

Transport Activity and Surface Expression of the Na⁺-Ca²⁺ Exchanger NCX1 Are Inhibited by the Immunosuppressive Agent Cyclosporin A and by the Nonimmunosuppressive Agent PSC833*

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Cyclosporin A (CsA) treatment of HEK 293 cells expressing the rat heart RHE-1 (NCX1.1, EMBL accession number X68191) or the rat brain RBE-2 (NCX1.5, GenBank™ accession number X68813) Na⁺-Ca²⁺ exchanger inhibited their transport activity in a concentration-dependent manner. The inhibition was detectable at 2 μM CsA, and exposure of the cells to 20 μM CsA resulted in a decrease of the Na⁺-dependent Ca²⁺ uptake to about 20% relative to that of untreated cells. Determination of the surface expression of the exchanger protein revealed a parallel concentration-dependent reduction in the amount of the immunoreactive protein. No reduction was detected in the amount of total immunoreactive exchanger protein in CsA-treated cells relative to untreated ones. Among the different drugs tested, only PSC833, an analog of cyclosporin D, mimicked the effects of CsA. Exposure of the transfected cells to the chemically related cyclosporin D and macrolide drugs (FK506 or rapamycin) had no effect on the transport activity or the surface expression of the Na⁺-Ca²⁺ exchanger. Co-expression of the human multidrug transporter P-glycoprotein (of which both drugs are modulators) with the cloned Na⁺-Ca²⁺ exchanger revealed that transport activity and surface expression of each transporter in the co-transfected system were similar to those of each transporter alone in both the presence and absence of CsA or PSC833. CsA and PSC833 inhibited the surface expression of the NCX1 protein but did not alter the surface expression of P-glycoprotein. Unlike some P-glycoprotein endoplasmic reticulum-retained mutants (Loo, T. W., and Clarke, D. M. (1997) *J. Biol. Chem.* 272, 709–712), CsA did not rescue RBE-2/F913→Stop, an endoplasmic reticulum-retained function-competent mutant of the Na⁺-Ca²⁺ exchanger (Kasir, J., Ren, X., Furman, I., and Rahamimoff, H. (1999) *J. Biol. Chem.* 274, 24873–24880) and did not induce its kinesis to the surface membrane, further demonstrating molecular differences between P-glycoprotein and NCX1 mutants for interaction with CsA.

contraction, transcription, and many others (1). Ca²⁺ signaling involves a rapid rise in free intracellular Ca²⁺ levels for brief periods of time, followed by a decrease and restoration of the low cytosolic resting Ca²⁺ levels. Among the different Ca²⁺ transporters present in the plasma membrane and intracellular organelles, the electrogenic Na⁺-Ca²⁺ exchanger plays a major and unique role in the regulation of cell [Ca²⁺] for two reasons: it can move Ca²⁺ across the membrane bidirectionally, depending on the net electrochemical driving force, and it has a very high turnover rate (2). Although the Na⁺-Ca²⁺ exchanger is expressed in most mammalian cell types, it is of especially high density in excitable tissues such as the heart, in smooth cells, and in the brain (2).

Cyclosporin A (CsA)¹ is an immunosuppressive drug that is widely used against graft rejection (3). Among the complications that accompany CsA treatment of transplant patients are hypertension (4) and nephrotoxicity (3), both of which can be linked to impaired Ca²⁺ homeostasis, an increase in intracellular Ca²⁺, and smooth muscle vasoconstriction (5).

In this work, we have studied the effects of CsA on the functional expression of the Na⁺-Ca²⁺ exchanger NCX1 to examine its possible involvement in CsA-related posttransplant complications. We show that treatment of cells expressing the Na⁺-Ca²⁺ exchanger with CsA or with the nonimmunosuppressant PSC833 (6), which is an analog of weakly immunosuppressant cyclosporin D (CsD) (7), results in a concentration-dependent inhibition of Na⁺-Ca²⁺ exchange activity and a corresponding decrease in surface expression of the protein. The effects are probably posttranslational because no significant differences in the amount of total immunoreactive protein derived from the Na⁺-Ca²⁺ exchanger are detected between cells expressing the transporter without and with CsA treatment. Other immunosuppressive agents such as FK506 and rapamycin (8) had no effect on either transport activity or surface expression of the Na⁺-Ca²⁺ exchanger. Similar results are found in cells expressing both the Na⁺-Ca²⁺ exchanger and P-glycoprotein (P-gp), with which CsA and PSC833 directly interact and function as modulator (9).

