Short-term Stimulation of the Renal Na-K-Cl Cotransporter (NKCC2) by Vasopressin Involves Phosphorylation and Membrane Translocation of the Protein*

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Na-K-Cl cotransporter (NKCC2)-mediated sodium chloride reabsorption in the thick ascending limb is stimulated by the antidiuretic hormone vasopressin. We investigate the mechanisms underlying the short term activation of NKCC2 by vasopressin in vivo, finding that administration of a vasopressin analogue (deamino-Cys-D-Arg vasopressin) causes a 2-fold increase in mouse kidney NKCC2 phosphorylation, as detected with a phosphospecific antibody, R5. Thesubset localization of the activation is defined by immunofluorescence. In vasopressin-treated animals, a dramatic increase in R5 immunostaining is observed in the initial segment of the thick ascending limb located in the inner stripe of the outer medulla, the region with a higher sensitivity to vasopressin. Although a pool of NKCC2 is present in cytoplasmatic vesicles, the distribution of the phosphorylated cotransporter seems to be restricted to the cell membrane compartment; morphometric analysis of electron microscope images demonstrates a 55% increase in NKCC2 molecules at the apical membrane, suggesting the administration of vasopressin induces trafficking of the cotransporter. Thus, the short term actions of vasopressin on the thick ascending limb cotransporter are mediated by both an effect on the translocation of the protein and an increase in phosphorylation of regulatory threonines in the amino terminus of NKCC2.

Vasopressin controls extracellular fluid osmolarity by adjusting the amount of free water excreted by the kidney. The main effect of the hormone is found in the collecting duct, where vasopressin causes insertion of aquaporin channels in the apical membrane (1) and up-regulation of urea transporters (2). However, the amount of water that is finally reabsorbed depends upon the osmolar gradient between the medullary interstitium and the luminal fluid. The existence of a medullary hypertonic interstitium is ensured by the reabsorption of NaCl against its electrochemical gradient in the thick ascending limb of the loop of Henle (TAL). ³ Transport of Na⁺ and Cl⁻ across the luminal membrane of the TAL is provided by the renal isoform of the Na-K-Cl cotransporter, NKCC2, which, in mammals, is exclusively expressed in TAL and macula densa cells (3–5). A rational design of the mechanism responsible for urine concentration would thus include vasopressin stimulation of NKCC2 for the generation of the hypertonic interstitium. The existence of V2-type vasopressin receptors in the TAL strongly supports this hypothesis (6).

Several effects of vasopressin on the thick ascending limb Na-K-Cl cotransporter have been reported. NKCC2 protein and messenger RNA levels increased in water deprived rats (7, 8); whereas treatment with a V2-receptor antagonist had the opposite effect (9). In Brattleboro rats, long-term administration of vasopressin restored the function (10) and expression levels of NKCC2 (8), which are significantly reduced in this vasopressin-deficient rat strain. More importantly, the short term effects of vasopressin on TAL electrolyte transport were demonstrated in early studies in isolated microperfused tubules. Addition of vasopressin to the bath stimulates CAMP production (11, 12) and transepithelial bumetanide-sensitive chloride fluxes (13–16). These effects of vasopressin are more pronounced in rodents, whereas they are weak or absent in species with a lesser urinary concentrating ability (17).

Vasopressin is just one of many hormonal and paracrine factors that have been shown to acutely up-regulate NaCl transport in the thick ascending limb (for review, see Ref. 18). Although the function of NKCC2 seems to be tightly regulated, little is known about the molecular mechanisms underlying its activation. Phosphorylation is involved in the activation of many membrane transport systems; indeed, the amino acid sequence of NKCC2 contains several potential consensus sites for protein kinases. However, before this study, a link between phosphorylation and activation of NKCC2 has not been demonstrated.

Activation of the secretory isoform of the Na-K-Cl cotransporter (NKCC1) involves phosphorylation of five to eight threonine and serine residues (19, 20). To date, three of these phosphoacceptors (Thr-184, Thr-189, and Thr-202; shark NKCC1) have been identified; these amino acids reside in a region of highly conserved amino acid sequence in the N terminus of the cotransporter (20). Phosphorylation of these residues correlates with transport activity, and their substitution dramatically affects the function of the protein. A phosphospecific antibody, R5, raised against two of the phosphothreonines (Thr-184, Thr-189), has recently proven to be a very useful tool for the study of NKCC regulation in vivo (21).

