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MINIREVIEW

Na^+/K^+-ATPase as a signal transducer

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Na^+/K^+-ATPase as an energy transducing ion pump has been studied extensively since its discovery in 1957. Although early findings suggested a role for Na^+/K^+-ATPase in regulation of cell growth and expression of various genes, only in recent years the mechanisms through which this plasma membrane enzyme communicates with the nucleus have been studied. This research, carried out mostly on cardiac myocytes, shows that in addition to pumping ions, Na^+/K^+-ATPase interacts with neighboring membrane proteins and organized cytosolic cascades of signaling proteins to send messages to the intracellular organelles. The signaling pathways that are rapidly elicited by the interaction of ouabain with Na^+/K^+-ATPase, and are independent of changes in intracellular Na^+ and K^+ concentrations, include activation of Src kinase, transactivation of the epidermal growth factor receptor by Src, activation of Ras and p42/44 mitogen-activated protein kinases, and increased generation of reactive oxygen species by mitochondria. In cardiac myocytes, the resulting downstream events include the induction of some early response proto-oncogenes, activation of the transcription factors, activator protein-1 and nuclear factor kappa-B, regulation of a number of cardiac growth-related genes, and stimulation of protein synthesis and myocyte hypertrophy. For these downstream events, the induced reactive oxygen species and rise in intracellular Ca^{2+} are essential second messengers. In cells other than cardiac myocytes, the proximal pathways linked to Na^+/K^+-ATPase through protein–protein interactions are similar to those reported in myocytes, but the downstream events and consequences may be significantly different. The likely extracellular physiological stimuli for the signal transducing function of Na^+/K^+-ATPase are the endogenous ouabain-like hormones, and changes in extracellular K^+ concentration.

Keywords: calcium ion; cardiac hypertrophy; cardiac myocyte; epidermal growth factor; mitogen activated protein kinase; Na^+/K^+-ATPase; ouabain; Ras; reactive oxygen species; Src kinase.

INTRODUCTION

In 1957, J. C. Skou reported the discovery of the Na^+/K^+-ATPase and proposed its role in the active extrusion of Na^+ from the nerve cell [1]. Soon thereafter, sufficient evidence was available to establish that this enzyme is indeed the molecular machine that uses the energy of hydrolysis of ATP for the coupled active transports of Na^+ and K^+ (the sodium pump) across the plasma membrane of nearly all animal cells [2]. In the ensuing decades, extensive work has been carried out on the structure–function of Na^+/K^+-ATPase as an energy transducing ion pump. This research has been summarized in numerous previous reviews and monographs, and is updated in one of the accompanying reviews [3]. Here, we address a newer aspect of the biology of Na^+/K^+-ATPase; i.e. the mechanisms and the pathways by which the enzyme acts as a signal transducer to relay messages, through protein–protein interactions, from the plasma membrane to the nucleus.

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Abbreviations: AP-1, activator protein 1; EGF, epidermal growth factor; Grb2, growth factor receptor-bound protein 2; MAPK, mitogen activated protein kinase; MEK, MAPK kinase; NF-κB, nuclear factor kappa B; PKC, protein kinase C; PLC, phospholipase C; Raf, a MAPK kinase kinase; ROS, reactive oxygen species; Shc, SH-2 domain-containing protein; Sos, mammalian homologue of Son-of-sevenless (a guanine nucleotide exchange factor).

Enzyme: Na^+/K^+-ATPase (EC 3.6.1.8).

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EARLY STUDIES ON THE ROLE OF NA^+/K^+-ATPASE IN GENE REGULATION AND CELL GROWTH

It has been known for a long time that Na^+/K^+-ATPase communicates with the nucleus to regulate genes and cell growth. What is new is the realization that this communication occurs through the properties of Na^+/K^+-ATPase that are distinct from its function as an ion pump.

