

invited review

Mechanisms of sodium pump regulation

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Therien, Alex G., and Rhoda Blostein. Mechanisms of sodium pump regulation. *Am J Physiol Cell Physiol* 279: C541–C566, 2000.—The Na⁺-K⁺-ATPase, or sodium pump, is the membrane-bound enzyme that maintains the Na⁺ and K⁺ gradients across the plasma membrane of animal cells. Because of its importance in many basic and specialized cellular functions, this enzyme must be able to adapt to changing cellular and physiological stimuli. This review presents an overview of the many mechanisms in place to regulate sodium pump activity in a tissue-specific manner. These mechanisms include regulation by substrates, membrane-associated components such as cytoskeletal elements and the γ -subunit, and circulating endogenous inhibitors as well as a variety of hormones, including corticosteroids, peptide hormones, and catecholamines. In addition, the review considers the effects of a range of specific intracellular signaling pathways involved in the regulation of pump activity and subcellular distribution, with particular consideration given to the effects of protein kinases and phosphatases.

γ -subunit; dopamine; norepinephrine; aldosterone; protein kinase A; protein kinase C

IN 1997, the Nobel Prize in Chemistry was shared by Danish researcher Jens C. Skou for his discovery of the Na⁺-K⁺-ATPase. Although the existence of an active “sodium pump” had been previously hypothesized, Skou was the first to suggest, in 1957, a link between transport of Na⁺ and K⁺ across the plasma membrane and a Na⁺- and K⁺-activated ATPase activity (307). The significance of this discovery is underscored by the subsequent publication, each year, of scores of reports relevant to various aspects of Na⁺-K⁺-ATPase structure and function. Although much information about the enzyme has become available in the years since its discovery, one area of pump research that is not completely understood, despite recent advances, is that of pump regulation.

The basic function of the Na⁺-K⁺-ATPase, or sodium pump, is to maintain the high Na⁺ and K⁺ gradients across the plasma membrane of animal cells. In particular, the sodium pump is the major determinant of cytoplasmic Na⁺. As such, it has an important role in regulating cell volume, cytoplasmic pH and Ca²⁺ levels through the Na⁺/H⁺ and Na⁺/Ca²⁺ exchangers, re-

spectively, and in driving a variety of secondary transport processes such as Na⁺-dependent glucose and amino acid transport. The sodium pump, in turn, is the target of multiple regulatory mechanisms activated in response to changing cellular requirements. The requirement for modulators of the Na⁺-K⁺-ATPase is likely to be greatest in tissues in which perturbations of the intracellular alkali cation content underlie their specialized functions, in addition to those processes mentioned above (see below for specific references). Prime examples are the changes in sodium pump activity that occur in response to physiological stimuli such as nerve impulse propagation, exercise, and changes in diet. Expression of various isoforms of the sodium pump may fulfill some of the requirements for altered pump behavior (for recent discussion, see Ref. 42). However, direct tissue-specific modulation of the enzyme also underlies mechanisms of pump regulation.

One of the primary needs for sodium pump adaptation comes from changes in dietary Na⁺ and K⁺. The mediators of natriuresis and diuresis, namely, hormones that control the volume and ionic composition of blood and urine, often act directly on the sodium pump of the kidney and intestine. The function of the pump in absorption or reabsorption of Na⁺

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and K^+ and, secondarily, other solutes, requires tight regulation of the enzyme to maintain normal levels of Na^+ and K^+ during altered salt intake (for reviews, see Refs. 101, 160). In addition, because water and Na^+ transport across epithelia are invariably linked, the work of the sodium pump is also critical to water absorption in the intestine and reabsorption in the kidney. Illustrating this are reports that impairment of the sodium pump in kidney and small intestine can be associated with the pathophysiology of hypertension (168) and chronic diarrhea (123), respectively.

In excitable tissues such as neurons (141), skeletal muscle cells (82), and pacemaker fibers of the heart (320), the sodium pump must reestablish the electrical potential across the plasma membrane following excitation-induced depolarization. Although part of this function is undoubtedly fulfilled by the presence and distinct kinetics of the α_3 -isoform in neurons, regulatory events are also likely to be involved as evidenced by the multiple effects of various hormones on Na^+ - K^+ -ATPase activity in these tissues. In skeletal muscle, regulation of sodium pump activity has widespread physiological implications. Continuous stimulation of muscle fibers during exercise leads to dissipation of the cation gradient necessary for muscle contraction. To offset excessive release of K^+ from the muscle cells, rapid activation of Na^+ - K^+ -ATPase activity under these conditions is an essential means of delaying the onset of muscular fatigue and reducing potentially toxic levels of plasma K^+ .

Na^+ - K^+ -ATPase regulation in cardiac muscle is particularly critical to the myocardium, where the enzyme acts as an indirect regulator of contraction (45). Thus the sodium pump controls the steady-state cytoplasmic Na^+ concentration, which then determines Ca^{2+} concentration via the Na^+ / Ca^{2+} exchanger. Ca^{2+} , in turn, is pumped into the sarcoplasmic reticulum (SR) by the sarco(endo)plasmic reticulum calcium (SERCA) pumps. Regulation of the sodium pump in these tissues is therefore paramount for determining the "set point" for cardiac muscle contraction and the steady-state contraction of vascular smooth muscle. Physiological regulators that act in a manner analogous to that ascribed to cardiac glycoside inhibitors of the Na^+ - K^+ -ATPase are likely to be critical for normal heart contraction. The aforementioned mechanism of increasing the force of contraction via increasing cell Na^+ is considered to be the basis of digitalis therapy for cardiac insufficiency (329).

This monograph focuses on mechanisms of tissue-specific regulation of the sodium pump, with emphasis on two areas. One deals with mechanisms involving signaling pathways that result in modulations in pump activity, and the other deals with the regulation resulting from the interaction of the pump complex with other membrane components, which, in turn, may or may not be subject to modulation via other signaling cascades.

SUBSTRATE CONCENTRATIONS AS DETERMINANTS OF PUMP ACTIVITY

The simplest and most straightforward determinants of pump activity are the concentrations of substrates. The sodium pump is activated by Na^+ and ATP at cytoplasmic sites and by K^+ at extracellular sites. The most dramatic effects involve variations in cytoplasmic Na^+ concentration. Half-maximal activation of the enzyme by intracellular Na^+ occurs at concentrations of ~ 10 – 40 mM, which, depending on the tissue, are often at or above the steady-state Na^+ concentration (for example, see Ref. 309). Accordingly, small changes in the cytoplasmic Na^+ concentration secondary to activation of either various Na^+ -dependent transporters or Na^+ channels can have dramatic effects on sodium pump activity. As described below, some hormones appear to alter sodium pump activity by changing its apparent affinity for Na^+ (K_{Na}). Aside from its direct effects on the Na^+ - K^+ -ATPase, Na^+ has been shown to induce other mechanisms of upregulation of the sodium pump. For example, Na^+ influx is thought to be the first signal leading to an increase in surface sodium pumps in one kind of aldosterone-mediated short-term regulation (302).

Whereas the high affinity of the enzyme for K^+ at activating sites generally precludes an effect of variations in extracellular K^+ concentrations on sodium pump activity except, perhaps, in some neuronal tissues (318), K^+ has been shown to act as a competitive inhibitor of Na^+ binding at cytoplasmic sites (134). Therefore, variation in cytoplasmic K^+ concentration, or, more likely, in the affinity of the enzyme for K^+ as an antagonist at cytoplasmic Na^+ -activating binding sites, is a plausible mechanism for determining the set point for the physiological concentration at half-maximal activation ($K_{0.5}$) for cytoplasmic Na^+ activation (327).

Because the $K_{0.5}$ of the Na^+ - K^+ -ATPase for ATP is between 300 and 800 mM (310), the ATP concentration in most cells is saturating for the enzyme. However, in some tissues and under certain conditions, ATP levels may fall to subsaturating levels. For example, cells of the kidney medulla are known to function under near anoxic conditions (56), and such conditions can lead to dramatic drops in ATP levels (310). Thus variations in ATP concentration or in the affinity of the sodium pump for ATP may be physiologically relevant mechanisms of pump regulation in this tissue.

MEMBRANE-ASSOCIATED COMPONENTS

Because the Na^+ - K^+ -ATPase is a membrane-embedded protein, the nature of constituents comprising the membrane components should be an important determinant of enzyme function. Unfortunately, this is an unclear aspect of pump research due mainly to the difficulty in separating such components from the enzyme complex. As a first step toward gaining some insight into the question of whether and to what extent tissue- rather than isoform-specific differences in kinetic pump behavior reflect pump modulation by com-

ponents of the membrane, Munzer and co-workers (240, 241) examined the kinetic behavior of kidney pumps delivered by polyethylene glycol-mediated fusion into another (red blood cell) environment. In the case of pumps incorporated into genetically low- K^+ (LK) red blood cells, they obtained unequivocal evidence of kinetic changes effected by the L_p antigen of these red cells (see below; Ref. 353). Using the same membrane fusion system, Therien and Blostein (324) recently showed that the membrane environment has highly specific effects on the interaction of kidney pumps with Na^+ and K^+ on the cytoplasmic side; specifically, fusion of kidney pumps into dog red blood cells abrogates, at least partly, the relatively high susceptibility of kidney α_1 pumps to K^+/Na^+ antagonism at cytoplasmic cation activation sites.

In general, there is little information on the nature and mechanistic basis of sodium pump modulation by specific membrane components. Many reports have focused on the role of membrane lipids. The main effects of lipids on the sodium pump are related to membrane fluidity and thickness. In general, lipids that promote bilayer formation of physiological thickness and increased fluidity tend to promote optimal Na^+-K^+ -ATPase activity (172, 186, 221), as do negatively charged lipids such as phosphatidylserine and phosphatidylglycerol (187). The effects of cholesterol on enzyme activity are often also related to membrane fluidity (140), although specific effects of cholesterol on the sodium pump have been reported (356). Free fatty acids present in the membrane or as the products of phospholipase A_2 (PLA $_2$)-dependent regulatory pathway tend to inhibit the Na^+-K^+ -ATPase (254).

The L_p Blood Group Antigen

A striking and mechanistically well-characterized tissue-specific modulator of the Na^+-K^+ -ATPase is the L_p antigen of LK ruminant red cells, in particular those of sheep. The L_p antigen is so called because of its association with the L blood group antigens and its highly specific effects on the sodium pump (reviewed in Ref. 103). Evidence for the existence of this inhibitor was derived from studies on the effects of an antiserum specific for the L_p antigen; treatment with anti- L_p stimulates Na^+-K^+ -ATPase of LK, but not of high- K^+ (HK), erythrocytes (104). In addition, trypsinization of intact cells reverses the effects of anti- L_p (199), providing evidence that the inhibitor is a peptide distinct from the sodium pump itself and that the anti- L_p epitope is removed upon trypsin treatment. Experiments using anti- L_p and trypsin have led to a model of L_p -mediated inhibition of Na^+-K^+ -ATPase whereby the antigen inhibits sodium pump activity in two distinct ways. One is secondary to an L_p antigen-induced increase in the susceptibility of pumps to noncompetitive inhibition by K^+ (102) and the other to an increase in pump protein turnover during red cell maturation (352). In the pump/red cell fusion experiments mentioned above, it was observed that rat kidney pumps fused into LK red blood cells were stimulated by anti-

L_p , providing unequivocal proof that the L_p antigen is a molecular entity distinct from the sodium pump. However, the exact molecular nature of the protein remains unknown.

