPase is indeed the receptor for ouabain-induced signal transduction. This notion is further supported by the findings presented in Fig. 8 C and D, showing that knock-in of the rat α1 not only restored the ouabain responses, but also shifted the dose-response curve to the right in AAC-19 cells.
DISCUSSION

In this report we not only introduced an effective and α1-specific RNA interference assay, but also provided a protocol for rescuing the Na/K-ATPase-depleted cells. These procedures have made it possible for us to demonstrate that the cellular Na/K-ATPase regulates Src and its effector FAK, and that the Na/K-ATPase/Src complex serves as a sole receptor for ouabain to activate Src and subsequently ERK1/2 in live cells.

Manipulation of the cellular Na/K-ATPase content by RNA interference assays: RNA interference is a cellular mechanism that was first discovered in 1998 in *C. elegans* and refers to the post-transcriptional gene silencing by double-stranded RNA-triggered degradation of a homologous mRNA (21). This has now been developed as a powerful tool for artificially silencing a specific gene in a variety of biological systems including cultured cells and whole organisms. Employing the strategy developed by Paul et al. (22) and transient transfection assay, we identified that A4 siRNA was effective for silencing the α1 expression. Thus, we transfected pig LLC-PK1 cells with the A4 siRNA expression vector and cloned several stable cell lines. Western blot analysis and immunostaining assay showed that the expression of the α1 in the cloned cell lines was significantly reduced (Figs. 1 and 2, and Table II). For example, the α1 in PY-17 cells is only about 8% of that in control P-11 cells. Functional analysis revealed that depletion of the α1 resulted in an 80% reduction in ouabain-sensitive ATPase activity in PY-17 cells (Table III). Clearly, we have developed
an effective protocol for silencing the expression of endogenous α1 in cultured cells.

To test whether the α1-depleted cells can be used to study the signaling functions of an exogenous/mutant α1, we transfected PY-17 cells with a rat α1 expression vector in which A4 siRNA-targeted sequence was silently mutated. By taking advantage of the availability of an antibody that specifically reacts with rat α1, we demonstrated that the exogenous rat α1 could be knocked in and that the expression of rat α1 restored not only the total cellular Na/K-ATPase protein, but also the Na/K-ATPase activity. Importantly, the rat α1-rescued cells (AAC-19) exhibited the same ouabain sensitivity as the rat cell lines that only express the Na/K-ATPase α1 subunit (Fig. 3). Taken together, the data indicate that we have developed an effective protocol for manipulating cellular Na/K-ATPase. It is important to note that this protocol offers additional advantages over the widely used ouabain-selection protocol for expression of mutated Na/K-ATPase in ouabain-sensitive cell lines (23-26). First, our protocol makes it possible to deplete endogenous Na/K-ATPase, which allows the investigators to study the effects of decreases in Na/K-ATPase expression on cellular function. Second, it does not require using ouabain to force the expression of the transfected Na/K-ATPase. This is important in view of recent studies showing that ouabain stimulates the signaling function of the Na/K-ATPase, and induces the endocytosis of the enzyme (4,9,27,28). Third, this protocol allows us to study the exogenous/mutant Na/K-ATPase in the cells that have very low (less than 10%)
endogenous Na/K-ATPase. Fourth, the identified A4 siRNA should be effective in silencing the α1 expression in cells derived from species other than human and pig because the human α1 cDNA sequence (nucleotide 2293 to nucleotide 2312) targeted by A4 siRNA is conserved among all identified α1 subunits (but not other isoforms) from fish to human. Finally, rescuing PY-17 cells with different isoforms of the Na/K-ATPase would make it possible for us to uncover the potential isoform-specific signaling functions.

**A pool of Src-interacting Na/K-ATPase:** Recently, we have shown that the Na/K-ATPase resides in caveolae with Src (11,29). FRET analysis indicates that the signaling Na/K-ATPase and Src are likely to interact and form a functional receptor complex. In vitro binding assay demonstrates that the α1 subunit and Src can interact directly via multiple domains and that the interaction keeps Src in an inactive state (10). These findings led us to propose that there may be a Src-interacting pool of Na/K-ATPase that not only regulates the basal Src activity, but also serves as a receptor for ouabain to stimulate Src-dependent tyrosine phosphorylation of multiple effectors. The data presented here provide further support to this hypothesis. First, since the signaling Na/K-ATPase binds and keeps Src in an inactive state (10), we expected that reduction of the endogenous Na/K-ATPase would deplete the Src-interacting pool of Na/K-ATPase, thus resulting in the Src activation. Indeed, as shown in Fig. 4, the α1 knock-down cells contain more active Src than the control P-11 cells. It is important to mention that the α1 knock-down did cause a significant increase in
intracellular Na\(^+\) concentration in PY-17 cells. However, when intracellular Ca\(^{2+}\) was measured by fura-2 as we previously described (30), the steady state Ca\(^{2+}\) in PY-17 cells was comparable to that in P-11 cells\(^2\). Thus, it is unlikely that increases in Src activity are due to changes in intracellular Na\(^+\) or Ca\(^{2+}\). Second, when the α1 knock-down PY-17 cells were rescued by the rat α1, we observed that the knock-in of the rat α1 was sufficient to replete the pool of Src-interacting Na/K-ATPase, leading to the restoration of basal Src activity. Finally, because our in vitro binding assay showed that the third intracellular domain of the α1 interacts and inhibits Src activity (10), we expected that a pumping-null mutant of the rat α1 should be able to bind and inhibit Src in live cells. Indeed, we found that knock-in of rat α1 mutant D371E into PY-17 cells was also able to replete this Src-interacting pool of Na/K-ATPase and reduce the amount of active Src (Fig. 5). In addition, both FRET analysis and co-immunoprecipitation assay showed that the pumping-null mutant could interact with Src in live cells (Fig. 6). Because expression of the pumping-null mutant would not reduce intracellular Na\(^+\) concentration in PY-17 cells, these data also indicate that the Na/K-ATPase can interact and regulate Src independent of changes in intracellular Na\(^+\) concentration.

FAK is involved in regulation of cell proliferation, cell survival and cell migration (16-18). It is also one of Src effectors. Binding of active Src to FAK leads to full activation of FAK and tyrosine phosphorylation of FAK Y\(^{925}\), which results in the assembly of several down-stream signaling modules including the activation of ERK1/2. Interestingly, we found that depletion of cellular Na/K-
ATPase not only activated Src, but also stimulated tyrosine phosphorylation of FAK. Inhibition of Src by either PP2 or knock-in a pump-null α1 mutant reduced pY\textsuperscript{925} FAK in PY-17 cells (Fig. 7). Consistently, we have also observed that ouabain stimulated Src, and subsequently FAK in the control LLC-PK1 cells\textsuperscript{3}. These findings are significant. First, they support the notion that the Na/K-ATPase is an important regulator of protein kinases. Second, the regulatory effects of the Na/K-ATPase on Src and Src effector FAK depend on its ability of protein interaction, but not the ion pumping function. Third, the α1 depletion-induced Src activation is capable of generating down-stream pathways. To this end, it is worth of noting that FAK plays a key role in regulation of cell motility. It has been reported that depletion of β1 in epithelial cells affects the formation of tight junction and cell motility (31,32). Thus, it will be of interest to further test the role of α1 depletion and subsequent activation of FAK in the regulation of cell migration.

We showed previously that ouabain-induced signal transduction appears to be initiated by the activation of Src (4). Because ouabain uses the Na/K-ATPase/Src complex as a functional receptor, we expected that the ouabain-induced activation of Src should correlate with the size of the pool of Src-interacting Na/K-ATPase. Indeed, we found that the effect of ouabain on Src activation correlated inversely with cellular levels of the Na/K-ATPase. While ouabain induced a modest activation of Src in A4-11 cells, it failed to activate Src in PY-17 cells. Because Src is required to transmit the ouabain signal to many down-stream effectors (5,10,11), the new findings support the notion that the
Na/K-ATPase/Src complex is the sole receptor for ouabain to provoke the protein kinase cascades. This notion is further supported by the following observations. First, rescuing PY-17 cells with the rat α1 restored the effect of ouabain on Src and ERK1/2. Second, because the rescued cells expressed the ouabain-insensitive rat α1, a much higher ouabain concentration was required to stimulate Src and subsequently ERK1/2 in AAC-19 cells (Fig. 8).

