Effect of Chronic Metabolic Acidosis on Calbindin Expression along the Rat Distal Tubule

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Abstract. Calbindin D28k has been reported to be involved in the transcellular calcium transport along the rat distal tubule. It has also been shown that chronic metabolic acidosis (CMA) induces significant hypercalciuria. The present study investigated whether CMA affects the mRNA and the protein expression of calbindin D28k along isolated distal tubule (DT) of rats. The animals were made acidic by adding 0.28 mol/L NH₄Cl to the drinking water for 7 d. This maneuver was associated with an increase in plasma ionized calcium. Inulin clearance experiments demonstrated that metabolic acidosis did not affect GFR, but it significantly increased both total and fractional urinary calcium excretion. To define the role of calbindin D28k, total RNA was extracted from DT, identified, and microdissected from collagenase-treated kidneys. cDNA was synthesized from RNA using reverse transcriptase and oligo(dT)₁₂₋₁₈ primers. Calbindin D28k mRNA abundance was semiquantified by a competitive reverse transcription-PCR, using an internal standard of cDNA that differed from the wild-type calbindin D28k by a deletion of 86 bp. The reverse transcription-PCR was performed starting from the same amount of total RNA. For each set of experiments, control and acidic rats were studied in parallel. The identity of the DT was further verified by the presence of the thiazide-sensitive NaCl cotransporter (rTSC1) mRNA. Calbindin D28k mRNA abundance was 0.89 ± 0.21 amol/ng total RNA in DT of CMA rats (n = 5) compared with 0.30 ± 0.12 amol/ng total RNA of control rats (n = 5) (P < 0.05). Using specific rabbit polyclonal anti-calbindin D28k antibody, Western blotting was performed starting from thin slices of outer cortex. Densitometric analysis revealed that in acidic rats (n = 7) there was a 17 ± 5% (P < 0.05) increase in calbindin D28k protein abundance compared with controls (n = 7). These results indicate that in the rat, ammonium chloride loading induces an increase in filtered ionized calcium load that is associated with a significant upregulation of calbindin D28k both at the mRNA and protein level. These last effects will help to reduce the concomitant hypercalciuria, thus mitigating the consequence of CMA on calcium metabolism.

Calbindin D28k belongs to a family of high-affinity calcium-binding proteins that comprises calmodulin, troponin C, parvalbumin, and S100 protein (1). Calbindin D28k was first reported in chick duodenum (2). It is present in the highest concentrations in several organs of the avians, in the mammalian kidney, and in the molluscan brain (1). In rat kidney, it is localized mainly in the distal tubule (DT) (3). Calbindin D28k has been shown to be regulated by several factors such as 1,25-dihydroxyvitamin D₃ (2,4,5), calcium intake (6), phosphorus (7), magnesium (8), glucocorticoids (2), thyroid hormones (9), and parathyroid hormone (10). Moreover, calbindin D28k gene expression is age-dependent: It increases during development (4,11), while it declines with age (12), thus contributing to the age-related decreased calcium transport in intestine and kidney. On the basis of its calcium-binding properties, various functions have been proposed for calbindin: facilitation of the diffusional flux of Ca²⁺ in the intestinal enterocyte (13), protection of neurons against excitatory Ca²⁺ toxicity (14), and action as a mobile Ca²⁺ buffer restricting evoked Ca²⁺ signals in nerve synapses and hair cells (15,16). In epithelial tissues, including the renal cells, calbindin D28k acts as a cytosolic Ca²⁺ buffer and presumably facilitates the diffusional flux of Ca²⁺ through the cytosol (13,17,18). The recent finding that the hypercalciuria induced by cyclosporin administration is associated with an inhibition of calbindin D28k expression (19) supports this hypothesis. Moreover, since intracellular Ca²⁺ concentration is implicated in the regulation of other ion transport processes (20,21), it is also possible that calbindin may indirectly tune the tubular transport rates of other electrolytes, such as, for example, sodium and potassium.

Several studies have shown that chronic metabolic acidosis (CMA) induces an increase in urinary calcium excretion (22). The reasons for the hypercalciuric effect of CMA have not been elucidated. It has been hypothesized to be due to stimulated release of calcium salts from the bone to buffer the excess.
of hydrogen ions (23); in addition, it has been proposed that CMA directly inhibits distal tubule Ca\(^{2+}\) reabsorption (24).