That the enzyme is capable of controlling expression of its own genes was suggested in 1974 [4]. Pressley [5] has reviewed the studies of various groups showing that chronic inhibition of the sodium pump of the cultured cells, either by the pump inhibitor ouabain or by lowering of $[K^+]_o$, leads to an increased abundance of functional Na^+/K^+-ATPase in the plasma membrane; and that this increase is due, in part, to the transcriptional up-regulation of the enzyme subunits. In these and subsequent studies on the adaptive upregulation of the enzyme due to its inhibition, the general conclusion has been that the intracellular ionic changes

resulting from pump inhibition are responsible for the noted regulation [5–8]. Over the years, the observed regulation by ouabain or low $[K^+]_o$ of a number of genes other than those of the pump subunits have also been ascribed to altered intracellular ionic concentration [9–14]. There is, however, no direct evidence for the transcriptional regulation of any of these genes by changes in $[Na^+]_i$ or $[K^+]_i$ that are expected to result from pump inhibition.

A large body of work reviewed by Kaplan [15] showed, as early as 1968 [16], that ouabain interaction with Na^+/K^+-ATPase inhibits mitogen-induced differentiation and proliferation of lymphocytes. Although these effects and subsequent proliferative ouabain effects on some cells [17] were also generally ascribed to altered intracellular ion concentrations, it is of interest to note that later observations clearly suggested that some growth-related effects of ouabain on lymphocytes may indeed be dissociated from ouabain-induced changes in intracellular ions [18–20].

CONTROL OF GROWTH AND GROWTH-RELATED GENES BY NA^+/K^+-ATPASE IN CARDIAC MYOCYTES

In mid-1990s, our laboratories became interested in the possible role of Na^+/K^+-ATPase in the nonproliferative growth (hypertrophy) of the heart. This was partly due to the established, but relatively ignored, effects of ouabain on lymphocyte growth mentioned above; and partly due to the growing realization that the well known hypertrophy of the failing heart may not be only an adaptive and beneficial response of the diseased heart as stated in textbooks, but rather a part of the continuum of the derangements leading to advanced heart failure. Because of the latter, at the time there was extensive ongoing research on the mechanisms by which a variety of hormones, neurotransmitters, cytokines, mitogens, and mechanical stimuli affect cardiac myocyte hypertrophy [21]. Interestingly, in this highly active field, there seemed to be no focus on how the cardiac Na^+/K^+-ATPase and its clinically used specific inhibitors, the digitalis drugs, may be involved in cardiac hypertrophy. Considering that digitalis drugs were, and remain to be, a mainstay of the treatment of the failing heart, it seemed to us that exploration of the effects of these drugs on cardiac hypertrophy deserved more attention. Using the cultured cardiac myocytes as a model, our studies of the past few years [22–26] have clearly indicated that the same nontoxic concentrations of ouabain that cause partial inhibition of Na^+/K^+-ATPase and an increase in cardiac contractility, also stimulate myocyte growth and protein synthesis, induce a number of early response proto-oncogenes, activate transcription factors activator protein 1 (AP-1) and NF-κB, and induce or repress the transcription of several late-response cardiac marker genes that are also regulated by other cardiac hypertrophic stimuli. These findings clearly establish that Na^+/K^+-ATPase indeed regulates the growth and the phenotype of the cardiac myocyte; and raise the intriguing question of whether the altered levels or properties of Na^+/K^+-ATPase, either drug-induced, or by the actions of endogenous digitalis-like compounds, or by other pathological down-regulatory mechanisms, are involved in the development of cardiac hypertrophy and failure. This important question is not likely to be resolved in the near future. In the highly active area of research on cardiac hypertrophy, there has been some tendency to overemphasize the importance of this entity or that pathway [27]. In view of the multiplicity of the stimuli and receptors that have been shown to regulate cardiac hypertrophy, and because of the evident overlap and superficial similarities of the signal pathways linked to such stimuli/receptors, it would be naively optimistic to think that focus on one entity and neglect of others may resolve the problem of cardiac hypertrophy/failure. We suggest that it is more important to recognize that multiple signals, receptors, pathways, and second messenger are involved in the control of myocyte growth, and that it is essential to delineate the pathways activated by each signal/receptor, and the nature of cross-talk among these, before the consequences of the pathological derangement of the interacting networks linked to different receptors can be understood. In this context, the mapping of the signaling pathways coupled to Na^+/K^+-ATPase, and the clarification of the mechanisms involved in pump interaction with neighboring receptors, has been the focus of our recent research.

