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The Expression of Plasma Membrane Ca^{2+} Pump Isoforms in Cerebellar Granule Neurons Is Modulated by Ca^{2+}*

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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 L-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. L-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 may be increased by the depolarization of the plasma membrane (1, 4). The procedure increases the influx of Ca^{2+} and thus raises its cytosolic concentration (4).

Cerebellar granule cells are frequently used to study physiological (*e.g.* development (7, 8)) and pathological (*e.g.* ischemic damage (9) and apoptosis (10)) aspects of nervous cell function. When isolated from the cerebella of newborn rats, they develop to mature neurons upon exposure to depolarizing concentrations of KCl (25 mM) or to the agonist of the glutamate receptor NMDA[1] (11). The expression of a number of genes is stimulated during the maturation process (12–16), and a complex switch in the expression of the NMDA receptor isoforms has been documented (14, 15).

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 the down-regulation of the isoforms depended on the sustained increase of intracellular Ca^{2+}.

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[1] The abbreviations used are: NMDA, *N*-methyl-D-aspartic acid; PMCA, plasma membrane Ca^{2+} ATPase; SERCA, sarcoplasmic/endoplasmic reticulum calcium; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MEM, minimum Eagle's medium; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; nt, nucleotide; PCR, polymerase chain reaction; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate.

MATERIALS AND METHODS

Chemicals—Poly-D-lysine, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Dulbecco's modified Eagle's medium, Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture, and other tissue culture supplements were form Sigma or Life Technologies, Inc. Ditocilpine (MK-801) was from Research Biochemicals, Inc. (Natick, MA). NMDA, nifedipine, and calcicludine were from Calbiochem. Oligonucleotides were purchased from MGW-Biotech, (Ebersberg, Germany). Ampli-*Taq* 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 Dulbecco's modified Eagle's medium (Hepes modification (Sigma), containing 1.8 mM $CaCl_2$) supplemented with heat-inactivated 10% fetal calf serum (Life Technologies or Sigma), 100 μg/ml gentamicin, 7 μM *p*-aminobenzoic acid, 100 μg/ml pyruvate, 100 microunit/ml insulin, on poly-D-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 48 h, 10 μM cytosine arabinofuranoside was added to the culture to inhibit mitotic active cell growth. After 3 additional days, the medium was replaced with serum-free Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (Sigma), containing 100 μg/ml transferrin, 20 nM progesterone, 50 units/ml penicillin, 50 μg/ml streptomycin, 5 μg/ml insulin, and 0.11 mg/ml pyruvate in the presence of 5.3 or 25 mM KCl. Neuronal survival was estimated by measuring the amount of colored formazan by the reduction of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (24). To this aim, the culture medium was replaced by Locke solution (134 mM NaCl, 4 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 10 mM Hepes, pH 7.5) 150 μg/ml 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, containing either 5.3 or 25 mM KCl and incubated for 15 min at 37 °C. The extent of contaminating astrocytes was estimated by immunocytochemistry using a glial fibrillary acidic protein-specific monoclonal antibody (Boehringer Mannheim). Immunocytochemistry has been performed as described earlier (25). The cells were in some cases incubated with 1 μg of calcein-AM/ml and 5 μg propidium jodide/ml (both from Molecular Probes, Eugene, OR) for 10–15 min at 37 °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).

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.

P-19 cells were routinely cultured in α-MEM (Life Technologies) supplemented with 2.5% fetal calf serum and 7.5% calf serum (both from Life Technologies). They were exposed to 0.3 μM retinoic acid (26) at a density of 10^5cells/ml and transferred to uncoated Petri dishes. After 2 days, cell aggregates were collected, reexposed to retinoic acid for 2 additional days, sedimented, and plated in medium without retinoic acid. After 2 days, 20–40% of the cells extended long neurites and could be kept in culture for up to 9 additional days.

Preparation of Membranes from Cerebellum—Cerebella 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 $\times$ *g* for 10 min at 4 °C. The supernatant was again centrifuged at 100,000 $\times$ *g* for 1 h at 4 °C. The 100,000 $\times$ *g* precipitated material was defined as the microsomal fraction.

Preparation of Membrane Proteins—Cells were resuspended at 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 freezing and thawing. 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.

Western Blotting—Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (27) and transferred to nitrocellulose or polyvinyldene 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) Polyclonal sera (donated by Dr. F. Wuytack, Leuven, Belgium) were used at a 1/100 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 1/2000. The blots were developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT). The reaction was stopped after 2–3 min for the SERCA2b antibody, after 5 min for the affinity-purified serum against the PMCA1, after 10 min for the affinity-purified antibodies for PMCA2 and PMCA3 and for the monoclonal 5F10 antibody, and after 15–25 min for the monoclonal antibody JA9. When the blots were incubated with CDP-Star™ (Tropix, Bedford, MA), they were exposed to the a chemiluminescence-sensitive photographic film. The times were 10–20 s for the SERCA2b antibody, 30–60 s for the affinity-purified serum against the PMCA1, 30–120 s for the affinity-purified antibodies for PMCA2 and PMCA3 and for the 5F10 monoclonal antibody, 60–120 s for the monoclonal antibody JA9. If needed, Western blots were scanned with Photoshop (Adobe Systems Inc.) and quantified with the help of the National Institutes of Health NIH Image program, Version 1.59.

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 Ca^{2+} plus 100 μM La^{3+}. La^{3+} was included to stabilize the phosphointermediate of the PMCA protein (30). The reaction was started with 0.3 μM [γ-^{32}P]ATP (300 Ci/mmol) on ice and stopped 30 s later by 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 H_2O and 0.5% SDS, 8 M 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 $\times$ *g* for 30 min. The supernatant was divided in four 250-μl aliquots. One was mixed with 2–3 μg of 5F10 monoclonal antibody (32) and the others with 3–6 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 centrifugation. The pellet was 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, twice with MEM buffer alone. The pellet was resuspended at room temperature and incubated for 30 min in 30–40 μl of 70 mM Tris-PO_4, pH 6.4, 5% SDS, 5% dithiothreitol, and 8 M urea, separated on acidic gels (31), stained with Coomassie Brilliant Blue, and dried prior the exposure to x-ray films (−70 °C for 2–5 days). The films were scanned with Adobe Photoshop and quantified with the help of NIH Image, Version 1.59. In some cases, they were scanned densitometrically.

