Plasma membrane Ca\(^{2+}\) ATPase (PMCA) pump isoforms 2, 3, and 1CII are expressed in large amounts in the cerebellum of adult rats but only minimally in neonatal cerebellum. These isoforms were almost undetectable in rat neonatal cerebellar granule cells 1–3 days after plating, but they became highly expressed after 7–9 days of culturing under membrane depolarizing conditions (25 mM KCl). The behavior of isoform 4 was different: it was clearly detectable in adult cerebellum but was down-regulated by the depolarizing conditions in cultured cells. 25 mM KCl-activated 1-type Ca\(^{2+}\) channels, significantly increasing cytosolic Ca\(^{2+}\). Changes in the concentration of Ca\(^{2+}\) in the culturing medium affected the expression of the pumps. 1-type Ca\(^{2+}\) channel blockers abolished both the up-regulation of the PMCA1CII, 2, and 3 isoforms and the down-regulation of PMCA4 isoform. When granule cells were cultured in high concentrations of N-methyl-D-aspartic acid, a condition that increased cytosolic Ca\(^{2+}\) through the activation of glutamate-operated Ca\(^{2+}\) channels, up-regulation of PMCA1CII, 2, and 3 and down-regulation of PMCA4 was also observed. The activity of the isoforms was estimated by measuring the phosphoenzyme intermediate of their reaction cycle: the up-regulated isoforms, the activity of which was barely detectable at plating time, accounted for a large portion of the total PMCA activity of the cells. No up-regulation of the sarcoplasmic/endoplasmic reticulum calcium pump was induced by the depolarizing conditions.

The messenger function of calcium is of particular interest in neuronal cells: processes as important and as diverse as gene expression (1), synaptic plasticity (2), the release of neurotransmitters (3), and the survival of neurons (4) are modulated by Ca\(^{2+}\). These aspects of neuronal regulation are conveniently studied on cultured neuronal cells (4–6), the survival of which was down-regulated by the depolarizing conditions.

Both the NMDA receptor and voltage-activated Ca\(^{2+}\) channels mediate the entry of Ca\(^{2+}\) that is necessary to the survival of cultured granule cells (7). It has been proposed that Ca\(^{2+}\) entering through the voltage-activated Ca\(^{2+}\) channels may modulate the expression of genes different from those controlled by Ca\(^{2+}\) penetrating through the NMDA receptor (1). The sustained increased influx of Ca\(^{2+}\) during the maturation demands the increased capacity of the cells to extrude it. The two classical plasma membrane Ca\(^{2+}\)-extruding systems, the Ca\(^{2+}\)-ATPase (PMCA) and the Na\(^{+}\)-Ca\(^{2+}\)-exchanger, have been documented in neurons (17–20). Two of the four PMCA basic gene products, PMCA2 and PMCA3, have in fact only been detected in significant amounts in neurons (19), although their transcripts have been detected in other tissues as well (17, 21). Induction of PMCA pump transcription as well as a switching of its isoforms is induced by the addition of NGF to PC-12 cells, which are also widely used as a neuronal cell model (22). A short incubation of IMR32 neuroblastoma cells with 56 mM KCl also induces the transcription of one of the PMCA2 alternatively spliced isoforms (PMCA2AII) (23).

Pilot experiments performed at the outset of this work had revealed that the PMCA2, PMCA3, and, to a lesser extent, PMCA4 pumps became up-regulated during the development of the cerebellum. It was thus interesting to investigate whether these isoforms also underwent regulation during the in vitro development of granule cells. The work presented here shows that the expression of the PMCA3 and of the PMCA2 genes increased markedly during the maturation process and was accompanied by the marked up-regulation of the expression of one of the alternatively spliced PMCA1 isoforms. At variance with the findings in intact cerebellum, the maturation process in granule cells induced instead an evident down-regulation of the PMCA4 pump. Both the up- and down-regulation of the isoforms depended on the sustained increase of intracellular Ca\(^{2+}\).
Expression of PMCA Pump Isoforms

MATERIALS AND METHODS

**Chemicals**— Poly-γ-lysine, 3,4-(5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Dubecco’s modified Eagle’s medium, Dubecco’s modified Eagle’s medium/Ham’s F-12 nutrient mixture, and other tissue culture supplements were form Sigma or Life Technologies, Inc. Ditocipline (MK-801) was from Research Biochemicals, Inc. (Natick, MA). NMDA, nifedipine, and calcudilene were from Calbiochem. 

