Cyclosporin A Inhibits Creatine Uptake by Altering Surface Expression of the Creatine Transporter*

Received for publication, June 27, 2000, and in revised form, August 28, 2000
Published, JBC Papers in Press, August 29, 2000, DOI 10.1074/jbc.M005636200

Thanh T. Tran, Wenxuan Dai, and Hemanta K. Sarkar‡
From the Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas 77030

The immunosuppressive drug cyclosporin A (CsA) inhibited the hCRT-1 cDNA-induced creatine uptake in Xenopus oocytes and the endogenous creatine uptake in cultured C_{6}C_{12} muscle cells in a dose- and time-dependent manner. FK506, another potent immunosuppressant, was unable to mimic the effect of CsA suggesting that the inhibitory effect of CsA was specific. To delineate the mechanism underlying, we investigated the effect of CsA on the K_{m} and V_{max} of creatine transport and also on the cell surface distribution of the creatine transporter. Although CsA treatment did not affect the K_{m} (20–24 μM) for creatine, it significantly decreased the V_{max} of creatine uptake in both oocytes and muscle cells. CsA treatment reduced the cell surface expression level of the creatine transporter in the muscle cells by ~60% without significantly altering its total expression level, and the reduction in the cell surface expression paralleled the decrease in creatine uptake. Taken together, our results suggest that CsA inhibited creatine uptake by altering the surface abundance of the creatine transporter. We propose that CsA impairs the targeting of the creatine transporter by inhibiting the function of an associated cyclophilin, resulting in an apparent loss in surface expression of the creatine transporter. Our results also suggest that prolonged exposure to CsA may result in chronically creatine-depleted muscle, which may be a cause for the development of CsA-associated clinical myopathies in organ transplant patients.

Cyclosporin A (CsA) is a potent immunosuppressive agent, which is used widely in organ transplants to prevent graft rejection and also to treat autoimmune disorders (1). A large body of evidence suggests that prolonged administration of cyclosporin induces a number of toxic side effects (2–4), including adverse effects of cyclosporin on skeletal muscle (5–9). The CsA-induced clinical myopathies were, however, reversed after cessation of cyclosporin and the muscle weakness returned with exacerbation upon reinstitution of cyclosporin therapy (5, 8). In vitro, CsA inhibited biochemical differentiation of cultured human myoblasts in a dose-dependent manner without significantly altering their proliferation (10). Moreover, the muscles of CsA-treated mice were found to be deficient in regenerated muscle fibers (10). Together, these studies suggest that CsA have profound biochemical and morphological effects on the skeletal muscle. Currently, the cellular mechanism(s) involved in the development of the CsA-induced toxicity, including the possibility of altered energy state of the muscle, is not fully understood.

Muscles store millimolar concentration of a high-energy phosphate intermediate, phosphocreatine. During high muscle activity, the enzyme creatine kinase replenishes the consumed ATP quickly by catalytic transfer of the phosphocreatine-phosphate group to the ADP (11, 12). Although phosphocreatine is synthesized in the muscle from creatine, the de novo synthesis of creatine occurs mainly in the human liver, kidney, and pancreas (11). Thus, to meet their energy demands and to maintain equilibrium between the intracellular concentration of creatine and phosphocreatine, muscles possess a mechanism to actively accumulate creatine from the circulating plasma (see Ref. 13 for a review).

Normally, muscles maintain a steep creatine concentration gradient, which is ~500–1000-fold higher inside than the plasma creatine concentration (14), primarily with the help of a high affinity creatine transporter (13, 15). This creatine concentration gradient (higher inside) is tightly regulated to maintain normal muscle function (12, 13). Conceivably, chronic dysfunction of the creatine transporter resulting in little or no creatine transport could adversely affect the energy metabolism in the muscle as a result of creatine depletion, which in turn might lead to muscle myopathy. Commensurate with the above idea, a number of studies documented both ultrastructural and functional abnormalities in the muscles of chronically creatine-depleted animals (16, 17).

Recently, we (18) and others (19–23) reported cloning of a high affinity creatine transporter (CRT-1) from a variety of mammalian tissues. The CRT-1 mRNA is most abundantly expressed in the skeletal muscle among all tissues examined (19, 20). Analysis of the encoded protein sequence predicts that the creatine transporter contains 12 transmembrane helical domains interconnected by extracellular and intracellular loops (19, 20). Expression of the cloned CRT-1 cDNAs in heterologous expression systems induced a Na"^{+} and Cl"^{-}-dependent creatine uptake that was both biochemically and pharmacologically similar to those obtained using cultured cells (for examples, see Refs. 18–20).

