SEMINARS IN
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Hormonal and Nonhormonal Mechanisms of Regulation of the Na,K-Pump in Collecting Duct Principal Cells

Manlio Vinciguerra, David Mordasini, Alain Vandewalle, and Eric Feraille

In the kidney, the collecting duct (CD) is the site of final Na^+ reabsorption, according to Na^+ balance requirements. In this segment of the renal tubule, principal cells may reabsorb up to 5% of the filtered sodium. The driving force for this process is provided by the basolateral Na,K-adenosine triphosphatase (ATPase) (sodium pump). Na,K-ATPase activity and expression in the CD are modulated physiologically by hormones (aldosterone, vasopressin, and insulin) and nonhormonal factors including intracellular [Na^+] and extracellular osmolality. In this article, we review the short- and long-term hormonal regulation of Na,K-ATPase in CD principal cells, and we analyze the integrated network of implicated signaling pathways with an emphasis on the latest findings.

KEYWORDS Na,K-ATPase, collecting duct principal cells, aldosterone, vasopressin, sodium

Kidneys play a major role in the homeostasis of body fluid compartments. Despite large qualitative and quantitative changes in the dietary intake of solutes and water, these bean-shaped organs are able to maintain the composition and the volume of the body fluids within a very narrow range. Another important function of the kidney is the clearing of waste products, such as urea and creatinine, generated by metabolic processes. To accomplish this clearing function, kidneys filter daily large amounts of fluid (close to 180 L) to maintain low circulating concentrations of these substances and to ensure elimination of waste products. Ultrafiltrate is generated during the passage of blood into the glomeruli. The reabsorption process taking place along kidney tubules results in the daily generation of 1 to 2 L of final urine containing 1% to 5% of the filtered Na^+ load. This tremendous work is accomplished by successive renal tubule segments, which exhibit specific functional properties and hormonal control.[1] Quantitatively, most of the H_2O and Na^+ reabsorption process takes place, in order, in the proximal tubule, loop of Henle, and distal convoluted tubule (altogether these segments reabsorb about 95% and 85% of the filtered Na^+ and water, respectively). Connecting tubules and collecting ducts (CDs) are responsible for the final, and fine tuning of, H_2O and Na^+ reabsorption, their function being controlled tightly by hormones and nonhormonal factors to meet water and Na^+ balance requirements.

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General Transport Properties of CDs

CDs are made up of 2 different cell types: intercalated cells and principal cells. Intercalated cells are involved in acid/base regulation and their role is not discussed here. On the other hand, principal cells are responsible for water and sodium reabsorption, and potassium secretion. The function of principal cells requires the presence of specific membrane transporters to drive unidirectional Na^+, K^+, and H_2O fluxes. Fig. 1 shows the functional organization of the CD principal cell: active electrogenic sodium transport generates a lumen-negative transepithelial voltage (0 to -60 mV), which is higher in the more cortical part of the CD (-10 to -60 mV), and which decreases in the deeper medullary portions as a result of decreased sodium reabsorption by principal cells combined with increased electrogenic proton secretion by

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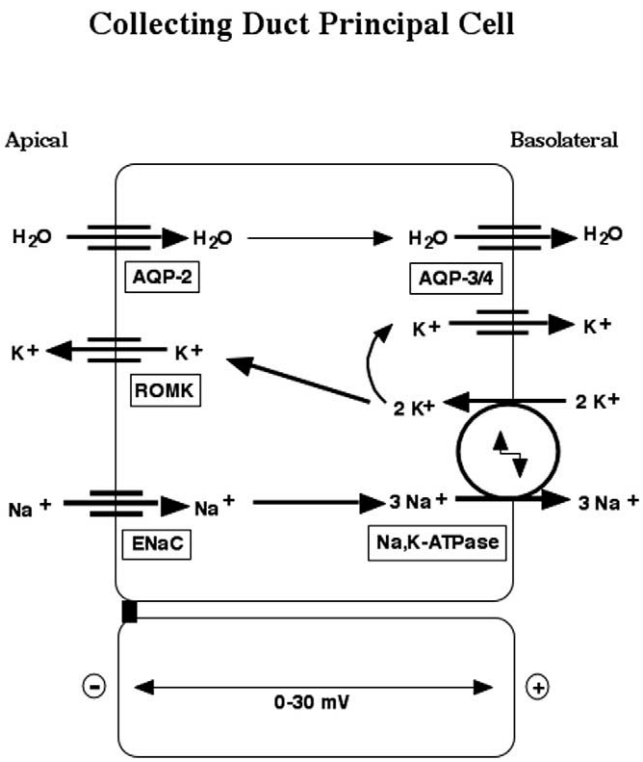

Figure 1 Mechanism of sodium, potassium, and water transport in principal cells of the CD. Arrows indicate net fluxes of water and ions. The names of the currently cloned transporters are shown in rectangular boxes.

intercalated cells. Water enters the luminal (apical) side via aquaporin 2 water channels and leaves the cell through basolateral aquaporin 3 and 4 water channels. Although the driving force for water reabsorption is provided mainly by the countercurrent concentrating mechanism in the loop of Henle, sodium reabsorption along the CD, especially in the cortical part, dilutes the luminal fluid and contributes to the generation of an osmotic gradient favorable to water reabsorption. As shown in Fig. 1, sodium reabsorption in principal cells is linked to potassium secretion through a 2-step mechanism: pumping of K^+ within and Na^+ out of the cell by the basolateral Na,K-pump generates driving forces for apical sodium entry and potassium exit.

