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REVIEW

The Na,K-ATPase Receptor Complex

Its Organization and Membership

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Abstract

A major difference between the Na,K-ATPase ion pump and other P-type ATPases is its ability to bind cardiotonic steroids such as ouabain. Na,K-ATPase also interacts with many membrane and cytosolic proteins. In addition to their role in Na,K-ATPase regulation, it became apparent that some of the newly identified interactions are capable of organizing the Na,K-ATPase into various signaling complexes. This new function confers a ligand-like effect to cardiotonic steroids on cellular signal transduction. This article reviews these new developments and provides a comparison of Na,K-ATPase-mediated signal transduction with other receptors and ion transporters.

Index Entries: Cardiotonic steroids; isoforms; c-Src; lipid kinase; caveolae; calcium.

INTRODUCTION

The movement of solutes across biological membranes catalyzed by various ion pumps, channels, and carriers is often regulated by an array of auxiliary proteins that form a regulatory complex. The cytoplasmic domains of a number of transporters interact with protein kinases and phosphatases, cytoskeletal tethers, and adaptor proteins that ultimately influence transport kinetics. Beyond their immediate impact on the regulation of transport, however, some of these assembled regulatory complexes are also involved in signal transduction, amplification, and integration that extend far beyond the membrane. Indeed, a growing number of transporting proteins have been implicated in downstream signaling, including the Na^+/H^+ exchangers (NHE) *(1,2)*, transient receptor potential (Trp) channels *(3,4)*, the red blood cell anion exchanger band 3 *(5)*, and IP3 receptors (IP3 R) *(6)*.

This role for transporters as initiators of signal transduction extends to central players in ionic homeostasis, the members of the plasma membrane Na,K-ATPase family. These enzymes are energy transducers that hydrolyze ATP to pump K^+ into and Na^+ out of the cell, thus establishing transmembrane ion gradients that are critical for the maintenance of membrane potential and cell volume. The energy stored in these gradients is used to absorb various nutrients into the cell and to regulate cytoplasmic pH and Ca^{2+} concentrations via secondary transporters or channels (7,8). From recent work, it has become evident that the Na,K-ATPases are also involved in multiple protein-protein interactions leading to the tethering of a number of kinases and phosphatases in caveolae of the cell membrane. Binding of either endogenous or exogenous cardiotonic steroids (CTS) to Na,K-ATPase regulates these interactions, resulting in the activation of protein kinases and assembly of multiple downstream signaling modules. Based on this new paradigm, several groups of investigators have unraveled new functions of the Na,K-ATPase over the last 10 years, and reinvestigated the molecular mechanisms underlying many earlier observations of critical importance in cell biology. This article reviews these new developments and provides a comparison of Na,K-ATPase-mediated signal transduction with other receptors and ion transporters.

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STRUCTURE OF Na,K-ATPases

The Na,K-ATPases are a family of isozymes composed of two major polypeptides, a catalytic α subunit and an auxiliary β subunit. These isozymes are members of the P-type ATPase superfamily, a group of integral membrane proteins sharing a common structure and transport mechanism. The formation of a transient phosphorylated aspartate residue during the catalytic cycle is a hallmark of all P-type ATPase family members. Ion movement across the membrane is coupled to ATP hydrolysis via a cation-dependent E1 to E2 conformational change. A simplified representation of the scheme that Albers and Post defined for the Na,K-ATPase is depicted in Fig. 1A (*9,10*). Earlier reviews dedicated to Na,K-ATPase structure and mechanism have outlined functional and structural similarities within the non–heavy metal (P2) subgroup of P-type ATPases that includes Na,K-ATPases and H,K-ATPases (group IIc), sarcoplasmic-endoplasmic reticulum calcium ATPases (SERCA, group IIa), and plasma membrane calcium ATPases (PMCA, group IIb). This classification has proven useful when applying conclusions drawn from one transporter to the others (*11–15*). Because direct structural data for the Na,K-ATPase has been difficult to obtain, the high-resolution structures of the Ca^{2+}-ATPase in different conformations have been particularly useful in our understanding of Na,K-ATPase. The legitimacy of such comparisons is supported by 1) patterns of similarity (*12*), 2) indirect structural information obtained through the use of proteolytic cleavage and other biochemical approaches (reviewed in *14* and *16*), and 3) low-resolution structures of Na,K-ATPase and SERCA in the E2 conformation that clearly show a similar architecture (*17*). It follows that the domain organization of SERCA depicted in Fig. 1B is thought to also reflect the structure of the Na,K-ATPase, including the organization of the three main cytoplasmic domains: N (nucleotide binding), A (actuator, composed of N-terminus and first intracellular loop), and P (phosphorylation). A recent comparison between high-resolution structures of the N domains of the Na,K-ATPase and SERCA further confirms these structural similarities, but also highlights secondary structural differences of undetermined significance (reviewed in *15*). Among the most noticeable differences revealed by superimposition of the two structures are extra electron-dense regions that appear on the extracellular side of Na,K-ATPase but are absent in the SERCA structure. These structures most likely correspond to the β polypeptide.

Subunit Isoforms

There are four isoforms of the α subunit, all multispanning transmembrane proteins responsible for the catalytic and transport properties of the Na,K-ATPase, as well as for providing binding sites for cations, ATP, and CTS. The β polypeptide, critical for the delivery of the α-polypeptide to the membrane, exists in three known isoforms and modulates the K^+ and Na^+ affinity of the enzyme. FXYD proteins, a group of seven structurally similar polypeptides, are expressed in a tissue-specific manner and appear to act as a third subunit of the Na,K-ATPase, at least in some tissues. Although FXYD proteins are not required for ATPase activity, they do associate with and regulate Na,K-ATPase function in a FXYD protein-specific manner (*18,19*).

