Reduced expression of Na-K-2Cl cotransporter in medullary TAL in vitamin D-induced hypercalcemia in rats

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Reduced expression of Na-K-2Cl cotransporter in medullary TAL in vitamin D-induced hypercalcemia in rats. Am J Physiol Renal Physiol 282: F34–F44, 2002. First published August 8, 2001; 10.1152/ajprenal.00101.2002.—Chronic hypercalcemia (HC) is accompanied by urinary concentration defects, and functional studies indicate defects in the thick ascending limb (TAL). We hypothesize that dysregulation of renal sodium transporters may play an important role in this. Vitamin D-induced HC in rats resulted in polyuria, natriuresis, and phosphaturia. Immunoblotting revealed a marked reduction in the abundance of rat type 1 bumetanide-sensitive Na-K-2Cl cotransporter (BSC-1) in inner stripe of the outer medulla (ISOM; 36 ± 5%) and whole kidney (51 ± 11%) in HC. Consistent with this finding, immunocytochemistry and immunoelectron microscopy demonstrated reduced BSC-1 labeling of the apical plasma membrane. Immunoblotting and immunohistochemical labeling of the K channel Kir 1.1 (ROMK) was also reduced in HC. In contrast, there were no reductions in the expression of Na/H exchanger (NHE)3 and Na,K-ATPase in ISOM. The abundance of the proximal tubule type II Na-P, cotransporter (NaPi-2) (but not Na,K-ATPase and NHE3) was significantly reduced (25 ± 4%), consistent with a dramatic increase in urinary phosphate excretion. In conclusion, J) the reduced abundance of BSC-1 and ROMK in TAL is likely to play a major role in the urinary concentration defects associated with HC and 2) the reduced abundance of NaPi-2 is likely to play a role in the increased urinary phosphate excretion.

IT IS WELL KNOWN THAT HYPERCALCEMIA in both humans and experimental animals is associated with urinary concentrating defects and significant polyuria (21, 53), but the exact mechanisms by which hypercalcemia exerts its effect on urinary concentration are not well understood. Alterations in hormone levels, especially arginine vasopressin (AVP) release or response (4), renal hemodynamics (8), and renal tubular function (19) have been examined as possible mediators of the defect. However, the pathogenic mechanisms involved in the impaired urinary concentration are likely to be multifactorial. Several studies showed that a renal concentrating defect persists in hypercalcemic animals despite elevated plasma AVP in response to dehydration (4, 39, 57). Thus reduced responsiveness of the collecting duct to vasopressin appears to be an important cause of polyuria in hypercalcemia, and this view has been supported by several lines of evidence, such as changes of adenylate cyclase activities, cAMP levels, and protein kinase activity (2, 9, 22, 47). Consistent with this view, Earm et al. (14) and Sands et al. (55) recently demonstrated that vasopressin-regulated water channel aquaporin-2 (AQP2) protein levels were downregulated and AQP2 targeting to the apical plasma membrane of the collecting duct principal cell was also somewhat reduced in hypercalcemia. This suggests that dysregulation of collecting duct AQP2 plays a role in the development of polyuria induced by hypercalcemia.

Although the ability of the kidney to conserve water is dependent on the water permeability of the collecting duct (especially in the presence of vasopressin), the urinary concentrating process is also largely dependent on the generation of hypertonic medullary interstitium by countercurrent multiplication. The countercurrent multiplication process is dependent on the active reabsorption of NaCl by the medullary thick ascending limb of Henle’s loop (mTAL) (34, 41). The key sodium transporters responsible for the secondary active transport of NaCl in the mTAL are the apical bumetanide-sensitive Na-K-2Cl cotransporter (BSC-1 or NKCC2; Refs. 20, 45, 66), type 3 Na/H exchanger (NHE3) (1), and basolateral Na,K-ATPase (29). In particular, the apically expressed Na-K-2Cl cotransporter in the TAL is known to be regulated by vasopressin (33), and this regulation may be involved in the long-
term regulation of the countercurrent multiplication system. Thus we hypothesize that changes in expression of BSC-1 (associated with reduced apical plasma membrane expression levels) may play an important role in the urinary concentrating defect as well as in the altered renal sodium handling in hypercalcemia. Also, the potassium channel Kir 1.1 (ROMK; Refs. 64, 67) plays a marked role in the efficiency of TAL sodium reabsorption. Thus a reduction in ROMK expression may potentially also contribute to the urinary concentrating defect associated with hypercalcemia. NHE3 is also present in TAL. Thus a potential downregulation of NHE3 may also be involved.