Components of the Cytoskeleton

Interactions of the Na^+-K^+ -ATPase with components of the cytoskeleton of cells are well documented. Specific cytoskeletal proteins thought to interact with the sodium pump, either directly or indirectly, include spectrin (182), actin (190), adducin (330), pasin (193), and ankyrin (245). Generally, ankyrin appears to mediate associations between the sodium pump and other cytoskeletal proteins, although direct associations of the enzyme with pasin and actin have also been observed. The two specific domains of the sodium pump that interact with ankyrin have been recently identified (96, 361). Of these, residues in the first cytoplasmic domain (142–166 of the rat α_1 -isoform) are especially intriguing because this region is highly conserved in all sodium pump isoforms and in H,K- and Ca^{2+} -ATPases, suggesting interactions of these P-type ion pumps with ankyrin. Ankyrin binding to the second cytoplasmic loop is likely mediated by a four-residue motif (ALLK) that has homology to a sequence of the anion exchanger, another ankyrin-binding transporter (174).

The main consequence of interactions between the Na^+-K^+ -ATPase and the cytoskeleton is believed to be the correct processing and targeting of sodium pumps to the appropriate membrane compartment. For example, disruptions in the cellular distribution of Na^+-K^+ -ATPase, induced either by ATP depletion or hypoxia, are linked to alterations in cytoskeletal proteins (233, 262), and a spectrin-ankyrin complex is required for transport of pumps from the endoplasmic reticulum to the Golgi apparatus (97). Recently, a role for cytoskeletal proteins in regulating sodium pump activity has been suggested. For example, monomeric, but not polymerized, actin has been shown to activate the sodium pump by a mechanism mediated by cAMP-dependent protein kinase (PKA) (60, 61). In addition, mutant forms of adducin have been shown to stimulate Na^+-K^+ -ATPase activity in transfected NRK-52E cells (330).

The identification of genetic polymorphisms in adducin in Milan hypertensive strain rats and in humans has led Manunta et al. (219) to suggest that adducin variants may affect kidney function by modulating the overall cation transport in renal epithelia, both by affecting assembly of the cytoskeleton and by modulating sodium pump activity. In a recent report, they showed that both rat and human adducins stimulate Na^+-K^+ -ATPase activity by increasing the apparent affinity for ATP (114). Interestingly, the mechanism appears to involve acceleration of the rate of the conformational change $E_2(K) \rightarrow E_1(Na)$ or $E_2(K).ATP \rightarrow E_1Na.ATP$. Stimulation is specific in that a stimulatory effect noted also with ankyrin, which also binds Na^+-K^+ -ATPase, is not additive. In general, these findings suggest a specific interaction between adducin and the

$\text{Na}^+\text{-K}^+\text{-ATPase}$ of the kidney. It is intriguing that the effect is similar to that effected by the γ -subunit of the pump (see below). Whether interaction of adducin with the pump involves the γ -subunit is relevant to the modulatory effect of adducin remains to be determined.

The γ -Subunit

The γ -subunit is a small transmembrane protein that specifically associates with the $\text{Na}^+\text{-K}^+\text{-ATPase}$ in a tissue-specific manner. Though its existence had been previously suggested (282), it was Forbush and co-workers (124) who first demonstrated that this small hydrophobic peptide is specific to the sodium pump by showing that it is specifically labeled, along with the α -subunit, by a photoactive derivative of ouabain. Although the peptide was at first thought to represent a third component of the $\text{Na}^+\text{-K}^+\text{-ATPase}$, recent evidence suggests that it is not an integral part of the enzyme complex.

Following the report of Forbush et al. (124), who studied the pig enzyme, experiments using various ouabain derivatives resulted in the identification of a small sodium pump-associated proteolipid in various tissues (151, 214, 284, 286). This peptide, initially referred to as “ γ component” or “ γ -subunit” (281), appeared to be present in approximately equimolar amounts compared with the α - and β -subunits (85, 155). The initial molecular cloning experiments indicated that the γ -subunit consisted of 58 amino acids and had a molecular mass of $\sim 6,500$ Da (228). Since then, cDNAs for the human (185) and *Xenopus laevis* (25) γ -subunits have also been cloned and sequenced. Sequence comparisons show strong homology (75%) among different species, which is further increased to 93% when only mammalian sequences are compared. Structural analysis has revealed that the γ -subunit contains a single transmembrane domain, with an NH_2 terminus-out, COOH terminus-in topology (25, 325). The NH_2 terminus, at least that of the rat sequence, has since been shown to be somewhat longer and different than originally reported. (For details, see Ref. 326 and GenBank accession no. AF129400.1).

An intriguing feature of the γ -subunit structure is that it is detected as two species with similar amino acid composition irrespective of the protein separation methods used (for examples, see Refs. 85, 228, 304). Early evidence suggested that the two bands detected on Western blots, henceforth referred to as γ_a and γ_b , are the products of a single mRNA species (228). Béguin and co-workers (25) later showed that in *Xenopus*, the two bands of γ are due to alternate usage of two distinct start codons in the γ -subunit message; only one appears relevant in vivo in this species. However, recent mass spectrometry analysis of the rat protein indicated that γ_a and γ_b are variants, most likely splice variants (194). γ_a has a mass of 7,184 Da, whereas the faster migrating γ_b species has a mass of 7,354 Da and contains only a different NH_2 terminus (6- replacing 7-residues). In fact, their amino acid sequences indicate that they correspond exactly to two splice variants

contained in the expressed sequence tag database as noted by Sweadner et al. (315). Recent expression studies show clearly that the γ_a and γ_b protein products of transcription/translation have the same mobilities as the upper and lower bands, respectively, of the kidney medulla (195). Depending on the cell line used, additional bands, presumably the products of posttranslational modification, are seen, namely, γ'_a with higher apparent mobility than γ_a in HEK and γ'_b with lower mobility than γ_b in HeLa, whereas only γ_a and γ_b are detected in HeLa and HEK, respectively.

The expression of γ -subunit mRNA has been investigated by Northern blot analysis in the rat, human, and *X. laevis*, and it was shown that the peptide is expressed in a tissue-specific manner in these species. Thus, in humans, γ -subunit mRNA was detected in kidney, pancreas, and fetal liver (185), and in *Xenopus*, it was detected mainly in kidney and stomach, with trace amounts in heart, skin, and oocytes (25). In rats, the situation is more complex, because two distinct mRNA species were detected by using the rat γ -subunit cDNA as a probe (228). The larger of the two, at 1.5 kb, corresponds in size to the *Xenopus* mRNA and was detected mainly in kidney and spleen, with lower amounts in lung, heart, and brain. The smaller transcript migrated at 0.8 kb, a size similar to that of human γ -subunit message, and was detected at high levels in the kidney and at very low levels in the spleen, lung, and heart. Also in the rat, Therien and co-workers (325, 326) have recently shown that at the protein level, the γ -subunit is expressed only in kidney tubules, with very low levels found in the spleen.

Most available data indicate that the γ -subunit is not expressed at the plasma membrane without the $\text{Na}^+\text{-K}^+\text{-ATPase}$, except perhaps in very early development, as described below. For example, γ -subunit is expressed at the surface of *Xenopus* oocytes only on coinjection of cRNA for the α - and β -subunits (25); immunocytochemical analysis has shown that the expression patterns of α - and γ -subunits are identical in renal proximal tubules and collecting ducts (228), although γ -subunit appears to be absent in other parts of the kidney (13, 325). In addition, coimmunoprecipitation of the γ -subunit with both the α - and β -subunits has been demonstrated (228). On the other hand, in their study on the role of the γ -subunit in mouse blastocyst development, Jones and co-workers (173) have shown that the γ -subunit is expressed at high levels at the apical membrane, whereas the α - and β -subunits are present only at the basolateral membrane.

The first attempts at defining a functional role for the γ -subunit indicated that this peptide is not essential for normal enzyme function. For example, Hardwicke and Freytag (155) were able to show that separation of the γ -peptide from the $\alpha\beta$ complex by nonionic detergent solubilization of shark rectal gland and avian salt gland membranes had no effect on $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity. More recently, it has been shown that the presence of the γ -subunit is not necessary for functional expression of the sodium pump in insect cells

(95), *Xenopus* oocytes (25), and yeast (296). In the latter system, the γ -subunit was shown to have no effect on either ouabain-sensitive Na^+ - K^+ -ATPase activity or $^{86}\text{Rb}^+$ influx. The failure to detect γ -subunit mRNA (25, 185, 228) or protein (325) in many tissues also supports the notion that the γ -subunit is not an essential component of the Na^+ - K^+ -ATPase.

Recent experiments have shown that the γ -subunit has a potentially important functional role in some systems. Treatment of mouse blastocysts with γ -subunit antisense oligodeoxynucleotide reduced the amount of expressed γ -subunit and caused a reduction in ouabain-sensitive $^{86}\text{Rb}^+$ transport as well as delayed blastocoele formation (173). In experiments on cRNA-injected *Xenopus* oocytes, the γ -subunit has been shown to influence the apparent affinity of the Na^+ - K^+ -ATPase for K^+ in a complex Na^+ - and voltage-dependent fashion (25), although the interpretation of these results remains unclear. A role of the γ -subunit in interactions of the Na^+ - K^+ -ATPase with K^+ had previously been suggested by Or et al. (259), who showed that the γ -subunit is a component of the protein complex found in so-called "19-kDa membranes." Such membranes are formed by tryptic digestion following occlusion of K^+ (or Rb^+) by the enzyme to form $\text{E}_2(\text{K})$ (181). More recently, Arystarkhova et al. (13) reported that the γ -subunit decreases both Na^+ and K^+ affinities of the sodium pump when transfected into NRK-52E cells transfected with γ_a cDNA (13). However, the decrease in Na^+ affinity is difficult to reconcile with the following: 1) the increase in K'_{Na} (~10-fold) is much larger than that (2-fold) observed for kidney compared with γ -subunit-free tissues (324, 327) if one takes into account the level of expression, and 2) a change in K'_{Na} could only be detected with cells expressing both γ'_a and γ_a , and not γ_a alone, despite the finding (195) that γ'_a appears to be a cell-specific modification of γ_a . In another recent report, the human γ -subunit has been shown to induce ouabain-independent ion currents in injected *Xenopus* oocytes and $^{86}\text{Rb}^+$ and $^{22}\text{Na}^+$ influx in baculovirus-infected Sf-9 cells (231). As described below, it is unclear whether this channel-like function is physiologically relevant, an artifact of high-level expression, or peculiar to human γ -subunit, for which the primary sequence at the extracellular amino terminus is notably different from that for several other species (231).

