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Modulation of Na^+-Ca^{2+} Exchanger Expression by Immunosuppressive Drugs Is Isoform-Specific

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ABSTRACT

The Na^+-Ca^{2+} exchanger (NCX) is a major Ca^{2+}-regulating protein encoded by three genes: *NCX1*, *NCX2*, and *NCX3.* They share a sequence homology of approximately 65%. NCX1 protein is expressed ubiquitously, and NCX2 and NCX3 are expressed almost exclusively in the brain. We have shown previously (Kimchi-Sarfaty et al., 2002) that treatment of *NCX1*-transfected human embryonic kidney (HEK) 293 cells with the immunosuppressive cyclosporin A (CsA) and its nonimmunosuppressive analog PSC833 (valspodar) results in down-regulation of surface expression and transport activity of the protein without a decrease in expression of cell NCX1 protein. In this study, we show that cyclosporin A and PSC833 treatment of *NCX2*- and *NCX3*-transfected HEK 293 cells also resulted in dose-dependent down-regulation of surface expression and transport activity of the two brain NCX proteins; however, whereas CsA had no effect on total cell NCX protein expression, PSC833 reduced mRNA and cell protein expression of NCX2 and NCX3. Moreover, tacrolimus (FK506), which had no effect on NCX1 protein expression, down-regulated NCX2 and NCX3 surface expression and transport activity without any significant effect on cell protein expression. Sirolimus (rapamycin) had no effect on NCX2 and NCX3 protein expression, yet it reduced NCX2 and NCX3 transport activity. Because all of the experimental conditions in our studies were identical, presumably the different drug response is related to structural differences between NCX isoforms. Clinical studies suggested that immunosuppressive regimes of patients who have received transplants resulted in complications related to Ca^{2+}. Expression of NCX genes is tissue-specific. Hence, our results can potentially provide a tool for choosing the immunosuppressive protocol to be used.

Cyclosporin A (CsA), FK506 (tacrolimus), and rapamycin (sirolimus) are widely used to prevent organ rejection by patients who have received transplants (Hariharan et al., 2000; Levy, 2000; First, 2004; Tsang et al., 2007). They bind to their respective immunophilin receptors, the cyclophilins and FK506 binding proteins (FKBPs) that are highly conserved families of proteins present in all cells and compartments (Barik, 2006). The immunosuppressive action of CsA and FK506 is based on the interaction of the immunophilin-CsA/FK506 complex with the Ca^{2+} and calmodulin-dependent phosphatase calcineurin (Liu et al., 1992) followed by inhibition of the dephosphorylation of nuclear factor of activated T cells and its translocation to the nucleus, which leads to subsequent suppression of the immune reaction. Rapamycin also interacts with FKBPs; however, the FKBP-rapamycin complex does not inhibit calcineurin but inhibits the target of rapamycin protein, which is a cell cycle-specific serine/threonine kinase involved in cell growth, proliferation, protein transcription, initiation, and translation (Proud, 2007).

Immunophilins are also involved in protein folding, mediated by two different activities localized within separate protein domains: peptidyl prolyl *cis-trans* isomerase (PPIase) activity, which is rate-limiting in acquisition of configuration of X-proline peptide bonds, and chaperone activity (Galat, 2003; Barik, 2006). The role of both activities in acquisition of functional conformation of proteins was supported by many studies, and the relevant protein segments responsible for these activities were identified (Fischer et al., 1989; Pirkl et

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ABBREVIATIONS: CsA, cyclosporin A; FKBP, FK506 binding protein; PPIase, peptidyl prolyl *cis-trans* isomerase; NCX, Na^+-Ca^{2+} exchanger; HEK, human embryonic kidney; MDR, multidrug transporter; PCR, polymerase chain reaction; FN, FLAG epitope tagged; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; NHS-SS-biotin, 2-(biotinamido)-ethyl-1,3-dithiopropionate; HRP, horseradish peroxidase; MFI, mean fluorescence intensity; PSC833, valspodar.

al., 2001; Galat, 2003; Barik, 2006; Mok et al., 2006). Binding of immunosuppressive drugs to their immunophilins receptors inhibits both PPIase and chaperon activity (Galat, 2003; Barik, 2006).

Reduction of functional expression by CsA treatment has been demonstrated for the homo-oligomeric acetylcholine receptor containing the α7 subunit, the homo-oligomeric 5-hydroxytryptamine type 3 receptor (Helekar et al., 1994; Helekar and Patrick, 1997), the Kir2.1 potassium channel (Chen et al., 1998), the creatinine transporter (Tran et al., 2000), and the insulin receptor (Shiraishi et al., 2001) in different cells.

The Na^+-Ca^{2+} exchanger is a major Ca^{2+}-regulating protein expressed in all excitable and many nonexcitable cells. It transports Ca^{2+} across the plasma membrane in a bidirectional manner in response to driving Na^+ gradient and changes in membrane potential (Blaustein and Lederer, 1999). Three separate genes, *NCX1*, *NCX2*, and *NCX3*, code for this activity (Philipson and Nicoll, 2000). Whereas NCX1 gene products are almost ubiquitously expressed, NCX2 and NCX3 are expressed mostly in the brain (Quednau et al., 1997; Blaustein and Lederer, 1999; Annunziato et al., 2004). NCX1, NCX2, and NCX3 share an overall sequence homology of 65% (Annunziato et al., 2004), which is highest in the transmembrane helices.

We have shown previously that treatment of human embryonic kidney (HEK) 293 cells transfected with the *NCX1.1* and *NCX1.5* genes with CsA and its nonimmunosuppressive analog PSC833 (valspodar; SDZ 215-833) (Boesch et al., 1991), results in a dose-dependent decrease of surface expression and Na^+-Ca^{2+} exchange activity without a significant change in total cell NCX1 protein (Kimchi-Sarfaty et al., 2002). Neither FK506 nor rapamycin treatment of transfected HEK 293 cells had any effect on expression of the Na^+-Ca^{2+} exchanger NCX1. Moreover, we have shown that CsA treatment of L6, H9c2, and primary cultured smooth muscle cells, all expressing the Na^+-Ca^{2+} exchanger NCX1, led to down-regulation of transport activity and surface expression (Rahamimoff et al., 2007). This result suggests that the phenomenon was not restricted to heterologous expression systems. However, not all membrane proteins are modulated by treatment with CsA. For example, no reduction in functional expression of the human multidrug transporter (MDR) P-glycoprotein, transfected into HEK 293 cells, was obtained by treatment with CsA (Kimchi-Sarfaty et al., 2002).

Clinical studies suggested that immunosuppressive regimes of patients who have received transplants resulted in complications related to Ca^{2+} such as nephrotoxicity, hypertension, bone loss, and neurotoxicity (Cameron et al., 1995; Cardenas et al., 1995; Bechstein, 2000; Hariharan et al., 2000). CsA, FK506, rapamycin, and PSC833 were shown to cross the blood-brain barrier (Shirai et al., 1994; Lemaire et al., 1996; Kochi et al., 1999; Tai, 2000; Pong and Zaleska, 2003; Hsiao et al., 2006). On the basis of these findings, we decided to assess the effects of CsA, FK506, rapamycin, and PSC833 on the expression of NCX2 and NCX3 proteins.

In this study, we show that, with the exception of CsA, which modulated the expression of all three NCX proteins (NCX1, NCX2, and NCX3) in a similar manner, all other immunosuppressive and nonimmunosuppressive drugs tested (PSC833, FK506, and rapamycin) modulated functional expression of NCX2 and NCX3 in transfected HEK 293 cells in a different manner than they modulated the expression of NCX1.