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 KCl, 1.3 mM $MgCl_2$, 0.9 mM $CaCl_2$. 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 Adamo and Penniston (34) were used for PMCA2, and those described by Keeton *et al.* (35) were used for PMCA4. For PMCA3, the following oligonucleotides were used: 5′-GCGCAAATCAGCAGACAAAG (nt 1448–1467, rat PMCA3) and 5′-TGCAGGACTGACTTCTCCTTC (nt 1715–1735, rat PMCA3). The glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) cDNA was amplified from rat tissue with the following oligonucleotides: 5′ CCAAAAGGGTCATCATCTCC (nt 371–391) and 5′ GTAGGCCATGAGGTCCACCAC (nt 994–1015). The oligonucleotides for c-*fos* were derived from the rat sequence (36) and were as follows: Fos-1, AAGTCTGCGTTGCAGACCGAG (nt 660–680); Fos-2, GTCTGCTGCATAGAAGGAACC (nt 1040–1020).

The conditions for the PCR were as suggested by Perkin-Elmer for the *Taq* Gold polymerase. The PCRs were performed under none saturation conditions, as described by Stauffer *et al.* (21) PCR standard

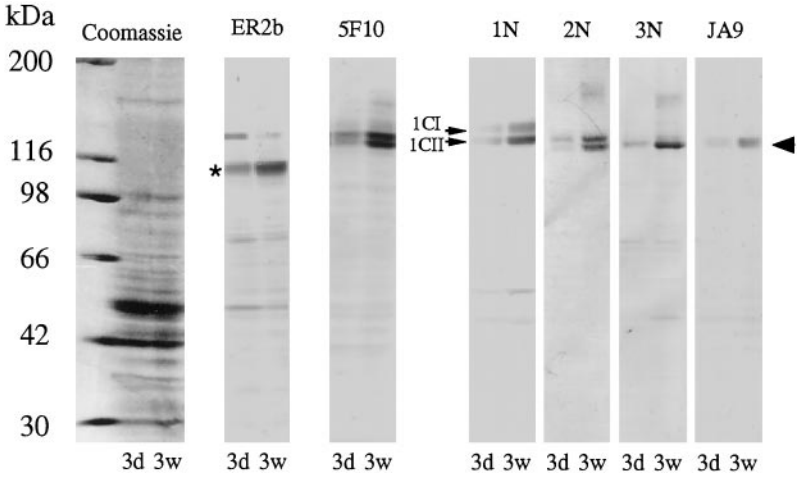


FIG. 1. **Expression of PMCA pump isoforms in cerebellum.** 15–20 μg of proteins from crude synaptosomal membranes prepared from the cerebella of 3-day-old (*3d*) or 3-week-old (*3w*) rats were separated by SDS-PAGE. The portion of the gel containing the markers was stained with Coomassie Brilliant Blue, and the remainder of the gel was transferred to nitrocellulose and probed with a polyclonal serum against the SERCA2b pump (*ER2b*); with affinity-purified polyclonal antibodies against the PMCA1 (*1N*), the PMCA2 (*2N*), and the PMCA3 pumps (*3N*), with a monoclonal antibody that recognizes rat PMCA4 pumps (*JA9*) (43); and with a monoclonal antibody (32) that recognizes all PMCA pump isoforms (*5F10*). The molecular mass of the protein markers is indicated on the left. The position of SERCA2b is indicated by an *asterisk* and that of PMCA1CI and PMCA1CII by *arrows* at the *left*, and the positions of of PMCA2CII, 3, and 4 are indicated by the *thick arrow* at the right. PMCA2CI comigrated with PMCA1CII. The blots were developed with NBT/BCIP as described under "Materials and Methods."

conditions were as follows: 12 min at 95 °C, 33 cycles of 1 min at 50–53 °C, 2 min at 72 °C, 30 s at 93 °C. The PCR products were separated on 1.5% agarose or by 8% PAGE. The identity of the PCR-generated fragments was verified by Southern blotting using oligonucleotides specific for the rat PMCA pump isoforms and by partially sequencing the PCR fragments cloned in the pGEM-T vector (Promega). Northern blotting was performed as described by Sambrook *et al.* (37). The following cDNA fragments were random primed labeled with ^{32}P-dCTP: PMCA1CI, 3546–3805 (17); PMCA2CI, 3663–3987 (38); PMCA3CI, 3813–4024 (17); PMCA4CI, 3456–3727 (17, 35); G3PDH, 371–1015 (39); and c-*fos*, 660–1040 (36). Competitive PCR experiments with PMCA2-, PMCA4-, and PMCA1-specific oligonucleotides were performed according to Siebert and Larrick (40): they essentially confirmed the Northern blotting experiments. The Northern and Southern blots were scanned with Adobe Photoshop and quantified with the help of NIH Image, Version 1.59. In some cases, the images were scanned densitometrically directly from autoradiographic films.