K^+ exit occurs through apical (belonging to the renal outer medulla K-channel (ROMK) family) and basolateral potassium channels, these latter being of several types of various conductance: their respective roles in potassium transport are not understood clearly. Through this mechanism, potassium secretion is coupled primarily to sodium reabsorption with the $2K^+$:$3Na^+$ stoichiometry of the Na,K-pump. The apical sodium influx is mediated by the amiloride-sensitive Na^+ channel (ENaC) and intracellular Na^+ then is extruded by basolateral Na,K-adenosine triphosphatase (ATPase), which provides the driving force for active Na^+ reabsorption and for secondary active transport of other solutes.[2] Long-term regulation of Na,K-ATPase relies mainly on alteration of the expression of its α and β subunits, whereas short-term control is mediated by changes in enzymatic turnover and/or redistribution between the cell surface and intracellular compartments. The 2 major hormonal factors that positively control sodium reabsorption and Na,K-ATPase activity and expression are aldosterone and vasopressin, although insulin may play a significant role in the postprandial period. Moreover, recently an important role of intracellular Na^+ and extracellular osmolality in controlling Na,K-pump expression has been highlighted. This article primarily reviews the current knowledge on the molecular events responsible for stimulatory effects on Na,K-ATPase and, secondarily, analyzes how these stimulatory effects on the Na,K-pump might be balanced by the negative influence of several mediators such as dopamine, α_2-adrenergic agonists, and prostaglandins.

Control of Na,K-ATPase Activity and Expression by Aldosterone

The major physiologic role of the mineralocorticoid hormone aldosterone is to increase extracellular volume in response to volume depletion signaled by the renin-angiotensin system. Aldosterone participates in the restoration of plasma volume mainly by stimulating the reabsorption of Na^+ from the lumen of the renal tubules and accessorily of other organs, such as the sweat glands and the distal colon. Aldosterone also plays an important role in K^+ homeostasis: on the one hand, high extracellular K^+ is a stimulus for aldosterone secretion and, on the other hand, the secretion of K^+ into the kidney tubule is linked directly to the aldosterone-regulated Na^+ reabsorption that generates the electrical driving force for K^+ secretion.[3] Aldosterone controls Na^+ reabsorption in CD principal cells by coordinated stimulation of the activities of apical ENaC and basolateral Na,K-ATPase (Fig. 2). In renal epithelial cells, the Na,K-ATPase works at about 20% of its maximal rate,[4] therefore, a large kinetic reserve allowing activation of Na,K-pump by intracellular Na^+ is available. However, a coordinated control of Na,K-ATPase that matches that of ENaC is necessary to maintain the stability of intracellular [Na^+], and therefore ENaC function, over a broad range of reabsorption rates. Indeed, in the absence of simultaneous stimulation of ENaC and Na,K-ATPase, an increased Na^+ influx through ENaC would increase intracellular [Na^+] and subsequently decrease ENaC activity by feedback inhibition.[5] The physiologic response to aldosterone action can be separated into short-term and long-term effects. The short-term (early) aldosterone effect on Na^+ reabsorption (and on K^+ secretion) can be observed after 30 minutes of aldosterone stimulation.[6,7] The long-term (late) effect of aldosterone induces a more sustained increase in the transport capacity of the target cells.[3]

In addition to these effects described earlier, mediated by the classic corticosteroid receptor/DNA interaction (reviewed by Féraille and Doucet[1] for the specificity and the localization of mineralocorticoid and glucocorticoid receptors along the nephron), aldosterone has been shown over the past few years to produce near-immediate cardiovascular effects whose physiologic role remains to be elucidated. These ef-

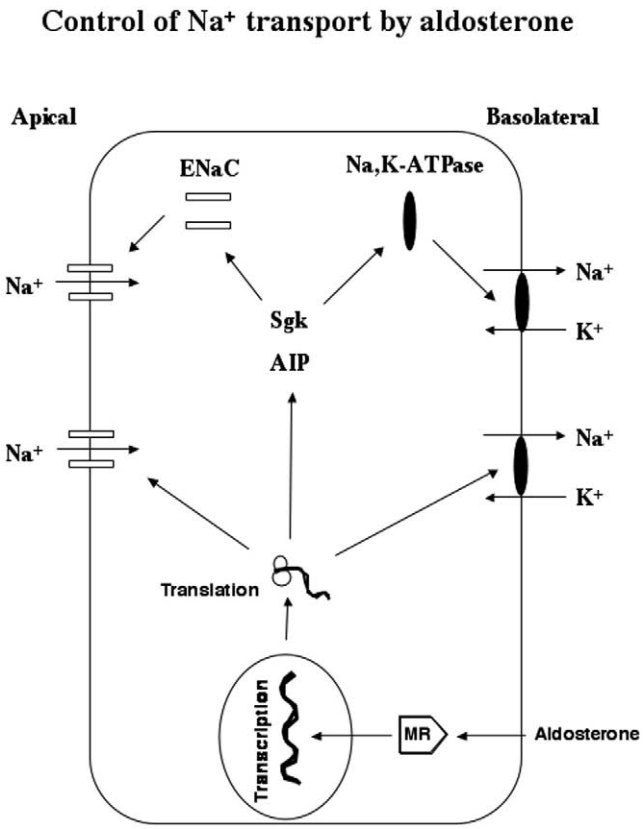

Figure 2 Overview of the control of sodium transport by aldosterone. Arrows indicate the direction of the signaling cascade. AIP, aldosterone induced protein; MR, mineralocorticoid receptor; SGK, serum and glucocorticoid-induced kinase.

fects are mediated by a nontranscriptional mechanism (also denominated *nongenomic* or *nonclassic*) that must involve receptor not yet identified.[8] Very recently, Le Moëllic et al[9] provided some experimental pieces of evidence indicating that aldosterone quickly stimulates transepithelial Na^+ transport through a nongenomic effect in cultured rat cortical collecting duct (CCD) cells.

