Cyclosporin A produces distal renal tubular acidosis by blocking peptidyl prolyl cis-trans isomerase activity of cyclophilin

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1Department of Pediatrics, Strong Children’s Research Center, University of Rochester School of Medicine, Rochester; 2Department of Clinical Pharmacology, Jichi Medical School, Minamikawachi, Tochigi, Japan; 3Department of Medicine, College of Physicians and Surgeons of Columbia University, New York, New York; and 4Max Planck Research Unit for Enzymology of Protein Folding, Halle/Saale, Germany

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Watanabe, Seiji, Shuichi Tsuruoka, Soundarapandian Vijayakumar, Gunter Fischer, Yixin Zhang, Akio Fujimura, Qais Al-Awqati, and George J. Schwartz. Cyclosporin A produces distal renal tubular acidosis by blocking peptidyl prolyl cis-trans isomerase activity of cyclophilin. Am J Physiol Renal Physiol 288: F40–F47, 2005. First published September 7, 2004; doi:10.1152/ajprenal.00218.2004.—Cyclosporin A (CsA), a widely used immunosuppressant, causes distal renal tubular acidosis (dRTA). It exerts its immunosuppressive effect by a calcineurin-inhibitory complex with its cytosolic receptor, cyclophilin A. However, CsA also inhibits the peptidyl prolyl cis-trans isomerase (PPIase) activity of cyclophilin A. We studied HCO3− transport and changes in β-intercalated cell pH on luminal Cl− removal in isolated, perfused rabbit cortical collecting tubules (CCDs) before and after exposure to media pH 6.8 for 3 h. Acid incubation causes adaptive changes in β-intercalated cells by extracellular deposition of hensin (J Clin Invest 109: 89, 2002). Here, CsA prevented this adaptation. The unidirectional HCO3− secretory flux, estimated as the difference between net flux and that after Cl− removal from the lumen, was ~6.7 ± 0.2 pmol·min−1·mm−1 and decreased to −1.3 ± 0.2 after acid incubation. CsA in the bath prevented the adaptive decreases in HCO3− secretion and apical Cl−·HCO3− exchange. To determine the mechanism, we incubated CCDs with FK-506, which inhibits cyclophilin activity independently of the host cell cyclophilin. FK-506 did not prevent the acid-induced adaptive decrease in unidirectional HCO3− secretion. However, [AD-Ser]6 CsA, a CsA derivative, which does not inhibit calcineurin but inhibits PPIase activity of cyclophilin A, completely blocked the effect of acid incubation on apical Cl−·HCO3− exchange. Acid incubation resulted in prominent “clumpy” staining of extracellular hensin and diminished apical surface of β-intercalated cells [smaller peanut agglutinin (PNA) caps]. CsA and [AD-Ser]6 CsA prevented most hensin staining and the reduction of apical surface; PNA caps were more prominent. We suggest that hensin polymerization around adapting β-intercalated cells requires the PPIase activity of cyclophilins. Thus CsA is able to prevent this adaptation by inhibition of a peptidyl prolyl cis-trans isomerase activity. Such inhibition may cause dRTA during acid loading.

bicarbonate secretion; cell pH; intercalated cell; hensin; cortical collecting duct

cyclosporin A (CSA) is a potent immunosuppressive cyclic undecapeptide whose use has changed the outcomes of transplantation and more recently autoimmune disease. Because this agent is administered for long periods and in the case of transplantation often permanently, it is not surprising that side effects began to appear. Among these complications, hypertension, renal fibrosis, and even the potential development of malignancies have clouded the salutary outcomes.

Cyclosporin can also cause distal renal tubular acidosis (dRTA) (28) independent of its “usual” nephrotoxic effects (1, 13, 17). Studies in patients undergoing treatment for liver transplantation as well as administration of CsA to experimental animals demonstrate the presence of dRTA without the presence of parenchymatous renal disease. Recently, we showed that net HCO3− absorption by isolated cortical collecting ducts (CCDs) from rats treated with CsA is reduced to 30% of the rate in CCDs from normal (32). The mechanism for this effect of CsA is also unknown. Cyclosporin exerts its immunosuppressive effect by binding to cyclophilin A, which then interacts with the Ser/Thr protein phosphatase calcineurin. It is calcineurin that mediates the blockade of transcriptional events needed for cellular immunosuppression. Besides their function as presenter proteins for CsA, cyclophilins also have an enzymatic activity; they catalyze the isomerization of peptidyl prolyl bonds, thereby accelerating slow steps in protein folding and oligomerization (9). However, it is thought that this activity is not relevant for the immunosuppressive action of CsA. In this paper, we show that CsA likely causes dRTA not by inhibiting calcineurin but rather by blocking the peptidyl prolyl cis-trans isomerase (PPIase) activity.

