Effects of Tacrolimus and Cyclosporin A on Peptide Transporter PEPT1 in Caco-2 Cells

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INTRODUCTION

Tacrolimus and cyclosporin A (CsA) are used as immunosuppressive agents in clinical medicine, particularly in the area of organ transplantation. Despite the beneficial immunosuppressive effects of tacrolimus and CsA in clinical transplantation, both immunosuppressants appear to have adverse effects such as nephrotoxicity, hepatotoxicity and neurotoxicity (1,2). Gastrointestinal side effects of these immunosuppressants such as nausea, bloating, and diarrhea have also been reported in humans (1,2). In liver transplant recipients, severe gastrointestinal toxicity was associated with chronic tacrolimus therapy (3). In normal rats, weight gain was reduced by high-dose CsA whether given orally or by subcutaneous injection (4). Oral CsA reduced *in vivo* fat absorption from the diet, and all doses and routes of administration of CsA caused reduction of both active glucose uptake and of passive fatty acid absorption by the intestine *in vitro* (4).

The peptide transporter in the small intestine has been considered to play a key role in maintenance of protein nutrition (5–7). This transporter is specific for peptides consisting of two or three amino acids. The physiological role of the peptide transport system is to mediate the absorption of small peptides generated by the digestion of dietary proteins. It has been shown that the absorption of protein digestion in the small intestine occurs primarily in the form of small peptides. In addition, it was suggested that enteral solutions containing small peptides may provide an absorptive advantage in patients with severely reduced intestinal absorptive area and in those who are acutely catabolic (6). This enteral peptide transporter was cloned as PEPT1 and it is considered as an intestinal peptide transporter (5–7).

In the present study, we investigated the effects of tacrolimus and CsA on the intestinal peptide transporter in the human colon adenocarcinoma cell line Caco-2, which is a useful model to study intestinal epithelial transport.

MATERIALS AND METHODS

Materials

Tacrolimus and CsA were kindly provided by Fujisawa Pharmaceutical Co. (Osaka, Japan) and Novartis Pharm K. K. (Tokyo, Japan), respectively. [^{14}C]Glycylsarcosine (1.7 GBq/mmol) was obtained from Daiichi Pure Chemicals (Ibaraki, Japan). D-[^{3}H]Glucose (566.1 GBq/mmol) was purchased from Moravek Biochemicals (Brea, CA). L-[^{3}H]Alanine (2.04 Tbq/mmol) was obtained from Amersham Int. (Buckinghamshire, UK). D-[^{3}H]Mannitol (728.9 GBq/mmol) was purchased from Du Pont-New England Nuclear Research Products (Boston, MA). Glycylsarcosine was from Sigma Chemical (St. Louis, MO). All other chemicals used were of the highest purity available.

Cell Culture and Cell Treatment

Caco-2 cells at passage 18 obtained from the American Type Culture Collection (ATCC HTB-37) were cultured as described previously (8). Stock solutions of tacrolimus and CsA were prepared in 20% cremophor and 80% ethanol solution. Tacrolimus or CsA was applied to culture medium and incubated for one day. The final concentrations of cremophor and ethanol during exposure were 0.05% and 0.2%, respectively. The control cells were incubated with the same concentrations of cremophor and ethanol in each experiment.

Uptake and Transport Studies

After treatment of the cells with tacrolimus or CsA, the accumulation of [^{14}C]glycylsarcosine, D-[^{3}H]glucose and L-[^{3}H]alanine by monolayers grown in 35 mm plastic dishes was measured as described previously (8). Transcellular transport of [^{14}C]glycylsarcosine was measured using monolayer cultures grown in Transwell chambers as described previously (9) with some modifications. Briefly, after preincubation for 10 min, 2 ml of incubation medium (pH 6.0) containing 20 μM [^{14}C]glycylsarcosine was added to the apical side, and 2 ml of non-radioactive incubation medium (pH 7.4) was added to the basal side. The monolayers were further incubated for 60 min at 37°C. For transport measurements, aliquots of the incubation medium on the basal side were taken and the radioactivity was counted. D-[^{3}H]Mannitol was used to calculate paracellular flux. For the accumulation study, the cells on the filters were solubilized with 0.5 ml of 1 N NaOH and the radioactivity of the solubilized cells was determined by liquid scintillation counting.