The region containing the three regulatory threonines identified by Darman and Forbush (20) is the most highly conserved ubiquitous Na-K-Cl cotransporter; isOM, inner stripe of the outer medulla; dDAVP, deamino-cysteine-ν-arginine vasopressin.

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sequence between NKCC1 and NKCC2 within the N terminus. This high degree of conservation has led us to suggest a common mechanism of activation between these proteins (21). To test this hypothesis and further elucidate the mechanisms responsible for regulation of Na-K-C1 cotransport in the thick ascending limb, we studied the short term stimulation of NKCC2 by vasopressin in vivo using the R5 antibody. Herein, we report the first evidence for a role of phosphorylation in the regulation of NKCC2, the renal Na-K-C1 cotransporter. Moreover, kidney-specific increased transcellular ion transport of the probe to the cell membrane accompanies the increase in NKCC2 phosphorylation after short term vasopressin administration in vivo. An abstract of this work has been presented previously (22).

MATERIALS AND METHODS

Animal Handling and Tissue Collection—All procedures employed in the present work involving animals were approved by Yale University Institutional Animal Care and Use Committee. Male 8-8-week-old CD-1 mice (Charles River Laboratories) were maintained at Yale’s School of Medicine facilities under standard conditions, including free access to food and water.

On the day of the experiment, 10 ng of dDAVP (a vasopressin analogue) per animal (Sigma) or vehicle alone was administered by subcutaneous injection. One hour after injection, animals were anesthetized with sodium pentobarbital (70 mg/kg body weight; intraperitoneal injection). Under deep anesthesia, the thoracic and abdominal cavities were exposed, and a catheter was placed in the left ventricle. Warmed Krebs-Henseleit-bicarbonate solution (140 mM Na+, 5 mM K+, 130 mM Cl−, 1.8 mM Ca2+, 0.9 mM Mg2+, 25 mM HCO3−, 1 mM PO43−, and 1mM SO42−, pH 7.4 after equilibration with 5% CO2/95% O2 at 37 °C) was perfused through the left ventricle and the blood drained through an incision in the Cava vein.

The right kidney was removed after placing a clamp around the renal vessels. To minimize tissue manipulation before phosphotissue blockage, we did not dissect the kidney into cortex and medulla. Whole kidneys were thus immediately homogenized in ice-cold antiphasphatase buffer (150 mM NaCl, 30 mM NaF, 5 mM EDTA, 15 mM Na2HPO4, 15 mM pyrophosphate, and 20 mM Hepes, pH 7.2) with 1% Triton X-100 and protease inhibitor cocktails added, using 25 strokes at 20,000 rpm with a Polytron tissue homogenizer and centrifuged at 4,000 rpm for 10 min at 4 °C to pellet debris. Membrane-containing supernatant was centrifuged for 40 min at 20,000 rpm at 4 °C, and the resulting membrane fraction resuspended in homogenization buffer and stored at −20 °C. Perfusion fixation of the left kidney was initiated after removal of the right kidney using prewarmed paraformaldehyde-lysine-phosphate solution (23). Blocks of fixed kidney were postfixed in the same solution for an additional 4–4 h at 4 °C.

To reduce the level of endogenous vasopressin, some animals were water-loaded by offering them a 5% dextrose/1% ethanol solution overnight. Water load was assessed by measuring solution intake. The response of the animals to dDAVP and water load was confirmed by assessing the changes in urinary concentration. Urinary osmolarity was measured with a freezing point osmometer (MicroOsmeter 3300; Advanced Instruments) in diluted urine samples collected directly from the bladder at the time of sacrifice.

