Calcineurin Controls the Expression of Isoform 4CII of the Plasma Membrane Ca\(^{2+}\) Pump in Neurons*

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The expression of the CII splice variant of the plasma membrane Ca\(^{2+}\) ATPase 4 (PMCA4) was down-regulated in granule neurons when they were cultured under conditions of partial membrane depolarization (25 mM KCl), which are required for long term in vitro survival of the neurons. These conditions, which cause a chronic increase of the resting free Ca\(^{2+}\) concentration in the neurons, have recently been shown to promote up-regulation of the PMCA2, 3, and 1CII isoforms. Whereas the chronic, i.e. >3 days, Ca\(^{2+}\) increase was necessary for the up-regulation of the PMCA1CII, 2, and 3, the down-regulation of the PMCA4CII mRNA was already evident 1–2 h after the start of culturing in 25 mM KCl. The immunosuppressant calcineurin inhibitor FK506 inhibited the down-regulation of the PMCA4CII at both the protein and the mRNA level but did not affect the changes of the other PMCA pumps. Direct evidence for the involvement of calcineurin in the down-regulation of the PMCA4CII was obtained by overexpressing a truncated, constitutively active, and Ca\(^{2+}\)-independent form of calcineurin; under these conditions, depolarization was not required for the down-regulation of the PMCA4CII pump. 

De novo synthesis of (transcription) factors was required for the down-regulation of the PMCA4CII mRNA. Calcineurin, therefore, controls the neuronal transcription of PMCA4CII, a splice variant of the pump isoforms that is found almost exclusively in brain.

The maintenance of a low intracellular Ca\(^{2+}\) concentration and the removal of Ca\(^{2+}\) from the cytosol after its transient increase require the concerted action of pumps and exchangers (1). These transporters are located in the membranes of intracellular organelles (the sarcoplasmic/endoplasmic reticulum and the mitochondria) and in the plasma membrane. The plasma membrane Ca\(^{2+}\) ATPase (PMCA) (also called the plasma membrane Ca\(^{2+}\) pump) shares with a Na\(^{+}/Ca\(^{2+}\) exchange the task of transporting Ca\(^{2+}\) out of the cell. The general biochemical properties of the pump have been established on the enzyme in membranes and in the purified state (2). The cloning of the pump cDNA has revealed an unexpected complexity in the genes encoding the ATPase and their products (3). The pump is encoded by four genes, and the primary transcripts are alternatively spliced at two independent sites (4, 5), resulting in the expression of more than 20 different proteins (3). The distribution of the four basic gene products and of the spliced isoforms has been extensively studied at the mRNA level and, more recently, at the protein level using isoform-specific antibodies (6). These studies have indicated that the transcripts for PMCA1 and PMCA4 are ubiquitously present in cells and tissues, whereas those for PMCA2 and PMCA3 have a restricted tissue distribution: i.e. they are expressed at levels comparable to those of PMCA1 and PMCA4 only in neurons (7, 8).

Only limited information is available on the function of the PMCA isoforms. However, it is known that PMCA2 has the highest calmodulin sensitivity, whereas the truncated variant PMCA4CII has much lower calmodulin affinity than the full-length PMCA4CII (9, 10). Information on the mechanisms that regulate the expression of the PMCA genes is equally limited, but a role of protein kinase C has been suggested for the case of PMCA1 (11). A recent study using primary cultures of rat cerebellar granule neurons has shown that the sustained increase of cytosolic Ca\(^{2+}\) occurring during maturation up-regulated the expression of the PMCA2 and PMCA3 proteins and of a splice variant of the PMCA1 protein (12). By contrast, the PMCA4CII isoform, which is the brain-specific splicing variant of PMCA4 (13), behaved differently (12).

To further explore the differences in the brain expression of the PMCA pump isoforms, experiments have been performed on the Ca\(^{2+}\) calmodulin-dependent protein phosphatase calcineurin, which has recently come to forefront as a modulator of gene transcription in a variety of systems (14, 15). The experiments have shown that the immunosuppressant FK506, which inhibits calcineurin, prevented the depolarization-mediated down-regulation of the PMCA4CII isoform but did not influence the expression of PMCA1, 2, and 3. The down-regulation of PMCA4CII was more rapid than the up-regulation of the other PMCA isoforms but was slower than the up-regulation of immediate early genes such as c-fos. In addition, it was prevented by cycloheximide, indicating the necessity of de novo protein synthesis. Finally, the overexpression of a Ca\(^{2+}\)-independent, constitutively active calcineurin down-regulated the expression of the PMCA4 protein in the absence of depolarization-mediated Ca\(^{2+}\) increases.

