Abstract. A recent study by Steiner et al. (Biochem Pharmacol 51: 253–258, 1996) demonstrated a decreased calbindin D$_{28K}$ expression in the kidneys of cyclosporin A (CsA)-treated rats. To evaluate the association of renal calcium handling with calbindin D$_{28K}$ expression in CsA-treated rats, two separate experiments (vehicle [VH] versus CsA groups, 1,25-dihydroxyvitamin D$_3$ [VitD] versus VitD + CsA groups) were done simultaneously. CsA (25 mg/kg per d, subcutaneously) and VitD (0.5 µg/kg per d, subcutaneously) were given for 7 d. The CsA group showed decreased serum calcium, increased urine calcium excretion, and decreased calbindin D$_{28K}$ protein level and immunoreactivity compared with the VH group. The VitD + CsA treatment decreased serum calcium, increased urine calcium excretion, and decreased calbindin D$_{28K}$ protein level and immunoreactivity compared with the VitD alone. CsA treatment did not affect the serum parathyroid hormone and VitD levels. This study demonstrates an association of calbindin D$_{28K}$ expression with the urinary calcium excretion in CsA-treated rats, and suggests that decreased calbindin D$_{28K}$ expression may play a role in renal calcium wasting.

Cyclosporin A (CsA) remains one of the most important immunosuppressive drugs in the management of organ transplantation and autoimmune disease. Despite its great benefits, a major side effect of CsA is nephrotoxicity (1,2). CsA causes renal tubular injury and tubular dysfunction. Of these, renal magnesium wasting is a well-known complication (3–6), but little is known about the effect of CsA on renal calcium handling.

In general, most of the filtered calcium (approximately 60%) is reabsorbed in the proximal tubule and the remainder is reabsorbed in the medullary thick ascending limb of Henle's loop (mTAL), the distal convoluted tubule, and the connecting segment. Calcium reabsorption in the proximal tubule and mTAL is largely passive and paracellular. In contrast, calcium reabsorption in the more distal segments is active and transcellular, and this is where the homeostatic control of calcium reabsorption occurs (7).

The calcium binding protein (calbindin) plays a significant role in the process of calcium transport (8–10). Two distinct subclasses of this protein with relative molecular masses of 9,000 and 28,000 have been described. The calbindin D$_{9K}$ is present in high concentrations in the proximal small intestine and facilitates intestinal calcium absorption (11). The calbindin D$_{28K}$ is expressed in the distal nephron segments (12,13) of the rat kidney and is assumed to take part in the process of calcium reabsorption (14,15).

1,25-dihydroxyvitamin D$_3$ (VitD) is an important inducer of calbindin D$_{28K}$ in the distal tubule (16–18). VitD, which regulates intestinal calcium absorption, bone calcium reabsorption, and renal calcium reabsorption, is activated in the renal proximal tubule by parathyroid hormone (PTH), and activated VitD induces the transcription of calbindin D$_{28K}$ in the distal tubule. This cascade process between VitD and calbindin D$_{28K}$ in the kidney strongly suggests a role for calbindin D$_{28K}$ in calcium absorption in distal tubule.

Recent studies by Steiner et al. (19,20) demonstrate a decreased calbindin D$_{28K}$ expression in the kidneys of long-term CsA-treated rats. However, the effects of CsA on segmental and cellular sites of calbindin D$_{28K}$ expression and renal calcium handling are unknown. In this study, we hypothesized that CsA might inhibit calbindin D$_{28K}$ expression in the distal nephron and that this might affect distal calcium reabsorption. To test the hypothesis, we examined the association of calbindin D$_{28K}$ expression and renal calcium handling in an acute model of CsA nephrotoxicity in rats treated with or without exogenous VitD, which is a well-known inducer of calbindin D$_{28K}$ expression.

