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

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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, tended to up-regulate the expression of NCX1. The latter system, which is particularly active in heart and neurons, uses the Na\(^+\)/K\(^+\) pump for the ATP-driven export of Ca\(^{2+}\) from the cytosol to the extracellular space. The latter system also 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.

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\(^+\)/K\(^+\) pump for the ATP-driven export of Ca\(^{2+}\) from the cytosol to the extracellular space. The latter system also 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).

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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 protein kinase II; 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 (GIBCO/Invitrogen, Carlsbad, CA) and cell 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. 4Ca++, [32P]dCTP, and [14C]ATP were from Amersham Pharmacia Biotech. Nitrocellulose filters were purchased from Tropix (Boston, MA). 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% CO₂ at 37 °C. Transient expression of NCX1 was achieved by infecting cells with 7 polymerase containing vaccinia virus for 48 h. The material bound to protein A-Sepharose was recovered by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The immunoprecipitates were analyzed by SDS-PAGE and exposed to a PhosphoImager plate or x-ray films.

**Membrane Preparations**—Cells were resuspended at a density of 5–10 × 10⁶ cells/ml in 10 ml Tris-HCl, pH 8.0, 1 ml EDTA, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 75 μg/ml phenylmethylsulfonyl fluoride, and 1 ml dithiothreitol and subjected to three cycles of freeze-thaw. The particulate fraction was sedimented at 15,000 × g for 15 min. The resulting protein pellet was resuspended in 4 ml Tris-HCl, pH 8.0, 10% sucrose and frozen at −70 °C. Cerebella were dissected from rat brains and homogenized in 5 ml 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 PhosphoImager plate or x-ray films.

**Preparation of Isoform-specific Na⁺/Ca²⁺ Exchanger Antibodies**—Portions of the NCX1 (amino acids 566–691) and the NCX2 (amino acids 486–661) sequences located in the large cytosolic loop were used to prepare the NCX1 and NCX2 peptides. The NCX1 and NCX2 peptides were purified on a nitrilo-triacetic acid-Sepharose CL-4B (20 μl pre-equilibrated in NET buffer) and 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 after 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 PhosphoImager plate or x-ray films.

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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. 1A) 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. 1B). Fig. 1B 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. 1C). 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. 2A). 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. 2B). 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. 2B, top). The experiment in Fig. 2C 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. 2B, 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 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. 2D), 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. 2E).

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. 3A, 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. 3A, compare lanes 1–4 with lanes 5 and 6). In cells cultured in 25...
The Down-regulation of NCX2 Is Dependent on Ca\textsuperscript{2+} Channels. The depolarization of granule cells by 25 mM KCl causes a sustained, albeit limited, increase of intracellular Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} influx. Cells were incubated in the presence of the L-type channel agonist BayK8644 (Fig. 4A), or the influx of Ca\textsuperscript{2+} was increased by manipulating the extracellular calcium concentration (Fig. 4B). The agonist
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. 4A). 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 mM KCl, and disappeared almost completely at 5 mM Ca\(^{2+}\) (Fig. 4B). 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. 5A); rapamycin did not, but it blocked the effect of FK506 when present at 2000-fold in excess of the latter. The expression of Ins\(_{3}P_{1}\) (inositol trisphosphate receptor isoform 1), which has recently been shown to be controlled by calcineurin (44), was used as a control in Fig. 5A; in contrast to NCX2, it was up-regulated by the depolarization (Fig. 5A).

Immunoprecipitation experiments of the NCX2 protein from

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**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 \(\mu M\)). Cells were cultured for 5 days in the presence (+) or absence (−) of the agonist and 5.3, 10, and 15 mM KCl, respectively. 20 \(\mu\)g 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. A, 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 \(\mu\)g 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 depolarization (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 \(\mu\)M rapamycin (Rap), or 1 mM FK506 plus 2 \(\mu\)M rapamycin (FK+R). 20 \(\mu\)g 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 mM FK506. After 4 days, cells were labeled overnight with [\(^{35}\)S]methionine (150 \(\mu\)Ci/ml), and crude membrane proteins were prepared. Aliquots corresponding to 5 \(\times\) 10\(^{6}\) cpm were immunoprecipitated with the anti-NCX2 polyclonal antiserum. The immunoprecipitates were separated on 8% PAGE and visualized by ethidium bromide staining. C, Northern blotting analysis of NCX2 transcripts. Cells were grown under depolarizing conditions for 5 days with 1 mM FK506 or 100 mM cyclosporin A (CsA). 20 \(\mu\)g of total RNA were subjected to Northern blotting. Random primer-amplified NCX2 or G3PDH-specific DNA fragments were used as probes.
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. 5B). When cells were cultured under depolarizing conditions, neither RTPCR 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 downregulation of the NCX2 transcripts introduced by the depolarizing treatment, whereas rapamycin had no effect unless present in a 2000-fold excess over FK506 (Fig. 5C). In contrast to NCX2, the immunosuppressants 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 or FK506 up-regulated the expression of NCX2 was observed instead (Fig. 7, compare Figs. 2C and 3C). 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 Ca2+ 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 Ca2+-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 isofrom 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
Granule cells were cultured for 3 days in 5.3 mM KCl and then 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.

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 calcmodulin-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 the cytosol and activation of 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 the cytosol and inhibition of its pro-apoptotic function.

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 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 T-cells is now being pursued in 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 calcmodulin-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 the cytosol and inhibition of its pro-apoptotic function.

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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 PSD-95 protein (55). It is therefore possible that one of the NCX transporters during the de-}