In short, we have developed a powerful protocol for manipulating the cellular Na/K-ATPase, which has allowed us to further characterize the signaling properties of the Na/K-ATPase. In addition, these new findings support the hypothesis that the Na/K-ATPase is an important receptor capable of transmitting ouabain signals via protein kinases (4,5,9,33,34). Finally, because Src is actively involved in control of cell growth (35), our new findings warrant the need for re-examining the issue whether the Na/K-ATPase-mediated repression of Src and ouabain-provoked activation of Src play a role in cancer biology.

REFERENCE


FOOTNOTES

* We thank Dr. Thomas Pressley for providing the polyclonal rat α1-specific antibody (anti-NASE) and rat-α1 expression vector pRc/CMV-α1AAC. We also appreciate the insightful comments from Dr. Sandrine Pierre regarding this
manuscript. This work was supported by National Institutes of Health Grants HL-36573 and HL-67963, awarded by the National Heart, Lung and Blood Institute, United States Public Health Service, Department of Health and Human Services.

1The abbreviations used are: EGFR, epidermal growth factor receptor; EYFP, enhanced yellow fluorescence protein; ECFP, enhanced cyan fluorescence protein; FRET, fluorescence resonance energy transfer; ERK, extracellular signal- regulated kinase. PLC, phospholipase C; PKC, protein kinase C; GST, glutathione S-transferase; siRNA, small interference RNA; RIPA, radioimmune precipitation assay; FAK, focal adhesion kinase.

2 Unpublished data by Liu,L and Xie,Z.

3 Unpublished data by Liang M and Xie Z.

FIGURE LEGENDS

Figure 1 Silencing of the endogenous Na/K-ATPase by siRNA: A. Total cell lysates (30µg/lane) from different cell lines were separated on SDS-PAGE and analyzed by Western blot for the expression of the α1 subunit of the Na/K-ATPase. A representative Western blot is shown [see quantitative data in table II]. B. P-11 and PY-17 cells were mixed and co-cultured for 24 h, and then immunostained with anti-α1 antibody (clone C464.6) as described in the Experimental Procedures. The scale bar represents 50 μm.
**Figure 2 Expression of the Na/K-ATPase in AAC-19 cells.** A. Clone AAC-19 was generated by transfecting PY-17 cells with a rat α1-expressing vector as described in the Experimental Procedures. Cell lysates (15 µg from P-11 and AAC-19; 60 µg from PY-17) were separated on SDS-PAGE and analyzed by Western blot. The blot was first probed with antibody α6F that recognizes both pig and rat α1 subunits, and then striped and re-probed with the anti-NASE that specifically reacts with rat α1. B. P-11 and AAC-19 cells were mixed and co-cultured for 24 h and immunostained with anti-α1 antibody (clone C464.6) as described in the Experimental Procedure. The scale bar represents 50 µm.

**Figure 3 Concentration dependent effects of ouabain on the Na/K-ATPase activity:** Whole cell lysates from P-11 and AAC-19 cells were prepared and assayed for the Na/K-ATPase activity as described in the Experimental Procedures. Data are shown as percentage of control and each point was presented as mean ± SEM of four independent experiments. Curve fit analysis was performed by Graphpad software.

**Figure 4 Regulation of Src activity by Na/K-ATPase:** A and B. Cell lysates (30 µg/lane) from different cell lines were separated on SDS-PAGE and analyzed by either anti-c-Src (B-12) or anti- pY418-Src antibody. The quantitative data are mean ± SEM from four separate experiments. *P < 0.05 versus P-11; C. Cultured P-11 and TCN23-19 cells were serum starved for 12 h and immunostained by
anti-pY\textsuperscript{418}-Src antibody. The images were collected as described in the Experimental Procedures. The scale bar represents 50 µm.

**Figure 5** Regulation of Src activity by the pumping-null Na/K-ATPase.  

**A and B.** Cell lysates (30 µg/lane) from different cell lines were separated on SDS-PAGE and analyzed by either anti-c-Src (B-12) or anti-pY\textsuperscript{418}-Src antibody. The quantitative data are mean ± SEM from four separate experiments. *P < 0.05 versus P-11;  

**C.** PY-17 cells were transiently transfected with either an empty vector (mock), silently mutated wt rat α1 (AAC), or the D371E mutant. After 36 h, the transfected cells were lysed and analyzed by Western blot using specific antibodies as indicated. A representative Western blot is shown and the same experiments were repeated four times.  

**D.** TCN23-19 cells were transiently transfected with a vector expressing EYFP-fused α1 D371E mutant (pEYFP-D371E). After 24 h, cells were serum starved for 12 h and then immunostained by anti-pY\textsuperscript{418}-Src antibody. Images from a representative experiment show that expression of mutant pEYFP-D371E reduced the intensity of red (pY\textsuperscript{418}-Src) fluorescence (comparing the green and nearby non-green cells). The quantitative data of pY\textsuperscript{418}-Src were collected from 40 different microscope vision fields in 4 independent experiments and expressed as mean ± SEM. **p<0.01. The scale bar represents 22 µm.

**Figure 6** Interaction between Src and the pumping-null Na/K-ATPase.  

**A and B.** TCN23-19 cells were co-transfected with Src-ECFP and EYFP-rat α1
mutant (D371E) expression vectors. After 24 h, FRET analysis was performed as described in the Experimental Procedures. Boxed ROI_1 (green) was photobleached, and the ROI_3 (yellow) membrane area was analyzed for FRET. The Box ROI_2 (purple) was selected and served as a non-bleaching control. The experiments were repeated three times and a total of 20 cells were analyzed.

C. TCN23-19 cells were transiently transfected as in Panel A with either silently mutated wt rat $\alpha_1$ (AAC) or rat $\alpha_1$ pumping-null mutant (D371E) expression vectors. After 36 h, cell lysates were prepared and subjected to immunoprecipitation using monoclonal anti-Src (clone GD11) antibody. Immunoprecipitants were then analyzed by Western blot using either anti-NASE antibody (for rat $\alpha_1$) or anti-c-Src (SRC2) antibody. The same experiments were repeated three times and a representative Western blot is shown.

**Figure 7 Regulation of FAK phosphorylation by Src-interacting Na/K-ATPase.** A. Cultured P-11 and PY-17 cells were serum starved for 12 h. Cell lysates were then immunoprecipitated using anti-phosphotyrosine antibody (4G10), and immunoprecipitates were analyzed by anti-FAK antibody. The combined quantitative data were from three independent experiments. B. Cell lysates from different cell lines were separated on SDS-PAGE and analyzed by anti-pY$^{925}$-FAK and anti-pY$^{418}$-Src antibody. The same membrane was stripped and reprobed with anti-c-Src (B-12) antibody. A representative blot of three independent experiments is shown. C. Cell lysates were analyzed by anti-pERK1/2 or anti-ERK1/2 antibody. The quantitative data (mean ± SEM) were
calculated from four separate experiments as relative ratio of pERK/ERK. D. P-11 and PY-17 cells were treated with 1µM PP2 for 0.5 and 2 h. FAK and Src activation were measured by using the specific antibodies. A representative Western blot is shown and the same experiments were repeated three times. E. PY-17 cells were transiently transfected with either an empty vector (mock) or the D371E mutant. After 36 h, the transfected cells were lysed and analyzed by Western blot using specific antibodies as indicated. A representative Western blot is shown and the same experiments were repeated three times.