In addition to the aforementioned studies on baculovirus-infected Sf-9 cells, cRNA-injected *Xenopus* oocytes, and transfected NRK-52E cells, the possible functional role of the γ -subunit has recently been investigated in human HeLa and HEK cells. The initial approach was to test what effects, if any, an anti- γ antiserum had on the function of the sodium pump of rat kidney. A specific effect was evidenced in the finding that anti- γ inhibits Na^+ - K^+ -ATPase turnover in kidney, but not in tissues that do not express γ -subunit (325), and that a peptide corresponding to the epitope of the antiserum can abrogate the effect (326). Further analysis of the functional effects of anti- γ showed that it stabilizes the E_2 form(s) of the enzyme. Thus the

pH-dependence of the anti- γ -mediated inhibition of activity, together with the observation that Rb^+ protects against tryptic digestion of the γ -peptide (325), are consistent with a role of anti- γ in shifting the equilibrium of the K^+ -deocclusion reaction [$\text{E}_2(\text{K}) \leftrightarrow \text{E}_1$] toward $\text{E}_2(\text{K})$. On the basis of the well-documented effects of anti- L_p antigen on the kinetics of the LK sheep red blood cell Na^+ - K^+ -ATPase (see above), it was hypothesized that anti- γ mediates its effects by disrupting interactions between the Na^+ - K^+ -ATPase complex and the γ -subunit such that the role of the γ -subunit is to shift the aforementioned equilibrium toward E_1 . By transfecting the γ -subunit into HEK cells, it was recently shown that this is indeed the case (326). These experiments with transfected cells showed that the γ -subunit stabilizes the E_1 conformation of the Na^+ - K^+ -ATPase by increasing the affinity of the enzyme for ATP at its low-affinity site and that anti- γ reverses this increase in affinity in transfected cells (326). These findings, taken together with the observation that inhibition of Na^+ - K^+ -ATPase activity by anti- γ in the renal enzyme was increased at subsaturating concentrations of ATP, provide strong support for the conclusion that anti- γ reverses γ -subunit-mediated effects. It should be noted that a γ -subunit-mediated increase in the affinity of the enzyme for ATP may lead to a secondary decrease in its apparent affinity for K^+ (328), which would agree with the results of Arystarkhova et al. (13) regarding γ -subunit-mediated decrease in K^+ affinity. However, it is likely to be the change in ATP affinity that is physiologically important, as described below.

What is the physiological importance of a regulator of the affinity of the sodium pump for ATP? In most cells, ATP levels are sufficient to saturate the Na^+ - K^+ -ATPase, and therefore a modest shift in ATP affinity should not have dramatic effects. However, there are cases where ATP levels in intact cells are dramatically lowered, such as during anoxic shock. The relationship between anoxia, or hypoxia, and cellular ATP concentration has been studied in many tissues (15, 171, 191, 210, 230, 260, 310). As might be expected, dramatic decreases in ATP levels (30–90%) have been reported following brief periods of oxygen and/or glucose deprivation. In many cases, ATP concentration under anoxic conditions falls to a value that will affect Na^+ - K^+ -ATPase activity, assuming a K'_{ATP} of 400–800 mM (310). For example, Koop and Cobbold (191) estimated that chemical hypoxia lowers the concentration of ATP in intact hepatocytes to 50–100 μM . In addition, Milushcheva et al. (230) reported that incubation of rat striatal brain slices under glucose-free, hypoxic conditions for a relatively short period of time (30 min) can decrease cytoplasmic ATP levels to 10% of control, which, even assuming a relatively high starting concentration of 5 mM, translates to <500 μM . Finally, a direct correlation between hypoxia and sodium pump activity was provided by Aw and Jones (15), who observed a near total inhibition of sodium pump-mediated Rb^+ uptake in hepatocytes under conditions where ATP levels dropped a mere 40%. It might be argued that in

the aforementioned studies, anoxia was induced artificially, and that such conditions may not be relevant to situations in vivo. However, recent studies have shown that even in normal, disease-free organisms, at least one tissue, the kidney medulla, must function under near-anoxic conditions (reviewed in Refs. 56, 81). As is the case in most segments of the nephron, water and solute reabsorption and secretion in the medulla are under the control of the sodium pump. As such, continued pumping is crucial for proper kidney function. Therefore, the existence of a reversible regulator of Na^+ - K^+ -ATPase ATP affinity would allow for fine tuning of sodium pump activity under ATP-depleted conditions. This regulator should alter the affinity of the pump for the nucleotide only moderately, because an excessive increase would effect even greater decreases in ATP concentration (310), leading to compromised cell viability.

The γ -Subunit as a Member of a Family of Proteins

In recent years, several small single-transmembrane-domain peptides with high sequence homology to the γ -subunit have been identified. As such, studies on these peptides may reveal interesting information on the structure and function of the γ -subunit. To date, in addition to the γ -subunit itself, three members of this family have been cloned: phospholemman (PLM) (263), channel-inducing factor (CHIF) (14), and mammary tumor-associated 8-kDa protein (Mat-8) (237). Cloned sequences of these peptides include PLM of the mouse (48), dog (263), rat, and human (72), CHIF of the rat (14), and Mat-8 of the human (238) and mouse (237). Two additional sequences with homology to the γ -subunit family of proteins are known, namely, a "phospholemman-like protein" in humans (HPLP; Ref. 17), and a "regulated ion channel homologue" (RIC; Ref. 128) in the mouse. The amino acid sequences of the rat γ -subunit, CHIF, PLM, and mouse Mat-8 are compared in Fig. 1. For the rat γ -subunit, the revised sequence of γ_a is shown (231, 326), whereas for PLM, CHIF, and Mat-8, the sequences for the mature proteins, after cleavage of their putative signal peptide (see below), are shown. As illustrated in Fig. 1, the latter three proteins have 38–43% homology with the γ -subunit, and this value increases to 74–80% in the transmembrane domain and immediate flanking sequences (P^{18} to C^{52} of the rat γ -subunit). There are several highly conserved motifs present in most of the known sequences of this family of proteins. With the use of numbering for the rat γ -subunit, these motifs include 1) P^{18}FXDY in the extracellular domain, 2) G^{29}G in the transmembrane domain, and 3) $\text{S}^{47}\text{X(R/K)C(R/K)C}$ flanking the transmembrane domain on the cytoplasmic side. It should be noted, however, that in the γ -subunit, the third motif described above contains a Phe residue instead of the first Cys. Interestingly, Gly-30, Gly-41, and Ser-47 are 100% conserved among all known sequences. Of these, Ser-47 is especially intriguing because the nearby presence of positive charges (either K or R) make it a possible target for phosphorylation by protein kinase C (PKC).

Functional studies on these γ -subunit-like proteins may yield valuable information on the role of the γ -subunit in regulating cation transport. PLM, CHIF, and Mat-8 have all been expressed in *Xenopus* oocytes and, similarly to the γ -subunit (231), have been found to induce ion channel activity in this system. Specifically, CHIF has been shown to induce K^+ fluxes (14) consistent with its putative role in K^+ homeostasis (341), Mat-8 induces Cl^- conductance (238), and PLM appears to have a broad substrate specificity as evidenced by its apparent permissiveness for cations, anions, and zwitterions (192). Mutational studies on PLM have shown that residues in the transmembrane domain (236) and COOH terminus (73) are important for the channel-forming ability of this peptide. Overall, the available data indicate that members of the γ -subunit family of proteins can induce or form ion channels in *Xenopus* oocytes and, in the case of PLM, in lipid bilayers (235). However, two recent observations have cast doubt on the physiological relevance of such channel-forming activity: 1) similar hyperpolarization-dependent Cl^- conductances were observed in *Xenopus* oocytes individually injected with the cRNA for a variety of structurally unrelated small membrane proteins including PLM, and 2) hyperpolarizing pulses, albeit of greater magnitude, induced similar currents in uninjected oocytes (304). It may well be that the ion channel properties of small transmembrane proteins are non-specific and that γ -subunit-like proteins have other roles in regulating ion transport.

CIRCULATING ENDOGENOUS INHIBITORS

The finding that endogenous cardiac glycosides (ECG) exist in animals and, indeed, may have a physiological role, is relatively recent. To date, little is known about these substances because they seem to be present only at very low concentrations in the blood, yet there is evidence to support the notion that they function as endogenous sodium pump regulators (for more in-depth discussions, see Refs. 99, 154).

ECG have been isolated from mammalian blood (153) and urine (142) as well as from various tissues, in particular, the hypothalamus (333). They are believed to be synthesized in the adrenal gland (153, 197). Structurally, ECG are generally homologous to ouabain, consisting of a cholesterol core conjugated to either a lactone or pyrone ring and containing various combinations of hydroxyl, sulfate, or carbohydrate groups (99). Several compounds have been identified as ECG, including derivatives of bufadienolides (a cardiac glycoside synthesized by some toads; Ref. 159), stereo- or regioisomers of ouabain (362), and, more recently, ouabain itself (297).

The main physiological role of ECG appears to be in regulating blood pressure. Thus hypertension has been linked to increased levels of plasma ECG (287) and can result from long-term treatment with cardiac glycosides (357). The mechanism by which ECG mediate increased blood pressure is linked to the transmembrane equilibrium between Na^+ and Ca^{2+} via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (reviewed recently in Ref. 44). Thus

A

<u>gamma</u>	MTELSANHGGG AKGTEN · PFEYDYET VRKGGLIFAGL AFVV	40
<u>PLM</u>	E APQEPD · PFTYDYHTLRIGGLTAGILLFIL	30
<u>CHIF</u>	NGPVDKGS PFYYDWESLQLGGMIFGGLLCIA	31
<u>Mat-8</u>	NDPENKND PFYYDWYSLRVGGLI CAGILCAL	31
<u>gamma</u>	GLLILLSKR FRCGGS KKHRQVNE DEL	66
<u>PLM</u>	GILILLSKRCRCKFNQQQ RTGEP DEEEGTFRSSIRRLSTRRR	72
<u>CHIF</u>	GIAMALSGKCKCRRNHTPSSLPEKVTPLITPGSAST	67
<u>Mat-8</u>	GIIVLMSGKCKCKFRQ KPSHRPGEGPPLITPGSAHNC	68