Recently, using competitive reverse transcription (RT)-PCR, we have established a method that allows us to semiquantify the gene expression of selected proteins at the level of isolated and well-identified single tubule segments of the nephron. In this article, we report a detailed description of the technique used. In addition, we present data demonstrating that CMA is associated with a significant upregulation of calbindin D28k mRNA expression at the level of rat distal tubules and with a 17% increase of protein abundance as measured by Western blot experiments using calbindin D28k-specific antibodies. It is concluded that the hypercalciuric effect induced by ammonium chloride loading cannot be attributed to a defect in the efficiency of distal tubular calbindin D28k.

**Materials and Methods**

**Preparation of the Animals and Tubule Microdissection**

The experiments were performed on male Sprague Dawley (200 to 300 g body wt) rats. Metabolic acidosis was induced by adding 0.28 mol/L NH\(_4\)Cl in the drinking water, whereas control rats received regular tap water. After 7 d, the animals were anesthetized (ketalar, 60 mg/kg body wt) and blood was drawn from the cannulated carotid artery for blood gas measurements. The left kidney was removed and washed in ice-cold dissection solution containing (mM): 140 NaCl, 0.4 KH\(_2\)PO\(_4\), 1.6 K\(_2\)HPO\(_4\), 3 H\(_2\)O, 1 MgSO\(_4\), 7 H\(_2\)O, 10 Na\(^{+}\) acetate, 2 glycine, 1 α-ketoglutaric acid, 1.3 Ca\(^{2+}\)-glucuronate, and 14 NaHCO\(_3\), pH 7.4. Small pieces of the renal cortex were cut under the stereomicroscope and incubated for 30 min at 37°C in the microdissection solution containing 0.5 mg/ml collagenase, continuously bubbled with 95% O\(_2\) 5% CO\(_2\). After the digestion, the tissue was washed with ice-cold, collagenase-free dissection solution containing 1 g/L albumin. The identification and microdissection of DT were done freehand under a stereo microscope (Wild M8, Heerbrugg, Switzerland). DT were identified for their appearance among the superficial tubules of the renal cortex. The use of collagenase eased the tubular dissection, thus allowing us to harvest enough material (five to seven tubule segments, corresponding to 1.5 to 3 mm tubule length) for the molecular biology experiments.

**Whole Kidney Clearance**

In similar groups of animals, GFR and urinary calcium excretion were measured. The animals received food and drinking water up to the time of the study. They were anesthetized intraperitoneally with Inactin (Sigma Aldrich, St. Louis, MO), using a dose of 120 mg/kg body wt, tracheostomized, placed on a thermoregulated table (37°C), and prepared for renal clearance. In brief, the right carotid artery was catheterized to record BP and take blood samples for measurements of acid-base parameters, inulin, and calcium concentrations. The left jugular vein was cannulated with polyethylene PE-50 tubing and used for intravenous infusion via a syringe pump (Braun, Melsungen, Germany). Inulin (Inutest, Fresenius Pharma, Graz, Austria) was infused through the jugular vein at a rate of 0.75 mg/min per 100 g body wt in isotonic saline (6 to 8 μl/min per 100 g body wt). The surgical procedure included also the bladder catheterization with PE-50 tubing. After a 60-min equilibration period, the first of three 30-min urine collections began. Arterial blood samples (100 μl) were taken at the start and end of each collection period.

**RNA Purification**

Total RNA was purified from isolated distal tubules. After digestion, the tubules were transferred into 350 μl of lysis buffer containing guanidium isothiocyanate and β-mercaptoethanol. The lystate material was loaded on a silica gel membrane (Qiagen, Chatsworth, CA) that binds specifically the RNA. The membrane was washed three times, and the RNA was eluted in 50 μl of diethylpyrocarbonate 1 g/L (DEPC-water). Possible contamination from genomic DNA was removed by incubation with 5 U of ribonuclease-free deoxyribonuclease I (Promega, Madison, WI), for 30 min at 37°C. The enzyme was inactivated by heating at 70°C for 5 min; thereafter, a second purification run was performed as detailed before. The concentration and purity of RNA was determined by measuring its absorbance at 260 and 280 nm, using a GeneQuant RNA/DNA calculator (Pharmacia Biotech, Freiburg, Germany).

**Reverse Transcription**

cDNA was synthesized starting from equal amounts of total RNA, using 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD), 0.5 μg of oligo(dT)\(_{12–18}\) (Life Technologies), 10 mM dithiothreitol, and 2.5 mM dNTP (Pharmacia in a total volume of 20 μl. Before the addition of reverse transcriptase, the reaction mixture was incubated at 65°C for 3 min to allow the primers to anneal to the poly(A) tail of mRNA. cDNA was synthesized at 37°C for 1 h. Controls, incubated as above, but without addition of reverse transcriptase, were included in each run.

