2011 Homer Smith Award: To Serve and Protect: Classic and Novel Roles for Na\textsuperscript{+},K\textsuperscript{+}-Adenosine Triphosphatase

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ABSTRACT
The ability of cells to maintain sharp ion gradients across their membranes is the foundation for the molecular transport and electrical excitability. Across animal species and cell types, Na\textsuperscript{+},K\textsuperscript{+}-adenosine triphosphatase (ATPase) is arguably the most powerful contributor to this phenomenon. By producing a steep concentration difference of sodium and potassium between the intracellular and extracellular milieu, Na\textsuperscript{+},K\textsuperscript{+}-ATPase in the tubules provides the driving force for renal sodium reabsorption. Pump activity is downregulated by natriuretic hormones, such as dopamine, and is upregulated by antinatriuretic hormones, such as angiotensin. In the past decade, studies have revealed a novel and surprising role: that Na\textsuperscript{+}, K\textsuperscript{+}-ATPase exerts tissue-protective effects. Ouabain-stimulated Na\textsuperscript{+},K\textsuperscript{+}-ATPase signaling has recently shown clinical promise by protecting the malnourished milieu, Na\textsuperscript{+},K\textsuperscript{+}-ATPase in the tubules provides the driving force for renal sodium reabsorption. Pump activity is downregulated by natriuretic hormones, such as dopamine, and is upregulated by antinatriuretic hormones, such as angiotensin. In the past decade, studies have revealed a novel and surprising role: that Na\textsuperscript{+}, K\textsuperscript{+}-ATPase exerts tissue-protective effects. Ouabain-stimulated Na\textsuperscript{+},K\textsuperscript{+}-ATPase exerts tissue-protective effects. Ouabain-stimulated Na\textsuperscript{+},K\textsuperscript{+}-ATPase is an activator of signals from extracellular to intracellular compartments. The signaling function of Na\textsuperscript{+},K\textsuperscript{+}-ATPase is activated by ouabain, a mammalian steroid hormone, at far lower concentrations than those that inhibit pump activity. By promoting growth and inhibiting apoptosis, activation of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase exerts tissue-protective effects. Ouabain-stimulated Na\textsuperscript{+},K\textsuperscript{+}-ATPase signaling has recently shown clinical promise by protecting the malnourished embryonic kidney from adverse developmental programming. A deeper understanding of the tissue-protective role of Na\textsuperscript{+},K\textsuperscript{+}-ATPase signaling and the regulation of Na\textsuperscript{+},K\textsuperscript{+}-ATPase pumping activity is of fundamental importance for the understanding and treatment of kidney diseases and kidney-related hypertension.


Recent reports of the high-resolution structure of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase complexes have, with support from studies of the functional effect of numerous mutations, provide in-depth understanding of the ion-transporting pathways and of the allosteric transformations Na\textsuperscript{+},K\textsuperscript{+}-ATPase undergoes as it cycles between sodium-transporting and potassium-transporting states.\textsuperscript{4,6} Na\textsuperscript{+},K\textsuperscript{+}-ATPase is a binary complex and consists of a catalytic α subunit and a β subunit, required for insertion of the α subunit into the plasma membrane. The α subunit consists of 3 cytoplasmic domains (the actuator, the nucleotide-binding, and the phosphorylation domains) and 10 transmembrane helices (Figure 1). Four isoforms of the α subunit have been identified in mammals, but only one, the ubiquitous α1, is expressed in the kidney (Figure 1B). The β subunit consists of a small N-terminal cytoplasmic domain; one transmembrane helix; and a large, glycosylated, extracellular domain that covers most of the extracellular surface of the α subunit. In epithelial cells, the β subunits from neighboring cells form bridges that are crucial for the integrity of the intracellular junctions.\textsuperscript{7} Glycosylation of the β subunit...
plays an important role for the formation of bridges. The bridges formed by the Na⁺,K⁺-ATPase β subunits between adjacent tubular cells prevent the translocation of Na⁺,K⁺-ATPase to the apical membrane.

The Na⁺,K⁺-ATPase complex is generally associated with a member of the family of FXYD proteins. These proteins consist of a single transmembrane helix. The kidney expresses FXYD-2 and -4, which can, in different directions, affect Na⁺,K⁺-ATPase sodium affinity.

