Biochemical Pharmacology, Vol. 53, pp. 723–731, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00772-1

Decrease in Kidney Calbindin-D 28kDa as a Possible Mechanism Mediating Cyclosporine A- and FK-506-induced Calciuria and Tubular Mineralization

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ABSTRACT. The use of the immunosuppressant cyclosporine A (CsA) is limited by its adverse renal effects. Most recently, we reported that the drug markedly decreases the levels of the calcium-binding protein calbindin-D 28kDa in kidneys of male Wistar rats. In the present study, the potential relationship between drug-induced nephrotoxicity and the decrease in kidney calbindin-D 28kDa was investigated. Four groups of male Wistar rats were treated for 10 or 31 days with either the immunosuppressant CsA (50 mg/kg/day), FK-506 (5 mg/kg/day), rapamycin (5 mg/kg/day) or with the nonimmunosuppressive cyclosporine derivative 3'keto-[Bmt^1]-[Val^2]-CsA (SDZ PSC-833) (50 mg/kg/day), and the effects on calcium homeostasis, kidney histology and renal calbindin-D 28kDa were examined. Similar effects were found with CsA and FK-506; both drugs strongly reduced kidney calbindin-D 28kDa protein levels, increased urine calcium excretion, caused intratubular calcification, and induced basophilic tubules. In contrast, rapamycin and SDZ PSC-833 caused no decrease in renal calbindin-D 28kDa levels, no noticeable alterations in calcium metabolism, and no renal calcification. The results provide evidence for a link between decreased renal calbindin, increased calcium urine excretion, and intratubular kidney calcification. The present data show no correlation between the decrease in renal calbindin and the induction of basophilic tubules; however, it needs to be investigated if these apparently independent kidney effects may have a common origin upstream of calbindin expression. BIOCHEM PHARMACOL 53;5:723–731, 1997.

KEY WORDS. cyclosporine A; FK-506; calcium-binding protein; calbindin-D 28kDa; calciuria; tubular mineralization; rat; kidney

CsA† is a potent immunosuppressant used to prevent organ graft rejection. Its immunosuppressive action is linked to the binding of the drug to the intracellular receptor cyclophilin [1] and the subsequent blockage of the calcium-dependent serine-threonine phosphatase calcineurin by the cyclophilin-drug complex [2]. The inactivation of calcineurin was shown to inhibit the calcineurin-dependent activation of transcription factors that ultimately regulate transcription of the IL-2 gene [3, 4].

The application of CsA is limited by its adverse side effects, the most clinically important being renal impairment [5]. CsA nephrotoxicity can be divided into 2 major subgroups: 1. functional toxicity with no significant morphological lesions; and 2. morphological forms of toxicity with tubular and/or vascular-interstitial lesions. Renal dysfunction is characterized by an acute decline in glomerular filtration rate and renal blood flow. The morphological changes seen in tubular toxicity in rat [6] and human [7, 8] are inclusion bodies in tubular epithelial cells corresponding to giant mitochondria, tubular vacuolization, and microcalcification. The most characteristic lesions of vascular-interstitial toxicity are interstitial fibroses with tubular atrophy [8]. Distinguishing between the different forms of cyclosporine nephrotoxicity is difficult, and it is not clear whether or not the nephrotoxic and immunosuppressive effects of cyclosporine can be completely separated [9]. The pathogenesis of drug-induced renal dysfunction, although widely studied, is still not completely understood. Interestingly, several studies have demonstrated the protective role of calcium-channel antagonists in CsA nephrotoxicity [10–12]. This may suggest that perturbation of calcium metabolism is crucial for CsA-induced adverse renal effects.

Most recently, we reported that CsA induces a marked decrease in the rat renal calbindin-D 28kDa protein [13,

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† *Abbreviations*: CsA, cyclosporine A; SDZ PSC-833, 3'keto-[Bmt^1]-[Val^2]-CsA; IL-2, interleukin-2; MDR, multidrug resistance; DTT, dithiothreitol; PVDF, polyvinylidene difluoride; calcitriol, 1,25-dihydroxyvitamin D; BCIP, 5-bromo,4-chloro,3-indolylphosphate; NBT, Nitroblue Tetrazolium; H&E, hematoxylin and eosin; PAS, periodic acid Schiff; CAB, Chromotrop Anilin Blue.

14]. Calbindin-D 28kDa is described as a cytosolic calcium-binding protein with characteristics of known calcium-sensitive proteins such as troponin C and calmodulin [15]. Calbindin has been postulated to function as a calcium transport molecule that facilitates the diffusion of calcium through the cell, and to serve as an intracellular calcium buffer, maintaining the ionized calcium below toxic levels during transcellular calcium transport [16]. This protein is found in many mammalian species and in various tissues, with highest concentrations in calcium-transporting tissues, such as intestine, kidney, and placenta [17]. Immunohistochemistry [18, 19] and *in situ* hybridization analysis [20] of calbindin-D 28kDa in mammalian kidney showed positive staining in distal tubules.

