MODULATION OF NCX (Na^+-Ca^{2+} EXCHANGER) EXPRESSION BY IMMUNOSUPPRESSIVE DRUGS IS ISOFORM SPECIFIC

Benayahu Elbaz, Ariella Alperovitch, Michael M. Gottesman

Chava Kimchi-Sarfaty and Hannah Rahamimoff

Department of Biochemistry Hebrew University – Hadassah Medical School, Jerusalem, Israel (B.E., A.A.,and H.R.), Laboratory of Cell Biology, NCI, NIH, Bethesda MD (B.E, M.M.G., and H.R.) and Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD (C.K.)

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Immunosuppressive drugs and NCX2 and NCX3 protein expression

Address correspondence to: Hannah Rahamimoff, Department of Biochemistry, Hebrew University-Hadassah Medical School, Jerusalem 91120, POB 12272, Israel

Phone: 972-2-6758511; FAX:972-2-6757379; E-mail: Hannah.Rahamimoff@huji.ac.il

Number of text pages – 32

Number of Tables – 1

Number of Figures - 8

Number of References - 49

Number of words:

Abstract - 252

Introduction - 727

Discussion - 1115

List of abbreviations: NCX- Na^{+}-Ca^{2+} exchanger; CsA- Cyclosporin A; NF-AT- Nuclear Factor of Activated T-cells; FKBP- FK506 Binding Protein; PPIase- Peptidyl Prolyl cis-trans Isomerase; TOR -Target of Rapamycin; BBB- Blood brain barrier; FN- flag epitope tagged; MFI- Mean Fluorescence Intensity.

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

The Na^+-Ca^{2+} exchanger is a major Ca^{2+} regulating protein encoded by three genes: *NCX1*, *NCX2* and *NCX3*. They share about 65% sequence homology. 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 HEK 293 cells with the immunosuppressive Cyclosporin A and its non-immunosuppressive analogue PSC833 results in down regulation of surface expression and transport activity of the protein without a decrease in expression of cell NCX1 protein. We show now that Cyclosporin A and PSC833 treatment of *NCX2* and *NCX3* transfected HEK 293 cells resulted also in dose-dependent down-regulation of surface expression and transport activity of the two brain NCX proteins; But 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. Since all 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 transplant patients resulted in complications related to Ca^{2+}. Expression of NCX genes is tissue specific. Hence, our results can potentially provide a tool for choice of the immunosuppressive protocol to be used.

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Cyclosporin A, FK506 (Tacrolimus) and Rapamycin (Sirolimus) are widely used to prevent organ rejection by transplant patients (First, 2004; Hariharan et al., 2000; Levy, 2000; Tsang et al., 2007). They bind to their respective immunophilin receptors the cyclophilins and 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 complex immunophilin-CsA/FK506 with the Ca^{2+} and Calmodulin dependent phosphatase Calcineurin (Liu et al., 1992) followed by inhibition of the dephosphorylation of NF-AT and its translocation to the nucleus, which leads to subsequent suppression of the immune reaction. Rapamycin also interacts with FKBPs but the complex FKBP-rapamycin does not inhibit calcineurin, but the TOR 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: PPIase activity, which is rate limiting in acquisition of configuration of X-proline peptide bonds and chaperone activity (Barik, 2006; Galat, 2003). 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 (Barik, 2006; Fischer et al., 1989; Galat, 2003; Mok et al., 2006; Pirkl et al., 2001) Binding of immunosupressive drugs to their immunophilins receptors, inhibits both PPIase and chaperon activity (Barik, 2006; Galat, 2003).

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

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

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 non-excitable 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 (Annunziato et al., 2004; Blaustein and Lederer, 1999; Quednau et al., 1997). 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 HEK 293 cells transfected with the *NCX1.1* and *NCX1.5* genes with CsA and its non-immunosupressive analogue 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, 2007). This suggests, that the phenomena was not restricted to heterologous expression systems. Not all membrane proteins however are modulated by treatment with CsA. For example, no reduction in functional expression of the human multidrug transporter p-

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

glycoprotein, transfected into HEK 293 cells, was obtained by treatment with CsA (Kimchi-Sarfaty et al., 2002).

Clinical studies suggested that immunosuppressive regimes of transplant patients resulted in complications related to Ca^{2+} such as nephrotoxicity, hypertension, bone loss and neurotoxicity (Bechstein, 2000; Cameron et al., 1995; Cardenas et al., 1995; Hariharan et al., 2000). CsA, FK506, Rapamycin and PSC833 were shown to cross the BBB (Hsiao et al., 2006; Kochi et al., 1999; Lemaire et al., 1996; Pong and Zaleska, 2003; Shirai et al., 1994; Tai, 2000). Based on all this, we have decided to asses the effects of CsA, FK506, Rapamycin and PSC833 on the expression of NCX2 and NCX3 proteins.

We show in this work, that except CsA, which modulated the expression of all three NCX proteins NCX1, NCX2 and NCX3 in a similar manner, all other immunosupressive and non immunosupressive 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.

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Cell line and cell culture. Human kidney embryonic HEK 293 cells (ATCC 1573) were used in all the transfection experiments and were grown in Dulbecco's Modified Eagle Medium (DMEM) (Biological Industries Kibbutz Beit Haemek, Israel or Gibco BRL Life Technologies) with 1% glutamine, 1% Penicillin-Streptomycin and 10% fetal bovine serum (Biological Industries Kibbutz Beit Haemek, Israel or Gibco BRL Life Technologies) at 37°C with humidity and 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). 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 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). All 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 protocol supplied by the manufacturer. One or two μg pDNA was used to transfect cells plated into 1- well of a 12 or 6 well- plate respectively. In some

