Na^+-K^+–ATPase-Mediated Signal Transduction: From Protein Interaction to Cellular Function

Foxglove (*Digitalis purpurea*), from which the cardiac glycoside compounds digitalis and digitonin are derived.

The Na^+-K^+–ATPase, or Na^+ pump, is a member of the P-type ATPase superfamily. In addition to pumping ions, Na^+-K^+–ATPase is engaged in assembly of multiple protein complexes that transmit signals to different intracellular compartments. The signaling function of the enzyme appears to have been acquired through the evolutionary incorporation of many specific binding motifs that interact with proteins and ligands. In some cell types the signaling Na^+-K^+–ATPase and its protein partners are compartmentalized in coated pits (i.e., caveolae) on the plasma membrane. Binding of ouabain to the signaling Na^+-K^+–ATPase activates the cytoplasmic tyrosine kinase Src, resulting in the formation of an active "binary receptor" that phosphorylates and assembles other proteins into different signaling modules. This in turn activates multiple protein kinase cascades including mitogen-activated protein kinases and protein kinase C isozymes in a cell-specific manner. It also increases mitochondrial production of reactive oxygen species (ROS) and regulates intracellular calcium concentration. Crosstalk among the activated pathways eventually results in changes in the expression of a number of genes. Although ouabain stimulates hypertrophic growth in cardiac myocytes and proliferation in smooth muscle cells, it also induces apoptosis in many malignant cells. Finally, the signaling function of the enzyme is also pivotal to ouabain-induced nongenomic effects on cardiac myocytes.

Zijian Xie[1,2] and Ting Cai[1], Department of Pharmacology[1] and Medicine[2], Medical College of Ohio, Toledo, OH 43614

Introduction

The Na^+-K^+–ATPase (EC 3.6.3.9), or Na^+ pump, is an energy-transducing ion pump first described by Skou in 1957 *(1)*. Since then, the structure, the pumping function, and the regulation of the enzyme have been well characterized *(2–4)*. In addition to pumping ions across the membrane, the enzyme, composed of both catalytic α and regulatory β subunits, functions as a receptor for cardiotonic steroids (CTS) *(5–8)*. Because CTS inhibit Na^+-K^+–ATPase, the physiological and pharmacological functions of CTS were thought secondary to their effects on intracellular ion concentrations. However, the concentration of endogenous ouabain, even under pathological conditions, is not high enough to cause changes in global cytosolic ion concentrations. This fact has prompted many laboratories to test whether ouabain-liganded Na^+-K^+–ATPase can generate secondary messages independent of its pumping function *(9–15)*. Subsequently, several mechanisms and pathways by which Na^+-K^+–ATPase transmits the ouabain signal to different intracellular compartments have been discovered *(16–22)*. It is the purpose of this review to provide a broad overview of Na^+-K^+–ATPase research with detailed discussions on the mechanisms and biological significance of Na^+-K^+–ATPase-mediated signal transduction.

Protein–Protein Interactions and Na^+-K^+-ATPase-Mediated Signal Transduction

Binding of CTS to the Na^+-K^+–ATPase affects gene expression and cell growth *(23, 24)*. Early studies indicated that the enzyme interacts with many soluble and membrane proteins *(25)*; however, only in recent years has research on Na^+-K^+–ATPase revealed that interactions of Na^+-K^+–ATPase with other proteins not only are important for regulation of pumping function, but also make it possible for the enzyme to function as a signal transducer *(25)*.

Evolving of Protein-Binding Motifs and Signal Transduction

P-type ATPases are expressed in all living organisms *(26)*. The primary structures of various P-type ATPase catalytic subunits contain eight conserved motifs solvated in the cytoplasm, where ATP binding and hydrolysis occur *(26, 27)*. The primary function of Na^+-K^+–ATPase is to transport ions across the cell membrane; the signaling function of Na^+-K^+–ATPase most likely arose from the separate evolution of specific binding motifs. Indeed, computational analyses have revealed that the human Na^+-K^+–ATPase α subunit contains multiple well-characterized protein-binding motifs (Table 1), all of which are located outside of the eight conserved core regions of P-type ATPases *(26)*. Because the interaction between Na^+-K^+–ATPase and caveolins—the protein constituents of caveolae located on the plasma membrane—plays a role in Na^+-K^+–ATPase-mediated signal transduction *(28, 29)*, the caveolin-binding motif (CBM), found on the Na^+ pump, has been used as a model for further analysis, leading to the following observations. First, human Na^+-K^+–ATPase α_1 subunit contains two potential CBMs (Table 1), one of which is in the cytosolic N-terminal domain near the first transmembrane region (M1), whereas the other CBM is located at the extracellular side of M10 (Figure 1). According to the phylogenetic analyses of the Na^+ pump *(26, 27)*, the N-terminal CBM of the Na^+ pump's α subunits was acquired quite early during evolution, as were those found in the epithelial growth factor receptor (EGFR) (Table 2). It first appeared in one of the mutated Na^+-K^+–ATPases in *C. elegans* (i.e., Eat-6) and is conserved in Drosophila *(26)* (Table 1). Second, the appearance of the N-terminal CBM correlates well with the occurrence of the domain for ouabain binding *(27)*, suggesting that ouabain regulates not only the ATPase activity but also the interactions of the enzyme with other proteins. In other words, evolutionary acquisition of this domain added a new function to Na^+-K^+–ATPase so that the enzyme can transduce the extracellular ouabain signal. Third, unlike CMBs in other proteins, the CBMs of the Na^+ pump's α subunits are located at sites far away from the catalytic P and N domains (Figure 1). Thus, it is conceivable that binding to caveolin will not affect the ion-pumping function of the enzyme. On the other hand, this interaction could serve to concentrate the enzyme and other signaling proteins into caveolae, resulting in the formation of a large signaling complex or signalosome (Figure 2).

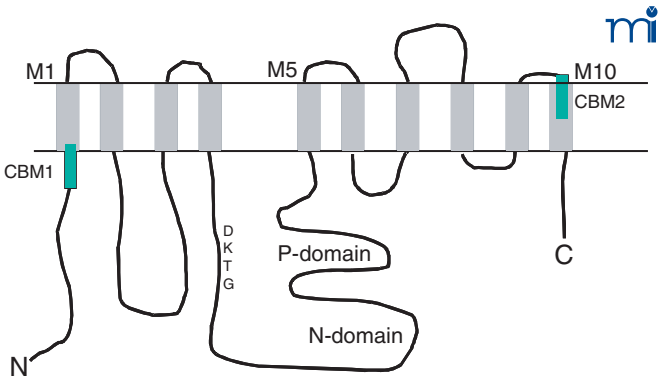

Figure 1. Schematic presentation of the α_1 subunit of Na^+-K^+–ATPase. The ten transmembrane domains are marked as M1 to M10. The third intracellular loop, connecting the M4 and M5, contains both P (phosphorylation) domain and N (nucleotide-binding) domain. The green bars indicate caveolin-binding motifs (CBMs).