In addition to the potential defect in the TAL sodium transporter, it remains possible that altered expression of sodium transporters in the proximal tubule may also play a role in the increased urinary sodium excretion associated with hypercalcemia. The proximal tubule is the main site for renal tubular sodium reabsorption, and NHE3 is believed to be responsible for ~75% of the apically absorbed sodium whereas the type II Na-phosphate (Pi) cotransporter (NaPi-2) accounts for 10% in this segment. Downregulation of NHE3 has been demonstrated in several conditions known to have proximal tubule defects in sodium reabsorption and increased renal sodium excretion (35). Thus we hypothesize that downregulation of NHE3 may also participate in the hypercalcemia-induced increase in urinary sodium excretion. Interestingly, it is now clear that transport of Pi through the proximal tubule apical plasma membrane is largely performed by NaPi-2 (5). Because Pi reabsorption and secretion are closely associated with calcium metabolism, it is possible that changes in NaPi-2 expression may occur in addition to the potential downregulation of NHE3 and participate in the increased urinary sodium and Pi excretion.

Therefore, we examined whether there are changes in the expression of several renal sodium transporters in rat kidney in response to experimental vitamin D-induced hypercalcemia, to advance the understanding of the molecular mechanisms that are responsible for the urinary concentrating defect, polyuria, and increased sodium excretion in hypercalcemia.

METHODS

Experimental animals and protocol. Studies were performed in 26 male Munich-Wistar rats, initially weighing 230–260 g. The animals were maintained on standard rat chow (Altromin, Lage, Germany) and were given free access to tap water throughout the experiment. After a period of acclimation, the animals were randomized into two groups matched for body weight: hypercalcemia (HC; n = 14) and control (n = 12) groups. During the entire experiment, rats were kept in individual metabolic cages, with a 12-h artificial light/dark cycle, a temperature of 21 ± 2°C, and humidity of 55 ± 2%.

To produce hypercalcemia, rats (n = 14) were fed for 8 days with rat chow containing 8.5 mg of dihydrotachysterol (DHT; D-9257, Sigma) per 1 kg of dry food. During the experimental period, each rat in the HC group had a consistent intake of 18–19 g of rat chow/day, corresponding to 153–162 μg of DHT/day. In the control group, rats were fed with rat chow without DHT for 8 days (n = 12), and they were offered the same amount of food as HC rats; thus the food intake was matched between the two groups.

Clearance studies. Daily urine output and water intake were determined throughout the study. Urine volume, osmolality, sodium, potassium, calcium, Pi, and creatinine concentration were measured. Plasma was collected from the abdominal aorta at the time of death for measurement of osmolality, sodium, potassium, calcium, Pi, and creatinine concentration.

Primary antibodies. For semiquantitative immunoblotting, previously characterized monoclonal and polyclonal antibodies were used as follows: 1) BSC-1: LL320, an affinity-purified polyclonal antibody to the apical Na-K-2Cl cotransporter of the TAL, characterized previously (16, 33); 2) NaPi-2: LL896, an affinity-purified polyclonal antibody to NaPi-2, characterized previously (37, 44); 3) Na,K-ATPase: a monoclonal antibody against the α1-subunit of Na,K-ATPase, characterized previously (29); 4) NHE3: LL546, an affinity-purified polyclonal antibody to NHE3, characterized previously (17, 32); 5) ROMK: LL567, an affinity-purified polyclonal antibody to ROMK, characterized previously (15).

Membrane fractionation for immunoblotting. Whole kidneys or dissected renal cortex with the outer stripe of the outer medulla (OSOM), the inner stripe of the outer medulla (ISOM), and inner medulla were homogenized (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2, containing 8.5 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride) with an ultra-turrax T8 homogenizer (IKA Labortechnik, Staufen, Germany), and the homogenate was centrifuged at 4°C in an Eppendorf centrifuge at 4,000 g for 15 min at 4°C to remove whole cells, nuclei, and mitochondria. The supernatant was then centrifuged at 200,000 g for 1 h to produce a pellet containing membrane fractions enriched for both plasma membranes and intracellular vesicles. Gel samples (Laemmli sample buffer containing 2% SDS) were made of this pellet.

Electrophoresis and immunoblotting. Samples of membrane fractions were run on 6–16% gradient polyacrylamide minigels (BioRad Mini Protean II) for BSC-1 or 12% polyacrylamide minigels for NHE3, NaPi-2, ROMK, and Na-K-ATPase. For each gel an identical gel was run in parallel and subjected to Coomassie blue staining to assure identical loading. The other gel was subjected to immunoblotting. After transfer by electroelution to nitrocellulose membranes, blots were blocked with 5% milk in PBS-T (80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h and incubated overnight at 4°C with primary antibodies (see Primary antibodies). The labeling was visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies (P447 or P448, diluted 1:3,000; DAKO, Glostrup, Denmark) with an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Little Chalfont, UK).

