Molecular Mechanisms of Na/K-ATPase–Mediated Signal Transduction

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ABSTRACT: Our recent work shows that in addition to pumping ions, Na/K-ATPase acts as a signal transducer. Binding of ouabain to Na/K-ATPase changes the interaction of the enzyme with neighboring membrane proteins and induces the formation of multiple signaling modules, resulting in activation of Src, transactivation of the EGF receptor (EGFR), and increased production of reactive oxygen species (ROS). Interaction of these signals leads to activation of several other cascades, including p42/44 and p38 MAPKs, phospholipase C, and protein kinase C isozymes, in a cell-specific manner. Ouabain also increases $[Ca^{2+}]_i$ and contractility, induces some of the early-response protooncogenes, and activates transcription factors AP-1 and NF-κB. Interplay among these pathways eventually results in changes in the expression of a number of growth-related genes and in cell growth. Significantly, inhibition of Src blocked many of the aforementioned ouabain-activated signaling pathways. Furthermore, Src binds to Na/K-ATPase directly and ouabain regulates the interaction between Src and the enzyme, resulting in Src activation. To address the possibility that the signaling Na/K-ATPase is concentrated in a separate pool on the plasma membrane, we have assessed interaction of the enzyme with caveolins. These studies indicated that Na/K-ATPase was concentrated in caveolae/rafts. In addition, caveolin-1 can be co-immunoprecipitated with Na/K-ATPase. Finally, we have shown that the signaling function of the enzyme is also pivotal to ouabain-induced nongenomic effects on cardiac myocytes.

KEYWORDS: Na/K-ATPase; ouabain; Src; caveolae; inter-receptor communication

INTRODUCTION

Na/K-ATPase was discovered as an energy transducing ion pump, and its pumping function and its regulation have been studied extensively since 1957.[1,2] Although early findings suggested that the enzyme also played a role in regulation of gene expression and cell growth,[3,4] only in recent years have studies been performed to investigate the molecular mechanisms by which this plasma membrane enzyme organizes signaling modules and regulates the functions of different proteins.[5-14] This work, done mostly on neonatal rat cardiac myocytes, shows that Na/K-ATPase has multiple signaling partners and that ouabain activates various signaling branches

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to send messages to various intracellular organelles via regulation of interactions between the enzyme and its partners. Significantly, many of these findings have also been reported recently in cells other than cardiac myocytes.[9,13,15–19] In addition, Na/K-ATPase was found to be essential for dopamine-induced activation of phosphatidylinositol 3-kinase in rat kidney proximal tubular cells.[20] Realization that Na/K-ATPase is a signal transducer has prompted us to ask "What are the possible endogenous ligands for Na/K-ATPase?" In principle, there are at least three major potential classes of chemicals that could serve as Na/K-ATPase ligands under both physiological and pathological conditions. The first group of chemicals is the cardiac glycosides (e.g., endogenous ouabain or marinobufagin-like factors).[21–23] The second class will be those that may chemically modify the enzyme either reversibly or irreversibly. We propose that ROS may fit in this category.[24] The third will be alterations in the concentrations of intracellular sodium and extracellular potassium. This article reviews some of our recent mechanistic studies that are related to the initiation, compartmentalization, and biological consequences of Na/K-ATPase–mediated signal transduction.