EXPERIMENTAL PROCEDURES

Expression Systems

HEK 293 cells (ATCC 1573) were used to express the cloned (in pcDNA3.1) Na⁺-Ca²⁺ exchangers *rbe-2/NCX1.5* (10), *rhe-1/NCX1.1* (11), their mutants, and the cloned *MDR1* (kindly given by Dr. Y. Zhou). Preparation of amino-terminal FLAG-tagged RBE-2 (*fn-rbe-2*) has been

Ca²⁺ ions regulate a wide spectrum of cellular processes such as signal transduction, neurotransmitter release, muscle

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¹ The abbreviations used are: CsA, cyclosporin A; CsD, cyclosporin D; FN, amino-terminal FLAG-tagged; P-gp, P-glycoprotein; ER, endoplasmic reticulum; FACS, fluorescence-activated cell-sorting; FKBP, FK506-binding protein.

described previously (12, 13). To prepare the amino-terminal FLAG-tagged rat heart $\text{Na}^+\text{-Ca}^{2+}$ exchanger NCX1.1 (*fn-rhe-1*), an *AgeI-Eco47III* fragment was excised from *rhe-1* (11) and subcloned into *AgeI-Eco47III*-digested *fn-rhe-2* vector. The fidelity of the subcloning procedure has been verified by sequencing. Transfection was carried out with LipofectAMINE Plus reagent (Invitrogen) according to the protocol supplied by the manufacturer. Two μg and 0.66 μg of pDNA were used to transfect cells plated into 60-mm dishes or 1 well of a 12-well plate, respectively. CsA (Calbiochem-Novabiochem Corp, La Jolla, CA), FK506 (Alexis Corp.), CsD (a gift from Dr. Gilbert Burckhardt, University of Pittsburgh School of Pharmacy), PSC833 (a gift from Dr. S. Bates, National Cancer Institute, National Institutes of Health), and rapamycin (a gift from Drug Repository, National Cancer Institute, National Institutes of Health) were dissolved in Me_2SO and added 3 h after transfection together with the 10% fetal calf serum-Dulbecco's modified Eagle's medium supplement to the transfected cells (Invitrogen). The experiments were repeated three to five times. Transport measurements were done in triplicate. In each experiment, the transport activity of *rbe-2*- or *rhe-1*-transfected cells with Me_2SO treatment was taken as 100%, and the transport activities measured in the drug-treated cells were calculated in relative values. Some of the experiments were also repeated by using the VTF-7/HeLa cell expression system (14, 15).

Determination of Na^+ -dependent Ca^{2+} Uptake

Determination of transport activity in whole cells was carried out exactly as described previously (10–13, 16). In principle, transfected cells were preloaded with 0.16 M NaCl and 0.01 M Tris-HCl, pH 7.4, using 25 μM nystatin (Sigma). Cells were washed with the same buffered NaCl solution (without MgCl_2) to remove nystatin. Transport was initiated by overlaying the cells with the same buffered Na^+ - or K^+ -containing solution, to which 25 μM $^{45}\text{Ca}^{2+}$ was added. All solutions also contained 1 mM ouabain (Sigma). Na^+ -dependent Ca^{2+} uptake was determined by subtracting the Ca^{2+} taken up in the absence of a Na^+ gradient from that taken up in its presence.

Determination of P-glycoprotein Pump Activity by Rhodamine 123 Dye Efflux Assay

P-gp pump activity was determined as described in Ref. 17. Transfected cells were equilibrated with a 150 ng/ml solution of rhodamine 123 in Iscove's modified Dulbecco's modified Eagle's medium-5% fetal calf serum for 20 min at 37 °C. After washing the cells with phosphate-buffered saline and 0.1% bovine serum albumin, the dye was allowed to efflux for 2 h at 37 °C. As a control for pump activity, the cells were preincubated with the P-gp inhibitor (reversing agent) verapamil (5 μM ; Sigma) for 30 min before and during both rhodamine 123 staining and the efflux phase of the assay. Immediately afterward, the cells were analyzed by FACSflow cytometer using CellQuest software (Becton Dickinson, Mountain View, CA).

Measuring Surface Expression of the $\text{Na}^+\text{-Ca}^{2+}$ Exchanger and P-glycoprotein by FACS Analysis

NCX1 Isoforms—Cells expressing the extracellular (12, 13) amino-terminal FLAG-tagged isoforms of the NCX1 gene were used to determine surface expression of the $\text{Na}^+\text{-Ca}^{2+}$ exchanger. As described previously, the FLAG epitope was introduced into the extracellular amino-terminal "tail" of the protein after Gly⁸ (instead of Asn⁹, the single glycosylation site of the protein), and it was accessible to externally added antibody without any permeabilization. Introduction of the epitope had no effect on the functional expression of the protein (12, 13, 18).