Quantitation of kidney levels of sodium transporters. ECL films with bands within the linear range were scanned with an AGFA scanner (ARCUS II) and Corel Photopaint software to control the scanner. The labeling density was determined for blots where samples from HC rats were run on each gel with samples from control rats. The labeling density was corrected by densitometry of Coomassie blue-stained gels.

Preparation of tissue for immunocytochemistry and immunoelectron microscopy. Kidneys from control and HC rats were fixed by retrograde perfusion via the aorta with 2% paraformaldehyde plus 0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). For immunoperoxidase staining, kidney blocks containing all kidney zones were dehydrated and embedded in paraffin. The paraffin-embedded tissue was cut at 2 μm on a rotary microtome (Micron). Staining was carried...
out using indirect immunoperoxidase. The sections were de-waxed and rehydrated, and endogenous peroxidase was blocked by 0.5% H2O2 in absolute methanol for 10 min at room temperature. To reveal antigens, sections were treated with a 1 mM Tris solution (pH 9.0) supplemented with 0.5 mM EGTA and were heated in a microwave oven for 10 min. Non-specific binding of immunoglobulin was prevented by incubating the sections in 50 mM NH4Cl for 30 min followed by blocking in PBS supplemented with 1% BSA, 0.05% saponin, and 0.2% gelatin. Sections were incubated overnight at 4°C with anti-BSC-1 antibodies diluted in PBS supplemented with 0.1% BSA and 0.3% Triton X-100. After being rinsed with PBS supplemented with 0.1% BSA, 0.05% saponin, and 0.2% gelatin for 3 × 10 min, the sections were incubated in HRP-conjugated goat anti-rabbit immunoglobulin (DAKO P448) diluted in PBS supplemented with 0.1% BSA and 0.3% Triton X-100. Microscopy was carried out using a Leica DMRE light microscope.

For immunoelectron microscopy, tissue blocks prepared from cortex, outer and inner stripe of outer medulla, and inner medulla were cryoprotected with 2.3 M sucrose containing 2% paraformaldehyde, mounted on holders, and rapidly frozen in liquid nitrogen (38, 46). The frozen samples were freeze-substituted in a Reichert AFS freeze substitution unit (38, 46). In brief, the samples were sequentially equilibrated over 3 days in methanol containing 0.5% uranyl acetate at temperatures gradually raised from −80 to −70°C, then rinsed in pure methanol for 24 h while the temperature was increased from −70 to −45°C, and infiltrated with Lowicryl HM20 and methanol 1:1, 2:1 and, finally, pure Lowicryl HM20 before ultraviolet polymerization for 2 days at 0°C. Immunolabeling was performed on ultrathin Lowicryl HM20 sections. Sections were pretreated with a saturated solution of NaOH in absolute ethanol (2–3 s), rinsed, and preincubated for 10 min with 0.1% sodium borohydride and 50 mM glycine in 0.05 M Tris, pH 7.4 containing 0.1% Triton X-100. Sections were rinsed and incubated overnight at 4°C with anti-BSC-1 antibodies diluted in 0.05 M Tris, pH 7.4 containing 0.1% Triton X-100 with 0.2% milk. After being rinsed, sections were incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated to 10-nm colloidal gold particles (GAR.EM10, 1:50; BioCell Research Laboratories, Cardiff, UK). The sections were stained with uranyl acetate and lead citrate before examination in Philips CM100 or Philips 208 electron microscopes.

Statistical analyses. Values are presented as means ± SE. Comparisons between groups were made by unpaired t-test. P values <0.05 were considered significant.

Results

Rats with hypercalcemia were associated with altered renal water and sodium handling. Rats treated with vitamin D (DHT) for 8 days developed significant hypercalcemia with an increase in total plasma calcium levels (3.0 ± 0.06 vs. 2.4 ± 0.04 mmol/l in control rats; P < 0.05). Consistent with previous reports (14, 55), hypercalcemia in rats was associated with marked alteration in water balance. In the basal period before vitamin D treatment, urine output was not different between the HC group and control rats (Fig. 1A). Three days after DHT treatment, urine output in HC rats started to increase compared with control rats (P < 0.05; Fig. 1A) and progressively increased during the period of treatment. Eight days after DHT treatment, urine output was significantly higher in HC rats (93 ± 8 vs. 46 ± 6 μl·min−1·kg−1 in control rats; P < 0.05; Fig. 1A). In contrast, urine output was unchanged in control rats (Fig. 1A). The marked increase in urine output was also associated with decreased urine osmolality as well as urine-to-plasma osmolality ratio (P < 0.05; Fig. 1B, Table 1), indicating that hypercalcemia is associated with reduced urinary concentration.