**Polymerase Chain Reaction**

PCR reactions were performed in a total volume of 50 μl in the presence of 10 pmol of each oligonucleotide primer, 200 mM dNTP, 5 μl of 10× PCR buffer, 1.5 mM MgCl\(_2\), and 1.25 U Taq polymerase. PCR was performed using the following primers: sense; 5′-GATGCCAGCAACTGAAGT-3′; antisense, 5′-GGGCTAAGCATAGACTTT-3′. The expected size of the PCR product was 732 bp. Samples were first denatured at 95°C for 3 min, and followed by 33 cycles consisting of denaturing at 95°C (1 min), annealing at 60°C (30 s), and extension at 72°C (1 min). After amplification, PCR products were subjected to size separation by agarose gel electrophoresis (18 g/L).

**Internal Standard Preparation**

Total RNA (200 ng) purified from isolated DT was submitted to RT using the oligo(dT)\(_{12–18}\) primer. Then the cDNA was amplified using the following primers: sense, 5′-GATGCCAGCAACTGAAGT-3′; linker antisense, 5′-GCATAGACTTTATTTCTCATATGCAG-3′. The expected size was 646 bp. PCR was performed as described previously, and the PCR product was reamplified a second time using the normal antisense primer: sense, 5′-GATGCCAGCAACTGAAGT-3′; antisense, 5′-GGGCTAAGCATAGACTTT-3′. This product was analyzed by agarose gel electrophoresis (18 g/L), and it was recovered from the gel using the agarose gel DNA extraction kit (Boehringer Mannheim, Mannheim, Germany). The concentration was determined by measuring the absorbance at 260 and 280 nm.

**Quantification of Calbindin D28k mRNA**

A competitive PCR was performed using as templates the internal standard of 646 bp and the cDNA obtained from 100 ng of total RNA, extracted from isolated DT. The primers yielded a product of 732 and 646 bp for wild-type and internal standard, respectively. Six to seven competitive PCR were performed by addition of decreasing amounts (from 158.3 to 7.5 amol) of the competitive template to replicate
reactions containing identical amounts of DT cDNA. A progressive
decrease of the competitive template PCR product (646 bp) corre-
sponds to a progressive increase of the wild-type template PCR
product (732 bp). The PCR products were separated on 18 g/L agarose
gel and stained by ethidium bromide. The gel was photographed,
and the quantification of the fluorescence intensity of PCR products was
performed using NIH Image 1.60 software. The amount of calbindin
D28k mRNA was calculated using a log-log scale plot of the ratio of
PCR products versus the known amount of internal standard used in
the competitive PCR reactions. Fluorescence data were multiplied by
732/646 to correct for the differences in molecular weight. When the
wild-type and competitive PCR products were equivalent, the amount
of wild-type present in the starting material was equal to the known
starting amount of internal standard. Results are expressed in attomoles (amol) of calbindin D28k per nanogram (ng) of total RNA.

**Western Blot Analysis**

Small pieces of external cortex (80 to 100 mg) were frozen at
−80°C and then disrupted with a potter homogenizer at 4°C in 50 mM
Tris-HCl buffer containing (μg/ml): 50 4-(2-aminoethyl)-benzene-
sulfonfluryl fluoride, 2 leupeptin, and 2 aprotinin. After centrifugation
at 10,000 × g for 15 min at 4°C, the supernatant was collected and
stored at −80°C until use. Before electrophoresis, the samples were
centrifuged again at 10,000 × g for 15 min at 4°C, and the protein
concentration of the supernatant was determined by Bradford assay.
To evaluate the abundance of calbindin D28k, 10 μg of proteins
obtained from control and treated groups were diluted in 5× loading
buffer (10 g/L sodium dodecyl sulfate [SDS], 20% glycerol, 2%
2-mercaptoethanol, 10 mM Tris-HCl, pH 6.8), boiled for 5 min, and
separated on two 10% SDS-polyacrylamide gel electrophoresis
(PAGE). After electrophoresis, the first gel was stained with Coomas-
sie blue and the second gel was submitted to electroblotting to transfer
the proteins on a polyvinylidene difluoride membrane that was
washed with phosphate-buffered saline. After the wash, the membrane
was incubated with blocking buffer (2 g/L high-purified casein, 1 g/L
Tween 20 in phosphate-buffered saline) for 1 h and then probed for
1 h with a rabbit polyclonal anti-calbindin D28k antibody diluted 1:1000 in 10 ml of blocking buffer. Secondary goat anti-rabbit IgG +
IgM alkaline phosphatase conjugate was diluted 1:5000 in 5 ml of
blocking buffer and added for 1 h after wash two times in 20 ml of
blocking buffer. The last washing was performed three times with 20
ml of blocking buffer and finally detection was obtained with a
CSPD® chemiluminescent substrate (Tropix, Bedford, MA). Calbi-
din D28k abundance in control and acidotic groups was quantified
by densitometric analysis.