Na^+-K^+–ATPase Signalosome

As illustrated in Figure 2, the Na^+-K^+–ATPase interacts with multiple signaling proteins to transmit ouabain signals *(14–22, 30–33)*. Prior studies also demonstrate the interactions of the Na^+-K^+–ATPase with the Na^+ pump's γ subunit (FXYD2),

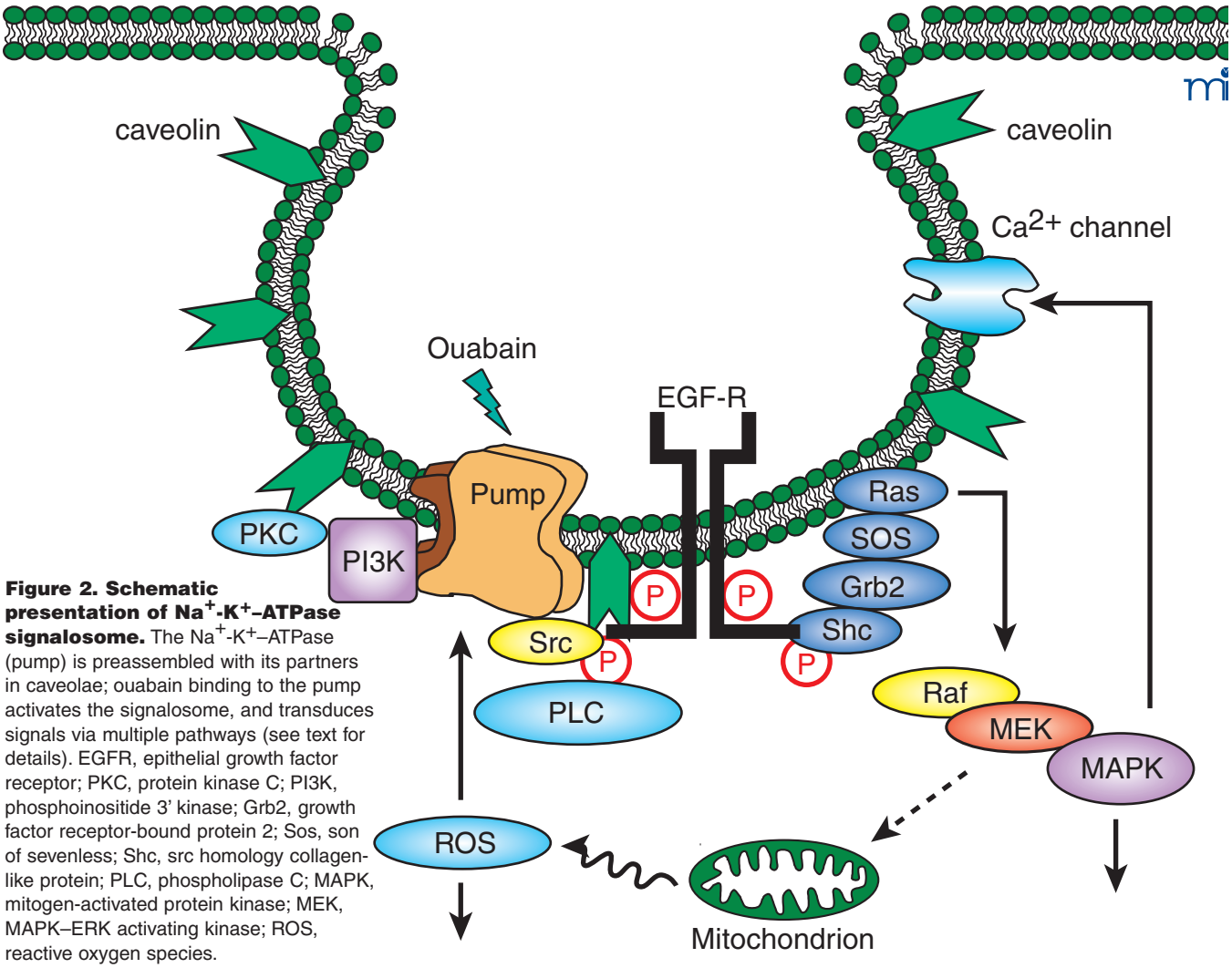

Figure 2. Schematic presentation of Na^+-K^+–ATPase signalosome. The Na^+-K^+–ATPase (pump) is preassembled with its partners in caveolae; ouabain binding to the pump activates the signalosome, and transduces signals via multiple pathways (see text for details). EGFR, epithelial growth factor receptor; PKC, protein kinase C; PI3K, phosphoinositide 3' kinase; Grb2, growth factor receptor-bound protein 2; Sos, son of sevenless; Shc, src homology collagen-like protein; PLC, phospholipase C; MAPK, mitogen-activated protein kinase; MEK, MAPK–ERK activating kinase; ROS, reactive oxygen species.

phospholemman (FXYD1), and other FXYD proteins in a cell-specific manner (*34*, *35*). The FXYD proteins share a signature sequence of six conserved amino acids that include the FXYD motif, where F, Y, and D represent amino acid residues of phenylalanine, tyrosine, and aspartic acid, respectively (*34*). The Na^+ pump also binds to ankyrin and adducin (*36–38*). Interestingly, polymorphisms in the sequence of adducin correlate with hypertension in both humans and Milan rats (*37*). These observations demonstrate the importance of protein–protein interactions in the regulation of both the ion-pumping and signal-transducing functions of the Na^+-K^+-ATPase, and have prompted several laboratories to isolate the intact Na^+-K^+–ATPase signalosome so that the composition of the Na^+ pump's interacting proteins can be identified using newly developed proteomic approaches (*14*, *22*, *39–43*). For example, using ouabain-affinity column, Yingst et al. demonstrated the feasibility of purifying Na^+-K^+-ATPase from various types of cells and tissue samples (*41*). In principle, when experimental conditions are controlled properly, this method could be used to isolate the intact signalosome of the enzyme. In addition, Caplan's laboratory proved GST (glutathione *S*-transferase) pull-down assays to be a useful tool for purification of the intact Na^+-K^+-ATPase signalosome from cultured cells (*42*). We also have performed experiments to test the feasibility of using a combination of protein purification, GST pull-down, immunoprecipitation, and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis to define the composition of Na^+-K^+-ATPase signalosome (*43*). From the identified proteins a picture emerges of a signaling module responsible for the ouabain-mediated regulation of intracellular calcium ($[Ca^{2+}]_i$). The transducer of this module is the Na^+-K^+–ATPase. The messengers are generated by the interaction of the enzyme with the tyrosine kinase Src and phospholipase C-γ (PLC-γ), and transmitted to the effector IP3 receptor (IP3R) (*43*). The involvement of IP3R in ouabain-induced calcium oscillation has been reported previously (*15*). We believe that the collective efforts of many laboratories in the field will eventually map the composition of Na^+-K^+–ATPase signalosome, which may reveal how Na^+-K^+–ATPase is organized spatially and temporally with its partners to transmit signals. This information will also allow us to test whether Na^+-K^+–ATPase participates in the

events activated by growth factors and other hormones; there is evidence that the enzyme plays a scaffolding role in other stimuli-induced signal transduction (e.g., dopamine-induced activation of PI3K) (*39*). Because protein–protein interactions are important for regulating the pumping function of the enzyme (*35*, *39*), the defined composition of the Na^+-K^+–ATPase signalosome under specific conditions may yield new insights into the mechanism by which Na^+-K^+–ATPase is regulated by various stimuli in a cell-specific manner.

Interaction of Na^+-K^+–ATPase with Src

The binding of ouabain to the Na^+-K^+–ATPase causes rapid activation of Src family kinases in many different types of cells, including cardiac myocytes, smooth muscle, and kidney epithelial cells (*14*, *21*, *22*). The kinases are 52–62-kDa membrane-associated non-receptor tyrosine kinases that regulate various signal transduction pathways (*44*), and their catalytic activity is controlled by tyrosine phosphorylation and protein–protein interactions (*44–46*). Phosphorylation of Tyr^{529} on Src holds the kinase in an inactive conformation through an intramolecular interaction with its Src homology (SH) 2 (SH2) domain, whereas phosphorylation of Tyr^{418} activates Src by disrupting the intramolecular interaction and creating the substrate-binding site. The complexities of the interactions that hold Src in an inactive conformation suggest that there are many mechanisms for activating the kinase (*45*, *46*).

Because the effects of ouabain on Src are independent of changes in intracellular ion concentrations (*14*, *21*, *22*), we proposed that Src might be activated by ouabain-induced changes in the interactions between Na^+-K^+–ATPase and Src. This hypothesis is supported by the following observations. First, ouabain stimulates Tyr^{418} phosphorylation but has no effect on Tyr^{529} phosphorylation of Src in different types of cells (*22*). In addition, when the α_1 subunit of the Na^+ pump is immunoprecipitated from both ouabain-treated and control LLC-PK1 cells, ouabain increases the binding of Src to the Na^+-K^+–ATPase signaling complex in a dose- and time-dependent manner (*22*). Third, GST pull-down assays show that the solubilized Na^+-K^+–ATPase binds to GST-Src in a dose-dependent manner (*47*), and that interaction involves both kinase domain and the SH3 and SH2 domains of Src. Fourth, purified Src interacts with a GST fusion protein encompassing the large central loop between transmembrane helices M4 and M5 of the α_1 subunit of the Na^+ pump. Several recent studies indicate that this domain may be involved in stabilizing the α–α subunit interactions of the Na^+ pump as well as the interaction of the α subunit with other proteins (*48–50*). In short, the above observations clearly demonstrate that Src interacts directly with Na^+-K^+–ATPase via multiple domains. However, it remains unclear how ouabain regulates these interactions and activates Src.