In this study, we investigated the potential link between CsA-induced functional and morphological renal changes and the drug-mediated decrease in kidney calbindin-D 28kDa. Rats were treated with the immunosuppressants CsA, FK-506, and rapamycin or with the nonimmunosuppressive cyclosporine derivative SDZ PSC-833, and the effects on renal calbindin-D 28kDa, calcium homeostasis, and kidney histology were examined. The macrolide FK-506, which is 10- to 100-fold more potent than CsA, is known to have a mode of action and toxicity profile similar to that of CsA [21]. Although the macrolide rapamycin has a potency similar to that of FK-506, its mode of action and toxicity profile differs, in that it does not appear to induce renal side effects [6, 22]. SDZ PSC-833, the 3'keto-[Bmt^1]-[Val^2]-CsA, is a nonimmunosuppressive active modifier of MDR that is described as nontoxic [23]. As shown in this work, CsA and FK-506 induced a marked decrease in kidney calbindin-D 28kDa protein levels and a significant increase in urine calcium excretion and renal calcifications. Rapamycin and SDZ PSC-833, however, exhibited none of the above disturbances in either renal structure or function.

MATERIALS AND METHODS

Animal Treatment Protocol

Fifty male Wistar rats (HanIbM:WIST) 8 weeks old and weighing between 180 to 250 g were purchased from Biological Research Labs., Füllinsdorf, Switzerland. The animals were fed commercial rodent food (KLIBA 32-343-4, rat/mouse maintenance diet, Klingentalmühle Ltd, Kaiseraugst, Switzerland) and tap water *ad lib*. The animals were divided randomly into 5 groups of 10. Each drug was dissolved in corn oil and administered daily by gavage. In each experimental group, 5 animals were killed after 10 days and 5 after 31 days of treatment. Group A received vehicle (corn oil), group B 50 mg/kg/day CsA, group C 5 mg/kg/day FK-506, group D 5 mg/kg/day rapamycin, and group E 50 mg/kg/day SDZ PSC-833. The animals were killed with carbon dioxide gas on the day following the final treatment.

Kidney Preparation

Renal cortex (150 mg) was taken from one of the kidney poles and homogenized in 8 volumes of 9 M urea, 4% Nonidet P-40, 1% dithiothreitol (DTT), and 2% carrier ampholytes pH 8–10.5 (Pharmacia, Uppsala, Sweden) using a 1 mL Wheaton glass homogenizer (B. Braun, Melsungen, Germany). The homogenates were centrifuged at 420,000 × *g* at 18°C for 12 min (TL100 ultracentrifuge, TLA 100.3 rotor, 100,000 rpm, Beckman Instruments, Palo Alto, CA). The supernatant was removed, divided into 4 aliquots and stored at −80°C until analysis.

Quantification of Renal Calbindin-D 28kDa by (ELISA)

The specificity of the monoclonal antibody against rat calbindin-D 28kDa (mouse ascites fluid, Sigma, St. Louis, MO) was shown by Western immunoblot analysis. Aliquots of the above kidney homogenates were diluted 1:4 in Laemmli buffer (0.32 M Tris, 0.07 M SDS, 10% glycerol, 5% 2-mercaptoethanol, and a trace of Bromophenol Blue). 10 μL samples (25 mg protein/mL) or molecular weight markers (Rainbow™ colored protein molecular weight markers, high-molecular-weight range, Amersham Life Science, Little Chalfont, Buckinghamshire, England) were loaded onto a 10% SDS-PAGE gel (Mini PROTEAN®II cell; Bio-Rad Laboratories, Hercules, CA) [24] and run for 50 min at 200 V. Proteins were transferred, in a 25 mM Tris buffer containing 5% methanol and 0.01% SDS, onto a PVDF membrane (Millipore Corporation, Bedford, MA) [25] using 100 V for 1 hr at 4°C. Membranes were then blocked for 2 hr in a 100 mM Tris-saline buffer pH 7.5 containing 5% nonfat dry milk. The membranes were incubated for 2 hr with a monoclonal anticalbindin-D 28kDa antibody (mouse ascites fluid, Sigma, St. Louis, MO), diluted 1:1000 with PBS and then with an alkaline phosphatase conjugated goat antimouse antibody 28kDa (DAKO A/S, Glostrup, Denmark) diluted 1:1000 with PBS. A BCIP/NBT chromogen kit (Biomeda Corp., Foster City, CA) was used to visualize the calbindin-D 28kDa protein. The membranes were developed until bands appeared, and the reaction was then stopped with 20 mM EDTA in PBS. The membranes were digitized under green light using an Ektron 1412 CCD scanner (Ektron, Bedford, MA) and a grey scale graphic of the digitized image was prepared using a Seikosha videoprinter (Seikosha Co. LTD, 4-1-1 Taihei Sumida-Ku Tokyo 130, Japan). The ELISA for calbindin-D 28kDa was performed as described previously [14, 26]. Calbindin-D 28kDa protein levels in rat kidney samples were calculated from the standard curve obtained with concentrations of 2 to 128 ng of reference calbindin-D 28kDa (SWant, Bellinzona, Switzerland) diluted in PBS, with tissue homogenisation buffer added to parallel the kidney sample.