For FACS analysis (19), 10^5 cells were washed and resuspended in 200 μl of Iscove's modified Dulbecco's modified Eagle's medium-5% fetal calf serum and 1 μg of mouse M2 (anti-FLAG) monoclonal antibody (Sigma) or control mouse IgG1 κ antibody (BD PharMingen, San Jose, CA) for 30 min at 37 °C. As a secondary antibody, 1 μg of fluorescein isothiocyanate-conjugated anti-mouse antibody IgG1 κ (BD PharMingen) was used. Cells were washed with phosphate-buffered saline and 0.1% bovine serum albumin and analyzed by FACS analysis (see above).

Median value was determined using CellQuest software (Becton Dickinson) histogram stat analysis for each curve. The average of two median fluorescence values (in arbitrary units) for similar curves from two independent experiments is presented under "Results."

For determination of the total amount of immunoreactive NCX1 protein, the cells were permeabilized before application of the primary antibody M2 or the control antibody using the IntraPrep permeabilization reagent (Immunotech, Marseille, France).

P-gp (Human)—To determine the surface expression of P-gp, MRK16 (a gift from Hoechst, Kawago City, Saitama, Japan), a monoclonal antibody against an external epitope of P-gp (20), was used. 1 μg of MRK16 or 5 μg of an isotype control antibody (IgG2a) was added to the washed cells in 200 μl of Iscove's modified Dulbecco's modified Eagle's medium and incubated at 37 °C for 20 min. As a secondary antibody, 1 μg of fluorescein isothiocyanate-conjugated anti-mouse IgG2 κ (BD PharMingen) was used.

Western Analysis

HEK 293 cells were lysed with M-PER mammalian protein extraction reagent (Pierce), to which 1 mM dithiothreitol and the following protease inhibitors were added: aprotinin (Sigma), 17 $\mu\text{g}/\text{ml}$; leupeptin (Sigma), 2 $\mu\text{g}/\text{ml}$; pepstatin (Sigma), 2 $\mu\text{g}/\text{ml}$; and AEBFSF (4-(2-aminoethyl)benzenesulfonyl fluoride) (Sigma), 1 mM. SDS-PAGE was carried out by standard procedures (21) using either 10% or 4–20% gradient precast polyacrylamide gels (Invitrogen). The following antibodies were used at a dilution of 1:1000 for Western blot analysis: anti-cyclophilin A (Calbiochem-Novabiochem Corp.), anti-FKBP59/HSP56 (Calbiochem-Novabiochem Corp.), anti-cyclophilin B (Affinity BioReagents, Golden, CO), and anti-FKBP12 (Affinity BioReagents). Detection of the antigen-antibody complexes was done with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Invitrogen) using ECL reagents purchased from Amersham Biosciences, Inc. or Pierce. NCX1 isoform-expressing HEK 293 cells were treated with or without CsA and analyzed similarly by Western blot. For analysis of total cell extracts, 30 μg of cell protein derived from a single well of a 12-well plate was used. Phenylmethylsulfonyl fluoride (0.1 μM), leupeptin (10 $\mu\text{g}/\text{ml}$), and pepstatin (10 $\mu\text{g}/\text{ml}$) were added to the cells before solubilization. To detect the level of $\text{Na}^+\text{-Ca}^{2+}$ exchanger protein AbO-8, a polyclonal antibody directed against a pentadecapeptide corresponding to amino acids 649–663 of the rat heart exchanger was used (12). Preparation, testing, and purification of the antibodies were done as described previously (12, 16). Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used to detect antigen-antibody complexes with the Amersham Biosciences, Inc. ECL kit.

RESULTS

CsA Inhibits the Transport Activity and the Surface Expression of the $\text{Na}^+\text{-Ca}^{2+}$ Exchanger NCX1—To examine the effects of CsA on the $\text{Na}^+\text{-Ca}^{2+}$ exchanger, we have transfected HEK 293 cells with *fn-rbe-2* or *fn-rhe-1*, two rat isoforms of the NCX1 gene. The transfected cells were exposed to 2–20 μM CsA or an equal volume of Me_2SO (which never exceeded 1% of the assay volume), and Na^+ -dependent Ca^{2+} uptake was determined 24 h after transfection, as described under "Experimental Procedures." Fig. 1 summarizes the results of these experiments. It can be seen that exposure of the cells expressing the rat heart exchanger RHE-1 (■) or the rat brain exchanger RBE-2 (●) to CsA results in inhibition of Na^+ -dependent Ca^{2+} uptake in a concentration-dependent manner. Inhibition of Na^+ -dependent Ca^{2+} uptake could be detected at 2 μM CsA. Exposure of the cells expressing the transporter to ≥ 20 μM CsA resulted in a decrease of transport activity to about 20% relative to that expressed in the absence of the drug. Higher concentrations of CsA could not be tested in transport experiments because at concentrations above 20 μM , the numerous washes included in the experimental protocol led to excessive cell peeling from the adherent surfaces. CsA had no effect on the transport assay itself because addition of the drug to the solutions used for the assay (but not to the transporter-expressing cells) did not result in a decrease in the Na^+ -dependent Ca^{2+} uptake (data not shown).