The "Binary Receptor" Relays Ouabain Signals from Na^+-K^+–ATPase to Other Signaling Cascades

Because Src interacts with the Na^+ pump directly, we suggest that the Na^+-K^+–ATPase–Src complex behaves as a "binary" receptor (Figure2). Once the receptor is activated by ouabain, it indirectly affects the phosphorylation of downstream proteins that are associated with or are proximal to the receptor. Indeed, when ouabain-treated LLC-PK1 cell lysates are immunoprecipitated with α_1-specific antibody, Src-dependent tyrosine phosphorylation of multiple Na^+-K^+–ATPase-associated proteins is observed (*47*).

The activated EGFR is a critical element in the signal transduction networks of cytokines, H_2O_2, and those pathways utilizing G protein–coupled receptors (*51*). Src family kinases can couple receptors that lack intrinsic kinase activity to receptor tyrosine kinases (RTKs), such as EGFR. Indeed, as depicted in Figure 2, activated Src transactivates EGFR, which in turn recruits adapter protein Shc to relay the initial ouabain signal to the Ras–Raf–MAPKs cascade (*14*, *22*). Furthermore, Src activation is also required for ouabain-induced stimulation of several other pathways including increased production of reactive oxygen species (ROS) and activation of PKC isozymes. Taken together, these findings support the proposal that the activation of the Na^+-K^+–ATPase–Src complex is the initial critical step that relays the signal emanating from the interaction of ouabain with the enzyme to the EGFR and other downstream pathways.

Although the above findings reaffirm the importance of protein–protein interaction in Na^+-K^+–ATPase-mediated signal transduction, it is important to note that large changes in intracellular ion concentrations can alter different ion concentration–sensitive signaling events (*23*, *52*, *53*). Furthermore, because local cytosolic Na^+ concentrations could be affected when a small percentage of Na^+ pumps is inhibited by low doses of ouabain (*54*), the impact of changes in local ion concentration on ouabain-activated signaling events should also be considered.

Are Na^+-K^+–ATPase Signalosomes Compartmentalized?

Because the Na^+-K^+–ATPase has to interact with Src, EGFR, and other proteins to transmit the ouabain signal, it is possible that the signaling enzyme may have to be preassembled with its signaling partners in caveolae. Caveolae are membrane microdomains that were first identified as flask-shaped vesicular invaginations of plasma membrane enriched in cholesterol, glycosphingolipids, and sphingomyelin (*55*, *56*). Caveolins are 21–24-kDa membrane-associated scaffolding proteins, with multiple cellular functions, that typify caveolae (*55*). Mammals express three different caveolin genes that encode five different protein isoforms, the expression and distribution of which are tissue-specific (*56*). Caveolins directly interact with cholesterol and many signaling proteins including receptors, Src family kinases, and adapter proteins. Recent studies have indicated that many of these interactions are mediated through

Table 1. Protein/Ligand-Binding Motifs in Na^+-K^+–ATPase α Subunits

Protein/Ligand-Binding Motifs	PI3K	Caveolin (CBM1)	Ouabain-binding Site	Ankyrin	AP-2	Caveolin (CBM2)
	PXX**P**	**F**XXXX**F**XX**F**	**Q**/**L**/**D**.....**P**.....**DN**	**ALLK**	**Y**XXf	**W**X**F**XXXX**Y**
Human-α1	**P**TT**P**	**F**CRQL**F**GG**F**	**Q**AATEEE**P**QN--**DN**	**ALLK**	**Y**lel	**WWF**CAFP**Y**
Human-α2	**P**TT**P**	**F**CRQL**F**GG**F**	**Q**AAMEDE**P**SN--**DN**	**ALLK**	**Y**mel	**WWF**CAFP**Y**
Human-α3	**P**TT**P**	**F**CRQL**F**GG**F**	**Q**AGTEDD**P**SG--**DN**	**ALLK**	**Y**lel	**WWF**CAFP**Y**
Rat-α1	**P**TT**P**	**F**CRQL**F**GG**F**	RSATEEE**P**PN--DD	**ALLK**	**Y**lel	**WWF**CAFP**Y**
Rat-α2	**P**TT**P**	**F**CRQL**F**GG**F**	**L**AAMEDE**P**SN--**DN**	**ALLK**	**Y**mel	**WWF**CAFP**Y**
Rat-α3	**P**TT**P**	**F**CRQL**F**GG**F**	**Q**AGTEDD**P**SN--**DN**	**ALLK**	**Y**lel	**WWF**CAFP**Y**
DUGJA_DJNAK[a]	KTTP	**F**CRQL**F**GG**F**	**Q**SGAYED**P**PK--**DN**	**ALLK**	**Y**lel	**WW**LPAIP**Y**
DROME-ATNA[b]	KQTP	**F**CKNL**F**GG**F**	**Q**ASTSEE**P**AD--**DN**	**ALLK**	**Y**mel	**WWF**PAIP**Y**
CTEFE_MTH1733[c]	KQTP	**F**CKNL**F**GG**F**	**Q**ASTVEE**P**AD--**DN**	**ALLK**	**Y**lel	**WWF**PAIP**Y**
CAEEL-Eat-6[d]	ETTP	**F**CKNL**F**GG**F**	**D**YFTMEY**P**SK--**DN**	**ALLK**	**Y**lel	**WW**LPALP**Y**
DROME-Q9W5Y2[b]	**P**TT**P**	**F**LKTM**F**GG**F**	**Q**LQTQHE**P**PD--**DN**	**ALLK**	**Y**lem	WWIYAFPF
CAEEL-O45240[e]	KQTS	LAGSIFGGF	DLSMSDDEEVPKDN	AIMR	**Y**etl	IGFLALPF
DICDI-IONA[f]	KEVP	LGKCFTNFF	DRNQR-------VN	AILR	**Y**eal	FWAYPLPM
BLAEM-PAT1[g]	KTKH	YLECLLALF	DPVSN------YAN	GLYR	**Y**eym	YLLIPFGV
CAEEL-O16436[e]	KVIS	FLRQFKNLL	DPSDL-------TN	ALLR	**Y**eaf	CWLVPIVV
CAEEL-O16331[e]	KTIS	FVRQFKNLL	DPTDA-------LN	ALLR	**Y**etf	CWLVPIVV

Potential functional protein/ligand-binding motifs are presented by highlighting their essential amino acid residues. All protein sequences are analyzed by ClustalW (EBI), representative sequences are shown in a phylogenetic manner, which is from *C. elegans* (bottom) to human (top) (*25*, *26*). [a]Na^+-K^+–ATPase α subunits found in planarian. [b]Na^+-K^+–ATPase α subunits found in *D. melanogaster*. [c]Na^+-K^+–ATPase α subunits found in cat flea. [d]A mutated Na^+-K^+–ATPase α subunit found in *C. elegans*. [e]Ancestor forms of Na^+-K^+–ATPase α subunit found in *C. elegans*. [f]Na^+-K^+–ATPase α subunit in Slime mold. [g]Na^+-K^+–ATPase α subunit in *Blastocladiella emersonii*.

the binding of caveolin scaffolding domains to caveolin-binding motifs of the target proteins. Two potential caveolin-binding motifs exist in the α_1 subunit of Na^+-K^+–ATPase (Table 1). Confocal imaging shows that Na^+-K^+–ATPase colocalizes with caveolins on plasma membrane in cardiac myocytes and other types of cells (*28*, *29*). Immunoprecipitation using caveolin-1–specific antibody-coated magnetic beads confirmes that the Na^+ pump is associated with caveolae, Src, and other signaling proteins. GST pull-down assays reveals that Na^+-K^+–ATPase binds to the N terminus of caveolin-1 (*29*). Significantly, ouabain appears to regulate the interaction of caveolin-1 with Na^+-K^+–ATPase and induces the formation of the Na^+-K^+–ATPase–Src–caveolin-1 complex. Ouabain also stimulates tyrosine phosphorylation of caveolin-1 in LLC-PK1 cells, and caveolar p42/44 MAPKs in cardiac myocytes (*28*, *29*). Furthermore, removal of cholesterol significantly reduces ouabain-induced activation of extracellular-regulated protein kinases (ERKs) in LLC-PK1 cells which is reversible after cholesterol repletion in these cells. These findings support the notion that ouabain activates the caveolar Na^+-K^+–ATPase to initiate different signal transduction pathways (*29*).

