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Inhibition of Na,K-ATPase by Dopamine in Proximal Tubule Epithelial Cells

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In the current report we review the results that lay grounds for the model of intracellular sodium-mediated dopamine-induced endocytosis of Na,K-ATPase. Under conditions of a high salt diet, dopamine activates PKCζ, which phosphorylates NKA α1 Ser-18. The phosphorylation produces a conformational change of α1 NH_2-terminus, which through interaction with other domains of α1 exposes PI3K- and AP-2-binding domains. PI3K bound to the NKA α1 induces the recruitment and activation of other proteins involved in endocytosis, and PI3K-generated 3-phosphoinositides affect the local cytoskeleton and modify the biophysical conditions of the membrane for development of clathrin-coated pits. Plasma membrane phosphorylated NKA is internalized to specialized intracellular compartments where the NKA will be dephosphorylated. The NKA internalization results in a reduced Na^+ transport by proximal tubule epithelial cells.
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A common feature often associated with cardiovascular diseases is a disorder in the regulation of kidney sodium (Na^+) reabsorption. Because this represents the major determinant of blood pressure, high blood pressure would necessarily result from an increase in the renal set point for Na^+ reabsorption. The cellular and molecular mechanisms responsible for increased renal Na^+ reabsorption largely are unknown. Dopamine is used widely in intensive care units to improve renal function. Dopamine, which is synthesized by the proximal tubule epithelial cells, regulates the activity of the 2 main proteins responsible for Na^+ reabsorption in proximal tubules: Na^+/H^+-exchanger (NHE) and Na^+,K^+-adenosine triphosphatase (ATPase) (NKA). Increased NKA activity has been associated with high blood pressure in Milan hypertensive rats;[1,2] and the NKA α1 locus (which expresses a mutated α1 responsible for reduced NKA activity) cosegregates with salt-sensitive hypertension.[3] Endogenous kidney dopamine accounts for 60% of the natriuretic response seen during acute volume expansion in normotensive rats, and an impaired natriuretic response to dopamine in spontaneous hypertensive rats has been reported.[4,5] Consistent with these observations, knockout of D_{1A} receptors produced transgenic mice that are hypertensive.[6] We have been working for several years to elucidate the molecular mechanism(s) controlling sodium transport across renal proximal tubules. In this review, we present some of our results on the mechanism by which inhibition of the Na^+,K^+-ATPase activity may contribute to the ability of intrarenal dopamine to reduce kidney sodium reabsorption under conditions of sodium load.

Kidney Sodium Reabsorption

Na^+ handling by the kidney represents the major determinant of blood pressure. In mammalian kidneys, more than 60% of the filtered Na^+ is reabsorbed in proximal tubule.[7] Of this total amount, most (~80%) of the luminal Na^+ enters the proximal tubule epithelial cells through the apical NHE; however, Na^+ also is cotransported into the proximal tubule epithelial cells with phosphate, glucose, amino acids, organic anions, and sulfate. Because the intracellular Na^+ concentration ($[Na^+]_i$) of proximal tubule epithelial cells is relatively low compared with tubular ultrafiltrate, Na^+ movement from the lumen of the prox-

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imal tubule into epithelial cells proceeds down an electrochemical potential gradient. The basolateral NKA maintains the Na^+ gradient across the cell membrane and provides the driving force for Na^+ reabsorption.[8,9] Therefore, under normal physiologic conditions, the NHE and NKA are the 2 main proteins involved in proximal tubule sodium reabsorption and therefore targets of hormones that regulate proximal tubule Na^+ reabsorption.[7,10-12] Although the role of the NHE in the regulation of proximal tubule Na^+ reabsorption has been recognized for many years, it is only in the past 10 years that the involvement of NKA in this regulation has received strong experimental support.[7,10,11] Considerable evidence indicates that hormones that regulate kidney Na^+ reabsorption also modulate the activity of kidney NKA, and that hormonal short-term regulation of NKA activity contributes to the ability of the kidneys to adjust Na^+ reabsorption.[7,10,11] Our hypothesis is that hormonal regulation of the NKA activity produces increased/decreased $[Na^+]_i$ of proximal tubule epithelial cells, independently of the NHE activity.[13] Because Na^+ reabsorption depends on the $[Na^+]$ gradient across the apical membrane of epithelial cells, any modulation of $[Na^+]_i$ should translate into modulation of proximal tubule Na^+ reabsorption. This hypothesis does not preclude the possibility that simultaneous action of hormones on both NHE and NKA may result in no significant change of $[Na^+]_i$.