In the present studies, we investigate the effects of CsA on creatine uptake in Xenopus oocytes and cultured muscle cells expressing recombinant and endogenous creatine transporter, respectively. We show that CsA selectively inhibited the activities of both the recombinant and endogenous creatine transporters. Our results further suggest that the inhibition in the
Inhibition of Creatine Transport by Cyclosporin A

Creative uptake is due to an alteration of the relative surface abundance of the creatine transporter.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cyclosporin A (CsA) and FK 506 were generous gifts from Dr. J. Clifford Waldrep and Dr. Albert Chang (Baylor College of Medicine), respectively. [4-14C]Creatine (50 mCi/ml) and L-[3H](G)-glutamic acid (30 mCi/ml) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and 1,2-[3H]taurine was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). All the other reagents were either ACS grade pure or ultrapure, and were purchased from various commercial sources.

**Culture of C_{2}C_{12} Muscle Cells—C_{2}C_{12} mouse myoblasts (American Type Culture Collection, Rockville, MD) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated bovine fetal serum and 1% penicillin/streptomycin in a humidified 5% CO2 atmosphere at 37 °C. For uptake studies, cells were seeded in 6-well culture dishes at a density of 10^5 cells/ml and were incubated for 2–3 days at 37 °C. Differentiation of myoblasts into myotubes was induced by replacing the growth medium of nearly confluent myoblasts with the differentiation medium (Dulbecco’s modified Eagle’s medium containing 5% heat-inactivated horse serum and 1% penicillin/streptomycin) followed by a 48-h period of incubation at 37 °C. Drugs were added directly to the differentiation medium 24 h after the cells were exposed to the differentiation medium, following which cells (nascent myotubes) were incubated at 37 °C for at least 24 h before they were used for uptake studies.

**Functional Expression of the CRT-1 Creatine Transporter**—The construction of the recombinant plasmid pCRT3 containing human CRT-1 (hCRT-1)-cDNA was described previously (18). Synthetic RNA (cRNA) was prepared from the pCRT3 plasmid using SP6 RNA polymerase (Epicerent Technologies, WI), and was used to microinject (5 ng/oocyte) defolliculated and healthy *Xenopus* oocytes (24). The microinjected oocytes were subsequently used for the functional studies as described (18, 25). Unless otherwise noted, uptake assays were carried out at least 48 and 72 h post-injection.

**Uptake Assays**—As described above, the creatine uptake studies in oocytes were performed essentially as described earlier (18) using 30 μM final concentration of [14C]creatine (stock solution: 10 mM; specific activity, 10 mCi/ml) as a substrate. The creatine uptake in cultured C_{2}C_{12} cells were measured as follows. Cells were first washed with the choline chloride buffer (ChCl-1 buffer: 135 mM choline chloride, 1 mM calcium chloride, 2 mM potassium chloride, 5 mM magnesium chloride, 5 mM HEPES-Tris, pH 7.5). The uptake was initiated by replacing the medium with 1 μM [14C]creatine. Cells were subsequently solubilized in 1 mL of 1% SDS and the amount of radioactivity in the extract was measured using a Beckman LS 3800 scintillation counter. Protein concentration in a portion of the extract was measured using the Pierce Protein Assay kit (Pierce, Rockford, IL).

To determine the effect of CsA and FK506 on creatine uptake, C_{2}C_{12} cells or microinjected oocytes were incubated in the presence of a given concentration of the drug for various time periods. The drug was added directly to the cell culture medium, or to the oocyte bathing medium (Barth’s solution: 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.33 mM Ca(NO3)2, 0.82 mM MgSO4, 0.41 mM CaCl2, 7.5 mM Tris-HCl, pH 7.6) supplemented with 1 mM sodium pyruvate and 0.01% penicillin/streptomycin). Control cells or oocytes were incubated in parallel with an equivalent amount of the vehicle solvent (ethanol). Pretreated cells and oocytes were subsequently used for uptake assays. K_{m} and V_{max} values for creatine uptake were determined as described previously (18) by measuring creatine uptake at various external concentrations of [14C]creatine.

**Creatine Transporter Anti-peptide Antibody**—A polyclonal antibody against a synthetic peptide (LEYRAQADVYRG), corresponding to an internal sequence of the creatine transporter C-terminal tail, was raised in rabbits using the commercial services offered by Research Genetics, Inc. (Huntsville, AL). Immunosorber from one of the rabbits showed high antibody titer against the synthetic peptide in an enzyme-linked immunosorbent assay. This immunosorber containing anti-C creatine transporter (anti-Crt-C) antibodies was used for immunoblot assay (26) without further purification.