**Oligonucleotides**—were purchased from MWG-Biotech, (Ebersberg, Germany). AmpliTaq Gold polymerase was from Perkin-Elmer. All other reagents were of the highest purity grade commercially available.

**Cell Cultures**—Granule cells were dissociated from the cerebella of 7-day old Wistar rats as described (7, 10). They were plated in Dubecco’s modified Eagle’s medium (Hepes modification (Sigma), containing 1.8 mM CaCl₂ supplemented with heat-inactivated 10% fetal calf serum (Life Technologies) in Dulbecco’s modified Eagle’s medium, Dulbecco’s phosphate-buffered saline (PBS) supplemented with 0.2% gelatin, 0.1% Nonidet P-40, 0.1% SDS; twice at 4 °C and viewed with a fluorescence microscope. Dead cells were stained by propidium iodide and showed a strong emission at 617 nm. Living cells were stained by calcein, resulting in a green fluorescent emission (517 nm).

**Materials and Methods**

**Expression of PMCA Pump Isoforms**

PC-12 cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (both from Life Technologies). They were seeded on laminin coated plates (Collaborative Biomedical Products and Becton Dickinson, Bedford, MA), before induction of differentiation with nerve growth factor (20 ng/ml). Three days later neurite outgrowth was observed in at least 30% of the cells, which could be maintained in culture for up to 21 days.

**Preparation of Membranes from Cerebellum**—Cerebellar were dissected from rat brains and homogenized in 5 mM Tris-HCl, pH 7.5, 320 mM sucrose, 5 μg/ml pepstatin, antipain, and leupeptin with a loose Potter homogenizer. A crude synaptosomal membrane fraction was obtained after centrifuging the postnuclear supernatant at 12,000 × g for 10 min at 4 ⁰C. The supernatant was again centrifuged at 100,000 × g for 1 h at 4 °C. The 100,000 × g precipitated material was defined as the microsomal fraction.

**Preparation of Membrane Proteins**—Cells were resuspended at 5–10 M 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 diithiothreitol and subjected to three cycles of freezing and thawing. The particulate fraction was sedimented at 150,000 × g for 30 min at 37 °C. The resulting supernatant was resuspended in 4 mM Tris-HCl, pH 8.0, 10% sucrose and frozen at −70 °C.

**Western Blotting**—Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (27) and transferred to nitrocellulose or polyvinylidene difluoride membranes (28). The membranes were incubated with affinity-purified isoform-specific polyclonal antibodies against the PMCA pump produced and used essentially as described by Stauffer et al. (19) Polyconal sera (donated by Dr. F. Wuytack, Leuven, Belgium) were used at a 1:500 dilution to detect the SERCA2b protein (29). The JA9 monoclonal antibody specific for the PMCA4 isoforms was provided by Dr. J. Penniston (Mayo Clinic, Rochester, MN). The monoclonal antibody directed to the NMDA receptor NR1 protein (Pharmigen, San Diego, CA) was used at a dilution ranging from 1:500 to 1:5,000. Oligonucleotides were purchased from MWG-BioTech, (Ebersberg, Germany). AmpliTaq Gold polymerase was from Perkin-Elmer. All other reagents were of the highest purity grade commercially available.

**Formation of the Phosphoenzyme Intermediate**—Membrane proteins were resuspended in 20 mM MOPS-KOH, pH 6.8, and 100 mM KCl in the presence of 100 μM Ca²⁺ plus 100 μM La³⁺. La³⁺ was included to stabilize the phosphoenzyme intermediate of the PMCA protein (30). The reactions were carried out with 0.5 μg of affinity-purified antibody and allowed to proceed for 20–30 s after the addition of 7% trichloroacetic acid. The proteins in the washed pellet were separated on acidic gels (31) stained with Coomassie Brilliant Blue, dried, and exposed for 1–5 days at 70 °C.