Overall, the αβ isozymes are characterized by a highly conserved and finely regulated pattern of expression that varies with species, cell type, developmental stage, and pathology, suggesting that they play a critical physiological role (reviewed in *20–22*). Unique enzymatic properties and distinctive interactions with regulatory proteins have been identified for the α polypeptides, and some insight has been gained into both the structural basis of those differences and the physiological role of Na,K-ATPase α isoform diversity (for reviews, see *21,22*). There is actually a high degree of identity (approx 87%) between α1, α2, and α3 isoforms and even the most divergent isoform, α4, shares 78% of identity with the α1 isoform. Those areas with the greatest structural variability are confined to three distinct regions of the α polypeptides. First, the amino terminus underlies isoform specificity in the rate of K^+ deocclusion and bears two protein kinase C phosphorylation sites in the α1 polypeptide. Second, the first extracellular loop is a critical component of the ouabain binding site (*23,24*), and finally, the large central loop (the third cytosolic domain, CD3, Fig. 1C) contains a so-called isoform-specific region. The isoform-specific region is an 11 amino acid sequence beginning with Lys-489 that protrudes into the cytoplasm from the N domain, and it is involved in isoform-specific regulation by protein kinase C (reviewed in *21*).

Most of what we know about downstream signaling mediated by the Na,K-ATPase has come from cultured renal cells or in vitro models that focus on the α1 isoform. The ubiquitous expression of α1 in tissues makes it difficult to study the other isoforms in isolation. Models that include additional isoforms, such as cardiac and skeletal muscle, pose technical problems when distinguishing between the various isoforms. The most popular strategy for discrimination among the isoforms takes advantage of natural or artificially induced differences in ouabain sensitivity. Unfortunately, studies on CTS-induced protein phosphorylation or tethering require a system in which only one isozyme is present at a time, because even a small amount of binding to α1 could result in a significant effect due to the phenomenon of amplification, which is a hallmark of signaling pathways (*25*). At this point, the potential role of β isoforms and FXYD family members in downstream signaling has not been investigated.