Figure 8 Effects of ouabain on Src and ERK1/2. A and B. Cells were exposed to 100 nM ouabain for either 5 or 15 min, and the cell lysates (50 µg/lane) were analyzed by Western blot for active Src or active ERK1/2. Blots were probed first with anti-pY418-Src or anti-pERK antibody, and then stripped and re-probed for total Src or ERK1/2 to ensure equal loading. C and D. Cells were treated with indicated concentrations of ouabain for 5 min, and total cell lysates were analyzed for pY418-Src and total Src or pERK1/2 and total ERK1/2 as in A and B. A representative Western blot and combined quantitative data are shown. The quantitative data (relative ratio of pSrc/Src or pERK/ERK) from three independent experiments (mean ± SEM) were calculated relative to the control condition of P-11 cells. *P < 0.05 versus the respective control condition of each cell line.
**Table I. Targets and oligo sequences of human Na/K ATPase-α1 subunit specific siRNAs.**

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Target Sequence</th>
<th>Oligo Inserts</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>467agatcatggaaccttcaaa</td>
<td>Sense: 5’tcagagatcatggaaccttcaatctaagagattgaaggatcattcagtctttt-3’&lt;br&gt;Anti-sense: 5’ctagagagatcatggaaccttcaatctcttggaaggattcattcagtctct-3’</td>
</tr>
<tr>
<td>A2</td>
<td>1469ctcaccaccaaaagtaccag</td>
<td>Sense: 5’tcagctcaccaccaaaagtaccagttcaagagacctgtaaccttgtggtggagttttt-3’&lt;br&gt;Anti-sense: 5’ctagagagaacctcaccaccaaaagtaccagttctctgtaacctgtggtggagc-3’</td>
</tr>
<tr>
<td>A3</td>
<td>1830gtgcatggtctacagga</td>
<td>Sense: 5’tcaggggtcatggtctacaggaattcaagagatctctgtgacccagtgaaccttttt-3’&lt;br&gt;Anti-sense: 5’ctagagaaaagttgcatggtctacaggaattctcttgacccagtgaaccct-3’</td>
</tr>
<tr>
<td>A4</td>
<td>2200gtgctgtgtctttgata</td>
<td>Sense: 5’tcagagggtcgtgtctttgataattcaagagcatcaagatcaagagtcagaccccttttt-3’&lt;br&gt;Anti-sense: 5’ctagagagagttgctgtgtctttgataattctcttgacccagtgaaccct-3’</td>
</tr>
</tbody>
</table>

**Table II. Relative α1 subunit protein content and the composition of DNA constructs used in different cell lines.**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Relative α1 content (mean ± SEM)</th>
<th>DNA Constructs used in transfection</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-11</td>
<td>100%</td>
<td>pSuppressor ; pBade-puro</td>
</tr>
<tr>
<td>A1</td>
<td>97.4 ± 2.1%</td>
<td>pSuppressor -A1 siRNA ; pBade-puro</td>
</tr>
<tr>
<td>A4-11</td>
<td>44.1 ± 2.3%</td>
<td>pSuppressor -A4 siRNA ; pBade-puro</td>
</tr>
<tr>
<td>TCN23-19</td>
<td>12.0 ± 4 %</td>
<td>pSuppressor -A4 siRNA ; pBade-puro</td>
</tr>
<tr>
<td>PY-17</td>
<td>7.5 ± 3.0 %</td>
<td>pSuppressor -A4 siRNA ; pBade-puro ; pEYFP</td>
</tr>
<tr>
<td>PY-17-AAC-M1-19 (AAC-19)</td>
<td>93.7 ± 9.9 %</td>
<td>pSuppressor -A4 siRNA ; pBade-puro ; pEYFP ; pRc/CMV-α1AACm1 (rat α1)</td>
</tr>
</tbody>
</table>
Table III: Na/K ATPase Activity in different cell lines.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>P-11</td>
<td>100%</td>
</tr>
<tr>
<td>PY-17</td>
<td>20.8 ± 3.7%</td>
</tr>
<tr>
<td>AAC-19</td>
<td>92.2 ± 6.4%</td>
</tr>
</tbody>
</table>
FIGURE 1

A.

\[ \alpha_1 \]

P-11  A4-11  A4-11  TCN23-19  PY-17

B.

EYFP  \[ \alpha_1 \]  Merge

![Images of EYFP, \[ \alpha_1 \], and Merge]
FIGURE 2

A. 

<table>
<thead>
<tr>
<th></th>
<th>P-11</th>
<th>PY-17</th>
<th>AAC-19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysate (µg)</td>
<td>15</td>
<td>60</td>
<td>15</td>
</tr>
</tbody>
</table>

Rat α1

α1

B. 

EYFP  α1  Merge

FIGURE 3

% of inhibition

<table>
<thead>
<tr>
<th>Oua log(nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>7</td>
</tr>
</tbody>
</table>

P-11

AAC-19
FIGURE 4

A. c-Src

B. pY418-Src

C. pY418-Src
FIGURE 6

A.  

Src-ECFP  

D pre  D post  

EYFP-D371E  

A pre  A post  

B.  

\[ FRET_{eff} = \frac{D_{post} - D_{pre}}{D_{post}} \]  

for all \( D_{post} > D_{pre} \)  

<table>
<thead>
<tr>
<th>ROI</th>
<th>ROI_2</th>
<th>ROI_3</th>
</tr>
</thead>
<tbody>
<tr>
<td>D pre</td>
<td>50.54</td>
<td>41.16</td>
</tr>
<tr>
<td>D post</td>
<td>52.23</td>
<td>49.40</td>
</tr>
<tr>
<td>A pre</td>
<td>30.07</td>
<td>27.24</td>
</tr>
<tr>
<td>A post</td>
<td>33.79</td>
<td>10.08</td>
</tr>
<tr>
<td>FRETeff (%)</td>
<td>3.23</td>
<td>16.67</td>
</tr>
</tbody>
</table>

C.  

IP c-Src  

<table>
<thead>
<tr>
<th></th>
<th>mock</th>
<th>AAC</th>
<th>D371E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat-α1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-Src</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 7

A. IP p-Tyrosine
IB FAK

phospho-FAK

Relative Units

0 100 200 300
P-11 PY-17

* P-11 PY-17

B. P-11 A4-11 PY-17

pY^{925}\_FAK

pY^{416}\_Src

c\_Src

C. P-11 PY-17

p-ERK1/2

Relative Level (% of control)

50 100 150
P-11 PY-17

* P-11 PY-17

D. mock D371E

pY^{925}\_FAK

α1

pY^{416}\_Src

FAK

PP2 (1μM) 0 0.5 2

P-11 PY-17

FAK
FIGURE 8

A. pY418-Src

B. p-ERK1/2

C. pY418-Src

D. p-ERK1/2
Identification of a Pool of Non-Pumping Na/K-ATPase*

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\textsuperscript{3} These authors contribute equally to this work.

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Running Title: Non-pumping Na/K-ATPase

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Summary

Recent studies have ascribed many non-pumping functions to the Na/K-ATPase which was initially discovered as an ion pump. In the current paper, we present experimental evidence demonstrating that over 65% of the plasma membrane Na/K-ATPase in LLC-PK1 cells are not involved in ion pumping. This “non-pumping” pool of Na/K-ATPase, like the pumping pump, binds ouabain. Depletion of either cholesterol or caveolin-1 moves some of “non-pumping” Na/K-ATPases into the pumping pool. Graded knock-down of the α subunit of the Na/K-ATPase eventually results in a loss of this “non-pumping” pool. Because prior studies indicate that loss of the non-pumping pool is associated with a loss of signaling function as evidenced by the failure of ouabain administration to induce additional activation of Src and/or ERK, these new findings suggest that, at least in some types of cells, a substantial amount of surface-expressed Na/K-ATPase may function as a ouabain-binding receptor, thus converting the extracellular ouabain signal into intracellular second messengers.

Introduction

Na/K-ATPase is an integral membrane protein found in most mammalian cells. It is composed of an equal molar α and β subunits. As an energy transducer, Na/K-ATPase translocates 3 Na⁺ out and 2 K⁺ into the cell by hydrolysis of one molecule of ATP (1,2). This process generates the transmembrane chemical and electrical gradients that are essential to the excitable
activity of muscle and nerve tissue, and the regulation of cell volume and a number of Na\(^+\) coupled transporters. Since its discovery by Skou in 1957 (3), numerous studies were carried out to elucidate the energy transducing function of the Na/K-ATPase. Indeed, these works have provided a wealth of detailed information about how these proteins carry out the coupled extrusion and uptake of Na\(^+\) and K\(^+\) across the membrane. However, evidence accumulated during the last ten years indicates that Na/K-ATPase also serves as a signal transducer. This function depends on interactions of the Na/K-ATPase with various proteins including protein and lipid kinases, membrane transporters, channels and cellular receptors (4-7). Binding of either endogenous or exogenous cardiotonic steroids such as ouabain to this protein complex evokes protein and lipid kinase cascades, thus generating a large number of secondary messengers (8-12). Studies from our and other laboratories have shown that the signaling Na/K-ATPase resides with its partners in caveolae (13) and this compartmentalized localization makes it possible for the signaling Na/K-ATPase to interact and assemble with Src into a functional receptor tyrosine kinase (14). Binding of ouabain to Na/K-ATPase activates the Na/K-ATPase-associated Src, resulting in tyrosine phosphorylation of downstream effectors and recruitment of additional kinases and adaptor protein to the activated receptors.