Rats with hypercalcemia also had significantly increased urinary sodium excretion. The urinary sodium excretion rate was significantly increased (6.2 ± 0.5 vs. 4.6 ± 0.3 μl·min−1·kg−1 in control rats; P < 0.05; Table 1). Moreover, the fractional excretion of sodium was significantly higher in HC rats (0.67 ± 0.05 vs. 0.44 ± 0.02% in control rats; P < 0.05; Table 1). This finding indicates that hypercalcemia is associated with decrease in the tubular reabsorption of filtered sodium.

Moreover, rats with hypercalcemia had significantly increased urinary calcium and Pi excretion. Urinary calcium excretion in HC rats was strikingly increased (121 ± 10 vs. 7.8 ± 0.7 μl·min−1·kg−1 in control rats; P < 0.05; Table 1), and phosphate excretion was also
creatinine clearance, was not significantly altered where the glomerular filtration rate, estimated by the
with significant calciuria as well as phosphaturia,
that the early phase of hypercalcemia is associated
UPV,
0.05 compared with control group.

No. of rats 14 12
P-osmolality, mosmol/kgH2O 297 ± 1.4 298 ± 1.6
P-sodium, mmol/l 139 ± 0.4* 141 ± 0.4
P-potassium, mmol/l 4.9 ± 0.2* 4.0 ± 0.1
P-calcium, mmol/l 3.0 ± 0.6* 2.4 ± 0.04
P-phosphate, mmol/l 2.1 ± 0.05 2.2 ± 0.03
P-creatinine, µmol/l 35 ± 0.9* 28 ± 0.8
U/P-osmolality 3.0 ± 0.2* 5.6 ± 0.7
Ccr, ml·min−1·kg−1 6.9 ± 0.6 7.8 ± 0.5
Ucr, µmol·min−1·kg−1 6.2 ± 0.5* 4.6 ± 0.3
UCr, µmol·min−1·kg−1 121 ± 10* 7.5 ± 0.7
UCaT, µmol·min−1·kg−1 312 ± 26* 121 ± 8
FENa, % 0.67 ± 0.05* 0.44 ± 0.02

Values are means ± SE. HC, hypercalcemia; Con, control; P-
sodium, plasma sodium; P-potassium, plasma potassium; P-calcium,
plasma calcium; P-phosphate, plasma phosphate; P-creatinine,
plasma creatinine; (U/P)-osmolality, urine-to-plasma osmolality ra-
tio; Ccr, creatinine clearance; Ucr, rate of urinary sodium excre-
tion; Ucr, rate of urinary calcium excretion; Ucr, rate of urinary
phosphate excretion; FENa, fractional excretion of sodium. *P <
0.05 compared with control group.

Table 1. Functional data

<table>
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<tr>
<th>Parameter</th>
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<th>Con</th>
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<tr>
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<td>12</td>
</tr>
<tr>
<td>P-osmolality, mosmol/kgH2O</td>
<td>297 ± 1.4</td>
<td>298 ± 1.6</td>
</tr>
<tr>
<td>P-sodium, mmol/l</td>
<td>139 ± 0.4*</td>
<td>141 ± 0.4</td>
</tr>
<tr>
<td>P-potassium, mmol/l</td>
<td>4.9 ± 0.2*</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>P-calcium, mmol/l</td>
<td>3.0 ± 0.6*</td>
<td>2.4 ± 0.04</td>
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<tr>
<td>P-phosphate, mmol/l</td>
<td>2.1 ± 0.05</td>
<td>2.2 ± 0.03</td>
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<tr>
<td>P-creatinine, µmol/l</td>
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<td>28 ± 0.8</td>
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<td>(U/P)-osmolality</td>
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<td>5.6 ± 0.7</td>
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<tr>
<td>Ccr, ml·min−1·kg−1</td>
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<td>7.8 ± 0.5</td>
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<tr>
<td>Ucr, µmol·min−1·kg−1</td>
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<td>4.6 ± 0.3</td>
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<tr>
<td>UCr, µmol·min−1·kg−1</td>
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<td>7.5 ± 0.7</td>
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<td>FENa, %</td>
<td>0.67 ± 0.05*</td>
<td>0.44 ± 0.02</td>
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Table 2. Densitometric analysis of immunoblots from
hypercalcemic and control rats

<table>
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<tr>
<th>Name</th>
<th>n</th>
<th>Whole Kidney</th>
<th>ISOM</th>
<th>Cortex + OSOM</th>
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<tbody>
<tr>
<td>BSC-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HC</td>
<td>14</td>
<td>51 ± 11*</td>
<td>36 ± 5*</td>
<td></td>
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<tr>
<td>Con</td>
<td>12</td>
<td>100 ± 14</td>
<td>100 ± 11</td>
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<tr>
<td>NaPi-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HC</td>
<td>14</td>
<td>22 ± 5*</td>
<td>25 ± 4*</td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>12</td>
<td>100 ± 10</td>
<td>100 ± 12</td>
<td></td>
</tr>
<tr>
<td>Na, K-ATPase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HC</td>
<td>14</td>
<td>90 ± 5</td>
<td>123 ± 17</td>
<td>101 ± 16</td>
</tr>
<tr>
<td>Con</td>
<td>12</td>
<td>100 ± 15</td>
<td>100 ± 12</td>
<td>100 ± 9</td>
</tr>
<tr>
<td>NHE3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HC</td>
<td>14</td>
<td>81 ± 21</td>
<td>108 ± 14</td>
<td>113 ± 27</td>
</tr>
<tr>
<td>Con</td>
<td>12</td>
<td>100 ± 19</td>
<td>100 ± 15</td>
<td>100 ± 6</td>
</tr>
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</table>