Endogenous Ouabain and Other Na^+-K^+–ATPase Ligands

CTS consist of a group of chemicals that specifically bind to Na^+-K^+–ATPase. They include plant-derived digitalis drugs such as digoxin and ouabain, and vertebrate-derived aglycones such as bufalin and marinobufagenin. Although they have been considered only as drugs for a long time, there is now ample evidence to indicate that some of these compounds can function as steroid hormones (*57–62*). Recent studies have identified both ouabain and marinobufagenin as endogenous steroids whose production and secretion are regulated by multiple pathological or physiological stimuli including angiotensin II and epinephrine (*55*, 58, *61*, *62*). In addition to CTS, changes in intracellular Na^+ or extracellular K^+ can cause conformational changes in the enzyme. These changes affect not only the enzyme conformation, but also membrane potential and other ion transporter–related activities; therefore, they could trigger other indirect signaling events mediated by ouabain. Indeed, although lowering of extracellular K^+ does activate protein kinases and raise intracellular calcium in cardiac myocytes (*21*), it does not mimic the effects of ouabain on smooth muscle and kidney epithelial cells (*14*, *15*). ROS are another group of molecules whose interaction with the Na^+ pump can cause a conformational change in Na^+-K^+–ATPase (*63*, *64*). This is of particular interest because both endogenous ouabain and ROS concentrations are elevated in several diseases. If ROS stimulates the signaling function of Na^+-K^+–ATPase, then a positive amplification loop would be established because activation of the signaling function of Na^+-K^+–ATPase stimulates the mitochondrial production of ROS (*12*, *19*, *65*) (Figure 2).

Regulation of Cellular Function by Na^+ Pump Signals

Binding of ouabain to Na^+-K^+–ATPase affects multiple cellular functions. The effects of ouabain on gene expression, cell attachment, formation of tight junctions and induction of polarity, modification of immune response, protein trafficking, and ion fluxes have been well documented (*20*, *66–71*). Although some of ouabain's effects are clearly mediated by Na^+-K^+–ATPase signaling

Table 2. Caveolin-Binding Motifs (CBMs) in G_α Subunits, Endothelial Nitric-Oxide Synthase (eNOS) and EGFR

Model Organism	$G_i\alpha$ subunits (190–200)	$G_o\alpha$ subunits (190–200)	$G_s\alpha$ subunits (123–130)	eNOS	EGFR	Caveolin in this organism
H. sapiens	**F**T**F**KDLH**F**KM**F**[a]	**F**T**F**KNLH**F**RL**F**	**F**D**F**PPEF**Y**	**F**PAAP**F**SG**W**	**W**S**Y**GVTV**W**	Yes
M. musculus	**F**T**F**KDLH**F**KM**F**	**F**T**F**KNLH**F**RL**F**	**F**D**F**PPEF**Y**	**F**PAAP**F**SG**W**	**W**S**Y**GVTV**W**	Yes
D. melanogaster	FSCKQLHFKLF	**F**S**F**KNLN**F**KL**F**	**F**N**Y**PPEF**Y**	**F**TATT**F**SG**W**	**W**A**F**GVTI**W**	Flotillin[c]
C. elegans	**F**T**F**KNLN**F**KL**F**	**F**T**F**KNLN**F**KL**F**	DNYPSEFY	N/A[b]	**W**A**F**GVTC**W**	Yes
A. thaliana	Not found	Not found	Not found	N/A	N/A	No
S. cerevesiae	Not found	Not found	Not found	N/A	N/A	No

[a]Core amino acids in CBMs are highlighted in black. [b]N/A, no molecules found in certain organism. [c]Flotillin, a novel ~45-kDa component of caveolae.

only (*67*, *69–71*), other effects involve both the pumping and signaling functions of the enzyme (*68*). Because of space limitations, we will only discuss the relationship between the signaling function of Na^+-K^+–ATPase and the effects of ouabain on cell growth and cardiac function.

Regulation of Cell Growth

As early as the 1960s, ouabain was found to inhibit the mitogen-induced differentiation and proliferation of lymphocytes as well as the growth of malignant cells (*24*, *72*). Significantly, there is evidence that administration of digoxin appears to reduce the mortality rate in breast cancer patients (*73*). In the laboratory, ouabain has been used in assays to identify mutagenized cell clones that express ouabain-resistant Na^+-K^+–ATPase mutants (*74*, *75*). More recently, ouabain and related CTS were found to cause either apoptosis or hypertrophic/proliferative growth in a cell type–specific manner (*76–87*).

Hypertrophic or Proliferative Growth

Cardiac tissue

In addition to their effects on cardiac contractility, digitalis drugs appear to promote cardiac hypertrophy. Based on clinical observations, Christian has argued that in patients with heart disease but not heart failure, digitalis retards cardiac enlargement and delays the appearance of symptoms of cardiac insufficiency (*88*). This observation prompted investigation into how digitalis affects growth and hypertrophy in the heart. Rather confusingly, early studies either confirmed that digitalis prevented overload-induced cardiac hypertrophy, showed that digitalis induced or amplified cardiac hypertrophy, or suggested that digitalis had no effect on cardiac growth (*89–92*). In retrospect, these apparent inconsistencies are not surprising, because overload-induced hypertrophy of the heart in an intact animal arises from a complex interplay of changes in a multitude of mechanic and neurohumoral stimuli. Treatment of intact animals with digitalis not only adds another growth stimulus that acts on myocardium directly, but through its well-established neural and vascular effects, can also exacerbate existing pathophysiological stimuli. Furthermore, the different ouabain sensitivities of the experimental animals add another layer of complexity to these studies. Consequently, we have used cultured cardiac myocytes as a model to demonstrate that ouabain stimulates hypertrophic growth in cardiac myocytes (*85–87*). Like other hypertrophic stimuli, ouabain regulates transcription of several hypertrophic marker genes in cardiac myocytes (*17*, *85*). Furthermore, the effects of ouabain on cardiac gene expression and growth depend on the signaling function of Na^+-K^+–ATPase and involve crosstalk among different signaling pathways (*18*, *19*). A recent study (*93*) supports our conclusion and demonstrates a correlation between plasma ouabain concentration and increases in left-ventricular mass in human subjects; this correlation exists even when the blood pressure is accounted for.

Cell lines

In addition to stimulating the hypertrophic growth of cardiac myocytes, ouabain also increases the proliferation of several different cell lines. Exposure of canine vascular smooth muscle cells to ouabain concentrations that cause no detectable inhibition (< 5%) of pump activity activates the signaling function of Na^+-K^+–ATPase (*14*, *84*). Concomitantly, ouabain also stimulates proliferation in these cells. These findings are significant in view of recent evidence whereby infused ouabain caused significant increases in blood pressure (*60*). Because these ouabain concentrations (nM) can affect the signaling (*14*, *15*) but not bulk cytosolic ion concentrations, the growth effects may explain some of the actions of ouabain on blood pressure.