Intrarenally produced dopamine causes a large increase in urinary Na^+ excretion that mainly is dependent on inhibition of tubule Na^+ reabsorption.[7,11] Dopamine and dopamine-receptor agonists, as well as parathyroid hormone and endothelin, inhibit proximal tubule NKA activity.[7,14,15] The antinatriuretic hormone angiotensin II (Ang II) stimulates NKA activity, and this effect is counteracted by dopamine.[15] It is important to note that dopamine-induced natriuresis and inhibition of NKA activity is prominent under salt-loading conditions, and small or negligible after salt depletion.[16-19] This is consistent with our observation that dopamine inhibition of NKA increases as $[Na^+]_i$ is increased.[20] After dopamine-receptor–mediated stimulation of phospholipase C and protein kinase C (PKC), production of the arachidonic acid metabolite 20-HETE represents a major pathway for inhibiting NKA in renal proximal tubules and inducing natriuresis.[5,12,21] It has been proposed that dopamine receptors are coupled to G proteins that are insensitive to both cholera and pertussis toxins, possibly Gq/11.[5,6,22] Although some insights into the mechanism of NKA inhibition by dopamine have been gained in recent years, important aspects including the organization of the intracellular signaling cascade still are unknown. We describe some aspects of the dopamine-induced inhibition of NKA that we have studied.

PKC Phosphorylation of NKA NH_2-Terminus α Subunit

Elimination of the α1 NH_2-terminus of purified kidney NKA by trypsin cleavage of the Lys-32 and Glu-33 bond produces a stable preparation called *invalid ATPase*.[23,24] At physiologic concentrations of ligands, the invalid ATPase has the same activity and kinetic properties as the native NKA.[25,26] However, when assayed at nonphysiologic concentrations of ligands, the invalid ATPase has defective K^+-stimulated dephosphorylation and K^+-dependent phosphatase activity.[23-26] While at physiologic ATP concentration, K^+ activates both the native NKA and invalid ATPase, at 10 μmol/L ATP, K^+ inhibits the native NKA and stimulates the invalid ATPase.[26,27] These observations suggested that the α1 NH_2-terminus might regulate the K^+-deocclusion pathway of the NKA reaction. Therefore, under physiologic conditions, the α1 NH_2-terminus does not affect the basal NKA activity but may participate in the regulation of this activity. Accordingly, we have observed that elimination of the α1 NH_2-terminus has no effect on the basal NKA activity and $[Na^+]_i$, but impairs the hormone-induced activation and inhibition of NKA activity.[28-30]

Phosphorylation of NKA α1 by PKC, in vitro and in vivo, is confined to the NKA α1 NH_2-terminus.[31-33] Ser-11 and Ser-18 of NKA α1 from different species and tissues were identified as the PKC targets in both intact cells and purified membranes.[32,33] Although 4 isoforms of the NKA α subunit are expressed in different tissues and species, α1 is the only isoform that is expressed significantly in kidney.[7,10,13,34] Stimulation of PKC either activates[28,29,35,36] or inhibits renal NKA.[36-38] We have observed that in rodent α1 this apparent discrepancy has its origin in the PKC isoform that is involved, and that either Ser-18 alone or both Ser-11 and Ser-18 are phosphorylated.[39] The PKC-dependent mechanism of NKA regulation may be different or not present in all types of cells.[40-42] Our studies mainly have examined the dopamine-induced NKA inhibition in both rodent proximal tubule cells and opossum kidney (OK) cells, which are a cell culture model of proximal tubule epithelia.[43-45]

Dopamine treatment of both rodent proximal tubule and OK cells results in inhibition of NKA activity.[7-12] This effect is not observed in cells expressing a NKA α1 mutant, similar to the invalid NKA, in which the first 26 N-terminal amino acids have been deleted.[30] This segment contains the putative PKC phosphorylation sites Ser-11 and Ser-18.[32,33,40] By comparing the level of NKA phosphorylation in OK cells transfected with the rodent α1 that lacks either Ser-11 or Ser-18 (S11A and S18A, respectively), we showed that dopamine treatment exclusively increases the phosphorylation of Ser-18.[46] Mutation of Ser-18 totally impairs the dopamine-induced phosphorylation and inhibition of NKA.