To study the effects of CsA on the surface and total expression of the $\text{Na}^+\text{-Ca}^{2+}$ exchanger NCX1, HEK 293 cells were transfected with the cloned amino-terminal FLAG-tagged $\text{Na}^+\text{-Ca}^{2+}$ exchangers. Surface expression was determined by measuring the fluorescence intensity using FACS analysis (for details, see "Experimental Procedures"). Cells were labeled with the monoclonal anti-FLAG M2 antibody or the control IgG1 κ antibody as primary antibodies. Fluorescein isothiocyanate-

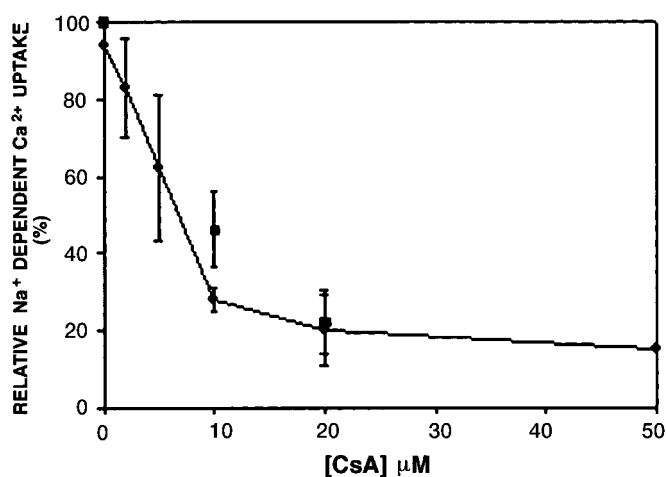


FIG. 1. The effect of different concentrations of CsA on Na⁺-dependent Ca²⁺ uptake in HEK 293 cells expressing the rat heart and rat brain isoforms of the NCX1 gene. Cells were transfected with plasmids encoding the rat heart Na⁺-Ca²⁺ exchanger *fn-rhe-1* (■) and the rat brain Na⁺-Ca²⁺ exchanger *fn-rbe-2* (●). Na⁺-dependent Ca²⁺ uptake was determined 24 h after transfection as described under "Experimental Procedures" without and with exposure of the isoform-expressing cells to different concentrations of CsA.

conjugated anti-mouse antibody was used as the secondary antibody. The total amount of immunoreactive Na⁺-Ca²⁺ exchanger protein in transfected cells was analyzed in a similar manner, except that the cells were permeabilized prior to application of the primary antibody or the control antibody.

Fig. 2, A–D, shows the surface expression of the Na⁺-Ca²⁺ exchanger FN-RBE-2 without (Fig. 2A) and with application of 0.1 (Fig. 2B), 10 (Fig. 2C), and 20 μM (Fig. 2D) CsA. It can be seen that the fluorescence of M2 antibody-labeled cells decreases in a concentration-dependent manner that parallels the decrease of transport activity (Fig. 1). The two population peaks shown in Fig. 2, A–D, represent the nontransfected cells (left peak, lower level of fluorescence) and the transfected cells (right peak). Although the efficiency of transfection might vary somewhat from dish to dish, in this experiment it did not change significantly, and there is no change in the extent of left peak, the population of cells exhibiting a lower level of fluorescence (nontransfected cells). CsA treatment changed only the surface expression of the exchanger protein, which is demonstrated by the change in fluorescence of the right peak (the population of transfected cells). Similar results were also obtained when FN-RHE-1 was expressed in HEK 293 cells (data not shown). We have also examined the effect of CsA on the amount of total immunoreactive Na⁺-Ca²⁺ exchanger protein. The total amount of immunoreactive protein detected in transfected cells did not change significantly when these cells were treated with 2–50 μM CsA. Fig. 3 shows the total exchanger protein expression detected by AbO-8, a polyclonal antibody directed against a pentadecapeptide corresponding to amino acids 649–663 of the rat heart exchanger (12). The same amount of protein (30 μg) was loaded in each lane. All transfections were done in parallel. Lane A shows control, *fn-rbe-2*-transfected cells, and lanes B–F show transfected cells exposed to 2, 5, 10, 20, and 50 μM CsA, respectively. Similar results were obtained when FACS analysis was used to detect the total amount of exchanger protein. The control antibody curve reveals an average median fluorescence (in arbitrary units) of 27.52. The average median value for M2 binding is 243.71 arbitrary units, whereas treatment of the cells with 10 μM CsA did not change significantly the median fluorescence value of M2 binding (229.79 arbitrary units). The inhibitory effects of CsA on the transport activity and surface expression of the