The protein content of the kidney samples was measured by a modified Bradford assay as described previously [27].

Urine and Blood Collection and Analysis

Urine samples were collected for 24 h at day 10 and day 29 of treatment. The urine was allowed to drip into dry ice-

cooled tubes and was, thereby, continuously deep frozen. During urine sampling, animals received no food but had free access to water.

Prior to blood sampling, animals fasted for 24 hr. Blood samples were collected from the retrobulbar plexus under light inhalation anesthesia with 100% isoflurane (Forene®, Abbott Laboratories SA, Cham, Switzerland) at day 11 and day 30 of treatment. The blood samples were collected without the addition of an anticoagulant. Serum was obtained after the blood was allowed to clot at room temperature for 30 min and after centrifugation at 10,000 × *g* at 24°C for 5 min (11,500 rpm, Eppendorf Centrifuge 5413).

All measurements were performed on a Synchron CX5® autoanalyzer (Beckman Instruments, Palo Alto, CA) using commercially available reagents. Calcium was measured using an o-cresolphthalein-timed endpoint kit [28] for calcium (Sigma Diagnostics®, St. Louis, MO). Creatinine was assayed using a creatinine reagent kit (Beckman) based on a picric acid method described by Jaffe [29]. Phosphorus was measured using a phosphomolybdate-time endpoint method [30] (Beckman).

Histological Techniques and Kidney Pathology

Both kidneys were collected at necropsy and fixed in 10% buffered formalin. One transverse cut of the right kidney and one longitudinal cut of the left kidney were embedded in paraffin wax. One representative, 4–5 μm thick section of each kidney was stained with hematoxylin and eosin (H&E), periodic acid Schiff (PAS), and Chromotrop Anilin Blue (CAB). The entire sections were examined by light microscopy and findings were scored subjectively according to the following severity codes: (0, no findings; 1, minimal/very few; 2, slight/few; 3, moderate/moderate number; 4, marked/many). Kossa's stain was used for the demonstration of microcalcification [31].

Statistical Analysis

Differences in renal calbindin levels and biochemical parameters in serum and urine between treated and control groups were computed using the Microsoft Excel 5.0 software package (Frontline Systems Inc., Incline Village, NV). Significance was assessed using the two-sample unequal variance Student's *t*-test followed by Holm-Bonferoni's procedure. Coefficients of determination with a p value < 0.05 were considered to be statistically significant.

RESULTS

Animal Weights and Clinical Signs

All experimental groups gained body weight throughout the treatment period. However, there was a slight inhibition of less than 10% for animals treated with CsA, FK-506, and rapamycin, and of approximately 10% for animals treated with SDZ PSC-833, compared to the control group. No treatment-related change in kidney weights was found. No relevant treatment-related clinical signs were noted. Individual animals treated with FK-506 and CsA showed slight sedation during the final week of treatment.

Kidney Calbindin-D 28kDa Protein Levels

The specificity of the monoclonal antibody against rat calbindin-D 28kDa (mouse ascites fluid) was shown by Western immunoblotting (Fig. 1).

In kidneys of control rats, calbindin-D 28kDa levels were approximately 3 μg/mg protein as measured by ELISA. After administration of CsA or FK-506, a marked decrease in