**Cell Surface Biotinylation**—Cell surface expression of the creatine transporter in control and CsA-treated C_{2}C_{12} cells was determined using the membrane impermeable biotinylation reagent NHS-Ss-biotin (Pierce) as described (27, 28). Briefly, control and CsA-treated myotubes (∼3 × 10^6 cells; passages 15–18) were grown as described above, following which cells were washed with phosphate-buffered saline containing 0.1 M NaCl, 1.0 M MgCl2, (phospho-buffed saline/CM). Biotinylation was carried out using 1.0 mg/ml NHS-Ss-biotin in biotinylation buffer (100 mM NaCl, 2 mM CaCl2, 10 mM triethanolamine, pH 8.0) for 20–25 min at 4 °C with gentle shaking. After labeling, cells were washed twice with ice-cold 100 mM glycine in phosphate-buffered saline/CM to quench the residual NHS-Ss-biotin. Subsequently, cells were washed with lysis buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5 containing Complete (protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN)) by gently shaking on ice for approximately 1 h. The extracts were clarified by centrifugation at 14,000 × g for 10 min at 4 °C and a portion of the extract (300 μl of extract containing 390 μg of protein) was incubated overnight with 50 μl of streptavidin-agarose beads (Sigma) at 4 °C with gentle agitation. After incubation, beads were washed three times with lysis buffer, twice with high salt wash buffer (0.1% Triton X-100, 500 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5), and finally once with no-salt wash buffer (50 mM Tris-HCl, pH 7.5). The captured biotinylated proteins were eluted from the beads with 40 μl of 2 × SDS sample buffer and a portion of it (20 μl) was analyzed by immunoblot assay.

**Immunoblot Assay**—Total cell extracts and captured biotinylated proteins were separated on a 12% SDS-polyacrylamide gel and subsequently electrotransferred onto a Hybond-P polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was blocked with 3% bovine serum albumin and 2% non-fat dry milk in TBST buffer (137 mM NaCl, 2.6 mM KCl, 25 mM Tris-HCl, pH 7.4, and 0.1% Tween 20) for 1 h at room temperature and incubated with 1:2000 dilution of the anti-Crt-C antibody for 1–3 h at room temperature. After washing with TBST buffer, the blot was incubated with a 1:15,000 dilution of horseradish peroxidase-conjugated anti-rabbit antibody (Pierce) for 1–2 h at room temperature. The immunoreactive bands were visualized using the ECL-Plus chemiluminescence detection kit (Amersham Pharmacia Biotech) and subsequently quantified by densitometric scanning of autoradiograms using the AlphaImager 2000 (Alpha Innotech Co., San Leandro, CA).

**RESULTS**

**Effect of CsA on Creatine Uptake in Oocytes Expressing hCRT-1**—To determine the effect of cyclosporin on creatine uptake CsA (1–30 μM final concentration) or ethanol (vehicle control, control) was added to the oocyte bathing medium 24 h after the oocytes were injected with the hCRT-1 cRNA. The oocytes were incubated in the absence and presence of CsA for an additional period of 72 h prior to using them for uptake assays. As shown in Fig. 1A, the creatine uptake in oocytes treated with 1 or 5 μM CsA for 72 h remained unchanged from that of the control uptake level. At higher concentrations, however, CsA inhibited the creatine uptake (Fig. 1A). Thus, in three independent experiments, treatment with 30 μM CsA for 72 h inhibited the creatine uptake in oocytes by −38.6 ± 4.5% (mean ± S.E.; results not shown).

Fig. 1B shows that the uptake level in oocytes pretreated with 1 μM CsA for 24 h remained unchanged from that of the control (ethanol, 24 h) uptake. However, the uptake was inhibited by −36% in oocytes treated with 30 μM CsA for 48 and 72 h, but not in vehicle solvent-treated control oocytes. Addition of 30 μM CsA directly to the uptake buffer of the untreated oocytes also had no effect on the induced creatine uptake (results not shown).

To test the specificity of CsA, we examined the effects of cyclosporin C (CsC), a cyclosporin A analog, and FK506, a structurally unrelated immunosuppressive drug (29), on creatine uptake. As shown in Fig. 2, neither CsC nor FK506 inhibited the creatine uptake. We used only 3 μM concentration of FK506 because at this concentration FK506 is known to inhibit the PPlase activity of FKBP, and at higher concentration FK506 is toxic for the oocytes (30).