Measurement of Alkaline Phosphatase, γ-Glutamyltransferase, and Lactate Dehydrogenase Activities

Activities of alkaline phosphatase (EC 3.1.3.1) and γ-glutamyltransferase (EC 2.3.2.2) of Caco-2 cells and the release of lactate dehydrogenase (EC 1.1.1.27) into the medium were determined as described previously (10,11). Protein was measured using a Bio-Rad protein Assay Kit with bovine γ-globulin as the standard.

Statistical Analysis

Data were analyzed for statistical significance by nonpaired t-test or one-way analysis of variance followed by Sheffe's test.

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RESULTS AND DISCUSSION

To investigate the effects of tacrolimus and CsA on glycylsarcosine uptake, we examined the concentration-dependence of tacrolimus or CsA on glycylsarcosine uptake in comparison with D-glucose and L-alanine uptake by Caco-2 cells. Since protein contents of Caco-2 cells were reduced by treating the cells with tacrolimus or CsA for more than 2 days (data not shown), Caco-2 cells were treated with various concentrations of tacrolimus or CsA for 1 day to evaluate their specific effects on transport systems without affecting cell viability or proliferation. As shown in Fig. 1, treatment with tacrolimus or CsA significantly decreased glycylsarcosine uptake in a dose-dependent manner, while L-alanine uptake was not changed by these treatments. Although D-glucose uptake was slightly decreased by treatment with 20 or 50 μM tacrolimus, no significant effects of 100 μM tacrolimus or of CsA at any concentration were observed. These results indicate that the effects of tacrolimus and CsA were not simply due to non specific effects on the transport activities of Caco-2 cells, and that the peptide transport system is more sensitive to treatment with tacrolimus and CsA than active glucose or amino acid transporters. Control cells were treated with 0.05% cremophor and 0.2% ethanol as vehicle alone; this treatment did not affect glycylsarcosine uptake by Caco-2 cells (data not shown).

We then examined the effects of tacrolimus or CsA on the kinetic parameters of glycylsarcosine uptake. Figure 2 shows the concentration-dependence of glycylsarcosine uptake by Caco-2 cells treated with or without tacrolimus and CsA. The specific uptake was calculated by subtracting the nonspecific uptake, which was estimated in the presence of excess unlabeled dipeptide, glycylleucine, from the total uptake. Using nonlinear least-squares regression analysis, kinetic parameters were calculated according to the Michaelis-Menten equation. Treatment with 100 μM tacrolimus decreased the maximal uptake rate (Vmax) to 49% of the control value (control, 15.0 ± 2.1 nmol/mg protein/10 min; tacrolimus treatment, 7.4 ± 1.4 nmol/mg protein/10 min, mean ± S.E. of three separate experiments, $p < 0.05$), whereas the apparent Michaelis-Menten constant (Km) was not affected (control, 0.83 ± 0.06 mM; tacrolimus treatment, 0.65 ± 0.22 mM, mean ± S.E. of three separate experiments). Treatment with 30 μM CsA also decreased the Vmax value (control, 15.0 ± 2.1 nmol/mg protein/10 min; CsA treatment, 11.5 ± 1.3 nmol/mg protein/10 min, mean ± S.E. of three separate experiments, $p < 0.05$) with no effect on the Km value (control, 0.83 ± 0.06 mM; CsA treatment, 0.77 ± 0.10 mM, mean ± S.E. of three separate experiments). The decrease in the Vmax value suggested that expression of the peptide transporter was reduced by either tacrolimus or CsA. We estimated the amount of human PEPT1 mRNA in Caco-2 cells by competitive PCR. In tacrolimus- or CsA-treated Caco-2 cells, the amounts of human PEPT1 mRNA were not significantly different from that of control cells (data not shown).