**Statistical Analyses**

Inulin and total calcium concentrations in plasma and urine were
measured by the anthrone and colorimetric method (Diacron), respec-
tively, while plasma ionized calcium was detected by an ionized
calcium analyzer (Nova 7). Arterial blood pH, CO₂, and calculated
plasma bicarbonate were measured with a blood-gas analyzer (ABL
300; Radiometer, Copenhagen, Denmark). GFR was calculated using
a standard inulin clearance equation, while fractional calcium excre-
tion (FE_{Ca}), i.e., the urinary calcium excretion expressed as % of
filtered calcium, was calculated according to the following formula:

\[ V \times U_{\text{Ca}} / GFR \times P_{\text{Ca}} \times 100 \]

where \( V \) is urine flow rate, and \( U_{\text{Ca}} \) and \( P_{\text{Ca}} \) are the calcium concen-
trations in urine and plasma, respectively. Statistical analysis was
performed using the paired \( t \) test. All data are expressed as means ±
SEM.

**Results**

**Data on Whole Kidney Functions**

Table 1 shows the data on systemic acid-base balance, GFR,
and fractional excretion of calcium in control rats and animals
with CMA. It has been shown by other investigators that rats
given NH₄Cl in the drinking water developed chronic meta-
bolic acidosis (25); however, after 7 d this metabolic disorder was
partially compensated by pulmonary hyperventilation. In-
deed, blood PCO₂ was significantly lower compared with con-
rol animals. With respect to renal hemodynamics, GFR, mea-
sured by inulin clearance, was not significantly affected by the
administration of NH₄Cl, while these animals showed a sig-
nificant hypercalciuria both in absolute and fractional terms. In
an additional group of control and CMA rats, plasma ionized
calcium was measured; the data show a significant increase of
plasma ionized calcium in acidic rats compared with control
animals (5.17 ± 0.06 versus 6.43 ± 0.05 mg/dl, respectively; \( n = 5, P < 0.001 \)).

**Distribution of D28k mRNA along the Nephron**

Figure 1 shows the distribution of calbindin D28k mRNA
along the nephron (representative of three experiments). It is clear
that the mRNA is present only at the level of the distal tubule,
while it is absent from the glomerulus, the proximal tubule, the thick
ascending limb, and the medullary collecting duct. The identity of
PCR products was confirmed by: (I) the size of the PCR products,
separated by agarose gel electrophoresis and ethidium bromide staining; and (2) their direct sequence. Moreover, since the distal tubule is a very difficult segment to dissect, to be completely sure that we had isolated distal tubules, we searched for the thiazide-sensitive NaCl cotransporter mRNA (rTSC1) that has been demonstrated to be expressed only in this segment (26). As illustrated in Figure 2, the tubules that expressed calbindin D28k mRNA showed the presence of rTSC1 mRNA. In the same experiments, we also checked that the PCR products were not due to material carryover from genomic DNA contamination. To this end, negative controls were included in the RT reaction; no PCR products were obtained when reverse transcriptase was omitted from the RT reaction (Figure 2) (representative of three experiments).

Figure 3A shows the typical gel of competitive PCR (representative of five experiments). The addition of decreasing amounts of internal standard (646 bp) resulted in a corresponding increase of the wild-type template products (732 bp). Figure 3B shows the corresponding log-log plot. The amount

Figure 2. Agarose gel electrophoresis of ethidium bromide-stained cDNA products from RT-PCR of mRNA for D28k (732 bp) and rTSC1 (549 bp) obtained from cortical distal tubules.
of wild-type mRNA is calculated when the PCR products of internal standard and wild type are equated (ratio = 1). In Figure 3C, we have reported the results obtained by competitive RT-PCR starting from known amounts of total RNA. The data show that in the range of total RNA used, there was a highly significant linear correlation versus the mRNA for calbindin D28k.