NA⁺,K⁺-ATPASE IS AN ADAPTIVE SERVANT

At the time of birth, the responsibility to control salt and water balance is transferred from the placenta to the kidneys, but birth does not bring about a dramatic maturation of kidney function. The capacity to adapt salt and water excretion to fluctuations in intake is not fully developed until age 18–24 months. The newborn infant is expected to have a relatively constant intake of fluid and electrolytes, and homeostatic disturbances are rarely encountered in healthy full-term infants; however, during the course of diarrheal disease they occur early, are profound, and are often associated with large fluctuations in serum sodium concentration. In preterm babies, disturbances in salt and water homeostasis are common even in the absence of pathologic losses.

Studies in rodents indicate that the immaturity of the newborn kidney and its inability to maintain a normal salt and water balance can be ascribed to low expression of sodium transporters in tubular cells. The activity of Na⁺,K⁺-ATPase in the proximal tubule and the loop of Henle increases approximately four-fold from 1 to 6 weeks of age. The most rapid increase occurs during the weaning period and coincides with an increase in serum corticosterone levels. The developmental pattern for urinary-concentrating capacity parallels the developmental increase of Na⁺,K⁺-ATPase activity in the thick ascending limb of Henle. As a consequence of low Na⁺, K⁺-ATPase activity, the fraction of energy consumed by the kidney will be much lower in infants than in adults. Infancy represents the fastest period of growth, and low renal oxygen consumption would allow the infant to spend more energy on growth and development.

HORMONAL CONTROL OF NA⁺, K⁺-ATPASE AFFECTS SALT HOMEOSTASIS

Na⁺,K⁺-ATPase is sometimes described as a housekeeping enzyme. This might lead to the erroneous conclusion that Na⁺,K⁺-ATPase does not play a dynamic and regulatory role for cell function. However, taking into account the extraordinarily important role of Na⁺,K⁺-ATPase for tubular sodium reabsorption, and thus for the overall control of sodium homeostasis, a fine-tuned regulation should be understood. Na⁺,K⁺-ATPase activity is substrate dependent, and increased intracellular sodium concentration will increase activity. Thus, Na⁺,K⁺-ATPase activity will be upregulated by increased sodium influx by sodium co-transporters and channels. Would this be sufficient for a well-controlled regulation of tubular sodium reabsorption, or is a direct regulation of Na⁺,K⁺-ATPase activity also required? In 1987, my colleagues and I reported that dopamine dose dependently and reversibly inhibits the activity of Na⁺,K⁺-ATPase in proximal tubular segments. Because the segments were permeabilized, we concluded that inhibition must be a direct effect on Na⁺,K⁺-ATPase and not secondary to inhibition of sodium entry into the cells. This was one of the first indications that Na⁺,K⁺-ATPase activity is under hormonal control.

The predominant dopamine receptor in proximal tubules is the dopamine D₁ receptor, which is a Gs-coupled receptor that activates protein kinase A (PKA). In follow-up studies we showed that PKA phosphorylates the catalytic Na⁺, K⁺-ATPase α subunit (Na⁺,K⁺-ATPase α), mapped the site of PKA phosphorylation, and showed that phosphorylation is associated with inhibition of enzyme activity and decreased sodium affinity of the enzyme.

Recent structure-function studies reveal that the PKA phosphorylation site, the serine 936 residue, is located close to one of the sodium entry sites in Na⁺,K⁺-ATPase α in the intracellular loop between the eighth and ninth transmembrane domains.