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

transfection experiments CaPi (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 4-6 times. The efficiency of the transfection was calculated from statistical values obtained from FACS analyses (see bellow- **Immunostaining to detect total and surface expression of the Na^+-Ca^{2+} exchanger by FACS**), 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 (CsA) (Calbiochem Corp. La Jolla, CA), FK506 (LC Laboratories, Woburn MA USA), Rapamycin (Calbiochem Corp. La Jolla, CA or LC Laboratories, Woburn MA, USA), PSC833 (a gift from Novartis Pharma AG Basel Switzerland to HR) were dissolved in DMSO and added 3 hours post-transfection together with the FBS-DMEM 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 bellow) 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, 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 - 0.01 M Tris-HCl pH 7.4 using 25 μM Nystatin (Sigma, 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

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

same buffered Na^+ or K^+ containing solution, to which 25 μM $^{45}Ca^{2+}$ (Amersham, UK) was added. All solutions also contained 1 mM ouabain (Sigma, Israel). 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 $MgCl_2$) –see above. 3 μl of the Na^+ loaded cells (about 40 μg 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 via Schleicher and Shuell 0.45 μm filters. 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 4-6 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 PBS. For determination of the immunoreactive NCX protein, cells were permeabilized and fixed using an IntraPrep permeabilization and fixation kit following the manufacturer's procedure (Beckman Coulter, Immunotech, France). Following permeabilization, or directly after harvesting for surface expression studies, cells were incubated with 1 μg mouse M2 (anti-Flag) monoclonal antibody (Sigma) or 1 μg control mouse IgG1κ antibody (BD

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

PharMingen, CA), in a total volume of 100 µl PBS with 0.1% BSA (Sigma), for 30 min at 37°C. After washing, cells were incubated with 1 µg FITC- conjugated anti-mouse antibody IgG1κ (BD PharMingen, CA), or Alexa-green 488 anti-mouse secondary antibody (Invitrogen) for 30 min at 37°C. Preliminary experiments show that incubation of cells with 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. Following the second incubation, cells were washed with PBS 0.1% BSA and 10^5 cells were analyzed by FACSCalibur (BD Sciences, San Jose, CA). Statistical analysis was performed using CellQuest software to determine the median fluorescence values (arbitrary units).

Biotinylation and Western 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; Ren et al., 2001) 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% TritonX100, 1% SDS and 0.1 mM PMSF (Sigma), 0.01 mg/ml Pepstatin A (Sigma) and 0.02 mM Leupeptin (Sigma). 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 Rockford, IL), and was gently shaken over night at 4°. Washing of the beads was done as described in (Kasir et al., 1999; Ren

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

et al., 2001). Biotinylated proteins were released from the beads by heating for 10 minutes at 85° with Laemmli sample buffer and separated by SDS PAGE. Western analysis was carried out by standard procedures. For analysis of total cell extracts, 20 μg out of about 600 μg 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) was used. HRP-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc., PA) was used to detect antigen-antibody complexes using ECL kit (Biological Industries, Kibbutz Beit Haemek, Israel). In all 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 4-6 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- that were without or with PSC833 treatment, using Tri reagent (Sigma, Israel) according to the manufacturer's instructions. cDNA synthesis was carried out with RobusT II RT-PCR kit (Finnzymes, Finland) according to the manufacturer's instructions.

Quantitative real time PCR was done using the TaqMan® Gene Expression assay (Applied Biosystems, Foster City CA USA). The reaction was carried out in an Applied Biosystems ABI Prism 7000 Spectrofluorometric Thermal Cycler. Assay IDs of the genes were the following Rn00589573_m1 (for NCX2); Rn01517854_m1 (for NCX3) 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

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

isolated from three separate transfections. Each assay was done in triplicates (for each NCX and respective TATA box binding protein).

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Cyclosporin A treatment of HEK 293 cells transfected with *FN-NCX2* and *FN-NCX3* results 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 10-30 μM CsA in DMSO or an equal volume of DMSO and Na^+ dependent Ca^{2+} uptake was determined 24 hours post transfection, as described in Materials and Methods. Fig. 1A and 1B 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). Figs. 2A and 2B show the surface and total FN-NCX2 protein expression and Figs. 2C and 2D show the surface and total FN-NCX3 at 0, 10, 20 and 30 μM CsA. It can be seen that the fluorescence of

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

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 in 0 CsA (DMSO treated cells) was taken as 100% and all other values were normalized. For FN- NCX2 expressing cell: 0-100; 10 μM- 74.6 (S.D. 14.1); 20 μM – 67.1 (S.D. 14.2); 30 μM- 25.8 (S.D. 8.9). For FN-NCX3 expressing cells: 0-100; 10 μM- 64.4 (S.D. 7.6); 20 μM – 46.3 (S.D. 15.9); 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 these were exposed to the same concentration of CsA.

FN-NCX2 and FN-NCX3 protein surface expression and transport activity is modulated by FK506. The macrolide compound FK506 (tacrolimus) 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 0-30 μM FK506, resulted in reduced Na^+-Ca^{2+} exchange activity (Figs. 3A and 3B, respectively) and surface expression (Figs. 4A and 4C) without a decrease in total cell NCX protein (Figs. 4B and 4D). The relative MFIs for FK506 treated surface expressed cells (calculated as that for CsA treated cells) were: For FN- NCX2 expressing cell: 0-100; 20 μM – 75.7 (S.D. 3.9); 30 μM - 69.06 (S.D. 6.8); 50 μM –(N.D.). For FN-NCX3 expressing cells: 0-100; 20 μM – 60.5 (S.D. 2.09); 30 μM- 55.9 (S.D. 4.06); 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^+-

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Ca^{2+} exchange activity was consistently detected. We could not calculate the MFI of the surface expressed FN-NCX2 exposed to 50 μM FK506, since the percentage of dead cells, which normally is 5% or bellow, was between 13-23%.

Rapamycin reduces Na^+ dependent Ca^{2+} uptake activity but has 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. 5A and Fig. 5B). Rapamycin treatment however had no effect on surface or total FN-NCX2 and FN-NCX3 protein expression (Figs. 6A-6D).

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 (see Table 1) .