We have demonstrated recently that there may be a pool of Src-interacting Na/K-ATPase since knock-down of the Na/K-ATPase α1 subunit by using siRNA-based approaches can decrease the interaction of the Na/K-ATPase with Src, and result in increase in basal Src activity and an inhibition of
ouabain-induced signal transduction (15). All of these novel findings have led us to propose that there may be two separate pools of Na/K-ATPase in cell membrane, one performing the canonical (pumping) function while the other carrying out non-canonical functions such as signal transduction and scaffolding. In this study, we characterized the ion-pumping properties of the Na/K-ATPase in the established α1 knock-down cells. The data presented here clearly demonstrated the existence of a pool of non-pumping Na/K-ATPase in the cell membrane.

**Experimental Procedures**

**Cell Culture:** The α1 knock-down stable cell lines were generated from LLC-PK1 cells as described (15).

**Cell Surface Biotinylation, Streptavidin Precipitation, and Immunoblot:** Labeling with biotin of plasmalemma proteins was performed according to the protocol described by Gattardi et al. (16) with minor modification (17). See more information in “Supplemental Data”.

**Ouabain-sensitive $^{86}$Rb$^+$ uptake:** Ouabain-sensitive $^{86}$Rb$^+$ uptake was employed to measure the transport function of Na/K-ATPase (18-21). Cells were cultured in 12 well plates to 90% confluence, washed, and serum-starved overnight. After washed with the fresh medium, the cells were incubated in the same medium at 37°C for 10 min in the presence of different concentrations of ouabain. Monensin (20 µM) was added to clamp intracellular Na$^+$ to ensure maximal capacity of active uptake (22). Then, $^{86}$Rb$^+$ as the tracer for K$^+$
(1 $\mu$Ci/well) was added to start the uptake experiment. After 10 min, 3 ml of ice-cold 100 mM MgCl$_2$ were added to stop uptake. Cells were then washed three times with the same solution. Trichloroacetic acid (TCA) soluble $^{86}$Rb$^+$ was counted by a liquid scintillation counter. TCA-precipitated protein was dissolved in SDS followed by the Lowry’s assay. Preliminary experiments show that the uptake was a linear function of time for up to 20 min.

$^3$H-ouabain binding: The number of ouabain binding sites in cultured cells was estimated by determining the specific binding of $^3$H-ouabain (23). Briefly, cells were seeded into 12 well plates and cultured for 2 to 3 days to 90% confluence. Then, cells were rinsed, and incubated in K$^+$ free Kreb’s solution (NaCl 137 mM; KCl 0 mM; CaCl$_2$ 2.8 mM; NaH$_2$PO$_4$ 0.6 mM; MgSO$_4$ 1.2 mM; dextrose 10 mM; Tris 15 mM; pH 7.4) for 15 min, and then exposed to various concentrations of $^3$H-ouabain for 15 min at 37˚C. At the end of incubation period, the cells were washed four times with ice-cold K$^+$ free Kreb’s solution, solubilized in 0.1M NaOH/0.2% SDS, and counted by a scintillation counter. Non-specific binding was measured in the presence of 5 mM unlabeled ouabain and subtracted from total binding. Curve fit analysis was performed by Graphpad software, and Bmax was calculated on the basis of formula: $Y = \frac{B_{max} \times X}{K_d + X}$, where $X$ is concentration of $^3$H-ouabain, $Y$ is the total binding and $K_d$ is the equilibrium dissociation constant.

$[\text{Na}^+]$ measurement: Estimation of relative amount of intracellular Na$^+$ was done according to Kim et al (24). In brief, cells were cultured in 12 well plates and incubated at 37˚C in the culture medium containing trace amounts of $^{22}$Na$^+$.
(0.5μCi/ml) for 60 min to fully equilibrate exchangeable intracellular Na⁺ with ²²Na⁺. Cells were washed in ice-cold 100 mM MgCl₂, and precipitated by TCA. The TCA extractable ²²Na⁺ was counted by scintillation counter.

**Data analysis:** Data presented are mean ± SEM and statistical analysis was performed using student’s t-test.

**Results and Discussion**

**The mismatch between the pumping activity and the protein amount of Na/K-ATPase:**

We have demonstrated recently that depletion of the intracellular Na/K-ATPase reduces the size of a pool of Na/K-ATPase which interacts with Src, resulting in increases in basal Src activity and an inhibition of ouabain-induced Src activation (15). Because cells need the pumping Na/K-ATPase to survive, we reasoned that knock-down of cellular α1 subunit might preferentially deplete the non-pumping pool of the Na/K-ATPase to preserve the pumping pool, resulting in a mismatch between the α1 protein level and the pumping activity in the knock-down cells. To test this hypothesis, we measured the Na/K-ATPase-mediated ⁸⁶Rb⁺ uptake in the established α1 knock-down cell lines that were derived from LLC-PK1 cell (15). As shown in Table I, the reduction of the α1 subunit content caused a roughly proportional decrease of the maximum pumping activity of A4-11 cells when compared to that of control P-11 cells (45.9
±1.9% of control). However, PY-17 cells that expressed less than 10% of the α1 subunits still exhibited about 40% of pumping activity.

It is important to note that the above ouabain-sensitive \(^{86}\text{Rb}^+\) uptakes were done in the presence of 20 µM monensin. Monensin increases the intracellular \(\text{Na}^+\) to a degree sufficient to maximally stimulate Na/K-ATPase activity (25). In the absence of monensin, PY-17 cells exhibited about 70% of pump activity compared to P-11 cells (Table I). As expected, when intracellular \(\text{Na}^+\) was measured, significant increases were detected in both A4-11 and PY-17 cells due to the decreased pump activity (Table I). PY-17 cells, for example, contained about twice more \(\text{Na}^+\) in comparison with that in P-11 cells when intracellular \(\text{Na}^+\) was measured by \(^{22}\text{Na}^+\) equilibrium assay (Table I). Importantly, while addition of monensin caused 90% increase in ouabain-sensitive \(^{86}\text{Rb}^+\) uptake due to the increased turnover rate (or reserved pump capacity) in P-11 cells (24), it failed to do so in PY-17 cells. These data indicate that the pumps in PY-17 cells operated at the maximal pump capacity, which is consistent with the fact that the \(\text{Na}^+\) content was doubled in these cells.

Based on the quantitation data of cell surface biotinylation assay, about 70% of α1 subunits in the control P-11 cells were distributed in the plasma membrane (Suppl fig. 1). If we assume that all of the α1 subunits in PY-17 cells are localized in the plasma membrane and function as pumps, it is clear that less than 35% of the surface Na/K-ATPase in control P-11 cells are directly involved in ion-pumping. Apparently, knock-down of Na/K-ATPase initially reduces both pumping and non-pumping pools as demonstrated in A4-11 cells. Once the
pumping activity is reduced to half, further reduction in \( \alpha_1 \) causes a preferential depletion of the non-pumping pool of the Na/K-ATPase, which is consistent with our prior observations (15).

To ensure that the \( \alpha_1 \) knock-down did not change other \( K^+ \) uptake mechanism, we measured Na/K/2Cl-cotransporter mediated-\( ^{86}\)Rb\(^+ \) uptake, observing that bumetanide-sensitive uptake activity in PY-17 cells was comparable to that in P-11 cells (data not shown). In short, PY-17 cells expressed less than 10\% of \( \alpha_1 \) subunits and were capable of retaining almost 70\% of basal pumping activity that is sufficient to maintain essential cellular functions for cells to survive and proliferate.

When Na/K-ATPase activity was measured in the \( \alpha_1 \) knock-down cells, we also found a mismatch between the ATPase activity and the protein amount of the Na/K-ATPase \( \alpha_1 \) subunit in these cells (15). Specifically, while PY-17 cells expressed only 8\% of the \( \alpha_1 \) protein, they exhibited 21\% of ouabain-sensitive ATPase activity in comparison to the control P-11 cells, indicating a pool of inactive \( \alpha_1 \) subunits in P-11 cells. Because the above ATPase assay was done with a crude membrane preparation in the presence of almethicin, the assay determined both the plasma membrane and intracellular pools of the Na/K-ATPase, which may increase the apparent total Na/K-ATPase activity in P-11 cells. In addition, the small fraction of ouabain-sensitive ATPase activity (less than 10\% of the total ATPase) in the crude membrane preparations made it difficult to run accurate assays. Thus, the apparent size of the non-pumping pool
of Na/K-ATPase may be underestimated by ouabain-sensitive ATPase activity assay.

