THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 27, Issue of July 7, pp. 20903–20910, 2000

Printed in U.S.A.

Calcineurin Controls the Transcription of Na^+/Ca^{2+} Exchanger Isoforms in Developing Cerebellar Neurons*

Received for publication, February 7, 2000, and in revised form, April 11, 2000
Published, JBC Papers in Press, April 14, 2000, DOI 10.1074/jbc.M000995200

Lei Li‡, Danilo Guerini‡§, and Ernesto Carafoli‡¶‖

From the ‡Institute of Biochemistry, Swiss Federal Institute of Technology, 8092 Zürich, Switzerland, the §Laboratory of Metabolic and Cardiovascular Diseases, Novartis AG, 4002 Basle, Switzerland, and the ¶Department of Biochemistry, University of Padova, 35121 Padova, Italy

The Na^+/Ca^{2+} exchanger (NCX) and the plasma membrane Ca^{2+}-ATPase export Ca^{2+} from the cytosol to the extracellular space. Three NCX genes (*NCX1*, *NCX2*, and *NCX3*), encoding proteins with very similar properties, are expressed at different levels in tissues. Essentially, no information is available on the mechanisms that regulate their expression. Specific antibodies have been prepared and used to explore the expression of NCX1 and NCX2 in rat cerebellum. The expression of NCX2 became strongly up-regulated during development, whereas comparatively minor effects were seen for NCX1. This was also observed in cultured granule cells induced to mature in physiological concentrations of potassium. By contrast, higher K^+ concentrations, which induce partial depolarization of the plasma membrane and promote the influx of Ca^{2+}, caused the complete disappearance of NCX2. Reverse transcription-polymerase chain reaction analysis showed that the process occurred at the transcriptional level and depended on the activation of the Ca^{2+} calmodulin-dependent protein phosphatase, calcineurin. The *NCX1* and *NCX3* genes were also affected by the depolarizing treatment: the transcription of the latter became up-regulated, and the pattern of expression of the splice variants of the former changed. The effects on the *NCX1* and *NCX3* genes were calcineurin-independent.

Hormonal and electrical stimuli promote the penetration of Ca^{2+} into cells to activate cellular responses. Ca^{2+} must then be continuously extruded, because its uncontrolled increase in the cytosol would lead to cell death. Two systems, a pump (1) and a Na^+/Ca^{2+} exchanger (2), eject Ca^{2+}. The latter system, which is particularly active in heart and neurons, uses the Na^+ gradient generated by the Na^+/K^+-ATPase to remove Ca^{2+} from the cytosol; under normal conditions 3 Na^+ ions are exchanged for 1 Ca^{2+}.

The cDNA of exchanger type 1 (NCX1)[1] has been cloned from mammalian (3–8), amphibian (9), and invertebrate (10, 11) tissues. A comparison of the sequences shows a high level of conservation. The mature NCX1 is a glycosylated protein (12) of 970 amino acids, the first 32 of which are post-translationally cleaved off (13–15). The original membrane topography model based on hydropathy analysis predicted 11 transmembrane domains, separated by small loops and by a large intracellular loop (>500 amino acids) between transmembrane domains 5 and 6. A more recent model based on cysteine accessibility studies has revised the number of predicted transmembrane domains down to 9 (16), eliminating 2 from the C-terminal half of the exchanger. Although the large intracellular loop is not strictly necessary for the activity of the exchanger, it contains important regulatory elements (17–19). Its C-terminal portion is subjected to alternative splicing (20), which also occurs at the 5′-untranslated region of the gene (21–23). Numerous splicing variants have been described for NCX1. The amount of the major variant present in neurons (the AD isoform) is altered by protein kinase A (24). Although α-adrenergic stimulation led to the increase of NCX1 mRNA in cultured cardiac myocytes (25), glucocorticoids and protein kinase A down-regulated it in vascular smooth muscle cells. Protein kinase C had the same effect in endothelial cells (26, 27). Changes in the expression of the *NCX1* gene have also been observed during cardiac development (28) and pressure overload (29).

Two additional exchanger genes encoding proteins with high homology to NCX1 have also been cloned: *NCX2* (30) and *NCX3* (31). Whereas *NCX1* is expressed at high levels in heart, and is thus normally referred to as the "cardiac form" of the protein even if also present in other tissues, significant amounts of *NCX2* and *NCX3* mRNAs have only been detected by Northern blots in brain and skeletal muscles. However, minor amounts were detected also in other tissues using more sensitive RT-PCR methods. Some splice variants have been detected for *NCX3* but none so far for *NCX2*.

Although the exchanger proteins have not been satisfactorily purified, comparisons of the biochemical properties of the NCX1, NCX2, and NCX3 exchangers have been made on membrane preparations and on overexpressing cells. Because no significant differences were detected (32), the rationale for the existence of three separate *NCX* genes is obscure. Brain cells, in particular neurons, contain large amounts of all three basic NCX isoforms and of their splice variants and are thus good models for study. In this research, their expression was investigated during the development of rat cerebellum and of cultured cerebellar granule neurons. The work has shown that Ca^{2+} and calcineurin are critical to the expression of the exchanger genes, supporting the idea that one of the major differences among the *NCX* genes is the regulation of their transcription.

* This work was made possible by the support of the Swiss National Science foundation, the Italian Ministry of University and Scientific Research (MURST-PRIN 1998), the National Research Council of Italy (Target Project on Biotechnology), and the Armenise-Harvard Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed. Tel.: 0039-049-8276137; Fax: 0039-049-8276125; E-mail: carafoli@civ.bio.unipd.it.

[1] The abbreviations used are: NCX1, NCX2, NCX3, exchanger types 1, 2, 3, cDNA; RT, reverse transcriptase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; CaMKK, calmodulin-dependent kinase-kinase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PMCA, plasma membrane Ca^{2+} pump.

EXPERIMENTAL PROCEDURES

Materials

The pTM3 vector and the vvT7 virus were gifts from Dr. B. Moss (National Institutes of Health, Bethesda, MD). Cyclosporin A and FK506 were a kind gift of Dr. Mauro Zurini (Novartis, Basle, Switzerland). Dulbecco's modified Eagle medium (DMEM or DME/F12) and other tissue culture supplements were from Sigma or Life Technologies. Poly-L-lysine and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were from Sigma. $^{45}Ca^{2+}$, [α-^{32}P]dCTP, and [^{14}C]ATP were from Amersham Pharmacia Biotech. Nitrocellulose filters for Western blotting and Nytran for Northern blotting were from Schleicher & Schuell. Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate and goat anti-rabbit alkaline phosphatase conjugate were from Promega (Madison, WI). Chemiluminescence substrates CDP-star™ and NitroblockII were from Tropix (Madison, WI). Oligonucleotides were purchased from MGW-Biotech (Ebersberg, Germany). Ampli-*Taq* Gold polymerase was from Perkin-Elmer.

Methods

Cell Cultures—HeLa cells were cultured in Dulbecco's modified Eagle medium supplemented with 5–10% fetal calf serum and 50 μg/ml gentamicin in 5.5% CO_2 at 37 °C. Transient expression of NCX1 was achieved by infecting cells with t7 polymerase containing vaccinia virus at a multiplicity of infection of 20 followed by transfection of the plasmid DNA (33).