The non-immunosuppressive PCS833 reduces surface expression, Na^+-Ca^{2+} exchange activity, mRNA and total immunoreactive cell FN-NCX2 and FN-NCX3 protein. PSC833 is a non immunosuppressive analogue of CsA. Hence, it was interesting to examine its effect on the expression of FN-NCX2 and FN-NCX3 in transfected HEK 293 cells. Fig. 7 and Fig. 8 summarize these data: In Fig. 7A (*FN-NCX2*-transfected cells) and 8A (*FN-NCX3*- transfected 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

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

proteins to PSC833 is higher than that to CsA: 20 μM PSC833 reduces about 20% of the relative transport activity and treatment of the cells with 30 μM PSC833 reduces the expression of the relative transport activity to about 10% as compared to untreated (DMSO only) transfected HEK 293 cells.

In a similar manner 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 to Fig. 7B) for *FN-NCX2*- transfected cells and the same 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 (inserts to Fig. 7B and 8B). This presumably represents two slightly different conformational forms derived both from the same cloned Na^{+}-Ca^{2+} exchanger, since 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 (Fig, 7C and Fig. 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.

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

In this study we have examined the effect of the commonly used immunosuppressive drugs CsA, FK506 and Rapamycin and the non-immunosuppressive PSC833, on the expression of NCX2 and NCX3 proteins in HEK 293 cells. This was done since many of the immunosupressive-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). We expected therefore that the effects of CsA, FK506, Rapamycin and PSC833, that we described previously on the expression of NCX1 (Kimchi-Sarfaty et al., 2002; Rahamimoff et al., 2002), would be similar. Surprisingly, we found 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 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,

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

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: Their response to the immunosuppressive FK506 treatment, their response to the immunosupressive Rapamycin treatment and their response to the non-immunosuppressive 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). When however *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.

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

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

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 does. Since 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 FN-NCX2 and FN-NCX3 protein and the impaired protein by-passed 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 HDL 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) which 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, Y., M.Sc. thesis) exhibited no Na^+ dependent Ca^{2+} uptake activity when expressed in HEK 293 cells, yet it had wilt 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). Further studies have to be carried out to provide an explanation how these altered proteins by-pass cellular quality control and express in the surface membrane. It is interesting also to speculate, that Rapamycin could have potentially impaired folding of FN-NCX2 and FN-NCX3 nascent chains in the ribosomal exit tunnel (Amit et al., 2005; Etchells and Hartl, 2004) to which they bind.

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

PSC833 is a non immunosuppressive analogue 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.

Taken together, we would like to suggest, that the different response of NCX2 and NCX3 proteins to immunosupressive and non immunosupressive drugs studied in this research, than 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 CaPi-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 both 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. These however have still to be elucidated.

The Na^+-Ca^{2+} exchanger genes and their multiple isoforms are expressed in a tissue selective manner (Annunziato et al., 2004; Quednau et al., 1997). 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 (Tacrolimus) and CsA suggest that clinical complications, patient and graft survival of kidney transplant patients are better when FK506 is used for immunosupression (First, 2004) rather then CsA. It is possible that one of the contributing

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

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.

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgments: We would like to thank Mr. George Leiman, NCI, NIH, for helping with preparation of the manuscript.

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

REFERENCES

Amit M, Berisio R, Baram D, Harms J, Bashan A and Yonath A (2005) A crevice adjoining the ribosome tunnel: hints for cotranslational folding. *FEBS Lett.* **579**:3207-3213. .

Annunziato L, Pignataro G and Di Renzo GF (2004) Pharmacology of brain Na+/Ca2+ exchanger: from molecular biology to therapeutic perspectives. *Pharmacol Rev.* **56**:633-654.

Barik S (2006) Immunophilins: for the love of proteins. *Cell Mol Life Sci* **31**:1-12.

Bechstein WO (2000) Neurotoxicity of calcineurin inhibitors: impact and clinical management. *Transpl Int* **13**:313-326.

Blaustein MP and Lederer WJ (1999) Sodium/calcium exchange: its physiological implications. *Physiol Rev* **79**:763-854.

Boesch D, Gaveriaux C, Jachez B, Pourtier-Manzanedo A, Bollinger P and Loor F (1991) In vivo circumvention of P-glycoprotein-mediated multidrug resistance of tumor cells with SDZ PSC 833. *Cancer Res* **51**:4226-4233.

Cameron AM, Steiner JP, Sabatini DM, Kaplin AI, Walensky LD and Snyder SH (1995) Immunophilin FK506 binding protein associated with inositol 1,4,5-trisphosphate receptor modulates calcium flux. *Proc Natl Acad Sci U S A* **92**:1784-1788.

Cardenas ME, Zhu D and Heitman J (1995) Molecular mechanisms of immunosuppression by cyclosporine, FK506, and rapamycin. *Curr Opin Nephrol Hypertens* **4**:472-477.

Chen H, Kubo Y, Hoshi T and Heinemann SH (1998) Cyclosporin A selectively reduces the functional expression of Kir2.1 potassium channels in Xenopus oocytes. *FEBS Lett* **422**:307-310.

Ellgaard L and Helenius A (2003) Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol.* **4**:181-191.

Etchells SA and Hartl FU (2004) The dynamic tunnel. *Nat Struct Mol Biol.* **11**:391-392.

First MR (2004) Tacrolimus based immunosuppression. *J Nephrol.* **17 Suppl 8**:S25-S31.

Fischer G, Wittmann - Liebold B, Lang K, Kiefhaber T and Schmid FX (1989) Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins [see comments]. *Nature* **337**:476-478.

Galat A (2003) Peptidylprolyl cis/trans isomerases (immunophilins): biological diversity--targets--functions. *Curr Top Med Chem* **3**:1315-1347.

Hariharan S, Johnson CP, Bresnahan BA, Taranto SE, McIntosh MJ and Stablein D (2000) Improved graft survival after renal transplantation in the United States, 1988 to 1996. *N Engl J Med* **342**:605-612.

Helekar SA, Char D, Neff S and Patrick J (1994) Prolyl isomerase requirement for the expression of functional homo-oligomeric ligand-gated ion channels. *Neuron* **12**:179-189.

Helekar SA and Patrick J (1997) Peptidyl prolyl cis-trans isomerase activity of cyclophilin A in functional homo-oligomeric receptor expression. *Proc Natl Acad Sci U S A* **94**:5432-5437.