**Effects of α1 knock-down on β1 expression:**

There is ample evidence that the formation of the functional Na/K-ATPase requires the β subunit (26). Thus, it is important to determine whether the apparent mismatch in the pumping activity between P-11 and PY-17 cells is due to either relative over-expression of the β in PY-17 cells or relative under-expression of the β in P-11 cells. As depicted in Suppl fig. 2A, control P-11 cells expressed at least three highly glycosylated β1 subunits. The expression of these glycosylated β1 was significantly reduced in both A4-11 and PY-17 cells, and the reduction is clearly proportional to the degrees of the α1 knock-down (Suppl fig. 2A). Quantitative analysis of the Western blots indicates that PY17 cells expressed about 25% of the total β1. However, when the two most glycosylated β1 species were compared, less than 5% were detected in PY-17 cells. These findings were consistent with the immunostaining images depicted in Suppl fig. 2B. While most of the cellular Na/K-ATPase β1 were localized in the plasma membrane in the control P-11 cells, they were largely retained in the intracellular compartments in TCN23-19 cells, which is another α1-depleted cell line that behaved like PY-17 (15). Interestingly, a portion of the retained Na/K-ATPase β1 subunits in TCN23-19 cells were clearly co-localized with the Golgi marker protein, giantin. Taken together, the data indicate that it is less likely that
changes in apparent pumping activity in the α1 knock-down cells are due to the over-expression of the β1.

**Ouabain binds non-pumping Na/K-ATPase:**

The above data suggest that the control P-11 cells contained a large pool of non-pumping Na/K-ATPase in the plasma membrane. To test if this non-pumping pool of the Na/K-ATPase can still bind ouabain, ^3^H-ouabain binding assay was performed. As depicted in Table I, P-11 cells contained about 800,000 ouabain binding sites per cell, which is consistent with prior observations (27). When ouabain binding was measured in PY-17 cells, we found that these cells contained about 125,000 sites per cell, thus 15% of the control P-11 cells. A4-11, on the other hand, expressed about 360,000 sites per cell. These data, taken together with the activity measurement, clearly indicate a mismatch between the number of functional Na/K-ATPase (measured as ouabain binding site) and the pumping activity in the knock-down cells. Quantitatively, the binding data again suggest that about 35% (15% of binding vs 40% activity in PY-17 cells) of ouabain-binding Na/K-ATPase belong to the pumping pool while the other 65% are capable of binding ouabain, but may perform cellular functions other than ion-pumping.

If control P-11 cells contain a pool of non-pumping Na/K-ATPase that can bind ouabain, we expect that PY-17 cells will be more sensitive to ouabain-induced pump inhibition than control P-11 cells. Indeed, when ouabain concentration curves were constructed, we found that 357 nM ouabain was
sufficient to cause 50% inhibition of ouabain-sensitive $^{86}\text{Rb}^+$ uptake in PY-17 cells whereas the same inhibition in control P-11 cells required 2131 nM of ouabain (Fig. 1). Consistently, the apparent $K_{1/2}$ for A4-11 cells was between P-11 and PY-17 cells. These data provide further support to the notion that there exists a large pool of non-pumping Na/K-ATPase in cultured cells. Importantly, this pool of Na/K-ATPase, like the pumping pump, is capable of binding ouabain. If this non-pumping pool is involved in transmitting extracellular ouabain signal, it is estimated that ouabain at physiological concentration (e.g. 0.1 nM) can activate about 2000 receptor sites per cell in LLC-PK1 cells. This activation shall generate enough second messengers to change cellular function, especially when the initial signal can be amplified via the protein kinase cascades.

It is known that purified renal Na/K-ATPase has a turn over rate of 8,000 to 10,000 cycles per min for ATP hydrolysis. However, when the same turnover rate was measured in cultured cells or isolated renal tubules, the number was much lower, and ranged from 2000 to 5000 cycles per min depending on cell type (28-30). Using the above ouabain-binding and $^{86}\text{Rb}^+$ uptake data, we calculated turnover rates of the Na/K-ATPase-mediated ATP hydrolysis (two K$^+$ per pump cycle per ATP hydrolysis) in the different cell lines. These calculations revealed that the Na/K-ATPase in P-11 cells operated at about 2,200 cycles per min while the pump in PY-17 cells ran at 5,000 cycles per min. These findings are consistent with the reported values in the literature, and with the notion that these control cells contain a large pool of non-pumping Na/K-ATPase. They also suggest that interaction of the Na/K-ATPase with other cellular proteins is a key
to generation of a pool of non-pumping Na/K-ATPase. Removal of the interacted proteins from the Na/K-ATPase during the purification may de-inhibit the enzyme, converting the non-pumping pump into pumping pump.

**Disruption of caveolae converts the non-pumping Na/K-ATPase into the pumping pump:**

We have shown that caveolae contain a pool of the Na/K-ATPase that interacts with caveolin-1, Src and other signaling proteins, thus serving as a functional receptor for cardiotonic steroids such as ouabain (13,31). Based on the structural information from SERCA and the Na/K-ATPase (32,33), both A (actuator) and N (nucleotide binding) domains of the α1 may undergo a large and rapid conformational change during each pump cycle. Because these domains are involved in interaction with the signaling and structural proteins (5-7), these interactions, in principle, will either slow down or inhibit the movement of these functional domains, thus the pumping activity of the Na/K-ATPase. Conceivably, this could generate a pool of non-pumping pump. By concentrating Na/K-ATPase and its signaling partners, caveolae could facilitate the interactions between the Na/K-ATPase and other proteins thus play a role in the generation of a pool of non-pumping Na/K-ATPase. We previously demonstrated that depletion of either cholesterol by methyl β-cyclodextrin (MβCD) or caveolin-1 by siRNA redistributed the Na/K-ATPase from caveolar fraction, and inhibited ouabain-activated signal transduction in LLC-PK1 cells (13). If caveolae are involved in assembly of the non-pumping pool of the Na/K-ATPase, we would
expect that depletion of cholesterol should increase Na/K-ATPase-mediated $^{86}\text{Rb}^+$ uptake. Indeed, as illustrated in Fig. 2A, pre-treatment of P-11 cells with 10 mM MβCD resulted in a significant increase (40% over control) in the Na/K-ATPase-mediated $^{86}\text{Rb}^+$ uptake without affecting the total ouabain-binding sites. The same effects were observed when the parent LLC-PK1 cells were exposed to 10 mM MβCD (data not shown). Significantly, when the same treatment was applied to the knock-down cells, we found no change in the pump activity in PY-17 cells. On the other hand, it caused a significant, but much smaller increase in A4-11 cells. These data are consistent with the notion that most of the Na/K-ATPase in PY-17 cells operates as a pump whereas A4-11 cells contain reduced pools of both pumping and non-pumping Na/K-ATPases. To further confirm the above findings, we also measured $^{86}\text{Rb}^+$ uptake and $^3\text{H}$-ouabain binding in both control P-11 cells and caveolin-1 knock-down C2-9 cells that were derived, like P-11, from LLC-PK1 cells (34). As depicted in Fig. 2B, depletion of caveolin-1 also resulted in a comparable increase in the pumping activity per ouabain binding site. Clearly, disruption of caveolae can convert a portion of non-pumping Na/K-ATPase into pumping pump. It is also clear that caveolae in LLC-PK1 cells only contain less than half of the cellular non-pumping Na/K-ATPase assuming that these treatments release all of the caveolar Na/K-ATPase from interactions with other proteins.

To test whether cells other than LLC-PK1 also contain a pool of non-pumping Na/K-ATPase, we treated cultured fibroblasts derived from the rat heart with MβCD, and then measured for the Na/K-ATPase-mediated $^{86}\text{Rb}^+$ uptake.
We found that the depletion of cholesterol doubled the pumping activity in cultured fibroblasts (231.5 ± 15.7%, n = 6). These results suggest that an even greater majority of plasma membrane Na/K-ATPase belongs to the non-pumping pool in fibroblasts than in the cells derived from renal epithelium. This is not surprising as renal epithelial cells express a large number of Na\(^+\) and K\(^+\) channels and transporters, thus requiring more “pumping pumps” to keep up with the inward sodium and outward potassium “leak”. Fibroblasts, on the other hand, are far less “leaky”, ergo their pump population would contain relatively less “pumping pumps”. Interestingly, reduction of cellular cholesterol has also been reported to increase the Na/K-ATPase activity in several other types of cells (35,36).