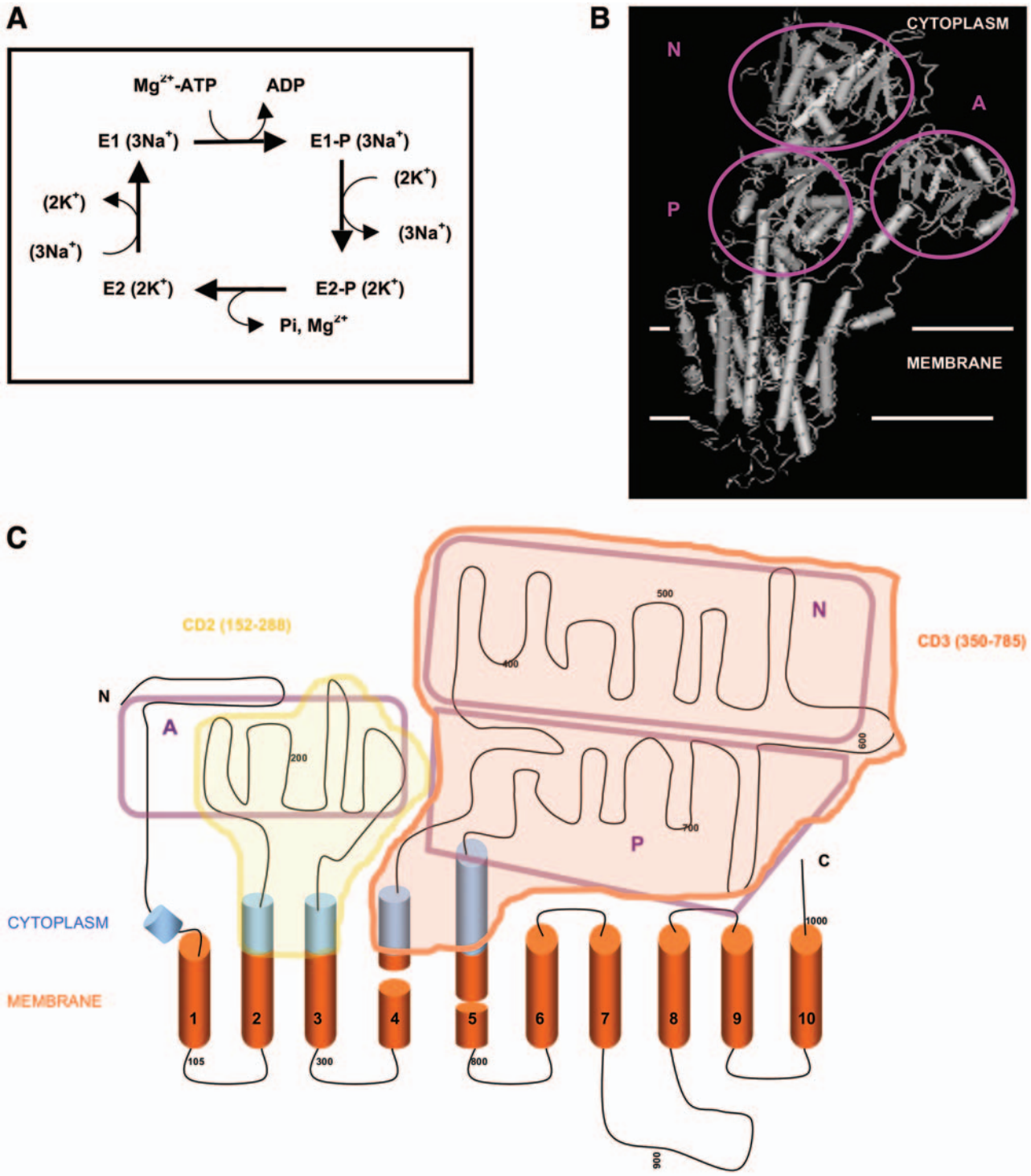

Fig. 1. **(A)** P-type ATPases enzymatic cycle, based on the post-Albers scheme for the Na,K-ATPase. Na^+ binds to E1 with high affinity on the intracellular side. This triggers the phosphorylation of the enzyme by Mg^{2+}-ATP (E1-P). Whereas E1-P conformation becomes E2-P, its affinity for Na^+ drops and its affinity for K^+ increases, leading to the release of Na^+ and the binding of K^+ on the extracellular side. After hydrolysis of the phosphorylated intermediate, K^+ is released on the intracellular side, whereas E2 conformation returns to the initial E1 state with high affinity for Na^+, ready to start another cycle. **(B)** Sarcoplasmic-reticulum (SR) Ca^{2+}-ATPase in the Ca^{2+} unbound E2 conformation stabilized by thapsigargin (PDB ID code 1IWO). Cytoplasmic domains: A (actuator or anchor composed of N-terminus and first cytosolic loop), P (phosphorylation), N (nucleotide-binding). **(C)** Na,K-ATPase α1 model (modified from *15*), showing the two cytoplasmic domains (CDs) involved in the interaction with c-Src. CD2 (yellow, residues 152–288) belongs to the A domain and constitutively interacts with the SH2SH3 domain of c-Src. CD3 (orange, residues 350–785) includes both N and P domains and its interaction with the kinase domain of c-Src is disrupted after ouabain binding.

CARDIOTONIC STEROIDS

Structure

CTS encompass a group of compounds that bind to the regulatory site on the extracellular surface of the Na,K-ATPase α subunits. They consist of a steroid ring system and a five- or six-membered lactone moiety attached to the steroid nucleus at position 17. Cardenolides are characterized by an unsaturated five-membered lactone ring, whereas bufadienolides contain a corresponding unsaturated six-membered lactone ring. Inhibition of ATPase activity by CTS is quite complex and does not reflect a simple competitive inhibitory mechanism. Rather, it has been proposed that CTS binding paralyzes the movement of the functional domains that accompanies ion translocation and thus stabilizes the ATPase in the E2-P conformation. CTS achieves this inhibition by binding to a shallow groove between TM1-TM2, TM3-TM4, TM5-TM6, and TM9-TM10 loops of the α-subunit of Na,K-ATPase (TM: transmembrane segment, *26,27*). In terms of activating downstream signal transduction, this mode of action suggests that the inability of the Na,K-ATPase to shift between conformations may be key to the initiation of the signaling cascade. If this is true, it seems unlikely that an active ion-pumping Na,K-ATPase could engage in signal transduction.

Non-Mammalian CTS

Cardenolides are naturally found in many different plants. Members include digitoxin, digoxin, and their derivatives, and the strophantidins. Krenn and Kopp have reviewed more than 250 naturally occurring bufadienolides, restricted to a few animal and plant families (*28*). In the animal kingdom, bufadienolides are most widespread in the Bufonidae (toad, 10 species of *Bufo*), but have also been found in Colubridae (snake, *Rhadbdophis tigrinus*) and Lampyridae (firefly, *Photinus* sp.). In the plant kingdom, they have been identified from at least six families of angiosperms (Crassulaceae, Hyacinthaceae, Iridaceae, Meliathaceae, Ranunculaceae, and Santalaceae). Cardenolides and bufadienolides are well known for their toxic and cardiotonic properties. More recently, they have been evaluated for their anticancer properties (*29–32*), and the cardenolide UNBS1450 is expected to enter phase I clinical trial as an anticancer drug this year (*33*).

Mammalian CTS

In the past few decades, several endogenous compounds have been identified in mammals that appear to be identical or closely related to the CTS reported previously and exhibit the same biological activity. They are now referred to as endogenous CTS (reviewed recently by Schoner and Scheiner-Bobis, *34*). Endogenous cardenolides include ouabain (found in human plasma and bovine adrenals and hypothalamus), an ouabain isomer (identified in bovine hypothalamus), and digoxin (found in human urine). Reported endogenous bufadienolides include marinobufagenin in human plasma and in urine of patients with myocardial infarction, and 19-norbufalin and its peptide derivative in cataractous human lenses. Strong evidence is pointing to the adrenal cortex as the site of synthesis of endogenous ouabain.

Our understanding of the role of endogenous CTS in mammalian physiology focuses on their role in maintenance of blood pressure. Circulating ouabain appears to work as a blood pressure–modulating factor at the systemic level, targeting the main components of the cardiovascular system (namely heart, vessels, and kidney). In addition, hypothalamic ouabain plays a role in the regulation of blood pressure at the central level. There is evidence that ouabain antagonists such as 17β-(-3-Furyl)-5β-androstane-3β,14β,17α-triol (PST2238) are antihypertensive (*35*). Somewhat surprisingly, endogenous digoxin opposes endogenous ouabain action. Finally, marinobufagenin seems to act as a natriuretic factor and has recently been shown to induce cardiac fibrosis (*36*). Direct evidence of the role of endogenous CTS/Na,K-ATPase interaction in the regulation of blood pressure has been provided by Lingrel's group, using an elegant model of genetically engineered mice. The investigators developed a mouse model in which Na,K-ATPase α2 was rendered insensitive to CTS using a well-known modification of the TM1-TM2 loop. Because rodent α1 sensitivity to CTS is naturally reduced, this results in decreased sensitivity of the entire Na,K-ATPase pool to endogenous CTS. Contrary to wild-type animals, adrenocorticotropic hormone fails to induce hypertension in this model. On the other hand, when the ouabain-insensitive α1 was converted to ouabain-sensitive isoform, the knock-in mice become super-sensitive to increases in endogenous CTS. Taken together, these findings support the hypothesis that adrenocorticotropic hormone–induced hypertension is mediated through an increase in endogenous CTS acting on the Na,K-ATPase (*37*). Therefore, an increasing body of compelling evidence is establishing the physiological and pathophysiological importance of endogenous CTS. Key issues such as their mechanism and site of synthesis are under intensive investigation.