The long-term treatment of animals with aldosterone was shown, already 20 years ago, to increase the amount of Na,K-pump α-subunit messenger RNA (mRNA), protein, and activity in the CCD (reviewed by Verrey et al[10]). This long-term regulation appears to depend also on other factors that play a permissive role, such as the presence of 3,3′,5-tri-iodothyronine.[11] Interestingly, this slow induction of Na,K-ATPase expression (able to be measured at a protein level only after 6-18 hours) can be traced back to a rapid transcriptional activation that was measured already 15 minutes after hormone treatment.[12,13] This observation is consistent with the presence of several glucocorticoid response elements along the 5′-flanking region of Na,K-pump α_1-subunit gene.[14] The long delay between the very early transcriptional response and the late accumulation of active pumps is accounted for by the fact that the pumps are numerous and represent a stable component of the transport machinery, with a half-life of approximately 1 day. Thus, it takes several hours until an approximately 3-fold increase in pump production impacts on the total pool level, as a result of accumulation of mRNA, translation, assembly of the α and β subunits, and insertion into the plasma membrane.[15]

The slow time course of cellular Na,K-ATPase accumulation obviously is not compatible with the functional short-term regulatory action of aldosterone described earlier. Indeed, early studies showed that the activity of the Na,K-ATPase, measured by hydrolytic activity after cell permeabilization by freeze thawing, slowly decreased after adrenalectomy but rapidly returned (1-3 h) to normal levels on aldosterone infusion in the mammalian CCD.[16,17] Subsequently, Barlet-Bas et al[18] and Blot-Chabaud et al[19] independently showed the presence of a mineralocorticoid-dependent pool of functionally silent Na,K-pumps. These observations raised the question of whether aldosterone activates this latent pool of preexisting Na,K-ATPase units or whether it induces de novo synthesis of Na,K-ATPase. Our laboratory, in collaboration with the Verrey laboratory, recently investigated the mechanism of the short-term stimulation of Na,K-ATPase by aldosterone in isolated rat CCDs and in mouse pyruvate kinase (mpk)CCD_{c14} cells,[20] a model of mammalian CD principal cells. The mpkCCD_{c14} cell model, derived from mouse CCD,[21] develop a tight epithelium and retained expression of transporters specific for CD principal cells, including ENaC and aquaporin 2, and controlled transepithelial Na^+ transport by aldosterone and vasopressin.[22-24] Intravenous infusion of aldosterone in adrenalectomized rats induced a nearly 3-fold increase in Na,K-ATPase activity. This stimulation was associated with a several-fold increase in Na,K-ATPase cell-surface expression measured by Western blot performed after biotinylation and streptavidin precipitation of cell-surface proteins. In contrast, the total cellular pool of Na,K-pumps increased to a much lesser extent. Similar results were obtained in mpkCCD_{c14} cells, which displayed an increase in maximal Na,K-pump current and cell-surface expression of Na,K-ATPase after 2 hours of incubation in the presence of aldosterone. On the other hand, Western blots of total mpkCCD_{c14} cell extracts revealed that the total pool of Na,K-ATPase increased later and to a lesser extent than cell-surface expression. Altogether, these results indicate that short-term aldosterone stimulates the Na,K-pump through an increase in cell-surface expression of Na,K-ATPase. This effect most likely relies on the translocation of an intracellular reservoir of Na,K-pumps to the plasma membrane and is independent of increased abundance of total Na,K-ATPase subunits. The rapid stimulatory effect of aldosterone originally observed at the level of Na,K-ATPase activity can be explained by the permeabilization technique used (freeze thawing), which allows ATP diffusion into the cytosol but not into intracellular vesicles.[25] The localization of this aldosterone-responsive intracellular pool of Na,K-pumps, whose presence in kidney cells already strongly has been suggested by cell-fractionation and cell-surface labeling studies,[25,26] remains to be investigated by morphologic studies.

The short-term stimulation of Na,K-ATPase activity and cell-surface expression is independent of apical Na^+ entry through ENaC and requires de novo transcription and translation.[20] These results indicate that aldosterone-induced re-