Apoptosis

Several CTS in nontoxic concentrations can induce apoptosis in different malignant cell lines (*76–82*). Bufalin, for example, induces apoptosis in leukemia cells and human skin squamous carcinoma cells (*76*, *81*). Furthermore, other digitalis-like drugs induce apoptosis or enhance the apoptotic effects of other drugs in human prostate cancer cells (*19*). Mechanistically, the apoptotic effects of CTS are related to the downstream effects of activated

MAPKs: increased $[Ca^{2+}]_i$ and ROS, and activation of transcription factors such as activator protein-1 (AP-1), and nuclear factor–κB (NF-κB). In view of the complexity of ouabain-activated signal transduction pathways, it is not surprising that so many factors could contribute to ouabain-induced apoptosis.

Regulation of $[Ca^{2+}]_i$ and Myocyte Contractility by Ouabain Involves p42/44 MAPKs and ROS

Although it is well established that the effects of digitalis-like drugs on cardiac contractility involve the pumping function of the enzyme and Na^+-Ca^{2+}-exchanger (NCX) *(5–8)*, early work did suggest that the activation of protein kinases and Ca^{2+} channels also participated *(93, 94)*. Our studies indicated that activation of protein tyrosine kinases precedes increases in $[Ca^{2+}]_i$ in response to ouabain *(21)*. We have tested whether the signal transducing function of Na^+-K^+–ATPase contributes to nongenomic effects of ouabain on myocytes *(13, 95)* and have found the following: First, inhibition of either Src or the small GTPase Ras abolishes ouabain-induced increases in both $[Ca^{2+}]_i$ and contractility. Second, although activation of p42/44 MAPKs is required for ouabain-induced increases in $[Ca^{2+}]_i$, both MAPKs and ROS contribute to ouabain regulation of cardiac contraction *(95)*. The effects of ouabain on $[Ca^{2+}]_i$, at least in part, are due to the activation of L-type Ca^{2+} channels *(93)*. This activation is most likely evoked by p42/44 MAPK–mediated phosphorylation of the channels, as demonstrated in neuronal cells *(96)*. Finally, ouabain regulation of cardiac contractility also involves opening of mitochondrial ATP-sensitive K channels ($mitoK_{ATP}$) and increases ROS production *(95)*. Inhibition of $mitoK_{ATP}$ by treating cells with 5-hydroxydecanoate significantly reduces ouabain-induced increases in ROS production and contractility. Thus, ouabain regulates cardiac contractility through the activation of at least two major pathways. Activation of p42/44 MAPKs and inhibition of the ion-pumping function of the Na^+-K^+–ATPase by ouabain increase $[Ca^{2+}]_i$, whereas opening of $mitoK_{ATP}$ stimulates, the production of ROS. Both $[Ca^{2+}]_i$ and ROS, in turn, work in concert and raise contractility in cardiac myocytes. These findings are supported by a recent report showing that ouabain stimulates contraction in myocytes incubated in Na^+-free medium *(97)*. These observations need to be replicated in the isolated heart and whole animals; nevertheless, it is important to note the significance of these studies. First, these findings suggest that stimulation of the signaling function of Na^+-K^+–ATPase alone may be sufficient to increase cardiac contractility. Second, they point to the possibility of developing agonists that can affect the signaling, but not pumping, function of the enzyme. Finally, because the opening of the $mitoK_{ATP}$ channels participates in ischemic preconditioning *(98)*, it will be of great interest to test whether digitalis drugs can protect the heart from ischemic injury.

Conclusion

The work of the past few years has demonstrated that Na^+-K^+–ATPase is an important signal transducer. Recent work has begun to define the Na^+-K^+–ATPase signalosome, to identify the signaling modules, and to reveal structural domains that are involved in organization of each module. It is important to emphasize that the above studies are critical for our understanding not only of the events regulated by the signaling Na^+-K^+–ATPase, but also of the enzyme ion-pumping activity in different signaling pathways. Identification of intact in vivo Na^+ pump signalosomes will ultimately allow us to test their role in ouabain-induced physiological and pharmacological changes in vitro as well as in transgenic or knock-out animal models. Continued study will also aid us in establishing new molecular targets for the development of therapeutics for cardiovascular diseases as well as cancer.

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References

1. Skou, J.C. The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim. Biophys. Acta* **23**, 394–401 (1957). **This article was the first to link ion transport to ATPase activity; a discovery that led to the awarding of the Nobel Prize to J.C. Skou in 1997 (http://www.nobel.se/laureates/chemistry-1997.html).**
2. Lingrel, J.B. and Kuntzweiler, T. Na^+,K^+-ATPase. *J. Biol. Chem.* **269**, 19659–19662 (1994).
3. Dunbar L.A. and Caplan M.J. Ion pumps in polarized cells: Sorting and regulation of the Na^+,K^+- and H^+,K^+-ATPases. *J. Biol. Chem.* **276,** 29617–29620 (2001).
4. Kaplan, J.H. Biochemistry of Na^+,K^+-ATPase. *Annu. Rev. Biochem.* **71,** 511–535 (2002).
5. Akera, T. and Brody, T.M. Inotropic action of digitalis and ion transport. *Life Sci.* **18**, 135–142 (1976).
6. Schwartz, A., Grupp, G., Wallick, E., Grupp, I.L., and Ball, W.J. Jr. Role of the Na^+,K^+-ATPase in the cardiotonic action of cardiac glycosides. *Prog. Clin. Biol. Res.* **268B**, 321–338 (1988).
7. Eisner, D.A. and Smith, T.W. *The Heart and Cardiovascular System (2nd ed.)*. Edited by H.A. Fozzard, E. Haber, R.B. Jennings, A.M. Katz, H.E. Morgan, Raven Press, New York, pp 863 –902 (1992).
8. Barry, W.H., Hasin, Y., and Smith, T.W. Sodium pump inhibition, enhanced calcium influx via

sodium–calcium exchange, and positive inotropic response in cultured heart cells. *Circ. Res.* **56**, 231 –241 (1985).