Taken together, the data indicate that caveolae play an important role in the organization of non-pumping pool of Na/K-ATPase. It also points out that the size of non-pumping pool may vary depending on the function of different cells. This is important because the size could determine the mode of ouabain action. For example, if the size of non-pumping pool is large enough, the physiological effects of ouabain are most likely mediated by the activation of protein kinase cascades as we demonstrated (5,15) or by stimulation/inhibition of other unknown protein complexes. On the other hand, if the non-pumping pool is small or depleted, the ouabain effect is most likely due to the pumping inhibition, and subsequent changes in intracellular ion concentration. Since Na/K-ATPase is very important in the economy of cells, the existence of a pool of “spare” Na/K-
ATPase could serve as a reservoir for cells to rapidly and appropriately adjust to different conditions.

In short, the evidence provided in the present study strongly suggests the existence of a large pool of non-pumping Na/K-ATPase in cultured cells. Apparently, this non-pumping pool of the Na/K-ATPase resides in the plasma membrane and is capable of binding ouabain. These findings are significant. First, they are consistent with recent studies from many laboratories, showing that the cellular Na/K-ATPase can transmit extracellular ouabain signal independent of its pumping function. Second, they suggest that the majority of plasma membrane Na/K-ATPase (over 60%), at least in some type of cells, is engaged in activities other than ion-pumping. It is important to note that these activities include, but are not limited to, these well-characterized signal transducing functions of the Na/K-ATPase. The list of the Na/K-ATPase-interacting proteins is getting longer and longer. Interaction of the Na/K-ATPase with phospholemman in the heart is known to keep the enzyme as a non-pumping pump (37,38). Interaction with cofilin appears to play a role in regulation of metabolic activity (39). Because ouabain can bind these protein complexes, cardiotonic steroids as endogenous hormones could have profound regulatory effects on various cellular functions. Third, the new findings bring about many new and important issues regarding the Na/K-ATPase. For example, how are these two pools of Na/K-ATPase assembled, delivered, and disassembled? Furthermore, is the size of these pools dynamically regulated? Finally, they call for the engagement of more investigators to unravel the
unknown non-canonical functions of the Na/K-ATPase and cardiotonic steroids, and delineate the roles of these functions in cell biology and animal physiology.

References


Figure Legends

Figure 1. Ouabain dose-response curve on $^{86}$Rb$^+$ uptake. Cells were pre-treated with different concentrations of ouabain as indicated for 10 min and assayed for $^{86}$Rb$^+$ uptake as described in “Experimental Procedures” section. Data are shown as % of respective control value and are presented as the mean ± SEM of 3 independent determinations. Curve fit analysis was performed by Graphpad software.

Figure 2. A. Effects of cholesterol depletion on Na/K-ATPase pumping activity. The control cells (P-11) and α1 knock-down cells (A4-11 and PY-17) were pre-treated with MβCD (10mM) at 37 °C for 1 h, then 1 mM ouabain were administrated for 10 min to analyze the ouabain sensitive $^{86}$Rb$^+$ uptake activity as described. Data are shown as % of control value for each cell line and are presented as the mean ± SEM (n = 3). * P<0.05 ** P<0.0001. B. knock-down of Caveolin-1 increases Na/K-ATPase pumping activity. The ouabain sensitive Na/K-ATPase $^{86}$Rb$^+$ uptake was assayed in both control P-11 cells and caveolin-
1 knock-down cells as described in “Methods”. Data are shown as % of P-11 and are presented as the mean ± SEM (n = 4). ** P<0.0001.

Table I. The Na/K-ATPase properties in different cell lines.

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<tr>
<th>Cell lines</th>
<th>P-11</th>
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<th>PY-17</th>
</tr>
</thead>
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<tr>
<td>α1 content (%), n=4</td>
<td>100</td>
<td>44.1 ± 2.3</td>
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<td>Pump activity (%), n=4</td>
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<td>(20 µM monensin)</td>
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<tr>
<td>Bmax (%), n =3</td>
<td>100</td>
<td>45.0 ± 3.0</td>
<td>15.6 ± 4.8</td>
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<tr>
<td>(Binding sites X10^4 /cell)</td>
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<td>(36.1 ±1.2)</td>
<td>(12.5 ±0.6)</td>
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<td>[Na^+]_i (%), n=4</td>
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<td>194.4 ± 7.1</td>
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<tr>
<td>Pump activity (%), n=4</td>
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<td>66.3 ± 8.8</td>
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<tr>
<td>(w/o monensin)</td>
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FIGURE 1

Sigmoidal dose-response (variable slope)

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Std. Error

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<td>HILLSLOPE</td>
<td>0.2151</td>
<td>0.1597</td>
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</tr>
</tbody>
</table>
Supplemental Data

Experimental Procedures:

Materials: Image-iT FX signal enhancer, Antifade kit, Alexa Fluor 488-conjugated anti-mouse/rabbit IgG and Alexa Fluor 546-conjugated anti-rabbit IgG antibodies were obtained from Molecular Probes (Eugene, OR). Anti-Na/K-ATPase β1 (cloneC464.8) was from Upstate (Lake Placid, NY); Anti-Giantin was from Covance (Berkeley, CA). The monoclonal anti-α1 antibody (α6F) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. All the secondary horseradish peroxidase-conjugated antibodies were from Santa Cruz (Santa Cruz, CA). Radioactive $^{86}\text{Rb}^+$ and $^{3}\text{H}$-ouabain were from NEN Life Science Products.

Cell Surface Biotinylation, Streptavidin Precipitation, and Immunoblot:

Cells grown on 60 mm dishes were placed on ice, rinsed twice with ice-cold PBS, and then incubated with 2 ml of NHS-ss-biotin freshly diluted into biotinylation buffer (10 mM triethanolamine pH9.0, 2 mM CaCl$_2$, 150 mM NaCl, and 250 mM sucrose) for 25 min at 4°C with very gentle horizontal motion to ensure mixing. Cells were then rinsed twice with PBS with 100 mM glycine, and washed in this buffer for 20 min at 4°C to make certain that all of the unreacted biotin was quenched. Monolayers were then rinsed twice more with PBS to wash away the quenched biotin, and solubilized in 900 µl lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5) for 60 min on ice. Lysates were clarified by centrifugation at 14,000 x g for 10 min at 4°C. The cleared lysate (750 µl) were collected and incubated overnight with 100 µl packed streptavidin-agarose
beads at 4°C with end-over-end rotation. The supernatant was then collected, and 1/10 of the total volume was loaded on the polyacrylamide gel as unbound fraction (U), together with 1/10 of the input cleared lysate as total lysate (T). Protein signal was detected using the ECL kit and quantified using Bio-Rad GS-670 imaging densitometer.

**Confocal fluorescence microscopy:** The subcellular localization of Na/K-ATPase β1 subunits and Giantin was analyzed by immunofluorescence staining. Cells cultured on the cover slips were washed twice with PBS and fixed with methanol pre-chilled at -20°C. The fixed cells were then rinsed with PBS for 3 times and blocked with 200 µl Image-iT FX signal enhancer for 30 min at room temperature. Cells were then incubated with the primary antibodies, anti-Na/K-ATPase β1 and anti-Giantin, diluted in PBS containing 1% BSA for 1 h followed by the incubation with secondary Alexa Fluor-conjugated antibodies. Image visualization was performed by Leica TCS-SP2 laser scanning microscope (Leica, Mannheim, Germany). Leica Confocal software was used in data analysis.

**Figure Legends**

**Supplemental figure 1. Cell surface biotinylation assay.** Biotinylation assay was performed in P-11 cells. Streptavidin-coated beads were used to pull down the biotinylated plasmalemma proteins. The same volume of the unbound fraction (U) and total cell lysate (T) was subjected to SDS-PAGE and probed with α6F antibody.
Supplemental figure 2. β1 expression in knock-down cells. A. Total cell lysates were used to detect the β1 expression level by using anti-Na/K ATPase β1 antibody. P-11 is the control cell line. A4-11, PY-17 and TCN23-19 are different α1 knock down cell lines. B. Immunostaining of β1 subunit in P-11 and TCN23-19 cells as described in “Methods”. Giantin was used as the Golgi marker.
SUPPLEMENTARY FIGURE 2

A.