Dopamine-Induced Inhibition of NKA Is Not Caused by Phosphorylation of NKA Molecules But by Their Endocytosis

Dopamine treatment of OK cells increased the amount of NKA molecules in early and late endosome fractions, and this was not observed in cells expressing an N-terminal deletion mutant of NKA α1, which did not contain Ser-18.[30] The plasma membrane NKA is phosphorylated rapidly after addition of dopa-

mine, but after 10 minutes all of the phosphorylated NKA molecules have been internalized into endosomes where the NKA is dephosphorylated.[30] Two inhibitors of phosphoinositide-3-kinase (PI3K), wortmannin and LY294009, prevented the internalization of the dopamine-induced phosphorylated α1.[30] Under this condition, phosphorylated NKA remained in the plasma membrane and no inhibition of NKA was observed. Wortmannin and LY294009, which had no effect on NKA activity or endocytosis by themselves, were used at a concentration that only inhibits PI3K and not other kinases involved in inositol phosphorylation.[47] Therefore, phosphorylation of α1 Ser-18 is essential for endocytosis of NKA molecules, but it is not phosphorylation but endocytosis that produces the inhibition of NKA.

Although NKA α1 phosphorylation and endocytosis induced by dopamine were blocked by the PKC inhibitor bisindolylmaleimide, inhibition of PKA has no effect on the inhibition of NKA by dopamine.[30] During the process of its activation, PKC molecules bind to intracellular structures at specific anchoring molecules named *RACKs*.[48] By using isoform-specific peptides that prevent the binding of PKC to RACKS, we showed that PKCζ is the isoform involved in the inhibition of NKA by dopamine.

The results described earlier indicate that PI3K is involved in dopamine-induced inhibition of NKA at a stage that is downstream from the phosphorylation of α1 Ser-18. Dopamine induced the activation of PI3K and increased coprecipitation of PI3K and NKA, indicating a direct interaction between NKA and PI3K molecules.[49] The fact that both PI3K activation and increased coprecipitation with NKA were not observed in cells expressing NKA α1-S18A, which cannot be phosphorylated by PKC, indicates that phosphorylation of Ser-18 is essential for activation of PI3K and its binding to the NKA. Although the general idea was that stimulation of PI3K (class IA subtype) results from its binding to phosphorylated tyrosine residues in target proteins,[50] we observed that inhibitors of tyrosine phosphorylation did not affect the dopamine-dependent inhibition of NKA. Moreover, elimination of NKA α1 Ser-18 phosphorylation prevented both PI3K activation and its coprecipitation with NKA.[49] We identified a polyproline domain in α1 (81-TPPPTTP-87) and observed that the α1-P78R mutation impaired the dopamine-induced NKA inhibition, PI3K activation, and PI3K-NKA coprecipitation without altering the basic NKA activity.[49] We also determined that a synthetic peptide, with the sequence of the polyproline site, interfered with the dopamine-induced inhibition of NKA, PI3K activation, and PI3K-NKA coprecipitation.[49]

Dopamine Induces the Formation of a Multiprotein Complex of NKA, AP-2, Clathrin, and Dynamin

Dopamine induces colocalization and coprecipitation of clathrin and NKA molecules,[51] and this is not observed in cells expressing the S18A or P78R α1 mutants.[49] Endocytosis of membrane proteins via clathrin-coated vesicles occurs through the interaction of adaptor protein 2 (AP-2) to specific sequences (endocytic sequence) in the target protein.[52] We have identified the endocytic sequence as the conserved sequence Y-537 LEL within α1, which represents the AP-2 binding site.[53] Dopamine induces the association of AP-2 with NKA in both OK and proximal tubule cells, and this interaction is prevented in cells expressing the α1-S18A mutant.[51] Dynamin is a protein that participates in the release of clathrin-coated vesicles from the plasma membrane. For this, dynamin must be activated by dephosphorylation and localization at the place of endocytosis. We observed that dopamine induces dephosphorylation of dynamin and coprecipitation of dynamin with PI3K.[54] By confocal microscopy, we determined that dopamine induces colocalization of NKA enhanced green fluorescent protein–α1, PI3K, and dynamin II. Under basal conditions, most of the NKA enhanced green fluorescent protein–α1 molecules were localized to the plasma membrane, and PI3K and dynamin were localized mainly in the cytosol. Treatment of the cells with dopamine induced colocalization of the 3 molecules in selected areas of the plasma membrane. Thus, the NKA-PI3K complex may help recruit dynamin to the site of NKA endocytosis.