**Immunoprecipitation of the Phosphorylated Proteins**—160 μg of membrane proteins were phosphorylated with ATP as described above. After precipitation with trichloroacetic acid, the pellet was washed with 200 μl of cold H₂O and 0.5% SDS, 8% urea, 150 mM NaCl, 50 mM MES-NaOH, pH 6.4. The resuspended proteins were diluted to 1000 μl with MEM buffer (50 mM MES-NaOH, pH 6.3, 150 mM NaCl, 1 mM EDTA) supplemented with 0.2% gelatin, 0.1% Nonidet P-40, 0.1% SDS and centrifuged at 10,000 × g for 30 min. The supernatant was divided in four 250-μl aliquots. One was mixed with 2–3 μg of 5F1 monoclonal antibody (26) and the others with 3–5 μg of 1N, 2N, or 3N affinity-purified isoform-specific antibodies. The samples were incubated overnight at 4 °C under gentle rocking prior to the addition of 15 μl of packed protein A-Sepharose CL-4B, (Amersham Pharmacia Biotech, Uppsala, Sweden). After an additional 2 h of incubation at 4 °C, the immunocomplexes bound to protein A-Sepharose were recovered by washing with buffer. The pellets were washed four times with MEM buffer supplemented with 0.2% gelatin, 0.1% Nonidet P-40, 0.1% SDS; twice with MEM buffer supplemented with 0.1% Nonidet P-40; and finally with MEM buffer alone. The pellet was resuspended at room temperature and incubated for 30 min in 30–40 μl of 70 μM Tris-Po₄, pH 6.4, 5% SDS, 5% dithiothreitol, and 8 μl urea, separated on acidic gels (31), stained with Coomassie Brilliant Blue, and dried prior the analysis in a gel dryer (Product No. 25-2,5 days). The gels were scanned with Adobe Photoshop and quantified with the help of the National Institutes of Health NIH Image program, Version 1.59.

**Isolation of RNA and Reverse Transcription PCR**—Total RNA was prepared form granule cells according to the method of Chomczynski and Sacchi (33). If required, the cells were depolarized with 75 mM KCl for 30 min at 37 °C by the addition of 10 mM Hepes-KOH (pH 7.2), 170 mM NaCl, 1.3 mM MgCl₂, 0.9 mM CaCl₂. Control cells were incubated with 75 mM NaCl. cDNA was synthesized using a random octamer primer (First Strand cDNA synthesis kit, Amersham Pharmacia Biotech) according to the manufacturer’s protocol. PCR was performed using the oligonucleotides described by Keeton et al. (17) for alternative splicing site C. To study splicing site A, the oligonucleotides described by Keeton et al. (17) for alternative splicing site were used. The following oligonucleotides were used: 5’-CCAAAAGGGTCATCATCTCC (nt 371–401) and 5’-GGGCCATGAGGTCCACCAC (nt 994–1015). The oligo-
were washed twice with calibration buffer (120 mM NaCl, 25 mM Hepes, medium supplemented with 1 mM EGTA) and equilibrated for 10 min with a buffer of the same ionic composition as the original growth medium and then left to equilibrate in the same medium for their complete maturation, and especially for their long term survival thereafter. As soon as after 5 days of culturing in low potassium culture (see the higher magnification field, in Fig. 2), cells begin to develop long and widespread processes shortly after plating (1–2 days), and become fully mature after 7–9 days of culturing under optimal conditions. Granule cells cultured in nondepolarizing concentrations of KCl survive for some days (8), particularly if growth factors are present, but in the experiments described here, the sustained depolarization of the plasma membrane was definitively found to be necessary for their complete maturation, and especially for their long term survival thereafter. As soon as after 5 days of culturing in low potassium, the total number of cells was lower than in high potassium (Fig. 2A), as confirmed by experiments in which the amount of living cells was determined by the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide method (not shown). Nevertheless, the morphology of the neurons in high KCl was indistinguishable from that of those surviving in the low potassium culture (see the higher magnification field, in Fig. 2B). After 5 days in culture, the ratio between viable and nonviable neurons was similar, as indicated by the staining with calcein and propidium iodide (Fig. 2, C and D). In addi-
tion, the cells surviving in low KCl were still able to produce Ca\(^{2+}\) transients (see below) and to up-regulate the \(c-fos\) mRNA when depolarized by the higher potassium concentration (not shown).