TABLE 1

The effect of immunosuppressive and nonimmunosuppressive drugs on Na^+-dependent Ca^{2+} uptake

HEK 293 cells were transfected with cloned *FN-NCX2* or cloned *FN-NCX3*. Twenty-four hours after transfection, CsA, FK506, rapamycin, and PSC833 (all in DMSO) or an equal volume of DMSO only were added to the buffered Ca^{2+}-containing NaCl and KCl solutions. Na^+-dependent Ca^{2+} uptake was determined as described previously (see *Materials and Methods*).

Drug	FN-NCX2	FN-NCX3
	%	%
DMSO		
Control	100	100
CsA		
10 μM	96.66307 ± 9.99	102.5609 ± 11.3
20 μM	95.842593 ± 4.98	95.0043 ± 6.159
FK506		
10 μM	102.63047 ± 6.23	94.7981 ± 3.038
20 μM	100.87448 ± 6.32	109.0749 ± 4.518
Rapamycin		
10 μM	109.89653 ± 7.85	93.66682 ± 3.246
20 μM	96.007516 ± 4.57	107.6892 ± 10.45
PSC833		
10 μM	92.36227 ± 8.65	98.87516 ± 1.386

Materials and Methods

Cell Line and Cell Culture. HEK 293 cells (American Type Culture Collection, Manassas, VA) were used in all of the transfection experiments, and they were grown in Dulbecco's modified Ea-

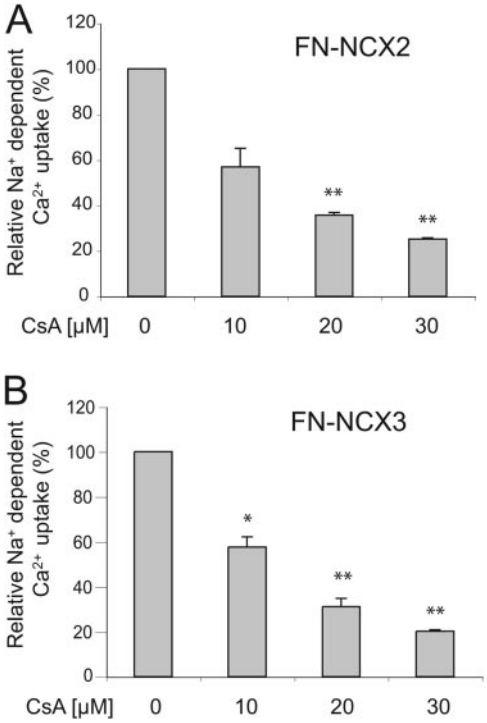

Fig. 1. The effect of different concentrations of CsA on Na^+-dependent Ca^{2+} uptake in HEK 293 cells expressing the FN-NCX2 and FN-NCX3 proteins. Cells were transfected with plasmids encoding the *FN-NCX2* (A) and *FN-NCX3* (B). Na^+-dependent Ca^{2+} uptake was determined 24 h after transfection, as described under *Materials and Methods*, without and with exposure of the expressing cells to 10, 20, and 30 μM CsA. Transport activities of FN-NCX2- and FN-NCX3-transfected cells with DMSO treatment were taken as 100%, and the transport activities measured in the drug-treated cells were calculated in relative values. The bars represent S.D. (*, $P < 0.05$; **, $P < 0.01$).

gle's medium (Biological Industries Ltd., Kibbutz Beit Haemek, Israel or Invitrogen, Carlsbad, CA) with 1% glutamine, 1% penicillin-streptomycin, and 10% fetal bovine serum (Biological Industries Ltd. or Gibco BRL Life Technologies) at 37°C in humid air containing 5% CO_2.

Expression System. HEK 293 cells were used to express the cloned Na^+-Ca^{2+} exchangers NCX2 and NCX3, kindly provided by Drs. Nicoll and Philipson (Li et al., 1994; Nicoll et al., 1996). To express them in HEK 293 cells, they were subcloned in the mammalian expression vector pcDNA3.1 (Invitrogen) by excision with HindIII/Ecl136II from cloned *NCX2* and NotI/ApaI from cloned *NCX3* in pBluescript SK (Stratagene, La Jolla, CA). They were subcloned in HindIII/EcoRV and in NotI/ApaI-digested pcDNA3.1(−). Preparation of N-terminal FLAG-tagged NCX2 and NCX3 was carried out by overlapping-ends polymerase chain reaction (PCR) (Ho et al., 1989). The FLAG epitope was inserted into NCX2 instead of N34 and into NCX3 instead of N45, the putative single extracellular glycosylation sites. The fidelity of the subcloning procedure and the mutagenesis have been verified by sequencing of the full-length genes (Center for Genomic Technologies, The Hebrew University, Jerusalem, Israel). All of the experiments described in this work were done with the N-FLAG-tagged exchangers FN-NCX2 and FN-NCX3.

Transfection Procedure. Transfection was carried out with the Lipofectamine and Plus reagents (Invitrogen) according to the manufacturer's protocol. One or 2 μg of pDNA was used to transfect cells plated into one well of a 12- or 6-well plate, respectively. In some transfection experiments, calcium phosphate (Sambrock et al., 1989) was used. No significant differences in the relative transport activities without or with drug treatment were observed between the two transfection reagents. Each type of the transfection experiments was repeated four to six times. The efficiency of the transfection was calculated from statistical values obtained from FACS analyses (see *Immunostaining to Detect Total and Surface Expression of the Na^+-Ca^{2+} Exchanger by FACS*, below), by measuring only the area under the curve of the positive transfected/treated sample (M2)—not including the area under the curve of the control sample (M1). The average number of transfected cells was 68% (range, 56–84%).

Drug Treatments. Cyclosporin A (Calbiochem Corp., San Diego, CA), FK506 (LC Laboratories, Woburn MA), rapamycin (Calbiochem Corp. or LC Laboratories), and PSC833 (a gift to H.R. from Novartis Pharma AG, Basel, Switzerland) were dissolved in dimethyl sulfoxide (DMSO) and added at 3 h after transfection together with the fetal bovine serum-Dulbecco's modified Eagle's medium supplement to the transfected cells. The amount of DMSO added to each well was equal, and the total volume never exceeded 1% of the volume in the wells.

The effect of each drug on the transport assay (see *Determination of Na^+-Dependent Ca^{2+} Uptake*) was examined as well. This was done by adding different concentrations of each drug to both the buffered Ca^{2+}-containing NaCl and KCl solutions directly, and no drug was added to the transfected cells (see Table 1).