Antibodies—Two antibodies of the T series of monoclonal antibodies raised against the last 310 residues of the human colonic Na-K-C1 cotransporter (hNKCC1) (24) were used in the present study. The T4 antibody is widely used to quantify NKCC protein expression in many tissues and species by Western blot. For immunofluorescence studies, we used the previously described R5 antibody (21). This antibody recognizes the phosphorylated cotransporter with high affinity and specificity over the non-phosphorylated cotransporter. This antibody was raised against the NKCC1 amino acid sequence (YLYKT*FGHNT–MDAHP) but because of the high conservation of the antigen, it also recognizes NKCC2 (YYLQT*FGHNT–MDAHP, where Q is the only residue that differs between the two sequences provided). Similarly, our attempt to produce an isoform-specific phoshoantibody by immunizing rabbits with the phosphopeptide sequence found in NKCC2 resulted in a serum (R78) that recognizes both the phosphorylated NKCC2 and NKCC1. Because the affinity of R78 for the cotransporter was somewhat lower than that of R5, we used the latter in our analysis. The same considerations mentioned above for T4 and T9 regarding discrimination between isoforms apply in this case; by immunofluorescence, only TAL cells showed R5 positive staining of the luminal membrane.

Staining with the T9 and R5 antibodies required antigen retrieval with SDS, which made these antibodies inadequate for ultrastructural studies. For electron microscopy studies, we used the L320 antibody (generous gift of Dr. M. Knepper), which has been successfully employed in previous studies of the ultrastructural localization of NKCC2 in the rat kidney (30). L320 is a polyclonal NKCC2 isoform-specific antibody, raised against a non-conserved 20-amino acid sequence in the initial N terminus of NKCC2. Finally, an anti-Tamm-Horsfall antibody (generous gift from Dr. J. R. Hoyer) was used as a marker for thick ascending limb cells.

Western Blotting—Equal amounts of protein (typically 40 μg) were subjected to SDS-PAGE using 7.5% acrylamide gels. After protein transfer to polyvinylidene difluoride membranes (Immobilon P; Millipore), Western blotting was performed as described previously (21) in duplicate blots, using R5 and T4 as primary antibodies. The presence of antigen-IgG complex was detected using an HRP-conjugated anti-IgG (generous gift from Dr. L. Knepper) and a chemiluminescence-based chemiluminescence assay (WestDura; Pierce). Light was recorded by means of a cooled charge-coupled device camera. For quantitative analysis, band densities were obtained using ImageQuant software (Amersham Biosciences).

Fluorescence Immunocytochemistry—Blocks of fixed tissue were cryoprotected by incubation for 1 h in 2.3 mM sucrose with 50% polyvinylpyrrolidone, mounted on aluminum nails, and stored in liquid nitrogen (21). Thin (2 μm) or semithin cryosections (0.5 μm) were cut with a Reichert Ultracut E ultramicrotome fitted with an FC-4E cryotatament. Sections were mounted on Superfrost Plus glass slides (Electron Microscopy Sciences, Fort Washington, PA). After antigen retrieval by incubation with 1% SDS for 5 min, sections were stained using primary antibodies (R5, T9, or Tamm-Horsfall) diluted in phosphate-buffered saline, 0.1% bovine serum albumin, and 10% goat serum. After washing the primary antibody, sections were incubated with secondary antibodies conjugated with Alexa fluorescent dyes (Molecular Probes). For double-label experiments, pilot studies were performed to ensure that the fluorochrome-conjugated secondary antibodies did not cross-react with the inappropriate primary antibody. Pictures were taken using a digital camera (Olympus), fitted on a fluorescence microscope (Nikon Eclipse E600).

Electron Microscopy—Ultrathin (200 Å) sections of the outer stripe of the medulla were sectioned with a Reichert Ultratoc E ultramicrotome fitted with an FC-4E cryotatament and placed on carbon-coated Formvar grids. Sections were labeled overnight with affinity-purified rabbit anti-NKCC2 (L320) diluted 1:100 in Tris-buffered saline containing 0.1% bovine serum albumin and 10% goat serum. After washing with Tris-buffered saline containing 0.1% bovine serum albumin, the sections were incubated for 1 h in the goat anti-rabbit gold conjugate, which was diluted 1:20 in Tris-buffered saline containing 0.1% bovine serum albumin and 10% goat serum. Sections were subsequently post-fixed in 2% glutaraldehyde and processed for electron microscopy and then stained and photographed with a Zeiss 902 electron microscope. Digital images were prepared by scanning the negatives with a Microtek scanner. The frequency distribution calculated to determine NKCC2 subcellular distribution was obtained using imaging software (LabWorks; UVP) to measure the shortest distance of each gold particle from the user-defined apical membrane.