Na,K-ATPase IN SIGNAL TRANSDUCTION

Identification of Caveolar Na,K-ATPase/Src Complex as a Functional Receptor: Activation of Protein and Lipid Kinase Cascades by CTS

Na,K-ATPase/Src Complex as a Functional Receptor

Many laboratories, including ours, have shown that the binding of CTS to the Na,K-ATPase stimulates tyro-

sine phosphorylation of multiple proteins in the absence of changes in intracellular Na^+ concentration. These increases in tyrosine phosphorylation play a pivotal role in CTS-induced changes in cell motility, metabolism, gene expression, and cell growth (*38–43*). Members of the Src family of kinases have been identified as key participants in this phosphorylation cascade. Src family kinases are 52–62 kDa membrane-associated nonreceptor tyrosine kinases that interact with many membrane proteins including ion pumps, channels, and transporters. Moreover, they regulate various signal transduction pathways (*44*). Each Src family kinase contains several functional domains. The acylated amino terminus mediates the association of the kinase with the membrane and a unique amino-terminal region contains multiple phosphorylation sites for protein kinases. The kinases are also characterized by a SH3 domain, a SH2 domain, a kinase domain, and a carboxy-terminal regulatory domain (*45,46,* Fig. 2). The kinase activity of Src, the best-understood member of this family, is regulated by tyrosine phosphorylation and intramolecular interactions.

The activation of Src may be the initiating event in downstream signaling invoked by interaction of the Na,K-ATPase with CTS. Early studies have demonstrated that Src and the Na,K-ATPase can be coimmunoprecipitated from cell lysates and tissue homogenates. In addition, ouabain stimulates Src activity in many different cell types, and appears to regulate the interaction between the Na,K-ATPase and Src. Many of the effects of CTS on cellular function are blocked by Src inhibitors or by knock-out of Src family kinases (*47–50*). These findings have led to the hypothesis that Na,K-ATPase and Src can directly interact and form a functional receptor complex.

Further support for an interaction of Src and Na,K-ATPase as an initiator of downstream signaling comes from more recent observations (*51*). Flourescence resonance energy transfer (FRET) analysis suggests that Na,K-ATPase and Src are colocalized in the cell membrane and are likely to form a functional complex. In vitro binding and kinase assays reveal that these two proteins interact via multiple domains. Whereas Src SH2SH3 domain binds to the second cytosolic domain (CD2) of the Na,K-ATPase α1 polypeptide, the Src kinase domain interacts with the third cytosolic domain (CD3) of α1 (Fig. 1C, 2). This latter interaction is regulated by CTS. Indeed, binding of ouabain was shown to promote the dissociation of pre-bound kinase domain from the Na,K-ATPase or inhibit the formation of the kinase domain-CD3 domain complex (*51*). Functionally, binding of Src kinase domain to the full length α1 polypeptide or its CD3 domain inhibits Src activity. Thus in nonstimulated cells, the signaling Na,K-ATPase interacts with Src, resulting in the formation of an inactive receptor complex.

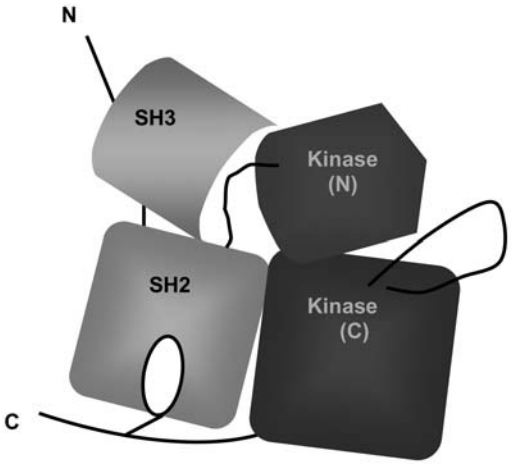

Fig. 2. Schematic diagram of c-Src, modified from *45*, showing the SH3-SH2 domain (Src homology domains 3 and 2) and the kinase domain (N: N-terminal; C: C-terminal).

These in vitro studies suggest that the Na,K-ATPase/Src receptor complex may transmit ouabain-invoked signals in a way similar to those of cytokine receptors (*52,53*). Although the Na,K-ATPase has no intrinsic kinase activity, its coupling to Src converts it into a functional receptor tyrosine kinase. Indeed, we found that addition of ouabain to the preformed Na,K-ATPase/Src complex in a test tube frees the kinase domain and restores Src activity (*51*). It is important to note, however, that the observed effect of ouabain on Src does not depend on overall inhibition of transport activity. Because these experiments were performed in test tubes under identical conditions, the results cannot involve changes in ion concentration. On the other hand, inhibition alone does not evoke the response. Vanadate, a P-type ATPase inhibitor that works by mimicking the phosphate-enzyme transitional state, has no effect on Src at a concentration that completely inhibits ATPase activity. Significantly, both FRET and BRET analyses indicate that the binding of ouabain releases the kinase domain from the Na,K-ATPase in live cells. The effects of ouabain on the Na,K-ATPase/Src-kinase domain interaction are dose-dependent and correlate with the known dose-response curve for ouabain binding to the Na,K-ATPase (*48*). Finally, as expected, ouabain stimulates tyrosine-phosphorylation of multiple proteins that are associated with or recruited to the signaling Na,K-ATPase complex via the activated Src in live cells.