cruitment of Na,K-ATPase is not mediated by an increase in intracellular Na^+ concentration brought about by ENaC stimulation and it most likely is mediated by one of several short-term aldosterone-induced and/or -repressed proteins. Corticosteroid Hormone Inducible Factor (CHIF), the first identified aldosterone-induced protein,[27] is expressed along the CD[28] and associates with the Na,K-ATPase to increase its apparent Na^+ affinity.[29] Although CHIF expression is induced by aldosterone in the distal colon, this is not the case in the kidney.[30] Therefore, CHIF is unlikely to play a major role in the early stimulation of CD Na,K-ATPase in response to aldosterone. By using a differential display strategy, Verrey et al[31] identified several aldosterone-induced genes in aldosterone-responsive epithelial A6 cells.[31] Among them, K-ras, a small G protein, was shown to stimulate ENaC current and surface expression in the *Xenopus laevis* oocyte expression system,[32] and plays a rate-limiting role for Na^+ transport activation by aldosterone in A6 epithelia.[33] However, K-ras mRNA is not induced by aldosterone in mammalian CD principal cells. Serial analysis of gene expression in cultured mouse mpkCCDcl4 cells[23] and substractive hybridization on isolated rat CCD[34] identified several aldosterone-induced genes, but their role in the modulation of transepithelial Na^+ transport remains to be established. Recently, the serum- and glucocorticoid-regulated kinase 1 (SGK1) has received much attention as an aldosterone-induced protein. SGK1 is a serine/threonine kinase that first was identified in mammary tumor cells and subsequently in hepatoma cell line HepG2 on the basis of its induction in response to high osmolarity.[35] SGK1 is a component of the phosphoinositide 3-kinase signaling pathway and requires phosphorylation by Phosphatidylinositol Dependent Kinase (PDK) for activity and nuclear translocation.[36] Experiments performed in adrenalectomized rats revealed that, in CD principal cells, SGK1 mRNA and protein are induced 2 hours after administration of aldosterone.[37,38] Moreover, co-expression of SGK1 and ENaC subunits in the *Xenopus* oocyte expression system strongly stimulates ENaC activity and cell-surface expression.[39] Recent studies indicated that SGK1 binds and phosphorylates the ubiquitin-ligase Nedd4 to 2, thereby reducing the interaction between ENaC and Nedd4 to 2, leading to enhanced ENaC cell-surface expression.[40,41] Taken together with in vivo experiments,[37] these results suggest a role of SGK1 in aldosterone-induced ENaC cell-surface recruitment in CD. However, induction of SGK1 per se is not sufficient to recruit ENaC to the cell surface: SGK1 was induced throughout the connecting tubule and CD whereas ENaC was translocated to the cell surface in the connecting tubule and CCD only.[37] In addition to the modulation of ENaC activity, the question has been raised whether SGK1 also modulates Na,K-ATPase cell-surface expression. Expression of SGK1 in *Xenopus* oocyte stimulates the endogenous Na,K-pump current without variation of intracellular $[Na^+]$, suggesting that SGK1 primarily stimulates Na,K-ATPase activity.[42] Co-expression of exogenous rat Na,K-ATPase and SGK1 in *Xenopus* oocyte increased both exogenous Na,K-pump current and Na,K-ATPase cell-surface expression, as visualized by Western blotting of surface-biotinylated proteins.[43] In contrast, a kinase-dead mutant SGK1 had no effect on Na,K-ATPase activity and expression. The SGK1-dependent increase in Na,K-ATPase cell-surface expression was independent of intracellular $[Na^+]$ and was specific because the cell-surface expression of Na/phosphate cotransporter NaPi-IIa or heterodimeric amino acid transporter LAT1-4F2hc were not altered by co-expression of SGK1. Taken together, these studies strongly suggest that SGK1 modulates ENaC and Na,K-ATPase cell-surface expression in a coordinated manner. However, these findings require validation by further experiments performed in a reliable model of mammalian CD principal cells. The physiologic control of renal Na^+ handling by SGK1 has been highlighted by the generation of SGK1-knockout mice, which exhibit impaired ability to reduce urinary Na^+ excretion in response to dietary Na^+ restriction.[44] It should be mentioned that the relatively mild phenotype observed in SGK1-knockout mice might be explained by functional redundancy of SGK isoforms.[45]

Finally, a recent study explored whether the short-term effect of aldosterone on Na,K-ATPase cell-surface expression might depend on α subunit isoform-specific structures.[46] Results obtained in $mpkCCD_{c14}$ cells expressing functional exogenous human $\alpha 1$ or $\alpha 2$ subunit showed that aldosterone stimulated only $\alpha 1$-subunit–containing Na,K-ATPase isozymes, indicating that aldosterone responsiveness is dictated by $\alpha 1$-subunit–specific sequences.

Control of Na,K-ATPase Activity and Expression by Vasopressin

Vasopressin (AVP), through its V_2 receptors coupled to adenylyl cyclase, stimulates the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling pathway in CD principal cells (Fig. 3). Stimulation of water reabsorption via increased water permeability of the apical membrane of principal cells is the major effect of AVP in CD. However, AVP also stimulates sodium reabsorption and potassium secretion along the CD. In vitro microperfusion studies performed in rat CCD have shown that vasopressin, as well as cAMP analogues, induce a rapid increase in Na^+ reabsorption.[47,48] AVP and cAMP stimulate Na^+ reabsorption by coordinate activation of ENaC and Na,K-ATPase. In isolated rat CCD, stimulation of Na^+ transport in response to AVP/cAMP is associated with a sustained increase in the lumen-negative transepithelial voltage and in apical Na^+ conductance.[49-51] The stimulatory effect of cAMP on ENaC was confirmed by patch-clamping experiments on the apical membrane of principal cells from isolated rat CCDs. In this preparation, cAMP treatment before formation of the patch increases the density of active ENaC.[52] The activation of ENaC by AVP also was observed in amphibian A6 cells in which AVP and cAMP analogues also increased the density of active ENaC.[53] This effect was prevented by brefeldin A, an agent that disrupts the Golgi apparatus (by disrupting the adenosine diphosphate (ADP) ribosylation factor 1–dependent vesicular traffic) and prevents the delivery of newly synthesized or recycling pro-