RESULTS AND DISCUSSION

NKCC2 Is Phosphorylated at N-terminal Threonine—A major finding of this work is to show for the first time evidence for phosphorylation of NKCC2, the kidney-specific Na-K-C1 cotransporter. We hypothesized that a regulatory site previously identified in NKCC1 is also present in NKCC2, so that in vivo activation of the protein can be measured by means of a phosphospecific antibody, R5. The experiments presented in this report (Figs. 1–4) demonstrate that phosphorylation of the
N-terminal threonines recognized by R5 also happens in NKCC2 in vivo.

**NKCC2 Phosphorylation Is Stimulated by Vasopressin**—To determine whether vasopressin causes activation of NKCC2 in the TAL, a V2-type receptor specific vasopressin analogue, dDAVP (10 ng per animal, subcutaneous injection), was administered to adult mice, and phosphorylation of NKCC2 was measured with the phosphospecific antibody R5. The antidiuretic effect of dDAVP was confirmed by measuring urinary osmolarity in samples collected at the time of sacrifice (2175 ± 477 versus 3139 ± 559 mOsm in control and vasopressin-treated animals, respectively; p = 0.0093, n = 7). As shown in Fig. 1b, in vivo short term administration of vasopressin induces a 2-fold increase in overall NKCC2 phosphorylation, determined as the ratio of R5 to T4 signal of parallel blots. Importantly, this short term administration of vasopressin does not increase expression of NKCC2 as measured by the T4 antibody (Fig. 1a). These data are the first to show regulatory phosphorylation of NKCC2 and suggest that, in vivo, vasopressin stimulates an increase in interstitial tonicity via the phosphorylation and activation of NKCC2 in the thick ascending limb.

**Vasopressin-mediated NKCC2 Phosphorylation Is Localized to the Medullary Section of the TAL**—Because of the well known differential sensitivity to vasopressin among segments from the cortical and medullary portions of the TAL, we used immunofluorescence to examine whether a subpopulation of NKCC2 was preferentially regulated in response to vasopressin. As shown in Fig. 2, a and b, micrographs of mouse kidney sections clearly demonstrate a dramatic increase in R5-de-
weakly stained the glomeruli and inner medullary collecting duct in the tip of the papilla (not shown), areas described previously to express the housekeeping isoform NKCC1 (26).

From analysis of micrographs, NKCC2 phosphorylation in the isOM increases more than 3-fold, although it is hard to be precise about the -fold-increase because of the very low R5 labeling usually present in this region in samples from control mice. In these animals, which have free access to water, the levels of vasopressin are sufficient to produce a moderately concentrated urine (\(\frac{\text{osm}}{\text{L}}\) 2000 mOsM). However, with regard to NKCC2 phosphorylation, the mouse isOM TAL seems to be rather insensitive to these levels of the antidiuretic hormone. Only after administration of exogenous vasopressin, which induces a degree of urinary concentration comparable with that observed in water-restricted animals (\(\text{osm}\) 3500–4000 mOsm), is the isOM NKCC2 phosphorylated to the same extent as the rest of the TAL cotransporter. This result is in good agreement with previous studies using microperfused isolated tubules (reviewed in Refs. 14, 27, 28) that had described a quantitatively larger response to vasopressin in the murine medullary TAL, compared with the cortical TAL.