It remains possible that tacrolimus and cyclosporin A injure Caco-2 cells and thus reduce viability of these cells. To examine whether the inhibitory effects of tacrolimus and CsA on glycylsarcosine uptake were due to injury of the plasma membrane, we measured the plasma membrane enzyme activities of Caco-2 cells. Figure 3A shows the release of lactate dehydrogenase from the treated cells, which was measured as a marker of the membrane permeability to characterize stress-induced membrane dysfunction. Lactate dehydrogenase release was increased by tacrolimus or CsA treatment in a concentration-dependent manner. In contrast, the activities of γ-glutamyltransferase and alkaline phosphatase, marker enzymes for the apical membrane, were not affected by tacrolimus or CsA treatment (Figs. 3B and 3C). Treatment with various concentrations of tacrolimus and CsA did not have any effects on protein contents of the Caco-2 cells (data not

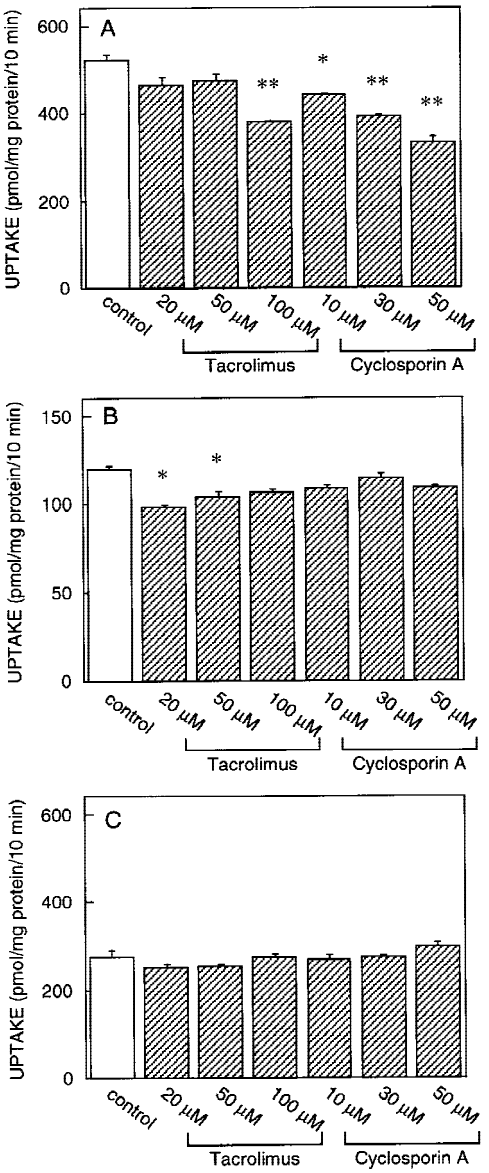

Fig. 1. Concentration-dependence of the effects of tacrolimus or CsA on [^{14}C]glycylsarcosine (A), D-[^{3}H]glucose (B) and L-[^{3}H]alanine (C) uptake by Caco-2 cells. Caco-2 monolayers were incubated with various concentrations of tacrolimus or CsA. After treatment, monolayers were rinsed once with incubation medium (pH 7.4) and then incubated for 10 min at 37°C with [^{14}C]glycylsarcosine (20 μM) in incubation medium at pH 6.0 (A), with D-[^{3}H]glucose (1 μM) in incubation medium at pH 7.4 (B) or with L-[^{3}H]alanine (20 μM) in incubation medium at pH 6.0 (C). Thereafter, the radioactivity of solubilized cells was determined. Each bar represents the mean ± S.E. of three monolayers. $^{**}p < 0.01$, $^{*}p < 0.05$, significant difference from control cells.