Protein kinase C (PKC) will also phosphorylate Na⁺,K⁺-ATPase α and, under appropriate intracellular conditions of intracellular calcium, inhibit Na⁺,K⁺-ATPase activity. In studies of cells expressing wild-type or mutant Na⁺,K⁺-ATPase α that lack the phosphorylation site, we found that activation of PKC increases intracellular sodium concentration two-fold in wild-type cells but not in cells expressing mutant Na⁺,K⁺-ATPase α; this finding indicates that regulation by phosphorylation affects the overall driving force for sodium influx (Figure 2). It remains to be determined to what extent this will influence each of the individual sodium in-transporters.
It is now well recognized that Na\(^{+}\), K\(^{+}\)-ATPase is a target for hormonal regulation by phosphorylation in many tissues, including the brain.\(^{29-31}\) Phosphorylation can affect Na\(^{+}\), K\(^{+}\)-ATPase pumping efficacy by an allosteric effect on the integral plasma membrane protein\(^{26}\) or by internalization of the protein.\(^{32}\) Recent studies indicate that phosphorylation might also require the participation of scaffolding and intermediary proteins. Dopamine regulation of Na\(^{+}\), K\(^{+}\)-ATPase in OK cells, a cell line derived from opossum kidney, requires the participation of Na\(^{+}\), K\(^{+}\)-ATPase α in the basolateral membrane, where it pumps three Na\(^{+}\) ions out and two K\(^{+}\) ions into the cell for each molecule of ATP that is hydrolyzed. This creates the gradient that allows sodium to diffuse into the cell via cotransporters, exchangers, and channels. Hormonal downregulation of Na\(^{+}\), K\(^{+}\)-ATPase turnover will reset basal intracellular sodium concentration and decrease the driving force for sodium diffusion.

Emerging evidence indicates that Na\(^{+}\), K\(^{+}\)-ATPase is located in a multiprotein complex together with receptors for natriuretic and antinatriuretic factors\(^ {45}\) and that binding of an antagonist to a member of the antinatriuretic family of receptors might lead to the activation of the natriuretic receptor family via allosteric modification.\(^ {46}\)

**Figure 2.** Na\(^{+}\), K\(^{+}\)-ATPase is an adaptive driving force for sodium reabsorption. In tubular cells, Na\(^{+}\), K\(^{+}\)-ATPase is exclusively located in the basolateral membrane, where it pumps three Na\(^{+}\) ions out and two K\(^{+}\) ions into the cell for each molecule of ATP that is hydrolyzed. This creates the gradient that allows sodium to diffuse into the cell via cotransporters, exchangers, and channels. Hormonal downregulation of Na\(^{+}\), K\(^{+}\)-ATPase turnover will reset basal intracellular sodium concentration and decrease the driving force for sodium diffusion.

**NA\(^{+}\), K\(^{+}\)-ATPASE IS A SIGNAL TRANSDUCER PROVIDING TISSUE PROTECTION**

Na\(^{+}\), K\(^{+}\)-ATPase is a target for cardio-tonsic steroids (CTSs), to which plant-derived digitalis, used for more than a century to treat heart disease, belongs.\(^ {57-59}\) The CTSs are composed of a steroid core, a lactone ring, and sugar moiety and are relatively cell impermeable. The CTSs bind with high specificity to the extracellular site of the catalytic subunit of Na\(^{+}\), K\(^{+}\)-ATPase. Ouabain is a mammalian CTS. The biologic significance of a highly specific Na\(^{+}\), K\(^{+}\)-ATPase ligand has been debated for many years. In high concentrations, CTSs inhibit the activity and ion-transporting capacity of Na\(^{+}\), K\(^{+}\)-ATPase. Yet there is little evidence that ouabain would act as a natriuretic hormone, and circulating levels of ouabain are too low to affect Na\(^{+}\), K\(^{+}\)-ATPase sodium transport.

Studies during the past decade reveal that Na\(^{+}\), K\(^{+}\)-ATPase activity is also dynamically regulated by direct interaction of Na\(^{+}\), K\(^{+}\)-ATPase α with the FXYD proteins,\(^ {8-10}\) which are targets for both PKA- and PKC-mediated phosphorylation.