It was thus decided to study whether the changes in the expression of the genes of the PMCA pumps observed in whole cerebellum during development also occurred during the maturation of granule cells. A marked and steady increase of the total PMCA pump protein (Fig. 3, \(5F10\)) was indeed detected in cells cultured for 2–9 days in 25 mM KCl. The membrane proteins were then analyzed using PMCA isoform-specific polyclonal and monoclonal antibodies (19, 43). In agreement with the findings on the intact tissue, the antibodies against the PMCA1 pump revealed that the intensity of the band at 135 kDa (Fig. 3), corresponding to the CI splice variant, remained approximately constant during the 5–9 days of the maturation process, whereas that of the CHI variant, which was only faint at plating time, increased very significantly (129 kDa; Fig. 3, \(1N\)). The expression of the PMCA2 (bands at 127–132 kDa; Fig. 3, \(2N\)) and PMCA3 (bands at 127–127 kDa; Fig. 3, \(3N\)) proteins, which was insignificant at the time of plating, also increased markedly during the same time. A weak band of higher mobility that was likely to be the 127-kDa PMCA2CI splice variant always accompanied the more prominent PMCA2 band at 132 kDa (Fig. 3, \(2N\)). A rather weak band corresponding to the PMCA4 protein was visible 2 days after plating (i.e., after 2 days in high KCl), the intensity of which, in contrast to the finding on the intact tissue, did not change during the KCl-induced maturation process (Fig. 3, \(JA9\)).

Granule cells that had survived for up to 5 days in low KCl
The amounts of the PMCA2 and PMCA3 proteins became significantly higher in cells cultured for 5 days in 25 mM KCl (Fig. 4, lanes 3 and 4), whereas the amount of PMCA4 decreased (Fig. 4, lane 3, JA9). If cells cultured in high potassium for 5 or 6 days were switched to the low KCl medium for 2 or 1 extra day, respectively, an evident decrease of the previously up-regulated PMCA2, PMCA3, and PMCA1CII proteins occurred (Fig. 4, lanes 4-6).

In the case of PMCA1, 2, and 3 an accumulation of PMCA-specific proteolytic products was observed (Fig. 4, bands indicated by the asterisks). As for the PMCA4 protein, once down-regulated for 5 days in high KCl, it did not come back if the cells were switched to the low KCl medium for 2 or 1 extra day, respectively (Fig. 4, JA9, lanes 4 and 5). Longer times after the switching were not tested because the neurons did not survive. No changes in the amount of the SERCA pump were observed in cells cultured in low or high KCl or switched to a low KCl medium after culturing (Fig. 4, SERCA, lanes 1-5).

Additional details on the pump isoforms, in particular on the alternatively spliced variants, were sought by performing PCR runs. Cells were cultured in the presence of 25 mM KCl for 5 days, i.e. until the isoform changes had become evident in the Western blots (Fig. 3). Total RNA was prepared from the cells, reverse transcribed and analyzed for alternative splicing at sites C and A (see Fig. 5 for a summary of the size of the expected products). In the case of splice site C, three PMCA1 isoforms were identified, whereas two PMCA1 isoforms were identified at site A (Fig. 3). The Western blots were developed with NBT/BCIP as described under "Materials and Methods."
bands were detected (Fig. 5A, lane 1) at 260, 350, and 410 bp, corresponding to the C-terminally spliced PMCA1CI, 1CIII (or CIV), and 1CII variants (17). In agreement with observations at the protein level (Fig. 3), 1CII, which corresponds to the shorter version of the PMCA1 pump, was the most prominent PMCA1 transcript (17, 21). The PMCA2 band at 320 bp was the PMCA2CI isoform, whereas the weak band at 550 bp corresponded to the PMCA2CII variant (Fig. 5A, lane 2). The two PMCA3 bands were the 3CI (210 bp) and 3CII (360 bp) products (Fig. 5A, lane 3). As for PMCA4, only the 270-bp CI isoform band (Fig. 5A, lane 4) was visible. A very weak band at 530 bp, corresponding to the 4CI isoforms, was only detected in Southern blots (not shown, but see Fig. 6B). The band of the AI isoform of PMCA2 was observed at 238 bp (Fig. 5B, lane 1), the two bands of the AI and AII isoforms of PMCA3 were detected at 288 and 330 bp (Fig. 5B, lane 2) and one band, corresponding to the AI isoform of PMCA4 at 385 bp (Fig. 5B, lane 3). These findings agree with previous observations in brain cells (21, 34, 35, 44). The identity of these isoforms was verified by Southern blotting and sequencing (not shown).

Effect of Depolarization on the PMCA Transcripts—Northern blotting analysis indicated that after 5 days, a KCl-dependent increase in the level of transcripts for isoforms 2 and 3 occurred (Fig. 6A). Competitive PCR experiments confirmed this observation (results not shown). In the case of PMCA1, cells cultured in high KCl showed a lower amount of the shorter (5000 nt) transcript (Fig. 6A, lane 1), whereas no changes were visible for the longer (7000 nt) transcript. Two distinct PMCA1 mRNAs had been previously observed in rat tissues (45). A signal specific for PMCA4 could only be detected after longer exposure of the autoradiogram. It showed a small but reproducible decrease in cells cultured for 5 days in the presence of high KCl.