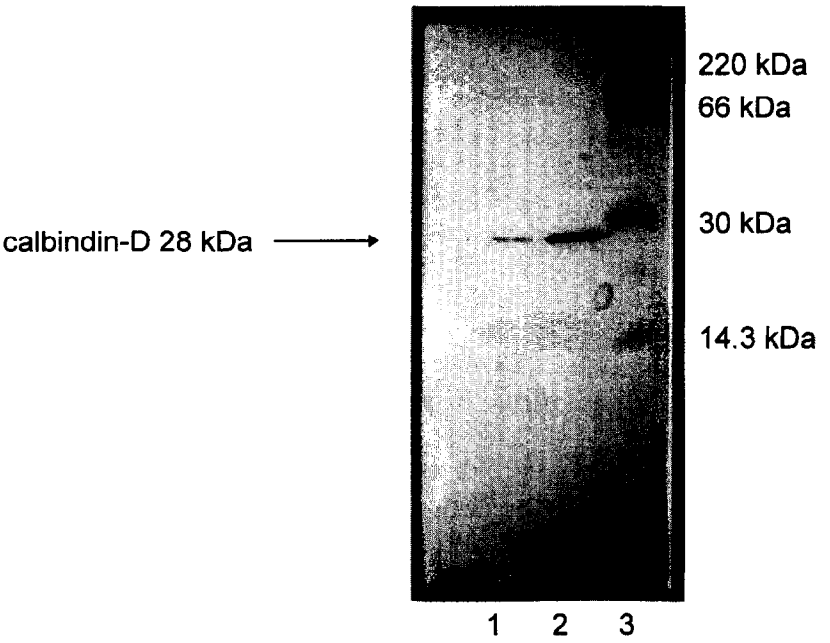

FIG. 1. Western immunoblot analysis of the calbindin-D 28kDa protein in rat kidney homogenates. Lane 1, kidney sample from a rat treated with 50 mg/kg/day CsA for 31 days; lane 2, control Wistar rat kidney sample; lane 3, molecular weight markers. Proteins were separated by 10% SDS-PAGE and blotted to PVDF membranes. Calbindin-D 28kDa was detected using an anticalbindin-D 28kDa antibody and an alkaline phosphatase-conjugated goat antimouse antibody. The anticalbindin-D 28kDa antibody bound to a single band at 28kDa, which was decreased in the kidney of the CsA-treated rat.

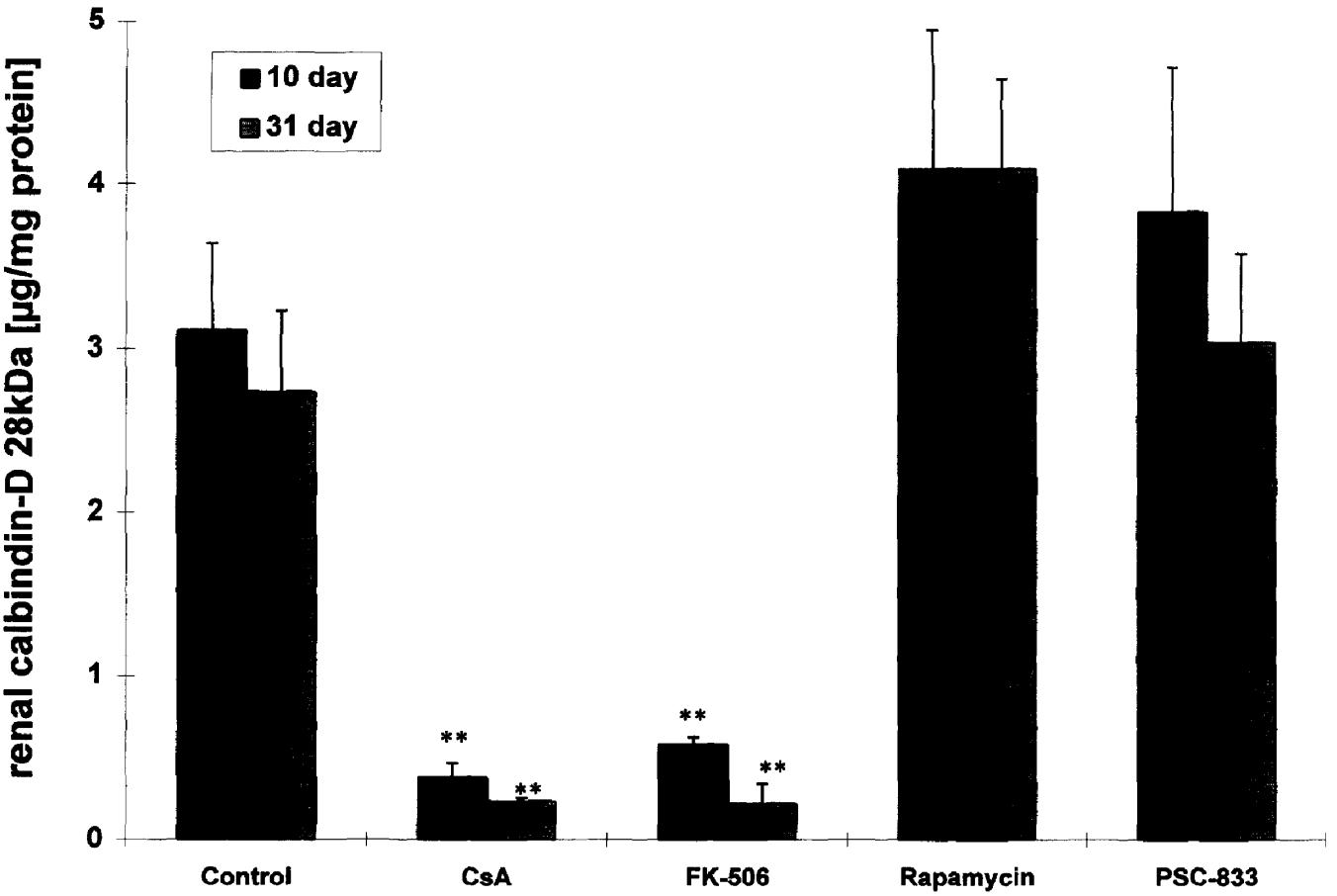

FIG. 2. Effects of 10- and 31-day oral treatment with CsA (50 mg/kg/day), FK-506 (5 mg/kg/day), rapamycin (5 mg/kg/day), and SDZ PSC-833 (50 mg/kg/day) on male Wistar rat kidney calbindin-D 28kDa protein levels as measured by ELISA. The drugs were dissolved in corn oil and control animals received only the vehicle. The bars represent the means ± SEM of 5 rats. **Significantly different from the corresponding control with $p < 0.01$.