Determination of Na^+-Dependent Ca^{2+} Uptake. Determination of transport activity in whole cells was carried out essentially as described previously (Kasir et al., 1999; Kimchi-Sarfaty et al., 2002). In principle, expressing cells were preloaded with 0.16 M NaCl and 0.01 M Tris-HCl, pH 7.4, using 25 μM nystatin (Sigma-Aldrich, Rehovot, Israel). 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 $^{45}Ca^{2+}$ (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) was added. All solutions also contained 1 mM ouabain (Sigma-Aldrich). 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. In some experiments, Na^+-preloaded cells were collected by centrifugation at 1500 rpm at 4°C and suspended in a minimal volume of the buffered NaCl solution (without $MgCl_2$). A total of 3 μl of the Na^+-loaded cells (approximately 40 μg of protein) were diluted into 100 μl of buffered K^+ or Na^+ to which 25 μM $^{45}Ca^{2+}$ was added (the same solution as described above). The transport reactions were terminated by filtration

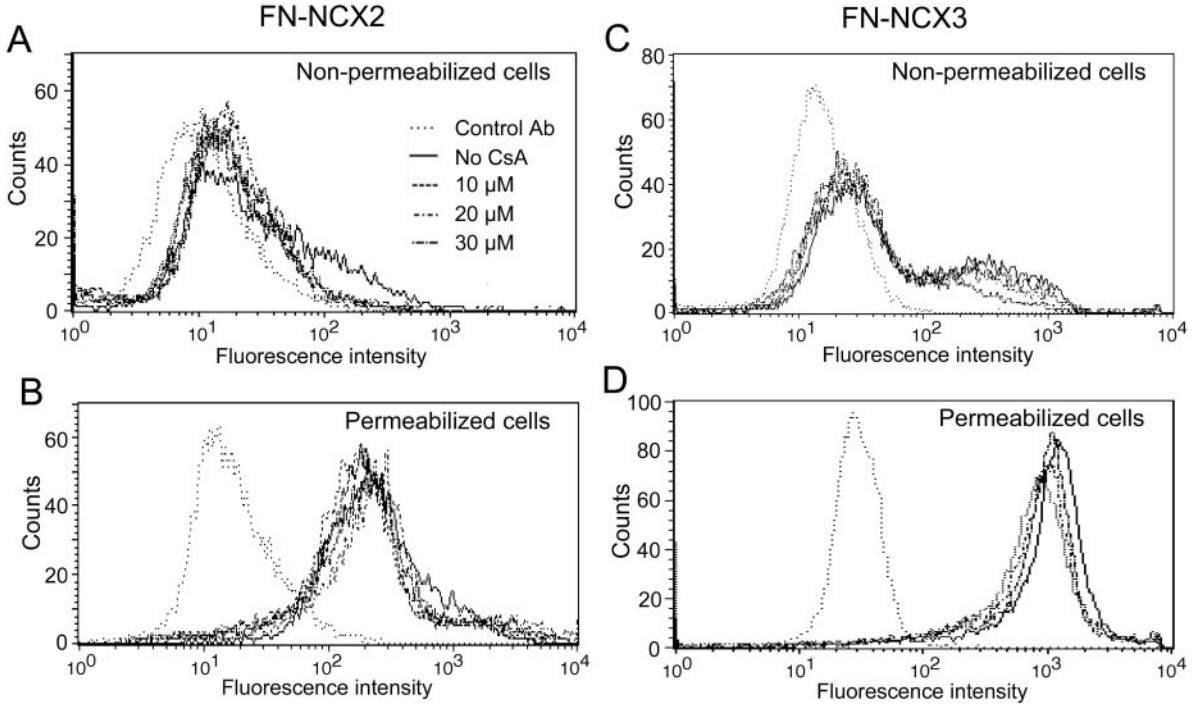

Fig. 2. The effect of various concentrations of CsA on the surface and total protein expression of the FN-NCX2 and FN-NCX3 proteins expressed in HEK 293 cells. Cells expressing the FN-NCX2 and FN-NCX3 were treated with various concentrations of CsA (10–30 μM) or equivalent volume of DMSO. Twenty-four hours after transfection, the cells were analyzed for cell surface and total expression using M2 anti-FLAG antibody and Alexa-Green 488 anti-mouse secondary antibody. A, nonpermeabilized *FN-NCX2*-transfected cells treated with 0 to 30 μM CsA. B, permeabilized *FN-NCX2*-transfected cells treated with 0 to 30 μM CsA. C, nonpermeabilized *FN-NCX3*-transfected cells treated with 0 to 30 μM CsA. D, permeabilized *FN-NCX3*-transfected cells treated with 0 to 30 μM CsA. The key to the lines used in all panels is identical (see A).

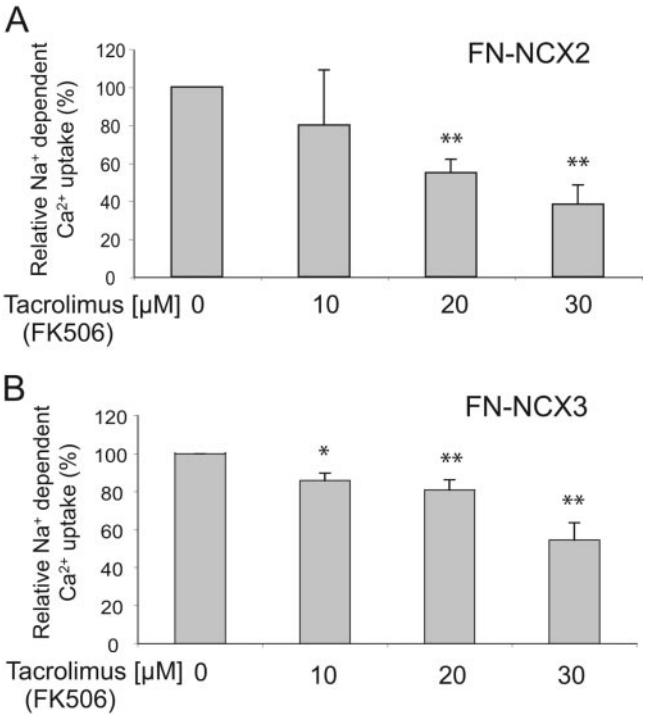

Fig. 3. Determination of the transport activities of HEK 293 cells expressing the FN-NCX2 and FN-NCX3 proteins exposed to different concentrations of FK506. Cells were transfected with plasmids encoding the *FN-NCX2* (A) and *FN-NCX3* (B). Na^+-dependent Ca^{2+} uptake was determined 24 h after transfection (as described under *Materials and Methods*) without and with exposure of the expressing cells to 10, 20, and 30 μM FK506. The transport activity of *FN-NCX2*- and *FN-NCX3*-transfected cells with DMSO treatment was taken as 100%, and the transport activities measured in the drug-treated cells were calculated in relative values. The bars represent S.D. (*, $P < 0.05$; **, $P < 0.01$).

via 0.45-μm filters (Whatman Schleicher and Schuell, Keene, NH). Washes of the filters and calculation of the net Na^+-dependent Ca^{2+} uptake was done as described above for adherent cells in situ. Transport measurements were done in triplicate, and each experiment was repeated four to six times. In each experiment, the transport activity of *FN-NCX2*- and *FN-NCX3*-transfected cells with DMSO treatment was taken as 100%, and the transport activities measured in the drug-treated transfected cells were calculated in relative values.