In our previous experiments, CsA was added to the oocyte
Inhibition of Creatine Transport by Cyclosporin A

A

**FIG. 1.** CsA inhibits creatine uptake in a dose- and time-dependent manner. A, dose dependence a day after the oocytes were injected with hCRT-1 cRNA (~5 ng/oocyte). CsA or ethanol was added directly to the medium bathing the oocytes, and the oocytes were incubated in the absence or presence of CsA for an additional ~72 h at 18 °C. Subsequently, the oocytes were used for a 30-min uptake assay at room temperature as described under “Experimental Procedures.” Results are expressed as mean creatine uptake ± S.E. (n = 6–8 oocytes). Uninjected oocytes were used as negative control. *, p < .0001. B, time dependence: microinjected oocytes were treated with CsA (30 μM) or vehicle solvent (ethanol; control) for 24, 48, and 72 h. In all cases, CsA was added a 24 h after the oocytes were injected. The control and CsA-treated oocytes were subsequently used for a 30-min creatine uptake assay. Results are expressed as mean uptake ± S.E. (n = 6 oocytes). *, p < .005.

bathing medium 24 h post-injection, and the inhibitory effect of CsA was not apparent for at least another 24 h. To gain further insight into the mechanism of CsA-mediated inhibition of creatine uptake, we examined the relative expression level of the creatine transporter in untreated control oocytes by measuring the appearance of the creatine uptake as a function of time. The creatine uptake levels at 24, 48, 72, and 96 h after microinjection were 47.7 ± 10.2 (mean ± S.E.; n = 7 oocytes), 148.1 ± 14.4, 195.8 ± 21.8, and 302.7 ± 38.3 pmol/60 min/oocyte, respectively (results not shown). Since the creatine uptake continued to increase during the time course of the experiment, it is reasonable to assume that the transporters were functionally assembled and targeted to the oocyte cell surface beyond 48 h. Thus, these results raise the possibility that, at least in oocytes, CsA might affect the targeting of the creatine transporter to the cell surface.

**Effect of CsA on Endogenous Creatine Uptake in C2C12 Cells**—To rule out the possibility that the effect of CsA on creatine uptake was cell specific, we examined the effect of CsA on the endogenous creatine uptake in cultured C2C12 muscle cells. Creatine uptake in C2C12 cells almost doubled after the myoblasts were differentiated into myotubes by serum deprivation (results not shown). The increased creatine uptake in myotubes is probably due to an increase in the creatine

porter expression as a result of muscle cell differentiation.

We did not observe any significant morphological difference between CsA-treated and control C2C12 myoblast (results not shown), which is in agreement with the previous report that CsA minimally affected C2C12 myoblast proliferation (10). Visual inspection of C2C12 cells treated with CsA for 24 h appeared slightly larger in size than the control myotubes, an effect that was more apparent at higher CsA concentration (results not shown). Moreover, less number of multinucleated differentiated myotubes were found when the differentiated cells were treated with 30 μM CsA in differentiation medium (results not shown), which is also consistent with the earlier observation by Abbott et al. (10). At 10 μM concentration of CsA, however, visually the number and the appearance of C2C12 myotubes were virtually similar to those in the control plate (results not shown). Therefore, we used differentiating C2C12 cells and 10 μM CsA for most of our studies.

The Na+-dependent creatine uptake in C2C12 nascent myotubes decreased when cells were treated with 10 μM CsA for 48 h (Fig. 3, compare 1 versus 2). In parallel experiments, CsA treatment also inhibited the Na+-dependent taurine uptake significantly (Fig. 3, compare 3 versus 4), while the Na+-dependent glutamate uptake remained mostly unaffected (Fig. 3, compare 5 versus 6) in C2C12 myotubes. Since the transport of creatine, glutamate, and taurine via their respective Na+-dependent transporters are driven by the sodium electrochemical gradient of the cell, our observation rules out the possibility that the effect of CsA on creatine uptake was due to a general decrease in the driving force.

CsA inhibited the endogenous creatine uptake in C2C12 cells both in a concentration- and time-dependent manner (Fig. 4). As shown in Fig. 4A, treatment with various concentrations of CsA for 24 h resulted in decreased creatine uptake, and the decrease in uptake was higher as the CsA concentration was increased. The inhibitory effect of CsA also increased with time of treatment. At 1 μM CsA, the inhibition in uptake was ~10% (n = 3; p < 0.04) at 24 h (Fig. 4A), which increased to ~28% (n = 3; p < 0.05) at 96 h (results not shown). At 10 μM CsA, the uptake was inhibited by ~40 and ~65% at 24 and 48 h, respectively (Fig. 4B). Compared with the results obtained with oocytes expressing the hCRT-1 cRNA, a lower dose and a shorter incubation time were required for CsA to inhibit the endogenous creatine uptake in the C2C12 muscle cells. This discrepancy may be due to a difference in the amount and/or rate of diffusion of CsA into these two different cell types. FK506,
expressed as % of control (solvent-treated cells) uptake.

Cyclosporin A (CsA) was added directly to the differentiation medium and the cells were incubated for an additional 48 h at 37 °C. Ethanol-treated control (1, 3, and 5) and CsA-treated (2, 4, and 6) cells were subsequently used for a 60-min uptake assay in NaCl-1 buffer containing 10 μM [3H]glutamate (1 and 2), 5 μM [3H]taurine (3 and 4), or 5 μM [3H]creatine (5 and 6) as a substrate. Results (mean of duplicate measurement ± deviation) are expressed as % of control uptake.