Granule cells were dissociated from the cerebella of 7-day-old Wistar rats as described (34). They were plated in Dulbecco's modified Eagle medium (Hepes modification, Sigma) supplemented with heat-inactivated 10% fetal calf serum (Sigma), 100 μg/ml gentamicin, 7 μM *p*-aminobenzoic acid, 100 μg/ml pyruvate, and 100 microunits/ml insulin on poly-L-lysine-treated plates at a density of 2–3 $\times$ 10^5 cells/cm^2 in the presence of 5.3 or 25 mM KCl. After 24 h, 10 μM cytosine arabinofuranoside was added to inhibit mitotic cell growth. Neuronal survival was estimated by measuring the amount of colored formazan in the cells by the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (35). The extent of contaminating astrocytes was estimated by immunocytochemistry using a monoclonal antibody specific for the glial fibrillary acidic protein (GFAP, Roche Molecular Biochemicals). Immunocytochemistry was performed as described earlier (36).

Isolation of RNA, RT-PCR, and Northern Blotting—Total RNA was prepared from granule cells according to the method of Chomczynski and Sacchi (37). cDNA was synthesized using a random primer (First-strand cDNA synthesis kit, Amersham Pharmacia Biotech) according to the manufacturer's protocol. PCR was performed using the following oligonucleotides: NCX1-F (rat NCX1, 1760–1782), atgttatcattccctataaaacc; NCX1-R (rat NCX1, 2117–2136), ctcctctttgctggtcagtg; NCX2-F (rat NCX2; 1453–1472), ctgcgtgtgggcgatgctc; NCX2-R (rat NCX2; 1965–1983), gacctcgaggcgacagttc; NCX3-F (rat NCX3, 2534–2555), gacagtagaaggaacagccaag; NCX3-R (rat NCX3; 2808–2828), tttagggtgttcaccaatac; G3PDH-F (rat G3PDH, 371–391), ccaaaaggggtcatcatctcc; G3PDH-R (rat G3PDH, 994–1015), gtaggccatgaggtccaccac; Fos-F (rat fos, 660–680), aagtctgcgttgcagaccgag; Fos-R (rat fos, 1040–1020), gtctgctgcatagaaggaacc; PMCA4CII (rat PMCA4CII, 3622–3647), gaggaggtgtaacggcagaag.

The conditions for the PCR reactions were as suggested by Perkin-Elmer for the *Taq* Gold polymerase. The identity of the PCR-generated fragments was verified by sequencing. The G3PDH fragment encompassed cDNA nucleotides 371–1015 (38). RNA was denatured by formaldehyde and formamide and fractionated on a 1% agarose gel containing 20 mM MOPS-NaOH, 8 mM sodium acetate, 1 mM EDTA, pH 7.0, and 6% formaldehyde. After separation, RNA was transferred to Nytran filters by capillary elution in 10× SSC buffer, prehybridized, and hybridized in 5× Denhardt's solution, 5× SSPE, 0.1% SDS, 0.1 mg/ml denatured salmon sperm DNA, 0.2–1 $\times$ 10^6 cpm/ml labeled DNA, and 50% formamide at 42 °C overnight. Nytran filters were washed in 0.1× SSC, 0.1% SDS twice at room temperature for 15 min, once at 55 °C for 30 min, and once at 65 °C for 20 min prior to the exposure to x-ray films or to PhosphorImager screens.

Western Blotting—The gel sample buffer contained 6 M urea, 5% SDS, 4% dithiothreitol, 50 mM Tris-HCl, pH 8.0, and 5 mM EDTA. After electrophoresis, proteins were blotted onto a nitrocellulose sheet (39). The membrane was blocked at room temperature in Tris-buffered saline (25 mM Tris-HCl, 500 mM NaCl) with 3% gelatin and then incubated for 60–90 min with exchanger polyclonal antibodies (diluted 1/500 or as indicated) in TBST (Tris-buffered saline containing 0.05% Tween 20 and 1% gelatin). After three washes with TBST, the membrane was incubated with a secondary antibody conjugated to alkaline phosphatase (Promega) for 1 h followed by washing. The staining reaction was carried out either with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate according to the ProtoBlot System (Promega) or with chemiluminiscence substrate CDP-star™ (Tropix) according to the manufacturer's instructions.

Immunoprecipitation—Granule cells were cultured under different conditions for 4 days at a density of 2.5 $\times$ 10^6 cells/well in a 6-well plate. The medium was replaced with methionine-free minimum essential medium supplemented with [^{35}S]methionine (150 μCi/ml) and incubated overnight. The cells were rinsed with phosphate-buffered saline, and crude membrane proteins were prepared. The labeled cells (corresponding to about 5,000,000 cpm) were solubilized in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% SDS. NET buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% gelatin, 0.1% Nonidet P-40, 1 mM EDTA) was added to dilute SDS to a final concentration of 0.2%. Triton X-100 and sodium deoxycholate were added to final concentrations of 0.3 and 0.5%, respectively. The mixture was incubated for 30 min at 4 °C. After centrifugation at 15,000 $\times$ *g*, the supernatant was incubated with the primary antibody (5 μl of serum) at 4 °C on a rocking plate for at least 1 h. To recover the immunoprecipitates, protein A-coupled Sepharose CL-4B (20 μl pre-equilibrated in NET buffer) was added to the mixture and incubated at 4 °C for at least 30 min under gentle rocking. The protein A-Sepharose-primary antibody complex was recovered by centrifugation (1–2 min in a microcentrifuge) and washed four times with 20 volumes of NET buffer, twice with NPT buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40), and once with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl. The material bound to protein A-Sepharose was released by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The immunoprecipitates were analyzed by SDS-PAGE and exposed to a PhosphorImager plate or x-ray films.

Membrane Preparations—Cells were resuspended at a density of 5–10 $\times$ 10^6 cells/ml in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 75 μg/ml phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol and subjected to three cycles of freeze-thaw. The particulate fraction was sedimented at 15,000 $\times$ *g* for 15 min. The resulting protein pellet was resuspended in 4 mM Tris-HCl, pH 8.0, 10% sucrose and frozen at −70 °C. Cerebella were dissected from rat brains and homogenized in 5 mM Tris-HCl, pH 7.5, 320 mM sucrose, 5 μg/ml each pepstatin, antipain, and leupeptin with a loose Potter homogenizer. A crude synaptosomal membrane fraction was obtained by centrifuging the post-nuclear supernatant at 12,000 $\times$ *g* for 10 min at 4 °C. The supernatant was then centrifuged at 100,000 $\times$ *g* for 1 h at 4 °C. The material precipitated at 100,000 $\times$ *g* was defined as the microsomal fraction.