9. Xie, Z. Ouabain interaction with cardiac Na^+-K^+–ATPase reveals that the enzyme can act as a pump and a signal transducer. *Cell. Mol. Biol.* **47**, 383–390 (2001).
10. Szamel, M., Schneider, S., and Resch, K. Functional inter-relationship between Na^+/K^+-ATPase and lysolecithin acyltransferase in plasma membrane of mitogen-stimulated rabbit thymocytes. *J. Biol. Chem.* **256**, 9198–9204 (1981). **The findings of this manuscript suggest that Na^+-K^+–ATPase regulate the function of other proteins via direct protein–protein interaction.**
11. Nakagawa, Y., Petricoin, E.F. 3rd, Akai, H., Grimley, P.M., Rupp, B., and Larner, A.C. Interferon-α–induced gene expression: Evidence for a selective effect of ouabain on activation of the ISGF3 transcription complex. *Virology* **190**, 210–220 (1992).
12. Liu, J., Tian, J., Haas, M., Shapiro, J.I., Askari, A., and Xie, Z. Ouabain interaction with cardiac Na^+/K^+-ATPase initiates signal cascades independent of changes in intracellular Na^+ and Ca^{2+}. *J. Biol. Chem.* **275**, 27838–27844 (2000). **A key reference that addresses whether Na^+-K^+–ATPase can transduce the ouabain signals in the absence of changes in intracellular ion concentration.**
13. Tian, J., Gong, X., and Xie, Z. Signal-transducing function of Na^+/K^+-ATPase is essential for ouabain's effect on $[Ca^{2+}]_i$ in rat cardiac myocytes. *Am. J. Physiol.* **281**, H1899–H1907 (2001). **This paper reexamines the molecular mechanism of digitalis-induced increases in intracellular calcium, showing, for the first time, that activation of Ras–MAPK cascade is required for ouabain to raise cytosolic calcium in adult cardiac myocytes.**
14. Aydemir-Koksoy, A., Abramowitz, J., and Allen, J.C. Ouabain induced signaling and vascular smooth muscle cell proliferation. *J. Biol. Chem.* **276**, 46605–46611 (2001). **A key reference addressing the signaling function of Na^+-K^+–ATPase in the regulation of vascular smooth muscle proliferation.**
15. Aizman, O., Uhlen, P., Lal, M., Brismar, H., and Aperia, A. Ouabain, a steroid hormone that signals with slow calcium oscillations. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 13420–13424 (2001). **The first publication to show that ouabain-liganded Na^+-K^+–ATPase can induce calcium oscillations in kidney epithelial cells independent of changes in intracellular Na^+ concentration.**
16. Nakagawa, Y., Rivera, V., and Larner, A.C. A role for the Na^+/K^+-ATPase in the control of human c-fos and c-jun transcription. *J. Biol. Chem.* **267**, 8785–8788 (1992). **This article suggests a role of non-ionic changes in ouabain-induced regulation of early response genes.**
17. Peng, M., Huang, L., Xie, Z., Huang, W.H., and Askari, A. Partial inhibition of Na^+/K^+-ATPase by ouabain induces the Ca^{2+}-dependent expressions of early-response genes in cardiac myocytes. *J. Biol. Chem.* **271**, 10372–10378 (1996).
18. Kometiani, P., Li, J., Gnudi, L., Kahn, B.B., Askari, A., and Xie, Z. Multiple signal transduction pathways link Na^+/K^+-ATPase to growth-related genes in cardiac myocytes: The roles of Ras and mitogen-activated protein kinases. *J. Biol. Chem.* **273**, 15249–15256 (1998). **A key reference showing, for the first time, that ouabain activates multiple signaling pathways in cardiac myocytes.**
19. Xie, Z., Kometiani, P., Liu, J., Li, J., Shapiro, J.I., and Askari, A. Intracellular reactive oxygen species mediate the linkage of Na^+-K^+–ATPase to hypertrophy and its marker genes in cardiac myocytes. *J. Biol. Chem.* **274**, 19323–19328 (1999). **This article demonstrates that ouabain increases production of reactive oxygen species (ROS) and that ROS are important signals for ouabain-induced cell growth in cultured cardiac myocytes.**
20. Contreras, R.G., Shoshani, L., Flores-Maldonado, C., Lazaro, A., and Cereijido, M. J. Relationship between Na^+,K^+-ATPase and cell attachment. *Cell Sci.* **112**, 4223–4232 (1999).
21 . Haas, M., Askari, A., and Xie, Z. Involvement of Src and epidermal growth factor receptor in the signal transducing function of Na^+/K^+-ATPase. *J. Biol. Chem.* **275**, 27832–27837 (2000).
22. Haas, M., Wang, H., Tian, J., and Xie, Z. Src-mediated interceptor cross-talk between the Na^+-K^+–ATPase and the EGF receptor relays the signal from ouabain to mitogen-activated protein kinases. *J. Biol. Chem.* **277**, 18694–18702 (2002). **The findings of this article led to the proposal that Na^+-K^+–ATPase–Src complex serves as a "binary receptor" for ouabain to transmit the signals to different intracellular compartments.**
23. Pressley, T.A. Ionic regulation of Na,K-ATPase expression. *Semin. Nephrol.* **12**, 67–71 (1992).
24. Kaplan, J.G. Membrane cation transport and the control of proliferation of mammalian cells. *Annu. Rev. Physiol.* **40**, 19–41 (1978).
25. Askari, A. Significance of protein-protein interactions to Na^+/K^+-ATPase functions. In: *Na^+-K^+–ATPase and Related ATPase*, K.

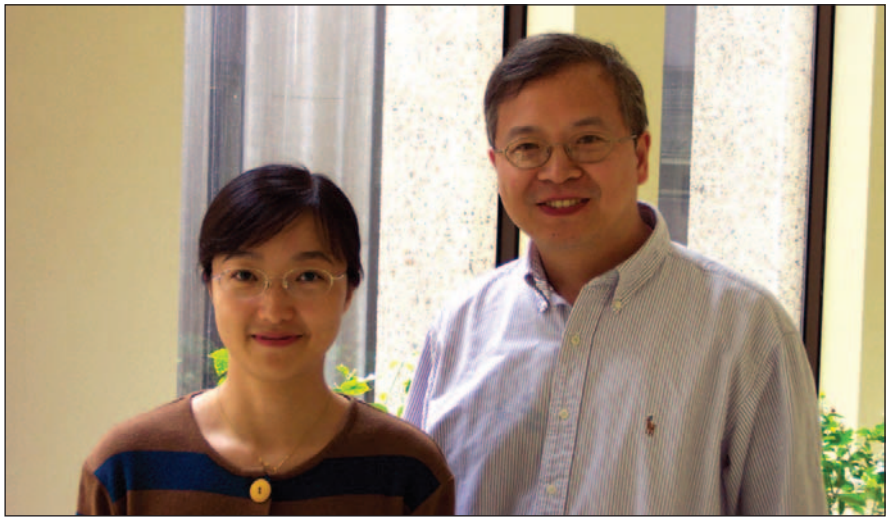
Taniguchi and S. Kaya, Eds., Excerpta Medica Internat. Congress Series 1207, Elsevier, Amsterdam, (2000).

26. Axelsen, K.B. and Palmgren, M.G. Evolution of substrate specificities in the P-type ATPase superfamily. *J. Mol. Evol.* **46**, 84–101 (1998). **This article provides the phylogenetic analysis of P-type ATPases: (http://biobase.dk/~axe/Patbase.html).**
27. Takeyasu, K., Okamura, H., Yasuhara, J.C., Ogita, Y., and Yoshimura, S.H. P-type ATPase diversity and evolution: The origins of ouabain sensitivity and subunit assembly. *Cell Mol. Biol.* **47,** 325–333 (2001).
28. Liu, L., Mohammadi, K., Aynafshar, B., Wang, H., Li, D., Liu, J., Ivanov, A.V., Xie, Z., and Askari, A. Role of caveolae in the signal transducing function of cardiac Na^+/K^+-ATPase. *Am. J. Physiol.* http://ajpcell.physiology.org/cgi/reprint/00555.2002v1 (2003). **This article provides evidence that Na^+-K^+–ATPase signalosome is pre-assembled in caveolae.**
29. Wang, H., Haas, M., Cai, T., and Xie, Z. Sodium pump as a signal transducer: A role of caveolae. *Biophysical J.* **83**, 268a (2003).
30. Mohammadi, K., Kometiani, P., Xie, Z., and Askari, A. Role of protein kinase C in the signal pathways that link Na^+,K^+-ATPase to ERK1/2. *J. Biol. Chem.* **276**, 42050–42056 (2001).
31. Done, S.C., Leibiger, I.B., Efendiev, R., Katz, A.I., Leibiger, B., Berggren, P.O., Pedemonte, C.H., and Bertorello, A.M. Tyrosine 537 within the Na^+,K^+-ATPase α-subunit is essential for AP-2 binding and clathrin-dependent endocytosis. *J. Biol. Chem.* **277**, 17108–17111 (2002).
32. Zhou, X., Jiang, G., Zhao, A., Bondeva, T., Hirszel, P., and Balla, T. Inhibition of Na^+,K^+-ATPase activates PI3 kinase and inhibits apoptosis in LLC-PK1 cells. *Biochem. Biophys. Res. Commun.* **285**, 46–51 (2001).
33. Feraille, E., Carranza, M.L., Gonin, S. et al. Insulin-induced stimulation of Na^+,K^+-ATPase activity in kidney proximal tubule cells depends on phosphorylation of the α subunit at Tyr^{10}. *Mol Biol Cell.* **10**, 2847–2859 (1999).
34. Sweadner, K.J. and Rael, E. The FXYD gene family of small ion transport regulators or channels: cDNA sequence, protein signature sequence, and expression. *Genomics.* **68,** 41-56 (2000). **This is the article first to define the FXYD protein family.**
35. Crambert, G. and Geering, K. FXYD proteins: New tissue-specific regulators of the ubiquitous Na^+,K^+-ATPase. *Sci. STKE.* **2003**, re1 (2003).
36. Nelson, W.J., Veshnock, P.J. Ankyrin binding to Na^+,K^+-ATPase and implications for the organization of membrane domains in polarized cells. *Nature* **328**, 533–536 (1987). **A key reference that shows a role of protein–protein interaction in regulation of Na^+-K^+–ATPase.**
37. Ferrandi, M., Salardi, S., Tripodi, G. et al. Evidence for an interaction between adducin and Na^+,K^+–ATPase: Relation to genetic hypertension. *Am. J. Physiol.* **277**, H1338–H1349 (1999).
38. Manunta, P., Barlassina, C., and Bianchi, G. Adducin in essential hypertension. *FEBS Lett.* **430**, 41–44 (1998).
39. Yudowski, G.A., Efendiev, R., Pedemonte, C.H., Katz, A.I., Berggren, P., and Bertorello, A.M. Phosphoinositide-3 kinase binds to a proline-rich motif in the Na^+,K^+-ATPase α subunit and regulates its trafficking. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6556–6561 (2000). **This article describes the molecular mechanism of dopamine-induced Na^+-K^+–ATPase trafficking.**
40. Vondriska, T.M., Klein, J.B., and Ping, P. Use of functional proteomics to investigate PKC epsilon–mediated cardioprotection: The signaling module hypothesis. *Am. J. Physiol.* **280**, H1434–H1441 (2001).
41. Yingst, D.R., Yang, S.Y., and Schiebinger, R. Purification of active Na^+-K^+-ATPase using a new ouabain-affinity column. *Am. J. Physiol.* **275,** C1167–C1177 (1998).
42. Caplan, M. Pagel, P., Zatti, A., Kimura, T., and Duffield, A. A search for novel pump-interacting proteins. *Ann. N.Y. Acad. Sci.* in press (2003)
43. Yuan, Z., Cai, T., Turner, D., Giovannucci, D., and Xie, Z. Sodium pump as a signal transducer: Proteomic identification of ouabain-activated signaling modules. *Biophysical J.* **83**, 267a (2003).
44. Thomas, S.M. and Brugge, J.S. Cellular functions regulated by Src family kinases. *Annu. Rev. Cell. Dev. Biol.* **13**, 513–609 (1997).
45. Young, M.A., Gonfloni, S., Superti-Furga, G., Roux, B., and Kuriyan, J. Dynamic coupling between the SH2 and SH3 domains of c-Src and Hck underlies their inactivation by C-terminal tyrosine phosphorylation. *Cell* **105,** 115–126 (2001).
46. Xu, W., Harrison, S.C., and Eck, M.J. Three-dimensional structure of the tyrosine kinase c-Src. *Nature* **385**, 595–602 (1997).
47. Tian, J., Cai, T., Haas, M., Li, D., and Xie, Z. Sodium pump as a signal transducer: Interaction with Src. *Biophysical J.* **83**, 267a (2003).
48. Costa, C.J., Gatto, C., and Kaplan, J.H. Interactions between Na^+,K^+-ATPase α-subunit ATP binding domains. *J. Biol. Chem.* [epub ahead of print] (2003).
49. Lee, K., Jung, J., Kim, M., and Guidotti, G. Interaction of the alpha subunit of Na^+,K^+-ATPase with cofilin. *Biochem J.* **353**, 377–385 (2001).