The effect of KCl on alternative splicing was analyzed by using the oligonucleotides described above (Fig. 5). The CI was the most prominent PMCA1 transcript at plating time or in cells grown in 5.3 mM KCl for 3 or 5 days (Fig. 6B, lanes 1, 2, and 4), whereas the high KCl medium induced an evident up-regulation of the 1CII transcript (Fig. 6B, lanes 3 and 5). Although the overall level of the PMCA4 transcripts was low and thus demanded a prolonged PCR, an additional splicing product (PMCA4CII) could be clearly seen in low potassium (Fig. 6B, lane 6); much lower amounts of the PMCA4 transcripts were detected in cells cultured in high potassium (Fig. 6B, lane 7). The KCl depolarization-mediated decrease of the
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FIG. 6. KCl dependence of the transcription of the PMCA pumps. A, 2 μg (lanes 1 and 2) or 11 μg (other lanes) of total RNA from cells incubated for 5 days in the presence of 5.3 (lane 3) or 25 mM KCl were separated on formaldehyde agarose gels. A portion of the gel was stained with ethidium bromide (lanes 1 and 2). The size of ribosomal RNA is indicated on the left. The remainder of the gel was transferred to Nytran and hybridized with \(^{32}\)P-random-primed labeled cDNA probes specific for the pump isoforms (indicated at the bottom). The upper portion of the blot contained the signal for the PMCA isoforms (the mRNAs for the pumps migrated between 5000 and 8000 nt) was exposed for 6 days (PMCA2 and PMCA3) or for 10 days (PMCA1 and PMCA4). As a control for the loading and integrity of the mRNA, the probes were hybridized with G3PDH (signal at 1800 nt) cDNA and exposed for 2 days. Only the lower portions of the Northern blots are shown for G3PDH. For PMCA1 and PMCA4, the blots that are shown were incubated with the specific probes for G3PDH and PMCA1 or for G3PDH and PMCA4 together. In the case of PMCA2 and PMCA3, the blots were incubated with the PMCA probe, stripped, and then incubated with the probe for G3PDH. Performing the hybridization with the probes together or sequentially did not influence the results. B, reverse transcription PCR of total cell RNA from cells performed as described under “Materials and Methods” and in the legend for Fig. 5 with oligonucleotides specific for the PMCA1 (lanes 1–5) or PMCA4 (lanes 6–9) pumps. The cells were cultured in the presence of 5.3 mM (lanes 1, 2, 4, 6, and 8) or 25 mM KCl (lanes 3, 5, 7, and 9) for 0 (lane 1), 3 (lanes 2 and 3), or 5 (lanes 4–9) days. PCR products were separated on PAGE gels and stained with ethidium bromide. DNA molecular mass markers are given on the left side of the gels. The splicing variants are indicated by arrows. The identity of the bands was confirmed by Southern blotting and DNA sequencing. C, reverse transcription PCR of total RNA obtained from cells cultured for 5 days in 5.3 mM KCl. The plasma membrane of the cells was depolarized for 30–40 min with 75 mM KCl (lanes 2, 4, and 6) or incubated with 75 mM NaCl (lanes 1, 3, and 5). The PCR samples were processed as described under “Materials and Methods” and in the legend for Fig. 5. Oligonucleotides specific for c-fos, lanes 1 and 2; for PMCA4, lanes 3 and 4; and for PMCA1, lanes 5 and 6. The c-Fos was used as positive control for effect of the KCl mediated of gene expression (1).

PMCA4 transcript was confirmed by the A-splice-specific oligonucleotide (Fig. 6B). A higher amount of the PMCA4-specific product was observed when cells were cultured in low KCl (Fig. 6B, lane 8) than in high KCl (Fig. 6B, lane 9).

Short exposures of IMR32 cells to KCl had been shown to produce changes in the splicing variants of the PMCA2 isoforms (46). Similar reverse transcription PCR experiments on granule cells have failed to show changes in the PMCA2 and PMCA3 splicing pattern at sites A and C (results not shown). The isoform switch of PMCA1 was only observed after 2–3 days in high KCl: no changes were induced by short time depolarization (Fig. 6C lanes 5 and 6). However, the RNA of isoform PMCA4CII started to be down-regulated by a short treatment (30–40 min) with high KCl (Fig. 6C, lanes 3 and 4).