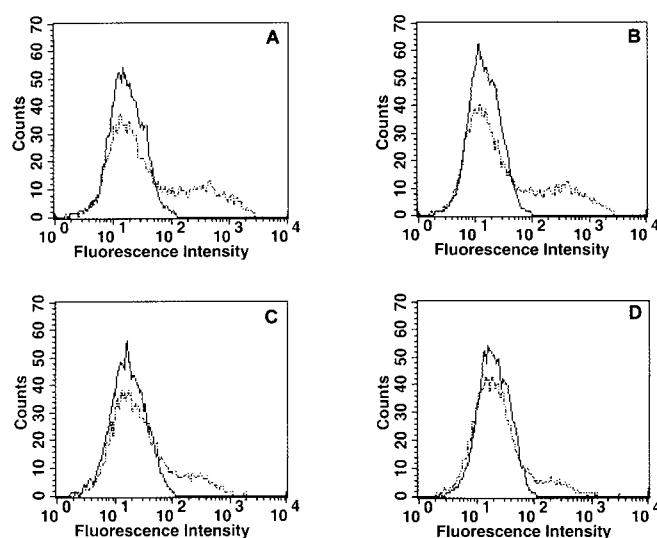


FIG. 2. The effect of different concentrations of CsA on the surface expression of the rat brain Na⁺-Ca²⁺ exchanger expressed in HEK 293 cells. Cells expressing the Na⁺-Ca²⁺ exchanger FN-RBE-2 were incubated with various concentrations of CsA or Me₂SO. 24 h after transfection, the cells were analyzed for cell surface expression using M2 (the anti-FLAG antibody) or IgG1 control antibody. A, transfected cells treated with Me₂SO only. Control antibody (—), M2 antibody (·····). B, transfected cells treated with 0.1 μM CsA. C, transfected cells treated with 10 μM CsA. D, transfected cells treated with 20 μM CsA.

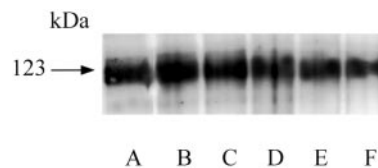


FIG. 3. Western analysis of HEK 293 cells expressing the Na⁺-Ca²⁺ exchanger protein without and with exposure to CsA. Protein derived from cells expressing FN-RBE-2 (lane A) or FN-RBE-2 after exposure to 2 (lane B), 5 (lane C), 10 (lane D), 20 (lane E), and 50 μM (lane F) CsA was separated by SDS-PAGE. 30 μg of protein derived from a single well of a 12-well plate was loaded in each lane. The polyclonal antibody AbO-8 (see "Experimental Procedures") was used for Western blot analysis. Horseradish peroxidase-conjugated goat anti-rabbit antibody was used to detect antigen-antibody complexes by ECL.

Na⁺-Ca²⁺ exchanger were not restricted to the HEK 293 expression system. Similar results were obtained when we expressed the cloned NCX1 gene in the recombinant vaccinia virus VTF-7/HeLa cell expression system (data not shown).

Not All Structurally Related Undecapeptide Analogues of CsA Inhibit Na⁺-Ca²⁺ Exchanger Transport Activity and Surface Expression—To examine whether the inhibitory effects of CsA on the Na⁺-Ca²⁺ exchanger are linked to its immunosuppressive properties, we have determined the effect of the weakly immunosuppressive analogue, CsD, and the nonimmunosuppressant analogue of CsD, PSC833, on the transport activity and the surface and total expression of the Na⁺-Ca²⁺ exchanger. CsD, added to transfected HEK 293 cells at concentrations of 5–50 μM, had no effect on Na⁺ gradient-dependent Ca²⁺ uptake, on the surface expression, and on the total amount of immunoreactive exchanger protein of both FN-RBE-2 and FN-RHE-1 (data not shown).

However, exposure of the transfected cells to PSC833 resulted in a concentration-dependent reduction in transport activity and surface expression. Fig. 4 shows the effect of 3–10 μM PSC833 on Na⁺ gradient Ca²⁺ uptake of the rat heart exchanger RHE-1 expressed in HEK 293 cells. The surface expression pattern in transfected cells exposed or not exposed to