Despite the developing argument for a Src/Na,K-ATPase signaling complex, several important issues remain to be resolved and are worthy of further discussion. Because Src family kinases are highly conserved, we speculate that the signaling Na,K-ATPase may interact

with other members of the Src family that are expressed in a tissue-specific manner. In addition, it is unclear what role is played by α isoforms other than α1. It is most likely that multiple α isoforms can interact with Src and form a functional complex because they share a highly conserved CD3 domain. To this end, it is of interest to note that Src also interacts with the CD3 domain of H,K-ATPase (*51*), potentially forming yet another group of functional receptors. Both Na,K-ATPase and H,K-ATPase may serve as Src effectors because recent studies have suggested a Src-mediated tyrosine phosphorylation of these P-ATPases (*54–58*). Therefore, interactions of different Src family kinases and different isoforms of the Na,K-ATPase (and possibly H,K-ATPase) could provide a diverse population of signaling receptor species and provide a tissue-specific response. Clearly, these hypotheses remain to be tested experimentally.

From the Na,K-ATPase/Src Complex to Downstream Protein and Lipid Kinase Cascades

Activation of a classical receptor tyrosine kinase by its ligand stimulates tyrosine kinases, which, in turn, signal the activation of lipid and Ser/Thr protein kinases, thus initiating the generation of second messengers such as specific lipids, Ca^{2+} and reactive oxygen species (*59*) (Fig. 3). Ouabain-activated signal transduction appears to operate in a similar manner via the Na,K-ATPase/Src complex. Activation of extracellular signal–regulated kinases and other Ser/Thr protein kinases by CTS has been well documented in the literature (*41–43,55*).

Receptor tyrosine kinases such as epidermal growth factor (EGF) receptor are central elements for cellular signal transduction (*59*), and as discussed previously, provide analogies with the signaling initiated by the Na,K-ATPase/Src complex. EGF receptor, however, may offer more than an understanding of ouabain-induced signaling: it may also play a more direct role. Binding of EGF to its receptor induces the formation of either homodimers or heterodimers which subsequently trigger the autophosphorylation of cytoplasmic tyrosine residues. These phosphorylated amino acid residues then function as docking sites for a variety of adaptor proteins and additional protein and lipid kinases. Working in concert, these signaling events convert the initial activation of protein tyrosine kinases to the stimulation of Ser/Thr and lipid kinases as well as other second messenger pathways.

In recent years, there has been a growing body of evidence that the EGF receptor cross-communicates with other signaling systems to integrate the variety of extracellular stimuli into a limited number of signaling pathways. For example, the activated EGF receptor has been identified as a critical element in the signal transduction network of cytokines, H_2O_2, and other stimuli signaling through G protein–coupled receptors (*60–62*). This process has been termed *EGF receptor transactivation* (*63*) to distinguish it from receptor activation by cognate ligand binding. Such transactivation of the EGF receptor appears to be a key linker that relays ouabain-induced activation of the Na,K-ATPase/Src receptor complex to downstream protein and lipid kinases (*48*). Several laboratories have demonstrated that binding of ouabain to the Na,K-ATPase stimulates tyrosine phosphorylation of the EGF receptor (*47*), and this appears to involve residues other than its major autophosphorylation site Y^{1173} (*64*). As expected, ouabain-induced transactivation of EGF receptor also requires the activation of the Na,K-ATPase/Src complex. Thus the data suggest that the ouabain-activated Na,K-ATPase/Src complex may be able to employ the phosphorylated (transactivated) EGF receptor as the functional scaffold to relay the message from protein tyrosine kinases to the stimulation of Ser/Thr kinases. Indeed, we demonstrated that the transactivated EGF receptor was capable of recruiting and phosphorylating the adaptor protein Shc, resulting in the assembly and activation of the Grb2/Ras/Raf/MEK/extracellular signal–regulated kinases cascade (*48*).

In addition to the activation of a protein kinase cascade, the ouabain-activated Na/K-ATPase/Src complex is also capable of recruitment and assembly of a lipid kinase cascade. PI3 kinases (PI3Ks) are a group of lipid kinases that catalyze the phosphorylation of phosphatidylinositol lipids at the D-3 position (*65*). Nine members of PI3K family have been identified from mammalian cells. They are divided into three classes: I, II, and III. Among them, Class Ia PI3K are well characterized and play an important role in regulation of cell survival, gene expression, cell metabolism, cytoskeleton rearrangement, and vesicle trafficking. Class Ia PI3K are heterodimeric proteins, each of which consists of a catalytic subunit and an associated regulatory subunit. Substrates of Class Ia PI3K include PtdIns, PtdIns4*P*, PtdIns5*P*, and PtdIns(4,5)P_2 that can be phosphorylated to form PtdIns3*P*, PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns PtdIns(3,4,5)P_3, respectively. The PtdIns*P*s produced by PI3K interact with downstream effectors such as Akt, protein kinase C, PDK1, and other signaling proteins that go on to regulate different cellular functions. Recent studies have shown that the Na,K-ATPase interacts with PI3K, which is essential for dopamine-induced activation of PI3K, as well as Na,K-ATPase-mediated regulation of cell mobility (*66,67*). In addition, ouabain was found to activate PI3K in cultured cells (*68,69*). Mechanistically, this activation of PI3K requires the formation and activation of a functional Na,K-ATPase/Src receptor complex. This dependence, however, cuts both