Determination of the Na^+/Ca^{2+} Exchanger Activity in Granule Cells—Granule cells (1.25 $\times$ 10^6/well) were plated on poly-lysine-coated, 12-well plates and cultured in the presence of 25 mM KCl or 25 mM KCl and 100 nM FK506 for 7 days. The cells were washed twice with 140 mM NaCl, 2 mM $MgCl_2$, 1 mM ouabain, 25 μM nystatin, 20 mM MOPS-Tris, pH 7.4, and incubated for 15 min in the same buffer at 37 °C. After two washes with 140 mM NaCl, 20 mM MOPS-Tris, pH 7.4, 2 mM $MgCl_2$, Ca^{2+} uptake was initiated by overlaying the cells with a buffer containing 140 mM KCl, 50 μM $CaCl_2$ ($^{45}Ca^{2+}$ 2–4.10^6 cpm/ml), 1 mM ouabain, 20 mM MOPS-Tris, pH 7.4 (uptake buffer). Control experiments were carried out by substituting 140 mM NaCl for KCl in the uptake buffer. The reaction was stopped at different time intervals with a buffer containing 10 mM $LaCl_3$, 100 mM $MgCl_2$, 20 mM MOPS-Tris, pH 7.4. The amount of Ca^{2+} taken up by the cells was determined after their lysis in 2% SDS, 10 mM Tris-HCl, pH 8.0.

Preparation of Isoform-specific Na^+/Ca^{2+} Exchanger Antibodies—Portions of the NCX1 (amino acids 566–691 (3)) and the NCX2 (amino acids 486–661 (30)) sequences located in the large cytosolic loop were chosen to raise isoform-specific antibodies (a portion of the sequence of NCX3 located in this region was also chosen). The corresponding cDNA fragments were amplified from rat brain RNA by RT-PCR using the following oligonucleotides: NCX1-F (1760–1782), atgttatcattccctataaaacc; NCX1-R (2117–2136), ctcctctttgctggtcagtg; NCX2-F (1453–1472), ctgcgtgtgggcgatgctc; NCX2-R (1965–1983), gacctcgaggcgacagttc. The fragments were cloned into the expression vector pRSET. The expression of the Histidine-tagged fusion peptides was performed according to the procedure suggested by Invitrogen (Leek, The Netherlands). The fusion proteins encompassed 36 amino acid residues deriving from the vector. The NCX1 and NCX2 peptides were purified on a nitrilo-triace-

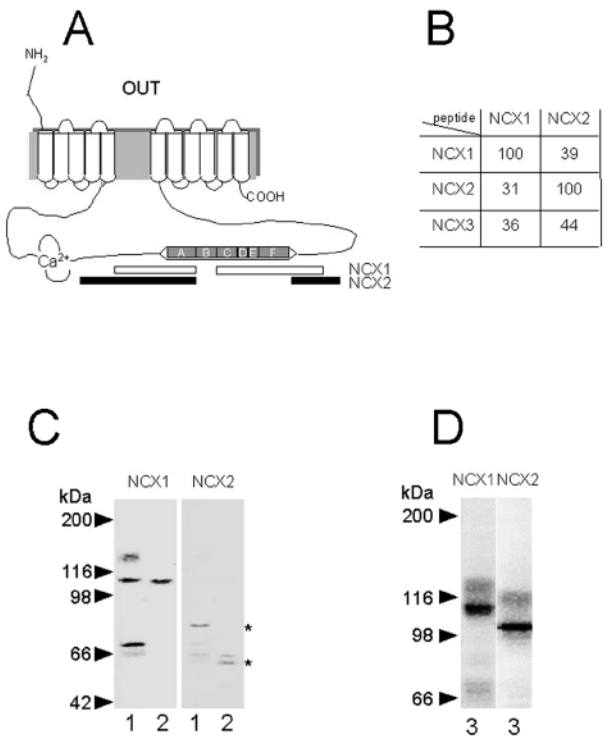

FIG. 1. **Isoform-specific polyclonal antibodies.** *A*, membrane model of the exchanger. The original topographic model of the exchanger, based on hydropathy plots, is shown. As mentioned in the text, an alternative model based on cysteine accessibility studies (16) has now also been proposed. Transmembrane domains are indicated by *cylinders* and the exons involved in the alternative splicing (documented for NCX1) by *boxes A–F*. The regulatory Ca^{2+} binding site in the N-terminal region of the large cytosolic loop is also shown. The location of the peptides used to generate the antibodies is indicated by the *open* (*NCX1*) and *closed* (*NCX2*) *ribbons*. *B*, percent sequence homology of the peptides of the isoforms used to generated the antibodies. The homology of a peptide from the sequence of NCX3, which had also been selected but failed to produce adequate antibodies, is also shown. *C*, 30 μg of proteins prepared from bovine heart sarcolemmal vesicles (*lane 1*) (57) and 10 μg of crude membrane proteins of HeLa cells overexpressing NCX1 using the vaccinia virus expression system (33) (*lane 2*) were separated by SDS-PAGE, transferred to nitrocellulose sheets, and incubated with antibodies against the NCX1 and NCX2 exchangers. The blot incubated with the NCX1 antibody was developed for 5 min, that with the NCX2 antibodies for 60 min. The *asterisks* indicate bands resulting from unspecific reactions that were observed when the blots were developed for longer than 60 min. The immunocomplexes were visualized as described under "Experimental Procedures." *D*, 30 μg of membrane proteins (microsomal fraction) from the cerebella of 21-day-old rats were immunoblotted with antibodies specific for the NCX1 and NCX2 exchangers. Similar results were obtained with synaptosomal fractions (not shown). The molecular masses of protein markers are given on the *left*.

tic acid (Ni^{2+}-NTA) column under denaturing conditions, yielding highly purified products (>95% according to Coomassie Brilliant Blue-stained gels). The polypeptides were utilized to immunize rabbits, using standard procedures (40). The antibodies were affinity-purified on an antigen-coupled Sepharose column as described earlier (40).

RESULTS

Antibodies Specific for the Na^+/Ca^{2+} Exchanger Isoforms—Antibodies specific for the NCX1 and NCX2 isoforms were prepared using peptides encompassing the region subjected to alternative splicing (Fig. 1*A*) as epitopes, because this region shows a low degree of sequence conservation in the three isoforms: The identity of the peptides was below 44% (Fig. 1*B*). Fig. 1*B* also lists a peptide derived from the main loop of NCX3. It had been planned originally to generate antibodies specific for NCX3 as well, choosing for this purpose a domain of low sequence conservation; however, none of the injected rabbits produced an adequate NCX3 antiserum. The NCX1-specific antiserum recognized the exchanger in dog cardiac sarcolemma (bands at about 110, 160, and 70 kDa) or the NCX1 expressed in HeLa cells (Fig. 1*C*). These three bands are typical for NCX1; the 70-kDa band is a proteolytic product (33), the 110-kDa band is the full-length protein, and the 160-kDa band is an internally locked variant of the exchanger that migrates with abnormal mobility (41). The amount of the internally locked version of the exchanger varies with the preparation and cell type and was not visible in overexpressing cells; this may have been a consequence of a different membrane composition of Hela as compared with muscle cells. No exchanger-specific bands were recognized by the NCX2 antiserum in these membranes. The NCX2 affinity-purified antibodies recognized instead a strong band at 102 kDa, which was the expected mass of NCX2 in brain membranes. Further experiments showed a very good correlation between the amount of NCX2-specific mRNA and the 102-kDa immunoreactive band. Blots with the NCX1 and NCX2 peptides used to immunize the rabbits and with the peptide derived from NCX3 indicated that the reaction of the NCX1 and NCX2 antibodies was isoform-specific (not shown), *i.e.* none of them recognized NCX3.