Immunostaining to Detect Total and Surface Expression of the Na^+-Ca^{2+} Exchanger by FACS. Cells expressing the extracellular N-FLAG-tagged clones were used to determine surface expression of the Na^+-Ca^{2+} exchanger. For FACS analysis (Zhou et al., 1999), cells were harvested and washed with phosphate-buffered saline (PBS). For determination of the immunoreactive NCX protein, cells were permeabilized and fixed using an IntraPrep permeabilization and fixation kit according to the manufacturer's procedure (Beckman Coulter, Immunotech, France). After permeabilization, or directly after harvesting for surface expression studies, cells were incubated with 1 μg of mouse M2 (anti-FLAG) monoclonal antibody (Sigma-Aldrich) or 1 μg of control mouse IgG1κ antibody (BD Biosciences Pharmingen, San Diego, CA), in a total volume of 100 μl of PBS with 0.1% bovine serum albumin (Sigma-Aldrich), for 30 min at 37°C. After washing, cells were incubated with 1 μg of fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody IgG1κ (BD Biosciences Pharmingen) or Alexa Green 488 anti-mouse secondary antibody (Invitrogen) for 30 min at 37°C. Preliminary experiments show that incubation of cells with the secondary antibody only revealed similar intensity background results as incubation of cells with the control mouse IgG1κ antibody. Therefore, experiments were run using control of secondary antibodies only for each treatment. After the second incubation, cells were washed with PBS containing 0.1% bovine serum albumin, and 10^5 cells were analyzed by a FACSCalibur cytometer (BD Biosciences, San Jose, CA). Statistical anal-

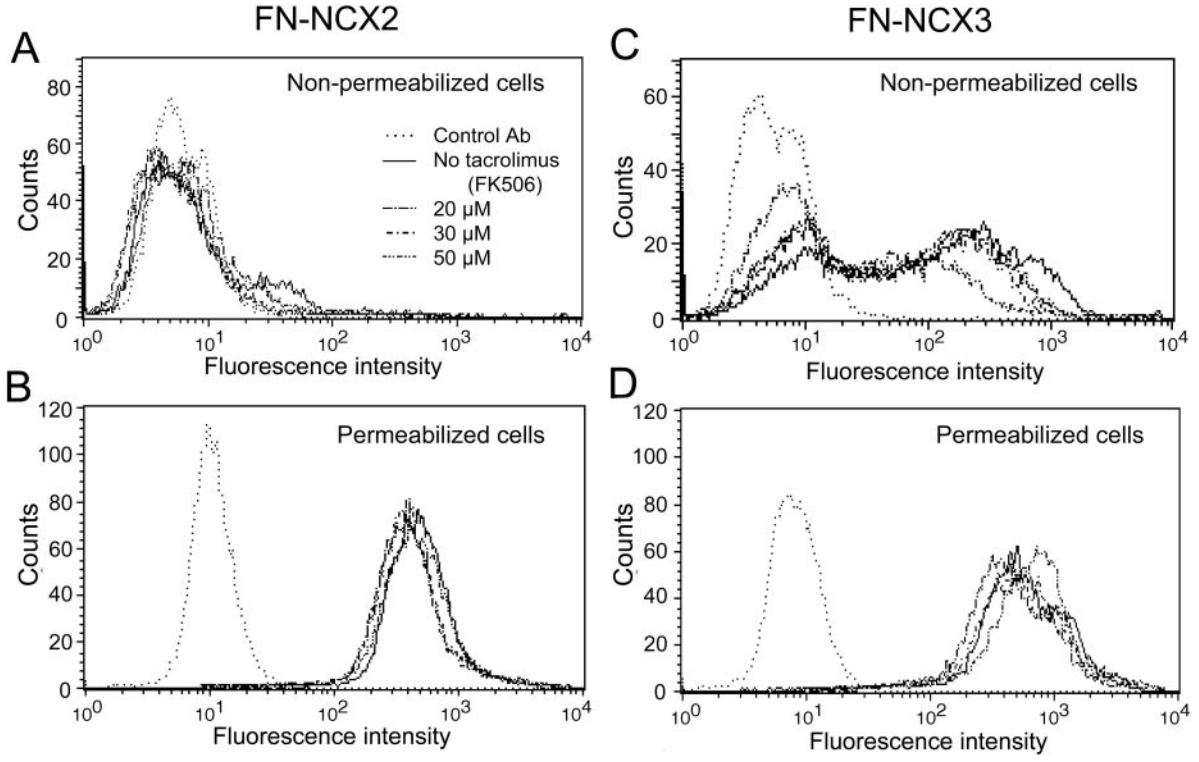

Fig. 4. The effect of FK506 on the surface and total FN-NCX2 and FN-NCX3 protein expressed in HEK 293 cells. Parallel transfections to those described in Fig. 3 were carried out. Twenty-four hours after transfection, the cells were analyzed for cell surface and total NCX protein expression using M2 anti-FLAG antibody and FITC-conjugated anti-mouse secondary antibody. A and C, nonpermeabilized *FN-NCX2* (A) and *FN-NCX3* (C)-transfected cells treated with FK506. B and D, permeabilized *FN-NCX2* (B) and *FN-NCX3* (D)-transfected cells treated with 0, 20, 30, and 50 μM FK506. The key to the lines used in all panels is identical to that in A.

ysis was performed using CellQuest software to determine the median fluorescence values (arbitrary units).

Biotinylation and Western Blot Analysis to Detect Total and Surface NCX2 and NCX3 Protein Expression. Biotinylation of surface membrane proteins of transfected HEK 293 cells was done in situ with NHS-SS-biotin (Pierce, Rockford, IL), essentially as described in Kasir et al. (1999) and Ren et al. (2001) and based on the protocol of Stephan et al. (1997). Adherent cells from a single well of a 12-well plate were used for surface biotinylation. The biotinylated cells were lysed with a solution containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% SDS, 0.1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), 0.01 mg/ml pepstatin A (Sigma-Aldrich), and 0.02 mM leupeptin (Sigma-Aldrich). The SDS concentration was lowered by a 10-fold dilution of the lysate with a solution of identical composition to that which was used to lyse the cells, except that it did not contain SDS. The lysate was loaded on streptavidin agarose beads (Pierce) and gently shaken overnight at 4°C. Washing of the beads was done as described in Kasir et al. (1999) and Ren et al. (2001). Biotinylated proteins were released from the beads by heating for 10 min at 85°C with Laemmli sample buffer and separated by SDS-polyacrylamide gel electrophoresis. Western blot analysis was carried out by standard procedures. For analysis of total cell extracts, 20 μg of approximately 600 μg of cell protein derived from the entire contents of a single well of a 12-well plate was used. To detect protein derived from the Na^+-Ca^{2+} exchanger, the anti-FLAG antibody M2 (Sigma-Aldrich) was used. Horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) was used to detect antigen-antibody complexes using the ECL kit (Biological Industries Ltd.). In all of the experiments presented in this work, transport activity, surface expression, and total immunoreactive protein were derived from cells transfected in parallel. Each experiment was repeated four to six times with different plasmid DNA preparations.

Determination of FN-NCX2 and FN-NCX3 mRNA by Quantitative PCR. RNA was isolated (1 μg) from *FN-NCX2* and from *FN-NCX3*-transfected HEK 293 cells, which were without or with PSC833 treatment, using TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions. cDNA synthesis was carried out with the RobusT II RT-PCR kit (Finnzymes, Espoo, Finland) according to the manufacturer's instructions.

Quantitative real-time PCR was done using the TaqMan Gene Expression assay (Applied Biosystems, Foster City, CA). The reaction was carried out in an ABI Prism 7000 spectrofluorometric thermal cycler (Applied Biosystems). The assay identification of the genes was as follows: Rn00589573_m1 (for NCX2); Rn01517854_m1 (for NCX3); and Hs99999910_m1 (for TATA box binding protein, used as endogenous control) (purchased from Applied Biosystems). Data analysis was done using ABI Prism 7000 software (Applied Biosystems). Each RNA sample was isolated from three separate transfections. Each assay was done in triplicate (for each NCX and respective TATA box binding protein).