Measurement of Intracellular Free Ca^{2+} in Granule Cells—$[Ca^{2+}]_i$ was measured by videoimaging as described in Ref. 41. Briefly, granule cells grown on coverslips were loaded for 30 min with 2.5 μM Fura-2-AM (Molecular Probes). Before the analysis the cells were washed twice for 10 min with a buffer of the same ionic composition as the original growth medium and then left to equilibrate at room temperature on the stage of a Leica DM-IRB microscope (Leica AG, Benzheim, Germany) equipped with a Dage-72 CCD camera (Dage-MTI, Michigan City, MI), a videoscope GEN-III image intensifier, and a computer-controlled filter wheel (Sutter, Novato, CA). Images were acquired at λ_{ex} 340 or 380 nm and λ_{em} 505 nm and handled for further processing by an imaging system (Imaging Corp., St. Catherines, Ontario, Canada). For the determination of steady state $[Ca^{2+}]_i$ 30 ratio images were recorded during 90 s. The $[Ca^{2+}]_i$ was calculated by *in situ* calibration using the equation $[Ca^{2+}]_i = K_d \times (R - R_{min})/(R_{max} - R) \times S_{f2}/S_{b2}$, with K_d (25 °C) = 264 nM as described (42). For the determination of R_{min}, cells were washed twice with calibration buffer (120 mM NaCl, 25 mM Hepes, 15 mM glucose, 25 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA) and equilibrated for 10 min in calibration buffer supplemented with 5 μM ionomycin. For the calculation of R_{max} 5 mM Ca^{2+} and 10 μM ionomycin were added. Autofluorescence was measured after the addition of 10 mM $MnCl_2$.

The measurements with Fluo3 were performed as follows. Granule cells grown on glass coverslips were incubated in the original culture medium supplemented with 1 μM Fluo-3-AM for 10 min at 37 °C. They were washed for 5 min at 37 °C in a solution of the same ionic composition as the culture medium and then equilibrated in the same medium without Mg^{2+} and supplemented with 500 μM tetradotoxin, 20 μM glycine for 10 min at room temperature. Cells were stimulated with 50 μM glutamate, and images were collected using 488 nm excitation and 520 nm emission. Data from 20 neurons were recorded at 2-s intervals with the system described above.

RESULTS

Expression of the PMCA Pump in Cerebellum—The amounts of PMCA1CII, PMCA2, PMCA3, and PMCA 4 proteins varied markedly in synaptosomal preparations from the cerebella of 3-day-old as opposed to 3-week-old rats. The Western blots of Fig. 1 show that PMCA proteins were very poorly detectable 3 days after birth (Fig. 1, *3d lanes*) but become very prominent after 3 weeks (Fig. 1, *3w lanes*). In the case of PMCA1, the increase was particularly marked in the faster migrating band, corresponding to the PMCA1CII isoform (Fig. 1, *1N, CII*). The overall extent of the up-regulation was particularly evident when synaptosomal membrane proteins were incubated with an antibody that recognized all four PMCA isoforms (Fig. 1, *5F10*): the increased intensity of the 130-kDa PMCA band evidently reflected the increased expression of the PMCA1CII, PMCA2, PMCA3 and to a lower extent PMCA4 proteins. At the same time a reproducible, if less dramatic, increase of the SERCA pump was observed (Fig. 1, *ER2b*).

Time-dependent Expression of the PMCA Pump Isoforms in Granule Cells—Granule cells routinely prepared from the cerebella of 6–8-day-old rats mature *in vitro* if they were cultured in the presence of depolarizing concentrations of KCl (25 mM). They 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. 2*A*), 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. 2*B*). 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-