Expression of NCX1 and NCX2 during the Development of Cerebellum and the Maturation of Granule Cells in Vitro—Analysis of the cerebellum from developing rats showed that the expression of NCX2 increased markedly during post-natal development, whereas only slight changes were observed for NCX1 (Fig. 2*A*). To simplify the study, experiments were then performed on cultured granule cells. Under appropriate conditions, these cells survive for a relatively long time, and their cultures contain more than 95% neurons (Fig. 2*B*). In the presence of physiological concentrations of KCl (5.3 mM), the cells matured to full neurons, but their numbers steadily decreased during the first days of culture, with only a few surviving after 7 days (Fig. 2*B, top*). The experiment in Fig. 2*C* shows that, in analogy to what was observed in whole cerebellum, the NCX2 protein became strongly up-regulated during the first days in culture in the 5.3 mM KCl medium. By contrast, as had been the case for the cerebellum, no evident changes were observed in the expression of NCX1. The long-term survival of granule cells in culture requires the chronic depolarization of the plasma membrane by higher concentrations of KCl (Fig. 2*B, bottom*) (34, 42). Recent studies have shown that under these conditions the expression of some of the Ca^{2+} transporting proteins, specifically, plasma membrane Ca^{2+} pumps and plasma membrane and internal Ca^{2+} channels, underwent significant changes (43–45). As preliminarily indicated in a recent review (46), the Na^+/Ca^{2+} exchanger was also affected by these conditions. Fig. 2D shows that a drastic reduction of the NCX2 protein occurred; After 5 days in 25 mM KCl, hardly any of the protein could be detected (Fig. 2*D*), whereas only marginal effects were observed for NCX1. The expression of NCX2 was very sensitive to the depolarizing treatment; an increase of KCl in the medium from 5.3 to 15 mM was sufficient to almost completely down-regulate it (Fig. 2*E*).

Effects of Depolarization on the Transcription of NCX Isoforms in Cerebellum and Cultured Granule Cells—RT-PCR with isoform-specific oligonucleotides was used to detect *NCX* transcripts, in particular their alternatively spliced variants (20, 47). In the case of *NCX1*, sequencing demonstrated that seven different splice isoforms were present after 3 days of culturing in non-depolarizing KCl concentrations (Fig. 3*A, lane M*). In the case of *NCX1*, up to four different PCR fragments were visible in gels (Fig. 3, *lane 1*). In both the cerebellum and the cells, the AD spliced variant was predominant (Fig. 3*A*, compare *lanes 1–4* with *lanes 5* and *6*). In cells cultured in 25

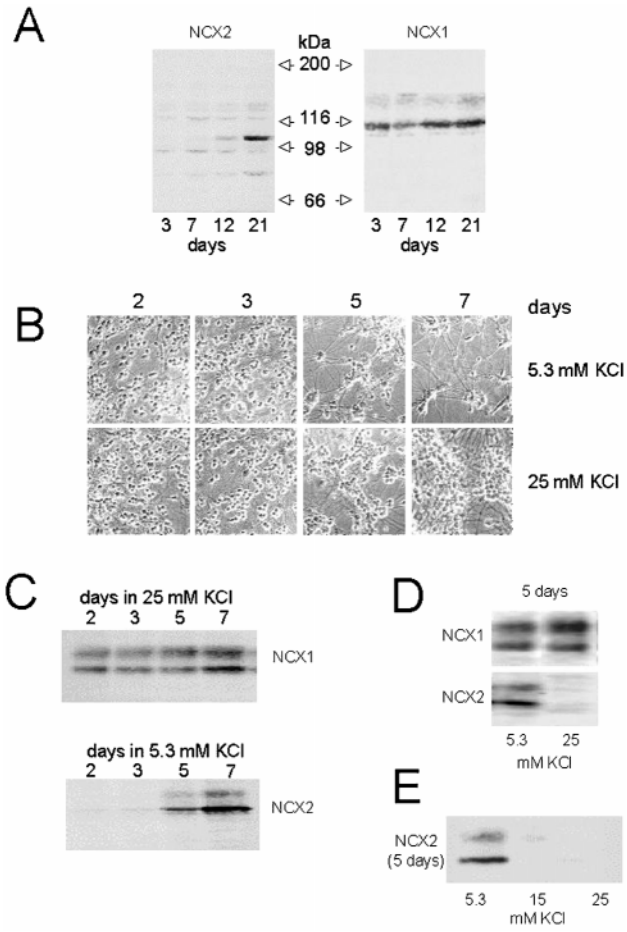

FIG. 2. **Expression of NCX1 and NCX2 during the development of cerebellum and during the maturation of cultured granule cells.** *A*, NCX1 and NCX2 proteins in the cerebellum. Crude membrane proteins (microsomal fraction) were prepared from 3-, 7-, 12-, and 21-day-old rats. 25–30 μg of membrane proteins were separated by SDS-PAGE (8%), blotted to nitrocellulose sheets, and incubated with antibodies specific for the NCX1 and NCX2 exchangers. The molecular masses of protein markers are given in the *center* of the *panel* (*arrows*). *B*, maturation and survival of cultured granule cells: phase contrast images of granule cells cultured for 2, 3, 5, and 7 days. Changes characteristic of neuronal development are observed after a few days in cells cultured either in low (5.3 mM KCl) or high (25 mM KCl) concentrations of KCl. However, survival improved markedly when cells were cultured under mild depolarizing conditions (25 mM KCl). *C*, crude membrane proteins (25–30 μg) from granule cells cultured at physiological KCl (5.3 mM) for different times were separated by SDS-PAGE (8%) and probed with isoform-specific antibodies. *D* and *E*, down-regulation of the expression of NXC2 by depolarization. *D*, crude membrane proteins (25–30 μg) from granule cells cultured for 5 days in the presence of 5.3 or 25 mM KCl were separated by SDS-PAGE and analyzed with isoform-specific antibodies. *E*, granule cells cultured for 5 days in the presence of either 5.3, 15, or 25 mM KCl. Crude membrane proteins (25–30 μg) were separated by SDS-PAGE and analyzed with the NCX2-specific antibodies.