Materials and Methods

Animals

Adult male Sprague Dawley rats weighing 200 to 225 g were housed in individual cages in a temperature- and light-controlled environment at the animal facilities of Catholic University Medical College. Animals were randomly assigned to different experimental groups. The rats were allowed to acclimate for 7 d prior to the start of the experiments. The rats were fed a standard rodent diet and water ad libitum. The rats were assigned to the following groups:

- **Vehicle Group (VH):** Rats received vehicle (saline) subcutaneously for 7 d.
- **Calbindin D$_{28K}$ Expression Group (VH + CsA):** Rats received VitD (0.5 µg/kg per d, subcutaneously) and VitD (0.5 µg/kg per d, subcutaneously) for 7 d.
- **Calbindin D$_{28K}$ Expression Group (VH + VitD + CsA):** Rats received VitD (0.5 µg/kg per d, subcutaneously) and VitD (0.5 µg/kg per d, subcutaneously) for 7 d.

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environment. All rats received a low-salt diet (0.05% sodium; Teklad, Madison, WI), which is known to accelerate the time course and magnify the structural and functional effects of CsA in rats (21).

**Drugs**

CsA, provided by Sandoz Research Institute (East Hanover, NJ), was diluted in olive oil to a final concentration of 25 mg/ml. VitD (Bonkey, Yu Han Pharmaceutical Co., Seoul, Korea) was dissolved in sterile water to a final concentration of 1 mg/ml.

**Experimental Groups**

Experiment 1 (vehicle versus CsA groups) and experiment 2 (VitD versus VitD + CsA groups) were done simultaneously. In the vehicle (VH) group, rats received a daily subcutaneous injection of olive oil, 1 mg/kg, for 7 d (n = 7). In the CsA group, rats received a daily subcutaneous injection of CsA, 25 mg/kg, for 7 d (n = 7). In the VitD group, rats received a daily subcutaneous injection of VitD, 0.5 μg/kg, for 7 d (n = 7). In the VitD + CsA group, rats received a daily subcutaneous injection of CsA, 25 mg/kg, and VitD, 0.5 μg/kg, for 7 d (n = 7).

**Experimental Protocol**

Weight-matched pairs of rats were randomly assigned to the different treatment groups. Daily body weight and food intake were recorded. Rats were fed the exact amount of food consumed the day before. At the end of each treatment period, 24-h quantitative urine samples were collected in metabolic cages (Nalge Co., Rochester, NY). The next day animals were anesthetized with ketamine, and a blood sample and tissue specimens were obtained. After opening the abdomen through a midline incision, the abdominal aorta was cannulated retrogradely below the renal arteries with an 18-gauge needle. With the aorta occluded by ligation above the renal arteries and renal veins opened by a small incision for outflow, the kidneys were preserved by in vivo perfusion fixation through the abdominal aorta. The kidneys were first perfused briefly with phosphate-buffered saline (PBS) to rinse away all blood and, subsequently, with 4% paraformaldehyde for 4 min.

**Functional Data**

Intact PTH was measured by an immunoradiometric assay specific for intact rat PTH (Nichols Institute, San Juan Capistrano, CA). Intra- and interassay coefficients of variation were 8.4 and 11.0%, respectively. VitD was extracted from plasma with diethyl ether, and extracts were chromatographed as described by Reinhardt et al. (22). The plasma VitD level was measured by a competitive protein assay using calf thymus as the source of binding protein. Plasma sodium was measured by a flame photometer (Instrumentation Laboratories, Lexington, MA). Serum blood urea nitrogen, creatinine, magnesium, calcium, and phosphorus were analyzed on the Ektachem 400 device (Eastman Kodak, Rochester, NY). Plasma CsA concentrations were measured by RIA (Incstar, Stillwater, MN).