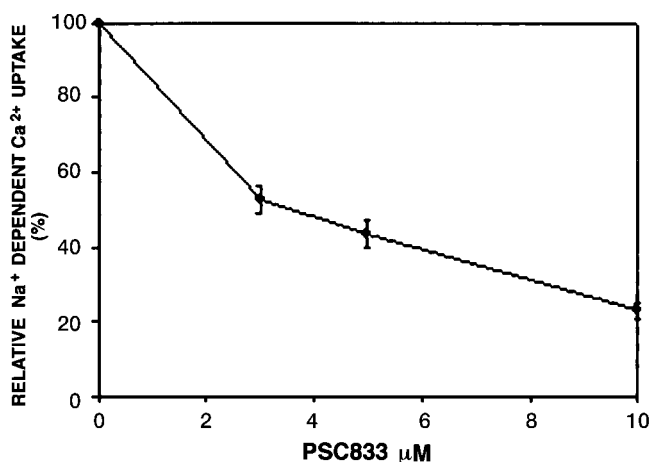


FIG. 4. The effect of different concentrations of PSC833 on the Na^+ - Ca^{2+} exchange activity of NCX1 expressed in HEK 293 cells. Cells were transfected with the plasmid encoding the rat heart Na^+ - Ca^{2+} exchanger *fn-rhe-1*. Na^+ -dependent Ca^{2+} uptake was determined 24 h after transfection as described under "Experimental Procedures" without and with exposure of the NCX1.1 expressing cells to different concentrations of PSC833.

10 μM PSC833 paralleled the transport data: control antibody curve reveals an average median of 45.68 arbitrary units. Average median value for surface M2 binding is 436.15 arbitrary units, whereas treatment of the cells with 10 μM PSC833 significantly changed the average median fluorescence value of M2 binding (193.39 arbitrary units). The corresponding amounts of total exchanger protein detected in permeabilized cells without and with exposure of the cells to 10 μM PSC833 were similar: average median fluorescence values of M2 binding were 268.0 and 225.26 arbitrary units, respectively (average median fluorescence value of control antibody binding is 115.23 arbitrary units). In a manner similar to CsA, PSC833 inhibited both the transport activity and surface expression of the protein in a concentration-dependent manner. No significant effects were observed on the total amount of exchanger protein. Similar results were also obtained when the rat brain isoform NCX1.5 was expressed.

The Effects of the Immunosuppressants FK506 and Rapamycin on the Transport Activity and Surface Expression of the Na^+ - Ca^{2+} Exchanger—To elucidate the cellular processes responsible for the effects of CsA on transport activity and surface expression of the Na^+ - Ca^{2+} exchanger, we exposed cells expressing the NCX1 or NCX1.5 gene products to FK506 (5–50 μM) and to rapamycin (2–50 μM). These drugs had no effect on the transport activity, surface expression, and total amount of exchanger protein expressed in transfected cells (data not shown). Similarly, exposure of transfected cells to rapamycin had no effect on Na^+ -dependent Ca^{2+} uptake, surface expression, or total expression of the Na^+ - Ca^{2+} exchanger (data not shown).

The Effects of CsA on Transport Activities and Surface Expression of P-glycoprotein and NCX1 Co-expressed in HEK 293 Cells—Because CsA and PSC833 are substrates of P-gp and are used as reversal agents to combat drug resistance in cancer cells (9), it was of interest to examine whether the effects of CsA and PSC833 on the transport activity and surface expression of the Na^+ - Ca^{2+} exchanger persist when HEK 293 cells are also co-transfected with both the cloned *fn-rbe-2* and *MDR1* genes. According to MRK16 and M2 monoclonal antibody staining (Fig. 5, B and C), a large majority of the transfected cells express both proteins.

The transport activity of FN-RBE-2 and P-gp was determined in cells transfected separately with each transporter and

in co-transfected cells, without and with CsA. Na^+ -dependent Ca^{2+} uptake in the absence and presence of 10 μM CsA was similar in *fn-rbe-2*-transfected cells and in cells co-transfected with *fn-rbe-2* and *MDR1*. The relative transport activity in cells exposed to 10 μM CsA was 28.7% (S.D. = 3.92%) and 36.12% (S.D. = 5.88%), respectively. RBE-2 expression did not change the activity of P-gp (using rhodamine 123 dye) in the absence (Fig. 5A) or presence (data not shown) of CsA. MRK16 and M2 staining revealed the same pattern: the surface expression of P-gp was not changed by co-transfection of *fn-rbe-2* with the *MDR1* (Fig. 5B). The surface expression of RBE-2 was similar in cells expressing this protein alone and in cells co-expressing this protein with P-gp. CsA inhibited the surface expression of RBE-2, regardless of whether RBE-2 was expressed alone or with P-gp (Fig. 5C). MRK16 staining of P-glycoprotein-expressing cells was not altered by CsA treatment. The curve of MRK16 binding reveals a median fluorescence of 154.78 arbitrary units. Treatment of the cells with 10 μM CsA did not significantly change the median fluorescence value of MRK16 binding (155.06 arbitrary units).