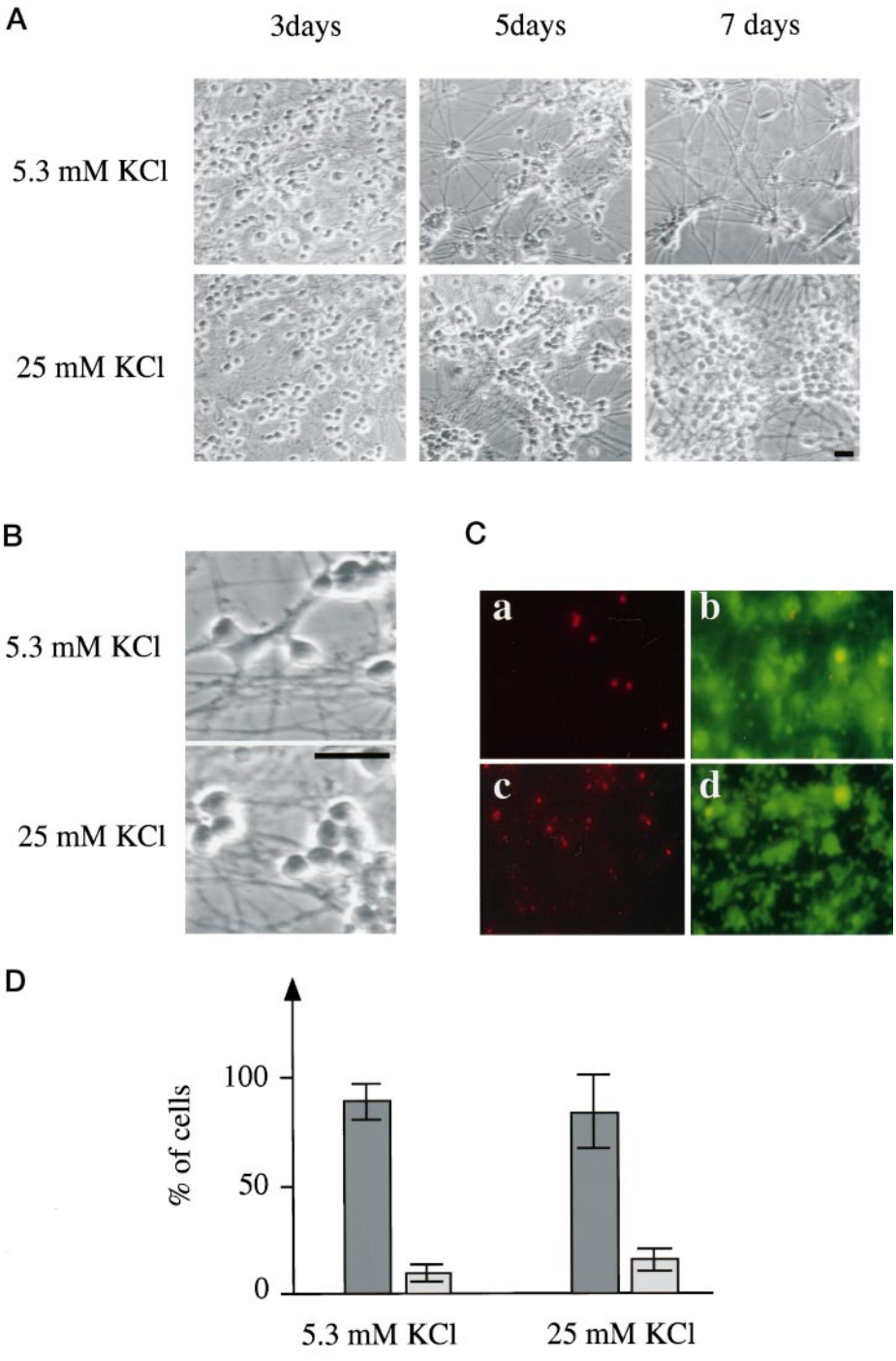


FIG. 2. **Viability of granule cells cultured in the presence of 5.3 or 25 mM KCl.** *A,* phase contrast microphotographs of cells cultured for 3, 5, and 7 days in 5.3 or 25 mM KCl. *Bar,* 10 μM. *B,* enlarged details of cells cultured for 5 days in 5.3 or 25 mM KCl. Notice the large numbers of dendrites surrounding the cells. *Bar,* 10 μM. *C,* cells cultured for 5 days in 5.3 (*a* and *b*) or 25 (*c* and *d*) mM KCl were incubated with propidium iodide (*a* and *c*) or calcein-AM (*b* and *d*) as described under "Materials and Methods." The images show the fluorescence at 617 nm (*a* and *c,* propidium bromide) and 517 nm (*b* and *d,* calcein). *D,* quantitation of images similar to those shown in *C*. 3000 cells were analyzed. The *dark gray columns* represent the percentage of living cells (stained by calcein), and the *light gray columns* represent the percentage of dead cells (stained by propidium iodide).

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 CII 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 PMCA2CII 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

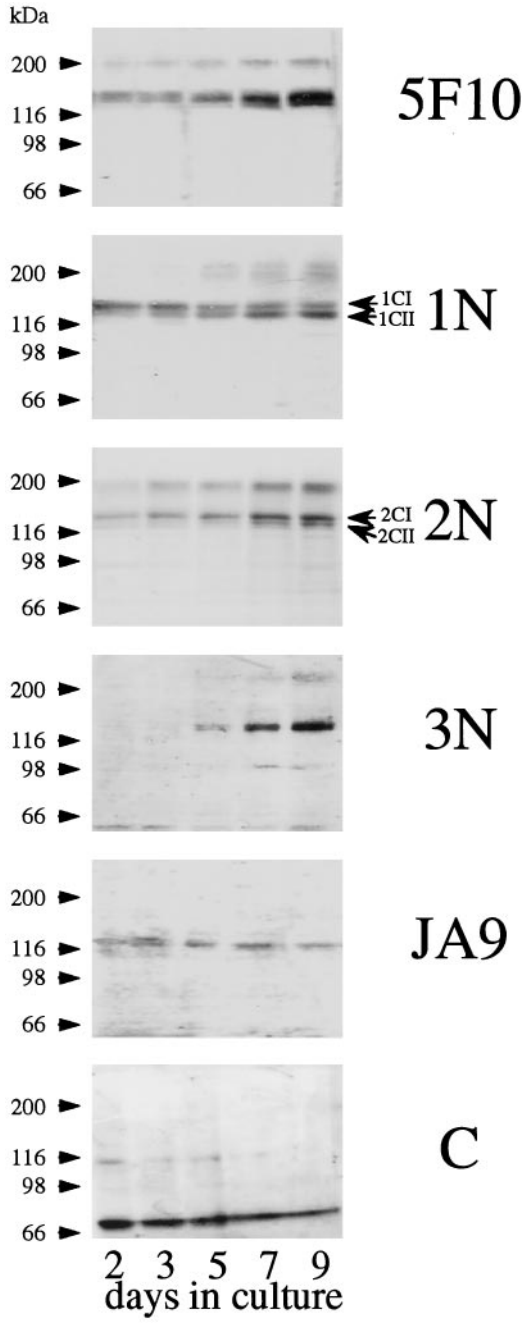


FIG. 3. **Expression of PMCA pump isoforms in granule cells.** 15–20 μg of crude membrane proteins from cells cultured in the presence of 25 mM KCl for 2, 3, 5, 7, and 9 days were separated by SDS-PAGE, transferred to nitrocellulose and incubated with the 5F10 monoclonal antibody, with affinity-purified PMCA1-specific (*1N*), PMCA2-specific (*2N*), or PMCA 3-specific (*3N*) antibodies, or with the monoclonal, PMCA4-specific JA9 antibody (43). An antibody specific for albumin was used as a control for the loading (*C*). The Western blots were developed with NBT/BCIP as described under "Materials and Methods." The predicted positions for the PMCA1CI and the 1CII proteins are indicated in the *1N* panel and those for the PMCA2CI and 2CII proteins in the *2N* panel.