The CCD is composed of two cell types, the principal cell (responsible for salt and water transport) and the intercalated cell, which mediates acid-base transport. Intercalated cells exist in a spectrum of types with two extremes: a β-form that secretes HCO3− and an α-form that secretes acid. Acid-base disturbances can cause a reversal of polarity of HCO3− flux in the CCD of rabbits (21, 24, 25, 27, 30), rats (4), and mice (22, 23). Metabolic acidosis in rats and rabbits results in a reduction in the number of β-cells and an increase in the number of α-cells (5, 25). In rabbit CCDs incubated in vitro in acidic media, we found that individually identified β-intercalated cells were remodeled to functionally resemble α-intercalated cells (24). A similar remodeling of β-intercalated cells, in which the polarity of H+ pumps and Cl−/HCO3− exchangers is reversed, occurs in rabbit intercalated cells in cell culture and requires the deposition of polymerized hensin in the extracel-

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ular matrix (ECM). Seeding β-intercalated cells on already polymerized hensin converts them to an α-phenotype (34, 36).

We recently discovered in rabbit CCDs exposed to acidic media in vitro that hensin was deposited underneath the intercalated cells that were undergoing the conversion. Furthermore, a blocking antibody to hensin prevented the adaptation of β-intercalated cells in isolated, perfused CCDs (27). Hence, the adaptive conversion of β-intercalated cells to α-intercalated cells during acid incubation depends on ECM-associated hensin. In the present study, we demonstrate that CSA also blocks the acid-induced adaptation of β-intercalated cells. Furthermore, CSA prevented the deposition of hensin in the ECM of isolated, perfused tubules. Finally, we show that blockade of peptidyl prolyl cis-trans isomerization but not the calcineurin pathway is responsible for this effect. These studies are the first to directly implicate the PPIase activity of cyclophilins in the maturation of a functional protein in cells.

METHODS

Animals. Female New Zealand White rabbits weighing 1.5 to 3 kg were maintained on laboratory chow and water (30). Each rabbit was anesthetized using an intracardiac injection of pentobarbital sodium (100 mg/kg) after premedication with intramuscular xylazine (5 mg/kg) and ketamine (44 mg/kg) (27).

Microperfusion of CCDs. CCDs were microdissected and microperfused as performed in this laboratory (27, 30). Equilibration, transport and cell pH studies were performed using Burg’s solution in the perfusate and bath, containing (in mM) 120 NaCl, 25 NaHCO3, 2.5 K2HPO4, 2 CaCl2, 1.2 MgSO4, 5.5 glucose, 1 trisodium citrate, 4 sodium lactate, and 6 l-alanine, 290 ± 2 mosmol/kgH2O, and gassed with 94% O2-6% CO2, yielding a pH 7.4 at 37°C (24, 27, 30).

In most experiments, the bath was continually exchanged at 11 ml/h to maintain constant composition. In the case of a limited amount of synthesized inhibitor, bath osmolality was maintained within 1% by adding 60 µl water to the bath every 10 min for 1 h and replacing the bathing solution (and drug) every hour (27).

Incubations for 3 h in acid (pH 6.8 in both luminal and bathing solutions) and control (pH 7.4) media at 37°C were previously described (24, 30). The incubation at pH 6.8 yields a physiology comparable to 3-day acidosis in vivo, whereas that at pH 7.4 sustains net HCO3 secretion (24, 25).