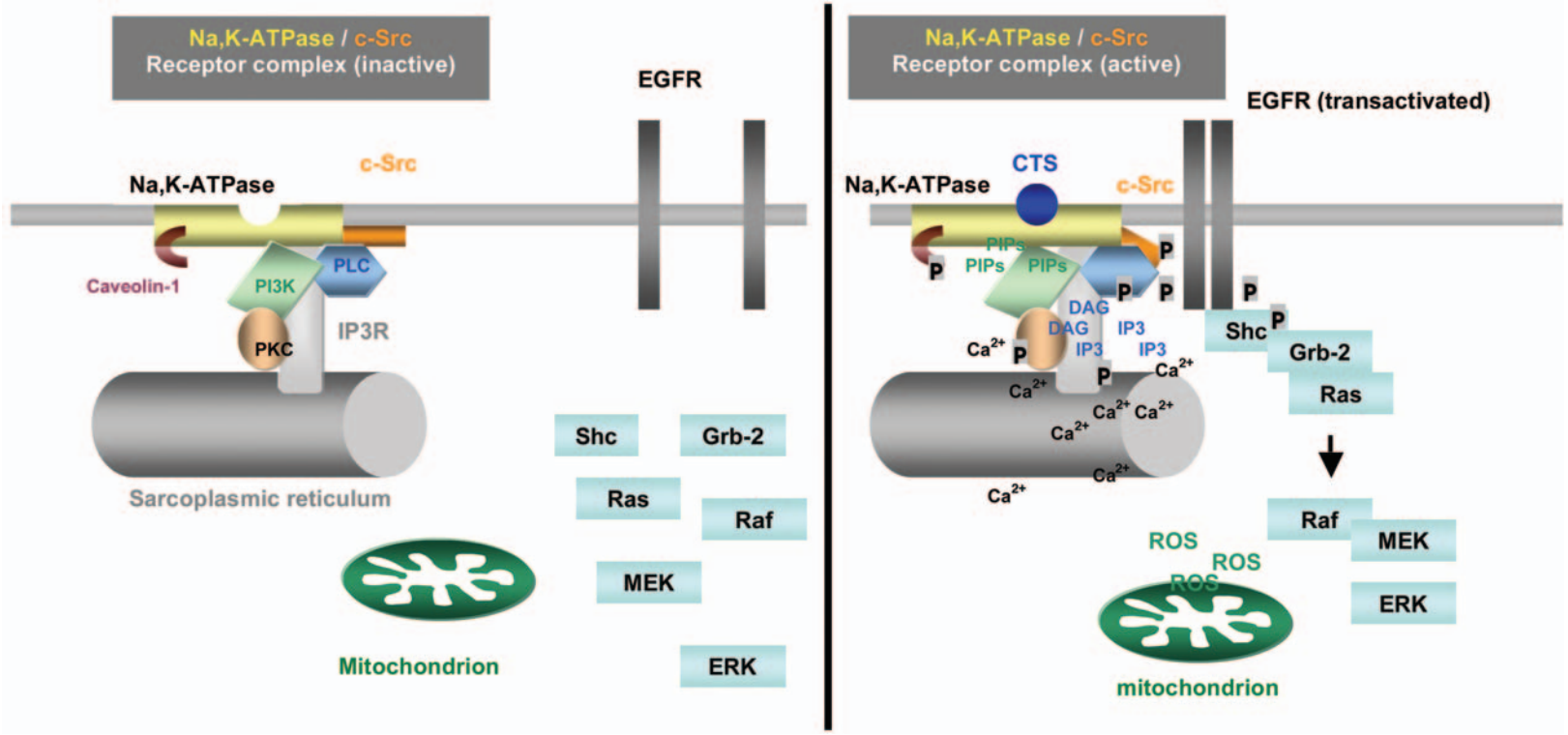

Fig. 3. Schematic of CTS signaling through the caveolar Na/K-ATPase/Src receptor complex. Left panel: Inactive complex. The multifocal Src/Na,K-ATPase interaction maintains Src in an inactive state. PLC γ1, PI3K, and IP3 R are tethered in a Ca^{2+} signaling complex through their interaction with Na,K-ATPase. Right panel: CTS-activated complex. After binding of CTS to its site on Na,K-ATPase, conformational changes release Src kinase domain, resulting in activation. Consequently, transactivation of EGFR, phosphorylation, recruitment, and activation of multiple proteins including protein and lipid kinases lead to the production of intracellular messengers ROS, PIPs, DAG, IP3, and Ca^{2+}. CTS: cardiotonic steroid; PI3K: phosphoinositide 3′ kinase, EGFR: epithelial growth factor receptor; PKC: protein kinase C; PLC : phospholipase C; Shc: Src homology collagen-like protein, Grb-2: growth factor receptor-bound protein 2; MEK: MAPK/ERK kinase; ERK: extracellular signal–regulated kinase; IP3R: IP3 receptor; IP3: Inositol 1,4,5-trisphosphate; PIP: phosphatidyl inositol phosphates; DAG: diacylglycerol; ROS: reactive oxygen species; P: Tyrosine or Ser/Thre phosphorylation.

ways, and the activation of PI3K is essential for ouabain-induced assembly of an endocytotic cargo and subsequent removal of the activated Na,K-ATPase/Src receptor complex from the plasma membrane (*69*). Apparently, this process not only terminates/targets the signaling complexes to intracellular compartments, but also reduces overall pumping capacity of the cells from the loss of plasma membrane Na,K-ATPase (*70*).