**Immunohistochemistry**

Perfused kidneys were removed and fixed additionally by immersion in the same fixative solution for 2 h at 4°C. Slices of kidney tissue were dehydrated and embedded in polyester wax, and sections were cut and mounted on gelatin-coated glass slides. The sections were dewaxed with xylene and ethanol, and, after rinsing in tap water, sections were treated with methanolic H₂O₂ for 30 min. Before incubation with primary antibody, the sections were permeabilized by incubation for 15 min in 0.5% Triton X-100 in PBS and, subsequently, blocked with normal goat serum diluted 1:10 in PBS for 15 min, and incubated overnight at 4°C in the mouse antiserum against calbindin D₂⁸K (Sigma, St. Louis, MO) diluted 1:10,000 in PBS or Na-K-ATPase a₁ subunit (Upstate Biotechnology, Lake Placid, NY) diluted 1:100 in PBS. The sections were rinsed in PBS and incubated for 2 h in peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) or donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories). Sections were then incubated with the peroxidase-substrate solution, a mixture of 0.05% 3,3'-diaminobenzidine and 0.01% H₂O₂ for 5 min at room temperature. After being rinsed with Tris-HCl buffer, the sections were counterstained with hematoxylin and examined with light microscopy.

**Western Blot Analysis**

For Western blot analysis, renal cortex was homogenized in lysis buffer containing 20 mM Tris-HCl, 1% Triton X-100, 150 mM sodium chloride, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.02% sodium azide, 1 mM ethylenediaminetetra-acetic acid, 10 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 3000 × g for 20 min at 4°C. After determination of protein concentration in the supernatant by the Coomassie method (Pierce, Rockford, IL), samples were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis in reducing condition. Proteins were then transferred to nitrocellulose membranes by electroblotting. To reduce nonspecific antibody binding, the membranes were blocked with 5% nonfat dried milk for 30 min at room temperature, and then incubated for 24 h at 4°C with affinity-purified anti-calbindin D₂⁸K (1:50,000). The membranes were then washed in several changes of blotting buffer containing 0.01 M PBS, pH 7.4, and 0.1% Tween 20, and incubated for 1 h with peroxidase-labeled donkey anti-mouse IgG (1:1000, Jackson ImmunoResearch Laboratories). Visualization was made after a 10- to 30-min exposure to enhanced chemiluminescence (Amersham Life Science, Buckinghamshire, United Kingdom) at room temperature. Densitometry analysis was performed using the Zero-Dscan software of Eagle EYETMII Still Video System (Stratagene, La Jolla, CA). The optical densities (mean ± SD) were obtained after three determinations for each band.

**Statistical Analyses**

Results are presented as mean ± SEM, and all statistical analyses were calculated with Statistical Package for Social Sciences version 6.1.1 for Macintosh. Comparisons between groups were done by unpaired t test. The level of statistical significance was chosen as P < 0.05.

**Results**

**Functional Studies**

Baseline parameters were not statistically different among the four groups (data not shown). Table 1 shows the body weight and biochemical parameters in experimental groups. Weight gains were progressive in the VH and CsA groups without significant difference throughout this experiment. Significant hypomagnesemia and inappropriately high fractional excretion of magnesium (FE_Mg) was observed in the CsA group, suggesting renal magnesium wasting (P < 0.05 versus VH) (Table 1). Blood urea nitrogen was significantly increased in the CsA group compared with the VH group (P < 0.05). The CsA group showed a significant decrease in serum calcium...
level (2.09 ± 0.07 versus 2.70 ± 0.12 mmol/L, P < 0.05) and an increase in urinary calcium excretion (0.028 ± 0.010 versus 0.017 ± 0.006 mmol/24 h, P < 0.05) compared with the VH group. The serum VitD and serum PTH levels were not significantly different between VH and CsA groups (43.3 ± 5.6 versus 45.6 ± 8.3 pg/ml and 14.7 ± 2.0 versus 15.6 ± 3.5 pg/ml, respectively) (Figure 1).