Bicarbonate transport. Triplicate collections of 12–15 nl of tubular fluid were made under water-saturated mineral oil and analyzed for HCO3 secretion (27, 30, 33). When JHCO3 was greater than 0, there was net HCO3 secretion; when JHCO3 was less than 0, there was net HCO3 secretion. To dissect out the coexisting fluxes of H+ secretion by α-intercalated cells and HCO3 secretion by β-intercalated cells, we reversibly removed Cl− from the luminal fluid (27, 30). CCDs were equilibrated for 5 min before collection of fluid during each experimental period. When Cl− is removed from the lumen, HCO3 secretion ceases because of the inhibition of apical Cl−/HCO3 exchange (30, 31); the uncovered flux is unopposed H+ secretion. The difference between net flux and H+ secretory flux is the HCO3 secretory flux (27). Measurements were repeated after the 3-h incubation (at pH 6.8 or 7.4) and compared with preincubation values. Transepithelial voltage was measured between calomel cells in 3 M KCl using the perfusion pipette as a luminal electrode (24, 27). In many of the incubations, CSA (Sigma, 5 or 10 µM) or tacrolimus (FK-506, 0.01–0.1 µM, Fujisawa) was added to the bath 10 min before the start of the acid incubation. Ten millimolar stock solutions of CSA and tacrolimus were prepared in methanol and diluted into the bathing fluid.

Cell pH studies. Cell pH was measured by excitation ratio fluorometry (490-nm/445-nm excitation; 520-nm emission) using 5–10 µM BCECF (Molecular Probes) (24, 27, 33). Fluorescence was detected in multiple intercalated cells and corrected for background (Photon Technology). Precision was enhanced by programming the averaging of two measurements during each snapshot. By examining cells in focus close to the perfusion pipette and in the wall of the tubule, we minimized movement and contaminating fluorescent signals. Readings were obtained in Burg’s solution, after the reversible removal of luminal Cl− and thereafter following the reversible removal of basolateral Cl−. Readings after Cl− removal were stable by 1 min and were obtained at 2 and 3 min of each period. These two readings were averaged for each period. The sequence of readings was repeated in the same identified intercalated cells after 3-h incubation.

In several of the incubations, CSA (5–10 µM), with a Ki value of 0.5 nM to inhibit the PPIase activity of cyclophilin A, and an IC50 of 100 nM of the CSA/cyclophilin A complex to inhibit calcineurin (6), the specific cyclophilin A inhibitor [O-[NH2(CH2)5NHC(O)CH2-]10-Cys-CsA, ([AD-Ser]5 CsA, or its inactive congener, CsH-Ac) was added to the bath. Ten micromolar [AD-Ser]5 CsA inhibited ≤5% of calcineurin in the presence of saturating concentrations of cyclophilin A, and the inhibition of the PPIase activity exhibited an IC50 of 3.2 nM (Zhang Y and Fischer G, unpublished observations). CsH-Ac is a derivative of the completely inert cyclosporine compound CsH (8).

The O-acetylation in position 1 of CsH allows us to remove traces of CsH in the CsH samples but did not change the inertness of the compound. Ten micromolar CsH-Ac showed <5% inhibition of cyclophilin PPIase activity and of calcineurin (11). Stock solutions of the latter agents (1 mM) were prepared in 50% ethanol/50% water and diluted into media on the morning of study. The maximum amount of ethanol added to the bath would have been 0.5%, a concentration previously found to have no effect on tubule function (25). For the studies in isolated, perfused tubules, we used concentrations of inhibitors that were much higher than the IC50 shown above. These concentrations prevented the phenotype switching of cultured intercalated cells in vitro (data not shown). One likely reason for the need for much higher concentrations is that these compounds are hydrophobic, and all of our media contained albumin from fetal bovine serum at substantial concentrations. Hence, the free concentration of these agents could not be determined.

Calibrations using the nigericin and high-potassium buffer technique (24, 33) were performed at the end of each experiment.

Confocal fluorescence microscopy. Microperfused CCDs were incubated for 3 h at pH 6.8 in the presence or absence of CsA or [AD-Ser]5 CsA, as described above, and then labeled extracellularly with anti-hensin antibody at 1:100 dilution in PBS/1% BSA by exposure at 4°C for 4–5 h, followed by fixation in Prefer (Anatech). After permeabilization by 0.1% Triton X-100, β-ICs in these CCDs were counterlabeled with rhodamine-peanut agglutinin (20 µg/ml) followed by secondary FITC-goat anti-guinea pig IgG (1:75) for 1–2 h at room temperature in the dark. Each tubule was transferred to a slide in 90% glycerol in PBS with 0.1% phenylendiamine (to prevent quenching), placed in coverslips, and examined using an Axiostar 100 laser-scanning confocal microscope (model LSM 410; Carl Zeiss) (15, 26, 27, 36). Images were collected using a x40 objective (real magnification ×100), and 1-µm optical sections were obtained and analyzed by the Zeiss LSM-PC software. The final images were processed with Adobe Photoshop software.