B.
MANUSCRIPT #3

The Potentiation Effects of Na/K ATPase Knock-Down On EGFR Phosphorylation

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**Introduction**

Na/K ATPase, or sodium pump, is an integral membrane protein contains equal molar of α and β subunit. Besides the canonical ion-pumping function to keep the transmembrane electrochemical gradients, sodium pump also plays an essential role in signal transduction. The preassembled Na/K ATPase-Src complex can serve as a functional signaling receptor to transmit ouabain signals. Upon ouabain binding to Na/K ATPase, a rapid activation of Src can be elicited and followed by the transactivation of the EGF receptor (EGFR) (Haas, Askari et al. 2000; Tian, Cai et al. 2006).

Recently, by taking advantage of the siRNA based approaches, we have generated several Na/K ATPase α1 subunit knock-down cell lines using LLC-PK1 cells as the parental. Functional analysis of the these cells indicated the existence of a pool of Src-interacting Na/K ATPase, which not only regulates Src activity, but also serves as a receptor for ouabain to stimulate down-stream protein phosphorylation (Liang, Cai et al. 2006). Furthermore, over 65% of the plasma membrane Na/K ATPase, which preserve the capability of binding with ouabain, are not involved in the process of ion pumping (Liang, Tian et al. 2006).

The establishment of a pool of non-pumping Na/K ATPase in live cells prompts us to investigate the functional role of these non-pumping pumps in more details and whether they are involved in the regulation of cellular signaling events induced by stimuli other than ouabain? In this paper, we presented some
of the initial evidence suggesting that knock-down of Na/K ATPase could affect EGF signaling by potentiating the EGF induced EGFR phosphorylation.

**Materials and Methods**

**Materials:** The polyclonal anti-EGFR and anti-phospho-EGFR (pY845, pY992, pY1045, pY1068) antibodies were from Cell Signaling (Danvers, MA). anti-phospho-EGFR (pY1173) antibody was from Upstate (Lake Placid, NY).

**Cell Culture:** The α1 knockdown stable cell lines were generated from LLC-PK1 cells as described (Liang, Cai et al. 2006).

**Immunoprecipitation and Immunoblot analysis:** Cells were washed with PBS and solubilized in modified and ice-cold RIPA buffer, and subjected to immunoprecipitation or Western blot analysis as previously described (Wang, Haas et al. 2004). Protein signal was detected using the ECL kit and quantified using Bio-Rad GS-670 imaging densitometer.

**Results**

As shown in Figure.1A, Na/K ATPase knock-down cells (A4-11), which contain about 40% of the sodium pump, have higher level of EGF induced EGFR phosphorylation on selected residues, such as Tyr-1045 or Tyr-1068, than that of control P-11 cells, whereas the phosphorylation on tyrosine residue 992 appears not to be affected by the down-regulation of Na/K ATPase. Specifically, when the time course and dose curve for EGF treatment of were conducted in
these cells, the EGF stimulated phosphorylation of Tyr-1068 in α1 knock-down cells was found to be consistently more than that in control P-11 cells under identical conditions (Figure 1B). Moreover, When PY-17 cells, which is another α1 knock-down cell line containing less than 10% of the Na/K ATPase, were tested for the ligand induced EGFR phosphorylation, we found that with less Na/K ATPase protein, these cells displayed more prominent stimulation of EGFR phosphorylation by EGF compared to A4-11 as well as control P-11 cells (Figure 1C).

Interestingly, both wild type rat α1 and its pumping-null mutant are able to recover the up-regulated phosphorylation of EGFR back to normal in response to EGF in α1 knock-down cells. AAC-19 is the stable cell line generated by transfecting the wild type rat α1 into PY-17 cells and expresses similar amount of α1 to that of P-11 cells. The immunoprecipitates of EGFR in AAC-19 cells showed a significant lower level of tyrosine phosphorylation than in PY-17 cells upon EGF treatment. Furthermore, the phosphorylated Tyr-1045 amount in AAC-19 cells decreased to a level comparable to that in P-11 cells (Figure 2A). Similarly, these potentiating effects of the knock-down of Na/K ATPase on EGFR activation can also be abrogated by transiently transfecting the pumping-null rat α1 mutant (D371E) into PY-17 cells (Figure 2B).

**Discussion and Future Work**

EGFR is an intrinsic membrane protein of 1186 amino acids. It contains a large highly glycosylated extracellular domain, a single transmembrane domain
and an cytoplasmic domain where multiple phosphorylation sites are located (Ullrich, Coussens et al. 1984). EGFR belongs to a family of receptor tyrosine kinase that functionally transmits extracellular cues to intracellular signal transduction pathways which mediate cell growth, differentiation and survival (Holbro and Hynes 2004). Specifically, upon ligand (EGF or TGFα) binding, EGFR undergoes dimerization followed by the activation of the intrinsic kinase and the phosphorylation of specific tyrosines located in the EGFR's cytoplasmic region. These phosphorylated residues provide a platform for EGFR to interact with a variety of the signaling proteins and stimulate intracellular signaling cascades.

It has been observed that EGFR is greatly enriched in specialized membrane domains known as caveolae or lipid rafts (Mineo, James et al. 1996; Wang, Haas et al. 2004). Depletion of cholesterol with methyl-b-cyclodextrin can increase the cell surface EGF binding by 40%, which is independent of the receptor externalization from intracellular pool (Pike and Casey 2002). Upon ligand binding, EGFR rapidly dissociates from caveolae and migrates to the bulk membrane. This process appears to be regulated by Src kinase and occurs independently of the clathrin-mediated endocytosis (Mineo, Gill et al. 1999). The sequestration of EGFR in the caveolae plays an important role in the EGF signaling. A prolonged or reduced residence of EGFR in this domain may contribute to abnormal cell behavior.

EGFR and Na/K ATPase α1 subunit can be co-immunoprecipitated by each other, however, the evidence for the direct interaction between α1 and
EGFR is still lacking. So, the enhancement of the EGFR activation in Na/K ATPase knock down cells is probably secondary to the regulatory effect of α1 on its immediate partner proteins or its surrounding micro-environment, which may cause the alterations in the kinetics of ligand binding to EGFR, or the receptor conformational state.

It has been shown that both caveolin-1 and -3 can directly interact with EGFR through the caveolin-binding motif within the kinase domain of EGFR. The scaffolding domains of caveolin-1 and -3 both functionally inhibit the EGFR kinase activity in vitro (Couet, Sargiacomo et al. 1997). Studies from our laboratory suggested that Na/K ATPase can regulate the caveolin-1 trafficking and the depletion of Na/K ATPase may cause the retaining of caveolin-1 in the intracellular compartment (unpublished data). Since both of the membrane and caveolar caveolin-1 amount is decreased in the Na/K ATPase knock-down cells, the inhibitory effect of caveolin-1 on EGFR could be released in these cells. This speculation can be verified by immunoprecipitating EGFR in both control and α1 knock-down cells and then detecting the amount of associated caveolin-1 or vice versa. To test whether the direct interaction between α1 and caveolin-1 also plays a role in the regulation of EGFR, we may transfect an α1 mutant, which is defective in caveolin-1 binding, into PY-17 cells. If the enhanced EGFR activation in Na/K ATPase knock-down cells can not be recovered by the caveolin-1 binding mutant, then the involvement of the α1-caveolin-1 interaction in the regulation of EGFR activation can be established.
Accompanying with the decrease of the membrane caveolin-1, the caveolar cholesterol amount in the Na/K ATPase knock-down cells was also found to be significantly lower than control cells (unpublished data), in which situation the caveolae may undergo a functional deregulation. These events are very like to result in the redistribution of the EGFR in the plasma membrane as well as the ensuing conformational rearrangement of EGFR, which makes some of the tyrosine residues more accessible and then the enhancement of the EGFR phosphorylation on specific residues can occur in these Na/K ATPase knock-down cells upon ligand binding. To test this hypothesis, we should examine the relative caveolar distribution of EGFR in both α1 knock-down and control cell and higher level of phospho-EGFR is expected to be seen in the non-caveolar fraction in knock down cells after EGF stimulation.

Since knock-down of the Na/K ATPase resulted in significant increase of basal Src kinase activity, it is also important to know whether the Src kinase is involved in the regulation of EGFR activation by Na/K ATPase. This question can be simply answered by pre-treating the cells with Src kinase inhibitor PP2 before EGF stimulation and then testing the phosphorylation of EGFR.