Studies on dopamine regulation of Na\(^{+}\), K\(^{+}\)-ATPase activity in single tubular segments have provided new information about the dopamine signaling pathway. DARPP-32, a dopamine and cAMP-regulated phosphoprotein first identified in brain tissue with an apparent molecular mass of 32 kD, was found abundantly expressed in the kidney and to be of key importance in mediating the inhibitory effect of dopamine on Na\(^{+}\), K\(^{+}\)-ATPase.\(^ {35,36}\)

The consequences of dopamine inhibition of Na\(^{+}\), K\(^{+}\)-ATPase activity for regulation of sodium reabsorption and excretion have been extensively studied.\(^ {37}\) L-dopa, filtered and reabsorbed in proximal tubular cells, is converted to dopamine by the enzyme aromatic L-amino acid decarboxylase. Inhibition of L-amino acid decarboxylase in salt-loaded rats decreases the availability of tubular dopamine, increases proximal tubular Na\(^{+}\), K\(^{+}\)-ATPase activity, and decreases sodium excretion.\(^ {38}\) Conversely, inhibition of catechol-O-methyltransferase, an enzyme that metabolizes dopamine, decreases proximal tubular Na\(^{+}\), K\(^{+}\)-ATPase activity and increases sodium excretion.\(^ {39}\) Emerging evidence also suggests that dysregulation of reninal tubular Na\(^{+}\), K\(^{+}\)-ATPase activity can result in salt-sensitive hypertension.\(^ {47}\) For example, oxidative stress, which attenuates signaling by dopamine receptor 1 and dopamine-mediated inhibition of Na\(^{+}\), K\(^{+}\)-ATPase, is associated with a decreased natriuretic response to salt loading and increased BP.\(^ {40}\)

Well controlled sodium balance depends on a well orchestrated cross-talk between natriuretic and antinatriuretic factors. Na\(^{+}\), K\(^{+}\)-ATPase is inhibited not only by dopamine but also by antinatriuretic factor and is activated by angiotensin and α2 adrenergic agonists.\(^ {41-44}\) Emerging evidence indicates that Na\(^{+}\), K\(^{+}\)-ATPase is located in a multiprotein complex together with receptors for natriuretic and antinatriuretic factors\(^ {45}\) and that binding of an antagonist to a member of the antinatriuretic family of receptors might lead to the activation of the natriuretic receptor family via allosteric modification.\(^ {46}\)
of ouabain. One nanomolar of ouabain should bind 1/10^3–1/10^5 Na^+,K^+-ATPase molecules, which would be sufficient to trigger Na^+,K^+-ATPase signaling function but would spare Na^+,K^+-ATPase pumping function. Because the ouabain off-rate exceeds the ouabain on-rate, the effect of low concentrations of ouabain becomes more apparent after long-term exposure.

We noted the first indication that the ouabain/Na^+,K^+-ATPase complex has a signaling function when we studied the interaction between Na^+,K^+-ATPase and the sodium-calcium exchanger in primary cultures of rat proximal tubular cells and made some completely unexpected observations. When cells were exposed to ouabain in concentrations that gave maximal inhibition of Na^+,K^+-ATPase activity and increased intracellular sodium concentration, we found, as expected, a sustained increase in intracellular calcium concentration that could be attributed to a negative feedback on the sodium-calcium exchanger. When, however, we used lower concentrations of ouabain that had little effect on intracellular sodium concentration, we observed slow, regular calcium oscillations. Release of calcium from intracellular stores by the inositol 1,4,5-triphosphate receptor (IP3R) was found to be the main source of the repetitive increases in intracellular calcium. When these studies were first performed, inositol triphosphate, generated from activation of phospholipase C, was the only known activator of IP3R. Inhibition of phospholipase C and intracellular absorption of IP3 with a peptide that had 10^7 higher affinity to IP3 than to its receptor, however, did not abolish the ouabain-triggered calcium oscillations. This finding prompted us to hypothesize that IP3R is activated by direct physical contact with Na^+,K^+-ATPase α, an hypothesis that proved to be correct.

We could show that the N-terminus tail of Na^+,K^+-ATPase α interacted with the IP3R N-terminus and that three amino acid residues, LKK, conserved in most species and most Na^+,K^+-ATPase α-isoforms, were essential for the interaction. Interaction between Na^+,K^+-ATPase and IP3R was strengthened by the scaffolding protein ankyrin. A schematic view of the ouabain/Na^+,K^+-ATPase/IP3R signaling pathway is shown in Figure 3.