Values (in %) are means ± SE, n, no. of rats. ISOM, inner stripe of the outer medulla; OSOM, outer stripe of the outer medulla; BSC-1, rat type 1 Na-K-2Cl cotransporter; NaPi-2, type II Na-Pi cotransporter; NHE3, type 3 sodium/hydrogen exchanger. *P < 0.05 compared with control rats.

whole kidney abundance of BSC-1 in HC rats was
significantly reduced (51 ± 11 vs. 100 ± 14% in control
rats; P < 0.05; Fig. 2, A and B, Table 1). Consistent
with this finding, in membrane fractions from ISOM,
BSC-1 abundance was significantly decreased to 36 ±
5% of control levels (100 ± 11%; P < 0.05; Fig. 2, C and
D, Table 2).

Immunocytochemical analysis of BSC-1 expression
also showed reduced BSC-1 labeling in TAL cells
in kidney of HC rats. In control rats, an intense BSC-1
labeling was seen in apical domains of cortical TAL
cells (Fig. 3A) and medullary TAL (Fig. 3, B and C),
consistent with previous observations (35, 36). In con-
tact, TAL cells in HC rats exhibited clearly reduced
labeling of BSC-1 (Fig. 3, D and F), consistent with the
decreased abundance determined by semiquantitative

Fig. 2. Semiquantitative immunoblotting of membrane fractions of whole kidney or inner stripe of the outer medulla (ISOM). A and C: immunoblots were reacted with anti-rat type 1 bumetanide-sensitive Na-K-2Cl cotransporter (BSC-1) antibodies and revealed a strong, broad band of molecular mass 146–176 kDa centered at ~161 kDa. HC, hypercalcemic; Con, control. B and D: densitometric analyses revealed that BSC-1 abundance in whole kidney as well as in ISOM was significantly decreased in hypercalcemic rats compared with control rats. *P < 0.05; n = no. of rats.
Immunoblotting. The reduction in BSC-1 labeling was most prominent in ISOM (Fig. 3F).

Immunoelectron microscopy further demonstrated the decrease in BSC-1 labeling in TAL cells in kidneys from HC rats. Immunoelectron microscopy was performed on ultrathin Lowicryl HM20 sections from the inner stripe of the outer medulla tissues of control rats (Fig. 4) and HC rats (Fig. 5). In normal controls, immunogold labeling of the BSC-1 in TAL cells was associated with apical plasma membranes as well as intracellular vesicles (Fig. 4A), consistent with previous observations (45). In contrast, the BSC-1 labeling of the apical plasma membranes and intracellular vesicles in TAL cells was markedly decreased in HC rats (Fig. 5A). The marked downregulation of BSC-1 expression strongly indicates that hypercalcemia may be associated with decrease in sodium and chloride reabsorption in TAL; hence, this may impair the generation of the hypertonic medullary interstitium.

Rats with hypercalcemia did not have altered NHE3 and Na,K-ATPase abundance in mTAL. NHE3 is expressed in the apical plasma membrane of TAL cells. To investigate whether NHE3 levels were altered in mTAL in response to chronic hypercalcemia, we performed semiquantitative immunoblotting of NHE3 in membrane fractions from ISOM. The results demonstrated that NHE3 abundance was not altered (108 ± 14 vs. 100 ± 15% in control rats; NS; Fig. 6). The TAL cells exhibited strong NHE3 labeling of apical plasma membranes in both control rats and HC rats (Fig. 7, A and B). Consistent with immunoblotting data, immunocytochemical analysis of NHE3 expression revealed that NHE3 labeling in TAL cells in kidney of HC rats is unchanged (Fig. 7, A and B).

Moreover, we investigated whether there were any changes in the abundance of Na,K-ATPase in mTAL by semiquantitative immunoblotting in membrane fraction from ISOM. As shown in Fig. 8, Na,K-ATPase abundance was not altered in the ISOM of HC rats (123 ± 17 vs. 100 ± 12%; NS; Table 2). Thus, of three major sodium transporters in TAL cells (i.e., BSC-1, NHE3, and Na,K-ATPase), a marked downregulation of BSC-1 was observed whereas NHE3 and Na,K-ATPase levels remained unchanged.
Rats with hypercalcemia were associated with marked reduction of ROMK abundance in kidney. In addition to the major sodium transporters in TAL cells, we also investigated the changes of expression of ROMK, which is localized at the apical domain of TAL cells (15, 67). This was performed as a control to assess whether changes in BSC-1 expression are accompanied by changes in other TAL proteins.