The Na,K-ATPase/Src Receptor Complex Resides in and Signals From Caveolae

Caveolae are plasma membrane microdomains that look like flask-shaped vesicular invaginations of different sizes. These microdomains are enriched in cholesterol, glycosphingolipids, and sphingomyelin and a number of receptors, kinases, phosphatases and scaffold proteins (*71,72*). Caveolins are 21–24 kDa membrane-associated scaffolding proteins that serve as protein marker of caveolae (*71*). Caveolins directly interact with cholesterol. They also bind and concentrate many signaling proteins in caveolae via the interaction of their scaffolding domains with the caveolin-binding motifs of the target proteins.

Given the need for the signaling Na,K-ATPase to interact with Src and other proteins for downstream transmission of the ouabain signal, we and others have recently proposed that Na,K-ATPase may reside in and signal from caveolae. A subpopulation of the enzyme is colocalized with caveolin-1 and concentrated in caveolae (*73–75*). For example, about 50% of Na,K-ATPase is found in caveolae in LLC-PK1 cells. Moreover, the α1 isoform contains two conserved caveolin-binding motifs, and in vitro assays have shown that the purified Na,K-ATPase can bind to the amino-terminus of caveolin-1. Ouabain regulates the interaction between the Na,K-ATPase and caveolins in a time-dependent and dose-dependent manner in cultured cells. Interestingly, the ouabain-activated Na,K-ATPase/Src receptor complex stimulates tyrosine phosphorylation of caveolin-1 in a Src-dependent manner in LLC-PK1 cells. Inhibition of Src not only blocks ouabain-induced tyrosine phosphorylation, but also the recruitment of caveolin-1 to the Na,K-ATPase signaling complex. These findings clearly demonstrate that the caveolar Na,K-ATPase/Src receptor complex can signal from caveolae and that caveolins serve as one of the Src effectors.

As an additional test of the importance of caveolae in the concentration of the signaling Na,K-ATPase and its partners during ouabain-evoked signal transduction, we compared the signaling properties of the isolated caveolae with noncaveolar membrane preparations (*73*). Similar to the results in live cells, ouabain stimulated tyrosine kinases in isolated caveolae, but failed to evoke a response in noncaveolar membrane preparations. In addition, we found that disruption of the caveolae structure through depletion of cholesterol using methyl β-cyclodextrin or caveolin-1 using siRNA redistributed the Na,K-ATPase and Src from the caveolae to other compartments. These manipulations also abolished ouabain-induced formation of the Na,K-ATPase/Src/caveolin signaling complex, and the subsequent activation of ERKs. These findings support the notion that caveolar Na,K-ATPase interacts with Src and forms a functional receptor complex.

Scaffolding Function of the Na,K-ATPase: Assembly of a Ca^{2+} Regulating Platform

Many membrane transporters and channels contain specific functional domains that can serve as scaffolds, bringing different proteins into a large signaling complex. Examples include IP3 R and NHE1 (*6,76*) (Fig. 3). IP3Rs are IP3-gated Ca^{2+} channels. In response to stimulation of G protein-coupled receptors or receptor-tyrosine kinases, either phospholipase C (PLC)-β or PLC-γ is recruited to the membrane and activated (*77*). The activated PLC, in turn, catalyzes the metabolism of PIP2, producing the second messenger IP3, with subsequent stimulation of IP3Rs. Structurally, the IP3-gated channel contains a small pore-forming carboxy-terminus and a large regulatory amino-terminus. In fact, the amino-terminus contains more than 2000 amino acid residues and has been found to interact with ion channels, protein kinases and phosphatases, and structural proteins. These interactions not only make it possible for the regulation of receptor function via various protein kinase cascades, but also for regulating the function of the interacting proteins. For instance, interaction with ankyrin-B ensures proper communication among IP3R, Na^+/Ca^{2+} exchanger, and SERCA (*78*). In addition, IP3 R functions as a central core for the formation of a mGluR1a/5-Homer-CASK-syndecan-2 signaling complex and links G protein–coupled receptors to TRPC channels (*79*). Similarly, NHE1 contains a long carboxy-terminus that interacts with many cytoskeletal and signaling proteins. This scaffolding function of NHE1 plays a key role in the organization of structural proteins and protein kinases at the leading edge of lamellipodia in fibroblasts, thus regulating the assembly of focal adhesions, the formation of actin stress fibers, and cell shape (*76*).

As with these membrane proteins, the Na,K-ATPase can serve as a scaffold, adding yet another role to the kinase-regulating and receptor functions discussed previously. It has been known for a long time that Na,K-ATPase interacts with many intracellular soluble enzymes, as well as structural and membrane proteins (*80,81*). Early studies focused on how these interactions regulate the ion pumping function of the Na,K-ATPase. Recent studies have begun to address the scaffolding function of the Na,K-ATPase. For example, interaction

with ankyrin is important for the trafficking and targeting of the Na/K-ATPase (*82,83*). Na,K-ATPase is also involved in regulation of cellular metabolism via its interaction with cofilin (*84*). Furthermore, several laboratories have identified the role of Na,K-ATPase in the formation of tight junctions and in regulation of cell attachment and motility (*67,85–87*). Finally, the Na/K-ATPase apparently interacts with many membrane transporters and channels to form a functional Ca^{2+}-regulatory platform (*88–90*).