Liu and Xie have described an additional Na^+,K^+-ATPase signaling pathway. They have shown that binding of ouabain to the signaling Na^+,K^+-ATPase activates the cytoplasmic tyrosine kinase Src, leading to transactivation of the epidermal growth factor receptor and a plethora of downstream effects that are to some extent cell specific. Xie and colleagues suggest the Na^+,K^+-ATPase/IP3R/calcium and Src signaling pathways are inter-related. Ongoing studies in our laboratory, showing that the calcium oscillatory response to ouabain in renal epithelial cells is dramatically downregulated in the presence of a Src inhibitor, support this notion.

**SLOW CALCIUM OSCILLATIONS ACTIVATE NF-κB AND PROTECT FROM APOPTOSIS**

Release of calcium from the intracellular stores by IP3R is generally organized in a spatial and temporal pattern (calcium oscillations), which makes it the most versatile of all cell signals because the cell can decode the frequency of the oscillations. Calcium oscillations with a periodicity of 2–5 minutes have been reported to activate the transcription factors NF activated T cells and NF-κB. We have found in studies performed on primary cultures of rat proximal tubular cells and on COS cells (a cell line derived from embryonic monkey kidney) that treatment with ouabain in concentrations that spare the pump function will result in translocation of NF-κB from the cytoplasm to the nucleus and reduced expression of I-κB, the cytosolic partner of NF-κB. NF-κB consists of five subunits and is a pleiotropic transcription factor. Depending on the mode of activation, it may trigger transcription of the antiapoptotic factor Bcl-xL or the apoptotic factor apoptotic factor Bax. Ouabain-triggered calcium oscillations have, in studies on rat renal cells, been found to have an antiapoptotic effect and to increase the expression of Bcl-xL.

**NA^+,K^+-ATPASE SIGNALING STIMULATES CELL GROWTH**

Low concentrations of ouabain have been shown to stimulate proliferation and increase the viability in many cell types, including renal epithelial cells. These effects depend on ouabain/Na^+,K^+-ATPase signaling by both Src and IP3R pathways. Na^+,K^+-ATPase stimulation of cell proliferation is a protective mechanism during adverse developmental conditions and in renal disease, but it might also have pathologic implications. Blanco and colleagues report that in...
epithelial cells derived from renal cysts of patients with autosomal-dominant polycystic kidney disease, the Na\(^+\),K\(^+\)-ATPase ouabain affinity is increased and the mitogenic response to nanomolar concentrations of ouabain is enhanced.\(^{65}\)

**NA\(^+\),K\(^+\)-ATPASE SIGNALING PROTECTS THE KIDNEY FROM ADVERSE DEVELOPMENTAL PROGRAMMING**

The kidney is extraordinarily sensitive to adverse fetal programming.\(^{66,67}\) Fetal malnutrition, the most common developmental challenge, results in reduced nephron formation.\(^{68}\) As nephron formation is completed toward the end of the gestational period in humans and within the first postnatal week in rodents, fetal malnutrition will result in a permanent reduction of nephron number.

The formation and organization of nephrons in the developing kidney require a well controlled balance between proliferation and apoptosis. Fetal malnutrition is a common cause of low nephron endowment and is associated with a relative increase in the apoptotic process.\(^{69}\) This observation led us to hypothesize that the antiapoptotic effect of ouabain is a signal transducer that protects from apoptosis of embryonic kidneys exposed to malnutrition was obtained from studies on pregnant rats given a low-protein diet and treated with ouabain or vehicle throughout pregnancy. Consistent with reports from other investigators,\(^{71,72}\) we found a reduction in the number of glomeruli in the low-protein group compared with the normal-protein group. This reduction was abolished in the offspring of low-protein-diet–fed dams that had been exposed to ouabain during pregnancy. Experimental studies indicate that loss of nephrons caused by low maternal caloric intake or by placental insufficiency encompasses increased risk for renal disease.\(^{73–75}\) Epidemiologic and morphologic studies of nephron number in autopsy material from individuals of different ages and ethnic backgrounds suggest this is also the case in humans.\(^{66,73–76}\) The finding that ouabain, belonging to the family of CTSs, rescues the development of fetal kidneys exposed to malnutrition may open a new avenue for protection against adverse developmental programming of the kidney and eliminate one risk factor for progressive renal disease.