Animals treated with VitD and CsA showed severe weight loss compared with the VitD group (P < 0.05) despite pair feeding (Table 1). The VitD + CsA group showed a significant increase in blood urea nitrogen and serum creatinine compared with the VitD group (P < 0.05). The VitD + CsA group showed decreased serum calcium (2.84 ± 0.17 versus 3.26 ± 0.22 mmol/L, P < 0.05) and increased urinary calcium excretion (0.1372 ± 0.044 versus 0.083 ± 0.012 mmol/24 h, P < 0.05) compared with the VitD group. There were no significant differences of serum VitD and PTH levels between VitD + CsA and VitD groups (195 ± 8 versus 201 ± 7 pg/ml and 4.5 ± 2.7 versus 5.7 ± 3.1 pg/ml, respectively) (Figure 1).

**Immunohistochemistry**

The intensity of the immunoreactivity of calbindin D$_{28K}$ in renal tubules in the four groups is summarized in Table 2. The immunoreactivity of calbindin D$_{28K}$ in the VH group was detected in the outer cortex, where it was localized in distal convoluted tubules and connecting tubules (Figure 3A). Immunostaining was much stronger in the connecting tubules than in the distal convoluted tubules (Figure 3B). The CsA group showed a decreased distribution of calbindin D$_{28K}$ immunoreactivity compared with the VH group (Figure 4A). In the distal convoluted tubules, immunoreactivity of calbindin D$_{28K}$ was almost undetectable, and connecting tubules showed weak immunoreactivity for calbindin D$_{28K}$ compared with the VH group (Figure 4B).

Exogenous administration of VitD (VitD group) increased the overall distribution of calbindin D$_{28K}$ immunoreactivity (Figure 5A). Immunoreactivity in the distal convoluted and connecting tubules was markedly increased compared with the VH group (Figure 5B). Immunoreactivity of calbindin D$_{28K}$ was also observed in the cortical collecting tubules (Figure 5B). However, combined treatment with VitD and CsA decreased the overall distribution of calbindin D$_{28K}$ compared with the VitD group (Figure 6A). Both the distal convoluted and connecting tubules showed weak immunoreactivity compared with the VitD group, and no immunoreactivity was detected in the collecting duct (Figure 6B).

To determine whether the observed effect of CsA was specific for calbindin D$_{28K}$, we also examined the immunostaining for Na-K-ATPase in the four experimental groups (Figure 7). In the VH group, immunoreactivity of Na-K-ATPase was detected in the distal convoluted tubules and thick ascending limb (Figure 7, A and B). The CsA group showed no significant change of Na-K-ATPase immunoreactivity compared with the VH group (Panels C and D). Exogenous administration of VitD increased the immunoreactivity of Na-K-ATPase in distal convoluted tubules and thick ascending limb compared with the VH group (Panels E and F). But the treatment with VitD

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**Table 1.** Body weight and biochemical parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
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<tbody>
<tr>
<td></td>
<td>VH (n = 7)</td>
<td>CsA (n = 7)</td>
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<tr>
<td>Body weight (g)</td>
<td>215 ± 10</td>
<td>210 ± 12</td>
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<td>Albumin (g/dl)</td>
<td>3.6 ± 0.2</td>
<td>3.5 ± 0.1</td>
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<td>Phosphorus (mg/dl)</td>
<td>6.8 ± 0.9</td>
<td>6.9 ± 0.6</td>
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<tr>
<td>Magnesium (mg/dl)</td>
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<td>1.2 ± 0.1c</td>
</tr>
<tr>
<td>FE$_{Mg}$ (%)</td>
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<td>11.7 ± 1.1c</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>16.8 ± 2.3</td>
<td>31.7 ± 3.0e</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.47 ± 0.08</td>
<td>0.47 ± 0.08</td>
</tr>
<tr>
<td>Sodium (mEq/L)</td>
<td>139 ± 2</td>
<td>137 ± 3</td>
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<tr>
<td>FE$_{Na}$ (%)</td>
<td>0.05 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>CsA conc. (mg/ml)</td>
<td>3450 ± 230</td>
<td>3622 ± 335</td>
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</table>

a VH, vehicle; CsA, cyclosporin A; VitD, 1,25-dihydroxyvitaminD$_3$; FE$_{Mg}$, fractional excretion of magnesium; BUN, blood urea nitrogen; FE$_{Na}$, fractional excretion of sodium; conc., concentration.

b $P < 0.05$ compared with VitD group.

c $P < 0.05$ compared with VH group.