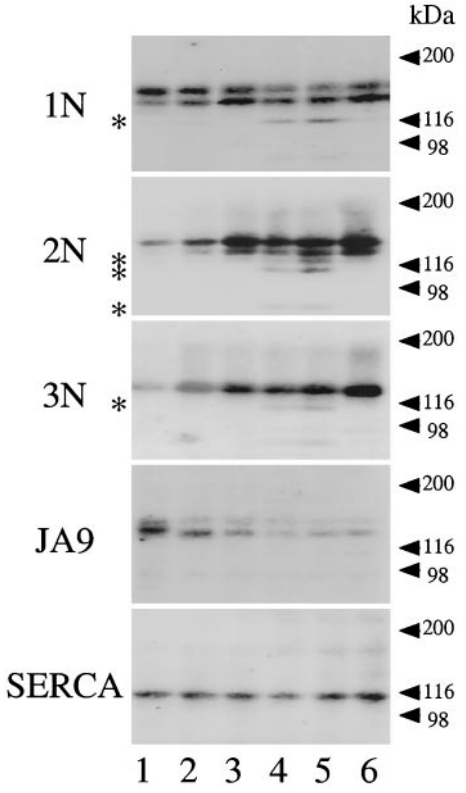
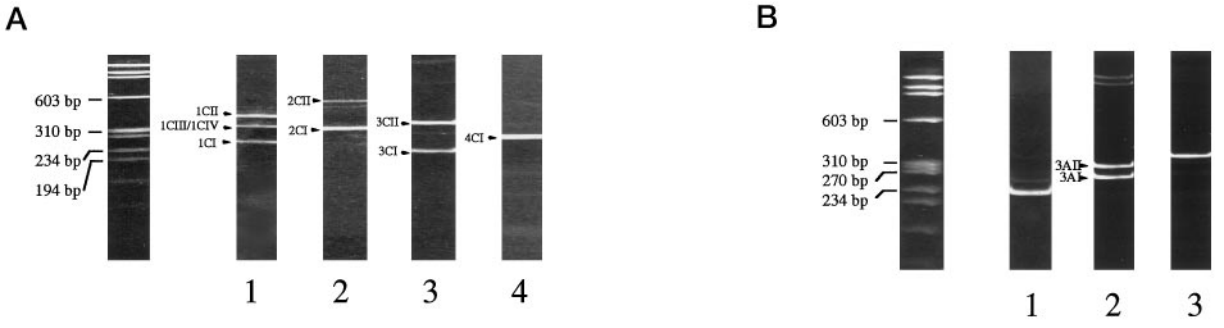
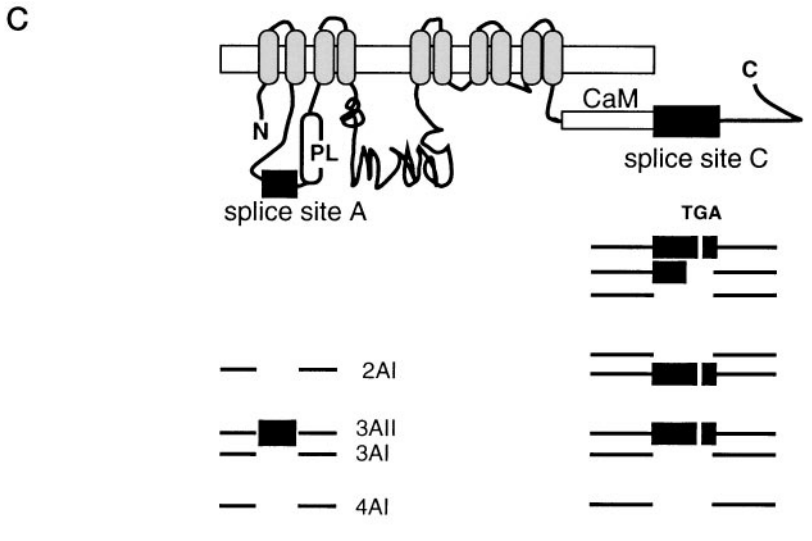


FIG. 4. **KCl concentration dependence of the expression of the PMCA pumps.** 15–20 μg of crude membrane proteins from cells cultured in 5.3 mM KCl for 3 (*lane 1*) or 5 (*lane 2*) days are compared with 15–20 μg of crude membrane proteins from cells cultured for 5 days in 25 mM KCl (*lane 3*). 15–20 μg of crude membrane proteins from parallel cultures of neurons kept for 5 days in the presence of 25 mM KCl and then switched to the low KCl medium (5.3 mM KCl) for 48 h (*lane 4*) or kept for 6 days in 25 mM KCl and then switched to the low KCl medium (5.3 mM KCl) for 24 h (*lane 5*).Gels of 15–20 μg of crude membrane proteins from cells cultured for 7 days in the presence of 25 mM KCl (*lane 6*) are also presented. Proteins were transferred to nitrocellulose after SDS-PAGE and analyzed with the PMCAspecific antibodies as described in Fig. 3. The panel labeled SERCA was incubated with a serum specific for the SERCA2b pump. The immunocomplexes were visualized with CDP-Star™ as described under "Materials and Methods."

contained minor amounts of PMCA2 and PMCA3 proteins (Fig. 4, *lanes 1* and *2*) and relatively high amounts of the PMCA1 and the PMCA4 proteins (Fig. 4, *lanes 1* and *2, panels 1N* and *JA9*), even if the amount of the latter tended to decrease somehow if culturing under low KCl was protruded for 5 days. The amounts of the PMCA2 and PMCA3 proteins became significantly higher in cells cultured for 5 days in 25 mM KCl (Fig. 4, *2N* and *3N, lane 3*). Under the same conditions, an increase was also observed for the PMCA1CII protein (Fig. 4, *1N, lane 3*), 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 1 or 2 days (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*C* for a summary of the size of the expected products). In the case of splice site C, three PMCA1





Isoform	Protein Predicted size in kDa	PCR products (in bp) Site A	Site C
1CII	129	none	410
1CIII/IV	138-139		350
1CI	135		260
2CI	132	238	320
2CII	127		550
3CII	131-133	330	360
3CI	126-128	288	210
4CI	129	385	270