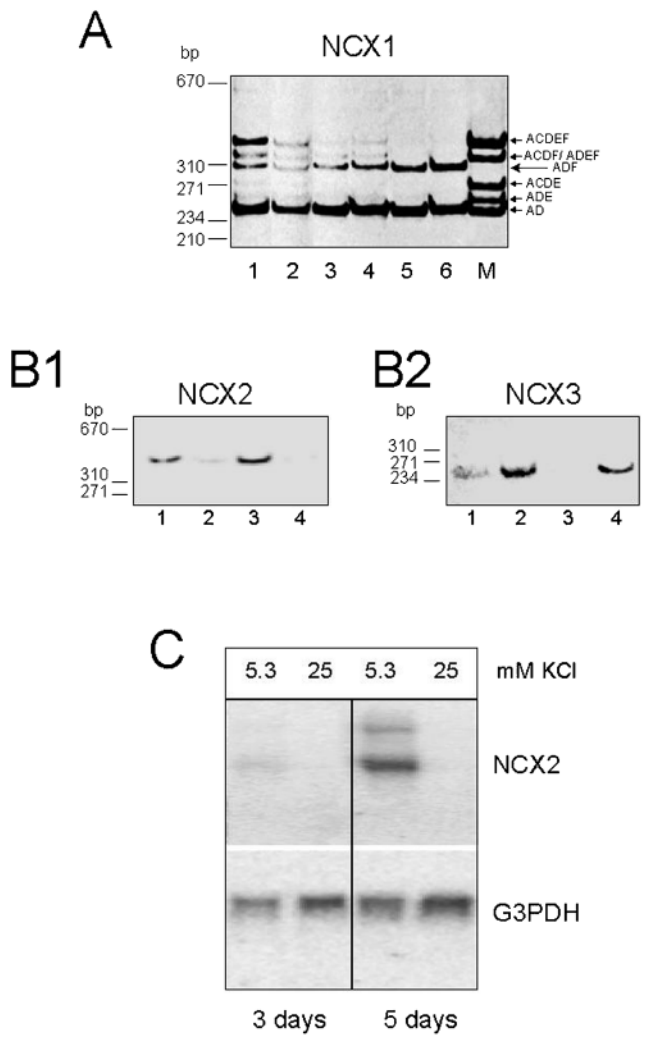

FIG. 3. **Effect of depolarization on the transcription of NCX isoforms.** *A*, NCX1 isoforms. RT-PCR on RNA isolated from granule cells and rat cerebellum with NCX1 isoform-specific oligonucleotides. RNA was prepared from granule cells after 3 (*lanes 1* and *2*) or 5 (*lanes 3* and *4*) days of culture in the presence of 5.3 (*lanes 1* and *3*) or 25 (*lanes 2* and *4*) mM KCl. RNA was also prepared from the cerebella of 7- and 21-day-old rats (*lanes 5* and 6, respectively). RNA was reverse-transcribed, amplified with primers specific for NCX1, and separated on 8% PAGE. PCR products obtained from granule cells were subcloned in pGEM-T vectors; 3–4 independent clones of the different fragments were sequenced. A mixture containing 6 of the 7 fragments found in granule cells, separated on 8% PAGE, is shown in *lane M* (fragment ADF was not included). DNA was visualized by staining with ethidium bromide. *B*, transcripts of the NCX2 (*B1*) and NCX3 (*B2*) isoforms in granule cells. RNA was isolated from cells cultured for 3 (*lanes 1* and *2*) or 5 (*lanes 3* and *4*) days in the presence of either 5.3 (*lanes 1* and *3*) or 25 (*lanes 2* and *4*) mM KCl, reverse-transcribed, and amplified in parallel with oligonucleotides specific for NCX2 (*B1*) and NCX3 (*B2*). The PCR products were separated on 8% PAGE and stained with ethidium bromide. *C*, Northern blotting of NCX2. 15 μg of total RNA from granule cells cultured for 3 or 5 days in the presence of 5.3 or 25 mM KCl were separated on a formaldehyde-agarose gel, transferred to a nylon membrane, and incubated with ^{32}P-labeled NCX2 or G3PDH DNA. The blots for G3PDH were exposed for 48 h and those for NCX2 for 6 days.

mM KCl for 3 to 5 days, the amounts of the AD and ADF isoforms increased, and this increase was accompanied by the disappearance of the larger variants (Fig. 3*A*).

At variance with *NCX1*, the RT-PCR experiment revealed a large, depolarization-dependent down-regulation of the *NCX2* transcript (Fig. 3*B1*), which was confirmed by Northern blotting (Fig. 3*C*). In contrast, the *NCX3* transcript became up-regulated by the depolarizing conditions (Fig. 3*B2*).

The Down-regulation of NCX2 Is Dependent on Ca^{2+}—The depolarization of granule cells by 25 mM KCl causes a sustained, albeit limited, increase of intracellular Ca^{2+} (45). This is because of the increased potential across the neuronal plasma membrane (from −70 to −40 mV) and the consequent opening of voltage-dependent Ca^{2+} channels. After 5 days in culture, the increase was about 3-fold (from about 50 to about 150 nM). Two experiments were carried out to verify whether the depolarization effects on NCX2 expression were the direct results of the increased Ca^{2+} influx. Cells were incubated in the presence of the L-type channel agonist BayK 8644 (Fig. 4*A*), or the influx of Ca^{2+} was increased by manipulating the extracellular calcium concentration (Fig. 4*B*). The agonist

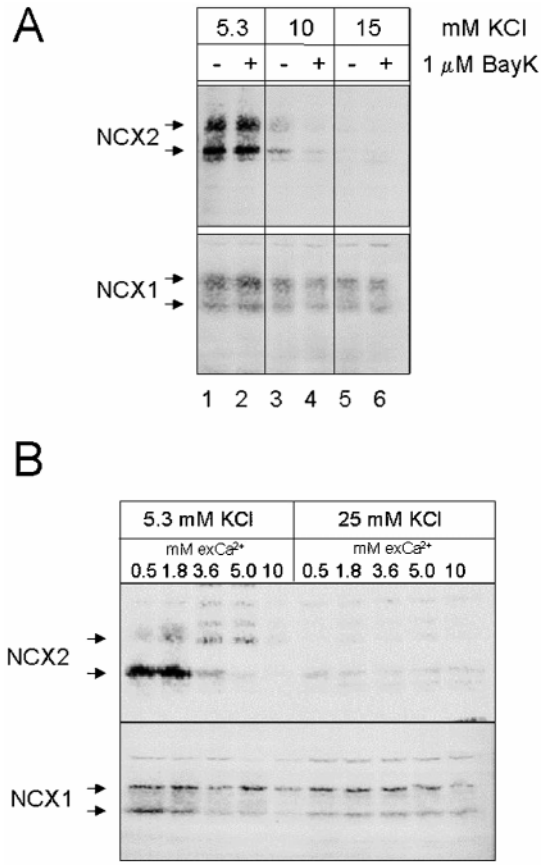

FIG. 4. **Ca^{2+}-dependent down-regulation of NCX2.** *A*, Western blotting analysis of NCX1 and NCX2 in granule cells cultured in the presence of the L-type calcium channel agonist BayK 8644 (1 μM). Cells were cultured for 5 days in the presence (+) or absence (−) of the agonist and 5.3, 10, and 15 mM KCl, respectively. 20 μg of crude membrane proteins from granule cells were separated by SDS-PAGE, transferred to nitrocellulose sheets, and subjected to Western blotting with isoform-specific antibodies. *B*, influence of extracellular Ca^{2+} on the expression of NCX2 and NCX1. Granule cells were cultured for 5 days in either low or high KCl in the presence of the extracellular calcium (*exCa*^{2}) at the concentrations indicated. 20 μg of crude membrane proteins from granule cells were separated on 8% SDS-PAGE, transferred to nitrocellulose sheets, and subjected to Western blotting with affinity-purified polyclonal antibodies against NCX1 and NCX2. The positions of the NCX1 and NCX2 bands are indicated by the *arrows*.