calbindin-D 28kDa was observed (Fig. 2). At day 10, average calbindin levels were reduced to 0.38 ± 0.086 µg/mg protein with CsA and to 0.58 ± 0.044 µg/mg protein with FK-506. At day 31, calbindin levels were decreased up to 0.23 ± 0.024 µg/mg protein with CsA, and 0.22 ± 0.122 µg/mg protein with FK-506. For both experimental groups and at both time points, the changes were statistically significant compared to the controls ($p < 0.01$, Student's t-test followed by Holm-Bonferroni's procedure). In the rapamycin and SDZ PSC-833 groups, an increase in calbindin levels was seen, but the changes were not statistically significant compared to the controls.

Biochemical Measurements

Urine calcium showed a high interindividual variability and ranged from 0.009 to 0.028 mmol/24 hr in control rats. A statistically significant increase in urine calcium was seen with CsA and FK-506 (Fig. 3, Table 1). At day 10, average urine calcium was 0.047 ± 0.015 mmol/24 hr with CsA and 0.029 ± 0.007 mmol/24 hr with FK-506 ($p < 0.05$) and, at day 29, 0.061 ± 0.017 mmol/24 hr with CsA and 0.070 ± 0.015 mmol/24 hr with FK-506 ($p < 0.05$ and $p < 0.01$, respectively). No significant changes were observed after treatment with rapamycin or SDZ PSC-833. Urine phosphorus and creatinine were not affected in any of the treatment groups. The urine volume was increased in the SDZ PSC-833 treatment group after 10 days, but the change was not statistically significant. At day 29, urine volumes were increased in a statistically significant manner in the CsA and FK-506 treatment groups ($p < 0.05$) and 2-fold increased in the SDZ PSC-833 group when compared to the control rats. Creatinine clearance was not affected in any of the treatment groups (Table 1).

Serum calcium, phosphorus and creatinine concentrations showed no treatment-related significant changes following treatment for 10 or 29 days (Table 2).

Renal Histopathological Findings

The major renal morphological changes after the 10- and 31-day treatments are summarized in Table 3. After 10 days, minimal intratubular mineralization, predominantly at the corticomedullary junction, was noted in all CsA animals. After 31 days, intratubular mineralization was found in all CsA and FK-506 animals. Mineral deposits were demonstrated to be positive for calcium phosphate by

TABLE 1. Drug effects on renal parameters

Drug	Dose (mg/kg/day)	10-day treatment					29-day treatment				
		Ca^{2+} (mmol/24hr)	PO_4^{3-} (mmol/24hr)	Creatinine (mmol/24hr)	Urine volume (mL/24hr)	Creatinine clearance (mL/min)	Ca^{2+} (mmol/24hr)	PO_4^{3-} (mmol/24hr)	Creatinine (mmol/24hr)	Urine volume (ml/24hr)	Creatinine clearance (mL/min)
Control	0	0.011 ± 0.004	0.74 ± 0.13	0.075 ± 0.010	32.5 ± 15.3	1.4 ± 0.3	0.016 ± 0.008	0.90 ± 0.12	0.091 ± 0.014	19.5 ± 9.3	1.4 ± 0.3
CsA	50	0.047* ± 0.015	0.69 ± 0.11	0.076 ± 0.003	35.7 ± 12.9	1.4 ± 0.1	0.061* ± 0.017	0.98 ± 0.43	0.106 ± 0.037	49.5* ± 13.4	1.8 ± 0.8
FK-506	5	0.029* ± 0.007	0.76 ± 0.05	0.064 ± 0.003	37.7 ± 3.0	1.1 ± 0.1	0.070† ± 0.015	1.36 ± 0.59	0.110 ± 0.043	51.4* ± 25.6	1.6 ± 0.5
Rapamycin	5	0.017 ± 0.007	0.64 ± 0.16	0.066 ± 0.008	26.3 ± 10.8	1.2 ± 0.2	0.020 ± 0.006	0.74 ± 0.13	0.087 ± 0.006	24.8 ± 7.6	1.3 ± 0.1
PSC-833	50	0.011 ± 0.001	0.94 ± 0.26	0.085 ± 0.021	49.7 ± 12.5	1.5 ± 0.3	0.016 ± 0.008	0.89 ± 0.07	0.095 ± 0.018	40.4 ± 13.2	1.7 ± 0.4

Note, The values represent the means ± SEM of 5 rats. * significantly different from the corresponding control with $p < 0.05$; † significantly different from the corresponding control with $p < 0.01$.