Src AND Na/K-ATPase–MEDIATED SIGNAL TRANSDUCTION

Interaction of Na/K-ATPase with Src

Src family kinases are 52–62-kDa membrane-associated nonreceptor tyrosine kinases that are key regulators of various signal transduction pathways.[25,26] The kinase activity of Src is regulated by tyrosine phosphorylation.[26] Phosphorylation of Tyr^{418} activates the kinase activity of Src. On the other hand, phosphorylation of a conserved Tyr^{529} induces formation of an inactive conformation through intramolecular interaction between the SH2 domain and the C-terminus. Interaction of the SH3 domain with the linker between the kinase domain and the SH2 domain further keeps the enzyme in the inactive state. Therefore, competitive binding of a regulatory protein to either the SH2 or SH3 domain will destabilize the inactive conformation of Src, resulting in increases in phosphorylation of Tyr^{418} and the kinase activity. We showed previously that binding of ouabain to Na/K-ATPase activated Src. Since Na/K-ATPase is not a receptor tyrosine kinase, we proposed that ouabain might regulate the interaction between Src and Na/K-ATPase, resulting in Src activation. To test this hypothesis, we have performed the following three sets of experiments. First, we measured the effects of ouabain on tyrosine phosphorylation of Src, since either stimulation of Tyr^{418} phosphorylation via a conformation change or Tyr^{529} dephosphorylation by a phosphotyrosine phosphatase can stimulate Src activity. We found that in both A7r5 cells and LLC-PK1 cells ouabain stimulated Tyr^{418} phosphorylation, but had no effect on Tyr^{529} phosphorylation.[13] These data support our proposition that ouabain regulates the interaction between Na/K-ATPase and Src. Therefore, in the second set of experiments we immunoprecipitated $\alpha 1$ from both ouabain-treated and control LLC-PK1 cells, and probed for Src. We found that ouabain increased Src binding to the Na/K-ATPase signaling complex in a dose- and time-dependent manner. In addition, when Src was immunoprecipitated from the LLC-PK1 cells, ouabain clearly increased the co-precipitated $\alpha 1$ subunit of the enzyme.[13] These findings indicate that there is indeed an interaction between Na/K-

ATPase and Src in response to ouabain. Because Src could bind to Na/K-ATPase through a scaffold protein, we have recently performed a third set of experiments to dissect the nature of the Src interaction with the enzyme. GST-Src was expressed in *E. coli*, purified, and used to pull down 1% Triton X-100 solubilized pig kidney Na/K-ATPase. The experiments showed that the solubilized Na/K-ATPase bound to GST-Src in a dose-dependent manner. In short, the above findings clearly demonstrate that Src can interact with Na/K-ATPase directly and that ouabain regulates the interaction between Src and Na/K-ATPase.

Src Relays Ouabain Signal from Na/K-ATPase to the EGFR and Other Signaling Cascades

It is well established that receptor tyrosine kinases (RTKs) are central elements for cellular signal transduction.[25] In recent years there is a growing body of evidence that RTKs cross-communicate with other signaling systems to integrate the variety of extracellular stimuli into a limited number of signaling pathways. The activated EGFR, for example, has been identified as a critical element in the signal transduction networks of cytokines, H_2O_2, and those using G protein–coupled receptors.[25] Since Src family kinases can couple the receptors lacking intrinsic kinase activity to RTKs, we proposed that activation of Src by ouabain might serve as a mediator for Na/K-ATPase to communicate with the EGFR so that the extracellular ouabain signal can be transmitted to the Ras/MAPK cascade. Indeed, our recent work showed that activation of Src was essential for the ouabain-induced activation of p42/44 MAPKs, since ouabain failed to activate MAPKs in A7r5 cells that were pretreated with Src inhibitor PP2 and herbimycin A.[13] This was further supported by the experiments performed in both SYF and SYF + Src cells.[13] The SYF cells are derived from mouse embryos harboring functional null mutations in both alleles of the Src family kinases Src, Yes, and Fyn. The SYF + c-Src cells are the stable transfectants of the SYF cells that express c-Src. While ouabain activated p42/44 MAPKs in a dose-dependent manner in the SYF + c-Src cells, it failed to stimulate MAPKs in the SYF cells. Furthermore, we showed that inhibition of Src also blocked ouabain-, but not EGF-induced Src binding to the EGFR and subsequent EGFR tyrosine phosphorylation. Finally, we found that the transactivated EGFR was capable of recruiting and phosphorylating the adaptor protein Shc, resulting in increased binding of the Grb2/Sos complex to the activated EGFR receptors. This led to the recruitment of Ras and the subsequent activation of the Raf/MEK/MAPK cascade. Significantly, we showed that the EGFR, but not the platelet-derived growth factor receptor, was involved in ouabain-induced activation of MAPKs. Taken together, these new findings support the proposal that activation of Src is the initial critical step that relays the signal emanating from the interaction of ouabain with Na/K-ATPase to the EGFR.