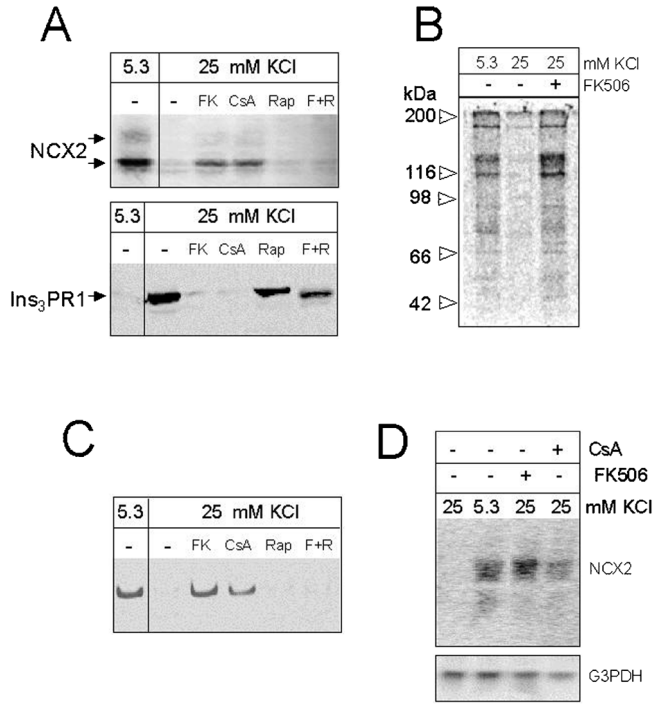

FIG. 5. **Depolarization mediated, calcineurin-dependent down-regulation of NCX2.** *A*, expression of NCX2. Cells were cultured under depolarizing (25 mM KCl) or physiological (5.3 mM KCl) conditions for 5 days in the presence of 1 nM FK506 (*FK*), 100 nM cyclosporin A (*CsA*), 2 μM rapamycin (*Rap*), or 1 nM FK506 plus 2 μM rapamycin (*F+R*). 20 μg of crude membrane proteins from granule cells were separated by SDS-PAGE (8%) and transferred to nitrocellulose sheets. Western blotting was carried out with affinity-purified polyclonal antibodies against NCX2 (*upper panel*) and the IP_3 receptor isoform 1 (*lower panel*). *B*, immunoprecipitation of NCX2 from granule cells. Cells were cultured in the presence of 5.3 mM KCl, 25 mM KCl, or 25 mM KCl and 100 nM FK506. After 4 days, cells were labeled overnight with [^{35}S]methionine (150 μCi/ml), and crude membrane proteins were prepared. Aliquots corresponding to 5×10^6 cpm were immunoprecipitated with the anti-NCX2 polyclonal antiserum. The immunocomplexes were recovered with protein A-Sepharose. The dried SDS-PAGE gel was exposed to a PhosphorImager plate. The sizes of the molecular markers are given to the *left* of the *panel*. *C* and *D*, calcineurin effects on NCX2 transcripts. *C*, RT-PCR analysis of NCX2 expression in granule cells in the presence of immunosuppressants. Cells were treated as described in *panel A*. 1.5–2 μg of total cell RNA was reverse transcribed and amplified by NCX2-specific primers. PCR products were separated on 8% PAGE and visualized by ethidium bromide staining. *D*, Northern blotting analysis of NCX2 transcripts in granule cells. Cells were grown under depolarizing conditions for 5 days with 1 nM FK506 or 100 nM cyclosporin A (*CsA*). 20 μg of total RNA were subjected to Northern blotting. Random primer-amplified NCX2 or G3PDH-specific DNA fragments were used as probes.

failed to influence the level of NCX2 protein at the physiological concentration of KCl (5.3 mM) but reproducibly reduced its level when the KCl concentration in the culturing medium was raised to 10 mM (Fig. 4*A*). Under these conditions, no effect on the level of NCX1 protein was observed. Similarly, increasing the extracellular concentration of Ca^{2+} had a dramatic effect on the expression of NCX2 even at non-depolarizing KCl concentrations. When the extracellular Ca^{2+} concentration was raised to 3.6 mM, the level of NCX2 protein decreased very markedly, even in 5.3 M KCl, and disappeared almost completely at 5 mM Ca^{2+} (Fig. 4*B*). Again, the effect was specific for NCX2, *i.e.* it was not observed with NCX1.

The Expression of NCX2 Is Controlled by Calcineurin—Prior to investigating the role of calcineurin, attempts were made to establish whether the Ca^{2+} effects on NCX2 expression could be mediated by calmodulin kinases. Unfortunately, the most widely used inhibitors of these enzymes, among them KN-92 and KN-93, proved highly toxic to granule cells, *i.e.* the great majority of the cells died after a few hours of incubation with these inhibitors. The time of survival was too short to reliably explore a possible function of calmodulin kinases.

To investigate the possible involvement of the Ca^{2+}-calmodulin-stimulated phosphatase, calcineurin experiments were carried out with the immunosuppressant drugs FK506 and cyclosporin, which bind to their respective immunophilins to become efficient inhibitors of calcineurin. The specificity of the effect of FK506 can be verified using rapamycin, an immunosuppressant that binds to the FK506-binding immunophilin, FKBP, but fails to inhibit calcineurin. However, high amounts of rapamycin compete with FK506 for binding to FKBP, therefore blocking its inhibitory effects. FK506 and cyclosporin A prevented the depolarization-mediated down-regulation of NCX2 (Fig. 5*A*); rapamycin did not, but it blocked the effect of FK506 when present at 2000-fold in excess of the latter. The expression of $InsP_3R_1$ (inositol trisphosphate receptor isoform 1), which has recently been shown to be controlled by calcineurin (44), was used as a control in Fig. 5*A*; in contrast to NCX2, it was up-regulated by the depolarization (Fig. 5*A*). Immunoprecipitation experiments of the NCX2 protein from

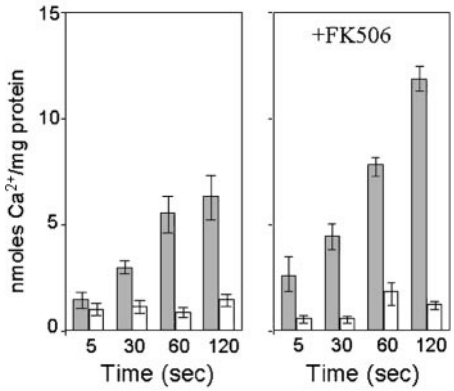

FIG. 6. **NCX activity of granule cells.** Cells were grown in medium containing 25 mM KCl or 25 mM KCl + 100 nM FK506 for 7 days and used for the $^{45}Ca^{2+}$ uptake experiments (see "Experimental Procedures" for details). Cells were preloaded with 140 mM NaCl, and Ca^{2+} uptake was initiated by adding $^{45}Ca^{2+}$ to a 140 mM KCl medium (*gray bars*). Controls were performed by adding $^{45}Ca^{2+}$ to cells diluted in 140 mM NaCl (*open bars*) The values were the average ± S.E. of three experiments on three different cell batches.