Ho SN, Hunt HD, Horton RM, Pullen JK and Pease LR (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene.* **77**:51-59.

Hsiao P, Sasongko L, Link JM, Mankoff DA, Muzi M, Collier AC and Unadkat JD (2006) Verapamil P-glycoprotein transport across the rat blood-brain barrier: cyclosporine, a concentration inhibition analysis, and comparison with human data. *J Pharmacol Exp Ther.* **317**:704-710. .

Kasir J, Ren X, Furman I and Rahamimoff H (1999) Truncation of the C-terminal of the rat brain Na+-Ca2+ exchanger RBE-1 (NCX1.4) impairs surface expression of the protein. *J. Biol. Chem.* **274**:24873-24880.

Kimchi-Sarfaty C, Kasir J, Ambudkar S and Rahamimoff H (2002) Transport activity and surface expression of the Na+-Ca2+ exchanger NCX1 is inhibited by the immunosuppressive agen cyclosporin A and the non-immunosupressive agent PSC833. *J. Biol. Chem.* **277**:2505-2510.

Kimchi-Sarfaty C, Oh JM, Kim IW, Sauna ZE, Calcagno AM, Ambudkar SV and Gottesman MM (2007) A "silent" polymorphism in the MDR1 gene changes substrate specificity. *Science.* **315**:525-528. .

Kochi S, Takanaga H, Matsuo H, Naito M, Tsuruo T and Sawada Y (1999) Effect of cyclosporin A or tacrolimus on the function of blood-brain barrier cells. *Eur J Pharmacol.* **372**:287-295.

Kopito RR (1997) ER quality control: the cytoplasmic connection. *Cell* **88**:427-430.

Lemaire M, Bruelisauer A, Guntz P and Sato H (1996) Dose-dependent brain penetration of SDZ PSC 833, a novel multidrug resistance-reversing cyclosporin, in rats. *Cancer Chemother Pharmacol* **38**:481-486.

Levy GA (2000) Neoral use in the liver transplant recipient. *Transplant Proc* **32**:2S-9S.

Li Z, Matsuoka S, Hryshko LV, Nicoll DA, Bersohn MM, Burke EP, Lifton RP and Philipson KD (1994) Cloning of the NCX2 isoform of the plasma membrane Na(+)-Ca2+ exchanger. *J-Biol-Chem* **269**:17434-17439.

Liu J, Albers MW, Wandless TJ, Luan S, Alberg DG, Belshaw PJ, Cohen P, MacKintosh C, Klee CB and Schreiber SL (1992) Inhibition of T cell signaling by immunophilin-ligand complexes correlates with loss of calcineurin phosphatase activity. *Biochemistry.* **31**:3896-3901.

Loo TW and Clarke DM (1999) The transmembrane domains of the human multidrug resistance P-glycoprotein are sufficient to mediate drug binding and trafficking to the cell surface. *J Biol Chem.* **274**:24759-24765.

Mok D, Allan RK, Carrello A, Wangoo K, Walkinshaw MD and Ratajczak T (2006) The chaperone function of cyclophilin 40 maps to a cleft between the prolyl isomerase and tetratricopeptide repeat domains. *FEBS Lett.* **580**:2761-2768. .

Nicoll DA, Quednau BD, Qui Z, Xia Y-R, Lusis AJ and Philipson KD (1996) Cloning of a third mammalian Na^+ -Ca^{2+} exchanger, NCX3. *J.Biol.Chem.* **271**:24914-24921.

Parathath S, Darlington YF, de la Llera Moya M, Drazul-Schrader D, Williams DL, Phillips MC, Rothblat GH and Connelly MA (2007) Effects of amino acid substitutions at glycine 420 on SR-BI cholesterol transport function. *J Lipid Res.* **48**:1386-1395. .

Philipson KD and Nicoll DA (2000) Sodium-calcium exchange: a molecular perspective. *Annu Rev Physiol* **62**:111-133.

Pirkl F, Fischer E, Modrow S and Buchner J (2001) Localization of the chaperone domain of FKBP52. *J Biol Chem.* **276**:37034-37041. .

Pong K and Zaleska MM (2003) Therapeutic implications for immunophilin ligands in the treatment of neurodegenerative diseases. *Curr Drug Targets CNS Neurol Disord.* **2**:349-356.

Proud CG (2007) Signalling to translation: how signal transduction pathways control the protein synthetic machinery. *Biochem J.* **403**:217-234.

Quednau BD, Nicoll DA and Philipson KD (1997) Tissue specificity and alternative splicing of the Na+/Ca2+ exchanger isoforms NCX1, NCX2, and NCX3 in rat. *Am-J-Physiol* **272**:C1250-1261.

Rahamimoff H, Elbaz, B., Alperovich, A., Kimchi-Sarfaty, C., Gottesman, M.M., Lichtenstein Y., Eskin-Shwartz,. M., and Kasir J. (2007) Cyclosporin A dependent down regulation of the Na+-Ca2+ Exchanger Expression *Ann N Y Acad Sci.* **1099**:204-214.

Rahamimoff H, Ren X, Kimchi-Sarfaty C, Ambudkar S and Kasir J (2002) NCX1 surface expression: a tool to identify structural elements of functional importance. *Ann N Y Acad Sci* **976**:176-186.

Ren X, Kasir J and Rahamimoff H (2001) The transport activity of the Na+-Ca2+ exchanger NCX1 expressed in HEK 293 cells is sensitive to covalent modification of intracellular cysteine residues by sulfhydryl reagents. *J Biol Chem* **276**:9572-9579.

Sambrock J, Fritsch E and Maniatis T (1989) *Molecular Cloning: A laboratory manual.* Cold Spring Harbor Press.

Shirai A, Naito M, Tatsuta T, Dong J, Hanaoka K, Mikami K, Oh-hara T and Tsuruo T (1994) Transport of cyclosporin A across the brain capillary endothelial cell monolayer by P-glycoprotein. *Biochim Biophys Acta.* **1222**:400-404.