Another potent immunosuppressive drug that acts via its own immunosuppressive dose. These results, in general, are similar to the results obtained using the oocytes expressing the hCRT-1 cDNA.

Effect of CsA on K_m and V_max of Creatine Uptake—We further examined whether CsA inhibited the creatine uptake by altering the K_m and/or the V_max of creatine transport. To this end, we measured the rate of uptake as a function of various extracellular concentrations of [14C]creatine. In both CsA-treated or ethanol-treated (control) oocytes expressing the hCRT-1 cDNA, the rate of uptake increased hyperbolically as the external creatine concentration was increased (Fig. 5). Similar results were also obtained using the control and the CsA-treated C2C12 cells (results not shown). These results were further analyzed using the Michaelis-Menten equation and the results are summarized in Table I. As shown, CsA treatment did not affect the K_m for creatine in both oocytes and C2C12 cells. Moreover, the observed K_m value is very similar to the K_m value we reported earlier for the recombinant hCRT-1 (18). However, CsA treatment resulted in reducing the V_max for creatine uptake in CsA-treated C2C12 cells by ~72% and in CsA-treated oocytes by ~36%. Currently, we do not know why CsA reduced V_max to different extents in cells and oocytes. One explanation is that the observed discrepancy might be simply due to a difference in the cell type (mammalian versus amphibian). Nevertheless, these results suggest that the V_max effect is probably due to a reduction of the amount of transporters in the cell surface (i.e. steady state levels at the plasma membrane).

Biotinylation of Cell Surface Creatine Transporter—The effect of CsA on the steady state levels of the transporter at the plasma membrane might be either due to an apparent decrease in the synthesis of the creatine transporter or due to a decrease in the relative number of the surface expressed transporters. To distinguish between these two possibilities, we first compared the relative expression level of the creatine transporter in control and CsA-treated C2C12 myotubes by immunoblot assay using a creatine transporter polyclonal antibody. We then analyzed the relative surface abundance of the creatine transporters in control and CsA-treated C2C12 myotubes using a surface biotinylation assay.

Our anti-CrT-C polyclonal antibody recognized two major immunoreactive protein bands (~55 and 75 kDa) in the detergent extracts of both control and CsA-treated cells, and the
relative intensities of these two immunoreactive bands were comparable in both extracts (Fig. 6, lanes 1 and 2). Incubation with tunicamycin for 24 h resulted in the partial loss of these two bands with a concomitant appearance of immunoreactive bands at ~60 and 40 kDa (Fig. 6, lane 3). We also independently detected the same immunoreactive bands in extracts prepared from HEK293 cells transiently expressing a hemagglutinin epitope-tagged human CRT-1 creatine transporter using the hemagglutinin-epitope specific monoclonal antibody 12CA5 (Roche Molecular Biochemicals) as a probe (results not shown). Our observations are in agreement with the observations of Guerrero-Ontiveros and Wallimann (31) and Neubauer et al. (32), who independently detected similar size (~55 and 70 kDa) immunoreactive bands in the extracts of rat and human muscles, respectively, using a different creatine transporter antibody. Thus, we believe that both the 55- and 75-kDa bands are directly related with the expression of the creatine transporter, and most likely represent the moderately N-glycosylated and more highly glycosylated forms of the creatine transporter.

The results of the surface biotinylation assay are also shown in Fig. 6 (lanes 4 and 5). Visual inspection of the results suggested that only the 55-kDa band binds to the streptavidin beads. Moreover, scanning of the 55-kDa bands from two independent experiments by densitometry revealed that the relative abundance of the streptavidin-bound 55-kDa band in the CsA-treated cells was 39.7 ± 2.7% (n = 2) of that of the untreated control cells. Mock biotinylation control, in which cells were processed in parallel without adding the sulfo-NHS-SS-biotinylation reagent, lacked any immunoreactive bands (result not shown), suggesting that surface biotinylation is required for the recovery of the 55-kDa band by the streptavidin beads. Since the 75-kDa immunoreactive band was not detected in the captured biotinylated proteins, we believe that this form of the creatine transporter is not expressed on the surface of the C₂C₁₂ cells. These observations collectively suggest that the creatine uptake activity in the C₂C₁₂ cells and the surface expression of the 55-kDa protein band are closely linked. Our results also suggest that CsA treatment decreased the relative abundance of the surface expressed creatine transporter, which paralleled the decrease in the $V_{\text{max}}$ of creatine uptake in CsA-treated cells.