Analysis and statistics. Data are presented as means ± SE. Paired and unpaired comparisons were performed using standard statistical software (Excel, Microsoft, Bellevue, WA). Significance was asserted when P values were <0.05.

RESULTS

Adaptation to in vitro acid incubation. When CCDs from normal rabbits are perfused and bathed in vitro in solutions simulating an ultrafiltrate of rabbit plasma at pH 7.4, they generally secrete HCO3" (25, 27, 30). After a 3-h incubation at
pH 6.8, these same tubules reverse their HCO$_3^-$ transport rates to net absorption (24, 27, 31). We confirmed these results in seven newly studied CCDs, finding that the baseline rate of HCO$_3^-$ transport reversed from a secretory flux of $-3.5 \pm 0.1$ pmol·min$^{-1}$·mm$^{-1}$ to a net absorptive flux of $2.6 \pm 0.1$ pmol·min$^{-1}$·mm$^{-1}$ (Fig. 1, panel 1). When Cl$^-$ was removed from the lumen before acid incubation, HCO$_3^-$ secretion was inhibited, revealing an absorptive H$^+$ flux of $3.3 \pm 0.1$ pmol·min$^{-1}$·mm$^{-1}$. The difference between this flux and baseline represents the HCO$_3^-$ secretory flux of $-6.7 \pm 0.2$ pmol·min$^{-1}$·mm$^{-1}$. After acid incubation, the H$^+$ flux was $3.9 \pm 0.2$ pmol·min$^{-1}$·mm$^{-1}$, and the computed HCO$_3^-$ secretory flux was significantly reduced to $-1.3 \pm 0.2$ pmol·min$^{-1}$·mm$^{-1}$, similar to what has been recently published from our laboratory (27). In sum, acid incubation in vitro again caused the CCDs to reverse polarity of HCO$_3^-$ flux from secretion to absorption, with the major adaptation being a reduction in HCO$_3^-$ secretory flux from $-6.7$ to $-1.3$ pmol·min$^{-1}$·mm$^{-1}$, and the minor adaptation being a significant 0.6 pmol/min increase in H$^+$ secretion. Also, there was a significant decrease in luminal electronegativity from $-2.9$ to $-2.4$ mV, compatible with increased electrogenic H$^+$ secretion.

CsA at 10 μM in the bath prevented the adaptive decrease in HCO$_3^-$ secretion (Fig. 1, panel 2). In five CCDs, the baseline net flux was $-3.5 \pm 0.3$ pmol·min$^{-1}$·mm$^{-1}$, and after acid incubation plus 10 μM CsA, it was $0.08 \pm 0.2$ pmol·min$^{-1}$·mm$^{-1}$. The H$^+$ secretory flux significantly increased from $3.4 \pm 0.2$ to $5.0 \pm 0.4$ pmol·min$^{-1}$·mm$^{-1}$, as has been observed previously after acid incubation (27). However, the adaptive decrease in HCO$_3^-$ secretory flux was attenuated by 75% ($-6.4 \pm 0.3$ to $-4.7 \pm 0.3$ pmol·min$^{-1}$·mm$^{-1}$) or a decrease of only 1.7 pmol·min$^{-1}$·mm$^{-1}$, compared with acid incubation alone. Transepithelial voltage significantly decreased from $-2.9 \pm 0.1$ to $-2.7 \pm 0.2$ pmol·min$^{-1}$·mm$^{-1}$, reflecting the increase in H$^+$ secretion.

Similar findings were obtained in six CCDs using 5 μM cyclosporin (Fig. 1, panel 3). The baseline net flux of $-3.3 \pm 0.2$ changed to $0.1 \pm 0.1$ pmol·min$^{-1}$·mm$^{-1}$, and this was accompanied by a significant increase in H$^+$ secretory flux from $3.3 \pm 0.2$ to $4.8 \pm 0.3$ pmol·min$^{-1}$·mm$^{-1}$. The HCO$_3^-$ secretory flux decreased only 29% from $-6.6 \pm 0.3$ to $-4.7 \pm 0.3$ pmol·min$^{-1}$·mm$^{-1}$, a change of 1.9 pmol·min$^{-1}$·mm$^{-1}$. Transepithelial voltage became significantly less negative ($-2.8 \pm 0.1$ to $-2.5 \pm 0.1$ mV), as H$^+$ secretion was increased in the adaptation.