Results

Cyclosporin A Treatment of HEK 293 Cells Transfected with *FN-NCX2* and *FN-NCX3* Resulted in Down-Regulation of the Na^+-Dependent Ca^{2+} Uptake and Surface Expression. To examine the effects of CsA on the Na^+-Ca^{2+} exchangers NCX2 and NCX3, we transfected HEK 293 cells with the cloned *FN-NCX2* or cloned *FN-NCX3*. The transfected cells were exposed to 10 to 30 μM CsA in DMSO or an equal volume of DMSO, and Na^+-dependent Ca^{2+} uptake was determined 24 h after transfection, as described under *Materials and Methods*. Figure 1, A and B, summarize the results of these experiments. As can be seen, exposure of the cells expressing the cloned exchangers to CsA resulted in a reduction of Na^+-dependent Ca^{2+} uptake in a concentration-dependent manner. Exposure of the cells expressing the transporter to 10 μM CsA or above resulted in a significant decrease of the transport activity relative to that expressed in the absence of the drug. Control experiments in which the cells were not exposed to the drug during transfection and the drug was added only to the transport solutions during the assay did not result in a decrease in the Na^+-dependent Ca^{2+} uptake (see Table 1).

To study the effects of CsA on the surface and total expression of the Na^+-Ca^{2+} exchangers FN-NCX2 and FN-NCX3, parallel transfections to those used for transport experiments were carried out. Surface and total protein expression was determined by measuring the fluorescence intensity using M2 antibody staining via FACS analysis (for details, see *Materials and Methods*). Figure 2, A and B, show the surface and total FN-NCX2 protein expression, and Fig. 2, C and D, show the surface and total FN-NCX3 at 0, 10, 20, and 30 μM CsA. It can be seen that the fluorescence of M2 antibody cell surface-labeled proteins decreases in a concentration-dependent manner that parallels the decrease of their respective transport activities (Fig. 1). The mean fluorescence intensity (MFI) was calculated by compiling the MFI data from three separate transfections. The MFI of surface-expressed protein

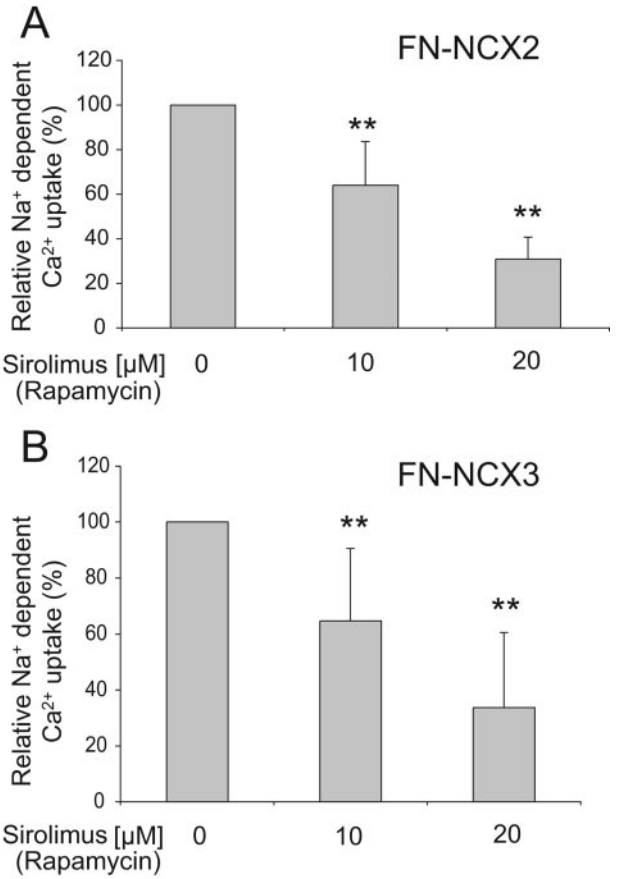

Fig. 5. Determination of the transport activities of HEK 293 cells expressing the FN-NCX2 and FN-NCX3 proteins exposed to different concentrations of rapamycin. Cells were transfected with plasmids encoding *FN-NCX2* (A) and *FN-NCX3* (B). Na^+-dependent Ca^{2+} uptake was determined 24 h after transfection (as described under *Materials and Methods*) without and with exposure of the expressing cells to 10 and 20 μM rapamycin. The transport activity of *FN-NCX2*- and *FN-NCX3*-transfected cells with DMSO treatment was taken as 100%, and the transport activities measured in the drug-treated cells were calculated in relative values. The bars represent S.D. (*, $P < 0.05$; **, $P < 0.01$).

in 0 CsA (DMSO-treated cells) was taken as 100%, and all other values were normalized. For FN-NCX2-expressing cells, calculations were as follows: 0 to 100; 10 μM, 74.6 (S.D. 14.1); 20 μM, 67.1 (S.D. 14.2); and 30 μM, 25.8 (S.D. 8.9). For FN-NCX3-expressing cells, calculations were as follows: 0 to 100; 10 μM, 64.4 (S.D. 7.6); 20 μM, 46.3 (S.D. 15.9); and 30 μM, 32.6 (S.D. 11.1). The immunofluorescence detected in permeabilized cells (total cell NCX protein) was calculated in the same manner. The average MFIs did not significantly change when they were exposed to the same concentration of CsA.

FN-NCX2 and FN-NCX3 Protein Surface Expression and Transport Activity Was Modulated by FK506. The macrolide compound FK506 is widely used as an alternative to CsA as an immunosuppressive agent. It binds to the family of cellular receptors, the FKBPs. Treatment of *FN-NCX2*- and *FN-NCX3*-transfected HEK 293 cells with 0 to 30 μM FK506 resulted in reduced Na^+-Ca^{2+} exchange activity (Fig. 3, A and B, respectively) and surface expression (Fig. 4, A and C) without a decrease in total cell NCX protein (Fig. 4, B and D). The relative MFIs for FK506-treated surface expressed cells (calculated as that for CsA-treated cells) were as follows: for FN-NCX2-expressing cells: 0 to 100; 20 μM, 75.7 (S.D. 3.9); 30 μM, 69.06 (S.D. 6.8); and 50 μM (N.D.); for FN-NCX3-expressing cells: 0 to 100; 20 μM, 60.5 (S.D. 2.09); 30 μM, 55.9 (S.D. 4.06); and 50 μM, 44.4 (S.D. 11.9). Although sensitivity of both transporters to FK506 was much lower than their sensitivity to CsA, the reduced surface expression and Na^+-Ca^{2+} exchange activity was consistently detected. We could not calculate the MFI of the surface-expressed FN-NCX2 exposed to 50 μM FK506, because the percentage of dead cells, which normally is 5% or below, was between 13 and 23%.

Rapamycin Reduced Na^+-Dependent Ca^{2+} Uptake Activity but Had No Effect on Surface Expression and Total FN-NCX2 and FN-NCX3 Protein Expression. We have also examined the effect of rapamycin treatment (5–20 μM) on the expression of *FN-NCX2*- and *FN-NCX3*-transfected HEK 293 cells. Rapamycin treatment of *FN-NCX2*- and *FN-NCX3*-transfected HEK 293 resulted in a decrease in FN-NCX2 and FN-NCX3 Na^+-dependent Ca^{2+} uptake activity (Fig. 5, A and B). However, rapamycin treatment had no effect on surface or total FN-NCX2 and FN-NCX3 protein expression (Fig. 6, A–D).

Rapamycin could have potentially inhibited the transport assay. To rule out this possibility, we have not added the drug to the cells during the transfection procedure but added it directly to the transport assay. Addition of rapamycin to the transport assay did not inhibit Na^+-dependent Ca^{2+} uptake activity (Table 1).