50. Zhang, Z., Devarajan, P., Dorfman, A.L., and Morrow, J.S. Structure of the ankyrin-binding domain of α-Na^+,K^+-ATPase. *J. Biol. Chem.* **273**, 18681–18684 (1998).
51. Prenzel, N., Fischer, O.M., Streit, S., Hart, S., and Ullrich, A. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. *Endocr. Relat. Cancer* **8**, 11–31 (2001).
52. Kuroki, D.W., Minden, A., Sanchez, I., and Wattenberg, E.V. Regulation of a c-Jun amino-terminal kinase/stress-activated protein kinase cascade by a sodium-dependent signal transduction pathway. *J. Biol. Chem.* **272**, 23905–23911 (1997).
53. Li, S. and Wattenberg, E.V. Differential activation of mitogen-activated protein kinases by palytoxin and ouabain, two ligands for the Na^+,K^+-ATPase. *Toxicol. Appl. Pharmacol.* **151**, 377–384 (1998).
54. Juhaszova, M. and Blaustein, M.P. Na^+ pump low and high ouabain affinity alpha subunit isoforms are differentlydistributed in cells. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 1800–1805 (1997). **This article first reported the different distributions and functional significance of low and high ouabain affinity α subunit isoforms in the same cell.**
55. Liu, P., Rudick, M., and Anderson, R.G. Multiple functions of caveolin-1. *J. Biol. Chem.* **277**, 41295–41298 (2002).
56. Razani, B., Woodman, S.E. and Lisanti, M.P. Caveolae: From cell biology to animal physiology. *Pharmacol. Rev.* **54**, 431–467 (2002).
57. Hamlyn, J.M., Blaustein, M.P., Bova, S., Ducharme, D.W., Harris, D.W., Mandel, F., Mathews, W.R., and Ludens, J.H. Identification and characterization of a ouabain-like compound from human plasma. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6259–6263 (1991). **A key reference that shows ouabain as a potential endogenous steroid.**
58. Fedorova, O.V., Doris, P.A., and Bagrov, A.Y. Endogenous marinobufagin-like factor in acute plasma volume expansion. *Clin. Exp. Hypertens.* **20**, 581–591 (1998).
59. Laredo, J., Shah, J.R., Lum Z.R., Hamilton, B.P., and Hamlyn, J.M. Angiotensin II stimulates secretion of endogenous ouabain from bovine adrenocortical cells via angiotensin type 2 receptors. *Hypertension* **29**, 401–407 (1997).
60. Manunta, P., Rogowski, A.C., Hamilton, B.P., and Hamlyn, J.M. Ouabain-induced hypertension in the rat: Relationships among plasma and tissue ouabain and blood pressure. *J. Hypertens.* **12**, 549–560 (1994).
61. Dmitrieva, R.I. and Doris, P.A. Cardiotonic steroids: Potential endogenous sodium pump ligands with diverse function. *Exp. Biol. Med.* **227**, 561–569 (2002).
62. Schoner, W. Endogenous cardiac glycosides, a new class of steroid hormones. *Eur. J. Biochem.* **269**, 2440–2448 (2002).
63. Xie, Z., Wang, Y., Askari, A., Huang, W., Klaunig, J.E., and Askari, A. Studies on the specificity of the oxygen free radical effects on cardiac sodium pump. *J. Mol. Cell. Cardiol.* **22**, 911–920 (1990).
64. Huang, W., Wang, Y., Askari, A., Zolotarjova, N., and Ganjeizadeh, M. Different sensitivities of the Na^+-K^+–ATPase isoforms to oxidants. *Biochim. Biophys. Acta* **1190**, 108–114 (1994).
65. Kajikawa, M., Fujimoto, S., Tsuura, Y. et al. Ouabain suppresses glucose-induced mitochondrial ATP production and insulin release by generating reactive oxygen species in pancreatic islets. *Diabetes* **51**, 2522–2529 (2002).
66. Bereta, J., Cohen, M.C., and Bereta, M. Stimulatory effect of ouabain on VCAM-1 and iNOS expression in murine endothelial cells: Involvement of NF-κB. *FEBS Lett.* **377**, 21–25 (1995).
67. Matsumori, A., Ono, K., Nishio, R. et al. Modulation of cytokine production and protection against lethal endotoxemia by the cardiac glycoside ouabain. *Circulation* **96**, 1501–1506 (1997).
68. Rajasekaran, S.A., Palmer, L.G., Moon, S.Y., Peralta Soler, A., Apodaca, G.L., Harper, J.F., Zheng, Y., Rajasekaran, A.K. Na^+,K^+-ATPase activity is required for formation of tight junctions, desmosomes, and induction of polarity in epithelial cells. *Mol. Biol. Cell* **12**, 3717–3732 (2001).
69. Baudouin-Legros, M., Brouillard, F., Tondelier, D., Hinzpeter, A., and Edelman, A. Effect of ouabain on CFTR gene expression in human Calu-3 cells. *Am. J. Physiol. Cell Physiol.* **284**, C620–C626 (2003).
70. Vega, C., Pellerin, L., Dantzer, R., and Magistretti, P.J. Long-term modulation of glucose utilization by IL-1α and TNF–α in astrocytes: Na^+ pump activity as a potential target via distinct signaling mechanisms. *Glia* **39**, 10–18 (2002).
71. McGowan, M.H., Russell, P., Carper, D.A., and Lichtstein, D. Na^+, K^+-ATPase inhibitors down-regulate gene expression of the intracellular signaling protein 14-3-3 in rat lens. *J. Pharmacol. Exp. Ther.* **289**,1559–1563 (1999).
72. Shiratori, O. Growth inhibitory effect of cardiac glycosides and aglycones on neoplastic cells: In vitro and in vivo studies. *Gann* **58**, 521–528 (1967).
73. Stenkvist, B., Pengtsson, E., Dahlqvist, B., Eriksson, O., Jarkrans, T., and Nordin, B. Cardiac glycosides and breast cancer, revisited. *N. Engl. J. Med.* **306**, 484 (1982).
74. Carver, J.H., Adair, G.M., and Wandres, D.L. Mutagenicity testing in mammalian cells. II. Validation of