**DISCUSSION**

At low nanomolar concentration (5–100 nM), CsA binds with its cellular target cyclophilin and inhibits the function of the protein phosphatase calcineurin (33, 34), a key step of an evolutionary conserved signal transduction pathway, and thus, suppresses immunoresponse. The drug FK506 mediates its immunosuppressive action via the same signaling pathway by inhibiting the function of calcineurin (34) after binding to its own cellular target, FKBP (33, 44), did not inhibit creatine uptake. Third, FK506, at a concentration known to inhibit the PPIase activity of FKBP (33, 44), did not inhibit creatine uptake. Fourth, addition of CsA to the transport mixture during the uptake assay also did not inhibit the creatine uptake, suggesting that the effect of CsA on creatine uptake was not due to a direct block of the creatine transporter.

The **cis-trans** isomerization of a peptidyl-prolyl bond may serve as the fulcrum of a conformational switch critical for either the folding or the function of a protein. Commensurate with this idea, Brandl and Deber (45) proposed a mechanistic role of the **cis-trans** isomerization of the peptidyl-prolyl bonds in active transport. Curiously, nine out of 30 Pro residues found in the primary sequence of the creatine transporter are predicted to be located in the putative transmembrane domains of its proposed topological model (19, 20). It is noteworthy that CsA also inhibits the activity of the endogenous (46) and the recombinant² taurine transporter, a highly homologous protein that contains Pro residues at places similar to those found in the creatine transporter (20). Although not proven, these results tend to suggest that the mechanism by which CsA affects creatine and taurine transport might be similar. It is conceivable that CsA inhibits the PPIase activity of an associated cyclophilin, which in turn blocks the **cis-trans** isomerization catalyze **cis-trans** isomerization around proline containing peptide bonds (for reviews, see Refs. 37 and 38). CsA and FK506 are also potent inhibitors of the PPIase activities of the cyclophilins and FKBP5, respectively (39–41). Several studies support the idea that CsA impairs protein folding by directly inhibiting the PPIase activity of an associated cyclophilin (42, 43).

Results described in this paper show that the creatine uptake is inhibited effectively by CsA at micromolar concentration, which is significantly higher than the concentration of CsA required to inhibit calcineurin function (33). We believe that the inhibition of the PPIase activity of an associated cyclophilin is the likely cause for the CsA-induced inhibition of creatine uptake because of the following reasons. First, CsA was effective at a concentration range that is known to inhibit the PPIase activity of cyclophilin (42, 43). Second, FK506, another potent immunosuppressive drug, did not mimic the effect of CsA at a concentration much higher than its immunosuppressive dose (10–100 nM), ruling out the possibility of calcineurin involvement in inhibiting the creatine uptake. Third, FK506, at a concentration known to inhibit the PPIase activity of FKBP (33, 44), did not inhibit creatine uptake. Fourth, addition of CsA to the transport mixture during the uptake assay also did not inhibit the creatine uptake, suggesting that the effect of CsA on creatine uptake was not due to a direct block of the creatine transporter.

### Table I

**Inhibition of Creatine Transport by Cyclosporin A**

|                  | Control | CsA-treated | CsA-treated |
|------------------|---------|-------------|-------------|
| $K_m$ (µM)       | 24.8    | 20.7        | 22.3        |
| % Decrease in $V_{\text{max}}$ | 72      | 22.6        | 36          |

**DISCUSSION**

At low nanomolar concentration (5–100 nM), CsA binds with its cellular target cyclophilin and inhibits the function of the protein phosphatase calcineurin (33, 34), a key step of an evolutionary conserved signal transduction pathway, and thus, suppresses immunoresponse. The drug FK506 mediates its immunosuppressive action via the same signaling pathway by inhibiting the function of calcineurin (34) after binding to its own cellular target, FKBP (33, 34, 35). We also independently detected the same immunoreactive bands in extracts prepared from HEK293 cells transiently expressing a hemagglutinin epitope-tagged human CRT-1 creatine transporter using the hemagglutinin-epitope specific monoclonal antibody 12CA5 (Roche Molecular Biochemicals) as a probe (results not shown). Our observations are in agreement with the observations of Guerrero-Ontiveros and Wallimann (31) and Neubauer et al. (32), who independently detected similar size (~55 and 70 kDa) immunoreactive bands in the extracts of rat and human muscles, respectively, using a different creatine transporter antibody. Thus, we believe that both the 55- and 75-kDa bands are directly related with the expression of the creatine transporter, and most likely represent the moderately N-glycosylated and more highly glycosylated forms of the creatine transporter.