Can CsA Modulate the Kinesis of Surface Expression-incompetent Mutants of the Na^+ - Ca^{2+} Exchanger?—We have also explored the possibility that application of CsA might correct the kinesis to the surface membrane of some ER-retained mutants of the Na^+ - Ca^{2+} exchanger in a manner similar to its effect on ER-retained maturation-impaired mutants of P-glycoprotein (22). FN-C13 (*fn-rbe-2/F913*→Stop), a FLAG epitope-tagged carboxyl-terminal-truncated, surface expression-incompetent mutant of RBE-2 (13), was expressed in HEK 293 cells (without and with CsA treatment). No transport activity was detected in whole HEK 293 cells transfected with FN-C13 without or with exposure of the transfected cells to 5–20 μM CsA (data not shown). No surface expression of FN-C13 was detected in the transfected cells without or with 5–20 μM CsA treatment (data not shown). The fluorescence intensity that was measured using control antibody overlapped the fluorescence intensity obtained by M2 binding to the cells that were not treated or treated with different concentrations of CsA, suggesting that the drug did not induce surface expression of this mutant protein.

Expression of Target Immunophilins in HEK 293 Cells—To assess whether the differences in sensitivity of Na^+ - Ca^{2+} exchanger NCX1 expressed in HEK 293 cells to CsA and FK506 resulted from differences in expression pattern of the respective immunophilin targets, we carried out Western blot analysis of HEK 293 cell extracts using antibodies against cyclophilin A (CyPA18), cyclophilin B (CyPB21), FKBP12, and FKBP59. All the immunophilins tested were expressed in HEK 293 cells before and after transfection (with the plasmid vector or the cloned *fn-rbe-2*), without and with CsA treatment (data not shown).

DISCUSSION

In this work, we have shown that CsA inhibited in a concentration-dependent manner the transport activity and surface expression of the rat heart and rat brain isoforms of Na^+ - Ca^{2+} exchanger NCX1 expressed in HEK 293 cells. CsA did not reduce the specific transport activity of the surface-expressed Na^+ - Ca^{2+} exchanger because addition of the drug to the assay rather than to the transfected cells did not have any effect on Na^+ - Ca^{2+} exchange activity. Moreover, immunofluorescence studies of CsA-treated cells clearly demonstrated a significant decrease in the amount of surface-expressed protein. Among the different drugs that we tested, only the nonimmunosuppressive PSC833 (23–25) mimicked the effects of CsA. The weakly immunosuppressive CsD (7), which is chemically related to both CsA and PSC833 (6), and the two macrolides,

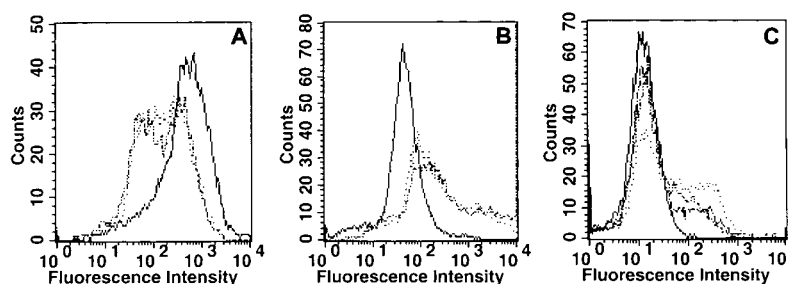


FIG. 5. Assessment of the transport function and surface expression of P-gp and RBE in P-gp- and/or RBE-2-expressing cells. A, 24 h after transfection, cells were harvested, washed, and loaded for 20 min with 150 ng/ml rhodamine 123, after which efflux was initiated for 2 h. The transport of rhodamine 123-transfected HEK 293 cells was determined by FACS analysis. Cells were transfected with *fn-rbe-2* only (control) (—), transfected with *MDR1* only (---), or co-transfected with *MDR1* and *fn-rbe-2* (·····). B, 24 h after transfection, the cells were analyzed for surface expression using MRK16 antibody or IgG2a control antibody. Cells expressed FN-RBE-2 only (—), P-gp and FN-RBE-2 (·····), or P-gp only (---). C, control IgG1 antibody staining of cells treated with 10 μ M CsA (—), M2 antibody staining of P-gp- and FN-RBE-2-expressing cells treated with Me₂SO only (·····), M2 antibody staining of P-gp- and FN-RBE-2-expressing cells treated with 10 μ M CsA (---), M2 antibody staining of FN-RBE-2-expressing cells treated with 10 μ M CsA (-·-·-).

FK506 and rapamycin, had no detectable effect on transport activity and surface expression of the Na⁺-Ca²⁺ exchanger, although we exposed the NCX1-expressing cells to concentrations as high as 50 μ M. The effects of CsA and PSC833 on the surface expression of the Na⁺-Ca²⁺ exchanger expressed transiently in HEK 293 cells are probably posttranslational and do not involve calcineurin-dependent transcriptional regulation (26). Several lines of evidence support this hypothesis. First, the total amount of Na⁺-Ca²⁺ exchanger protein expressed in CsA- and PSC833-treated cells was similar to the amount of immunoreactive exchanger protein detected in untreated cells. Second, surface expression was reduced by both the immunosuppressive CsA and the nonimmunosuppressive PSC833. Third, it was recently shown (27) that calcineurin is not involved in developmental regulation of transcription of NCX1 and NCX3 gene products in cerebellar granule cells. Fourth, the concentrations of CsA that inhibit calcineurin-mediated transcriptional regulatory effects are in the nanomolar range (26), whereas a micromolar range of CsA was required to inhibit transport activity and surface expression of the Na⁺-Ca²⁺ exchanger.