SECOND MESSENGERS, SIGNALING INTERMEDIATES, AND PATHWAYS LINKED TO CARDIAC NA^+/K^+-ATPASE THROUGH PROTEIN–PROTEIN INTERACTIONS

To date, most of the work on signal transduction by Na^+/K^+-ATPase has been carried out on cardiac myocytes. It is convenient to present the information on myocytes first, and then discuss the emerging data on other cell types. Figure 1 depicts a summary of the signal transducing function of Na^+/K^+-ATPase and its consequences in cardiac myocytes. It is appropriate to point out that while all of our research cited below has been carried out on rat cardiac myocytes, recent work on cardiac preparations from other species (K. Mohammidi, L. Liu, P. Komentiani, Z. Xie & A. Askari, unpublished results) shows that the indicated conclusions are not limited to rat myocytes.

Pathways that are independent of changes in intracellular ions

Because the cardiac myocyte plasma membrane contains a highly active Na^+/Ca^{2+}-exchanger, the most significant intracellular ionic change that results from the partial, but nontoxic, inhibition of cardiac Na^+/K^+-ATPase is a rise in $[Ca^{2+}]_i$, which has been known for decades to be the cause of the positive inotropic action of a digitalis compound such as ouabain [28]. When we first noted the hypertrophic and the gene regulatory effects of ouabain on myocytes [22,23], we made the reasonable assumption that these effects were also caused by the rise in $[Ca^{2+}]_i$. It has turned out, however, that an increase in $[Ca^{2+}]_i$ is necessary but not sufficient for the ouabain-induced hypertrophy and the associated gene regulation. It is now evident that large segments of the early events that result from ouabain interaction with cardiac Na^+/K^+-ATPase are indeed independent of any changes in intracellular Na^+, K^+, and Ca^{2+} concentrations, but depend on the enzyme's interaction with other proteins [29,30]. The present state of knowledge about these proximal pathways may be summarized as follows. The earliest