Kossa's stain. No mineralization was observed in the rapamycin or SDZ PSC-833 treatment groups or in the controls.

At both day 10 and day 31, increased incidence and severity of renal tubular basophilia was observed in the rats of the FK-506 and CsA groups when compared to the control groups. A slight increase in basophilic tubules was also found in animals treated with rapamycin for 31 days. The basophilic tubules were often accompanied by minimal to slight chronic interstitial inflammatory infiltrates, most pronounced in the FK-506 group treated for 31 days. In controls and SDZ PSC-833-treated rats, basophilic tubules were noted at a minimal to slight degree in some animals.

Minimal to slight vacuolization of the proximal tubular epithelium was noted in 2 animals treated for 10 days with CsA. No vacuolation was seen in any of the other experimental groups.

DISCUSSION

It was shown that the immunosuppressants CsA and FK-506, which induce alterations in the kidneys, also markedly decreased the renal calbindin-D 28kDa protein levels and increased calcium urine excretion in rats, whereas rapamycin and SDZ PSC-833 produced neither noticeable kidney lesions nor changes in calbindin-D 28kDa when compared to control rats.

The data suggest a correlation between a drug-induced decrease in kidney calbindin-D 28kDa protein and an increase in urine calcium excretion. After the 10-day CsA treatment, renal calbindin-D 28kDa protein levels in rats were reduced by approximately 90% and urine calcium excretion was increased more than four-fold when compared to control rats. Similar figures were obtained after the 31-day treatment with CsA. The response to FK-506 was slightly delayed compared to that in CsA. After the 10-day FK-506 treatment, renal calbindin was decreased in rats by approximately 80% and calcium urine excretion was increased almost 3-fold. Following the 31-day treatment with FK-506, calbindin levels were similar to those in CsA rats and urine calcium excretion was increased more than 4-fold when compared to the controls. A relatively high interindividual variation in severity of the pathological response was observed, most likely reflecting the known interindividual differences in the absorption of hydrophobic drugs [32]. In particular, one rat in the 31-day FK-506 treatment group showed a calcium excretion almost twice as high as the group average and, most interestingly, renal calbindin levels of this individual were 10 times lower than the group average.

The treatment-related increase in calcium urine was observed in the absence of any measurable changes in creatinine clearance. Phosphorus urine excretion was slightly increased after 29-day treatment with FK-506, although the extent of this increase was much lower than that seen with calcium. Following the 29-day treatment with CsA, FK-506, and SDZ PSC-833, the 24-hour urine volumes in rats

TABLE 2. Drug effects on serum parameters

Drug	Dose (mg/kg/day)	10-day treatment Ca^{2+} (mmol/L)	10-day treatment PO_4^{3-} (mmol/L)	10-day treatment Creatinine (μmol/L)	29-day treatment Ca^{2+} (mmol/L)	29-day treatment PO_4^{3-} (mmol/L)	29-day treatment Creatinine (μmol/L)
Control	0	2.54 ± 0.05	3.05 ± 0.31	38 ± 7	2.08 ± 0.09	2.02 ± 0.89	49 ± 18
CsA	50	2.61 ± 0.10	3.08 ± 0.40	38 ± 4	1.98 ± 0.07	2.21 ± 0.29	43 ± 4
FK-506	5	2.60 ± 0.10	2.85 ± 0.31	41 ± 4	2.06 ± 0.09	2.47 ± 0.22	48 ± 7
Rapamycin	5	2.60 ± 0.09	2.71 ± 0.16	38 ± 5	1.99 ± 0.04	2.15 ± 0.29	47 ± 4
PSC-833	50	2.56 ± 0.07	2.86 ± 0.23	39 ± 1	2.00 ± 0.06	2.11 ± 0.12	40 ± 5

The values represent the means ± SEM of 5 rats.

were increased approximately 2-fold. The increase in urine volumes did not seem to be related to the drug-induced changes in calcium excretion, because SDZ PSC-833 caused no increase in urine calcium.

Taken as a whole, these observations suggest a link between the decrease in the renal calcium-binding protein calbindin-D 28kDa and the increase in urine calcium excretion, one supported by the known role of calbindin-D 28kDa as a facilitator of calcium transport. The cytosolic protein is known to enhance the uptake of calcium at the luminal surface of the distal convoluted tubule by binding of the divalent cation. By diffusion through the cytoplasm, the liganded protein then moves to the basolateral membrane, where the calcium is transferred to a pump or Ca-ATPase for extrusion into the vascular system [33]. Consequently, a decrease in kidney calbindin levels leads to an impaired reabsorption of calcium in the tubule and an increased excretion of calcium in the urine. The serum calcium concentrations for all treatment groups were in the control range, confirming that impaired calcium reabsorption, rather than high blood calcium levels, was responsible for the increased calcium excretion.