B

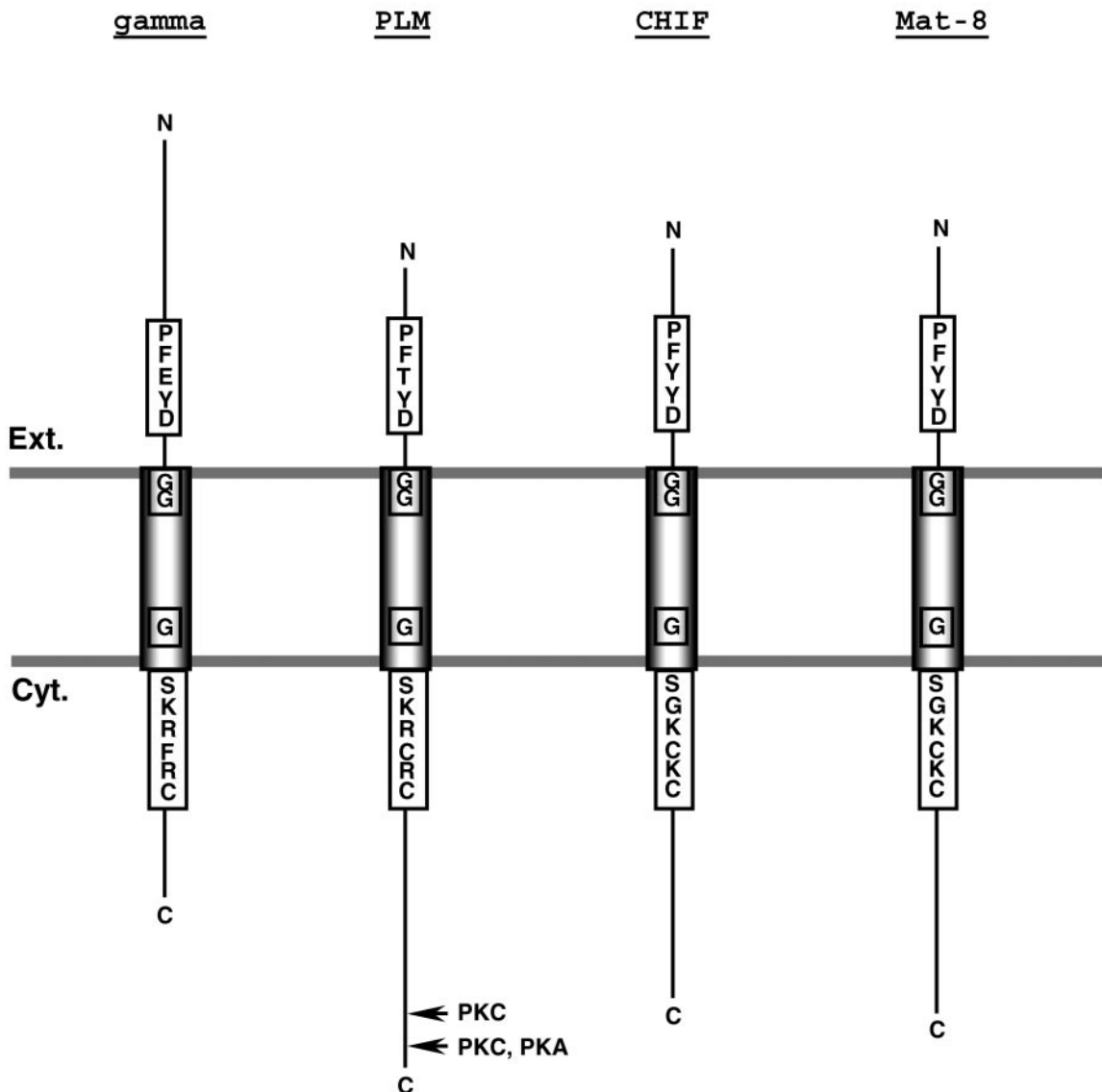


Fig. 1. Comparison of the members of the γ -subunit family of proteins. *A*: amino acid sequences of rat γ -subunit (γ_a), rat phospholemmann (PLM), rat channel-inducing factor (CHIF), and mouse 8-kDa mammary tumor-associated protein (Mat-8). For PLM, CHIF, and Mat-8, the putative cleaved amino-terminal signal peptide is not shown (see text and Ref. 263), whereas for γ_a , the recently revised sequence of γ_a is shown (see Refs. 231, 326). Dark-shaded X, identical residues; light-shaded X, conserved residues compared with γ_a . *B*: topology of γ_a and putative topologies of PLM, CHIF, and Mat-8. Conserved domains (shaded) as well as PKC and PKA phosphorylation sites (arrows) for PLM are indicated. N, NH₂ terminus; C, COOH terminus; Ext, extracellular side of membrane; Cyt, cytoplasmic side of membrane.

inhibition of the sodium pump in vascular smooth muscle cells and myocytes by ECG leads to an increase in the cytoplasmic Na^+ concentration, causing Ca^{2+} to enter the cell and be sequestered in the SR. Increased Ca^{2+} in the SR results in greater and sustained contractions of the vascular and heart muscle fibers, directly increasing blood pressure. Such a mechanism is also believed to be the basis for the partial reversal of cardiac insufficiency following treatment with cardiac glycosides (329). It should be noted, however, that such a mechanism of blood pressure regulation by ECG is only a hypothesis. Many investigators hold the view that hypertension is primarily a renal problem and that it does not result from changes in peripheral tension (for recent discussions, see Refs. 152, 207). The properties of ECG must therefore be investigated further before a consensus can be reached regarding the physiological role of these molecules.

HORMONAL REGULATION

The Na^+ - K^+ -ATPase is subjected to both short- and long-term regulation by a variety of hormones. Short-term regulation involves either 1) direct effects on the kinetic behavior of the enzyme or 2) translocation of sodium pumps between the plasma membrane and intracellular stores. On the other hand, long-term regulatory mechanisms generally affect *de novo* Na^+ - K^+ -ATPase synthesis or degradation. Of the various hormones that have been shown to alter sodium pump activity, the ones whose effects are best understood are catecholamines, peptide hormones, and steroid hormones. The regulatory role of many of these hormones as well as the known cellular mechanisms by which this regulation is achieved are described below. The focus is on short-term regulation, with a brief overview of the long-term regulatory effects of steroid hormones.

Corticosteroids

Steroid hormones, in particular, corticosteroids, have specific long- and short-term regulatory effects on the Na^+ - K^+ -ATPase. Long-term effects are generally mediated by changes in mRNA/protein synthesis induced by direct interactions of receptor/corticosteroid complexes with nuclear DNA. Though many types of corticosteroids have been shown to mediate regulation of the Na^+ - K^+ -ATPase (reviewed in Ref. 338), the most widely studied are the mineralocorticoid aldosterone and the glucocorticoid dexamethasone.

Corticosteroids are synthesized in and released by the adrenal cortex. Aldosterone in particular has long been known to have an important role in Na^+ and K^+ transport in epithelial tissues such as the kidney, and its physiological role is thought to be in long-term adaptation to decreases in Na^+ or increases in K^+ intake (reviewed in Refs. 49, 258). It has been shown that the main effect of aldosterone and dexamethasone on the Na^+ - K^+ -ATPase is to sustain a long-term increase in expression of sodium pumps, observed directly or as an increase in ouabain binding. This effect is widespread and has been observed in toad bladder

(137) and in many mammalian tissues including kidney (346) and kidney-derived cell-lines (302, 339, 347), colon (131), skeletal muscle (100), brain (144), heart (276), inner ear (272), cultured liver cells (41), vascular smooth muscle cells (252), and cultured cardiocytes (169). Experiments have shown that both steroid hormones can increase mRNA expression of the α - and β -subunit genes: aldosterone increases sodium pump mRNA expression via mineralocorticoid (type I) receptors in toad bladder (136), mammalian kidney (347), and hippocampus (107), whereas dexamethasone, presumably bound to glucocorticoid (type II) receptors, has similar effects in colon (131, 343), skeletal muscle (100), and cultured liver cells (41). In addition, the glucocorticoid betamethasone was shown to have an age-dependent effect on sodium pump mRNA in rat kidney and lung (68).

It has been shown that corticosteroid/receptor complexes mediate mRNA synthesis by interacting with regulatory elements 5' of both the α_1 - (252) and β_1 -subunit (92) genes. Corticosteroid-mediated increases in protein synthesis of sodium pumps may be dependent on changes in cytoplasmic Na^+ concentrations, as illustrated by abrogation of the effects in the presence of blockers of Na^+ transport (156, 169, 242). In addition, corticosteroid effects may be facilitated by the thyroid hormone triiodothyronine (T3) in mammals (349), but not in amphibians (137). Interestingly, long-term stimulation of the sodium pump by aldosterone is abrogated by inhibitors of the protein phosphatase calcineurin in cultured *Xenopus* kidney (A6) cells (285). In addition, there is evidence that cAMP-inducible factors have a role in mediating aldosterone-dependent increases in both α - and β -subunit mRNA (3, 348). These findings suggest the involvement of a protein phosphorylation cascade in long-term regulation by corticosteroids.

Recent experiments have shown that long-term up-regulation of Na^+ - K^+ -ATPase by corticosteroids can be isoform specific. Oguchi and co-workers (252) first showed that the α_1 -isoform, but not the α_2 - and α_3 -isoforms, is upregulated by aldosterone in cultured vascular smooth muscle cells (252). In contrast, α_3 -isoform is the main target for aldosterone-mediated regulation in brain (107, 144), whereas α_2 -isoform responds to aldosterone/salt treatment in heart (276).

Whereas the classic effects of aldosterone on the Na^+ - K^+ -ATPase are on long-term expression of the enzyme as described above, this mineralocorticoid has also been shown to have specific short-term effects on Na^+ - K^+ -ATPase activity. These short-term effects may be mediated by specific membrane-associated receptors, rather than the well-known nuclear mineralocorticoid receptors (345). Specifically, two distinct types of aldosterone-mediated short-term effects have been described. The first type is dependent on increases in cytoplasmic Na^+ concentration, because it is inhibited by amiloride (264, 269, 280, 302). The mechanism is hypothesized to involve an increase in membrane permeability to Na^+ , leading to an increase in cytoplasmic Na^+ concentration, a signal for translocation of pumps

to the plasma membrane (47, 302). This mode of regulation does not involve synthesis of new protein, because it is not sensitive to either actinomycin D or cycloheximide, inhibitors of nucleic acid and protein synthesis, respectively (47, 302). A second type of short-term aldosterone-mediated upregulation of $\text{Na}^+\text{-K}^+\text{-ATPase}$ has been observed in the rat cortical collecting tubule (18, 129) and A6 cells (30, 268). It is not inhibited by amiloride, nystatin, or amphotericin B or by incubation in the absence of extracellular Na^+ , and thus it is not dependent on increases in cytoplasmic Na^+ concentration. This type of modulation is sensitive to actinomycin D and cycloheximide and is partly stimulated by the hormone T3 (18, 29, 129, 268). The increase in activity may be secondary to changes in the number of plasma membrane sodium pumps (268) or to an increase in the intrinsic affinity of the enzyme for Na^+ (29). Recent findings suggest that the Na^+ -independent aldosterone-induced increase in $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity is isoform specific because α_1 pumps, but not α_2 pumps, transfected into A6 cells were affected (270).

Catecholamines

Although many catecholamines have been shown to affect $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity, the two most studied catecholamine regulators are norepinephrine and dopamine. They often act antagonistically as illustrated by their distinct roles in regulating salt reabsorption in the kidney (for reviews, see Refs. 8, 226).

Dopamine is a natriuretic factor synthesized in the kidney proximal tubule. It acts in both paracrine and autocrine fashion (for reviews, see Refs. 6, 168, 203). Dopamine was first shown to be an inhibitor of $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in the kidney proximal convoluted tubule (PCT; Ref. 7), but similar effects have since been observed in other regions of the kidney, namely, the medullary thick ascending limb (mTAL; Ref. 9) and cortical collecting duct (CCD; Ref. 292), as well as in cultured Madin-Darby canine kidney (MDCK) cells (301), neurons (37), arteries (279), retinal cells (306), aortic smooth muscle (278), small intestine (340), and lung (19). The overall consensus is that dopamine inhibits the $\text{Na}^+\text{-K}^+\text{-ATPase}$, and in the kidney, this represents a physiologically important mechanism for regulating salt reabsorption during high salt intake (see for examples Refs. 16, 36, 248). Illustrating this point is the observation that mechanisms of dopamine-dependent sodium pump modulation are often compromised in old (179, 340) and hypertensive (71, 149, 167, 178, 248, 249) rats.

Dopamine-dependent inhibition of $\text{Na}^+\text{-K}^+\text{-ATPase}$ appears to be both age related and cell specific (127). In the kidney, inhibition of sodium pumps in proximal segments of the nephron (for example, the PCT) is mediated through both types of dopamine receptors, DA_1 and DA_2 , and involves G protein-linked, PKC-dependent pathways (7, 32, 34, 35, 130, 177, 291), whereas in distal segments (mTAL and CCD), mainly DA_1 receptors and PKA-associated pathways appear to

be involved (9, 291, 292, 321). However, this receptor-type assignment is probably an oversimplification, because PKA-mediated pathways seem necessary for modulation of the enzyme in the PCT (32) and PKC-mediated inhibition has been observed in MDCK cells, a cell line derived from the distal part of the nephron (300, 301). A recent study has shed some light on this issue by showing that PKC-mediated pathways may be involved in short-term responses to dopamine inhibition, whereas PKA may have a role in long-term responses (271). Throughout the nephron, PLA_2 -activated elements, specifically, arachidonic acid and its metabolites, also have a role in dopamine-mediated inhibition (167, 292, 294). The recent observation that dopamine inhibits the ouabain-sensitive component (α_3 -isoform), but not the relatively ouabain-resistant component (α_1 -isoform), of rat rod cells (306) has raised the further possibility that dopamine may act in an isoform-dependent fashion in some systems.