The results of the surface biotinylation assay are also shown in Fig. 6 (lanes 4 and 5). Visual inspection of the results suggested that only the 55-kDa band binds to the streptavidin beads. Moreover, scanning of the 55-kDa bands from two independent experiments by densitometry revealed that the relative intensity of the streptavidin-bound 55-kDa band in the CsA-treated cells was 39.7 ± 2.7% (n = 2) of that of the untreated control cells. Mock biotinylation control, in which cells were processed in parallel without adding the sulfo-NHS-SS-biotinylation reagent, lacked any immunoreactive bands (result not shown), suggesting that surface biotinylation is required for the recovery of the 55-kDa band by the streptavidin beads. Since the 75-kDa immunoreactive band was not detected in the captured biotinylated proteins, we believe that this form of the creatine transporter is not expressed on the surface of the C₂C₁₂ cells. These observations collectively suggest that the creatine uptake activity in the C₂C₁₂ cells and the surface expression of the 55-kDa protein band are closely linked. Our results also suggest that CsA treatment decreased the relative abundance of the surface expressed creatine transporter, which paralleled the decrease in the $V_{\text{max}}$ of creatine uptake in CsA-treated cells.

**DISCUSSION**

At low nanomolar concentration (5–100 nM), CsA binds with its cellular target cyclophilin and inhibits the function of the protein phosphatase calcineurin (33, 34), a key step of an evolutionary conserved signal transduction pathway, and thus, suppresses immunoresponse. The drug FK506 mediates its immunosuppressive action via the same signaling pathway by inhibiting the function of calcineurin (34) after binding to its own cellular target, FKBP (34–36). Both cyclophilin and FKBP are widely expressed members of a highly conserved family of proteins known as peptidyl-prolyl isomerases (PPIase), which

**Experimental Procedures.** Experimental data points were subsequently fitted to Michaelis-Menten equation using the Sigma Plot program to determine the respective $K_m$ and $V_{\text{max}}$ values.

**Inhibition of Creatine Transport by Cyclosporin A**

|                  | Control | CsA-treated | CsA-treated |
|------------------|---------|-------------|-------------|
| $K_m$ (µM)       | 24.8    | 20.7        | 22.3        |
| % Decrease in $V_{\text{max}}$ | 72      | 22.6        | 36          |

**FIG. 6.** Cell surface biotinylation of untreated (control) and CsA-treated C₂C₁₂ cells. Immunoblots were performed using the anti-CrT-C polyclonal antibody as described under "Experimental Procedures." A, total cell extracts were prepared from the control and CsA-treated C₂C₁₂ cells (lanes 1 and 2), and also from control cells treated with tunicamycin (5 µg/ml) for 24 h (lane 3). Cell extract applied to each lane (lanes 1–3) contains ~26 µg of protein. B, cell extracts were prepared from biotinylated control and CsA-treated cells. A portion of each extract containing a total of 390 µg of protein was used for incubation with the streptavidin-agarose beads. Surface biotinylated proteins captured by streptavidin-agarose beads are shown in lane 4, control cells; and lane 5, CsA-treated cells.

² W. Dai and H. K. Sarkar, unpublished observation.
around a critical peptidyl-prolyl bond of the transporter, creating an improperly folded transporter protein.

Our results show that the $V_{\text{max}}$ of creatine uptake in CsA-treated cells decreased without affecting the $K_m$ for creatine. There are several potential mechanisms by which impaired folding of the creatine transporter may lead to an apparent decrease in the $V_{\text{max}}$ of creatine uptake. One possibility is that the misfolded transporter is not at all targeted to the plasma membrane, resulting in an apparent decrease in surface abundance of the transporter. A second possibility is that both the properly folded (active) and the misfolded (inactive) forms of the creatine transporter are targeted to the plasma membrane, resulting in an apparent decrease in the $V_{\text{max}}$ without altering the relative number of the surface expressed transporters. A third possibility is that the misfolded transporters are degraded by proteolysis, resulting in a reduction in the relative expression level of the transporter. Based on our results, we can rule out the third possibility since the relative intensities of the immunoreactive protein bands (i.e. 55 and 75 kDa) remained unchanged both in control and CsA-treated muscle cells. We can also rule out the second possibility since our results show that the relative number of the surface expressed creatine transporters decreased significantly in the CsA-treated cells than that in the control cells. The simplest interpretation of our results is that CsA appears to affect the steady state levels of the creatine transporter at the plasma membrane, possibly by interfering with its membrane targeting mechanism. Our results, however, cannot completely rule out the possibility that the decrease in the $V_{\text{max}}$ is due to CsA-induced internalization of surface expressed transporters. Additional experiments are required to unambiguously resolve this question.

In organ transplant patients, CsA is therapeutically used at a daily dose of 200–250 mg and the treatment usually lasts from several weeks to several months (47, 5). Clinically relevant muscular disorders associated with CsA are generally mild and develop on chronic treatment (5). Even though the therapeutic concentrations of CsA in the blood of organ transplant patients ranges generally between 0.1 and 0.6 μM, occurrences of 1 μM and higher concentrations also have been reported (48, 49). As shown in Fig. 4A, treatment with 1 μM CsA for 24 h inhibited the creatine uptake in cultured muscle cells by ~10% ($p < 0.04$); the inhibition increased to ~28% when the cells were treated with CsA for 96 h (results not shown). At a concentration of 3 μM, 24 h treatment with CsA inhibited the creatine uptake by ~30% (Fig. 4A). The experiments described here were performed using creatine concentration close to the normal physiologic range (25–100 μM) of plasma creatine concentration (14). Taken together, our results suggest the CsA, at or near its therapeutic range, selectively impairs creatine transport.