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**Table 2.** Immunoreactivity of calbindin D$_{28K}$ expression in the distal nephron

<table>
<thead>
<tr>
<th>Group</th>
<th>DCT</th>
<th>CNT</th>
<th>CD</th>
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</thead>
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<tr>
<td>VH (n = 7)</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>CsA (n = 7)</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VitD (n = 7)</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>VitD + CsA (n = 7)</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

* Staining intensity: −, no staining; ±, equivocal positive staining; +, weak positive staining; ++, moderate positive staining; ++++, strong positive staining. CNT, connecting tubule; DCT, distal convoluted tubule; CD, collecting duct.

Other abbreviations as in Table 1.
Figure 1. Serum calcium, 24-h urinary calcium excretion, serum parathyroid hormone (PTH), and 1,25 dihydroxyvitamin D3 (VitD) concentrations in vehicle (VH) and cyclosporin A (CsA) groups. *P < 0.05 compared with vehicle group.

and CsA (VitD + CsA group) did not show any significant difference of the overall distribution and immunoreactivity of Na-K-ATPase compared with the VitD group (Panels G and H).

Western Blot Analysis
Immunoblot and relative optical densities of calbindin D28K are shown in Figure 8. Western blot analysis against calbindin D28K showed a single band at 28 kD (lane 1), which was decreased in CsA-treated rats (lane 2). When relative optical densities of the calbindin D28K band in each lane were compared using normal adult cortex as 100% reference, exogenous VitD (VitD group; lane 3) treatment increased the calbindin D28K protein significantly compared with the VH group (130.3% versus 100%, P < 0.05). In the CsA group (lane 2), calbindin D28K protein was markedly decreased compared with the VH group (23.5% versus 100%, P < 0.05). A comparison between the VitD + CsA and VitD groups revealed similar results (61.5% versus 130.3%, P < 0.05).

Discussion
Our study demonstrates an association between urinary calcium excretion and calbindin D28K expression in the CsA-treated rat. In rats that received CsA, there was a decrease in serum calcium concentrations, an increase in urinary calcium excretion, and a decrease in calbindin D28K protein levels and immunoreactivity compared with the VH group. In animals that received VitD, the addition of CsA resulted in a decrement in serum calcium concentration, an increase in urinary calcium excretion, and a decrease in calbindin D28K protein level and immunoreactivity when compared with animals that had received VitD alone. These results demonstrate an association of calbindin D28K expression with renal calcium handling in CsA-treated rats, and suggests that CsA-mediated downregulation of calbindin D28K might be a critical factor in the renal calcium wasting.

Until now, little was known about the association of CsA and calbindin D28K expression. Recently, Steiner et al. (19) reported decreased levels of calbindin D28K in kidneys of long-term CsA-treated rats, using enzyme-linked immunosorbent assay and two-dimensional protein electrophoresis. These investigators also reported an association of calbindin D28K expression with hypercalciuria in CsA-treated rats (20). However, their study did not examine the localization of calbindin D28K in kidney tissue, and did not provide data on the circulating concentration of VitD and PTH, which are involved in calcium regulation. In the present study, the influence of CsA...
on calbindin D\textsubscript{28K} expression and renal calcium handling was examined using four different approaches. First, calbindin D\textsubscript{28K} localization and calbindin D\textsubscript{28K} protein amount were studied by immunohistochemistry and Western blot analysis. Second, the serum VitD and PTH levels were measured to evaluate the association of these hormones with CsA. Third, calbindin D\textsubscript{28K} and Na-K-ATPase expressions were compared to evaluate the specific action of CsA on calbindin D\textsubscript{28K}. Fourth, the effect of CsA on calbindin D\textsubscript{28K} expression was observed at both basal and VitD-stimulated states to better understand the inhibitory action of CsA on calbindin D\textsubscript{28K} expression.