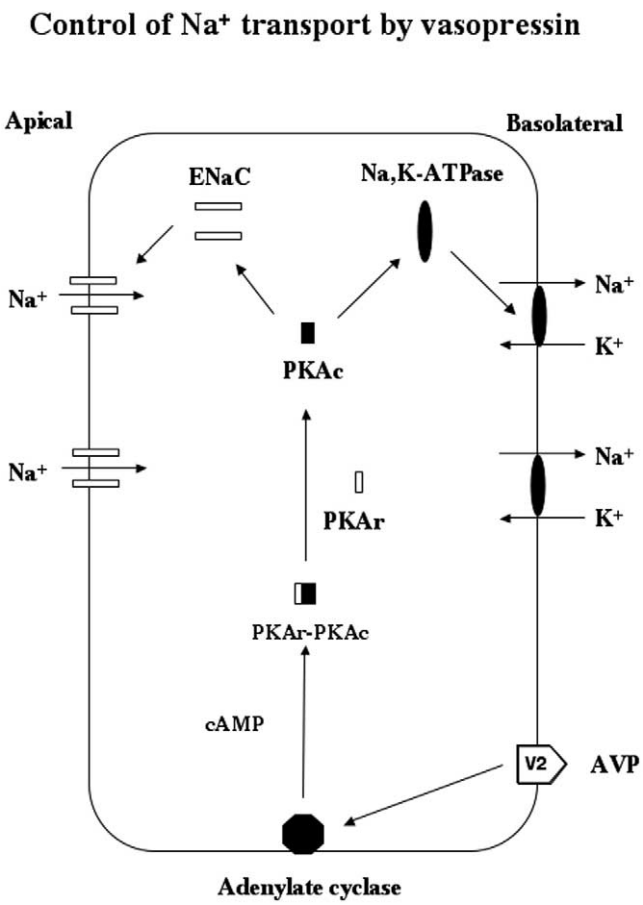

Figure 3 Overview of the control of sodium transport by vasopressin. Arrows indicate the direction of the signaling cascade. PKAR, protein kinase A regulatory subunit, V_2 receptor.

teins from this intracellular compartment. In addition to these short-term (minutes) effects on apical Na^+ conductance, long-term (hours) vasopressin stimulation augmented the translation rate of ENaC subunits in rat CCD cultured cells[54] and increased β- and γ-ENaC subunit expression in AVP-supplemented Bratleboro rats (a rat strain exhibiting a spontaneous knockout of the AVP gene).[55] These reports showed that AVP controls the synthesis, the plasma membrane expression, and the activity of ENaC in mammalian CD principal cells.

Stimulation of Na,K-ATPase is a prerequisite for an increased Na^+ reabsorption, but initial studies reported an inhibitory effect of AVP and cAMP analogues on Na,K-ATPase activity in isolated rat CCD.[56] However, results from our laboratory indicated that this inhibitory pathway was promoted by artifactual metabolic stress[57]: the inhibitory effect of cAMP is indirect and relied on the PLA2/arachidonate/cytochrome P-450–monoxygenase pathway,[56] and, indeed, in well-oxygenated isolated rat CCDs, cAMP analogues induced a 2-fold stimulation of both transport and hydrolytic Na,K-ATPase activity.[25] The stimulation of the Na,K-pump in response to cAMP analogs and AVP was observed within minutes and was associated with a proportional increase in Na,K-ATPase cell-surface expression[25,58] and without alteration of the total cellular pool of Na,K-ATPase. Similar results were obtained in $mpkCCD_{c14}$ cells (Fig. 3).

Similarly to the regulation of ENaC, described earlier, cAMP analog (db-cAMP) increased the cell-surface expression and activity of Na,K-ATPase through a brefeldin A–dependent process in CD principal cells: pretreatment of microdissected rat CCDs and of $mpkCCD_{c14}$ with 20 μg/mL of brefeldin A abolished the db-cAMP–induced increase in Na,K-ATPase activity and cell-surface expression. Therefore, taken together, these findings suggest that similar intracellular events are involved in both apical and basolateral steps of the Na^+ reabsorption process mediated by cAMP in the collecting duct. Moreover, in the same study[25] we showed that the cAMP-dependent recruitment of Na,K-pumps subunits to the cell surface, and the increase in activity strictly was dependent on temperature and on the intracellular free calcium: incubation of rat CCDs and of $mpkCCD_{c14}$ at 20°C or pretreatment with BAPTA-AM (a Ca^{2+} chelator) prevented the effect of db-cAMP. Finally, we showed that cAMP did not alter the internalization rate of Na,K-ATPase. Altogether these results showed that cAMP rapidly mobilized an intracellular pool of Na,K-pumps that might be resident in vesicles derived from the trans-Golgi network toward the plasma membrane in mammalian CD principal cells. Permeabilization of rat CCDs with saponin mimicked stimulation of Na,K-ATPase activity by db-cAMP: saponin allowed the measurement of the activity of the silent pool of Na,K-pumps. This agent had no stimulatory effect on vasopressin-sensitive adenylyl cyclase (embedded in the basolateral plasma membrane of principal cells) and the stimulatory effect of db-cAMP was no longer observed in saponin-permeabilized CCDs. Therefore, the effect of saponin on Na,K-ATPase was not accounted for by a detergent effect but most likely by permeabilization of cellular membranes containing the reservoir pool of Na,K-ATPase, thereby allowing measurement of its activity. The 90% increase in Na,K-ATPase activity observed with saponin permeabilization indicated that the reservoir pool of Na,K-pumps accounts for about 50% of the total cellular pool of the enzyme. The 75% increase in cell-surface expression of Na,K-ATPase in response to cAMP also indicated that this intracellular pool is constituted by active units that can be recruited to the cell surface almost entirely in response to a physiologic stimulus, suggesting a rapid mechanism of modulation of Na^+ reabsorption according to requirements of the Na^+ balance in CD principal cells. The localization and identity of the cAMP-responsive pool of Na,K-ATPase and its relation to an aldosterone-controlled reservoir remains also to be determined.