Many of the mechanistic details of regulation of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ by protein kinases will be discussed below, but two aspects particular to regulation by dopamine should be mentioned at this point. First, it was recently demonstrated by Chibalin et al. (77) that dopamine-activated PKC signaling pathways result in endocytosis of pumps and that direct phosphorylation of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ at a specific serine residue (Ser-23 of the rat enzyme, a putative PKC phosphorylation site) is involved (78). Second, the PKA-activated pathway of dopamine inhibition seems to involve phosphorylation of both the sodium pump and the so-called dopamine and cAMP-regulated phosphoprotein (DARPP-32), the latter being an inhibitor of protein phosphatase 1 (PP1; Refs. 9, 126). In combination, the two mechanisms help to keep the enzyme in an inactive phosphorylated state.

Despite the present consensus that dopamine is a specific inhibitor of the $\text{Na}^+\text{-K}^+\text{-ATPase}$, at least when it binds to DA_1 receptors, two studies have shown that DA_2 agonists coupled to a pertussis toxin-sensitive G protein can stimulate $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity through a decrease in cellular cAMP levels (166, 354). Aizman et al. (4) have recently resolved this apparent dichotomy by showing that activation of DA_1 receptors in striatal neurons results in sodium pump inhibition, whereas DA_2 stimulation activates sodium channels, thereby increasing cytoplasmic Na^+ and presumably activating the $\text{Na}^+\text{-K}^+\text{-ATPase}$.

Besides dopamine, other catecholamines have marked effects on $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity. In particular, adrenergic agents such as epinephrine and norepinephrine have been found to specifically stimulate sodium pump activity (for examples, see Refs. 1, 12, 20, 69, 94, 150, 162, 175, 314, 342). Like dopamine, their effects on activity are probably tissue specific. For example, epinephrine seems to be involved in stimulating K^+ uptake by skeletal muscle after exercise-induced hyperkalemia (reviewed in Refs. 84, 208), whereas norepinephrine, acting as a dopamine antagonist, appears to have a role in Na^+ reabsorption in the nephron (reviewed in Refs. 8, 226). In addition, several

catecholamines, including norepinephrine, act as neurotransmitters in the central nervous system. Their likely importance as stimulators of $\text{Na}^+\text{-K}^+\text{-ATPase}$ in neural tissue is to reestablish the electrochemical cation gradient across the cell membrane following transmission of electrical impulses (reviewed in Ref. 158).

In addition to these tissue-specific effects, adrenergic catecholamines may increase the susceptibility of the sodium pump to inhibition by ethanol (176, 277), although the physiological relevance of this observation remains unknown.

Adrenergic catecholamines modulate $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity through two general mechanisms. The first is nonreceptor mediated and involves direct effects on the enzyme or chelation of inhibitory divalent metals (86, 283, 313). The physiological relevance of this mode of regulation is unknown, but the effects seem to occur only at very high concentrations of catecholamine (313). The second pathway, more likely to be physiologically important, is more complex and involves stimulation via α -adrenergic or β -adrenergic receptors and both PKC and PKA pathways. The role of different protein kinases in catecholamine stimulation of $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity appears to be tissue specific. Thus catecholamine-dependent increases in cAMP levels, and, therefore, stimulation of PKA, have been shown to activate $\text{Na}^+\text{-K}^+\text{-ATPase}$ of brown adipose tissue (162), ventricular myocytes (132), kidney cortex (139), smooth muscle of the stomach (234) and arteries (344), skeletal muscle (206), and macrophages (98), whereas PKC-mediated pathways appear to be responsible for sodium pump stimulation in hepatocytes (217), ventricular myocytes (342), and skeletal muscle (206). Regulation can be mediated through α -adrenergic receptors (12, 342), β -adrenergic receptors (1, 175), or both (162, 314). Generally, β -adrenergic stimulation is associated with activation of PKA pathways, whereas α -adrenergic agents stimulate PKC-dependent effects. Paradoxically, the β -adrenergic receptor agonist isoproterenol stimulates $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in most tissues but inhibits it in kidney medulla (139), brain (121), and COS-7 cells (75). These contradictory results have been explained recently, at least for the mTAL enzyme, where PKA agonists were found to stimulate the pump under oxygenated conditions and inhibit it under nonoxygenated conditions (188). The mechanism of catecholamine regulation of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ was investigated recently by Bertorello and co-workers (40), who showed that in lung alveolar cells, isoproterenol increases the number of sodium pumps at the plasma membrane through a PKA-mediated mechanism involving the cytoskeleton but not direct phosphorylation of the pump. Isoproterenol has, however, been shown to mediate direct phosphorylation of the sodium pump, either at a PKA site, as observed with the rat enzyme transfected into COS cells (75), or at a PKC site, as seen with the brain enzyme (121). Interestingly, both effects appear to be mediated through PKA activation. These complexities are not surprising in view of the varied nature of protein kinase effects as described below.

In the kidney proximal tubules, stimulation of the sodium pump by α -adrenergic agents has been shown to involve protein phosphatase 2B (PP2B), a Ca^{2+} - and calmodulin-dependent phosphatase also called calcineurin. For example, an inhibitor of calcineurin, FK-506, blocks oxymetazoline-dependent stimulation of the pump, whereas a calcium ionophore, A-23187, mimics it (12). Because the actions of norepinephrine in the kidney appear to counter the inhibitory effects of dopamine, it has been suggested that the sodium pump is regulated in this organ by the antagonizing actions of calcineurin, which would serve to keep the pump in an active, dephosphorylated state, and protein kinases, which would keep the enzyme in an inactive, phosphorylated form (8, 11, 226).

Although it is clear that catecholamines have highly specific effects on the $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in most tissues and cells, the role of specific signaling pathways in catecholamine regulation of the sodium pump remains controversial. An example in point is the recent report showing that both adrenergic (α and β) as well as dopaminergic (DA_1) receptors transfected into COS-7 cells are linked to PKA-activated pathways (23). It is unclear how receptors that activate similar signaling mechanisms can mediate opposite effects.

Peptide Hormones

Peptide hormones comprise a major class of $\text{Na}^+\text{-K}^+\text{-ATPase}$ regulators. The peptide hormone whose effects on the pump have been best characterized is insulin, a major metabolic hormone that regulates glycolytic storage and plays an important role in K^+ homeostasis. In particular, increased uptake of K^+ by various tissues is a well-known effect of insulin and has been ascribed mainly to stimulation of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ (reviewed recently in Ref. 316; see also Ref. 106). Insulin modulates cell functions by binding to the insulin receptor, which results in activation of a variety of intracellular signaling processes.

There are several mechanisms of short-term effects of insulin on the $\text{Na}^+\text{-K}^+\text{-ATPase}$. One example is the insulin-mediated translocation of sodium pumps from intracellular stores to the cell surface. The first evidence was obtained, and later substantiated, in experiments with frog skeletal muscle (145, 256). Such rapid translocation is considered to be the main mechanism of pump stimulation in skeletal muscle (reviewed in Refs. 106, 316). Insulin-dependent increases in surface pump expression are independent of amiloride (83, 135) and cycloheximide (145) and are thus not secondary to changes in cytoplasmic Na^+ concentration and protein synthesis, respectively. Experiments on rat skeletal muscle have shown that the effect of insulin on cell-surface expression of pumps is specific to oxidative slow-twitch muscles, rather than glycolytic fast-twitch muscles (200), and to pumps comprising $\alpha_2\beta_1$ heterodimers, with increases in α_1 and β_2 not detected (165, 222). Short-term insulin-mediated sodium pump stimulation can also be secondary to an increase in the cytoplasmic Na^+ concentration. For example, increases

in cell Na^+ are a consequence of insulin stimulation of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter or Na^+ channels in adipocytes (57, 289) or of the $\text{Na}^+\text{/H}^+$ exchanger in hepatocytes (216). Another short-term route of insulin-mediated upregulation of $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity has been observed in the kidney. In studies of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ of kidney cortical tubules, insulin appeared to increase the apparent affinity of the enzyme for Na^+ (111, 113). As with insulin-mediated increases in Na^+ concentration, this can result in stimulation of the sodium pump in normally low Na^+ cells.

In addition to the aforementioned short-term mechanisms of regulation, insulin also has long-term effects on the $\text{Na}^+\text{-K}^+\text{-ATPase}$. These effects are complex and have been evidenced in both increases and decreases in pump activity, the latter being particularly relevant to diabetes (for review, see Ref. 316).

Despite the clear evidence for short-term regulation of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ following the administration of insulin, the mechanisms remain largely unknown. It has been shown that PKC may have a role in the insulin-mediated activation of $\text{Na}^+\text{-K}^+\text{-ATPase}$ in cultured rat skeletal muscle cells (288). More recently, Sweeney and Klip (316, 317) have shown that inhibition of specific kinases, namely, 1) the phosphatidylinositol 3-kinase, 2) a specific isoform of PKC (PKC- ζ), and 3) p38 MAP kinase, all abrogate the insulin effect on $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in 3T3-L1 fibroblasts. In addition to their independent cellular roles, signaling cascades effected by these kinases converge on the PLA_2 pathway, indicating that regulation of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ by insulin may involve arachidonic acid and its metabolites as described below. A role for tyrosine phosphorylation in insulin-mediated pump regulation has also been demonstrated (112).

As mentioned above, insulin is the most widely studied peptide hormone regulator of the sodium pump. However, many other such hormones have specific regulatory effects on the enzyme. In particular, parathyroid hormone has been shown to specifically inhibit the pump through a pathway that involves a Ca^{2+} -independent PLA_2 (93). Another peptide whose effects on the pump have been widely studied is angiotensin II, which appears to increase the affinity of the enzyme for intracellular Na^+ in a PKC-dependent mechanism (59). Other peptide hormones that modulate pump activity are insulin-like growth factor I (205), epithelial growth factor (112), vasopressin (125, 350), atrial natriuretic peptide (26, 295), the cytokine interleukin-1 (358), and endothelin (359).

SIGNALING EVENTS INVOLVED IN HORMONE ACTION

Most of the hormones that regulate the $\text{Na}^+\text{-K}^+\text{-ATPase}$ do so through signaling mechanisms that modulate the activities of a group of protein kinases, phospholipases, and phosphatases. The interplay between the main effectors of regulation of the sodium pump and their effects on the $\text{Na}^+\text{-K}^+\text{-ATPase}$ are shown in Fig. 2 and described below.