In summary, the data presented here clearly indicated that Na/K ATPase is involved in the EGFR signaling pathway and knock-down of α1 subunit can potentiate the EGF induced phosphorylation of EGFR. However, the downstream effects of this regulation are still unknown and more studies need be done to identify the underlying mechanisms.
References


**Figure Legends**

**Figure 1. phosphorylation of EGFR in Na/K ATPase knock-down cells.** A and C. Cells were exposed to EGF (50ng/ml) for 2 min and tested for the phosphorylation of EGFR on specific tyrosine residues. B. Treat P-11 and A4-11 cells with indicated concentration of EGF for 2, 15 or 30 min and cell lysates were analyzed for phospho-Tyr-1068.

**Figure 2. phosphorylation of EGFR in rat α1 rescued PY-17 cells.** A. Cell lysates from EGF stimulated PY-17 and P-11 cells were immunoprecipitated using polyclonal anti-EGFR antibody and the immunoprecipitants were then analyzed by anti-phosphotyrosine antibody (4G10). B. PY-17 cells were transiently transfected with either an empty vector (mock) or the rat α1 pumping-null mutant (D371E), after 24 h, the transfected cells were treated with EGF and then assayed by Western Blot using the specific antibodies as indicated.
FIGURE 1

A. 

<table>
<thead>
<tr>
<th></th>
<th>P-11</th>
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<th>P-11</th>
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FIGURE 2

A.

EGF (50ng/ml)

IP-EGFR
IB-pTyr (4G10)

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B.

EGF (50ng/ml)

EGFR-pY1046
EGFR

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DISCUSSION AND SUMMARY

This dissertation is composed of three major parts:

For the first part of work, by using RNA interference-based techniques, we established several Na/K-ATPase α1 subunit knock-down cell lines. Previous studies have shown that Na/K-ATPase can directly interact with Src to form a signaling receptor complex through binding to its SH2 domain and kinase domain. This interaction keeps Src in an inactivate state. When ouabain binds to Na/K-ATPase, the associated Src kinase domain is released and activated. By analyzing how the alterations in the amount or properties of Na/K-ATPase can affect ouabain signal transduction, we found that there exists a pool of Src-interacting Na/K-ATPase in LLC-PK1 cells that regulates Src activity and serves as a receptor for ouabain to activate downstream signaling molecules such as FAK and ERK1/2. The RNA interference techniques we were using to manipulate the cellular Na/K-ATPase content offers additional advantages over the widely used ouabain selection procedure which forces the expression of the transfected ouabain-insensitive Na/K-ATPase in ouabain-sensitive cells. By rescuing PY-17 cells with different isoforms, we also will be able to uncover the potential isoform-specific signaling function. Functionally, we have provided further evidence to support the notion that Na/K-ATPase is an important regulator of protein kinases. Both Src and the Src downstream effector FAK can be activated by either ouabain or Na/K-ATPase depletion. These regulatory effects of Na/K-ATPase depend on its capability of protein-protein interaction, but not the ion-pumping function. However, the significance of the enhanced FAK phosphorylation
induced by Na/K-ATPase depletion remains to be determined. It has been known for a long time that the coordinated activation of FAK is critical in diverse cellular processes including the focal adhesion formation/turnover, cell spreading and migration. Specifically, FAK can regulate the interactions of focal adhesion with caveolae and microtubules, and the formation of focal adhesion at the front of the cell and disassembly at the rear to promote the cell migration. Thus, it will be valuable to determine how the manipulation of α1 subunit expression affects the FAK mediated cell migration. Besides the aforementioned, this part of work also calls for the need to examine the role of Na/K-ATPase-mediated Src repression and activation in cancer biology. Usually, upon activation, the endogenous Src is localized to the sites of cell adhesion from where to initiate signals to influence cell growth and adhesion strength. The strategy featured by targeting Na/K-ATPase to directly or indirectly inhibit Src kinase activity and its downstream FAK may have profound effects on cancer cell growth and differentiation.

In the second part, we mainly characterized the ion-pumping properties of the Na/K-ATPase in the established α1 knock-down cells and found that there is a mismatch between the protein amount of the Na/K-ATPase and its pumping activity. These data suggested to us that it is very likely that there exists a pool of non-pumping Na/K-ATPase in the plasma membrane. $^3$H-ouabain binding assay further indicated that about 35% of membrane Na/K-ATPase belong to the non-pumping pool of Na/K-ATPase whereas the other 65% are capable of binding ouabain but perform cellular functions other than ion-pumping. Caveolae have been found to play an important role in the organization of non-pumping pool of
Na/K-ATPase. Either the cholesterol depletion or knock-down of caveolin-1 can convert a portion of the non-pumping Na/K-ATPase to the pumping pump. In different types of cells, the size of the non-pumping pool may also vary depending on the specific physiological function of cells. For example, fibroblasts appear to contain a greater majority of non-pumping Na/K-ATPase in the plasma membrane than that in renal epithelial cells. The cholesterol depletion can double the pumping activity in cultured cardiac fibroblast while only 40% of increase was observed in LLC-PK1 cells. Based on the fact that the sodium pump is essential in the economy of cells, the significance of the existence of a pool of “spare” Na/K-ATPase is self-evident. These non-pumping pumps not only serve as a reservoir for cells to rapidly and appropriately respond to various physiological conditions, but also provide the basis for another layer of control in the regulation of multiple signaling pathways. More and more proteins are found to be able to interact with the Na/K-ATPase and then form a series of signaling complexes. Cardiotonic steroids, by binding to these protein complexes, should have more profound regulatory effects on various cellular functions. Currently, we still don’t know how the pumping and the non-pumping pool of Na/K-ATPase are assembled, delivered, disassembled and converted. And many potential non-pumping functions of Na/K-ATPase still wait for further exploration.

It has been established for a long time that Na/K-ATPase can mediate ouabain induced signal transduction and the underlying mechanisms also have been documented in much detail, thus, it is tempting for us to investigate whether, and if so how, Na/K-ATPase is involved in the regulation of any other essential
cellular signaling events. In the third part of this dissertation, we basically initiated a project to examine the potential role of Na/K-ATPase in EGF signaling pathways. The preliminary data suggested that EGF stimulated EGFR phosphorylation can be potentiated by the depletion of Na/K-ATPase α1 subunit. Both wild type rat α1 and its pumping-null mutant are able to reverse the “over-reacted” EGFR back to normal.

Even though the downstream effects of these increased EGFR stimulation is still unknown, several possible underlying mechanisms have been proposed. First, Na/K-ATPase appears to regulate the caveolin-1 trafficking and membrane expression, as well as the cholesterol metabolism or distribution, both of which can modulate the time and strength of the EGFR activation. The involvement of the lipid rafts or caveolae in this regulatory process should be tested by the cell membrane fractionation analysis to examine the relative distribution of the active EGFR. Second, since EGFR is also a substrate of Src kinase, the direct regulatory effects of Na/K-ATPase on Src kinase have brought up the need to verify the potential role of Src in the Na/K-ATPase regulated EGFR activation. However, the Src specific phosphorylation site of EGFR Tyr-845 seems insensitive to Na/K-ATPase regulation.

In summary, in this dissertation, we presented extensive data to identify the regulatory effects of Na/K-ATPase on several essential cellular molecules, such as Src, FAK and EGFR. The existence of different pools of Na/K-ATPase in live cells ensured these regulations to occur promptly and properly. This work
has laid the fundamental basis for us to further explore the unknown functions of Na/K-ATPase in multiple signaling pathways.
REFERENCES


ABSTRACT

Several α1 subunit knock-down LLC-PK1 cells were generated by using RNA interference assay. While the knock-down of α1 resulted in significant decrease in Na/K-ATPase activity, it increased the basal Src activity and tyrosine phosphorylation of a Src effector FAK. This Src-interacting pool of Na/K-ATPase may serve as the receptor for ouabain to activate protein kinases. Ouabain induced activation of Src or ERK1/2 was inhibited in the α1 knock down cells whereas the EGF induced EGFR phosphorylation on several tyrosine residues were found to be potentiated by the α1 depletion. Reconstitution of the knock-down cells with wild type α1 or its pumping-null mutant can restore the above cellular functional changes induced by α1-depletion back to normal. When the ion-pumping properties were characterized in these α1 knock-down cells, we found a mismatch between the cellular Na/K-ATPase amount and its pumping activity. More than 65% of the plasma membrane Na/K-ATPase in LLC-PK1 cells are not involved in ion pumping. The size of this pool of non-pumping Na/K-ATPase appears to be regulated by the integrity of caveolae.