It is well established that the Na,K-ATPase serves as a functional receptor for ouabain and other CTS to regulate intracellular Ca^{2+} in cardiac myocytes (*91*). This regulation apparently involves the functional and physical coupling between the Na,K-ATPase and Na^+/Ca^{2+} exchanger (*90*) as well as the activation of protein kinases (*51*). Such a link between ouabain binding and Ca^{2+} regulation may extend to cell types other than cardiomyocytes. A fascinating and unanticipated observation made initially by Aperia's laboratory showed that the Na,K-ATPase is physically coupled to IP3 receptor in epithelial cells (*92*). Binding of ouabain to the Na,K-ATPase regulates this interaction and induces Ca^{2+} release in renal epithelial cells. Using a different approach, we have recently confirmed the interaction between the Na,K-ATPase and IP3 receptors in renal epithelial cells (*50*). Significantly, in vitro GST-pull down assays indicate that the amino-terminus of the α1 subunit binds directly to the IP3 receptor. We also noted that CD3 of the α1 interacts with PLC-γ1. Because PLC generates the ligand IP3 for the IP3 receptor, these findings suggest that the Na,K-ATPase may bring into close juxtaposition the proteins involved in intracellular Ca^{++} signaling. In a study using cultured LLC-PK1 as a model of renal epithelial cells, we observed that ouabain induced the interaction between the signaling Na,K-ATPase and PLC-γ1, and stimulated PLC-γ1 and subsequent generation of IP3 via the activated Na,K-ATPase/Src complex. It also increased the formation of the Na,K-ATPase/PLC-γ1/IP3 receptor complexes. Furthermore, ouabain stimulated Src-dependent tyrosine phosphorylation of IP3R. Functionally, ouabain increased Ca^{2+} release via the IP3-mediated opening of IP3R in LLC-PK1 cells. Taken together, these data imply that the Na,K-ATPase is not only a provider of second messenger (via activation of Src/PLC-γ and generation of IP3), but also plays an integrative role via scaffolding domains that tether the affector (PLC-γ1) and effector (IP3R) together for efficient and specific signal transmission.

Regulation of Ion Transporting Activity by the Signaling Na/K-ATPase: Pump-Leak Coupling

The concept of active transport (pumping) and passive diffusion (leak) of ions across cell membrane was first introduced by August Krogh in 1946. This idea was further developed by the discovery of the Na pump and various ion transporters and channels (*93,94*). It is recognized that pump and leaks must be coordinately regulated or coupled to maintain the normal cellular activity and ionic balance between intracellular and extracellular compartments. Experimental evidence for this pump-leak coupling has been well documented (*95,96*). For example, in renal principal cells, the apical and basolateral transport activities are tightly correlated, and changes in basolateral Na,K-ATPase activity directly affects apical cation conductance (*97*). A tight coupling mechanism also operates to link the basolateral Na,K-ATPase activity with the apical renal outer medullary potassium (ROMK) channel function (*98*). Mechanistically, there is evidence that the Na,K-ATPase-induced decrease in intracellular ATP concentration may serve as a coupling factor for regulating apical ROMK channel conductance (*99*).

Realization that the Na,K-ATPase has a pump-independent signaling function has led us in recent years to test whether the Na,K-ATPase/Src receptor complex plays a role in regulation of apical transporters. It is known that volume expansion increases circulating endogenous CTS. This increase appears to be responsible for reduced Na^+ reabsorption in the kidney (*100*). We found that the addition of nanomolar concentrations of ouabain to cultured and polarized LLC-PK1 cells at the basolateral, but not the apical side, produced a dose-dependent decrease in transcellular $^{22}Na^+$ movement (*69,70*). These findings support the notion that ouabain may signal through the Na,K-ATPase/Src complex to reduce apical Na^+ transport because these low doses of ouabain do not cause significant inhibition of the pumping activity of the Na,K-ATPase. Because NHE3 plays an important role in mediating apical Na^+ transport, we further tested whether ouabain-evoked signals at the basolateral membrane could regulate NHE3 activity in polarized LLC-PK1 cell cultures. These studies showed that ouabain decreased NHE3 activity when it was added to the basolateral, but not the apical side of chamber (*101*). This decrease in NHE3 activity was mediated by at least two independent mechanisms. First, ouabain decreased exocytosis (or increased endocytosis) of NHE3. Second, ouabain reduced the expression of NHE3 via a transcriptional mechanism in the polarized LLC-PK1 cells. Finally, these ouabain effects required the activation of Src and the assembly of the caveolar Na,K-ATPase/Src receptor complex (*101*). Taken together, the new findings indicate that the signaling function of the Na,K-ATPase may also be involved in coupling of cellular pumping activity to the leaks mediated by channels and transporters. To this end, it is of interest to note that the activation of Src by low K^+ can lead to decreased exocytosis of ROMK (*102*), bringing about the possibility that ouabain-induced activation of Src may reduce ROMK activity via a mechanism independent of changes in intracellular ATP concentration.

CONCLUSION AND PERSPECTIVE

Over the past 10 years, studies from many laboratories have confirmed that Na,K-ATPase has scaffolding/ receptor functions independent of its role as an ion pump. Meanwhile, an increased appreciation of other membrane transporters in scaffolding and signal transduction has emerged. These studies mark the beginning of a fascinating new field of investigation, evidenced by the rapid growth of protein interaction research concerning these membrane proteins. Many important issues related to molecular mechanisms of action and dynamic interactions among these pumps, channels, and transporters remain to be investigated. Furthermore, only a few studies have been performed to assess the significance of the scaffolding/receptor functions of the pump, channels, and transporter to cell biology and animal physiology. Clearly, further development of this new field requires the engagement of many more investigators, not only those with long-lasting interest and expertise in pump, channel, and transporter biology, but also outside players, so that new ideas and approaches can be introduced and applied.

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