The expression of the pump isoforms is dependent on Ca\(^{2+}\) influx—25 mM KCl causes partial depolarization of the plasma membrane, promoting the influx of Ca\(^{2+}\) into granule cells (4). The free Ca\(^{2+}\) concentration in cells cultured in 25 mM KCl was indeed significantly higher than in cells cultured in 5.3 mM (149 ± 30 versus 58 ± 7 nM, see also Fig. 7A, upper panel). Both cell types showed typical and very large Ca\(^{2+}\) transients upon further depolarization with 75 mM KCl, although the effect was smaller in cells that had already been partially depolarized by 25 mM KCl (Fig. 7A, upper panel). Nifedipine abolished both the increase of [Ca\(^{2+}\)], induced by 25 mM KCl and the much larger Ca\(^{2+}\) transient induced by 75 mM KCl: the number of viable cells after 5 days of culturing with nifedipine was similar to that of cells incubated with low KCl, i.e. about 60–70%. The treatment with nifedipine (Fig. 7A, lower panel) abolished the 25 mM KCl-dependent up-regulation of the PMCA2, PMCA3, and PMCA1CII pumps and the down-regulation of the PMCA4 pump (not shown). Cadicludine (another \(\alpha\)-type Ca\(^{2+}\) channel inhibitor (47)) behaved essentially like nifedipine (not shown). Under these conditions, the glutamate-operated Ca\(^{2+}\) channel was probably inactive, because the NMDA channel inhibitor MK801 did not affect the expression of the pumps (Fig. 7A, lower panel, lane 4). It did, however, completely inhibit Ca\(^{2+}\) transients induced by the addition of the excess glutamate (not shown).

Ca\(^{2+}\) may also enters into granule cells through glutamate-activated NMDA channels if high concentrations of NMDA are present in the culture medium. 140 μM NMDA in the presence of only 10 mM KCl have been found to support the survival of granule cells (11). Culturing the granule cells in 140 μM NMDA and 10 mM KCl increased the intracellular Ca\(^{2+}\) over the levels found in cells cultured in 10 mM KCl alone (110 ± 14 nM versus 41 ± 6 nM). Consistently, 140 μM NMDA decreased the concentration of KCl needed to up-regulate the expression of PMCA1CII, PMCA2, and PMCA3 pumps (Fig. 7B, compare lanes 1–5 with lanes 6–10). At the same time, NMDA down-regulated the expression of the PMCA4 pump (Fig. 7C). These effects, however, were partially inhibited by nifedipine (Fig. 7C, lane 3), indicating that the 10 mM KCl, which was included to remove the Mg\(^{2+}\) block on the NMDA receptor (15), promoted a (limited) entry of Ca\(^{2+}\) through \(\alpha\)-type channels. The up-regulation of the PMCA pump isoforms in the presence of 140 μM NMDA and 10 mM KCl thus resulted from the entry of Ca\(^{2+}\) through NMDA receptors and \(\alpha\)-type channels. In agreement with these observations, stepwise increases in the concentration of KCl up-regulated the expression of the PMCA1CII, PMCA2 and 3 pumps in a concentration-dependent way (Fig. 7B). The use of potassium concentrations higher than 50–75 mM resulted in a marked reduction of the number of cells (15 ± 5% at 100 mM KCl) and in the disappearance of the PMCA2 and 3 pumps (not shown). In contrast to the PMCA pumps, the SERCA2b pump was practically insensitive to the changes in the cellular Ca\(^{2+}\) free concentration.