FIG. 5. **The alternative splicing variants of the PMCA pumps present in granule cells.** *A* and *B,* total RNA from cells cultured for 5 days in 25 mM KCl was reverse transcribed and subjected to PCR with oligonucleotides specific for the PMCA isoforms. In the case of PMCA4, a longer (36 cycle) PCR had to be performed. Aliquots corresponding to 100–150 ng of DNA were then separated by PAGE and visualized by ethidium bromide. *Panel A* shows the PCR products obtained with oligonucleotides specific for splice site C of rat PMCA1 (*lane 1*), PMCA2 (*lane 2*), PMCA3 (*lane 3*), and PMCA4 (*lane 4*) pumps. The splice variants are indicated by *arrows*. *Panel B* shows the PCR products obtained with oligonucleotides specific for splice site A of rat PMCA2 (*lane 1*), PMCA3 (*lane 2*), and PMCA4 (*lane 3*) pumps. DNA molecular mass markers are given on the *left*. The splice variants of PMCA3 are indicated by *arrows*. *C,* the splice variants of the PMCA pumps found in granule cells. For sake of clarity, a topographical model of the pump is given at the *top* of the panel. Transmembrane domains are shown in *gray*. *PL,* acidic phospholipid binding domain; *CaM,* calmodulin binding domain. Splice sites A and C are indicated by the *black boxes;* note that a portion of the calmodulin binding domain overlaps splice site C. Details on the PMCA isoform nomenclature can be found elsewhere (55).

bands were detected (Fig. 5*A, 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. 5*A, lane 2*). The two PMCA3 bands were the 3CI (210 bp) and 3CII (360 bp) products (Fig. 5*A, lane 3*). As for PMCA4, only the 270-bp CI isoform band (Fig. 5*A, lane 4*) was visible. A very weak band at 530 bp, corresponding to the 4CII isoforms, was only detected in Southern blots (not shown, but see Fig. 6*B*). The band of the AI isoform of PMCA2 was observed at 238 bp (Fig. 5*B, lane 1*), the two bands of the AI and AII isoforms of PMCA3 were detected at 288 and 330 bp (Fig. 5*B, lane 2*) and one band, corresponding to the AI isoform of PMCA4 at 385 bp (Fig. 5*B, 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. 6*A*). 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. 6*A*), 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 1CI was the most prominent PMCA1 transcript at plating time or in cells growth in 5.3 mM KCl for 3 or 5 days (Fig. 6*B, lanes 1, 2,* and *4*), whereas the high KCl medium induced an evident up-regulation of the 1CII transcript (Fig. 6*B, 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. 6*B, lane 6*); much lower amounts of the PMCA4 transcripts were detected in cells cultured in high potassium (Fig. 6*B, lane 7*). The KCl depolarization-mediated decrease of the

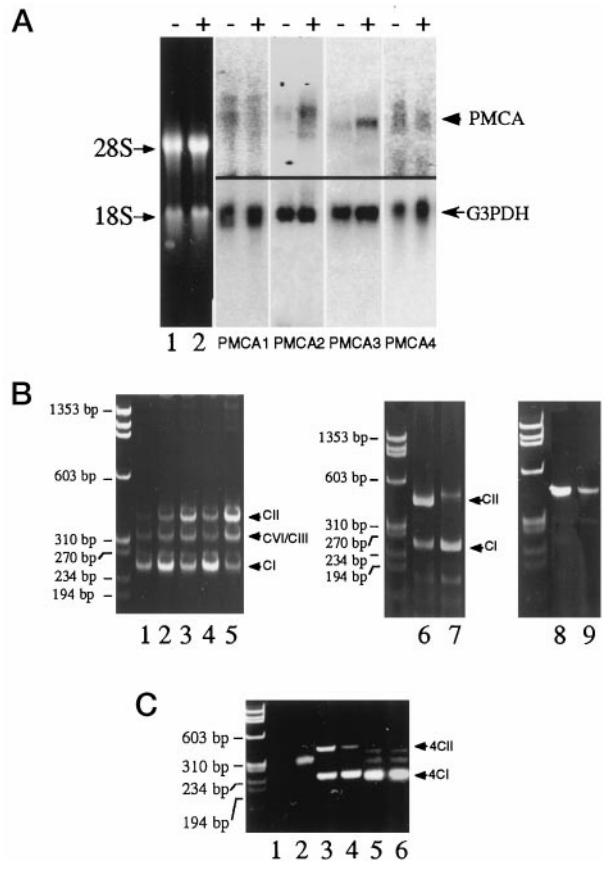


FIG. 6. **KCl dependence of the transcription of the PMCA pumps.** *A,* 2 μg (*lanes 1* and *2*) or 17 μg (*other lanes*) of total RNA from cells incubated for 5 days in the presence of 5.3 (−) or 25 (+) mM KCl were separated on formaldehyde agarose gels. A portion of the gel was stained with ethidium 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 blots containing the signal for the PMCA isoforms (the mRNAs for the pumps migrated between 5000 and 8000 nt) was exposed at −70 °C 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 blots 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. 6*B*). A higher amount of the PMCA4-specific product was observed when cells were cultured in low KCl (Fig. 6*B, lane 8*) than in high KCl (Fig. 6*B, 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. 6*C 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. 6*C, 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. 7*A, 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. 7*A, upper panel*). Nifedipine abolished both the increase of $[Ca^{2+}]_i$ 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. 7*A, 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). Calcicludine (another L-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. 7*A, 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. 7*B,* compare *lanes 1–5* with *lanes 6–10*). At the same time, NMDA down-regulated the expression of the PMCA4 pump (Fig. 7*C*). These effects, however, were partially inhibited by nifedipine (Fig. 7*C, 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 L-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 L-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. 7*B*). 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 depolariza-