The Nonimmunosuppressive PCS833 Reduced Surface Expression, Na^+-Ca^{2+} Exchange Activity, mRNA, and Total Immunoreactive Cell FN-NCX2 and FN-NCX3 Protein. PSC833 is a nonimmunosuppressive analog of CsA. Hence, it was interesting to examine its effect on the expression of FN-NCX2 and FN-NCX3 in transfected HEK 293 cells. Figures 7 and 8 summarize these data. In Figs. 7A (*FN-NCX2*-transfected cells) and 8A (*FN-NCX3*-transfected

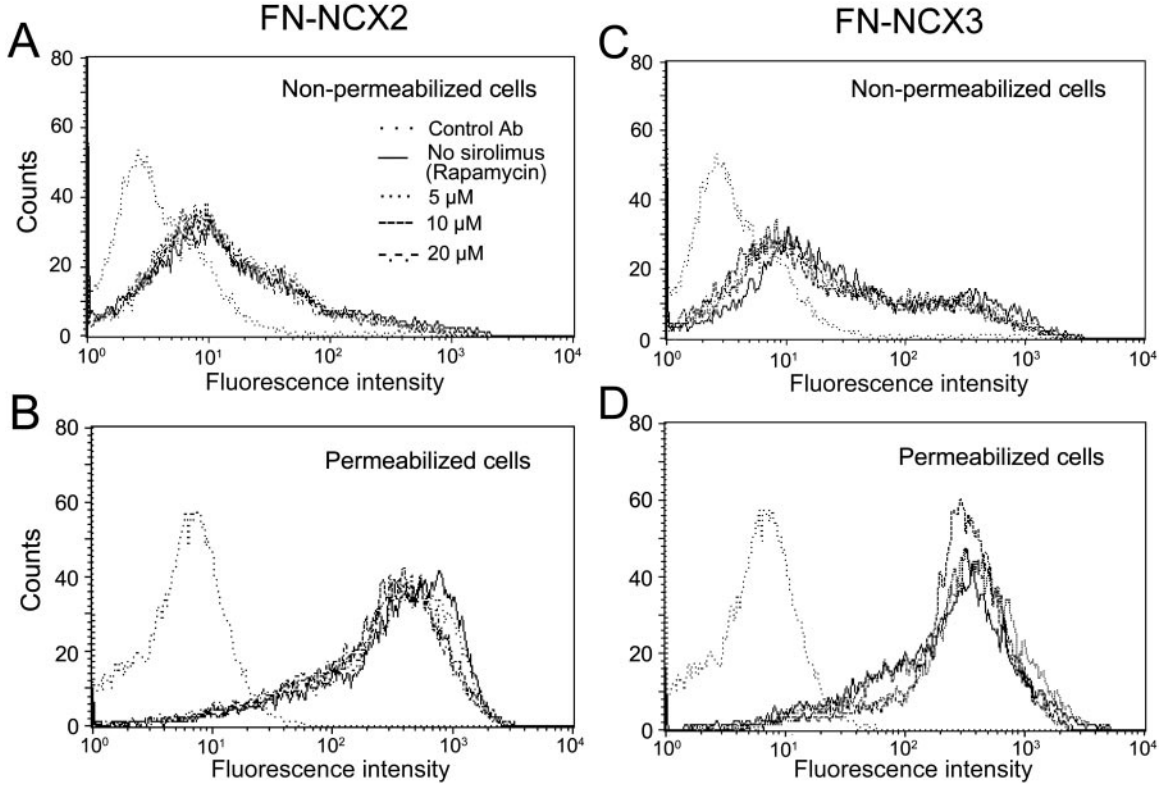

Fig. 6. The effect of rapamycin on surface and total FN-NCX2 and FN-NCX3 protein expressed in HEK 293 cells. Parallel transfections to those described in Fig. 5 were carried out. Twenty-four hours after transfection, the cells were analyzed for cell surface and total NCX protein expression using M2 anti-FLAG antibody and FITC-conjugated anti-mouse secondary antibody. A and C, nonpermeabilized *FN-NCX2* (A) and *FN-NCX3* (C)-transfected cells. B and D, permeabilized *FN-NCX2* (B) and *FN-NCX3* (D)-transfected cells. The key to the lines used in all panels is identical to that shown in A.

cells), it can be seen that treatment of HEK 293 cells with PSC833 leads to dose-dependent reduction in Na^+-Ca^{2+} exchange activity. The sensitivity of the FN-NCX2 and FN-NCX3 proteins to PSC833 is higher than that to CsA: 20 μM PSC833 reduces approximately 20% of the relative transport activity, and treatment of the cells with 30 μM PSC833 reduces the expression of the relative transport activity to approximately 10% compared with untreated (DMSO only) transfected HEK 293 cells.

In a manner similar to the effect of CsA treatment of transfected *FN-NCX2* and *FN-NCX3* HEK 293 cells, PSC833 treatment resulted in reduced dose-dependent surface protein expression. This was shown by immunostaining and FACS analysis (Fig. 7B) and surface biotinylation (insert in Fig. 7B) for *FN-NCX2*-transfected cells, and the same result is shown in Fig. 8B for *FN-NCX3*-transfected cells. Immunostaining with M2, the anti-FLAG antibody, shows that FN-NCX2 and FN-NCX3 proteins migrate as a double band (insert in Figs. 7B and 8B). This presumably represents two slightly different conformational forms derived both from the same cloned Na^+-Ca^{2+} exchanger, because only the cloned constructs bear the FLAG epitope, which is stained by the monoclonal antibody.

Unlike the effect of CsA on FN-NCX2 and FN-NCX3 expression, PSC833 treatment resulted in dose-dependent reduction of total cell FN-NCX2 protein (Fig. 7D) and respective FN-NCX3 and protein (Fig. 8D). To elucidate the mode of action of PSC833 on total FN-NCX2 and FN-NCX3 cell protein expression, we have measured the corresponding mRNA levels (Figs. 7C and 8C) without (DMSO-treated cells) and with PSC833 treatment by quantitative PCR. The PSC833-dependent reduction in mRNA levels suggests that the effect of PSC833 on FN-NCX2 and FN-NCX3 expression is at the transcriptional level.

Discussion

In this study, we have examined the effect of the commonly used immunosuppressive drugs CsA, FK506, and rapamycin and the nonimmunosuppressive PSC833 on the expression of