CsA- and FK-506-induced corticomedullary intratubular mineralization was shown to be positive for calcium phosphate by Kossa's stain. Similarly to calcium urine excretion, calcification with FK-506 was delayed when compared to that with CsA. Following 10-day treatment, calcium deposits in rats were detected only with CsA; however, after 31 days, similar amounts were found with CsA and FK-506. Intratubular calcium deposits may be one consequence of calbindin-related impaired tubular calcium uptake, which may lead to both increased calcium urine excretion and saturated intratubular calcium concentrations. Because kidney calbindin-D 28kDa is described to be localized predominantly in distal tubules [18, 20], calcification would be expected to occur mainly in the distal part of the tubule. With the present stained kidney sections, the mineral deposits could not be clearly confined to proximal or distal regions.

The basophilic tubules found in all animals treated with CsA and FK-506 were disseminated throughout the cortical area of the kidney. At present, it is not possible to determine whether randomly distributed tubular basophilia may be related to calbindin-induced corticomedullary tubular mineralization or whether an alternative mechanism may be responsible for the induction of basophilic tubules. One could well imagine that the above two kidney effects may be linked to a common pathway upstream of calbindin expression (e.g. impaired signal transduction) leading to the alterated expression of several genes.

To gain more insight into the mechanisms involved in CsA and FK-506 toxicity, a study of the molecular events leading to a decrease in renal calbindin might well be illuminating. It is known that expression of the kidney calbindin-D 28kDa is induced by the hormonal and biologically active form of vitamin D, calcitriol [17]. The depletion of the hormone may not be the mechanism by which renal calbindin protein levels are decreased, because it has been reported that plasma calcitriol levels are increased by CsA [34], although CsA and FK-506 may interfere with the

TABLE 3. Histopathological findings in rat kidney

Drug	Dose (mg/kg/day)	10-day treatment basophilic tubules	10-day treatment intratubular mineral	31-day treatment basophilic tubules	31-day treatment intratubular mineral
*Grade**		*0 1 2 3 4*	*0 1 2 3 4*	*0 1 2 3 4*	*0 1 2 3 4*
Control	0	3† 2 – – –	5 – – – –	2 2 1 – –	5 – – – –
CsA	50	– – 1 2 2	– 2 3 – –	– – 1 3 1	– 1 2 1 1
FK-506	5	– 4 1 – –	5 – – – –	– – 1 – 4	– – 1 2 2
Rapamycin	5	3 2 – – –	5 – – – –	– 3 2 – –	5 – – – –
PSC-833	50	3 2 – – –	5 – – – –	4 1 – – –	5 – – – –

Each group consisted of five rats.

* 0, no finding; 1, minimal/very few; 2, slight/few; 3, moderate/moderate number; 4, marked/many. †The number of rats with the finding.