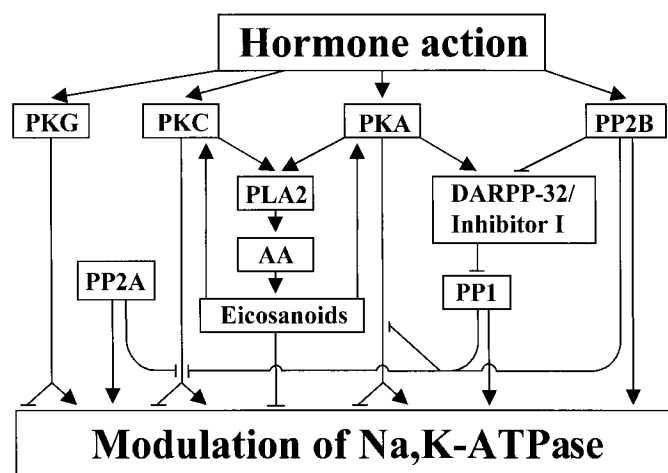


Fig. 2. Summary of the major mechanisms of hormonal regulation of the $\text{Na}^+\text{-K}^+\text{-ATPase}$. The main effectors of hormonal regulation of the sodium pump and their interactions are shown. The scheme is summarized from published reports of the various effects in different tissues, as described in the text. Activation (arrow) and inhibition (crossbar) are indicated. PKA, PKC, and PKG, protein kinases A, C, and G; PLA_2 , phospholipase A_2 ; AA, arachidonic acid; PP1 and PP2B, protein phosphatases 1 and 2B; DARPP-32, dopamine and cAMP-regulated phosphoprotein.

PKA

cAMP-activated protein kinase, or PKA, is activated by the intracellular accumulation of cAMP (reviewed in Ref. 22). The enzymes that regulate cAMP levels in the cell are adenylate cyclase, which synthesizes it, and cAMP phosphodiesterase, which degrades it. Therefore, signals that activate or inhibit these two enzymes affect cAMP levels and thus PKA activation. Increases in cAMP concentration can be effected by incubation with various hormones (as described in HORMONAL REGULATION), cAMP or cAMP analogs (such as bromo-cAMP or dibutyryl-cAMP), stimulators of adenylate cyclase (e.g., forskolin), or inhibitors of phosphodiesterase (e.g., IBMX). Effects of cAMP levels on $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity have been observed in various tissues, and the nature of the effect varies in a tissue-specific manner as shown in Table 1. The reason for this variability is unclear, although Cheng et al. (74) have recently shown that in COS cells, the concentration of Ca^{2+} ions is an important determinant of whether PKA inhibits or stimulates the pump. This finding is especially intriguing in light of the relationship between cytoplasmic Ca^{2+} and Na^+ concentrations (see CIRCULATING ENDOGENOUS INHIBITORS; see also Ref. 45). In addition to tissue-specific effects, there is evidence that PKA affects the $\text{Na}^+\text{-K}^+\text{-ATPase}$ in a species-dependent manner. For example, following incubation with cAMP, sodium pump activity of salivary glands is stimulated in the dog (189) but unchanged in the rat (223).

The mechanisms by which PKA alters $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity are varied and complex and have only recently begun to be investigated. The most straightforward effect of PKA is through direct phosphorylation of the sodium pump, which is suggested to be the mechanism of action of enzyme inhibition by β -adren-

Table 1. Summary of $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ regulation by PKA

Tissue/Cell	Effectors*	Effect*†	Ref.‡
Brown adipose tissue (rat)	cAMP, adrenergic agents	↑	162
Liver (rat)	(Chlorpropamide, phenformin)	(↑)	215
Skin (frog)	cAMP, oxytocin	↑	2
Reconstituted renal enzyme (human)	cAMP	↓	54
Thyroid (guinea pig)	cAMP	↑	170
Colon (rat)	cAMP, bisacodyl	↑	299
Tail artery (rat and pig)	cAMP, isoproterenol	↑	344
Swiss 3T3 cells	BrcAMP	↑	265
Brain (rat)	cAMP, PKA	↓	209
Sperm (hamster)	cAMP, PKA	↓	239
Kidney CCT, cTAL (rabbit)	db-cAMP, isoproterenol, vasopressin	↓	350
Kidney medulla (rat)	db-cAMP, forskolin, IBMX, isoproterenol	↓	139
Kidney cortex (rat)	db-cAMP, forskolin, IBMX, isoproterenol	↑	139
Macrophage (mouse)	cAMP, IBMX	↑	98
Hepatocytes (rats)	db-cAMP	↑	52
Rectal gland (shark)	cAMP	↑	224
Urethral smooth muscle (guinea pig)	cAMP, PGE, forskolin, IBMX	↑	337
Diaphragm muscle fibers (rat)	db-cAMP, theophylline, aminophylline	↑	90
Retinal pigment epithelium (frog)	cAMP	↑	164
Submandibular gland (dog)	db-cAMP	↑	189
Pancreatic islets (rat)	db-cAMP, theophylline, caffeine	↓	332
Rectal gland (shark)	Purified PKA	↓	39
Kidney cortex (rat)	Purified PKA	↓	39
Ciliary epithelium (rabbit)	db-cAMP	↓	88
Rectal gland (shark)	db-cAMP, theophylline	↑	202
MDCK cells (dog)	db-cAMP, PGE	↓	323
Kidney CCD (rat)	db-cAMP, dopamine, forskolin, others	↓	291
Stomach smooth muscle cells (toad)	BrcAMP, forskolin	↑	234
Sciatic nerves (rat)	db-cAMP, cilostazol, iloprost	↓	305
Sensory neurons (leech)	db-cAMP, forskolin, IBMX	↓	67
Kidney PCT (rat)	db-cAMP, forskolin	↓	55
Skeletal muscle (rat)	BrcAMP, isoproterenol	↑	206
Ventricular myocytes (guinea pig)	Forskolin, IBMX	↑	132
Transfected COS cells (rat α_1)	Forskolin, IBMX	↑	120
Motor nerve (rat)	db-cAMP, aminophylline, PGE	↓	355
Kidney PCT (rabbit)	db-cAMP	↑	21
Kidney PCT (rat)	db-cAMP, BrcAMP, forskolin, IBMX	↑	63
Rectal gland (shark)	PKA	↓	87
Transfected COD cells (rat α_1)	Isoproterenol, forskolin, IBMX	↓	75
Transfected HeLa (rat $\alpha_1, \alpha_2, \alpha_3$)	Forskolin, IBMX	↓	247
Aortic smooth muscle cells	BrcAMP, forskolin, IBMX, isoproterenol	↓	51
Infected Sf-9 cells (rat α_1, α_2)	db-cAMP	↓	43
Infected Sf-9 cells (rat α_3)	db-cAMP	↑	43
RN22 Schwann cells	BrcAMP, forskolin, cholera toxin	↑	312
Skeletal muscle (squirrel)	cAMP	↑	218
Kidney mTAL (rat)	db-cAMP, forskolin, IBMX (+ oxygen)	↑	188
Kidney mTAL (rat)	db-cAMP, forskolin, IBMX (- oxygen)	↓	188

* Kinase inhibitors and their effects are shown in parentheses. † Activation (↑) or inhibition (↓) of [^3H]ouabain binding or strophanthidin/ouabain-sensitive ATPase activity, $^{86}\text{Rb}^+$ or $^{22}\text{Na}^+$ transport, PNPPase activity, current, or oxygen consumption. ‡ References are listed in chronological order. In cases where several studies have led to the same conclusion, only the first is cited. PKA, protein kinase A; BrcAMP, δ -bromo-cAMP; db-cAMP, dibutyryl-cAMP; CCT, cortical collecting tubule; cTAL, cortical thick ascending limb; CCD, cortical collecting duct; PCT, proximal collecting tubule; mTAL, medullary thick ascending limb; MDCK, Madin-Darby canine kidney.

ergic agents, such as isoproterenol (see, for example, Ref. 75). Bertorello et al. (39) first showed that the shark rectal gland and rat kidney enzymes are phosphorylated by PKA in vitro, with 1 mole of phosphate incorporated per mole of enzyme. Similar results were obtained with the enzymes of duck salt gland, *Bufo marinus*, and *X. laevis* (80). It was later shown that PKA phosphorylates the pump in vivo and that the site of PKA phosphorylation is at Ser-943 (note that the numbering of amino acids used in this monograph includes the posttranslationally cleaved NH_2 -terminal 5 amino acids) in the enzyme of rat (120) and *B. marinus* (24). In the former study, Fisone and co-

workers (120) also showed that phosphorylation of Ser-943 results in inhibition of enzyme activity, an effect abrogated by mutation of the serine residue to alanine. Similar experiments by Andersson et al. (5) showing that PKA-induced phosphorylation and inhibition of activity in rat α_1 -transfected COS-7 cells is not associated with internalization of the pumps have led these authors to suggest direct effects on the catalytic turnover of the enzyme. Finally, Kiroytcheva et al. (188) recently showed that there is a correlation between PKA-dependent phosphorylation of the sodium pump and activation of ouabain-sensitive Rb^+ uptake and $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity in oxygenated, but not hy-

poxic, conditions. However, the role of direct phosphorylation by PKA in regulating sodium pump activity is not straightforward. Recent experiments have shown that phosphorylation of Ser-943 plays a permissive role in allowing phosphorylation of the pump by PKC at Ser-23 (76). Consistent with a dependence of PKA-mediated phosphorylation on enzyme conformation, Feschenko and co-workers (116, 119), using rat enzyme and purified PKA, found that phosphorylation of Ser-943 occurs mainly in the presence of stabilizers of the E₁ enzyme conformation. Although direct phosphorylation of the Na⁺-K⁺-ATPase appears to correlate with the well-documented PKA-mediated stimulation of enzyme phosphorylation and ouabain-sensitive ⁸⁶Rb⁺ uptake in renal proximal tubules (63), there is evidence to support the notion that the activation is secondary to an increase in plasma membrane pumps (64). Perhaps related to this are the observed PKA-induced increases in plasma membrane pumps of MDCK (323) and Schwann cells (312).

Although direct phosphorylation of the Na⁺-K⁺-ATPase by PKA is an attractive simple mechanism for PKA-mediated regulation of the enzyme and appears to apply to at least some systems, other more complex mechanisms have been observed. Lingham and Sen (209) were the first to suggest that PKA required the presence of an intermediate protein to mediate its effects on the sodium pump in rat brain. More recently, Satoh et al. (293) showed that PKA inhibits Na⁺-K⁺-ATPase activity in the renal collecting duct by activating the PLA₂ pathway, specifically by increasing synthesis of eicosanoids that presumably downregulate Na⁺-K⁺-ATPase activity. Activation of PLA₂ is also suggested to be the mechanism of PKA-mediated pump inhibition in mTAL under nonoxygenated conditions (188). In other systems, PKA appears to activate a protein phosphatase inhibitor, which in turn alters sodium pump activity (9). In addition to the foregoing, the cytoskeletal protein actin has been postulated to have a role in mediating PKA regulation of the rat kidney sodium pump. Cantiello (61) showed that phosphorylation of monomeric actin by PKA prevented the actin-mediated stimulation of the sodium pump, whereas phosphorylation of polymeric actin promoted it. Finally, in some cases, PKA does not regulate the sodium pump directly but, rather, alters the function of other Na⁺ transporters, leading to changes in cytoplasmic Na⁺ concentration, which in turn alter Na⁺-K⁺-ATPase activity (164, 312).

In recent years, isoform-specific effects of PKA have been reported in some systems. Whereas Nestor et al. (247) showed that the PKA activators forskolin and IBMX effect a significant inhibition of the rat α₁-, α₂-, and α₃-isoforms in transfected HeLa cells, Blanco and co-workers (43) later reported that treatment of Sf-9 cells transfected with the individual rat isoforms with dibutyryl-cAMP results in inhibition of α₁- and α₂-isoform pumps, activation of α₃-isoform pumps, and direct phosphorylation of all three isoforms (43). In studies with ventricular myocytes, Gao et al. (133) have shown that the targets of PKA-dependent effects

of β-adrenergic agents are pumps comprising the α₁- but not the α₂-isoform (133).