Shiraishi S, Yamamoto R, Yanagita T, Yokoo H, Kobayashi H, Uezono Y and Wada A (2001) Down-regulation of cell surface insulin receptors by sarco(endo)plasmic reticulum Ca2+-ATPase inhibitor in adrenal chromaffin cells. *Brain Res* **898**:152-157.

Smith AJ, Mayer U, Schinkel AH and Borst P (1998) Availability of PSC833, a substrate and inhibitor of P-glycoproteins, in various concentrations of serum. *J Natl Cancer Inst* **90**:1161-1166.

Stephan MM, Chen MA, Penado KM and Rudnick G (1997) An extracellular loop region of the serotonin transporter may be involved in the translocation mechanism. *Biochemistry* **36**:1322-1328.

Tai HL (2000) Technology evaluation: Valspodar, Novartis AG. *Curr Opin Mol Ther.* **2**:459-467.

Tran TT, Dai W and Sarkar HK (2000) Cyclosporin A inhibits creatine uptake by altering surface expression of the creatine transporter. *J Biol Chem* **275**:35708-35714.

Tsang CK, Qi H, Liu LF and Zheng XF (2007) Targeting mammalian target of rapamycin (mTOR) for health and diseases. *Drug Discov Today.* **12**:112-124. .

Zhou Y, Gottesman MM and Pastan I (1999) Domain exchangeability between the multidrug transporter (MDR1) and phosphatidylcholine flippase (MDR2). *Mol Pharmacol* **56**:997-1004.

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

FOOTNOTES

a. This study was supported in part by research grants from The Israel Science Foundation and by The Israel Ministry of Health to HR and by the Intramural Research Program of the National Institutes of Health, National Cancer Institute (M.M.G.).

Lichtenstein, Y., M.Sc. thesis, 2004, The Hebrew University, Jerusalem

b. Hannah Rahamimoff, Department of Biochemistry, Hebrew University-Hadassah Medical School, Jerusalem 91120, POB 12272, Israel

Phone: 972-2-6758511; FAX:972-2-6757379;

E-mail: Hannah.Rahamimoff@huji.ac.il

c. [1]Department of Biochemistry Hebrew University – Hadassah Medical School, Jerusalem, Israel, [2]Laboratory of Cell Biology, NCI, NIH, Bethesda MD and [3]Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Legends For Figures

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 hours post-transfection as described in Materials and Methods without and with exposure of the expressing cells to 10, 20 and 30 μM of 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.(standard deviations). ($*P<0.05$; $**P<0.01$).

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 post-transfection the cells were analyzed for cell surface and total expression using M2 the anti-Flag antibody and Alexa-green 488 anti-mouse secondary antibody.

A: Non-permeabilized *FN-NCX2*-transfected cells treated with 0-30 μM CsA. B: Permeabilized *FN-NCX2*-transfected cells treated with 0-30 μM CsA. C: Non-permeabilized *FN-NCX3*-transfected cells treated with 0-30 μM CsA. D: Permeabilized *FN-NCX3*-transfected cells treated with 0-30 μM CsA. The key to the lines used in all panels is identical (see panel A).

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

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 hours post-transfection as described in Materials and Methods without and with exposure of the expressing cells to 10, 20 and 30 μM of FK506. Transport activities 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.(standard deviations). ($*P<0.05$; $**P<0.01$).

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 post-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): Non-permeabilized *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 of FK506. The key to the lines used in all panels is identical to that in panel (A).

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

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

with plasmids encoding *FN-NCX2* (A) and *FN-NCX3* (B). Na^+-dependent Ca^{2+} uptake was determined 24 hours post-transfection as described in Materials and Methods without and with exposure of the expressing cells to 10 and 20 μM of Rapamycin. Transport activities 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.(standard deviations). ($*P<0.05$; $**P<0.01$).

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 post-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): Non-permeabilized *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 panel (A).

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 hours post-transfection as described in Materials and Methods without and with exposure of the transfected cells to 0-30 μM PSC833. A. Transport activity of *FN-NCX2*- transfected cells with DMSO treatment was taken as 100%, and

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

the transport activities measured in the drug treated cells, were calculated in relative values. The bars represent S.D.(standard deviations). (*P<0.05; **P<0.01).

B. Surface expression was determined by FACS analysis and by Surface Biotinylation (insert to B). M2 the anti-Flag antibody and FITC anti-mouse secondary antibody was used for detection of FN-NCX2 by FACS analysis and HRP-conjugated anti-mouse secondary antibody for detection of surface biotinylated FN-NCX2. C. *FN-NCX2* mRNA levels were measured by quantitative PCR as described in Materials and Methods. All results were normalized to untreated samples and TATA box binding protein values. Standard deviations represent results from three independent experiments. D. Western analysis was used to determine total immunoreactive FN-NCX2 protein using M2 the anti-Flag antibody and HRP conjugated anti-mouse secondary antibody. 20 μg of transfected cell extract was loaded on each lane. Immunodetection of Actin was used as an equal loading control.

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 hours post-transfection as described in Materials and Methods without and with exposure of the transfected cells to 0-30 μM PSC833.

Experimental conditions and statistical analysis are identical to those described in detail in Fig. 7.

Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1

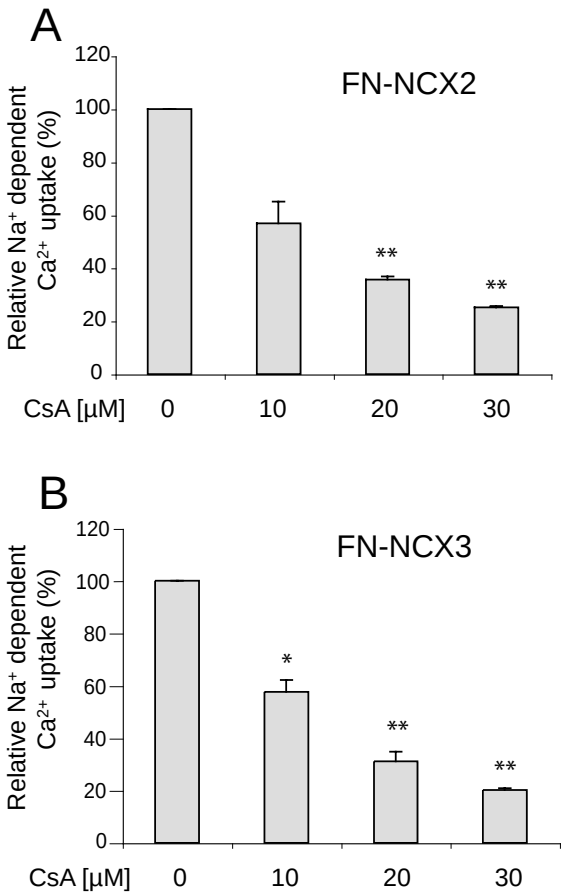
The effect on immunosupressive and non-immunosupressive drugs on Na^+-dependent Ca^{2+} uptake

HEK 293 cells were transfected with cloned *FN-NCX2* or cloned *FN-NCX3*. 24 hours post transfection, CsA, FK506, Rapamycin and PSC833 (all in DMSO) or 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 (see Methods).

Relative Na^+ dependent Ca^{2+} uptake (%)					
Drug	μM		S.D		S.D
DMSO	control	100		100	
CsA	10	96.66307	9.99	102.5609	11.3
	20	95.842593	4.98	95.0043	6.159
FK506	10	102.63047	6.23	94.7981	3.038
	20	100.87448	6.32	109.0749	4.518
Rapamycin	10	109.89653	7.85	93.66682	3.246
	20	96.007516	4.57	107.6892	10.45
PSC833	10	92.36227	8.65	98.87516	1.386
		FN-NCX2		FN-NCX3	

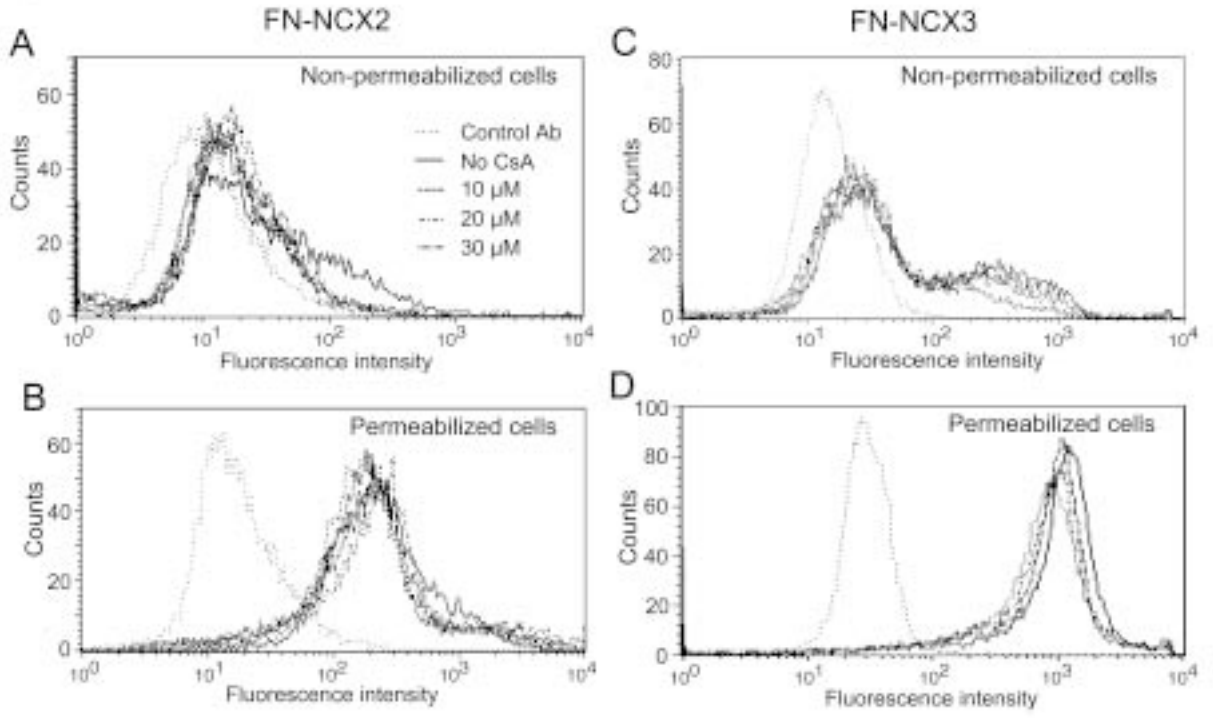
Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1



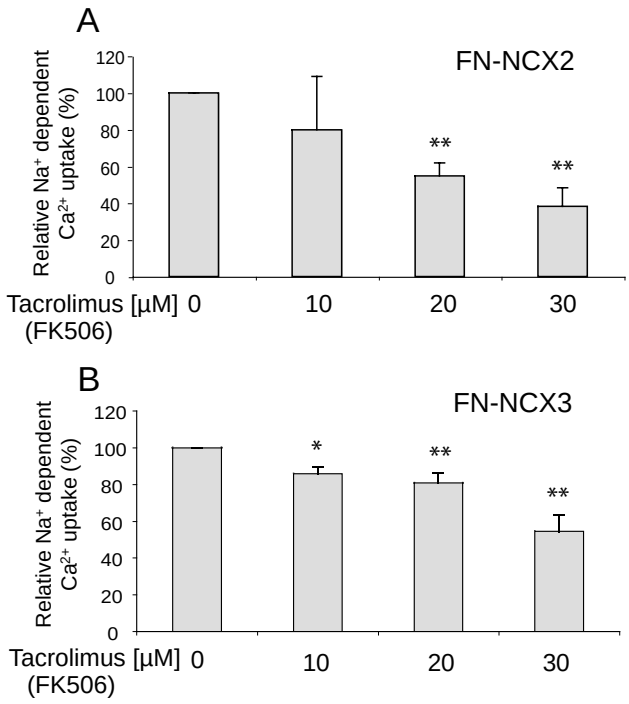
Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2