Effect of extracellular Ca\(^{2+}\) on the expression of the pump—The results in the preceding section had shown that the increased penetration of Ca\(^{2+}\) induced by the partial depolarization—...
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Fig. 7. Effect of Ca\textsuperscript{2+} influx on the expression of the PMCA pump isoforms. A, upper panel, measurement of the intracellular Ca\textsuperscript{2+} concentration in granule cells cultured for 5 days in 5.3 or 25 mM KCl or in 25 mM KCl and 10 \mu M nifedipine (25 nif). Open columns, resting Ca\textsuperscript{2+} concentration; closed columns, peak cytosolic Ca\textsuperscript{2+} concentration after the addition of 75 mM KCl. The Ca\textsuperscript{2+} concentrations (in nm) are given under the columns. Lower panel, 25 \mu g of crude membrane proteins from granule cells cultured for 5 days in the presence of 5.3 mM KCl (lane 1), of 25 mM KCl (lane 2), of 25 mM KCl plus 10 \mu M nifedipine (lane 3), or of 25 mM KCl plus 1 \mu M MK801 (lane 4) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with PMCA isoform-specific antibodies. The immunocomplexes were visualized with NBT/BCIP. The 0.1% dimethyl sulfoxide used as the solvent for nifedipine did not affect either the viability of the cells or the expression of the pumps. B, Western blot analysis of the expression of PMCA pump isoforms in cells cultured in different concentrations of Ca\textsuperscript{2+} in the absence or presence of 140 \mu M NMDA. The gel shows PMCA1, PMCA2, PMCA3, and SERCA2b proteins in 20 \mu g of crude membrane proteins from granule cells cultured for 5 days in the presence of 5.3 mM KCl (lane 1), 10 mM KCl (lane 2), 15 mM KCl (lane 3), 25 (lane 4), and 50 mM KCl (lane 5). Lanes 6–10 are like lanes 1–5, except that 140 \mu M NMDA was present in the culture medium. The immunocomplexes were visualized by CDP-Star™ as described under “Materials and Methods.” The asterisk indicates a nonspecific reaction of the SERCA antibody. C, nifedipine suppresses the effect of NMDA on PMCA pump expression. 20 \mu g of proteins from crude membranes of granule cells were used. Cells cultured in 10 mM KCl (lane 1), 10 mM KCl/140 \mu M NMDA (lane 2), and 10 mM KCl/140 \mu M NMDA/10 \mu M nifedipine (lane 3) were separated by SDS-PAGE and transferred to polyvinylidene difluoride filters. The immunocomplexes were visualized by NBT/BCIP. D, influence of extracellular Ca\textsuperscript{2+} on the expression of the PMCA2 and PMCA3 isoforms. 20 \mu g of crude membrane proteins from granule cells were separated by SDS-PAGE and transferred to nitrocellulose. They came from cells cultured for 5 days in 25 mM KCl in the presence of an extracellular concentration of 0.5 (lane 1), 1.8 (lane 2), 3.6 (lane 3), 5 (lane 4), or 10 (lane 5) mM Ca\textsuperscript{2+} for 3 days in 25 mM KCl in the presence of 1.8 mM Ca\textsuperscript{2+} followed by 2 days in 25 mM KCl, 2 mM EGTA (lane 6). The immunocomplexes were visualized by CDP-Star™ as described under “Materials and Methods.” The asterisk indicates a nonspecific reaction of the SERCA antibody.
isoforms in cultured granule cells. 200 µg of crude membrane proteins from cells cultured for 5 days in 25 (+) or 5.3 (−) mM KCl were phosphorylated with 0.3–0.4 µM [γ-32P]ATP (300 Ci/mmol) in the presence of Ca2+ and La3+ (see under “Materials and Methods”). Three changes were only observed in the presence of depolarizing concentrations of KCl. These changes were identical to those observed in postnatal cerebellum. In the presence of high concentrations of KCl, they exhibit the typical morphological changes of the fully mature granule neurons (e.g., formation of synapses and migration to form aggregates). The development of active synapses has been suggested to be critical to the survival of the cells in vivo because the synaptic connections would provide cells with the continuous extra-depolarization that is mimicked by the incubation with high KCl in vitro (7, 14) It may also be speculated that this low level of depolarization would keep the enzymes responsible for apoptosis repressed. Indeed, once committed to full maturation by high KCl the cells cannot be switched back to low KCl without undergoing fast apoptotic death.

The up-regulated pump isoforms (PMCA2, PMCA3, and PMCA1CII) that have been detected in granule cells are those typical of adult brain (17, 19, 21, 49). The most significant increase was that of PMCA2, the increase of which reached a plateau of at least 30% of the total pump protein after 7 days in high KCl. The up-regulation was a relatively slow process: short term (1–2 h) exposures to KCl concentrations of up to 75 mM failed to induce it. This, however, was not true of PMCA4 (the CII variant), the down-regulation of which by high potassium followed in a reverse fashion the kinetics of up-regulation of c-fos. The lower amounts of total PMCA4 protein in cells cultured in high potassium was consistent with the recent finding of the virtual absence of one of its major splicing isoforms, PMCA4CII, from adult rat cerebellum (43).