cAMP classically binds to the regulatory subunits of the PKA holoenzyme and releases active catalytic PKA (PKAc) subunits by alleviating autoinhibitory contacts.[59] In line with this classic mechanism of action of cAMP, the stimulation of ENaC activity is dependent on PKA activity in A6 cells.[60] The PKA-dependent activation of ENaC requires phosphorylation of actin filaments[61] and also may involve direct phosphorylation of the β and γ subunits of ENaC.[62] The PKA dependence of the Na,K-ATPase cell-surface expression in response to cAMP analogs has been shown in isolated rat

proximal tubule.[63] In mpkCCD$_{cl4}$ cells, inhibition of PKA by either H89 or mirystoylated protein kinase A inhibitor (PKI) prevents the AVP-induced recruitment of Na,K-ATPase to the cell surface (our unpublished results). However, the exact role of PKA in the short-term Na,K-ATPase up-regulation in CD principal cells remains to be elucidated. PKA may induce recruitment of Na,K-ATPase to the cell surface by phosphorylating directly the α subunit of intracellular Na,K-pump units. Transient expression of wild-type and mutant human α1 subunits in mpkCCD$_{cl4}$ cells revealed that phosphorylation of the Na,K-ATPase α1 subunit on Ser943, the PKA phosphorylation site[64] is not involved in the recruitment of Na,K-pumps to the cell surface in response to PKA activation (our unpublished results). Therefore, the effect of PKA may rely on phosphorylation of an intermediate target(s) that ultimately leads to increased cell-surface expression of Na,K-ATPase.

Control of Na,K-ATPase Activity and Expression by Insulin

An antinatriuretic effect of insulin that is independent of the glycemic status was shown in healthy humans by DeFronzo et al[65] and confirmed in the isolated perfused dog kidney preparation,[66] indicating that insulin may control renal sodium handling directly. Insulin subsequently was shown to alter sodium transport in various parts of the renal tubule including the CD.[1] However, opposite effects of insulin were reported in rabbit and rat CCD. In the in vitro perfused rabbit, CCD insulin was shown to inhibit both K^+ secretion and Na^+ reabsorption,[67] whereas it stimulates Na,K-pump–mediated cation transport in a time- and concentration-dependent manner in the isolated rat CCD.[68] The reason for this discrepancy remains unexplained because in every system studied to date, insulin stimulated Na,K-ATPase-coupled Na^+ transport.[1] The stimulation of Na,K-ATPase activity by insulin was independent of apical Na^+ entry through ENaC in rat CCD.[69] In amphibian A6 cells, insulin also stimulated ENaC, suggesting a coordinated control of the apical and basolateral steps of vectorial transepithelial Na^+ transport.[70]

In contrast to the proximal tubule, in which insulin did not alter the maximum velocity (V_{max}) but increased the apparent sodium affinity of Na,K-ATPase,[71] in CD the transport activity of the Na,K-pump was stimulated both under rate-limiting and -saturating Na^+ concentrations, revealing a V_{max} effect. Insulin did not induce a change in the number of active pump units measured by specific ouabain binding, therefore an increase in turnover of Na,K-ATPase has been proposed. In addition, the effect of insulin was abolished by permeabilization of cells, suggesting the requirement of soluble cofactors. These findings point out the cellular specificity of the mechanisms of control of Na,K-ATPase activity. Interestingly, in the proximal tubule insulin stimulates Na,K-ATPase activity through direct tyrosine phosphorylation of the α1 subunit Tyr-10.[72] The role of tyrosine phosphorylation of the Na,K-ATPase remains to be investigated in CD principal cells.

Control of Na,K-ATPase Activity and Expression by Intracellular [Na^+] and Extracellular Hypotonicity

Intracellular [Na^+] may be the most important nonhormonal factor regulating Na,K-ATPase activity. The Na,K-pump is stimulated kinetically by Na^+, acting at the cytosolic face of the membrane, with an apparent affinity constant ($K_{0.5}$) in the 5- to 15-mmol/L range in the presence of 5 to 10 mmol/L of K^+, and under these conditions the V_{max} is achieved with 60 to 100 mmol/L of Na^+. Because intracellular [Na^+] ranges between 5 and 20 mmol/L, Na,K-ATPase works well below its V_{max} (20%-30%) and intracellular [Na^+] is the major rate-limiting factor for Na,K-ATPase activity in intact cells. Thus, any increase in intracellular [Na^+] stimulates the Na,K-pump, which, in turn, pumps more Na^+ out of the cell and thereby contributes to restore the initial intracellular [Na^+]. Symmetrically, any decrease in intracellular [Na^+] slows down the Na,K-pump. This autoregulatory process is highly efficient because Na^+ activation of Na,K-pump displays a marked positive cooperativity; thus, small variations of [Na^+] around the $K_{0.5}$ induce large variations of Na,K-ATPase activity. On the extracellular side, Na,K-ATPase is stimulated by K^+ with an apparent K_m in the millimolar range (0.5-1.5 mmol/L). Thus, extracellular K^+ is not rate limiting for ATPase activity, except in the case of severe hypokalemia.

Na,K-ATPase in CD exhibits an apparent affinity for Na^+ that is twice as high as in the proximal tubule and thick ascending limb.[69,71] Therefore, the kinetic reserve for an intracellular [Na^+] activation of Na,K-ATPase is much lower in the CD than in the more proximal nephron segments. Thus, principal cells must face the challenge to maintain intracellular [Na^+] within a narrow range, a priority with respect to many cellular functions and ENaC activity, despite a lower affinity of the Na,K-pump for Na^+ and a lower extracellular [Na^+] (about 95% of the filtered Na^+ is reabsorbed by the more proximal renal tubule segments). In mammalian CCD an increase in intracellular [Na^+] was shown to increase rapidly and proportionally the Na,K-ATPase activity and the number of functional Na,K-pump units independently of transcriptional activation and/or de novo protein synthesis.[18] These reports raised the possibility that silent Na,K-pumps already located at the cell membrane are activated or, alternatively, that pre-existing intracellular Na,K-ATPase units are shuttled to the cell surface. We recently showed that in microdissected rat CCDs and in mpkCCD$_{cl4}$ cells, an increase in intracellular [Na^+] increases Na,K-ATPase activity and recruits Na,K-pump units to the cell surface,[73] similarly to the regulation of the Na,K-pump by cAMP[25] and aldosterone,[20] as discussed earlier. Therefore, sustained changes in intracellular [Na^+] would control the number of active Na,K-pumps present at the cell surface of principal cells and