Previous studies have demonstrated calbindin D\textsubscript{28K} in the distal convoluted tubule and in the connecting tubule (12,13), where it is evenly distributed through the cytosol. In this study, the VH group showed strong immunostaining for calbindin D\textsubscript{28K} in the connecting tubule and weaker labeling of the distal convoluted tubule. However, CsA treatment decreased the overall distribution and intensity of calbindin D\textsubscript{28K} immunoreactivity, which was almost undetectable in the distal convoluted tubules and weak in the connecting tubules compared with the VH group. In the VitD group, overall immunoreactivity for calbindin D\textsubscript{28K} was markedly increased, and its expression was extended to the cortical collecting duct. This immunohistochemical study shows the inhibitory effect of CsA on calbindin D\textsubscript{28K} expression in the distal convoluted and connecting tubules, and stimulatory effect of VitD treatment on calbindin D\textsubscript{28K} expression in different nephron segments.

Microperfusion experiments support an active role of PTH in the fine-tuning of tubular calcium reabsorption, which takes place in segments of the distal nephron (23). Therefore, we measured the serum PTH levels for a possible link between calbindin D\textsubscript{28K} and serum PTH in CsA-treated rats. However, there was no significant difference in serum PTH levels between the VH and CsA groups. This finding suggests that short-term treatment of CsA does not affect the serum PTH levels. In addition to PTH, a relationship between CsA and VitD has been reported, although the results are conflicting (24–28). In general, low-dose CsA (5 mg/kg per d in humans) does not affect the circulating VitD levels, whereas a moderate dose of CsA (15 mg/kg per d) increases VitD levels in rat and humans by stimulating 1-α hydroxylase synthesis (28,29). In this study, CsA dose (25 mg/kg per d) was larger than in previous studies (28,29) on the presumption that CsA might decrease the calbindin D\textsubscript{28K} immunoreactivity by inhibiting 1-α hydroxylase synthesis in the proximal convoluted tubules.
Figure 3. Immunolocalization of calbindin D$_{28K}$ protein in the VH group. (A) At low magnification, positive tubular profiles are seen mainly in the cortical labyrinth. (B) Immunostaining for calbindin D$_{28K}$ was weak in distal convoluted tubules (stars) and heavy in connecting tubules (CNT). Arrows indicate the intercalated cells with no immunoreactivity. Magnification: ×66 in A; ×400 in B.

Figure 4. Immunolocalization of calbindin D$_{28K}$ protein in the CsA group. (A) Positive tubular profiles were significantly decreased in the cortical labyrinth compared with the VH group. (B) Intensity of calbindin D$_{28K}$ immunoreactivity was markedly decreased in distal convoluted tubules (stars) and connecting tubules (CNT) compared with the VH group. Arrows indicate the negative intercalated cells. Magnification: ×66 in A; ×400 in B.
Figure 5. Immunolocalization of calbindin D$_{28K}$ protein in the VitD group. (A) Overall distribution of calbindin D$_{28K}$-positive tubular profiles was increased compared with VH group. In addition to distal convoluted tubules and connecting tubules, cortical collecting ducts (arrowheads) in medullary rays were also positive for calbindin D$_{28K}$. (B) Intensity of calbindin D$_{28K}$ immunoreactivity is markedly increased in distal convoluted tubules (stars) and connecting tubules (CNT) compared with the VH group. Arrows indicate the negative intercalated cells. Magnification: $\times66$ in A; $\times400$ in B.