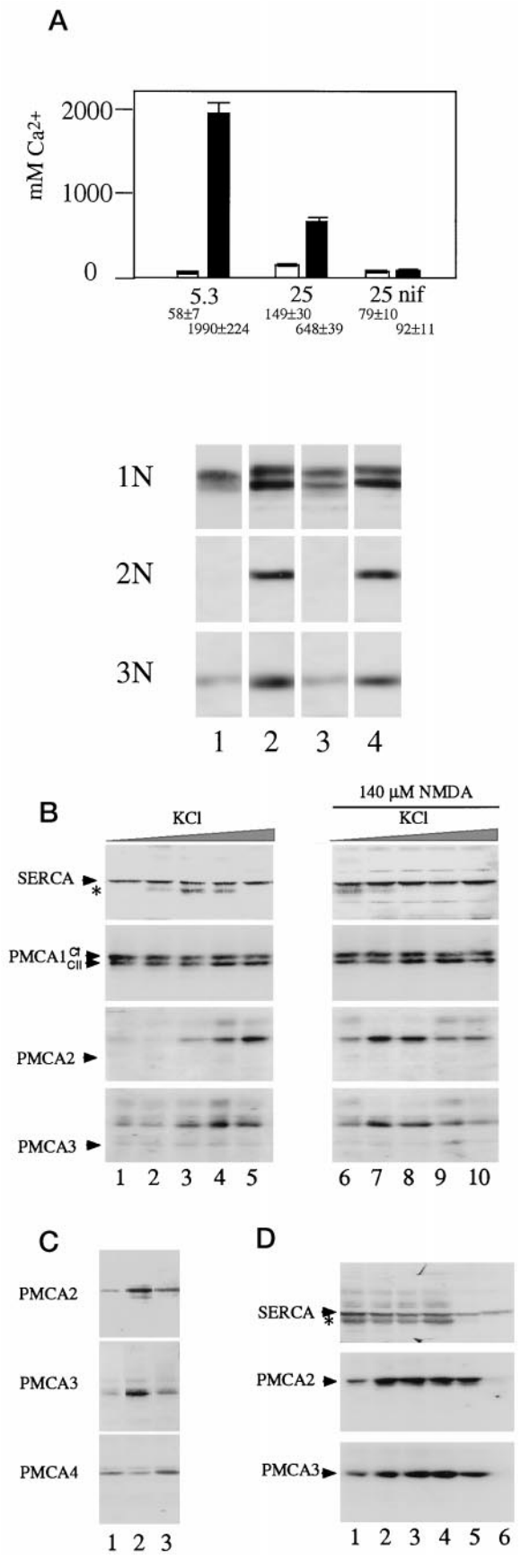


FIG. 7. **Effect of Ca^{2+} influx on the expression of the PMCA pump isoforms.** *A, upper panel,* measurement of the intracellular Ca^{2+} concentration in granule cells cultured for 5 days in 5.3 or 25 mM KCl or in 25 mM KCl and 10 μM nifedipine (*25 nif*). *Open columns,* resting Ca^{2+} concentration; *closed columns,* peak cytosolic Ca^{2+} concentration after the addition of 75 mM KCl. The Ca^{2+} concentrations (in nM) are given under the *columns*. *Lower panel,* 25 μg 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 μM nifedipine (*lane 3*), or of 25 mM KCl plus 1 μM 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 KCl in the absence or presence of 140 μM NMDA. The gel shows PMCA1, PMCA2, PMCA3, and SERCA2b proteins in 20 μg of crude membrane proteins from granule cells cultured for 5 days in the presence of 5.3 (*lane 1*), 10 (*lane 2*), 15 (*lane 3*) 25 (*lane 4*), and 50 mM KCl (*lane 5*). *Lanes 6–10* are like *lanes 1–5,* except that 140 μM 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 μg of proteins from crude membranes of granule cells were used. Cells cultured in 10 mM KCl (*lane 1*), 10 mM KCl/140 μM NMDA (*lane 2*), and 10 mM KCl/140 μM NMDA/10 μM nifedipine (*lane 3*) were separated by SDS-PAGE and transferred to polyvinyldene difluoride filters. The immunocomplexes were visualized by NBT/BCIP. *D,* influence of extracellular Ca^{2+} on the expression of the PMCA2 and PMCA3 isoforms. 20 μg 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^{2+} or for 3 days in 25 mM KCl in the presence of 1.8 mM Ca^{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.

tion was responsible for the changed pattern of PMCA pump expression. The experiments, however, were all performed on cells incubated with the standard external concentration of 1.8 mM Ca^{2+}: Fig. 7*D* shows that after 5 days in culture, the two isoforms chosen as example, PMCA2 and 3, indeed became optimally overexpressed at 1.8 mM Ca^{2+}. 0.5 mM Ca^{2+}, however, was insufficient for optimal overexpression. Because the attachment of the cells to the culture dish required Ca^{2+}, it proved impossible to carry out a control in the total absence of Ca^{2+}. Fig. 7, however, shows that no PMCA pump was detected when cells exposed to 1.8 mM Ca^{2+} for 3 days were switched for 2 days to a medium in which the concentration of external Ca^{2+} was decreased to the nanomolar range with EGTA. The expression of the SERCA2b pump, which was run as a control, only became affected at very high (10 mM) Ca^{2+} concentrations or when the cells were switched to the EGTA medium.

Pattern of PMCA Protein Expression in P-19 and PC-12 Cells Cultured in High KCl—Experiments were performed on P-19 and PC-12 cells, which also differentiate into neurons in culture, to test the effect of KCl on the expression of PMCA pumps. PC-12 cells up-regulate the expression of c-Fos when their plasma membrane is depolarized (1). Transcripts for the PMCA1 and PMCA2 pumps were present in P-19 and PC-12 cells. In both lines, however, only isoform PMCA1CI could be detected as a protein before and after differentiation: the amounts of the PMCA2 and the PMCA3 proteins were below detection level. Addition of 25 mM KCl had no evident changes in the pattern of pump expression.