Increased $[Na^+]_i$ Increases Dopamine Inhibition of NKA

We hypothesized that an augmented $[Na^+]_i$ is required for dopamine inhibition of NKA.[20] To test this hypothesis, $[Na^+]_i$ was increased with monensin. Although most of the Na^+ ionophores change the $[Na^+]_i$ by equilibrating intracellular and extracellular Na^+ concentrations, monensin is the only known Na^+ ionophore that can increase $[Na^+]_i$ without dissipating the Na^+ gradient across the plasma membrane. The free $[Na^+]_i$ of OK cells was determined in situ by digital imaging fluorescence microscopy.[20] As monensin increases $[Na^+]_i$, basal (without dopamine) NKA activity is increased.[20] Dopamine has no effect on the NKA activity at basal (without monensin) $[Na^+]_i$. However, an increase of only 4 mmol/L in the $[Na^+]_i$ is enough to induce a significant inhibition of NKA activity by dopamine. Ca^{2+} is not involved in this process because both the basal NKA activity, and the inhibition of this activity by dopamine, were not affected by the removal of external Ca^{2+} and loading the cells with the Ca^{2+} chelator BAPTA.[20] Thus, an increased $[Na^+]_i$ may exert a permissive effect on one or more steps of the dopamine intracellular signaling cascade.

At basal $[Na^+]_i$, Ang II induces an activation of NKA activity. However, we observed that the activation of NKA by Ang II is reduced at increasing $[Na^+]_i$. This effect is not caused by monensin because monensin by itself activates the NKA.[20] Therefore, a small increase in $[Na^+]_i$ facilitates the inhibitory effect of dopamine and counteracts the activating effect of Ang II. Consistent with this conclusion, when cells were treated simultaneously with both dopamine and Ang II, whether activation or inhibition of NKA was observed de-

pended on the concentration of $[Na^+]_i$. These results support the hypothesis that increased $[Na^+]_i$ favors the action of hormones that inhibit proximal tubule Na^+ reabsorption (dopamine) and reduces the action of hormones that activate proximal tubule Na^+ reabsorption (Ang II). Because an increased tubular Na^+ concentration should result in an increased $[Na^+]_i$ of proximal tubule cells, we hypothesized that increased $[Na^+]_i$ triggers and modulates the dopaminergic response so that less Na^+ is reabsorbed in proximal tubules. At the same time, the increased $[Na^+]_i$ reduces the effects of hormones (similar to Ang II) that activate Na^+ reabsorption. Brismar et al[55] suggested that, under basal conditions, proximal tubule cells have a low abundance of D_1 receptors at the plasma membrane and that dopamine induces a recruitment of D_1 receptor to the plasma membrane. Indeed, we observed that a 5-minute treatment with dopamine increased by 60% the abundance of plasma membrane D_1 receptors. However, this increase is small as compared with the increase (250%) of plasma membrane receptors produced by an 8-mmol/L increase of $[Na^+]_i$ in the absence of dopamine.[56] Therefore, we hypothesize that $[Na^+]_i$ is a major modulator of hormones that regulate proximal tubule Na^+ reabsorption.