Semi-quantitative immunoblotting demonstrated that whole kidney abundance of ROMK [the 45-kDa band indicated in previous studies to correspond to ROMK (15)] in HC rats was significantly reduced (62 ± 10% vs. 100 ± 9% in control rats; \(P = 0.05\), \(n = 8\) for HC rats and \(n = 6\) for control rats; not shown). Consistent with this finding, immunocytochemical analysis of ROMK also revealed reduced ROMK labeling in TAL cells in kidneys from HC rats. In control rats, ROMK labeling was seen in apical domains of cortical TAL cells (not shown) and mTAL cells (Fig. 9A). In contrast, TAL cells in HC rats exhibited reduced labeling of ROMK (Fig. 9B).

Changes in proximal tubule sodium transporter abundance in rats with hypercalcemia. Semiquantitative immunoblotting revealed that there were no significant changes in whole kidney abundance of NHE3 between HC rats and control rats (81 ± 21% vs. 100 ± 19%; \(P = 0.05\), \(n = 8\). Moreover, in membrane fractions of kidney cortex and the outer stripe of the outer medulla, NHE3 abundance was not altered between HC rats and control rats (101 ± 16% vs. 100 ± 9%; \(P = 0.05\), \(n = 8\). Thus, like NHE3, there appeared not to be a change in proximal tubule Na,K-ATPase abundance levels.

In contrast, NaPi-2 abundance was significantly decreased in kidneys of rats with hypercalcemia. Semiquantitative immunoblotting revealed that whole kid-
ney abundance of NaPi-2 was significantly decreased in HC rats (22 ± 5 vs. 100 ± 10% in control rats; *P*, 0.05; Fig. 10, A and B). Moreover, in membrane fractions of kidney cortex and the outer stripe of the outer medulla NaPi-2 abundance was markedly reduced (25 ± 4 vs. 100 ± 12% in control rats; *P* < 0.05; Fig. 10, C and D). Thus the reduced abundance of NaPi-2 may participate in the increased urinary sodium excretion and is also likely to play a major role in the increased P<sub>i</sub> excretion.

Fig. 6. Semiquantitative immunoblotting of membrane fractions of ISOM. A: immunoblots were reacted with anti-type 3 Na/H exchanger (NHE3) antibodies and revealed a single ~87-kDa band. *B*: densitometric analyses revealed that NHE3 abundance in ISOM was unchanged in hypercalcemic (HC) compared with control (Con) rats (*n* = no. of rats).

Fig. 7. Immunocytochemical analyses of NHE3 in proximal tubule and TAL of control (A and C) and hypercalcemic (B and D) rats. In normal control rats, abundant NHE3 labeling was seen of the apical plasma membrane domains of proximal tubule cells in cortex (C) and TAL (arrow) in ISOM (A). In hypercalcemic rats, the immunolabeling as well as subcellular distribution of NHE3 in proximal tubule cells and TAL cells in cortex (D) and ISOM (B) were similar to those observed in the control rats (C and A, respectively). G, glomerulus; P, proximal tubule; T, TAL. Magnification: ×1,000.

Fig. 8. Semiquantitative immunoblotting of membrane fractions of ISOM. A: immunoblots were reacted with anti-Na,K-ATPase (α1-subunit) and revealed a ~96-kDa band. *B*: densitometric analyses revealed no changes in Na,K-ATPase abundance in ISOM in hypercalcemic (HC) compared with control (Con) rats (*n* = no. of rats).
DISCUSSION

We demonstrated that hypercalcemia is significantly associated with polyuria and decreased urinary concentration as well as increased urinary sodium, calcium, and Pi excretion. This indicates that rats with hypercalcemia have altered water and sodium metabolism, in addition to changes in calcium and Pi metabolism. In the TAL of hypercalcemic rats, both abundance and apical plasma membrane expression levels of BSC-1 and ROMK were significantly decreased, whereas NHE3 and Na,K-ATPase levels remained unchanged. These findings strongly suggest that downregulation of Na-K-2Cl cotransporter is likely to play a major role in the urinary concentration defects in hypercalcemia by reducing the reabsorption of NaCl in the TAL and impairing the generation of hypertonic medullary interstitium. In proximal tubule of hypercalcemic rats, NaPi-2 was significantly downregulated, whereas NHE3 and Na,K-ATPase remained unchanged. These results strongly suggest that downregulation of NaPi-2 may participate in the increased urinary Pi excretion associated with hypercalcemia.