Figure 6. Immunolocalization of calbindin D$_{28K}$ protein in the VitD + CsA group. (A) Overall distribution of calbindin D$_{28K}$ was markedly decreased compared with VitD group. (B) Intensity of calbindin D$_{28K}$ immunoreactivity also decreased significantly both in distal convoluted tubules (stars) and in connecting tubules (CNT) compared with VitD group. Arrows indicate the negative intercalated cells. Magnification: $\times66$ in A; $\times400$ in B.
Figure 7. Immunolocalization of Na-K-ATPase in cortex (A, C, E, and G) and inner stripe of outer medulla (B, D, F, and H) of VH (A and B), CsA (C and D), VitD (E and F), and VitD + CsA (G and H) groups. Positive immunoreactivity of Na-K-ATPase in the VH group was observed in the distal convoluted tubules (stars) and thick ascending limb (asterisks). In the CsA group, there was no significant change of immunoreactivity in both nephron segments (C and D) compared with VH group. In the VitD group, immunoreactivity of Na-K-ATPase was increased significantly in the distal convoluted tubules (stars) and thick ascending limb (asterisks) compared with VH group, but there was no difference of immunoreactivity between VitD and VitD + CsA groups (G and H). Magnification: ×330 in A through H.
CsA-treated rats, the expression of calbindin D$_{28K}$ (lane 2) was cortex treated with VH (lane 1) as 100% reference. In the kidney of the distal tubules and connecting segments (30). Our study on the effect of CsA on calbindin D$_{28K}$ expression in these segments is specific for calbindin D$_{25K}$, we also examined the effect of CsA treatment completely prevents VitD-induced expression of calbindin D$_{28K}$ in the collecting duct.

In this study, significant hypomagnesemia was observed with CsA treatment, but hypocalcemia was marginal, albeit statistically significant. The hypomagnesemia caused by CsA is usually thought to be associated with a physiologic defect in the mTAL, which is the most vulnerable to CsA (34,35) and the major site for renal magnesium reabsorption (60% of the filtered load). In contrast, marginal hypocalcemia caused by CsA seems to be related to the functional defect of the distal convoluted tubule, which is less vulnerable to CsA and the minor site for renal calcium reabsorption (36). Therefore, the discrepancy between renal magnesium and calcium wasting after administration of CsA may be related to the different sites of reabsorption of the two ions and the preferential site of action of CsA.

However, there was no significant difference in circulating VitD levels between the VH and CsA groups. This finding shows that decreased calbindin D$_{28K}$ expression by CsA is not related to the inhibition of 1-$\alpha$ hydroxylase production.

CsA is a well known nephrotoxic drug. Therefore, it is possible that decreased calbindin D$_{28K}$ expression by CsA represents one aspect of a general disturbance of cellular function in some parts of the nephron. To determine whether the effect of CsA on calbindin D$_{28K}$ expression in these segments is specific for calbindin D$_{28K}$, we also examined the effect of CsA on the expression of Na-K-ATPase, which is abundant in the distal tubules and connecting segments (30). Our study showed no significant change of Na-K-ATPase immunoreactivity in CsA-treated rats compared with the calbindin D$_{28K}$ expression. Similar results were obtained with the VitD and VitD + CsA groups. These results indicate that the inhibitory effect of CsA on calbindin D$_{28K}$ expression is not due to a nonspecific toxic effect in the distal convoluted tubule and connecting tubule. One interesting finding in this study is that VitD has a stimulatory effect not only on calbindin D$_{28K}$ expression, but also on Na-K-ATPase expression. This finding is consistent with previous reports that VitD stimulates the Na-K-ATPase activity in chondrocyte (31) and small intestine (32,33).