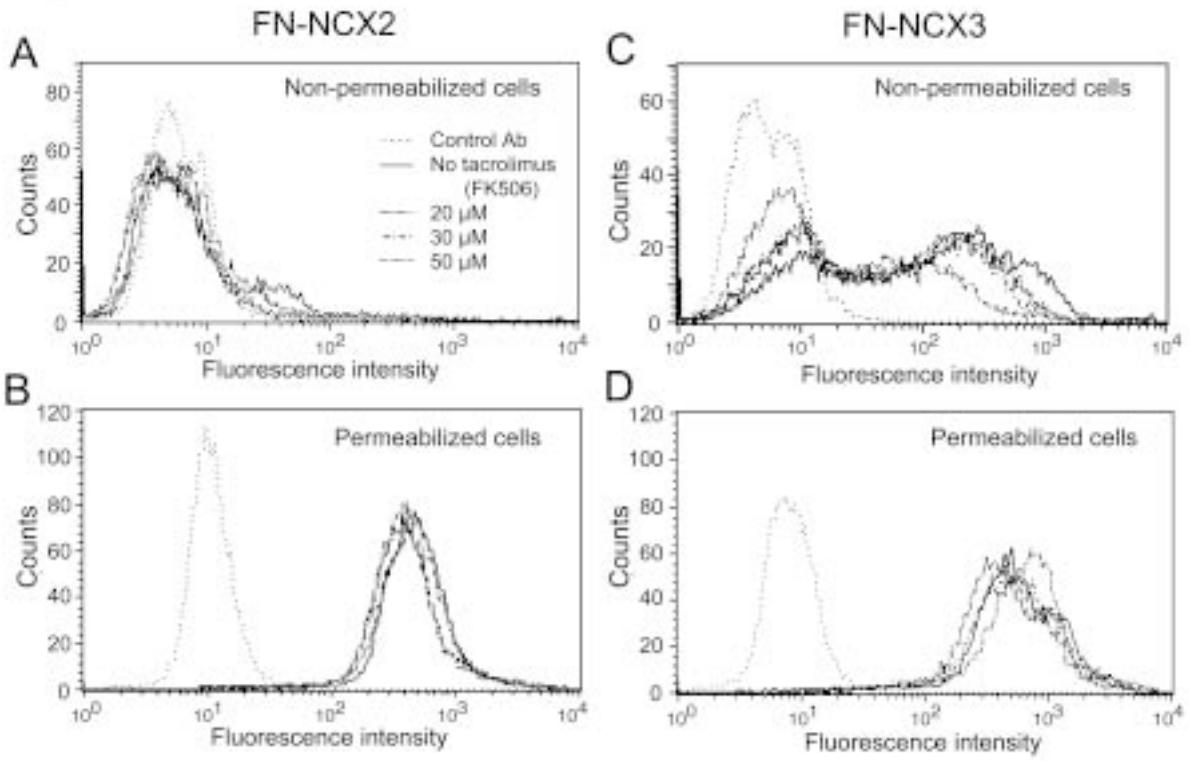
Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3



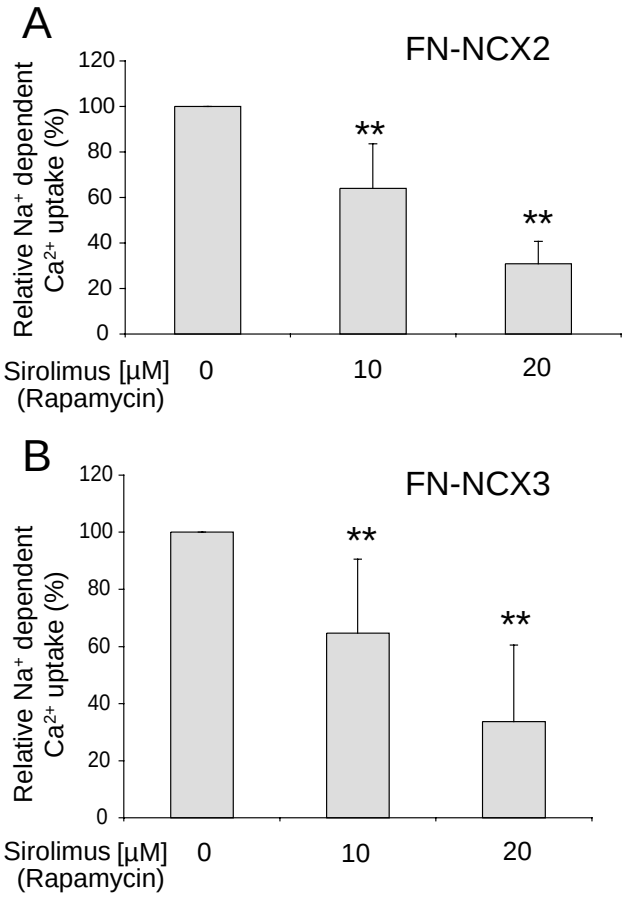
Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4