The finding that the changes in the expression of the PMCA pumps during maturation induced by 25 mM KCl were abolished by nifedipine clearly indicated that the effects were linked to the entry of Ca2+ through L-type channels. The Ca2+ imaging experiments have provided direct demonstration of this: after 5 days in 25 mM KCl, cells contained up to 3 times more Ca2+ than those grown in 5.3 mM KCl. Although the essential process controlled by Ca2+ evidently was the transcription of the PMCA genes, other yet unidentified posttranscriptional and/or posttranslational Ca2+ sensitive mechanisms may also have played a role in the effects described. For instance, the stability of the pump mRNA and/or its translation efficiency could also be specifically affected by a Ca2+-dependent mechanism. The stability of the translated protein could also become modified during the cell maturation process. Although these considerations may be valid for the three up-regulated isoforms, the much faster kinetics of the response of PMCA4 is very likely to reflect a different mechanism of regulation by Ca2+.

At the end of the maturation process, the total amount of PMCA protein, despite the down-regulation of isoform 4, was markedly increased. Because both 25 mM KCl and the NMDA-glutamate receptor raise [Ca2+]i, it would be logical to imagine that the increase reflects the need of the cells to deal with the augmented level of cytosolic Ca2+. Despite the PMCA up-regulation, cell Ca2+ nevertheless increased during the maturation process: evidently, the increased influx of Ca2+ into the cells offset their increased exporting ability. This apparent paradox could be rationalized by assuming that the maturation process could demand the increase of intracellular Ca2+ (perhaps to regulate the transcription of the genes). The up-regulation of the pumps, considering that their pumping ability only becomes optimal at 200–300 nm Ca2+, would be a safety device to prevent Ca2+ from rising to levels incompatible with the normal life of the cells.

Observations on Ca2+-mediated gene expression have so far mostly concerned immediate early genes, the induction of which can be detected minutes to hours after stimulation. In the case of the c-fos gene, the increase in transcription has been detected minutes after the initiation of depolarization; the effect disappears after 1–2 h (50). As mentioned above, an effect of this type may have prevailed in the down-regulation of the expression of PMCA4CII. In the case of the PMCA2 pump isoform, an effect of the early gene type was observed in a neuroblastoma cell line (46): depolarization by KCl induced a
witch in the isofrm pattern, linked to an alternative splicing change at splice site A. Because no isofrm switch of this type was observed in granule cells, some of these gene induction processes may be cell specific. This would be consistent with the observation that in the neuroblastoma cell line mentioned above no changes were observed at the more commonly used C splice site (46). It is also consistent with the finding that PMCA4 was rapidly down-regulated in maturing granule cells but not in intact cerebellum. In the latter, PMCA4 in other cell types evidently obscured the down-regulation in the granules. The up-regulation of the PMCA pump isoforms was not an unexpected specific of the increased concentration of KCI in the medium, because PC-12 and P-19 neurons treated under similar conditions failed to show changes in the pattern of PMCA pump expression.

The changes in the PMCA isoforms pattern are likely to reflect the demand of cells in terms of specific functional aspects of Ca\(^{2+}\) pumping. After 7–9 days in culture, a major portion of the total pump protein in granule cells was represented by the PMCA2 (and PMCA3) proteins. The analysis of the phosphoenzyme intermediate showed that the increase of the PMCA2 protein largely paralleled that of the total pump activity. Even after 5 days of depolarization, when the maturation of the cells was still incomplete (7, 8), at least 30% of the total phosphoenzyme of the PMCA pumps was that of the PMCA2 isoform. Because this isoform has the highest affinity for calmodulin of all PMCA pumps (51), its increase may be useful in situations in which the latter would become limiting. Because the Ca\(^{2+}\)-calmodulin-PMCA complex is the active state of the pump, the increased affinity for calmodulin should also result in the improved response of the pump to lower concentrations of Ca\(^{2+}\) (51). After 5 days in high KCl, the contribution of PMCA3 to the total cell phosphoenzyme was quantitatively minor, but this was expected because the PMCA3 pump was only expressed at optimal levels at later times, i.e. after 7–9 days in culture. Unfortunately, the functional consequences of the increase of the PMCA3 pump cannot be assessed, because this pump isoform has not yet been functionally characterized.

Acknowledgments—We are indebted to Dr. F. Wuytack (Leuven, Belgium) for the polyclonal antibody against the SERCA2b pump. We thank Dr. P. Nicotera (Konstanz, Germany) for numerous stimulating discussions and Dr. J. T. Penniston (Mayo Clinic, Rochester, MN) for the JAS monoclonal antibody.