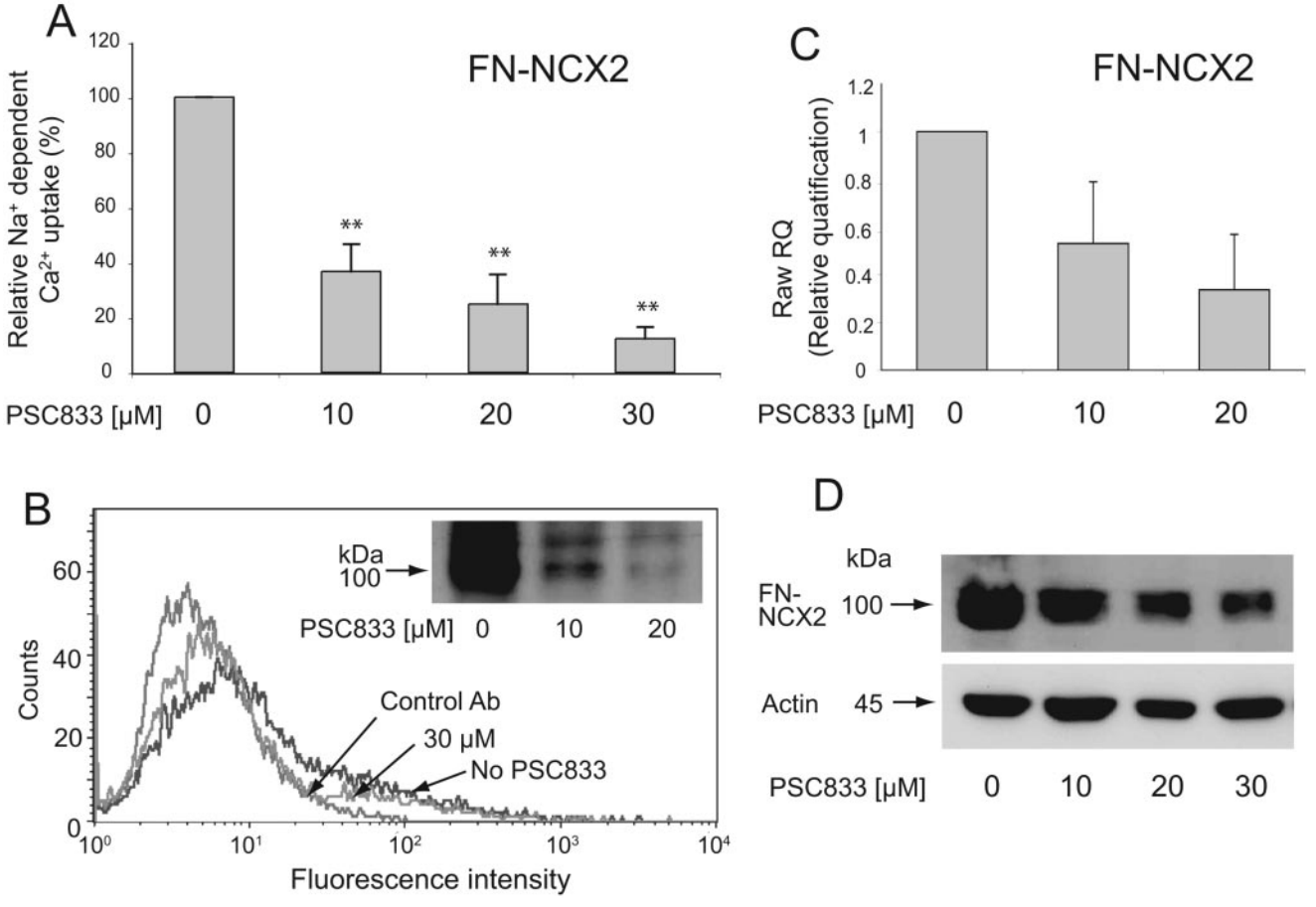

Fig. 7. The effect of different concentrations of PSC833 on the expression of FN-NCX2 in HEK 293 cells. Na^+-dependent Ca^{2+} uptake, surface expression, mRNA expression, and total FN-NCX2 protein expression were determined 24 h after transfection (as described under *Materials and Methods*) without and with exposure of the transfected cells to 0 to 30 μM PSC833. A, The transport activity of *FN-NCX2*-transfected cells with DMSO treatment was taken as 100%, and the transport activities measured in the drug-treated cells were calculated in relative values. The bars represent S.D. (*, $P < 0.05$; **, $P < 0.01$). B, surface expression was determined by FACS analysis and by surface biotinylation (insert in B). The M2 anti-FLAG antibody and FITC anti-mouse secondary antibody were used for detection of FN-NCX2 by FACS analysis, and the HRP-conjugated anti-mouse secondary antibody was used for detection of surface-biotinylated FN-NCX2. C, FN-NCX2 mRNA levels were measured by quantitative PCR as described under *Materials and Methods*. All results were normalized to untreated samples and TATA box binding protein values. The S.D. represents results from three independent experiments. D, Western blot analysis was used to determine total immunoreactive FN-NCX2 protein using the M2 anti-FLAG antibody and HRP-conjugated anti-mouse secondary antibody. Twenty micrograms of transfected cell extract was loaded on each lane. Immunodetection of actin was used as an equal loading control.

NCX2 and NCX3 proteins in HEK 293 cells. This was done because many of the immunosuppressive drug-related clinical complications are linked to impaired homeostasis of cell Ca^{2+}, and the Na^+-Ca^{2+} exchanger is a major Ca^{2+} regulator. The exchanger is the only surface membrane Ca^{2+} handling protein that transports Ca^{2+} in a bidirectional manner. Direction of Ca^{2+} flux changes in response to changes in membrane potential and respective Na^+ and Ca^{2+} gradients. Hence, changes in NCX expression can potentially modulate Ca^{2+} efflux, influx, and homeostasis (Blaustein and Lederer, 1999).

Na^+-Ca^{2+} exchange activity is encoded by three genes: *NCX1*, *NCX2*, and *NCX3*. They share considerable sequence homology (Quednau et al., 1997). Therefore, we expected that the effects of CsA, FK506, rapamycin, and PSC833, which we described previously on the expression of NCX1 (Kimchi-Sarfaty et al., 2002; Rahamimoff et al., 2002), would be similar. We were surprised to find that only the effect of CsA treatment of *NCX2*- and *NCX3*-transfected cells is similar to that observed with *NCX1*-transfected cells, leading to down-regulation of the expression of each one of the NCX proteins in the surface membrane in a dose-dependent manner, parallel reduction in the Na^+-Ca^{2+} exchange activity, and no significant change in immunoreactive total cell NCX protein. These findings can be explained by CsA binding to cyclophilin and inhibition of either the *cis-trans* isomerization of target X-Pro peptide bonds and/or chaperone activity. This would result in impaired post-translational NCX protein maturation, folding, and cell retention (Kopito, 1997). In addition, CsA treatment could indirectly impair trafficking between organelles, increase membrane retrieval, or modify any other post-translational process that could reduce surface expression of the protein and not change total NCX protein (Ellgaard and Helenius, 2003).

Our studies show three major differences, elucidated by the drug treatments, between NCX1-expressing cells and cells expressing NCX2 and NCX3 proteins: 1) their response to the immunosuppressive FK506 treatment, 2) their response to the immunosuppressive rapamycin treatment, and 3) their response to the nonimmunosuppressive PSC833 treatment. NCX1 expression was not modulated by FK506 or by rapamycin treatments of transfected HEK 293 cells (Kimchi-Sarfaty et al., 2002; Rahamimoff et al., 2002). However, when *FN-NCX2*- and *FN-NCX3*-transfected cells were treated with FK506, surface expression and Na^+-dependent Ca^{2+} uptake of both FN-NCX2 and FN-NCX3 decreased in a concentration-dependent manner without any change in total cell NCX protein. This finding suggests that FK506 treatment in a similar manner to CsA treatment of transfected cells could have impaired NCX2 and NCX3 protein folding and/or maturation, in a post-translational manner, presumably by inhibition of PPIase and/or chaperon activity of FKBP or any other unknown mechanism as suggested for the mode of action of CsA.