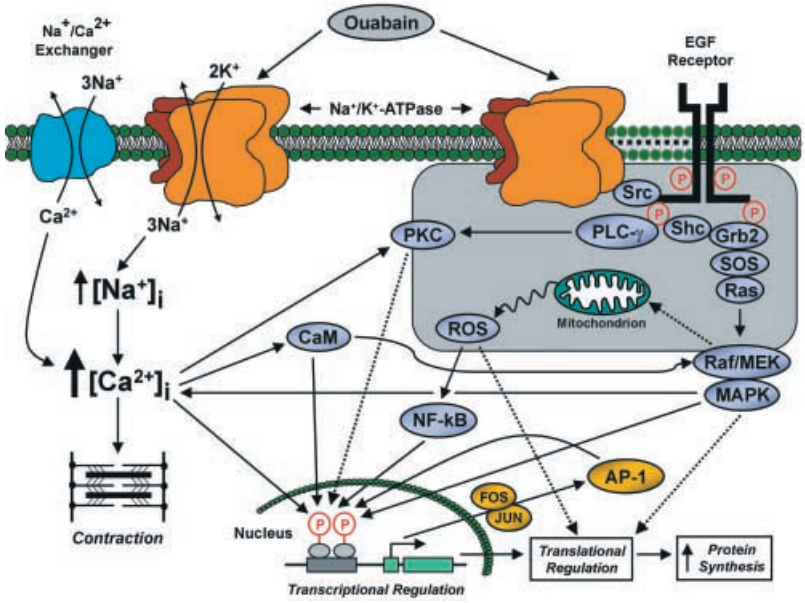

Fig. 1. The signal transducing function of Na^+/K^+-ATPase and its consequences in cardiac myocytes. Two pools of the enzyme, one pumping ions and the other interacting with neighboring proteins are suggested by the data. The partial inhibition of the pump by ouabain causes a modest change, if any, in $[Na^+]_i$ and $[K^+]_i$, but a significant change in $[Ca^{2+}]_i$ due to the presence of the Na^+/Ca^{2+}-exchanger. Ouabain interaction with the other pool alters protein–protein interactions to activate the indicated signaling pathways. The events placed in the grey box have been shown to be independent of changes in $[Na^+]_i$, $[K^+]_i$, and $[Ca^{2+}]_i$ that may occur. These activated pathways, the resulting increase in ROS, and the concomitant increase in $[Ca^{2+}]_i$ lead to activations of NF-κB and AP-1, transcriptional regulation of early response genes (c-fos, c-jun), and cardiac growth-related genes (those of atrial natriuretic factor, skeletal α-actin, and the α_3 subunit of Na^+/K^+-ATPase), stimulation of protein synthesis, and myocyte hypertrophy. The solid arrows indicate experimentally supported events induced by ouabain in myocytes, and the broken arrows indicate those with limited or indirect support. In several cell types other than cardiac myocytes, some of the same signaling events are induced by ouabain, but there are also significant cell-specific differences between the ouabain-induced pathways and the down-stream consequences (see text).

specific ouabain-induced event that has been identified is the activation of Src kinase which leads to tyrosine phosphorylation of a number of cellular proteins, including the epidermal growth factor (EGF) receptor [29]. Although it is possible that other receptor or nonreceptor tyrosine kinases may also be activated by ouabain, the transactivation of the EGF receptor by Src is sufficient to account for the recruitments of Shc (SH-2 domain-containing protein), Grb2, Sos (a mammalian homologue of Son-of-sevenless), and Ras to the plasma membrane [25,29]. Whether Src interacts with Na^+/K^+-ATPase directly or indirectly is not known [29]; this is the subject of ongoing studies. Upon ouabain-induced activation of the G-protein Ras, a pathway is initiated (the details of which are not known), which clearly extends from the plasma membrane to the mitochondria where it leads to increase in generation of ROS [26,30]. This increase in ROS is an essential second messenger for many, but not all, of the downstream events that are linked to Na^+/K^+-ATPase. The antioxidants, *N*-acetylcysteine or vitamin E, which prevent this increase in reactive oxygen species (ROS) block ouabain-induced transcriptional regulation of the late-response marker genes, but not the induction of c-fos [26]. Consistent with the latter, ouabain-induced activation of the transcription factor AP-1, which contains Fos, is also insensitive to antioxidants [26]. However, activation of NF-κB by ouabain is prevented by antioxidants [26], suggesting that ouabain-induced regulation of some late-response genes may involve this transcription factor. Most significantly, antioxidants block ouabain-induced stimulation of protein synthesis [26], suggesting that the pathway beginning with Ras and leading to ROS generation and NF-κB activation is essential to the ouabain-induced hypertrophy of the cardiac myocyte. Antioxidants attenuate but do not abolish ouabain-induced p42/44 mitogen-activated protein kinase (MAPK) activation [26], suggesting the existence of a signal amplification cycle consisting of Ras-dependent ROS generation and ROS-dependent activation of Ras.

The role of increased $[Ca^2+]_i$

Ouabain-induced activation of Ras, through the transactivation of the EGF receptor by Src, not only leads to mitochondrial ROS generation, but also to the activation of p42/44 MAPK (also called $ERK_{1/2}$) through the Ras/Raf/MAPK kinase/MAPK cascade (where Raf is a MAPK kinase kinase and MEK is a MAPK kinase) [29,30]. However, the activation of this cascade, unlike that of ROS generation, also requires the rise in $[Ca^{2+}]_i$ that is caused by ouabain's inhibition of the ion transporting function of Na^+/K^+-ATPase [25]. One locus of this Ca^{2+} effect has now been identified. It turns out that ouabain-induced activation of Ras is necessary but not sufficient for the activation of Ras/Raf/MEK/MAPK cascade. Ouabain-induced activation of protein kinase C (PKC) is also required for MAPK activation [31], most likely due to PKC activation of Raf whose recruitment to the membrane has been induced by Ras. Unsurprisingly, activation of PKC seems to be due to activation of phospholipase C (PLC)-γ that is also recruited to the ouabain-activated Src/

EGF receptor complex [31]; it is this PKC activation that requires the elevation of $[Ca^{2+}]_i$ by ouabain [31]. There is, however, evidence to suggest additional mechanisms by which a rise in $[Ca^{2+}]_i$ regulates the signaling events initiated by ouabain. The Ca^{2+}-calmodulin kinase also seems to be involved in ouabain-induced activation of MAPK and regulation of early and late-response genes [23–25] by mechanisms yet to be clarified.