CAVEOLAE AND COMPARTMENTALIZATION OF Na/K-ATPase

Caveolins are 21–24 kDa membrane-associated scaffold proteins and are major structural components of caveolae, which exist as flask-shaped vesicular invaginations of plasma membrane and are rich in cholesterol, glycosphingolipids, and sph-

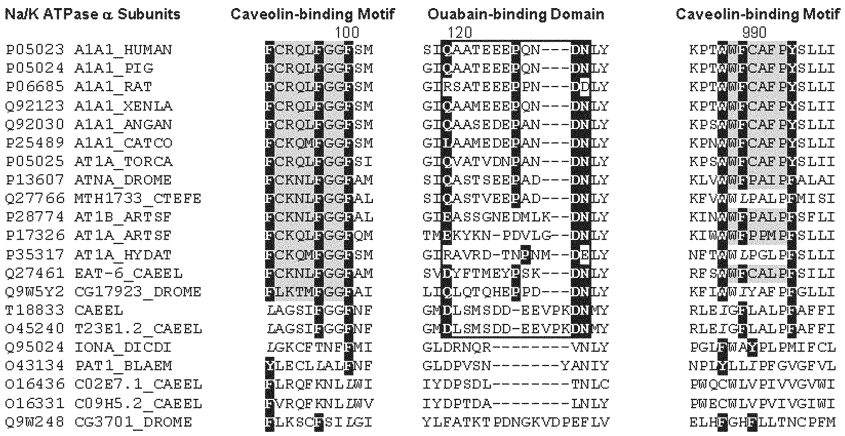

FIGURE 1. CBMs and ouabain binding domains in IIC Na,K-ATPase α subunits.

ingomyelin.[27] Three major groups of caveolins have been identified and named as caveolin-1, 2, and 3. The primary sequence of caveolin-1 contains three palmitoylation sites, a central hydropholic domain (residues 102–134) that anchors to membranes, an oligomerization domain (residues 61–101), and a scaffolding domain (residues 82–101).[27] Interaction between the oligomerization domains and the C-terminal domains results in formation of high molecular oligomers containing 14 to 16 caveolins, which is important for the scaffolding function of caveolins.[27] Interestingly, multiple proteins such as EGFR, Src, PKC, and Ras contain caveolin-binding motifs (CBM),[27] and interaction of caveolin-1 with these proteins plays an important role in clustering of these signaling proteins in the compartment of caveolae. Database searches indicate that both α and β subunits of mammalian Na/K-ATPase contain conserved CBM (e.g., ΦXXΦXXXXΦ and ΦXΦXXXXΦ), where Φ represents an aromatic amino acid residue. Interestingly, the CBM in the α subunits was acquired quite early during evolution. It first appeared in one of the mutated Na/K-ATPases in *C. elegans,* and has been conserved since *Drosophila* (FIG. 1). Significantly, the appearance of the CBMs correlates well with the occurrence of the domain for ouabain binding. These findings led us to propose that caveolae may cluster the signaling Na/K-ATPase with its partners. Confocal imaging of immunostained LLC-PK1 cells showed that Na/K-ATPase co-localized with caveolin-1 on plasma membrane. Both density gradient fractionation experiments and immunoprecipitation using anti-cav-1–coated magnetic beads revealed that Na/K-ATPase was enriched in caveolae, together with Src and other signaling proteins.[28] To test whether the ouabain-activated signal transduction can originate from caveolae, we immunoprecipitated caveolin-1 from ouabain-treated LLC-PK1 cells, and probed for α1 and Src by Western blot analysis of the immunocomplex. We found that 100 nM ouabain (2 min exposure) increased the binding of both Na/K-ATPase and Src to caveolin-1. More importantly, ouabain stimulated tyrosine-phosphorylation of caveolin-1. Taken together, these findings clearly demonstrated that the ouabain-bound Na/K-ATPase was capable of recruiting and assembling of both Src and caveolin-1 into

signaling modules in LLC-PK1 cells. In addition, these findings provide strong evidence that at least some of the signaling events of ouabain must be originated from caveolae in these cells.