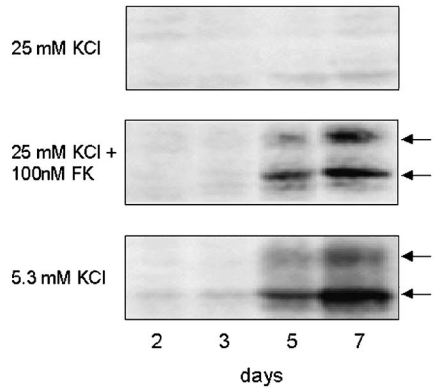

FIG. 7. **The expression of NCX2 increases with the maturation of granule cells and is inhibited by calcineurin.** Granule cells were cultured under non-depolarizing (5.3 mM KCl) or depolarizing (25 mM KCl) conditions for 2, 3, 5, and 7 days in the presence or absence of 100 nM FK506. 20 μg of crude membrane proteins were separated on SDS-PAGE (8%) and subjected to Western blotting with the affinity-purified NCX2 antibodies. The positions of the NCX2 bands are indicated by the *arrows*. The same material was analyzed with the NCX1 specific antibody, showing no changes in the concentration of NCX1 (not shown).

granule cells incubated for 5 days with FK506 using the NCX2-specific antiserum further confirmed that the down-regulation of NCX2 was reversed by calcineurin inhibitors (Fig. 5*B*). When cells were cultured under depolarizing conditions, neither RT-PCR nor Northern blot analysis (Fig. 5, *C* and *D*) revealed NCX2 transcripts, which were present in cells cultured in 5.3 mM KCl. In agreement with the observations at the protein level, FK506 and cyclosporin A partially prevented the down-regulation of the NCX2 transcripts introduced by the depolarizing treatment, whereas rapamycin had no effect unless present in a 2000-fold excess over FK506 (Fig. 5*C*). In contrast to NCX2, the immunosupressants failed to affect the depolarization-mediated expression of the NCX1 splice variants and the up-regulation of the NCX3 transcripts (results not shown).

The large changes in NCX2 protein and transcripts could not be used to evaluate quantitatively the contribution of NCX2 to the total exchanger activity of granule cells. To address this important question, the activity of the exchanger was measured in cells cultured for 7 days in the presence of 25 mM KCl and in the presence or absence of FK506. Cells cultured in 100 nM FK506 had 30–40% more exchanger activity than cells grown under the same conditions but in the absence of the immunosuppressant (Fig. 6). Attempts were made also to measure the activity of the exchanger in cells cultured in 5.3 mM KCl. Unfortunately, they were unsuccessful because the few cells remaining after 5–7 days were very fragile and did not survive the washes required to measure exchanger activity. Thus, at the end of the maturation process one-third of the exchanger activity of granule cells was evidently due to NCX2.

The experiments presented above have shown that calcineurin plays a role in the down-regulation of NCX2. When the phosphatase was inactive, *i.e.* high KCl plus FK506 or low KCl, a strong up-regulation of the expression of NCX2 was observed instead (Fig. 7, compare Figs. 2*C* and 3*C*). The increase in NCX2 protein in cells cultured in low KCl was equivalent to that observed in 25 mM KCl and 100 nM FK506 (Fig. 7), indicating that calcineurin was insufficiently active in the low Ca^{2+} medium prevailing within cells cultured in low KCl. Alternatively, factors that counteracted the presumably limited activity of calcineurin in granule neurons cultured in 5.3 mM KCl could have permitted the up-regulation of the expression of NCX2.

Calcium Regulation of NCX2 Expression Is Fast and Does Not Require de Novo Protein Synthesis—Kinetics studies were performed next on the expression of NCX2 in granule cells initially cultured for 3 days in 5.3 mM KCl and then submitted to different treatments (Fig. 8). These studies showed that the down-regulation of the transcript was fast; at 1 h after the addition of 25 mM KCl, the NCX2 signal had already disappeared (Fig. 8). The decrease of the transcript was as fast as the up-regulation of that of the immediate early gene c-*fos*, which was used as a control (Fig. 8). Inhibition of protein translation by cycloheximide failed to affect the change in NCX2 transcript, showing that its down-regulation did not require *de novo* protein synthesis. This finding was in sharp contrast to the transcript of PMCA4CII (plasma membrane Ca^{2+}-ATPase isoform 4), which was also found to be down-regulated in granule cells upon depolarization (48). The PMCA4CII transcripts disappeared more slowly than the NCX2 transcripts; in the case of the PMCA, the disappearance was prevented by cycloheximide (Fig. 8).

DISCUSSION

Numerous NCX isoforms are expressed in different tissues. It is possible that 12 *NCX1*, 1 *NCX2*, and 4 *NCX3* variants are generated by alternative splicing of the primary transcripts (47). In addition, the use of three independent promoters produces three NCX1 transcripts differing in the 5′-untranslated sequence (22, 23, 47, 49). The expression of the isoforms appears to be regulated by independent mechanisms; understanding them would be important and would help in rationalizing both the regulation mechanisms and the changes in isoform composition during development.

Western blot analysis showed that the NCX2 protein increased during the early development of the cerebellum. Following this finding, the work then concentrated on the expression of the NCX genes in granule cells, which undergo very significant morphological changes during the first days in culture. Mild depolarizing conditions (25 mM KCl) amplify these phenotypic changes and, most importantly, prevent the onset of early apoptosis. At plating time, rat granule cells contained all three basic NCX isoforms. Seven spliced variants of the transcripts were detected in the case of NCX1; The depolarizing treatment influenced their expression pattern, although the total amount of expressed NCX1 protein did not change appreciably during this time. Depolarization also up-regulated the NCX3 transcripts, but the most striking behavior was in the NCX2 isoform. Whereas under physiological (non-depolarizing) conditions its expression (transcripts and protein) showed an

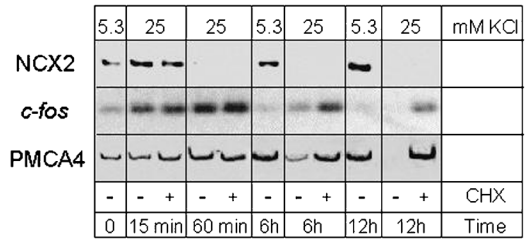

FIG. 8. **RT-PCR analysis of the kinetics of NCX2 down-regulation.** Granule cells were cultured for 3 days in 5.3 mM KCl and then divided into two portions. One portion was kept in 5.3 mM KCl, and cells were collected after 0, 6, and 12 h. The other was depolarized with 25 mM KCl for 15 min, 60 min, 6 h, or 12 h in the presence or absence of 10 μg/ml cycloheximide (*CHX*). The treatment with cycloheximide lasted 10 min, and then depolarization was initiated. After the cells were collected, total RNA was prepared and an aliquot was subjected to RT-PCR with NCX2-, c-*fos*-, or PMCA4CII-specific primers (see details under "Experimental Procedures"). A typical experiment is shown, which was repeated three times with the same results.

evident time-dependent increase (10–20-fold as protein), the isoform decreased dramatically instead in cells cultured under conditions (depolarization) that promoted the influx of Ca^{2+}. An important corollary of the down-regulation of NCX2 was its complete dependence on calcineurin, which had no effect on the expression of the other two NCX isoforms. The experiments strongly suggest a transcriptional effect of calcineurin, even if it could be argued that they have not rigorously demonstrated it; the phosphatase could have influenced instead the processing of the transcript to mature mRNA or affected the stability of the latter. However, the complete disappearance of the NCX2 transcript soon after initiating the depolarization appears more in line with a promoter effect. The rapid kinetics of NCX2 down-regulation and its independence on *de novo* protein synthesis suggest a direct phosphatase-mediated modulation of the activity of the nuclear protein complex controlling the transcription of the *NCX2* gene.