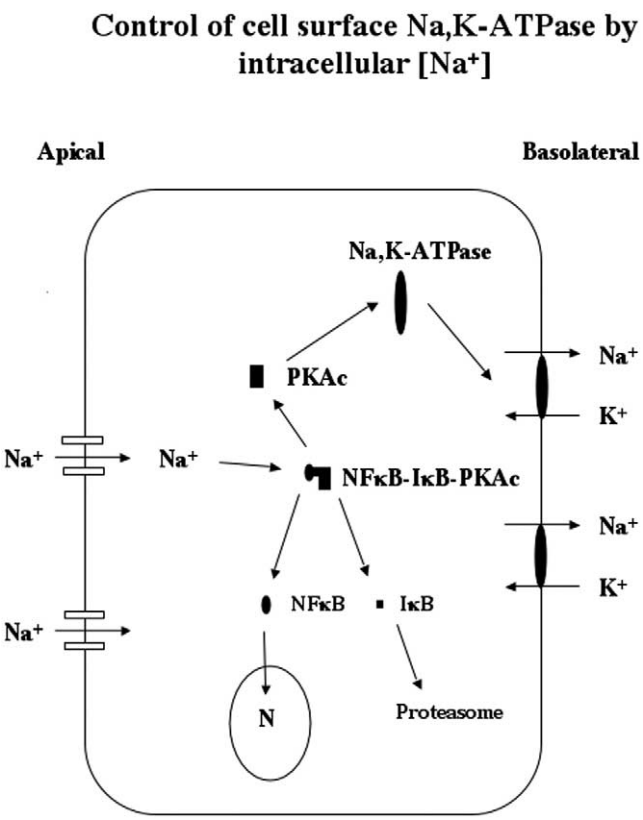

Figure 4 Overview of the putative intracellular sodium-dependent signaling pathway controlling the recruitment of Na,K-ATPase. Arrows indicate the direction of the signaling cascade. N, nucleus; V2, vasopressin.

thereby the rate of basolateral Na^+ extrusion. The Na^+-induced increase in Na,K-ATPase cell-surface expression is aldosterone dependent,[18,19,73] suggesting the requirement of an aldosterone-dependent expression of regulatory protein(s) exerting a permissive effect. Conversely, the early stimulation of Na,K-ATPase activity by aldosterone is independent of an increment of the intracellular [Na^+] brought about by increased apical Na^+ conductance.[11]

As shown in Fig. 4, we showed that the cellular mechanism leading to the shuttling of Na,K-pumps to the surface of CD principal cells relies at least in part on cAMP-independent PKA activation and requires the proteasomal degradation of an as yet unidentified regulatory factor that maintains PKAc in an inactive state.[73] Pharmacologic inhibition of PKA by either H89 or mirystoylated PKI abrogated the Na^+-induced shuttling of Na,K-ATPase to the cell surface and high intracellular [Na^+] increased PKA activity. Because cellular cAMP concentration and adenylate cyclase activity were not altered in response to increased intracellular [Na^+], the classic cAMP-mediated activation of PKA was not involved.[59] Recently, a cAMP-independent mechanism of PKA activation has been shown in response to cytokines. In this setting, free active PKAc is released on dissociation of a multiprotein complex containing PKAc, IκBα and NF-κBp65, and subsequent proteasomal degradation of IκBα.[74] This mechanism would be consistent with the fact that inhibitors of proteasome degradation pathway (MG-132 and lactacystin) abolished the Na^+-induced recruitment of Na,K-ATPase to the cell surface,[73] and its role currently is under investigation.

Because CD cells are exposed physiologically to variations of interstitial and tubular fluid osmolarities, we studied the effects of extracellular anisotonicity on Na,K-ATPase cell-surface expression (unpublished results). Results obtained in mpkCCD$_{cl4}$ cells indicate that extracellular hypotonicity increases both cell volume and Na,K-ATPase cell-surface expression. In contrast, extracellular hypertonicity, which induces cell shrinkage, did not alter the subcellular Na,K-pump localization. The effect of hypotonicity was not related directly to cell-volume variations. The effect of extracellular hypotonicity is mediated by a proteasomal-dependent PKA activity that is reminiscent of the effect of increased intracellular [Na^+].[73] We therefore addressed the role of Na^+ entry in the hypotonicity-induced recruitment of Na,K-pump and showed that increased Na^+ influx through ENaC plays a causal role. Therefore, increased apical Na^+ influx brought about by ENaC activation induces a coordinated increase in basolateral Na,K-ATPase activity that may rely on the activity of an intracellular [Na^+] sensor that remains to be identified.