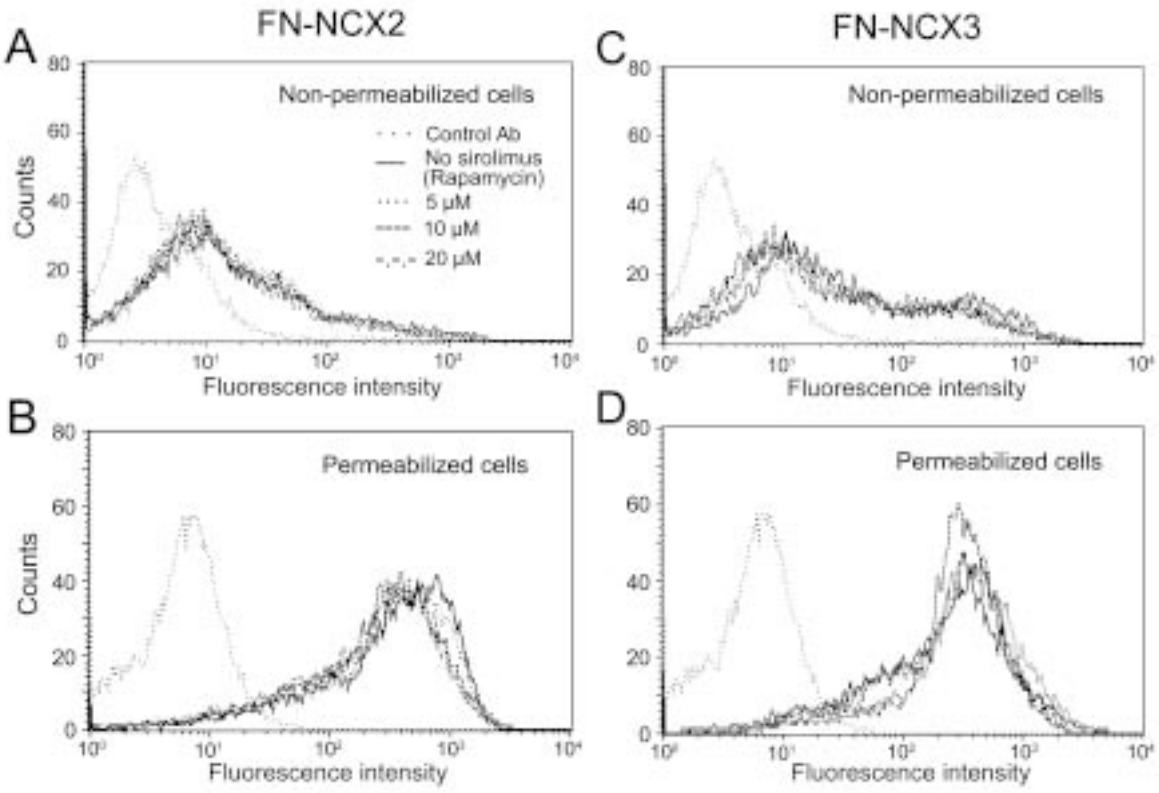
Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5



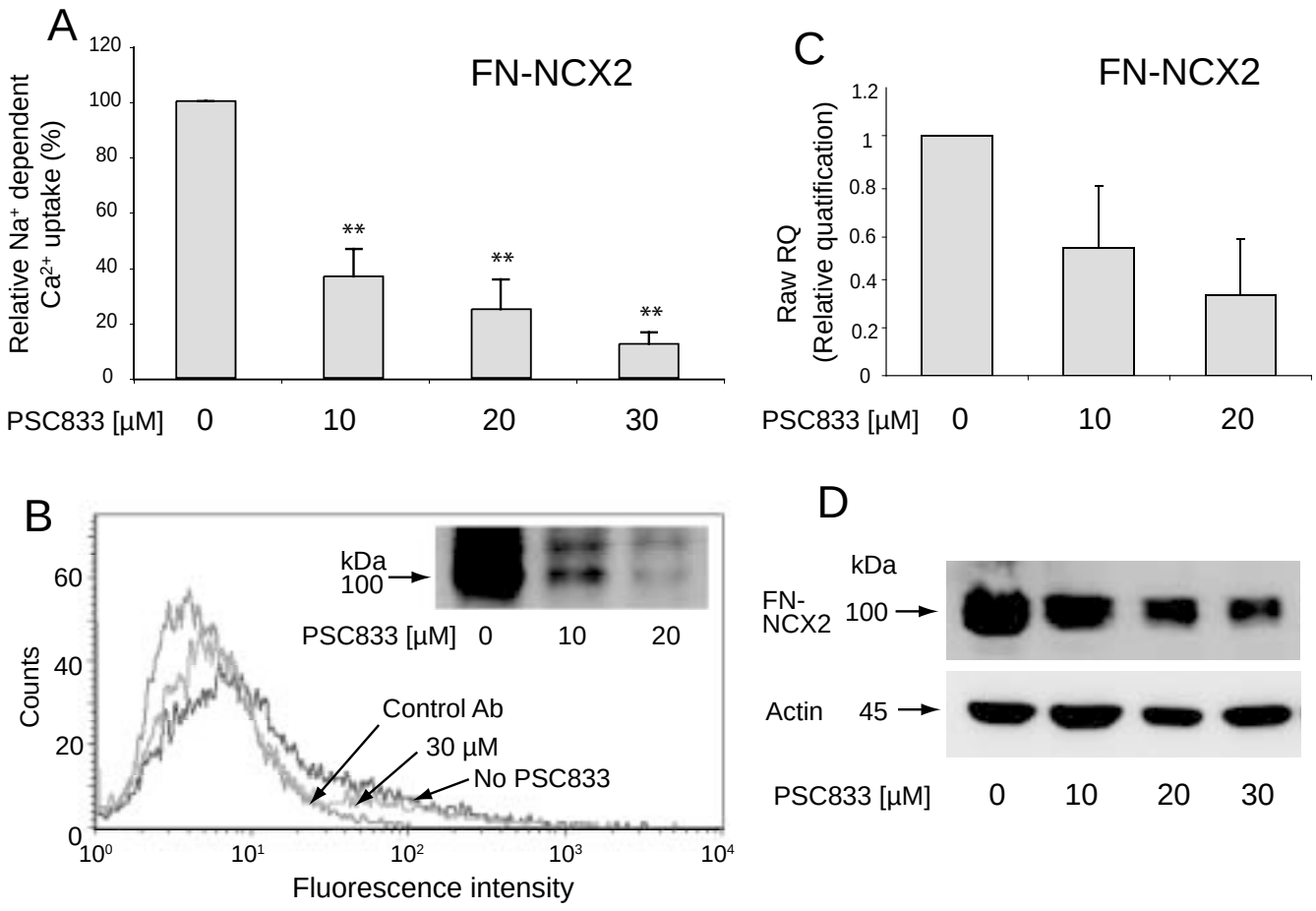
Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 6



Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 7



Molecular Pharmacology Fast Forward. Published on January 8, 2008 as DOI: 10.1124/mol.107.041582
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 8