Decreased abundance of BSC-1/NKCC2 and ROMK in kidneys of rats with hypercalcemia. The Na-K-2Cl cotransporter BSC-1 (or NKCC2), which is localized at the apical plasma membrane domains of mTAL and cortical TAL segments (16, 28, 45), mediates the apical NaCl transport in these water-impermeable segments. This pathway is critical for the generation of the hypertonic medullary interstitium for concentrating urine (41). We demonstrated that the abundance of BSC-1 was significantly decreased in kidneys of rats with hypercalcemia. Immunohistochemistry and immunoelectron microscopy confirmed this finding and revealed reduced apical plasma membrane levels of BSC-1. These findings strongly indicate that the reduced expression of BSC-1 plays a key role in the decreased reabsorption of sodium and chloride in the TAL in response to hypercalcemia. Moreover, the reduced expression is likely to contribute to the decreased urinary concentration and increased urinary sodium excretion. Consistent with this possibility, several previous reports revealed that the hypercalcemic state is associated with decreased renal medullary

Fig. 9. Immunocytochemical analyses of K channel Kir 1.1 (ROMK) in TAL in kidneys of control (A) and hypercalcemic (B) rats. In normal control rats, abundant ROMK labeling was seen of apical plasma membrane domains (arrows) of TAL cells in ISOM (A). In hypercalcemic rat, labeling densities of ROMK in TAL cells (arrows) were markedly decreased in ISOM (B). Note that some TAL cells lack detectable ROMK labeling (arrowheads). Magnification: A, ×1,000; B, ×650.

Fig. 10. Semiquantitative immunoblotting of membrane fractions of whole kidney or kidney cortex with OSOM. A and C: immunoblots were reacted with anti-type II Na-Pi co-transporter (NaPi-2) antibodies and revealed a ~85-kDa band. B and D: densitometric analyses revealed that NaPi-2 abundance in whole kidney as well as in the cortex and OSOM was significantly decreased in hypercalcemic (HC) compared with control (Con) rats. *P < 0.05; n = no. of rats.
hypertonicity (39, 40, 60) as well as reduced TAL NaCl reabsorption (50). Therefore, this will impair the formation of maximally concentrated urine.

Several studies have shown that vasopressin increases the rate of net NaCl absorption in microperfused TAL segments of the mouse and rat (23, 25, 56). Moreover, vasopressin increases NaCl absorption from the TAL in vasopressin-deficient Brattleboro rats (13, 65), further demonstrating that the absorption of NaCl in the TAL is regulated by vasopressin. Consistent with this view, the abundance of Na-K-2Cl cotransporter in the TAL is increased in response to 1-deamino-[8-D-arginine]vasopressin (33, 35); thus this regulation may be involved in the long-term regulation of the countercurrent multiplication system. Because V2 receptor is coupled to activation of adenyl cyclase, it is possible that the upregulation of BSC-1 by vasopressin is a result of elevated levels of cAMP levels. Consistent with this, a cAMP-regulatory element was identified in the 5′-flanking region of the mouse NKCC2 gene (27).

In contrast to the vasopressin action in the enhancement of NaCl reabsorption in this segment, PGE2 decreases the rate of vasopressin-stimulated NaCl reabsorption (11, 18, 59). EP3 receptor, presumably the major receptor for PGE2 in the TAL (6, 63), may couple to adenyl cyclase via the heterotrimeric G protein G, which inhibits cAMP production (7). Thus endogenous PGE2 in the kidney may have an inhibitory effect on TAL cAMP levels; hence, this may influence the abundance of Na-K-2Cl cotransporter.

Chronic hypercalcemia induced by vitamin D in rats was shown to be associated with inhibition of TAL NaCl reabsorption (50). Several possible mechanisms for this hypercalcemia have been suggested. In vitro studies demonstrated that increased extracellular calcium concentration inhibited vasopressin-dependent cAMP production in the outer medulla of mouse kidney (62). Interestingly, renal excretion of PGE2 is significantly increased in rats with hypercalcemia (39, 57), and it has been suggested that elevated PG in kidney may participate in the vasopressin-resistant urinary concentration in hypercalcemia (57). Consistent with this possibility, it was demonstrated that outer medullary PGE2 concentration is significantly high in hypercalcemic rats, and fractional chloride reabsorption percentage (FRCOCl%) in isolated loop segments microperfused in vivo was markedly low compared with control rats (51). This possibility was further supported by an observation that acute systemic treatment with indomethacin in hypercalcemic rats returned FRCOCl% to normal values as well as reducing PGE2 concentration in the outer medulla (51). This finding indicated that elevated PGE2 in kidneys in hypercalcemia is likely to decrease chloride reabsorption in TAL, probably because of decreased expression of the Na-K-2Cl cotransporter in TAL. Consistent with this view, Fernández-Llama et al. (17) demonstrated that cyclooxygenase inhibitors increase the abundance of Na-K-2Cl cotransporter in membrane fractions of renal cortex and outer medulla in normal rats. It is therefore possible that 1) the expression of Na-K-2Cl cotransporter is regulated by vasopressin; 2) PGE2 may have an inhibitory effects on cAMP production via EP3 receptor; and 3) in hypercalcemia, elevated PGE2 levels in kidney may be associated with significantly reduced Na-K-2Cl cotransporter expression in TAL via its inhibitory actions on cAMP production.