An important recent development is the finding that when ouabain-induced activation of MEK and MAPK is prevented, the ouabain-induced increase in $[Ca^{2+}]_i$ is also blocked [32], establishing the existence of another positive feed-back cycle; i.e. the requirement of rise in $[Ca^{2+}]_i$ for MAPK activation, and the necessity of MAPK activation for rise in $[Ca^{2+}]_i$.

Two pools of Na^+/K^+-ATPase with two distinct but coupled functions

Based on the findings summarized above, it is clear that interaction of nontoxic concentrations of ouabain with the cardiac myocyte Na^+/K^+-ATPase leads to the generation of two intracellular second messengers, increased ROS and increased $[Ca^{2+}]_i$, both of which are essential for the full expression of the hypertrophic and gene regulatory actions of ouabain; and each is generated in parallel with the other [30]. The inescapable conclusion is that there are two pools of Na^+/K^+-ATPase within the plasma membrane with two distinct functions: one being the classical pool of the enzyme as an energy transducing ion pump whose partial inhibition by ouabain initiates the increase in $[Ca^{2+}]_i$, and the other the signal transducing pool of the enzyme which, through protein–protein interactions, leads to the activation of a host of signaling intermediates and a rise in intracellular ROS. In cardiac myocytes, the functions of these two pools are tightly coupled through feed-back cycles to regulate cardiac contractility and growth.

SIGNAL TRANSDUCING ROLE OF NA^+/K^+-ATPASE IN CELLS OTHER THAN CARDIAC MYOCYTES

Relevant information on cell types other than cardiac myocytes is more limited, but rapidly increasing. It is already clear that linkage to signaling intermediates and pathways through protein–protein interactions is a common property of Na^+/K^+-ATPase in most, if not all, cells. Using partially inhibitory concentrations of ouabain, activation of parts or all of the pathways that begin with protein tyrosine phosphorylation and lead to ROS generation and MAPK activation (Fig. 1) has been shown in A7r5 cells and HeLa cells [29,30]. The findings on HeLa cells are of particular interest for two reasons. First, activation of signal pathways in these cells of human origin are obtained at ouabain concentrations that are about two to three orders of magnitude lower than those that elicit similar effects in rodent cells [29–32]. This is in keeping with the relative ouabain sensitivities of the predominant Na^+/K^+-ATPase isoforms of these cells; thus establishing firmly that ouabain effects on signaling pathways indeed begin at the Na^+/K^+-ATPase, and are not due to unidentified ouabain interactions with other receptors. Second, because HeLa cells contain little or no Na^+/Ca^{2+}-exchanger, the demonstration of the independence of the signal transducing role of Na^+/K^+-ATPase using altered intracellular ion concentrations has been easier in these cells than in cardiac myocytes [30].

Ouabain has also been shown to stimulate proliferation, induce early response proto-oncogenes, and activate p42/44 MAPK in primary cultures of vascular and prostatic smooth muscle cells at ouabain concentrations that cause little or no change in intracellular ion concentrations [33–35]. This also supports a signal transducing role of the enzyme through protein–protein interactions. Significantly, in vascular smooth muscle cells, the proximal events of ouabain-induced signaling also involve the activation of Src and the transactivation of the EGF receptor [35]. Ouabain signaling distinct from ouabain's effect on $[Na^+]_i$ and $[K^+]_i$ is also indicated by the intriguing recent demonstration of ouabain-induced slow calcium oscillations and associated NF-κB activation in renal epithelial cells, suggesting the possibility of ouabain-regulated Na^+/K^+-ATPase interactions with neighboring Ca^{2+} handling proteins [36]. We have already mentioned the older work [18–20] showing inhibitory ouabain effects on lymphocyte proliferation at ouabain concentrations shown to be without effect on intracellular ions. Although these studies were carried out before the development of many of the current concepts of signal transducing pathways, it is appropriate to note that with remarkable insight these ouabain effects were ascribed to protein–protein interactions between Na^+/K^+-ATPase and other plasma membrane proteins [19].