Activity of the PMCA Pump: the Phosphoenzyme Intermediate—The formation of the phosphoenzyme intermediate is routinely used as a reliable indication of the PMCA and SERCA pump activity. The activity of the Ca^{2+} pumps was thus explored in experiments on the formation of their phosphorylated intermediates. Two strongly radioactive bands at 100 and 135 kDa, corresponding to the SERCA and PMCA pumps (30), were detected in the membranes of granule cells (Fig. 8, *lanes 1* and

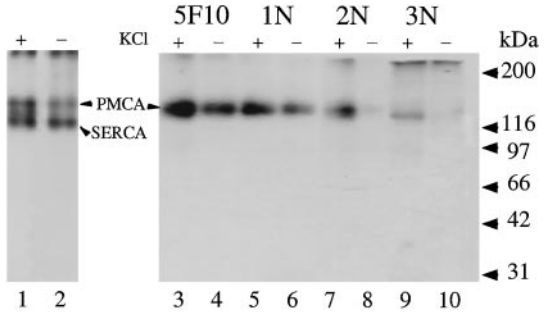


FIG. 8. **The phosphoenzyme intermediate of the PMCA pump isoforms in cultured granule cells.** 200 μg of crude membrane proteins from cells cultured for 5 days in 25 (+) or 5.3 (−) mM KCl were phosphorylated with 0.3–0.4 μM [γ-^{32}P]ATP (300 Ci/mmol) in the presence of Ca^{2+} and La^{3+} (see under "Materials and Methods"). 1/5 of the reaction volume was used to analyze the sample before immunoprecipitation (*lanes 1* and *2*); the remainder of the suspension was divided into four aliquots and incubated with 2 μl of antibody 5F10 (1–2 μg/μl) (*lanes 3* and *4*) or with 3–6 μg of the affinity-purified antibodies specific for the PMCA isoforms: PMCA1 antibody, *1N, lanes 5* and *6*; PMCA2 antibody, *2N, lanes 7* and *8*; PMCA3 antibody, *3N, lanes 9* and *10*. The immunocomplexes were bound to protein A-Sepharose; released by treatment with 6 M urea, 0.5 M dithiothreitol, and 5% SDS; and separated on acidic gels. After staining with Coomassie Brilliant Blue, the gels were dried and exposed for autoradiography for 24–72 h at −70 °C.

2). As expected, the incubation of cells in 25 mM KCl for 5 days significantly increased the amount of the 135 kDa band (PMCA). In contrast, the band at 100 kDa (SERCA) remained unchanged (Fig. 8, *lanes 1* and *2*). Aliquots of the same membranes were immunoprecipitated with isoform-specific antibodies and with a monoclonal antibody able to recognize all four isoforms under conditions that prevented its degradation (30). All antibodies precipitated proteins of 130–140 kDa (Fig. 8). Because the acidic gel conditions required to preserve the phosphorylated intermediate failed to resolve the alternatively spliced isoforms, no conclusions on the alternatively spliced variants of PMCA1 were possible. The increase of the phosphoenzyme of the PMCA2 pump was the most evident, whereas that of PMCA3 was minor but also clear (Fig. 8). Quantification from these and similar data has indicated that the phosphoenzyme intermediate of the PMCA2 pump, which was only 2–6% of the total at plating time, represented as much as 40% of it after 5 days of culturing in 25 mM KCl.

DISCUSSION

PMCA pump isoforms 1CII, 2, and 3 are expressed in a limited number of rat tissues, and PMCA2 and PMCA3 have only been detected in significant amounts in brain (19, 48). The present work has shown that these isoforms became strongly up-regulated in cerebellum during the first weeks of life. PMCA4, which is present in cerebellum at birth, also became up-regulated during the first weeks of life. The work described here has used cerebellar granule cells as a model and has shown that during maturation *in vitro,* the pattern of expression of the PMCA 2, 3 and 1CII pump isoforms underwent changes identical to those observed in postnatal cerebellum. In contrast, however, isoform 4 became rapidly down-regulated. These changes were only observed in the presence of depolarizing concentrations of KCl.

Granule cells survive for some days in the presence of low concentrations of KCl (about 5 mM), acquiring some of the phenotypic properties of neurons (7, 8). When cultured 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 extradepolarization 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 Ca^{2+} through L-type channels. The Ca^{2+} imaging experiments have provided direct demonstration of this: after 5 days in 25 mM KCl, cells contained up to 3 times more Ca^{2+} than those grown in 5.3 mM KCl. Although the essential process controlled by Ca^{2+} evidently was the transcription of the PMCA genes, other yet unidentified posttranscriptional and/or posttranslational Ca^{2+} 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 Ca^{2+}-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 Ca^{2+}.

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 $[Ca^{2+}]_i$, it would be logical to imagine that the increase reflects the need of the cells to deal with the augmented level of cytosolic Ca^{2+}. Despite the PMCA up-regulation, cell Ca^{2+} nevertheless increased during the maturation process: evidently, the increased influx of Ca^{2+} 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 Ca^{2+} (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 Ca^{2+}, would be a safety device to prevent Ca^{2+} from rising to levels incompatible with the normal life of the cells.

Observations on Ca^{2+}-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 isoform pattern, linked to an alternative splicing change at splice site A. Because no isoform 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 unspecific effect of the increased concentration of KCl 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 expected to provide the cells with higher sensitivity to lower concentrations of Ca^{2+}-calmodulin, which would 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 only became 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. As for isoform 1CII, its properties could be tentatively derived from those of the well characterized homologous 4CII isoform. Alternative splicing at the C-site, generating the 4CII and the 1CII isoforms (52), results in pumps that have weaker binding affinity to calmodulin (53, 54), but also higher basal (*i.e.* calmodulin-independent) ATPase activity (54). The up-regulation of the PMCA1CII isoform could thus confer to cells higher calmodulin-independent Ca^{2+} pumping capacity (PMCA1CII). A separate problem is that of the isoform 4, the down-regulation of which during the maturation process cannot be adequately interpreted along similar reasoning lines. It could be cautiously speculated that its down-regulation is the response to a different demand generated by the exposure to high KCl concentrations.

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