One of the biological effects of VitD action is the stimulation of biosynthesis of calbindin D$_{28K}$ in the distal convoluted tubule and connecting tubule (16–18). On the basis of these reports, we examined the effect of CsA on calbindin D$_{28K}$ expression with or without VitD treatment. In our study, CsA treatment decreased calbindin D$_{28K}$ expression significantly compared with the VH group (23.5% versus 100%), and the combined treatment of VitD and CsA resulted in a significant decrease in calbindin D$_{28K}$ expression compared with VitD alone (61.5% versus 130.3%). This observation indicates that CsA has an inhibitory action on calbindin D$_{28K}$ expression in VitD-stimulated states, as well as under normal conditions. However, immunohistochemistry still shows a larger increase and Western blot shows a greater band intensity in the VitD + CsA group than CsA group, suggesting incomplete inhibition of calbindin D$_{28K}$ by CsA in the VitD-stimulated state. Whether CsA can completely inhibit the VitD-stimulated calbindin D$_{28K}$ expression remains to be established. However, the results of our immunohistochemical studies indicate that CsA treatment completely prevents VitD-induced expression of calbindin D$_{28K}$ in the collecting duct.

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In this study, significant hypomagnesemia was observed with CsA treatment, but hypocalcemia was marginal, albeit statistically significant. The hypomagnesemia caused by CsA is usually thought to be associated with a physiologic defect in the mTAL, which is the most vulnerable to CsA (34,35) and the major site for renal magnesium reabsorption (60% of the filtered load). In contrast, marginal hypocalcemia caused by CsA seems to be related to the functional defect of the distal convoluted tubule, which is less vulnerable to CsA and the minor site for renal calcium reabsorption (36). Therefore, the discrepancy between renal magnesium and calcium wasting after administration of CsA may be related to the different sites of reabsorption of the two ions and the preferential site of action of CsA.

It is difficult to evaluate the effect of CsA on renal calcium handling in a clinical setting. Hypocalcemia in renal transplant recipients might be caused by several pathogenetic factors (e.g., hypoalbuminemia, decreased production of calcitriol, hypomagnesemia, immunosuppressive drugs [steroids], volume status, and various clinical events [ischemia and rejection]) (37). Most clinical studies have focused on hyperparathyroidism and steroid use as the causes of bone and mineral disorders after renal transplantation (38). Little is known about the effect of CsA on bone turnover and renal calcium handling.

**Figure 8.** Western blot analysis for calbindin D$_{28K}$ in renal cortex of four different study groups. (A) Representative immunoblot for calbindin D$_{28K}$ in cortex prepared from kidney of VH (lane 1), CsA (lane 2), VitD (lane 3), and VitD + CsA (lane 4) groups. Five micrograms of protein were loaded in each lane. A band of 28 kD was observed in all types of tissues examined, which corresponds to molecular weight of calbindin D$_{28K}$. Note the decrease in calbindin D$_{28K}$ levels in CsA group compared with the VH group. Induction of calbindin D$_{28K}$ with exogenous VitD was confirmed with significant increase in calbindin D$_{28K}$ levels (lane 3), and this was significantly decreased with concomitant treatment of VitD and CsA (lane 4). (B) Relative optical densities of calbindin D$_{28K}$ band in each lane, using renal cortex treated with VH (lane 1) as 100% reference. In the kidney of CsA-treated rats, the expression of calbindin D$_{28K}$ (lane 2) was markedly lower than in VH-treated rat kidney (23.5% versus 100%, $P < 0.05$). Induction of calbindin D$_{28K}$ by VitD (lane 3, 130.3%) was significantly decreased in the VitD + CsA group (lane 4, 130.3% versus 61.5%, $P < 0.05$).
However, our experimental data raise the possibility that CsA-induced renal calcium wasting might be one of the causes of hypocalcemia after renal transplantation.

In summary, the results of our study showed decreased calbindin D28K expression in distal convoluted and connecting tubules of rats with acute CsA nephrotoxicity. This finding was closely related to a decrease in serum calcium level and an increase in urinary calcium excretion, but was not associated with changes in circulating PTH or VitD. This observation suggests that decreased calbindin D28K expression in the distal tubule may play a role in renal calcium wasting after CsA administration.

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References