Negative Modulators of Na,K-ATPase

The stimulatory effect of AVP on CD principal cells Na,K-ATPase is counteracted by several negative modulators such as prostaglandins, α_2-adrenergic agonists, endothelin, dopamine, and bradykinin. Most of these mediators modulate the intracellular concentration of cAMP at the level of its production and/or degradation, therefore controlling indirectly Na,K-ATPase activity. Both rabbit and rat CCD synthesize prostaglandin 2, and this process is stimulated by vasopressin, through V_1 receptors.[75] Vasopressin-induced synthesis of prostaglandins is part of a regulatory feedback mechanism that limits vasopressin action. This may be important for limiting water transport because it may prevent excessive swelling and dilution of cell compartments. In agreement with the stimulatory effect of AVP on Na,K-ATPase activity, chronic inhibition of prostaglandin 2 synthesis by indomethacin treatment increased Na,K-ATPase activity in rabbit CCD.[76] However, the very high doses of prostaglandin 2 used (micromolar range) preclude any physiologic relevance to this effect.

Dopamine has no effect per se on Na^+ transport by the CD, but it inhibits the AVP-induced Na^+ reabsorption in the in vitro microperfused rat CCD.[77] This effect is mediated through D_4 receptors (D_2-like) and likely is secondary to a decrease in cAMP generation in response to AVP.[78] However, in contrast to the D_1-like agonist fenoldopam, dopamine did not increase intracellular cAMP content in the rat CCD.[79] These observations can be accounted for by a balanced activation of D_1-like and D_2-like receptors, which are coupled negatively and positively to adenylyl cyclase, respectively.[80] An inhibition of Na,K-ATPase activity in response to dopamine has been reported in isolated rat CCD.[81] The cellular mechanism of dopamine effect on Na,K-ATPase has been investigated recently in proximal tubule cells. Activation of D1-like receptors by dopamine was reported to

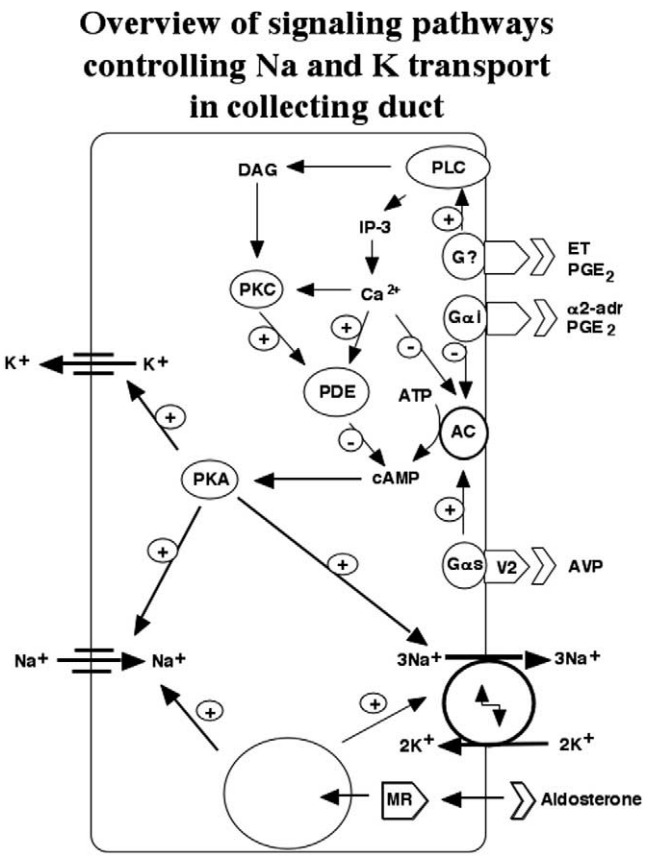

Figure 5 Overview of the main signaling pathways controlling the active sodium and potassium transport in CD principal cells. Arrows indicate the direction of the signaling cascade and the resulting stimulatory (+) or inhibitory (-) effect on their targets. AC; adenyl cyclase; α2-ADR, α2-adrenergic agonist; DAG, diacylglycerol; ET, endothelin; G, G protein, MR, mineralocorticoid receptor; PDE, phosphodiesterase; PLC, phospholipase C; V2, vasopressin V_2 receptor.

induce adaptor protein 2–and clathrin-dependent endocytosis of Na,K-ATPase.[82,83] This effect is finely modulated by intracellular $[Na^+]$.[84] Activation of D_2-like receptors was reported to stimulate Na,K-ATPase activity via tyrosine kinase and extracellular regulated kinase (ERK)1/2 activation.[85,86] The mechanism responsible for the down-regulation of Na,K-ATPase activity by dopamine in CD principal cells remains to be investigated.

The AVP-induced stimulation of water and Na^+ reabsorption is antagonized by α_2-adrenergic agonists[87] and endothelin through its endothelin B receptors.[88] Inhibition of cAMP production by α_2-adrenergic agonists in CD principal cells largely results from $G\alpha_1$-mediated inhibition of adenylyl cyclase.[89] However, a post-cAMP effect also may be involved in the functional inhibition of Na^+ transport by α_2-adrenergic agonists. In CCD, endothelin reduces AVP-induced cAMP production through a calcium-dependent stimulation of PKC.[90]

Conclusions

In this review, we summarized the current knowledge on the regulatory mechanisms that control Na,K-ATPase activity and expression in CD principal cells (Fig. 5). These cells, although quantitatively responsible for only up to 5% of the tubular Na^+ reabsorption process, are the site of fine tuning of Na^+ transport by aldosterone, vasopressin, and other important regulators of blood pressure at the kidney level. Na,K-pump plays a fundamental role in these processes, as shown by the large interest of clinicians and scientists and by the prolific literature on this enzyme since its discovery some 40 years ago by 1997 Nobel prize winner J. C. Skou.[2] The study of the interactions between the Na,K-pump, signaling molecules, cytoskeleton, and vesicular transport machinery is an exciting area for future research in cell physiology and conceivably will lead to therapeutic implications for the treatment of Na^+-sensitive hypertension and other renal pathologies.

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