Control incubation of four CCDs for 3 h at pH 7.4 in the presence of 10 μM cyclosporin did not change the net, H$^+$ secretory, or HCO$_3^-$ secretory fluxes (Fig. 1, panel 4) or the transepithelial voltage (data not shown).

Adaptation of apical and basolateral Cl$^-$-base exchange. We examined the same intercalated cells before and after acid incubation to determine their adaptation to low pH. The vast majority of cells loaded from the lumen with BCECF-AM showed a reversible alkalization in response to the removal of luminal Cl$^-$ (Fig. 2), compatible with their being β-intercalated cells (27). Incubation at pH 6.8 × 3 h inhibited apical Cl$^-$/HCO$_3^-$ exchange, manifested by a complete loss of this alkalization (Fig. 2, top tracing, and Fig. 3, panel 3). The ΔpH before incubation averaged 0.42 ± 0.03 pH units, and this was reduced to 0.03 ± 0.02 units in 20 cells from four CCDs. In contrast, incubation at pH 7.4 × 3 h did not change apical anion activity (ΔpH 0.50 ± 0.03 to 0.46 ± 0.03; Fig. 3, panel 4) in 10 cells from 3 CCDs.

CsA (10 μM) in the bath prevented the loss of apical Cl$^-$/HCO$_3^-$ activity (Fig. 2, bottom tracing, and Fig. 3, panel 4). The ΔpH before acid incubation averaged 0.50 ± 0.02, and after incubation was 0.44 ± 0.02 pH units in 21 cells from 4 CCDs. Similarly for a 3-h incubation at pH 7.4, CsA did not change the ΔpH in response to removal of luminal Cl$^-$ (0.38 ± 0.02 to 0.42 ± 0.02 pH units) in 30 cells from 3 CCDs (Fig. 3, panel 2).

In response to the removal of bath Cl$^-$, β-intercalated cells show a decrease in cell pH (27) as shown in Fig. 2. This sensitivity was lost after pH 6.8 incubation (before: $-0.45 \pm 0.04$, after: $-0.04 \pm 0.03$ pH units; Fig. 4, panel 3). In addition, some cells now showed an alkalization in response
CsA inhibits calcineurin. On the other hand, it catalyzes peptidyl cis-trans isomerizations, which often represent rate-limiting steps in the folding of proteins. Because the active site of cyclophilin A is involved in these processes, CsA blocks the PPIase and protein phosphatase catalytic functions. We used another presenter protein complex known to block the PPIase activity of cyclophilins and was used as a control. In 12 cells from 3 CCDs, CsH-Ac did not prevent the adaptation to low pH: before incubation, the increase in cell pH on luminal Cl⁻ removal was 0.46 ± 0.01 pH units, and after pH 6.8 incubation the increase in pH was completely blunted: 0.03 ± 0.03 pH units (Fig. 6, bottom tracing, and Fig. 7, bottom). Similarly, the effect of the inactive congener, CsH-Ac, was comparable to that of pH 6.8 alone on removal of bath chloride. Before incubation with CsH-Ac, cell pH decreased by 0.45 ± 0.03, after: −0.49 ± 0.3 pH units; Fig. 4, panel 1).

CsA in the bath prevented the loss of response to basolateral removal of Cl⁻ (Fig. 4, panel 4). The ΔpH before acid incubation was −0.43 ± 0.03, and after incubation was −0.43 ± 0.03 pH units. In a control study, 3-h incubation at pH 7.4, CsA slightly but significantly increased the ΔpH in response to the removal of basolateral Cl⁻ (before: −0.41 ± 0.03, after: −0.49 ± 0.3; Fig. 3, panel 2).

Mechanism of action of CsA. Cyclophilin A, the cytosolic receptor of CsA in immunosuppression, has two biochemical activities. In the cytosol, it forms the presenter protein for CsA to removal of bath Cl⁻ (see Fig. 2, top tracing). In contrast, incubation at pH 7.4 did not affect this sensitivity (before: −0.45 ± 0.03, after: −0.49 ± 0.3 pH units; Fig. 4, panel 1).