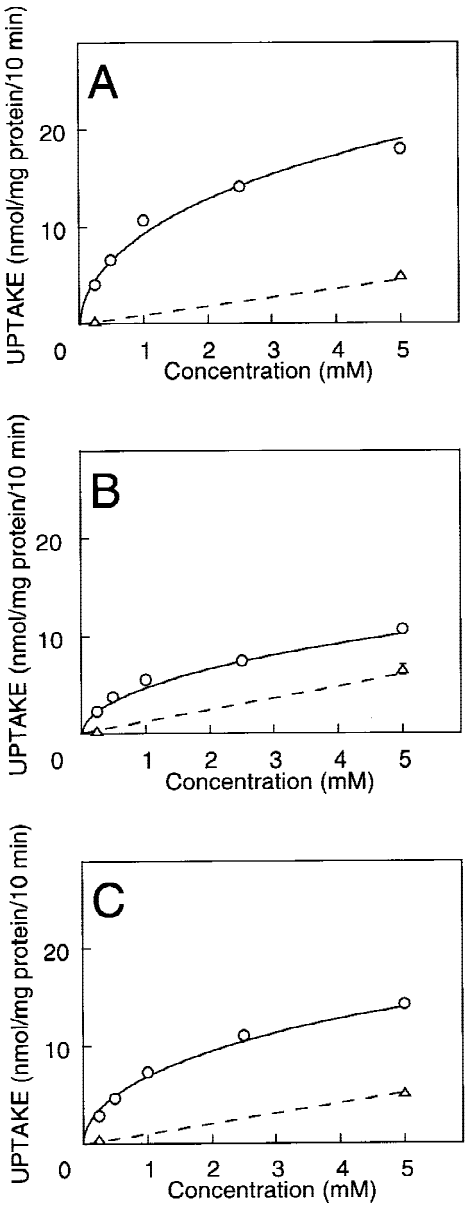

Fig. 2. Concentration-dependence of [^{14}C]glycylsarcosine uptake by Caco-2 cells treated with tacrolimus or CsA. Caco-2 monolayers were incubated with 0.05% cremophor and 0.2% ethanol as vehicle (A), 100 μM tacrolimus (B), or 30 μM CsA (C). After treatment, monolayers were rinsed once with incubation medium (pH 7.4) and then incubated at 37°C for 10 min with varying concentrations of [^{14}C]glycylsarcosine in incubation medium at pH 6.0 in the presence (△) or absence (○) of 50 mM unlabeled glycylleucine. Thereafter, the radioactivity of the solubilized cells was determined. Each point represents the mean ± S.E. of three monolayers. When error bars are not shown, they are smaller than the symbol.