PKC

The cascade that results in activation of PKC is usually initiated by activation of the membrane-bound phospholipase C, which cleaves phospholipids into two components: phosphatidylinositol trisphosphate, which in turn increases cytosolic Ca²⁺, and diacylglycerol (DAG; for a recent review, see Ref. 211). DAG allows the inactive, cytoplasmic form of PKC to bind to the membrane and increases its affinity for Ca²⁺ and phospholipids, its final activators. Activated PKC is a potent regulator of many enzymes, including the Na⁺-K⁺-ATPase. Experimentally, increases in PKC can be achieved in the cell by incubation in the presence of phorbol esters or DAG analogs (66). As is the case with cAMP/PKA-mediated regulation of the Na⁺-K⁺-ATPase and as shown in Table 2, the effects of PKC activation on the enzyme are varied and tissue specific. In particular, Table 2 highlights discrepancies in the effects of PKC on the Na⁺-K⁺-ATPase of renal proximal tubules (33, 38, 62, 109, 257) and OK cells (a cell line derived from proximal tubules of opossum kidney) (78, 79, 229, 267), where PKC has been shown to mediate either stimulation or inhibition of the enzyme, as discussed below.

The question of the mechanisms of PKC regulation of the Na⁺-K⁺-ATPase is controversial. The aforementioned dichotomy regarding the enzyme of proximal convoluted tubules illustrates the many contradictions present in the literature. Efendiev et al. (105) have recently shed some light on the subject by showing that the nature of the effect of PKC on the sodium pump depends on the isoform of PKC involved. In addition, and similarly to PKA, the nature of PKC effects is dependent on the Ca²⁺ concentration, at least in COS cells (74). Mechanistically, PKC-dependent activation of the Na⁺-K⁺-ATPase in the proximal nephron appears to be secondary to an increase in Na⁺ influx, possibly via the Na⁺/H⁺ exchanger (38), and seems to be an oxygen-dependent process (109). Inhibition of proximal tubule enzyme by PKC, on the other hand, is mediated by one of two mechanisms. The first involves activation of the PLA₂ pathway (257) and is discussed below. The second involves direct phosphorylation of the sodium pump by PKC at Ser-23 of the α-subunit, leading to endocytosis of pumps as observed by Chibalin and co-workers using α₁-transfected OK cells (78, 79). Endocytosis secondary to direct phosphorylation of the sodium pump is also the suggested mechanism of PKC-mediated inhibition of the Na⁺-K⁺-ATPase in *Xenopus* oocytes (335, 336). As already mentioned, phosphorylation of the rat enzyme at Ser-23 appears to require prior phosphorylation at the PKA site, Ser-943 (76). Taken together with the fact that PKC-mediated internalization of sodium pumps is postulated to be the mechanism for dopamine-dependent inhibition of activity in proximal tubules (78), this observation may explain the requirement of both DA₁-activated PKA- and DA₂-activated PKC-mediated pathways for the full

Table 2. Summary of $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ regulation by PKC

Tissue/Cell	Effectors*	Effect*†	Ref.‡
Nerve (diabetic rat)	PMA, dioctanoylglycerol	↑	143
Hepatocytes (rat)	PMA, mezerein	↑	217
Pancreatic acinar cells (guinea pig)	TPA	↑	161
Erythrocytes (hypertensive human)	TPA	↑	274
Brain (rat)	(ET-18-OCH ₃ , BM 41.440)	(↓)	255
Tracheal smooth muscle (rabbit)	PDB	↓	298
Kidney PCT (rat)	OAG, PDB	↓	33
Nerve (diabetic rabbit)	DOG, PMA	↑	198
Oocytes (frog)	PMA	↓	336
Ileal smooth muscle (guinea pig)	PDB, PDA, PMA	↑	290
Aorta (rabbit)	PDB, endothelin	↑	148
Rectal gland (shark)	Purified PKC	↓	39
Kidney cortex (rat)	Purified PKC	↓	39
Sciatic nerve (diabetic mouse)	(H7)	(↓)	157
L 1210 cells (mouse leukemia)	PMA	↑	183
Kidney PCT (rat)	OAG (short-term)	↑	38
	OAG (long-term)	↓	38
MDCK cells (dog)	OAG, DOG, PMA	↓	301
Ciliary epithelium (rabbit)	PMA	↓	243
OK cells (opossum)	PDB	↓	229
Skeletal muscle (rat)	PMA	↑	206
Cultured ciliary epithelial (human)	PDB	↑	232
Skeletal muscle cells (rat)	PMA, insulin	↑	288
Brain (rat)	PDB, serotonin	↓	122
Kidney PCT (rat)	PDB (+ oxygen)	↑	109
	PDB (- oxygen)	↓	109
Vascular smooth muscle cells (rat)	PMA	↓	351
Kidney PCT (rat)	PDB, DOG, dopamine, PTH	↓	257
Cerebellar neurons (rat)	PMA	↓	220
Arterial endothelial cells (cow)	(Calphostin, staurosporine, H7)	(↑)	70
Transfected oocytes (toad α_1)	PMA	↓	28
Transfected HeLa (rat $\alpha_1, \alpha_2, \alpha_3$)	PMA	↓	247
Transfected OK cells (rat α_1)	PMA	↑	267
Transfected COS cells (rat α_1)	20-HETE	↓	250
Mucociliary cells (frog)	TPA, DOG	↓	138
Aortic smooth muscle cells	PDB, PMA, AVP	↑	51
Transfected <i>Xenopus</i> oocytes (rat α_1)	PMA (endogenous PKC activation)	↓	335
	PKC (rat)	↓	335
<i>Xenopus</i> oocytes (endogenous)	PMA (endogenous PKC activation)	↓	335
	PKC (rat)	↑	335
Ciliate epithelial cells (rabbit)	PDB	↑	89
Infected Sf-9 cells (rat $\alpha_1, \alpha_2, \alpha_3$)	PMA	↓	43
Kidney cortex (rat)	PDB	↓	204
Ventricular myocytes (guinea pig)	(staurosporine)	(↓)	342
Aorta (rat)	PDB	↑	196
3T3 fibroblasts (mouse)	(bisindolylmaleimide)	(↓)	317
Vascular smooth muscle cells (rat)	(bisindolylmaleimide)	(↓)	205
Transfected COS cells (toad α_1)	PDB (37°C)	↓	108
	PDB (18°C)	↑	108

*Kinase inhibitors and their effects are shown in parentheses. †Activation (↑) or inhibition (↓) of [³H]ouabain binding or strophanthidin/ouabain-sensitive ATPase activity, ⁸⁶Rb⁺ or ²²Na⁺ transport, PNPPase activity, current, or oxygen consumption. ‡References are listed in chronological order. In cases where several studies have led to the same conclusion, only the first is cited. PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TPA, 12-O-tetradecanoylphorbol 13-acetate; DPB, phorbol 12,13-dibutyrate; OAG, 1-oleoyl-2-acetate-*sn*-glycerol; PTH, parathyroid hormone; 20-HETE, 20-hydroxyeicosatetraenoic acid; AVP, arginine vasopressin; DOG, 1,2-dioctanoyl-*sn*-glycerol; PDA, phorbol 12,13-diacetate.

dopamine effect in this tissue (32, 37). The observation that PKA mediates PKC phosphorylation in nerves (50) shows that this type of mechanism may not be restricted to the kidney.

As mentioned above, direct phosphorylation of the $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ is one of the mechanisms by which sodium pump activity is regulated by PKC. Such phosphorylation was first shown in vitro for the duck salt gland and dog kidney enzymes (213) and subsequently for the enzyme of shark rectal gland and of rat kidney

(39) and *B. marinus* and *X. laevis* kidney (80). Middleton and co-workers (229) showed that phosphorylation of the sodium pump by PKC can occur in vivo. Their results showed that treatment of intact OK cells with the PKC activator phorbol dibutyrate results in phosphorylation of a protein that comigrates on SDS-PAGE with the α -subunit of the sodium pump, as well as inhibition of $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity. Similar treatment of the enzyme of LLC-PK cells was without effect. Identification of the PKC-phosphorylated residue has

been hampered by the presence of several putative cytoplasmic PKC phosphorylation sites on the α -subunit of the sodium pump (see, for example, Ref. 118). Nevertheless, the general consensus is that PKC phosphorylation occurs primarily at the NH_2 terminus of the catalytic subunit *in vivo*. For example, the *B. marinus* enzyme is phosphorylated by PKC in intact transfected COS-7 cells mainly at Thr-15 and Ser-16 (24), whereas the mammalian enzyme is phosphorylated at low levels on Ser-16, and in the rat, at higher levels on Ser-23 (118). Feschenko et al. (119) have recently examined two interesting aspects of phosphorylation of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ by PKC. They found that *in vitro* phosphorylation of the rat α_1 -enzyme by purified PKC is facilitated by agents that stabilize the E_2 conformation of the enzyme and that the $\text{Na}^+\text{-K}^+\text{-ATPase}$ itself can stimulate PKC autophosphorylation. The physiological consequences of these observations have yet to be determined. Although the absence of Ser-23 in dog and pig enzyme shows that PKC may not have a major role in direct phosphorylation of the sodium pump *in vivo* in these species, as mentioned earlier, phosphorylation of Ser-23 appears to be an important mechanism by which PKC modulates the rat kidney enzyme. Other experiments supporting this conclusion include recent studies showing that neither a Ser-23 \rightarrow Ala mutant transfected into COS cells (27) nor a deletion mutant lacking the first 31 amino acids transfected into OK cells (267) is modulated by PKC activators, even though the wild-type enzyme is affected in both systems. Other experiments have shown that inhibition of the rat α_1 -enzyme by phosphorylation of Ser-23 is due to a shift in the conformational equilibrium toward E_1 , leading to a decreased apparent affinity for K^+ (212). In experiments on the *B. marinus* enzyme transfected in COS-7 cells, Féraille and co-workers (108) have recently shown that PKC-dependent phosphorylation of the pump at Ser-16 results in a stimulatory effect that is attributable to an increase in the affinity of the enzyme for Na^+ (108). In addition, direct phosphorylation of the sodium pump is the proposed mechanism of action of PKC in rat choroid plexus (122), aorta (196), and nerves (50).