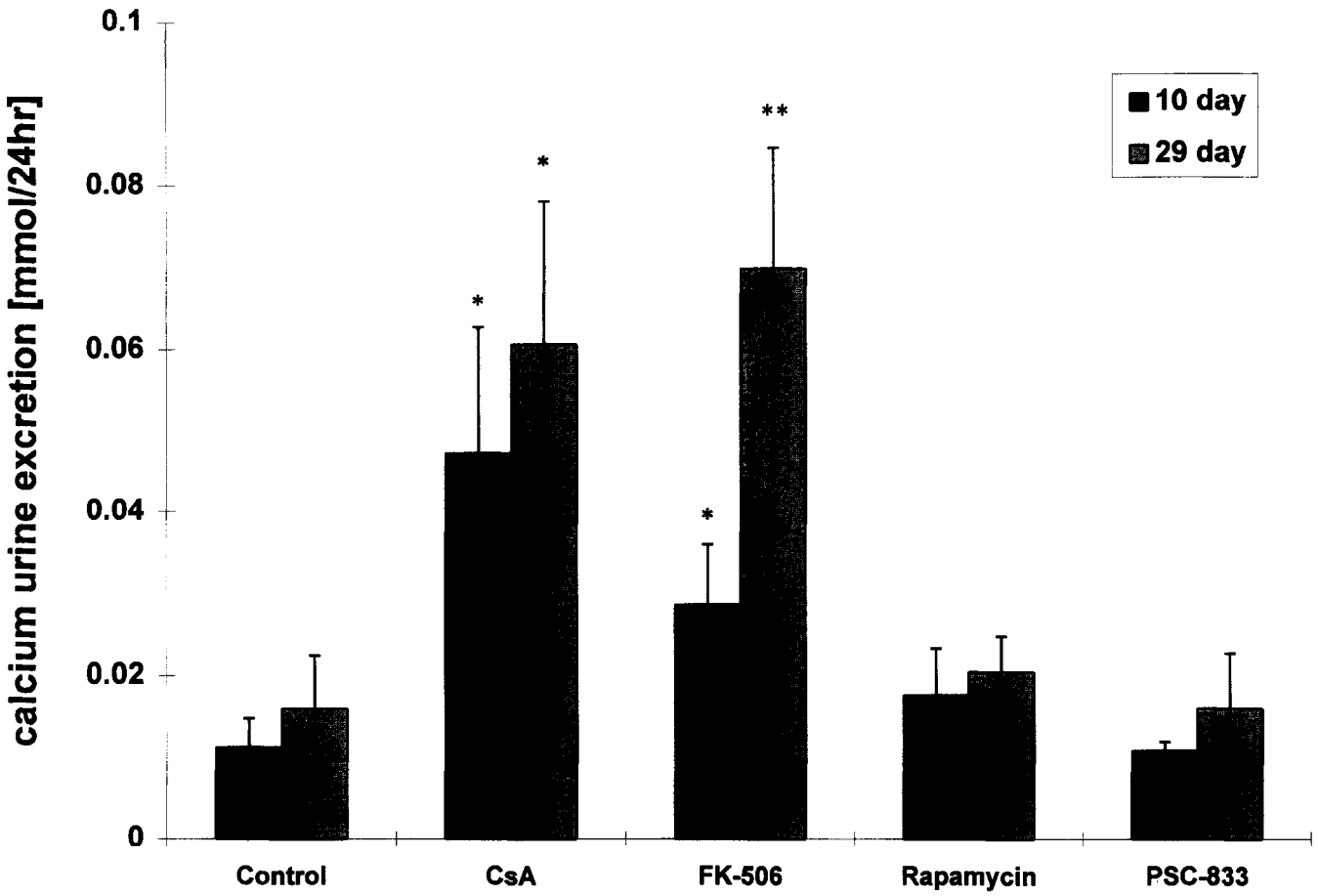

FIG. 3. Effects of 10- and 29-day oral treatment with CsA (50 mg/kg/day), FK-506 (5 mg/kg/day), rapamycin (5 mg/kg/day), and SDZ PSC-833 (50 mg/kg/day) on urine calcium excretion in male Wistar rats. The drugs were dissolved in corn oil and control animals received only the vehicle. Measurements were performed on a Synchron CX5 autoanalyzer using an o-cresolphthalein-timed endpoint kit for calcium. The bars represent the means ± SEM of 5 rats. *$p < 0.05$; **$p < 0.01$, significantly different from the corresponding control.

calcitriol signal transduction mechanism mediated by a cytosolic/nuclear receptor. To confirm this hypothesis, it needs to be demonstrated that the calbindin m-RNA and the transcription of the calbindin gene are decreased in kidneys of CsA-treated rats. If no effect is found at this level, one could assume that the stability of the calbindin protein, rather than its transcription, may be impaired by CsA.

Calcitriol is known to regulate a variety of genes or gene products, among which are the biosynthesis of calcium-binding proteins and lymphokines [35]. If the calcitriol signal transduction pathway is impaired locally, the expression of other kidney proteins known to be regulated by this hormone should also be changed. If, on the other hand, CsA induces a systemic blockage of the calcitriol signal, calcitriol-regulated proteins, including the calbindin of other calcium-transporting tissues such as the intestine or the placenta, should also be altered. The theory that holds that impaired signal transduction is part of the nephrotoxic mechanism is supported by the mode of action of these drugs involving inhibition of calcium-dependent calcineurin signal transduction [3, 4]. The results obtained from the immunosuppressive rapamycin and the nonimmunosuppressive cyclosporine SDZ PSC-833, neither of which decreased renal calbindin levels or had any effect on calcium homeostasis and kidney toxicity, support this hypothesis. The immunosuppressive action of rapamycin is known to be mediated by a calcium-independent event not related to calcineurin inhibition [36]. Likewise, the inactive cyclosporine derivative SDZ PSC-833 was shown to be a poor inhibitor of calcineurin [37]. The fact that rapamycin and FK-506 both bind to the FK-binding protein indicates that binding to the immunophilin is insufficient to induce nephrotoxicity. It remains to be elucidated whether or not inhibition of the phosphatase calcineurin or inactivation of other phosphatases or kinases by the cyclophilin-drug complex is critical for triggering nephrotoxicity.

In conclusion, the data showed that not only the immunosuppressant CsA, but also FK-506, decreased renal calbindin-D 28kDa protein levels in rats. Furthermore, an increase in calcium urine excretion, intratubular calcification, and basophilic tubules was observed with both drugs. Evidence was provided that a decrease in kidney calbindin-D 28kDa in rats is linked to increased calcium urine

excretion and to intratubular calcification, the latter being a consistent finding in CsA-induced tubular toxicity [6–8]. With the present data, the induction of basophilic tubules cannot be correlated with the decrease in renal calbindin. It needs to be investigated in future studies whether or not an event upstream of calbindin expression may be a shared feature of these two kidney findings. In contrast, the immunosuppressant rapamycin and the nonimmunosuppressant cyclosporine SDZ PSC-833 produced no decrease in renal calbindin, no increase in calcium urine, and no renal calcification.

The authors thank Dr. Robin S. Goldstein for critical review of the manuscript.

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