CsA-induced distal renal tubular acidosis (DRTA) is a condition characterized by an inability to acidify the urine to a pH below 5.5, despite a normal or increased ability to excrete bicarbonate. The mechanism involves the inhibition of the basolateral Na⁺,K⁺-ATPase, which is responsible for maintaining the intracellular pH (pHi) in the apical domain of the renal tubular cells. CsA inhibits calcineurin, a Ca²⁺-dependent serine/threonine phosphatase that activates nuclear factor of activated T-cells (NF-AT), which in turn binds to the promoters of genes involved in immune responses. Inhibition of calcineurin leads to the inhibition of NF-AT, which results in the downregulation of genes involved in acid-base balance, particularly genes encoding for the Na⁺,K⁺-ATPase. This leads to a decrease in the ability of the kidney to acidify the urine, resulting in DRTA.

Fig. 2. Tracings of pH of individual cells studied under baseline conditions (Burg's solution in bath and lumen), Cl⁻-free lumen (−ClL), baseline, Cl⁻-free bath (−ClB), before and after the 3-h incubation (3-h Inc.). Top: 2 cells studied before and after 3-h incubation at pH 6.8. Note that 1 cell now alkalinizes on removal of bath Cl⁻ (−ClL) after the 3-h incubation. Bottom: tracings of 2 cells studied before and after 3-h incubation at pH 6.8 in the presence of 10 μM CsA (6.8 + CsA).

Fig. 3. Summary data showing the effect on delta intracellular pH (ΔpHi) of removing Cl⁻ from the lumen (ΔpH was calculated from the difference in cell pH before and after removing Cl⁻ from the lumen). Open bars are preincubation and filled bars are postincubation values. Panel 1: incubation at pH 7.4 (n = 10 cells in 3 CCDs). Panel 2: incubation at pH 7.4 plus 10 μM CsA (n = 30 cells in 3 CCDs). Panel 3: incubation at pH 6.8 (n = 20 cells in 4 CCDs). Panel 4: incubation at pH 6.8 plus 10 μM CsA (n = 21 cells in 4 CCDs). *Significantly different from preincubation value by paired t-test, P < 0.05.

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H11002 0.46 H11006 0.02 pH units in response to removal of bath Cl−, but after incubation the decrease was completely attenuated (0.01 ± 0.03 pH units, n = 12 cells from 3 CCDs) (Figs. 6 and 8, bottom).

In contrast the potent monofunctional PPIase inhibitor [AD-Ser]8 CsA prevented the adaptation. In 36 cells from 4 CCDs, the removal of luminal Cl− raised cell pH by 0.45 ± 0.05 pH units before incubation. After a 3-h incubation at pH 6.8 in the presence of 10 µM [AD-Ser]8 CsA, adaptation of β-intercalated cells was prevented: cell pH increased by a similar value of 0.42 ± 0.04 pH units (Fig. 7, top). The tracing of representative cells shows virtually no effect of the pH 6.8 incubation in the presence of [AD-Ser]8 CsA (Fig. 6, top tracing). Incubation at pH 6.8 plus 10 µM [AD-Ser]8 CsA in the bath also

−0.46 ± 0.02 pH units in response to removal of bath Cl−. but after incubation the decrease was completely attenuated (0.01 ± 0.03 pH units, n = 12 cells from 3 CCDs) (Figs. 6 and 8, bottom).

To summarize the effect on ΔpH of removing Cl− from the bath (ΔpH was calculated from the difference in cell pH before and after removing Cl− from the bath). Open bars are preincubation and filled bars are postincubation values. Top panel 1: incubation at pH 7.4. Panel 2: incubation at pH 7.4 plus 10 µM CsA. Panel 3: incubation at pH 6.8. Bottom panel 4: incubation at pH 6.8 plus 10 µM CsA. The numbers of cells and CCDs are the same as in the legend to Fig. 3. *Significantly different from preincubation value by paired t-test, P < 0.05.

Fig. 4. Summary data showing the effect on ΔpH of removing Cl− from the bath (ΔpH was calculated from the difference in cell pH before and after removing Cl− from the bath). Open bars are preincubation and filled bars are postincubation values. Top panel 1: incubation at pH 7.4. Panel 2: incubation at pH 7.4 plus 10 µM CsA. Panel 3: incubation at pH 6.8. Bottom panel 4: incubation at pH 6.8 plus 10 µM CsA. The numbers of cells and CCDs are the same as in the legend to Fig. 3. *Significantly different from preincubation value by paired t-test, P < 0.05.