In the mouse, the rise in interstitial tonicity resulting from the increased NKCC2-mediated NaCl reabsorption in the isOM TAL is apparently only required under conditions that maximize water reabsorption in the medullary collecting duct. It remains a puzzle how the mouse isOM TAL is kept at a lower level of activation at rest. This observation is in marked contrast with the uniform activation of NKCC2 along the TAL present under normal conditions in the rat, a species with a lower ability to concentrate urine.²

The distinctive sensitivity of the isOM TAL to vasopressin adds another layer of specialization to this segment of the nephron, characterized by the exclusive expression of the NKCC2 alternative splice variant F. NKCC2F exhibits low affinities for the cotransported ions such that its transport rates can be affected by physiological changes in ion concentration in the lumen of the initial TAL (29). Taken together, these results strongly suggest that the murine medullary TAL has evolved to serve as a buffer of Na⁺/H⁺ and Cl⁻/H⁺ reabsorption.

Short Term Vasopressin Administration Increases NKCC2 Traffic to the Cell Membrane—When viewing higher magnification images of double-stained mouse kidney sections, we noticed that the R5 signal consistently appears as a sharp line at the surface of the TAL cells, whereas T9 staining extends from the membrane into the cytosol (Fig. 3a). We wondered whether this represented a redistribution of NKCC2 within the cell in response to vasopressin. NKCC2 has previously been shown to be present in intracellular vesicles, leading Nielsen and Knepper and colleagues (30) to speculate that vasopressin

\[\text{FIG. 3. Subcellular localization of NKCC2 in isOM TAL cells. a, immuno-fluorescence images of TAL cells from a vasopressin-treated mouse using the phosphospecific R5 antibody (left, green) and T9 antibody (middle, red) demonstrate that the signals do not colocalize in the cytoplasm (overlay). b, electron microscopy micrographs of control (left) and vasopressin-treated (right) mouse kidney tissue stained with L320, an NKCC2-specific antibody. c, frequency distribution of NKCC2 immunogold in control (black columns) and vasopressin-treated animals (white columns). d, analysis of data in c showing the cumulative percentage of NKCC2 molecules found at increasing distances from the apical membrane in control (triangles) and vasopressin-treated animals (squares) (15–20 images per condition from three separate experiments; *, } p < 0.05).\]
may induce the shuttling of these vesicles to the cell membrane in a manner reminiscent of its actions in the collecting duct.

To quantitate the intracellular distribution of NKCC2, we analyzed medullary TAL cells in sections from control and vasopressin-treated mice using electron microscopy. For this experiment, NKCC2 protein was detected with an NKCC2-specific antibody (L320) that has previously been used in ultrastructural studies (30, 31). Micrographs illustrating the distribution of NKCC2 molecules in control and vasopressin-treated animals are shown in Fig. 3b. The presence of gold particles in intracellular areas compatible with vesicular compartments was higher in control animals compared with vasopressin-treated animals.

Data from the morphometric analysis of ~100 images like those in Fig. 3b shows that in control conditions, 40.1% of the NKCC2 molecules were found within 0.10 μm from the cell membrane; the rest were in regions ranging from 0.10 to 1.5 μm (Fig. 3, c and d). With administration of vasopressin, the percentage of gold particles within 0.10 μm from the apical membrane rose to 62.0% (Fig. 3, c and d). This change represents a 1.55-fold increase in the absolute number of NKCC2 transporters available at the membrane for solute transport.

Two different TAL cell types, rough and smooth, have been distinguished in the thick ascending limb according to their ultrastructural features (32). Subapical vesicles are thought to be more abundant in the smooth-cell type (30, 32), and because this is the predominant type in medullary TAL, we hypothesized that vasopressin may have a selective effect within the cell type. However, we found no noticeable differences in either cotransporter expression levels or vasopressin-induced membrane translocation when comparing smooth and rough TAL cells (smooth cells, 44.6% vs 69.9%; rough cells, 34.2% vs 62.5%; particles at the luminal membrane in control and vasopressin-treated TAL cells, respectively). We conclude from these data that the function and regulation of NKCC2 by vasopressin does not differ between the two subtypes of TAL cells.

Evidence linking the activation of NKCCs with translocation to the cell surface is scarce and limited to NKCC1 (33, 34). Interestingly, an intact cytoskeleton seems to be necessary to achieve vasopressin stimulation of Na-K-Cl cotransporter in cultured mouse TAL cells (35), and a C terminus truncated variant of NKCC2 has been shown, in Xenopus laevis oocytes, to modulate the traffic of full-length NKCC2 molecules to the cell membrane (36). We now demonstrate that, in addition to its effects on NKCC2 phosphorylation, vasopressin increases the traffic of NKCC2 to the apical membrane of TAL cells in vivo.