**Absolute Quantification of Calbindin D28k mRNA**

To examine whether calbindin D28k mRNA increases in chronic metabolic acidosis under *in vivo* conditions, we measured calbindin D28k mRNA expression by competitive RT-PCR in NH4Cl-treated and control rats. The quantification was performed on a small amount (about 5 to 7 DT) of starting material. The total RNA was purified simultaneously from acidic and control rats, and the competitive RT-PCR reactions were performed starting from the same amounts of total RNA (about 100 ng). For each experiment, the acidic and control rats were studied in parallel. The results of the competitive RT-PCR reactions are showed in Figure 4. Calbindin D28k mRNA abundance was 0.89 ± 0.21 amol/ng total RNA in DT of CMA rats (*n* = 5) compared with 0.30 ± 0.12 amol/ng total RNA of control rats (*n* = 5) (mean ± SEM) (*P < 0.05).

**Calbindin D28k Protein Abundance as Measured by Western Blot**

Calbindin D28k protein abundance was determined by Western blot analysis using a rabbit polyclonal D28k antibody. In Figure 5A, using slices of renal outer cortex, it is shown that this antibody recognized a 28-kD protein (compared with correspondent Coomassie blue-stained SDS-PAGE). Results obtained by densitometric analysis demonstrated that in control animals, calbindin D28k abundance was 2416 ± 166 (integrated optical density units) (*n* = 7) in controls, while it increased (17 ± 5%) to 2823 ± 254 (*n* = 7) (*P < 0.05) in acidotic rats (Figure 5B).

**Discussion**

**Mechanisms of Renal Calcium Transport and the Importance of Calbindin D28k**

The tubular reabsorption of calcium proceeds through two types of processes: (1) a passive, nonsaturable component that uses the paracellular pathway as the consequence of Na\(^+\) and Cl\(^-\) absorption; and (2) an active, saturable process using the transcellular route (27). These two processes have different locations along the nephron. The first is largely confined to the proximal tubule, and the latter is exclusively localized in the distal tubule. Active Ca\(^{2+}\) absorption is a two-step process. Ca\(^{2+}\) enters the polarized distal tubule cell down a steep electrochemical gradient. The molecular nature of the Ca\(^{2+}\) entry step has been recently elucidated; it appears to be an epithelial channel, named ECaC, of 730 amino acids containing six putative membrane-spanning domains (28). In the kidney, it is highly expressed in the apical membrane of Ca\(^{2+}\) transporting cells. Ca\(^{2+}\) exits the cell through the basal lateral membrane. At this level, two main transporters have been identified: a Ca\(^{2+}\)-ATPase and a Na\(^+\)-Ca\(^{2+}\) exchanger. In addition, calcium-binding proteins and, in particular, calbindin D28k, may play a role in the active transcellular Ca\(^{2+}\) transport. This protein belongs to the calmodulin family of intracellular calcium-modulated proteins that reversibly bind cal-

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*Figure 4.* Effect of chronic metabolic acidosis (CMA) on distal tubule D28k mRNA abundance. *P < 0.05 versus control.

*Figure 5.* (A) Representative Western blot results for calbindin D28k expression in renal cortex of control and acidotic rats. (B) Effect of CMA on distal tubule D28k protein abundance. *P < 0.05 versus control.
cium with dissociation constants in the micromolar range (1). It has been proposed that calbindin D28k acts as a carrier protein facilitating the diffusion of Ca$^{2+}$ from the luminal to the basolateral site and maintaining a low intracellular calcium concentration (18). This hypothesis is strongly supported by our observation that calbindin D28k mRNA is expressed only in those cells characterized by active Ca$^{2+}$ transport, i.e., distal tubular cells (Figures 1 and 2), and by other reports showing that calbindin D28k gene is turned on by 1,25-(OH)$_2$D$_3$ (29), a substance that has been demonstrated to increase the transcellular active Ca$^{2+}$ reabsorption (30). Our findings indicating the lack of calbindin D28k mRNA in other nephron segments, such as the proximal tubule, the thick ascending limb, and the medullary collecting duct, cannot be explained by a loss of tissue or degradation of mRNA during sample processing. Indeed, the mRNA for β-actin, an indicator of tissue presence and amplifiable mRNA, was present in all of the tubular structures examined (Figure 1). Taken together, these results confirm that in the rat kidney, the gene for calbindin D28k is only transcribed at the level of distal tubule cells, i.e., the only tubular cells suited for active Ca$^{2+}$ reabsorption. These data are in agreement with previous findings obtained by Rhoten and Christakos in mouse kidney, using the in situ hybridization technique (31).