Calcineurin is now attracting wide attention as a regulator of gene transcription. Its action has been characterized in T-lymphocytes (50), whose activation is linked to the entry of Ca^{2+}, resulting in the stimulation of the phosphatase and in the dephosphorylation of the transcription factor NFAT. The factor then translocates to the nucleus together with calcineurin, where it up-regulates the transcription of a set of T-cell-specific genes (50). The finding is not limited to T-cells; evidence for the presence of a variant of NFAT (NFAT-3c) in hippocampal cells has been recently published (51). Also in this case calcineurin controlled its dephosphorylation and translocation to the nucleus. A similar situation also prevails in *Saccharomyces cerevisiae*, where the transcription factor Crz1p is dephosphorylated by calcineurin and then translocated to the nucleus (52). In an alternative mode of action, calcineurin promotes the activation of protein phosphatase-1, leading to the dephosphorylation of the transcription factor CREB (cAMP response element-binding protein) (50), which controls the expression of immediate early genes like c-*fos* (53). Although the activation of protein phosphatase-1 could still play a role in granule cells, the effect of calcineurin on the expression of NCX2 was more reminiscent of that observed in T-cells in hippocampal neurons and *Saccharomyces*. The very rapid onset of NCX2 down-regulation (only 1 h after initiating the depolarizing treatment) and the finding that de *novo* protein synthesis was not required strongly suggest that the calcineurin effect was mediated by the dephosphorylation of a (pre-existing) transcription factor. Possibly, analogously with T-cells, hippocampal neurons, and yeast, this would permit the transfer of this putative factor to the nucleus.

The functional tests performed in this study have shown that the activity of NCX2 in granule neurons was high, *i.e.* it accounted for up to 30–40% of the total exchanger activity. Granule cells also contain the NCX1 and NCX3 isoforms. The multiplicity of isoforms in brain is not easily rationalized, the chief difficulty being the very scarce information on their differential functional properties (*e.g.* their regulation characteristics). Despite this difficulty, however, granule cells evidently have the option of modifying their total exchanger activity in response to the increase in intracellular Ca^{2+}. Even if no information is available on the differential functional properties of the three exchanger types, the results clearly support the suggestion that the NCX2 exchanger is functionally different from the other isoforms. The (moderate) up-regulation of NCX3 expression and the reshuffling of the splice variants of NCX1 are not likely to compensate for the down-regulation of NCX2 occurring under these conditions. In contrast, neurons cultured under conditions that did not lead to the sustained increase of intracellular Ca^{2+} (and to the activation of calcineurin) strongly up-regulated NCX2. Evidently, Ca^{2+} acts as a switch that can reverse the expression of NCX2 when conditions prevail that lead to the increase of Ca^{2+} in the cell. This discussion must, for the moment, be restricted to the total quantitative aspects of NCX activity; but the NCX2-NCX1-NCX3 shift is also likely to have qualitative consequences, the full assessment of which will be possible only in the future.

The conditions that led to the down-regulation of NCX2 are those that promoted the long term survival of cultured granule cells. In the developing cerebellum, granule cells survive instead under conditions that promote the up-regulation of NCX2. This finding may indicate that these cells in the cerebellum avoid apoptosis by mechanisms unrelated to membrane depolarization (unless the up-regulation of NCX2 in the tissue also reflected the contribution of other cell types). It would be important to understand why Ca^{2+}, which is not strictly necessary for maturation, is instead essential to protect granule cells against (apoptotic) death. Clues to this question have come from recent work on the activation of the protein kinase B pathway (54), which is controlled by the calmodulin-dependent kinase-kinase (CaMKK). A modest increase of cell Ca^{2+} activates CaMKK, and leads to the phosphorylation of protein kinase B. In turn, activated protein kinase B phosphorylates the pre-apoptotic protein BAD, leading to its sequestration to protein 14-3-3. The important point is that the increase of cell Ca^{2+} to activate CaMKK must be modest, precisely as observed in granule neurons depolarized by 25 mM KCl; a massive, uncontrolled Ca^{2+} overload would evidently be incompatible with cell survival. Thus, one could relate the changes in NCX isoform pattern induced by the depolarizing treatment to the necessity of maintaining the Ca^{2+} increase within the limits required to switch off the apoptotic signals, effectively preventing its uncontrolled increase to intolerable levels.

In cultured granule neurons, the large amounts of Ca^{2+} that penetrate through the plasma membrane channels are eventually extruded by Ca^{2+}-ATPases and Na^+/Ca^{2+} exchangers. Recent work has shown that two isoforms of the former, present in low amounts at plating time, became slowly (*i.e.* 3–5 days) up-regulated during the depolarizing treatment, whereas a third experienced a switch of its spliced variants up-regulating a less active truncated form (45). In analogy with the findings on NCX, one PMCA isoform underwent instead a rapid, Ca^{2+}-calcineurin-mediated down-regulation. One could thus envisage a situation in which, at early stages of *in vitro* development, the contribution of the exchanger(s) to the total Ca^{2+} extrusion from granule cells predominates, because relatively small amounts of the PMCA pumps are present. However, after

maturation has occurred in a few days, the contribution of the strongly up-regulated pumps is likely to become predominant. Because the depolarizing treatment eventually increases the total pump activity (45), it would be reasonable to expect that at this stage cells would have a lower cytosolic Ca^{2+} concentration. The fact that the opposite was found to be true was probably because of the compensation of the increased pump activity by the down-regulation of the NCX2 exchanger. Thus, granule cells apparently react to the persistent increase of Ca^{2+} influx not only by changing the pattern of NCX expression but also by changing that of other Ca^{2+} extruding systems, *i.e.* they switch from exchangers to pumps. Future work will possibly detect (subtle) functional differences among the variants of the pumps and the exchangers, leading to a better understanding of the physiological implications of the findings described here.

However, other points are probably also important in discussing the results in this study, *in primis* possible differences in the subcellular localization of the NCX (and PMCA) proteins. Some plasma membrane proteins, in particular in neurons, have a very defined subcellular localization, which is controlled by specific proteins. For instance, the synaptic localization of the NMDA (N-methyl-D-aspartate) receptor is mediated by the PSD-95 protein (55). It is therefore possible that one of the NCX isoforms is localized predominantly in a selected region of the neuronal plasma membrane, *e.g.* in the synapse. The enrichment or decrease of the exchanger proteins in particular domains of the plasma membrane would naturally significantly affect local Ca^{2+} swings. The same would apply to the PMCA pumps, where evidence for their dishomogeneous distribution along the plasma membrane has actually been provided; the PMCA2 pump is specifically concentrated in the spines of the dendrites of Purkinje neurons (56).

In closing, it may be appropriate to mention again that the up-regulation of the inositol 1,4,5-trisphosphate receptor (44) and the down-regulation of one of the PMCA isoforms in granule cells are also dependent on calcineurin. Calcineurin is thus crucial to the expression of Ca^{2+} transporters during the development of granule neurons and emerges as a key actor in the expression of genes involved in (Ca^{2+}-linked) neuronal signal transduction. It might even emerge as a major player in the expression of genes underlying cognitive processes in the brain.

Acknowledgments—We thank Dr. B. Moss (National Institutes of Health, Bethesda, MD) for the kind gift of the recombinant virus and the pTM3 vector.

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Lei Li, Danilo Guerini and Ernesto Carafoli

J. Biol. Chem. 2000, 275:20903-20910.
doi: 10.1074/jbc.M000995200 originally published online April 14, 2000