Based on this evidence, one might propose a model for the action of vasopressin in the mouse TAL that reflects that found in the collecting duct, where protein kinase A-mediated phosphorylation of aquaporin-2 seems to be involved in the increased translocation of the water channel elicited by vasopressin (37). However, a causal relationship between phosphorylation and trafficking of NKCC2 has yet to be established. Although the effect of the truncated variant of NKCC2 mentioned above is observed after the elevation of cAMP in the oocyte (36), evidence for direct phosphorylation of NKCC2 by protein kinase A is lacking. Although we cannot rule out at this time a causal relationship between phosphorylation and increased NKCC2 traffic, we postulate that phosphorylation of N-terminal threonines and changes in protein traffic may work independently to regulate the activity of NKCC2. The increase in phosphorylated cotransporter brought about by vasopressin seems to exceed that observed for the protein translocation. This observation suggests that, in vivo, phosphorylation of cell membrane-resident NKCC2 molecules acts as the primary regulatory step in the short-term activation of the cotransporter.

**NKCC2 Phosphorylation in Mice Undergoing Water Diuresis—**NKCC2 phosphorylation, as measured by R5, was found in the isOM of untreated, control animals, albeit at low levels (Fig. 2, a and b). This suggests either that endogenous levels of vasopressin were maintaining a basal NKCC2 activation or the existence of an additional non-vasopressin-mediated component to NKCC2 phosphorylation. To distinguish between these two possibilities, mice were subjected to water loading to abolish the secretion of endogenous vasopressin. Surprisingly, despite a significant reduction in urinary osmolality (822 ± 573 versus 2510 ± 830 mOsm, control versus vasopressin-treated, respectively), NKCC2 phosphorylation state in water-loaded mice was increased compared with animals under normal water balance (Fig. 4a). The levels of NKCC2 phosphorylation were not significantly different in control or vasopressin-treated water-loaded mice (Fig. 4b). Immunofluorescence studies confirmed the increased NKCC2 phosphorylation in mice undergoing water diuresis. Strong R5 labeling, comparable with that observed after administration of vasopressin, was observed in the TAL of water-loaded mice, even in the region of the isOM (Fig. 4b).

This result suggests the extracellular conditions imposed during water-loading trigger a separate activation pathway for NKCC2. We hypothesize that this non-additive, alternative NKCC2 stimulatory mechanism acts to compensate for the decrease in net ion reabsorption expected from the higher tubular flow and reduced luminal substrate in water-loaded animals, which underscores the role of the medullary portion of the TAL as a buffer for electrolyte reabsorption, as discussed above. Therefore, the increase in sodium and chloride reabsorp-
tion in the medullary TAL would assist the kidney in the clearance of a large amount of electrolyte-free water.

The phosphorylation of N-terminal threonines in NKCC2 is thus shown to represent the end point for different stimuli regulating ion transport in the TAL. It is well understood that sodium chloride transport in the TAL is sensitive to many regulatory factors, including those involved in renal handling of calcium and magnesium (18, 38). We propose that the molecular mechanisms of NKCC2 regulation described here for vasopressin underlie the activation of NKCC2 by any known stimuli of ion reabsorption in TAL and macula densa cells.

CONCLUSION

In this report, we show that conditions previously described to increase NKCC2 activity also increase the phosphorylation of two threonines in NKCC2 N terminus. In addition, we demonstrate an increase in translocation of NKCC2 to the apical membrane upon administration of vasopressin. The phosphorylation of N-terminal threonines is emerging as a common regulatory process shared between isoforms of the Na-K-Cl cotransporter. The conservation of this sequence in the thiazide-sensitive Na-CI cotransporter, NCC, leads us to speculate that these transport systems play in keeping constant body electrolyte and water content. These mechanisms, especially the as-yet unidentified cotransporter-kinase, are expected to occupy a central spot in the regulation, at the cellular level, of sodium and chloride reabsorption in the distal nephron.

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