Fig. 5. Effect of tacrolimus on HCO3− transport. Open bars are preincubation and filled bars are postincubation. Net HCO3− flux is left pair, unidirectional H+ secretory flux is middle pair, and unidirectional HCO3− secretory flux is right pair (n = 6 CCDs). *Significantly different from preincubation value by paired t-test, P < 0.05.

Fig. 6. Tracings of pH of individual cells studied under baseline conditions (Burg’s solution in bath and lumen). −ClL, baseline, −ClB, before and after the 3-h Inc. Top: 3 cells studied before and after 3-h Inc. at pH 6.8 in the presence of 10 µM [AD-Ser]8 CsA. Bottom: tracings of 3 cells studied before and after 3-h Inc. at pH 6.8 in the presence of 10 µM CsH (inactive agent).

Fig. 7. Summary data showing the effect on ΔpH of removing Cl− from the lumen (ΔpH was calculated from the difference in cell pH before and after removing Cl− from the lumen). Filled bars are preincubation and slashed bars are postincubation values. Top: incubation at pH 6.8 in the presence of 10 µM [AD-Ser]8 CsA (n = 36 cells in 4 CCDs). Bottom: incubation at pH 6.8 plus 10 µM CsH (inactive agent, n = 12 cells in 3 CCDs). *Significantly different from preincubation value by paired t-test, P < 0.05.
and immediately stained for extracellular hensin and then incubated at pH 6.8. Hensin staining was increased and prominent red PNA caps were more prominent; the appearance resembled that at pH 7.4 in the presence of 10 μM CsH (inactive agent). The numbers of cells and CCDs are the same as in the legend to Fig. 7. *Significantly different from preincubation value by paired t-test, P < 0.05.

Deposition of extracellular hensin. CCDs were incubated 3 h at pH 6.8 in the presence or absence of CsA and stained for extracellular hensin (green) and peanut agglutinin (PNA, red). Control CCDs were dissected in pH 7.4 media, not incubated, and immediately stained for extracellular hensin and then rhodamine PNA (Fig. 9, top left). There was minimal staining for extracellular hensin and prominent red PNA caps in a confocal 1-μm image. Figure 9, top right, shows a CCD after incubation at pH 6.8. Hensin staining was increased and appeared “clumpy” or clustered. The PNA caps were less prominent. Figure 9, bottom left, shows a CCD after incubation at pH 6.8 in the presence of 10 μM CsA. Hensin staining appeared decreased compared with Fig. 9, top right, and the red PNA caps were more prominent; the appearance resembled more closely that at pH 7.4 in the top left.

Incubation of CCDs at pH 6.8 in the presence of the PPIase inhibitor [AD-Ser]8 CsA (bottom) also prevented acid-induced increase in extracellular hensin staining (less green staining in Fig. 9, bottom right). Note also that the acid-induced reduction in PNA labeling (red) was also prevented.

DISCUSSION

Identification of the genes responsible for human dRTA has recently been complemented by a number of gene deletions that cause the same syndrome in mice. Of the human genes, several subunits of the H+-ATPase, the anion exchanger AE1 and carbonic anhydrase II, all cause defects in the well-described H+-secretory pathway by the α-intercalated cell. Studies in human dRTA have identified further heterogeneity of the loci where the loci were not linked to the ATPase, AE1 or carbonic anhydrase (19). Identification of these genes promise to identify new mechanisms that mediate acid-base transport in the CCD. Our finding that the mechanism by which cyclosporin causes dRTA provides an additional new mechanism for the causation of dRTA, namely, a defect in the adaptation of β-intercalated cells to acidosis. Other studies in collecting duct function have recently identified pendrin as an apical anion exchanger expressed in the β-intercalated cell. Mutations in this gene would not be expected to cause acidosis, rather they ought to cause distal renal tubular alkalosis, although this has not been seen in either humans or mice except under conditions of mineralocorticoid loading (35).