**PROTECTIVE EFFECTS OF NA\(^+\), K\(^+\)-ATPASE SIGNALING IN RENAL DISEASE**

The identification of a novel signaling system that protects from apoptosis raises the question: Might activation of this signaling system protect from permanent renal damage in AKD and CKD associated with apoptosis? Our ongoing studies indicate this is the case. Hemolytic uremic syndrome, triggered by shigatoxin2-producing *Escherichia coli*, is associated with massive apoptosis in the acute stage.\(^{77}\) Hemolytic uremic syndrome is a common cause of renal insufficiency in children and young adults, and there is as yet no therapy that prevents the acute insult. We have in ongoing studies found that exposure of rat proximal tubule cells to shigatoxin2 results in massive apoptosis and that nanomolar concentrations of ouabain have a rescuing effect.

Apoptosis is a common feature in CKD associated with progressive loss of renal function. The progressive loss of function can be ascribed not only to glomerulosclerosis but also to glomerular-tubular disconnection.\(^{78–80}\) This phenomenon, described more than 70 years ago by Oliver, has recently received attention as a potential target for renal-protective therapy. Atubular glomeruli are particularly abundant in diseases associated with proteinuria.\(^{81}\) A narrowing of the glomerular tubular junction and apoptosis of the proximal tubular cells within the junction precede the disconnection. In an ongoing study performed on rats with passive Heymann nephritis, a model of human membranous nephritis, we have found that long-term treatment with ouabain attenuates apoptosis of proximal tubular cells in the glomerular tubular junction as well as the formation of atubular glomeruli.

**IS THERE AN ENDOGENOUS ACTIVATION OF THE NA\(^+\), K\(^+\)-ATPASE/IP3R NEPHROPROTECTIVE SIGNAL?**

HPLC, immunoassay methods, and mass spectrometry studies show beyond doubt the presence of ouabain in biologic fluids and the adrenal gland.\(^{47,48}\) It is now generally agreed that ouabain is an endogenous hormone, and recent studies indicate the existence of at least two more human CTSs, marinobufagenin and digoxin. Circulating levels of CTS are in the picomolar-nanomolar range, which would be sufficient to activate the Na\(^+\),K\(^+\)-ATPase signaling function but would spare the Na\(^+\),K\(^+\)-ATPase pumping function. Circulating levels of ouabain are increased during pregnancy.\(^{82}\) Patients with renal failure have increased levels of endogenous CTSs, and there is evidence that the high levels of CTS may be deleterious for
the cardiovascular system. This would particularly be the case for patients who have also been given CTS drugs. Recent studies focus on these negative effects of endogenous CTS, but it seems very unlikely that the human organism would, under normal physiologic conditions, produce circulating hormones that would only be harmful. Currently there is little information about the levels of CTS in apoptosis-associated kidney diseases with moderate reduction of renal function. The question of whether there is a compensatory increase of these tissue protective hormones will be an important topic for future studies.

CONCLUSIONS

All vital mechanisms have a common goal: to maintain the uniformity of the interior environment, particularly the conditions of life in the cell. This concept, described by Claude Bernard more than 150 years ago, remains essential to the understanding of physiology. Na⁺,K⁺-ATPase plays an essential role, maybe the most essential role, for the maintenance of uniformity of the normal conditions of the cell. In the kidney, where Na⁺,K⁺-ATPase is more abundantly expressed than in most other organs, Na⁺,K⁺-ATPase also plays an essential role in the maintenance and stability of the extracellular environment through its role in the sodium reabsorptive process. The discoveries that the pumping activity of Na⁺,K⁺-ATPase can be regulated to adapt to the needs of a constant sodium homeostasis and that Na⁺,K⁺-ATPase has a signaling function that can act to support cell viability emphasizes the enormous importance of this multifunctional protein for maintaining the uniformity of the conditions of life in the cell. More research regarding the regulation of Na⁺,K⁺-ATPase activity and of Na⁺,K⁺-ATPase signaling function under normal and pathologic conditions and during development will without doubt lead to new insights into the pathophysiology and treatment of many kidney diseases and salt-dependent hypertension.

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DISCLOSURE

None.

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