The foregoing results notwithstanding, the physiological relevance of direct phosphorylation of the pump by PKC in regulating the $\text{Na}^+\text{-K}^+\text{-ATPase}$ has recently been questioned. Thus a PKC-mediated decrease in plasma membrane sodium pumps of A6 cells transfected with the *B. marinus* enzyme is not associated with phosphorylation of residues 15 and 16 (31). Consistent with this are the observations that 1) a deletion mutant of the rat α_1 -enzyme lacking the first 32 amino acids was inhibited by PKC activators to the same extent as the wild-type enzyme (247) and 2) phosphorylation of Ser-23 by activators of PKC in a rat kidney cell line, NRK-52E, had no effect on either maximum velocity or apparent Na^+ affinity of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ (117). These experiments represent unequivocal evidence that direct phosphorylation of the sodium pump by PKC, at least at the NH_2 terminus, cannot explain many of the PKC-mediated effects on the en-

zyme and that other mechanisms must be involved, especially in species such as the dog and pig, where Ser-23 is absent. Indeed, there is considerable evidence for PKC-dependent mechanisms of $\text{Na}^+\text{-K}^+\text{-ATPase}$ regulation independent of pump phosphorylation. One mechanism involving stimulation of the pump secondary to increases in cytoplasmic Na^+ via the $\text{Na}^+\text{/H}^+$ exchanger has been suggested to result in activation of the pump in cultured ciliary epithelial cells (232) as well as kidney proximal tubules (38). Another plausible mechanism of PKC-mediated stimulation, without direct phosphorylation, involves stimulation by PKC of the PLA_2 pathway. As described below, PLA_2 produces arachidonic acid, whose metabolites, the eicosanoids, can have highly specific effects on the sodium pump. PLA_2 -mediated PKC regulation has been observed not only in kidney proximal tubules (257), as already mentioned, but also in vascular smooth muscle cells (351), transfected COS-7 cells (108), and pancreatic β -cells (261). In the latter case, however, both PLA_2 -specific effects and PKC-mediated phosphorylation of the sodium pump were reported, suggesting that the two mechanisms may act in concert to inhibit $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity. Such a model is compatible with the observed dual mechanism of PKC-mediated pump inhibition in proximal tubules (for example, see Refs. 78, 257). Another mechanism of PKC-mediated regulation of the sodium pump has been recently described. Nemoto and co-workers (246) showed that PKC-dependent mechanisms mediate the serum-induced increase in β_1 -subunit mRNA in vascular smooth muscle cells, which implies a role of PKC in long-term regulation of the sodium pump.

Effects of PKC on the sodium pump are not restricted to the α_1 -isoform. For example, PKC-dependent inhibition of the rat α_1 -, α_2 -, and α_3 -isoforms has been described in transfected HeLa cells (247) as well as Sf-9 cells (43). In the latter system, direct phosphorylation of the α -isoforms was observed. In those studies, differences in the extents of inhibition of the different isoforms were not detected. However, more recent work suggests that, like PKA, PKC can affect enzyme activity in an isoform-specific fashion. Thus, in experiments with guinea pig ventricular myocytes, PKC-dependent effects were shown to modulate α_2 but not α_1 pumps (133), whereas in experiments with frog mucociliary cells, PKC effected almost complete inhibition of the ouabain-sensitive activity without a change in the ouabain resistant activity, also suggesting isoform-specific effects (138).

PKG

cGMP-dependent protein kinase (PKG) is another kinase that appears to have highly specific effects on the $\text{Na}^+\text{-K}^+\text{-ATPase}$. In a mechanism similar to the one involved in PKA activation, PKG is activated by cGMP, the cytoplasmic concentration of which is regulated by synthesis by guanylate cyclase, and degradation by cGMP phosphodiesterase (reviewed in Ref. 334). Increases in cGMP have been shown to inhibit

the $\text{Na}^+\text{-K}^+\text{-ATPase}$ in colon (299), skeletal muscle (206), brain (273), cultured alveolar cells (146), and infected Sf-9 cells (43). Conversely, cGMP is involved in activation of the enzyme in duck salt gland (311), mammalian aorta and arteries (115), pulmonary arterial smooth muscle (322), ciliary epithelium (65), Purkinje neurons (244), and NB-OK-1 cells (91). In the kidney, cGMP and PKG have been shown to inhibit (26, 319, 360) or stimulate (225, 295) the $\text{Na}^+\text{-K}^+\text{-ATPase}$. Although the basis for these conflicting results is unknown, the effects of cGMP/PKG are sometimes antagonistic to those of cAMP/PKA as, for example, in ciliary epithelium (65), rat skeletal muscle (206), and hamster sperm (239). The mechanism of PKG-activation appears to involve activation of guanylate cyclase by nitric oxide (NO). For example, there are reports that increases in NO via hormonal activation or incubation with NO donors such as sodium nitroprusside increase cGMP levels in cultured vascular smooth muscle cells (147), aorta and arteries (115), brain (273), renal proximal tubules (360), and alveolar cells (146). In intact cells, NO-stimulated cGMP synthesis is mediated by the neurotransmitters acetylcholine (225) and glutamate (244) as well as by atrial natriuretic peptide (26, 225, 295). Whether PKG regulates the pump through secondary modulators or by direct phosphorylation of the pump is unknown, although in one system cGMP appears to stimulate the sodium pump indirectly by increasing Na^+ influx via the $\text{Na}^+\text{-K}^+\text{-Cl}^-$ cotransporter (251).

Isoform-specific effects of PKG on the $\text{Na}^+\text{-K}^+\text{-ATPase}$ have also been observed. Thus PKG modulates $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity of α_3 - but not α_1 -isoform in Purkinje neurons (244), α_1 - but not α_2 - or α_3 -isoform in brain endothelial cells (273), and α_1 - and α_3 - but not α_2 -isoform in infected Sf-9 cells (43).

Tyrosine Kinases

In addition to the serine/threonine kinases mentioned above, tyrosine kinases have been shown to mediate $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity. Specifically, tyrosine kinases appear to have a role in the stimulatory effects of insulin and epithelial growth factor in kidney proximal tubules (112). Recent experiments on transfected OK cells have shown that the mechanism of stimulation involves direct phosphorylation of Tyr-10 of the rat enzyme (110).

Protein Phosphatases

Many of the effects of protein kinases on the $\text{Na}^+\text{-K}^+\text{-ATPase}$ can be reversed by protein phosphatases. Regulation of the sodium pump by the antagonistic actions of protein kinases and phosphatases has been studied extensively in the kidney and brain (reviewed in Ref. 126; see also Refs. 121, 204) and has also been observed in skeletal muscle (218) and ventricular myocytes (132). The major participants in protein phosphatase-dependent modulation of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ are PP1 and PP2B.

The role of PP1 in countering the effects of protein kinases is thought to represent an important mechanism of pump inhibition by dopamine through the DA_1 receptor and isoproterenol via the β -adrenergic receptor. Such inhibition is mediated in part by the activation of the PP1 inhibitors DARPP-32 and inhibitor-1 (I1) (see, for example, Refs. 10, 121). Thus it has been shown that the increase in cAMP levels mediated by dopamine or isoproterenol in kidney and brain leads to phosphorylation of DARPP-32, which in turn becomes a potent inhibitor of PP1 (9, 121, 227). Therefore, the inhibition of $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity by stimulation of PKA in these two organs involves the synergistic effects of 1) direct phosphorylation of the enzyme by protein kinases and 2) inhibition of PP1 by DARPP-32 and I1 (8, 121). Although DARPP-32 is involved in sodium pump regulation in most parts of the kidney and in brain, its low expression in renal PCT precludes such a role in this segment of the nephron (308). In addition to its role in regulating the kidney enzyme, inhibition of PP1 activity by okadaic acid or calyculin A has been shown to affect $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in ventricular myocytes (132) and both pump activity and phosphorylation level in the rat skeletal muscle cell line L6 (275).

The physiological role of PP2B, or calcineurin, in the kidney has recently been reviewed (331). It is a Ca^{2+} - and calmodulin-dependent enzyme that, upon activation by norepinephrine and α -adrenergic receptor agonists, activates the $\text{Na}^+\text{-K}^+\text{-ATPase}$ of most segments of the nephron (201), although its main effects are on the enzyme of PCT (12). Other activators of calcineurin in the kidney include neuropeptide Y and the connecting peptide of proinsulin, C-peptide (253). It has also been suggested that the role of calcineurin in the kidney is to counter dopamine-induced inhibition of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ and that it does this by dephosphorylating targets of dopamine-stimulated protein kinases (8). It has been suggested that calcineurin mediates its stimulatory effects at least in part by increasing the apparent affinity of the sodium pump for Na^+ (12). In addition to its role in the kidney, calcineurin mediates ouabain-induced upregulation of surface expression of $\alpha_1\beta_1$ pumps in cultured astrocytes (163) and has a role in sodium pump activation during glutamate toxicity in rat neurons (220) and in the long-term upregulation of the sodium pump by aldosterone in A6 cells (285).

Another protein phosphatase shown to modulate $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity is protein phosphatase 2A (PP2A), which increases pump plasma membrane expression in cortical collecting duct (46) and counters PKC-mediated inhibition of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ in Sf-9 infected cells (43). Paradoxically, inhibitors of PP2A stimulate the pump in hepatocytes (216). Finally, tyrosine phosphatases may also modulate $\text{Na}^+\text{-K}^+\text{-ATPase}$ function in PCT (112) and liver (58), as evidenced by the stimulatory effect of vanadate ions acting as tyrosine phosphatase inhibitors.

PLA₂

As discussed above, the PLA₂ pathway of pump regulation can be activated by both PKA and PKC. Activated PLA₂ can cleave phospholipids in the membrane to generate lysophospholipids and arachidonic acid, both of which have been shown to have specific effects on the Na⁺-K⁺-ATPase. Arachidonic acid is further metabolized in the cell by a variety of oxygenases to form eicosanoids, including prostaglandins (PG), thromboxanes (TX), and oxygenated compounds such as hydroxyeicosatetraenoic acids (HETE) and epoxyeicosatrienoic acids (EET), all of which are modulators of the Na⁺-K⁺-ATPase (293).

The consequence of lysophospholipids as well as arachidonic acid and its metabolites on the Na⁺-K⁺-ATPase are generally inhibitory. Thus addition of lysophosphatidylcholine to sarcolemmal membranes of mammalian heart caused a 50% inhibition of Na⁺-K⁺-ATPase activity (180). Similarly, arachidonic acid has been shown to be one of the mediators of dopamine-induced inhibition of the sodium pump in the kidney (291). Further studies using this system revealed that the effectors of arachidonic acid-mediated inhibition are its metabolites, specifically prostaglandin E (PGE) and the various products of cytochrome P-450-dependent monooxygenase-mediated cleavage of arachidonic acid, including HETE and EET (293). In addition to effects on the renal enzyme, PG can alter Na⁺-K⁺-ATPase activity in other tissues (for examples, see Refs. 89, 184, 266). Satoh and co-workers (293) showed that PGE inhibits the pump by decreasing intracellular Na⁺, whereas HETE and EET have direct effects on the sodium pump. The precise mechanism whereby eicosanoids inhibit sodium pump activity is unknown.

In addition to acting directly on the sodium pump, eicosanoids have also been shown to stimulate protein kinases, resulting in modulation of the pump via mechanisms described above. For example, PG modulates cAMP levels, thereby affecting sodium pump activity in several mammalian tissues and cells, including small intestine (303), smooth muscle (337), nerves (355), macrophages (53), and MDCK cells (323). Recently, a role of PKC in eicosanoid-mediated sodium pump regulation was also observed in rat α₁-transfected COS cells (250) and pancreatic β-cells (261).

CONCLUSIONS

The need for the ubiquitous Na⁺-K⁺-ATPase to adapt to the diverse needs of different tissues underscores the importance of mechanisms for regulating its activity. The signaling cascades involved in hormonal regulation, in particular, are varied and complex. Alterations in activity may be the result of posttranslational modification such as phosphorylation. It remains to be determined whether and to what extent such modifications affect the Na⁺-K⁺-ATPase α-subunit, per se, or some regulatory component. An added complexity is the question of whether various kinase isoforms such as those of PKC can have differential effects on sodium pump activity, offering a possible

explanation for differences in regulation in various tissues and of the various pump isoforms.

In certain instances, alterations in Na⁺-K⁺-ATPase activity and kinetic behavior result from specific interaction with other membrane components. These include proteins intrinsic to the plasma membrane as well as those of the cytoskeleton. Such interactions are clearly tissue specific, and studies of the nature and mechanism of regulation by these components are a current topical and exciting area of investigation.

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