Because total cell NCX2 and NCX3 protein was not reduced by exposing the transfected HEK 293 cells to CsA and

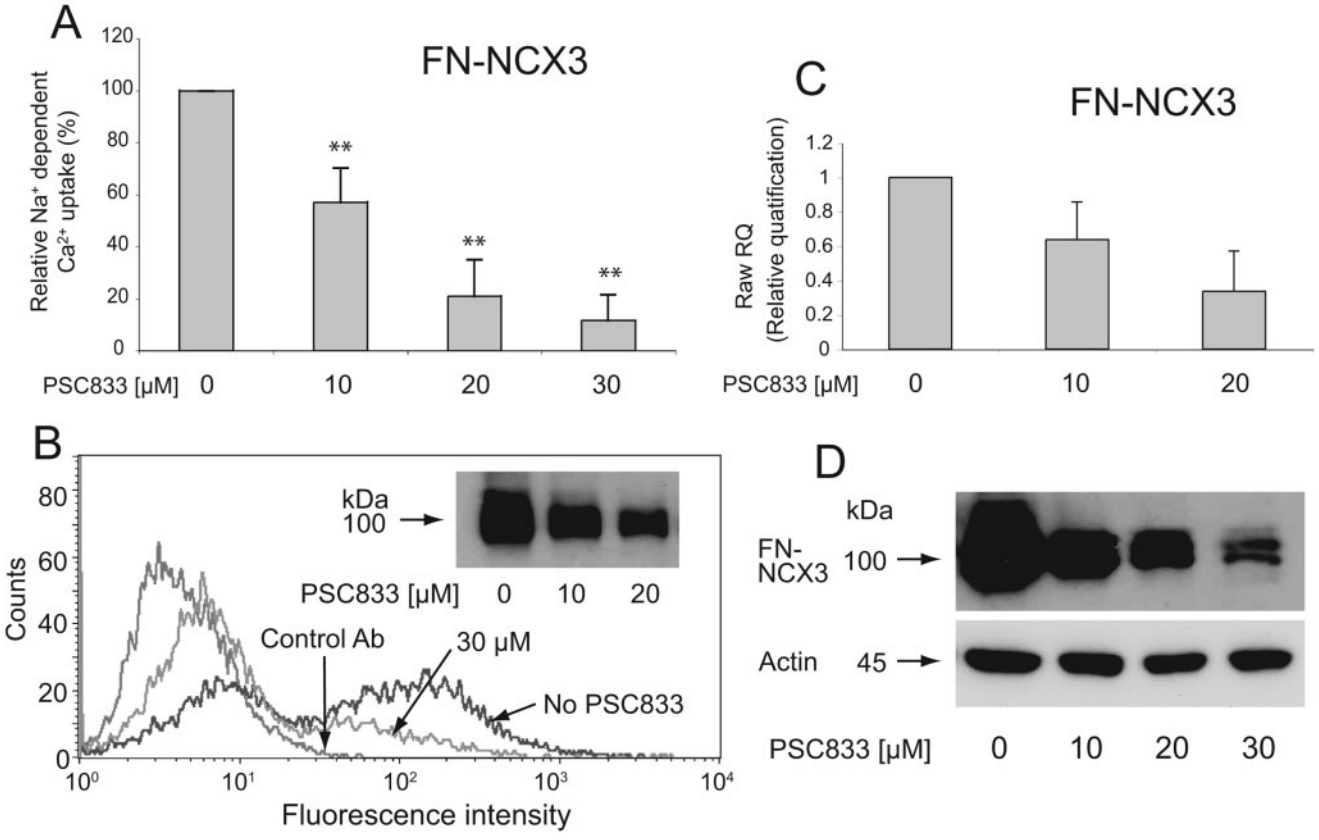

Fig. 8. The effect of different concentrations of PSC833 on the expression of FN-NCX3 in HEK 293 cells. Na^+-dependent Ca^{2+} uptake, surface expression, mRNA expression, and total FN-NCX3 protein expression were determined 24 h after transfection (as described under *Materials and Methods*) without and with exposure of the transfected cells to 0 to 30 μM PSC833. Experimental conditions and statistical analysis are identical to those described in detail in Fig. 7.

to FK506, presumably drug-dependent inhibition of transcription via the calcineurin pathway (or another unknown pathway) was not involved. Although both FK506 and rapamycin bind to immunophilins from the FKBP family, rapamycin treatment of *FN-NCX2*- and *FN-NCX3*-transfected HEK 293 cells did not result in any decrease in either surface or total immunoreactive protein expression, yet a decrease in Na^+-dependent Ca^{2+} uptake was consistently obtained. This suggests that rapamycin modulates FN-NCX2 and FN-NCX3 expression in a different manner than FK506. Because addition of rapamycin had no effect on the transport assay itself, it is possible that it impaired (in an unknown yet mode of action) the correct functional expression of the FN-NCX2 and FN-NCX3 protein, and the impaired protein bypassed the quality control in the endoplasmic reticulum (Kopito, 1997) and trafficked to the surface membrane. Wild-type-like surface expression of impaired protein has been described previously in several cases. For example, mutant G420H of the scavenger receptor class B type I (Parathath et al., 2007) exhibited reduced high-density lipoprotein cholesterol ester uptake but had wild-type-like surface expression and total receptor protein expression. A deletion mutant of P-glycoprotein (Loo and Clarke, 1999) that was cell retained did express in the surface membrane after transfected HEK 293 cells were treated with verapamil, vinblastine, capsaicin, or CsA. P112L mutant of NCX1 protein (Lichtenstein, 2004) exhibited no Na^+-dependent Ca^{2+} uptake activity when expressed in HEK 293 cells, yet it had wild-type-like surface expression. Haplotype C1236T-G2677T-C3435T of MDR1 exhibited altered function but wild-type-like total protein and cell surface expression (Kimchi-Sarfaty et al., 2007). Additional studies are needed to provide an explanation of how these altered proteins bypass cellular quality control and express in the surface membrane. In addition, it is interesting to speculate that rapamycin could have potentially impaired folding of FN-NCX2 and FN-NCX3 nascent chains in the ribosomal exit tunnel (Etchells and Hartl, 2004; Amit et al., 2005) to which they bind.

PSC833 is a nonimmunosuppressive analog of CsA, mostly studied as a potent MDR modulator (Smith et al., 1998). To provide an explanation for our results, its involvement in modulating protein expression directly or by binding to immunophilins without inhibition of calcineurin needs to be studied.

Taking these data together, we suggest that the different response of NCX2 and NCX3 proteins to immunosuppressive and nonimmunosuppressive drugs studied in this research, compared with the response of NCX1 protein that we described previously (Kimchi-Sarfaty et al., 2002), is due to the structural differences among the three NCX exchangers. In every other respect, the experiments were done using identical experimental conditions: Lipofectamine and Plus reagent or calcium phosphate-mediated transfection of HEK 293 cells and pcDNA 3.1, the mammalian expression vector encoding each one of the NCX genes. The cells express a repertoire of immunophilins that can bind CsA, FK506, and rapamycin (Rahamimoff et al., 2002). Therefore, the interaction of the immunophilins-drug complex with NCX and the modulation of its expression, which involves protein-protein interaction, are specific for the appropriate NCX protein and the appropriate drug. However, these still have to be elucidated.

The Na^+-Ca^{2+} exchanger genes and their multiple isoforms are expressed in a tissue-selective manner (Quednau et al., 1997; Annunziato et al., 2004). The drug concentrations that were used in our study are medically relevant. In this respect, it is interesting that clinical research studies comparing the action of FK506 and CsA suggest that clinical complications, patient survival, and graft survival in patients who have received kidney transplants are better when FK506 is used for immunosuppression (First, 2004) rather than CsA. It is possible that one of the contributing factors to this observation could be our finding that the surface expression of NCX1, the major and most abundant Na^+-Ca^{2+} exchanger, is not modulated by FK506 treatment.

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