Regulation of NKA in Animal Models of High Blood Pressure

Abnormal regulation of sodium excretion is an important determinant for the development of high blood pressure. Therefore, the mechanisms regulating renal sodium excretion have been studied extensively. Alterations in the NKA activity and its regulation along the kidney nephron have been described in different animal models of hypertension. Previous studies showed that during a high-salt diet (0.9% saline) there is a decrease in NKA activity in the proximal and distal segments of the nephron.[16] This effect was mediated by an increase in renal production of dopamine and its interaction with specific membrane receptors. Interestingly, intrarenal dopamine failed to decrease NKA activity in the kidney tubules from Dahl-sensitive rats that developed high blood pressure after being placed on a high-salt diet (0.9% saline).[57-59] Mutations within the NKA α subunit have been described in these animals,[60,61] however, functional kinetic studies performed in intact proximal tubule cells did not show any abnormality of the NKA activity, except that dopamine failed to down-regulate its activity.[62] These results suggested the possibility that a deficient intracellular signaling network might be responsible for the lack of response to dopamine. Several lines of investigation performed in spontaneously hypertensive rats, another animal model of hypertension, support this hypothesis. In renal tubule cells obtained from these animals, there is a deficient coupling between activation of dopamine receptors and their ability to generate cyclic adenosine monophosphate.[63,64] We have studied the NKA activity and its regulation by dopamine in Milan hypertensive rats, another animal model of hypertension. NKA activity in proximal tubules from Milan hypertensive rats was higher when compared with their normotensive counterpart.[2] Detailed studies revealed that this abnormality was not associated with a defective dopamine receptor or inactive NKA molecules, but that dopamine failed to promote the endocytosis of active NKA molecules present at the plasma membrane. It appears that the defect resides within the mechanisms responsible for controlling the endocytic network: specifically, the status of AP-2 phosphorylation and its association with the NKA α subunit and their incorporation into clathrin-coated vesicles.[65] Under basal conditions, AP-2 already existed in a phosphorylated form and dopamine failed to increase further the level of AP-2 phosphorylation. The increased basal phosphorylation of AP-2 may be associated with its abnormal interaction with a protein phosphatase that failed to be recruited to the endocytic site by a mutated form of α-adducin that cosegregates with high blood pressure. These results point to a primary cellular defect within the renal tubule cells, deficient NKA endocytosis in response to G protein-coupled receptor (GPCR) signals, which could contribute to establish an abnormal sodium balance and the development of high blood pressure.

Conclusions

The 3-dimensional structure of the NKA molecule is not known. Recently, the structure of a related protein, the sarcoplasmic reticulum Ca^{2+} ATPase has been resolved.[66] In this protein, the NH_2 terminus is approximately 3 nm from (and pointing toward) the plasma membrane lipid bilayer. However, the first 30 amino acids corresponding to the NKA α1 are not present in the NH_2 terminus of the sarcoplasmic reticulum Ca^{2+} ATPase.[67] It is possible that this segment reaches the plasma membrane. The α1 NH_2-terminus has an accumulation of basic charged amino acids, which may interact with phospholipids in the internal face of the plasma membrane.[68] Then, α1 Ser-18 may be in close proximity to the polyproline motif. Interestingly, the α1 polyproline motif that binds PI3K is very close to the cell membrane and may be just in front of the sequence through which α1 binds to ankyrin.[67] Thus, phosphorylation of Ser-18 may produce a conformational change of the α1 NH_2 terminus, which then interacts with other domains of α1 and exposes both the polyproline domain (where PI3K binds) and the endocytic sequence (that interacts with AP-2). By binding to the polyproline site, PI3K is activated, and it may hydrolyze plasma membrane phospholipids to produce phosphoinositides. This may help to recruit and/or organize other inositide-binding proteins such as AP-2, clathrin, and dynamin. Phosphoinositides also may affect the local membrane cytoskeleton to favor the formation of clathrin-coated pits and the release of phosphorylated NKA targeted for endocytosis.

Therefore, our results are consistent with the following model of the dopamine-induced endocytosis of NKA. (1) An increase of the $[Na^+]_I$ in the proximal tubule epithelial cells induces the dopaminergic response; (2) dopamine induces the activation of PKCζ, which phosphorylates NKA α1 Ser-18; (3) this produces a conformational change of the α1 NH_2 terminus, which interacts with other domains of α1 and gen-

erates a signal that exposes both the 76-TPPPTTP-82 domain (for binding and activation of PI3K), and the endocytic sequence at 537-YLEL (for binding of AP-2); (4) PI3K binds to the NKA α1 and induces the recruitment and activation of other proteins involved in endocytosis (AP-2 and its kinase, clathrin, and dynamin and its phosphatase protein phosphatase 2); (5) generation of 3-phosphoinositides by PI3K enhances the binding of AP-2 and dynamin to their targets, affects the local cytoskeleton, and modifies the biophysical conditions of the membrane for development of clathrin-coated pits; (6) plasma membrane–phosphorylated NKA are internalized to specialized intracellular compartments where the NKA will be dephosphorylated; and (7) endocytosis of phosphorylated NKA molecules to intracellular compartment(s) results in a reduced capacity of the cell to transport Na^+.

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