The ubiquitously expressed immunophilins, the cyclophilins and the FKBP (8, 28), are involved in protein folding in at least two ways: catalysis of the rate-limiting (29) isomerization of imide peptide bonds preceding prolines via their peptidyl prolyl cis-trans isomerase domain and chaperone activity via their conserved protein binding domain (8, 30, 31). "Quality control" of newly synthesized secretory and membrane proteins takes place in the ER. The process ensures that only mature and properly folded proteins reach their target destination. Misfolded and immature proteins are retained in the ER and subsequently degraded (32). Because both immunosuppressive CsA and nonimmunosuppressive PSC833 inhibit the surface expression of the Na⁺-Ca²⁺ exchanger NCX1 posttranslationally, the involvement of their cyclophilin targets in the folding of the exchanger protein is implied.

We do not know at present whether this is mediated by inhibition of the peptidyl prolyl cis-trans isomerase activity of cyclophilin A (cytosolic) or B (ER-resident) (8), inactivation of the chaperone-like activity of cyclophilin A or B (28), or both activities in concert. Each of these could provide a plausible explanation for the reduction of surface expression and transport activity of the Na⁺-Ca²⁺ exchanger NCX1.

Surface expression reduction by immunosuppressive CsA but not SDZ-211-811, its nonimmunosuppressive analogue, was demonstrated for the homo-oligomeric acetylcholine receptor containing the α 7 subunit and the homo-oligomeric 5-hydroxytryptamine type 3 receptor expressed in *Xenopus* oocytes (33, 34). The effect could be reversed by overexpression of

exogenous rat brain cyclophilin but not by expression of cyclophilin bearing a mutation within the peptidyl prolyl cis-trans isomerase domain. Reduction of surface expression by CsA was also reported for the Kir2.1 potassium channel (35), the creatinine transporter (36), and the insulin receptor (37). Like the homo-oligomeric acetylcholine and 5-hydroxytryptamine receptors (33) and the creatinine transporter (36), but unlike the insulin receptor (37) and the Kir2.1 potassium channel (35), the Na⁺-Ca²⁺ exchanger studied in this work was not sensitive to micromolar concentrations of FK506 treatment of the NCX1-expressing cells. Because FKBP12 and FKBP59 are expressed in HEK 293 cells, the absence of these two target immunophilins is not responsible for the insensitivity of our system to this immunosuppressant. It is interesting that surface expression of Na⁺ channels in adrenal chromaffin cells was up-regulated by all three drugs (CsA, FK506, and rapamycin) (38).

At drug concentrations (6, 9, 41, 42) similar to those used in this study, CsA is commonly used in transplant patients to protect against graft rejection, and PSC833 is used as a reversal agent for up-regulated P-gp (39, 40) in cancer cells. Reduction of surface expression by CsA and PSC833 of the Na⁺-Ca²⁺ exchanger NCX1, by the same or a different pathway, might have considerable effects on cellular Ca²⁺ homeostasis in view of the transporters' major role in the regulation of cell Ca²⁺ ion concentration. Based on the changing membrane potential values, the Na⁺-Ca²⁺ exchanger is involved in both Ca²⁺ extrusion and Ca²⁺ influx (2). Its reduced surface expression can lead to increased intracellular Ca²⁺ levels in resting cells. It can also result in reduced Ca²⁺ influx in above reversal potential-depolarized cells, which is of importance in the heart (2). Both CsA and PSC833 were shown to cross the blood-brain barrier (43, 44). They were shown in neuronal cells to be highly neurotoxic (45, 46). The Na⁺-Ca²⁺ exchangers NCX1, NCX2, and NCX3 have about 65% structural homology (47–49). Their proline residues are highly conserved. Whether the actions of CsA and PSC833 are mediated by inhibition of the peptidyl prolyl cis-trans isomerase activity of cyclophilin or by its chaperone-like activity, the surface expression of the three gene products might be affected by exposure of the Na⁺-Ca²⁺ exchanger-expressing cells to CsA or PSC833. However, further research has to be done, both in heterologous expression systems and in cells endogenously expressing the Na⁺-Ca²⁺ exchangers, to elucidate the mechanism of action of CsA and PSC833 on modulation of cell surface expression of the Na⁺-Ca²⁺ exchanger protein.

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