Changes in $[Na^+]_i$ or $[K^+]_i$ that are caused by means other than inhibition or activation of Na^+/K^+-ATPase are known to affect intracellular signal pathways; e.g. a rise in $[Na^+]_i$ induced by gramicidin activates a stress-activated protein kinase [37]. An important question is whether changes in $[Na^+]_i$ and $[K^+]_i$ that may be induced by inhibition of the transport function of Na^+/K^+-ATPase can also co-operate with the signal transducing function of the enzyme as $[Ca^{2+}]_i$ does in cardiac myocytes. This question can not be answered in myocytes, or other cells that have a highly active plasma membrane Na^+/Ca^{2+}-exchanger, because partial inhibition of Na^+/K^+-ATPase causes an increase in $[Ca^{2+}]_i$ with little or no change in $[Na^+]_i$ and $[K^+]_i$, and higher levels of inhibition lead to loss of viability due to Ca^{2+}-overload before significant changes in $[Na^+]_i$ or $[K^+]_i$ can be obtained and sustained [30]. However, cells that do not express the plasma membrane Na^+/Ca^{2+}-exchanger, such as HeLa cells, can tolerate even complete inhibition of the transport function of Na^+/K^+-ATPase for hours, thus exhibiting large changes in $[Na^+]_i/[K^+]_i$ ratio. In such cells, using high ouabain concentrations, or palytoxin, which also alters $[Na^+]_i/[K^+]_i$ ratio by interaction with Na^+/K^+-ATPase, activation of a number of protein kinase signaling pathways have been demonstrated [37–39]. This suggests that in some cells other than cardiac myocytes, changes in $[Na^+]_i$ or $[K^+]_i$ or both may also modulate the signal transducing function of Na^+/K^+-ATPase that is initiated by protein–protein interactions.

THE PHYSIOLOGICAL STIMULI FOR SIGNAL TRANSDUCTION BY NA^+/K^+-ATPASE

The signal transducing receptors of the plasma membrane respond to specific extracellular stimuli such as hormones

and neurotransmitters. Ouabain and related digitalis compounds bind to the extracellular domains of Na^+/K^+-ATPase with exquisite specificity. Although they have been considered only as drugs for a long time, as discussed in the accompanying review [40] there is now ample evidence to indicate that these compounds are indeed hormones. The other highly selective physiological ligand for the extracellular domain of Na^+/K^+-ATPase is K^+. Lowering of $[K^+]_o$ has been shown to act in a manner similar to ouabain and activate the proximal segments of the signaling pathways in cardiac myocytes [29]. In smooth muscle and epithelial cells, however, lowering of $[K^+]_o$ does not mimic the signaling effects of ouabain [35,36], emphasizing the diversity of the signal transducing functions of Na^+/K^+-ATPase (see below).

CONCLUSIONS AND FUTURE PROSPECTS

The work of the past few years, built on the foundation of a number of excellent but somewhat ignored studies of the past three decades, has clearly shown that in addition to its established role as an ion pump, Na^+/K^+-ATPase also functions as a signal transducer to relay messages from the plasma membrane to the intracellular organelles through stimulus-induced protein–protein interactions involving the Na^+/K^+-ATPase, the neighboring plasma membrane proteins, and the organized cytoplasmic protein assemblies. An important point that is already evident from the work carried out to date is that while Na^+/K^+-ATPase pumps ions by the same basic mechanism in all cells, there are both similarities and differences in the mechanisms and the consequences of its signal transducing function in different cell types. The differences may be due to the different protein–protein interactions of the various Na^+/K^+-ATPase isoforms, or the different sensitivities of the isoforms to a stimulus, or the different expression levels of the proteins that interact with Na^+/K^+-ATPase in various cells, or the cell-specific down-stream events within the activated signal pathways. This diversity is an added complexity that makes the task of clarifying the signal transducing mechanism(s) of Na^+/K^+-ATPase more difficult, but it also provides vast opportunities for significant expansion of research in a field that seemed to have matured. This new direction of research on Na^+/K^+-ATPase is clearly underdeveloped. The number of unanswered issues is so large that any list of 'important remaining questions' drawn up by one interested investigator would look woefully inadequate to others with different perspectives.

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