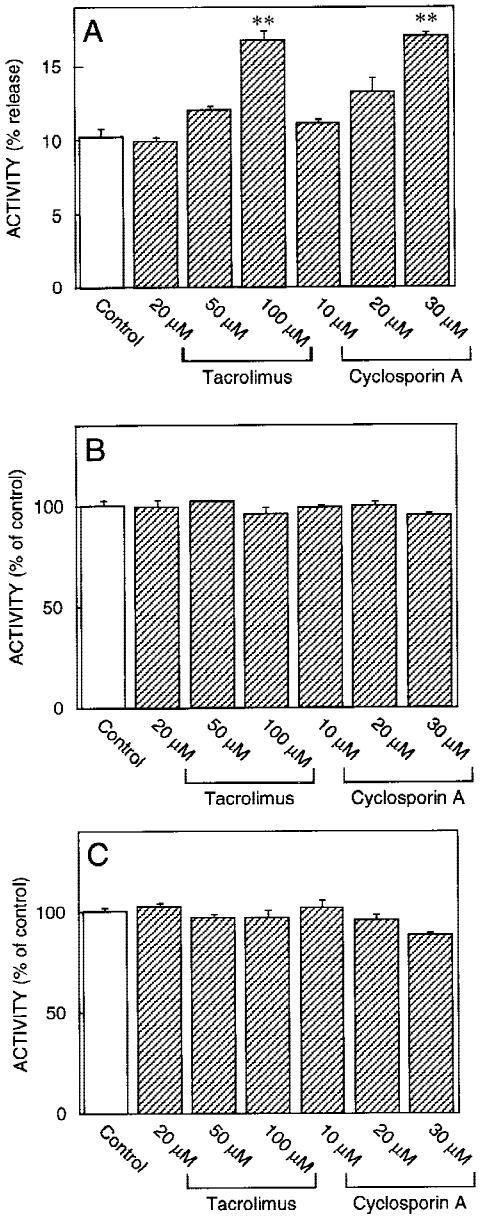

Fig. 3. Effects of various concentrations of tacrolimus or CsA on the release of lactate dehydrogenase (A), alkaline phosphatase (B), and γ-glutamyltransferase (C) activities of Caco-2 monolayers. Caco-2 monolayers were incubated with various concentrations of tacrolimus or CsA. After treatment, lactate dehydrogenase activities in the culture medium and cell homogenates were determined and the lactate dehydrogenase activities released into the culture medium are represented as percentages of total lactate dehydrogenase activity. Alkaline phosphatase activities (B) and γ-glutamyltransferase activities (C) in cell homogenates were determined and are represented as percentages of those of control cells. Each bar represents the mean ± S.E. of three monolayers. $^{**}p < 0.01$, significant difference from control cells.

shown). Although these findings suggest that the apical membrane was damaged to some extent by these treatments, the effects of both tacrolimus and CsA on the plasma membrane were not severe enough to cause dysfunction of all carrier-mediated transport systems and membrane-bound enzymes.

In MDCK (C7) cells, the transport activity of Na^+/K^+-ATPase was significantly decreased by CsA treatment, while $Na^+/K^+/2Cl^-$ cotransporter activity was not affected (12). It was suggested that each plasma membrane protein was affected differently by CsA. This speculation may account for the different effects of both tacrolimus and CsA on glycylsarcosine, D-glucose and L-alanine uptake. Galàn et al. (13) reported that administration of CsA changed liver plasma membrane composition and fluidity in rats, which influenced carrier-mediated transport processes and membrane-bound

enzyme activities. Therefore, it is possible that tacrolimus and CsA decrease the peptide transport activity across the apical membrane by impairing the apical membrane composition and/or fluidity. The induced moderate damage of the apical membrane, which did not cause dysfunction of all transporters and enzymes, is likely to be the cause of the decrease in glycylsarcosine uptake by Caco-2 cells.

We then examined the effects of tacrolimus (Fig. 4A,B) and CsA (Fig. 4C,D) on the transcellular transport of glycylsarcosine from the apical to the basolateral side of Caco-2 cells. Transcellular transport of [^{14}C]glycylsarcosine was decreased by treatment with tacrolimus and CsA in a dose-dependent manner (at 60 min, 53% or 23% decrease by 50 μM tacrolimus or 30 μM CsA treatment, respectively). Paracellular flux of mannitol was not affected by these treatments (data not shown). The accumulation of glycylsarcosine at 60 min was also decreased by tacrolimus or CsA treatment in a dose-dependent manner. These results imply that the decrease in transcellular transport of glycylsarcosine was due to impairment of transport across the apical membrane.

The adverse effects of tacrolimus and CsA have been reported (1–3) and the impairment of intestinal function was shown to be induced by these drugs in humans and in experimental animals (4,14). In the present study, we found that tacrolimus and CsA decreased PEPT1 activity, and reduced the transcellular transport of glycylsarcosine from the apical to the basal side. Although further studies are needed to clarify the effects of tacrolimus and CsA on intestinal PEPT1 *in vivo,* our findings suggest that these agents decrease peptide absorption in the small intestine. This is the first report of peptide transport system dysfunction caused by the drug treatment.