ROMK has been shown to be present in the apical domains of cortical and medullary TAL cells (15, 67) and has been shown to be regulated in response to changes in potassium intake, vasopressin, and hydration (see references in Ref. 15). In the present study we demonstrated a marked reduction in TAL ROMK expression levels [determined by immunoblotting revealing a reduction in the 45-kDa band previously shown to correspond to ROMK (15) and by immunocytochemistry]. Thus the reduction in ROMK may participate with the reduced expression in BSC-1 to reduce the urinary concentrating ability in hypercalcemia.

**Hypercalcemic rats exhibited hyperkalemia.** In the present study, hypercalcemia was associated with hyperkalemia. The explanation for this is not simple. Potassium secretion by connecting tubule cells and principal cells of the cortical collecting duct (CCD) is affected by several factors, including urine flow rate and luminal sodium concentration, two parameters that are changed in rats with hypercalcemia (54). Moreover, Reilly and Ellison (54) emphasized the important role of distal convoluted tubule (DCT) and renal connecting tubule (CNT) cells in K+ homeostasis, and many factors also regulate K+ secretion along the distal tubule. This also includes Ca2+ levels in the distal tubule, which inhibit K+ secretion indirectly, by blocking Na+ channels (48, 49). ROMK has been found to be expressed in TAL, DCT, CNT, and CCD (64, 67), and the observed reduction in ROMK in association with hypercalcemia may also contribute to reduced potassium secretion, thereby contributing to the development of hyperkalemia.

**Decreased abundance of NaPi-2 in kidneys of rats with hypercalcemia.** NaPi-2, a Na-Pi cotransporter located in the proximal tubule, is associated with proximal tubular sodium and P(i) reabsorption (42, 58). We demonstrated that vitamin D-induced hypercalcemia in rats was associated with marked reduction in abundance of the NaPi-2 in kidney; hence this may participate in the increased urinary sodium and P(i) excretion. Parathyroid hormone (PTH), which is the major hormonal regulator in P(i) reabsorption, has been shown to decrease the expression of NaPi-2 in proximal tubule cells of rat and rabbit (31, 43, 52). Consistent with these observations, we recently demonstrated that NaPi-2 expression in the proximal tubule was significantly decreased in rats with chronic lithium treatment, which has previously been reported to raise serum calcium and lower serum P(i) concentrations and to increase urinary calcium excretion in human and rat, presumably because of hyperparathyroidism (3, 12, 37).

In the present study, NaPi-2 abundance was significantly downregulated in hypercalcemic rats, although during hypercalcemia plasma PTH levels are supposed to be lower than in the normal state. However, acute in-
creases in plasma calcium concentration are associated with a number of systemic alterations that may affect the reabsorption of P,", independent of PTH levels (61). Interestingly, calcium infusion affects the filtered load of P, and is associated with an increase in total plasma P, levels, independent of PTH levels (24, 26). This is probably because of an exodus of P, from erythrocytes (10). Moreover, chronic administration of 1,25(OH)2D3 results in phosphaturia, also independent of the effect of PTH (30), which may be secondary because of increased intestinal absorption. Thus it is not clear whether vitamin D directly regulates NaPi-2 expression in the apical part of the proximal tubules (43) or this change is caused by other factors. Further studies are needed to determine the mechanisms involved in the regulation of proximal tubular sodium transporter NaPi-2 expression in rats with hypercalcemia.

In summary, we demonstrated that hypercalcemia in rats is associated with altered abundance of renal sodium transporters, accompanied by polyuria, reduced urinary concentration, increased urinary sodium excretion, calcium, and phosphaturia. The abundance of the Na-K-2Cl cotransporter in TAL was significantly decreased in hypercalcemic rats, and this response is likely to play a significant role in decreasing NaCl reabsorption as well as generation of hypertonic medullary interstitium in hypercalcemia and thereby lead to the severe impairment in urine concentration associated with hypercalcemia. In contrast, the abundance of NHE3 and Na,K-ATPase in TAL was not associated with hypercalcemia. In contrast, the abundance of Na,K-ATPase in TAL was not associated with hypercalcemia. In contrast, the abundance of Na,K-ATPase in TAL was not associated with hypercalcemia.