We recently discovered that the mechanism of conversion of a β- to α-intercalated cell is critically dependent on the deposition of an extracellular matrix protein, hensin (reviewed in Ref. 2). Hensin is expressed in intracellular vesicles of all cells of the collecting duct but only underneath the α-intercalated cells of the CCDs and in medullary collecting tubules is it present in the ECM (27). Moreover, during the adaptation of β-intercalated cells to lowering the bath pH, hensin becomes deposited in the ECM under these β-cells. Deposition of hensin in the ECM requires extensive polymerization (14, 15). We discovered that addition of labeled monomeric hensin to α-phenotype intercalated cells in the in vitro cell culture system resulted in the formation of higher order multimers. In other words, there exists an ECM activity capable of polymerizing hensin (14). We propose that this activity is among the cyclophilins, enzymes known to assist protein folding and oligomerization. At present, this speculation is entirely based on the fact that acid treatment in the presence of specific inhibitors of the PPIase function of cyclophilins did not lead to deposition of hensin in the ECM and consequently no adaptation of β-intercalated cells. A structurally related CsA derivative, CsH-Ac, which lacks active site blockade of cyclophilin A, was completely inactive in preventing the adaptation of β-intercalated cells. All we know at present is that CsA and PPIase inhibitors are blocking the process “upstream” of the deposition of hensin. But the following arguments suggest that the speculation is plausible.

Mature hensin contains eight SRCR domains separated from each by a proline-rich string of 23 amino acids. Productive formation of polymerized hensin from monomers with the underlying slow prolyl cis-trans isomerization reactions of the proline-rich segments of hensin might benefit from PPIase catalysis by avoiding aggregation-prone folding intermediates. Importantly, prolyl trans-to-cis isomerization was shown to be the prerequisite for dimerization of the antibody domain C(H3). In refolding experiments, the species with the wrong trans isomerization state accumulated as a monomeric folding intermediate (29).

For the extracellular polymerizing activity to be a PPIase of the cyclophilin type, the protein would obviously have to be secreted. There are three cyclophilins known to be directed to the extracellular space. The original member, cyclophilin A, was found to be a cytosolic enzyme. Two homologs, cyclophilins B and C, were discovered, and each of these proteins has a signal peptide allowing it to be secreted by the classical protein transport pathway. Both of these proteins can be secreted, although it is thought that cyclophilin B is most often located in the endoplasmic reticulum. Cyclophilin C was dis-
covered as a protein that binds to a 77-kDa protein whose sequence reveals that it contains an SRCR domain (10). Some studies have shown that cyclophilin B is secreted and acts to mediate adhesion of lymphocytes to the ECM (3). There has been a recent report that even cyclophilin A can be secreted (18). These studies have shown that oxidative stress causes release of cyclophilin A from vascular smooth muscle. We point out that there are a number of similarities between oxidative stress and acidosis.

CsA exerts its immunosuppressive effect by binding to cyclophilin A, a cytoplasmic protein with IIPase enzymatic activity. CsA also binds to and inhibits the enzymatic activity of the several other cyclophilin homologues present in mammalian genomes. The cyclophilin:cyclosporin complex interacts with the calcium-regulated Ser/Thr protein phosphatase calcineurin, blocking its dephosphorylating activity. Calcineurin is critical for the immune response where it dephosphorylates the cytoplasmic form of the transcription factor nuclear factor of activated T cells, allowing it to translocate to the nucleus and activate the transcription of immune response genes including IL-2 (8, 20).

FK-506 (tacrolimus) is another immunosuppressive agent whose targets are the protein family of FK binding proteins (FKBP's) with the most prominent cytosolic member FKBP12.
FK-506 is completely inert toward cyclophilins (7, 12). However, the FK-506/FKBP complex also interacts with calcineurin, blocking its enzymatic activity and interrupting the same pathway as CsA/Cyclophilin A. It is well known that cyclophilins are widely distributed in cells and that calcineurin is critical for a number of physiological actions in a variety of cells and tissues. There is at present no doubt that the mechanism of immunosuppressive effect of CsA and FK-506 is mediated through the calcineurin pathway. However, despite a wealth of biochemical and molecular studies on this system it has been difficult to define the mechanism of the side effects of CsA. For instance, CsA is associated with hyperkalemia, but it is not possible to conclude whether the effect is simply due to the acidosis (with its effect on re-distribution of cell K+ or inhibition of K+ secretion) or is a direct effect on a specific K+ transporting cell. There is increasing evidence that nephrotoxicity associated with immunosuppressive therapy by CsA can be mediated by inhibition of the PPlase activity of cyclophilin A (16). However, could blockade of this enzymatic activity be responsible for at least some of the other complications? Our present studies are the first to directly suggest a role for this enzymatic activity in causing one of the known complications of CsA treatment. Future work will have to determine whether the other complications are also related to this effect.

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REFERENCES