multiple drug-resistance markers having practical application for screening potential mutagens. *Mutat. Res.* **72**, 207–230 (1980).
75. Price, E.M. and Lingrel, J.B. Structure-function relationships in the Na^+,K^+-ATPase alpha subunit: Site-directed mutagenesis of glutamine^{111} to arginine, and asparagine^{122} to aspartic acid generates a ouabain-resistant enzyme. *Biochemistry* **27**, 8400–8408 (1988).
76. Watabe, M., Kawazoe, N., Masuda, Y., Nakajo, S., and Nakaya, K. Bcl-2 protein inhibits bufalin-induced apoptosis through inhibition of mitogen-activated protein kinase activation in human leukemia U937 cells. *Cancer Res.* **57**, 3097–3100 (1997).**This article shows the apoptotic effects of a cardiotonic steroid.**
77. Watabe, M., Ito, K., Masuda, Y., Nakajo, S., and Nakaya, K. Activation of AP-1 is required for bufalin-induced apoptosis in human leukemia U937 cells. *Oncogene* **16**, 779–787 (1998).
78. Kawazoe, N., Watabe, M., Masuda, Y., Nakajo, S., and Nakaya, K. Tiam1 is involved in the regulation of bufalin-induced apoptosis in human leukemia cells. *Oncogene* **18**, 2413–2421 (1999).
79. McConkey, D.J., Lin, Y., Nutt, L.K., Ozel, H.Z., and Newman, R.A. Cardiac glycosides stimulate Ca^{2+} increases and apoptosis in androgen-independent, metastatic human prostate adenocarcinoma cells. *Cancer Res.* **60**, 3807–3812 (2000).
80. Chueh, S.C., Guh, J.H., Jun, C., Lai, M.K., and Teng, C.M. Dual effects of ouabain on the regulation of proliferation and apoptosis in human prostatic smooth muscle cells. *J. Urol.* **166**, 347–353 (2001).
81. Akiyama, M., Ogura, M., Iwai, M., Iijima, M., Numazawa, S., and Yoshida, T. Effect of bufalin on growth and differentiation of human skin carcinoma cells in vitro. *Hum. Cell.* **12,** 205–209 (1999).
82. Xiao, A.Y., Wei, L., Xia, S., Rothman, S., and Yu, S.P. Ionic mechanism of ouabain-induced concurrent apoptosis and necrosis in individual cultured cortical neurons. *J. Neurosci.* **22**, 1350–1362 (2002).
83. Murata, Y., Matsuda, T., Tamada, K., Hosoi, R., Asano, S., Takuma, K., Tanaka, K.-i., and Baba, A. Ouabain-induced cell proliferation in cultured rat astrocytes. *Jpn. J. Pharmacol.* **72**, 347–353 (1996).
84. Golomb, E., Hill, M.R., Brown, R.G., and Keiser, H.R. Ouabain enhances the mitogenic effect of serum in vascular smooth muscle cells. *Am. J. Hypertens.* **7**, 69–74 (1994).
85. Huang, L., Li, H., and Xie, Z. Ouabain-induced hypertrophy in cultured cardiac myocytes is accompanied by changes in expressions of several late response genes. *J. Mol. Cell. Cardiol.* **29**, 429–437 (1997).**A key reference that shows the growth effect of ouabain on cardiac myocytes.**
86. Huang, L., Kometiani, P., Xie, Z. Differential regulation of Na^+-K^+–ATPase alpha-subunit isoform gene expressions in cardiac myocytes by ouabain and other hypertrophic stimuli. *J. Mol. Cell Cardiol.* **29**, 3157–3167 (1997).
87. Priydarshi, S., Valentine, B., Han, C. et al. Effect of green tea extract on cardiac hypertrophy following 5/6th nephrectomy in the rat. *Kidney Int.* [*in press*] (2003).
88. Christian, H.A. The use of digitalis other than in the treatment of cardiac decompensation. *JAMA* **100**, 789–792 (1933).
89. Williams, J.F. Jr. and Braunwald, E. sStudies on digitalis XI. Effects of digitoxin on the development of cardiac hypertrophy in the rat subjected to aortic constriction. *Am. J. Cardiol.* **16**, 534–539 (1965).
90. Cutilletta, A.F., Rudnik, M., Arcilla, R.A., and Straube, R. Effect of prophylactic digitalization on the development of myocardial hypertrophy. *Am. J. Physiol.* **233**, H600–H604 (1977).
91. Wendt, L., Geppert, M.P., and Hesse, H. Uber die beziehungen zwischen herzhypertrophie und digitalis wirkung. *Virchows Archiv Abt B Zellpathol.* **210**, 291–325 (1951).
92. Pierdomenico, S.D., Bucci, A., Manunta, P., Rivera, R., Ferrandi, M., Hamlyn, J.M., Lapenna, D., Cuccurullo, F., Mezzetti, A. Endogenous ouabain and hemodynamic and left ventricular geometric patterns in essential hypertension. *Am. J. Hypertens.* **14**, 44–50 (2001).
93. Marban, E. and Tsien, R.W. Enhancement of calcium current during digitalis inotropy in mammalian heart: Positive feed-back regulation by intracellular calcium? *J. Physiol.* **329**, 589–614 (1982).
94. Lederer, W.J. and Eisner, D.A. The effects of sodium pump activity on the slow inward current in sheep cardiac Purkinje fibres. *Proc. R. Soc. Lond. B Biol. Sci.* **214**, 249–262 (1982).
95. Tian, J., Liu, J., Shapiro, J.I., Garlid, K., and Xie, Z. Involvement of mitogen-activated protein kinases and reactive oxygen species in the inotropic action of ouabain on cardiac myocytes. A potential role for mitochondrial K_{ATP} channels. *Mol. Cell. Biochem.* **242**, 181–187 (2003).
96. Fitzgerald, E.M. Regulation of voltage-dependent calcium channels in rat sensory neurones involves a Ras-mitogen-activated protein kinase pathway. *J. Physiol.* **527**, 433–444 (2000).
97. Nishio, M., Ruch, S.W., Wasserstrom, J.A. Positive inotropic effects of ouabain in isolated cat ventricular myocytes in sodium-free conditions. *Am. J. Physiol. Heart Circ. Physiol.* **283**, H2045–H2053 (2002).
98. Garlid, K.D., Paucek, P., Yarov-

Yarovoy, V., Murray, H.N., Darbenzio, R.B., D'Alonzo, A.J., Lodge, N.J., Smith, M.A. and Grover, G.J. Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K^+ channels. Possible mechanism of cardioprotection. *Circ. Res.* **81**, 1072–1082 (1997).



Zijian Xie, PhD, (right), is an Associate Professor of the Departments of Pharmacology and Medicine. **Ting Cai, MD, MS**, (left), is a Research Associate of the Department of Pharmacology. Both are at the Medical College of Ohio. Address correspondence to ZX.
Email: zxie@mco.edu
FAX: 419-383-2871