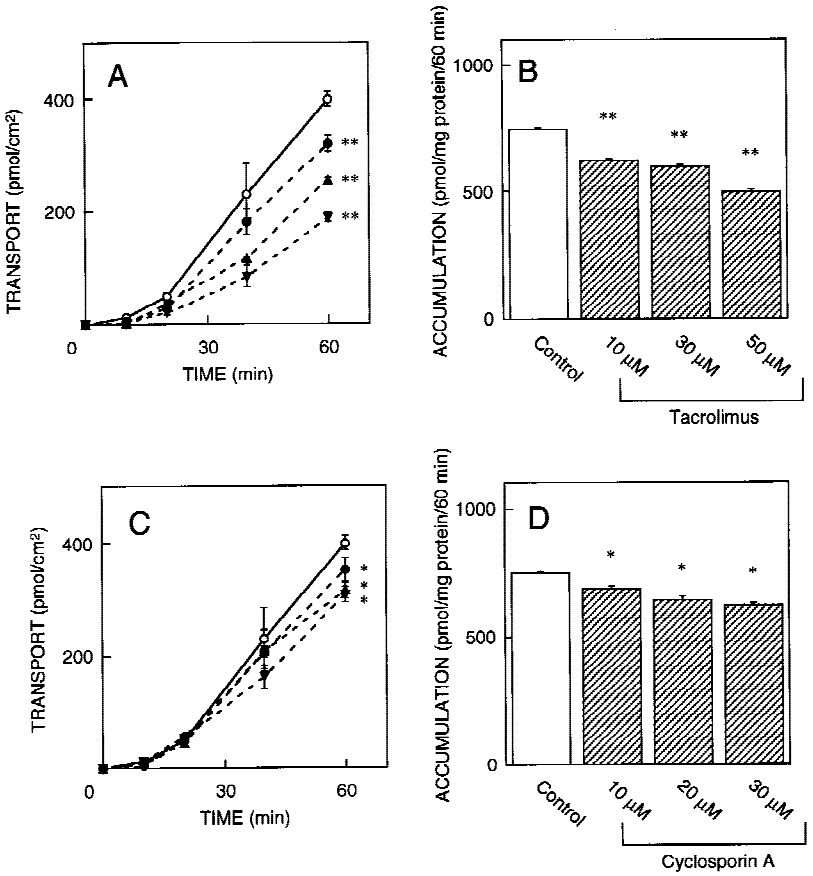

Fig. 4. Effects of tacrolimus (A, B) or CsA (C, D) on the apical-to-basal transport (A, C) and cellular accumulation (B, D) of [^{14}C]glycylsarcosine by Caco-2 cells. (A) Confluent monolayers were incubated for one day with 0.02% cremophor and 0.08% ethanol as vehicle (○), 10 μM (●), 30 μM (▲) or 50 μM (▼) tacrolimus. After treatment, [^{14}C]glycylsarcosine (20 μM) and D-[^{3}H]mannitol (50 μM) were added to the apical side of monolayers. The appearance of [^{14}C]glycylsarcosine and D-[^{3}H]mannitol on the basal side was measured periodically. (B) After 60 min incubation, accumulation of [^{14}C]glycylsarcosine in Caco-2 monolayers was determined. (C) Confluent monolayers were incubated for one day with 0.02% cremophor and 0.08% ethanol as vehicle (○), 10 μM (●), 20 μM (▲) or 30 μM (▼) CsA. After treatment, [^{14}C]glycylsarcosine (20 μM) and D-[^{3}H]mannitol (50 μM) were added to the apical side of monolayers. The appearance of [^{14}C]glycylsarcosine and D-[^{3}H]mannitol on the basal side was measured periodically. (D) After 60 min incubation, accumulation of [^{14}C]glycylsarcosine in Caco-2 monolayers was determined. Each point and bar represent the mean ± S.E. of three monolayers. $^{**}p < 0.01$, $^{*}p < 0.05$, significant difference from control cells.

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