BIOLOGICAL CONSEQUENCES OF THE SIGNAL TRANSDUCING FUNCTION OF Na/K-ATPase

Regulation of Cardiac Growth and Growth-Related Genes by Na/K-ATPase in Cardiac Myocytes

Several years ago we became interested in the role of Na/K-ATPase in the nonproliferative growth (hypertrophy) of cardiac myocytes. This stemmed from the growing realization that cardiac hypertrophy plays an important role in the development of heart failure. Our early studies demonstrated that exposure of the cultured cardiac myocytes to ouabain stimulated cardiac growth.[5–8] Like other hypertrophic stimuli, ouabain also regulated transcription of several hypertrophic marker genes in cardiac myocytes.[5–8] Clearly, Na/K-ATPase must now be considered as a potential signal transducer for hypertrophic growth in the heart along with other membrane receptors.

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

Since activation of PTKs preceded the increases in $[Ca^{2+}]_i$ in response to ouabain,[12] we have tested if the signal transducing function of Na/K-ATPase contributes to nongenomic effects of ouabain on myocytes. These studies showed the following:[12,14] First, inhibition of either Src or Ras abolished ouabain-induced increases in both $[Ca^{2+}]_i$ and contractility. Second, while activation of p42/44 MAPKs was required for ouabain-induced rise in $[Ca^{2+}]_i$, both MAPKs and ROS contributed to ouabain regulation of cardiac contraction.[14] Finally, ouabain stimulated mitochondrial ATP-sensitive K channel ($mitoK_{ATP}$) activity, resulting in increases in mitochondrial ROS production. Inhibition of $mitoK_{ATP}$ by 5-hydroxydecanoate significantly reduced ouabain-induced increases in contractility. Thus, ouabain regulates cardiac contractility via 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 increased $[Ca^{2+}]_i$, whereas opening of $mitoK_{ATP}$ stimulated the production of ROS. Both $[Ca^{2+}]_i$ and ROS, in turn, worked in concert, resulting in increases in contractility in cardiac myocytes. Although most of the above studies need to be repeated in the isolated heart and whole animals, the significance of these studies should be noted. First, the above findings provide a new insight into the mechanism of ouabain action on contractility. Second, they point to a possibility that blocking of certain pathways may separate the therapeutic effect from the toxicity of ouabain on the heart.

Ouabain Protects the Perfused Rat Heart against Ischemia-Reperfusion Injury

Ischemia preconditioning describes an experimental treatment of the normal heart that reduces myocyte damage from a subsequent ischemia-reperfusion event. We were struck by the fact that elements of the ouabain signaling pathway, including

Src, PKC, and mitoK_{ATP}, overlap with those involved in the signaling pathway of preconditioning.[29] Accordingly, we carried out experiments on perfused rat hearts to determine whether pretreatment with ouabain is cardioprotective. Results from ten preparations indicate that 80 μM ouabain administered 15 min before 30-min ischemia is cardioprotective. These data show that ouabain protection is equal to protection by ischemia preconditioning, with respect to enzyme release during the first 10 min of reperfusion. To ensure that the cardioprotective effects of ouabain are not limited to these ouabain-insensitive rodents, we also measured whether ouabain protects rabbit cardiac myocytes from ischemia. Rabbit cardiac myocytes express ouabain-sensitive Na/K-ATPase, and 50% inhibition occurs when the cells are exposed to 1 μM ouabain. Using an *in vitro* ischemic model, we showed that preincubation of cardiac myocytes with 0.1 to 1 μM ouabain gave a dose-dependent protection of these cells from ischemia-induced cell death. Since digitalis glycosides are still widely used in the treatment of congestive heart failure, the above findings have significant clinical implications.

CONCLUSIONS AND FUTURE PERSPECTIVES

The work of the past few years has clearly demonstrated that Na/K-ATPase is an important signal transducer. Some of our recent work has begun to identify the various signaling partners of Na/K-ATPase and the domains that are involved in such interactions. It is important to emphasize that the above studies are not only important for us to understand the events regulated by the signaling Na/K-ATPase, but also the regulation of the enzyme as an energy transducer by different signaling pathways. Clearly, this new line of investigation will provide vast opportunities for significant expansion of research in the Na/K-ATPase field.

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