Regardless of its mode of action, our finding that CsA specifically inhibits creatine transport may have important clinical implications with regard to its toxic side effects. The CRI-1 creatine transporter mRNA, although found in a wide variety of tissues, is most abundantly expressed in the skeletal muscle (20). This tissue distribution pattern of the creatine transporter mRNA is consistent with the idea that about 95% of the total body creatine is found in the muscles, where it is utilized as a dynamic source of stored energy (11, 13). When in demand, muscles maximally utilize the stored phosphocreatine to maintain muscle energy homeostasis. In the muscle, phosphocreatine is in a dynamic equilibrium with its precursor creatine. Creatine entry into the muscle via the creatine transporter is a key step that maintains this equilibrium between the phosphocreatine and creatine. Thus, an impairment of the creatine transporter function due to prolong exposure to CsA may chronically deplete muscle creatine. Chronic creatine depletion may adversely affect the muscle energy homeostasis (13) and may subsequently lead to the development of CsA-associated unwanted clinical myopathies seen in organ transplanted patients.

**Acknowledgments**—We thank Cynthia Edwards for technical help in cell culture, and Raynard Cockrell and Fredelina Pieri for oocyte microinjection.

**REFERENCES**

1. Borel, J. F. (1989) *Pharmacol. Rev.* 41, 259–371
2. Bennett, W. M., Burchmann, E., Andoh, T., Elzinga, L., and Franceschini, N. (1994) *Molec. Cell. Biochem.* 201, 133–141
3. Garcia-Escrib, M., Matinez, J., Fernandez-Ponsati, J., and Soto, O. (1994) *Clin. Neuropharmacol.* 17, 298–302
4. Hoyer, P. F. (1995) *Contr. Nephrol.* 114, 111–123
5. Arellano, F., and Krupp, P. (1991) *Lancet* 337, 915
6. Buring, M. S., Fournier, M., Ross, D. J., and Lewis, M. I. (1998) *J. Appl. Physiol.* 84, 1967–1975
7. Budak-Alpoglan, T., Kalayoglu-Besisik, S., Sargin, D., and Tangan, Y. (1998) *Bone Marrow Transplant.* 23, 115–116
8. Fernandez-Sola, J., Campistol, J., Casademont, J., Grau, J. M., and Urbano-Marquez, A. (1990) *Lancet* 333, 362–363
9. Hardiman, O., Sklar, R. M., and Brown, R. H., Jr. (1995) *Neurology* 43, 1432–1434
10. Abbott, K. L., Friday, B. B., Thaloor, D., Murphy, T. J., and Palvath, G. K. (1998) *Mol. Cell. Biochem.* 193, 295–305
11. Walker, J. B. (1979) *Adv. Enzymol.* 50, 177–242
12. Bessman, S. P., and Geiger, P. J. (1981) *Science* 211, 448–451
13. Wyse, M., and Wallimann, T. (1994) *Molec. Cell. Biochem.* 133/134, 51–66
14. Beis, I., and Newsholme, E. (1975) *FEBS Lett.* 45, 275–279
15. Raynard, Cockrell, and Fredelina, Pieri for oocyte microinjection.
Inhibition of Creatine Transport by Cyclosporin A

43. Lodish, H. F., and King, N. (1991) J. Biol. Chem. 266, 14835–14838
44. O'Keefe, S. J., Tamura, J., Kincaid, R. L., Toce, M. J., and O'Neill, E. A. (1992) Nature 357, 692–694
45. Brandl, C. J., and Deber, C. M. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 917–921
46. Ramamoorthy, S., Leibach, F. H., Mahesh, V. B., and Ganapathy, V. (1992) Pediat. Res. 32, 125–127
47. Goy, J. J., Stauffer, J. C., Deruaz, J. P., Gillard, D., Kaufmann, U., Kuntzer T., and Kappenberger, L. (1989) Lancet 1, 1446–1447
48. Shaw, L. M., Yatscoff, R. W., Bowers, L. D., Freeman, D. J., Jeffery, J. R., Keown, F. A., McGilveray, I. J., Rosano, T. G., and Wong, P.-Y. (1990) Clin. Chem. 36, 1841–1846
49. Tegzess, A. M., Doorenbos, B. M., Minderhoud, J. M., and Donker, A. J. M. (1988) Transplant. Proc. 20, Suppl. 3, 530–533