**Competitive RT-PCR Technique**

PCR is a very useful technique to examine renal tubular function. Because of its incredible sensitivity, it is the ideal method to detect nucleic acids from few cells. This property, combined with the tubular microdissection, allows the detection of the distribution of the specific RNA along a very heterogeneous structure such as the nephron. However, two main problems are associated with the use of this technique: the poor reproducibility and the intrinsic difficulty to obtain quantification data of gene modulation. The first complication is clearly related to the method itself: The final yield of a PCR reaction is connected to the exponential amplification of starting template; small variance in the amplification effectiveness will result in a massive difference of the final product, particularly if the quantity of the starting material is very low. On the other hand, to obtain data on the abundance of selected RNA, most authors have used other RNA as a standard. The intensity of the gene expression is measured as a relative ratio factored by the expression of another gene that is supposed to be unaffected by the experimental maneuver. β-actin and GAPDH have been commonly used for this purpose. This method is clearly flawed by the assumption that the expression of the reference gene is not altered. A way to overcome these two limitations of PCR technique is to coamplify, in the same test tube, a reference template (internal standard) that will differ from the wild-type template by a short deletion, but shares the same primer sites. Under these conditions, the two templates will compete for the same primers and will amplify at the same primer sites. Under these conditions, the two templates for each point displays a linear relationship (Figure 3B), allowing the measurements of unknown calbindin D28k mRNA abundance.

**Metabolic Acidosis-Induced Hypercalciuria and Calbindin D28k Expression**

The clearance studies (Table 1) clearly show that after ammonium chloride loading, the fractional calcium excretion is increased. These results may indicate that at some point along the nephron, Ca$^{2+}$ transport is inhibited by metabolic acidosis. However, the tubular segment and the mechanism(s) responsible for the hypercalciuric effect of metabolic acidosis have not yet been identified.

Several authors have shown that metabolic acidosis alters vitamin D metabolism. Although in humans ammonium chloride loading has been associated with an increase in the serum concentration of 1,25-(OH)$_2$D$_3$ (33), in the rat it has been repeatedly demonstrated that CMA reduces circulating 1,25-(OH)$_2$D$_3$ levels by inhibiting renal proximal tubule 25-hydroxyvitamin D3-1-hydroxylase (1-OHase) (34), the key enzyme responsible for the conversion of 25-(OH)-D$_3$ to 1,25-(OH)$_2$-D$_3$, and by enhancing the activity of the renal 24-hydroxylase (35), the enzyme partially responsible for the degradation of 1,25-(OH)$_2$-D$_3$. Therefore, it is clear that the enhancement of calbindin D28k mRNA, described herein, cannot be attributed to a vitamin D$_3$ effect, since there is general agreement that in the rat this substance is decreased during CMA.

Another consequence of acidosis is an increase in ionized calcium probably related to the lower blood pH. Our findings are in close agreement with those of Cunningham et al. (35), showing that after 6 d of NH$_4$Cl administration, while total calcium was comparable in control and acidic rats (as in our present experiments), there was a significant increase in plasma ionized calcium. This finding has been confirmed by other authors (7,36). The increase in plasma ionized calcium may be responsible for the altered vitamin D metabolism. Indeed, when it was blocked with ethyleneglycol-bis(β-aminoethyl ether)-N,N’-tetra-acetic acid, a calcium chelator, the effect of metabolic acidosis on vitamin D metabolism disappeared (36). Our present results, showing a significant increase in mRNA for calbindin and, to a lesser extent, in calbindin D28k protein abundance, indicate that this protein may be involved in the effort to compensate the concomitant hypercalciuria. It is therefore tempting to speculate that the increase in filtered load of ionized calcium may enhance the distal calcium load, leading to an increased calcium flux through the
luminal membrane, and thus stimulating calbindin D28k synthesis. This hypothesis is very attractive and suggests that luminal Ca\(^{2+}\) activity may be responsible for the stimulation of the calbindin gene. In all cases, it is evident that the hypercalciumia induced by ammonium chloride loading cannot be explained by a downregulation of calbindin D28k along the distal tubule, since we have clearly shown that under this condition, calbindin D28k expression is upregulated both at the mRNA and protein level. From a clinical point of view, this effect will help to reduce the hypercalciumia generated by metabolic acidosis, thus mitigating the consequence of this acid-base disorder on calcium metabolism.

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