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‡ The abbreviations used are: PMCA, plasma membrane Ca\(^{2+}\) ATPase; CnA, calcineurin A; ΔCnA, truncated CnA; CREB, cAMP-response element-binding protein; GDPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NFAT, nuclear factor of activated T-lymphocytes; NMDA, N-methyl-D-aspartic acid; PCR, polymerase chain reaction; PP, protein phosphatase; RT, reverse transcription.
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MATERIALS AND METHODS

**Chemicals**—Poly-D-lysine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT). N-Methyl-D-aspartic acid (NMDA), nifedipine, calyculin A, and okadaic acid were from Calbiochem (San Diego, CA). Oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany). Ampli-Taq Gold polymerase was from Roche Molecular Systems, Inc. All other reagents were of the highest purity grade commercially available. Cyclosporin A and FK506 were a kind gift of Dr. Mauro Zurini (Novartis, Basel, Switzerland).

**Cell Cultures**—Granule cells were dissociated from the cerebella of 7-day old Wistar rats as described (16). They were plated in Dulbecco’s modified Eagle’s medium (Hepes modification, Sigma) supplemented with heat-inactivated 10% fetal calf serum (Sigma), 100 μg/ml gentamicin, 7 μM p-amino benzoic acid, 100 μg/ml pyruvate, 100 microunits/ml insulin, on poly-D-lysine-treated plates at a density of 2–3 x 10^5 cells/cm^2 in the presence of 5.3, 10, or 25 mM KCl. 10 mM KCl has been used in the experiments with NMDA, to permit a limited penetration of Ca^{2+} to remove the block by Mg^{2+} at the inner mouth of the NMDA channel. After 24 h, 10 μM cytosine arabinofuranoside was added to the culture to inhibit mitotic cell growth. Neuronal survival was estimated by measuring the amount of colored formazan in the cells by the reduction of the tetrazolium-bromide (MTT).

**Western Blotting**—Cells were resuspended in 5–10 x 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 phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and subjected to freeze and thaw. The particulate fraction was sedimented at 15,000 x 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.

**Results**

The expression of the PMCA4 protein is down-regulated by membrane depolarization—Previous studies had indicated that very low amounts of PMCA4 were present in granule cells matured in the presence of depolarizing concentrations of KCl (25 mM), which increases their long term survival (12). This was at variance with the brain-specific isoforms 2 and 3 and isoform 1CII, which were poorly represented at plating time but became strongly up-regulated during the maturation process (Fig. 1A). The behavior of the PMCA4 isoform was similar to that of PMCA1CII, which did not change significantly during the maturation of the neurons. A further decrease in the already low concentration of PMCA4 was even observed in some cases, after longer times of culturing in high KCl (Fig. 1A, lane 4). In sharp contrast to this, if the cells were cultured for a few days in the presence of low depolarizing concentrations of KCl (5–10 mM), higher amounts of the PMCA4 isoform became expressed (Fig. 1B). Thus, the isoform was down-regulated by treatments that induced a chronic increase of intracellular Ca^{2+} (12), e.g. in addition to depolarization, the exposure of the cells to the glutamate agonist NMDA (Fig. 1B, lanes 2 and 4). Consistent with this, t-type Ca^{2+} channel inhibitors prevented the down-regulation (Fig. 1B, lanes 3 and 5).
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Fig. 1. The expression of the PMCA4 protein in granule cells is controlled by Ca\(^{2+}\) and calcineurin. A, expression of PMCA4 during the maturation of the cells in vitro. 30 μg of crude membrane proteins from granule cells cultured for 2 (lane 1), 3 (lane 2) 5 (lane 3), and 7 (lane 4) days in vitro in the presence of 25 mM KCl were separated by SDS-polyacrylamide gel electrophoresis and incubated with the PMCA4-specific monoclonal antibody JA9 (8) (panel A) or with antibodies specific for PMCA1 (1N), PMCA2 (2N), and PMCA3 (3N) (7). The immunocomplexes were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. B, the expression of PMCA4 is down-regulated by treatments that increase cytosolic Ca\(^{2+}\). 30 μg of crude membrane proteins from granule cells cultured in vitro for 5 days were immunoblotted and incubated with the PMCA4- (JA9 monoclonal antibody), PMCA2-, or PMCA3-specific antibodies. The cells were cultured in the presence of 10 mM KCl (lane 1), 10 mM KCl and 140 μM NMDA (lane 2), 10 mM KCl/140 μM NMDA and 10 μM nifedipine (lane 3), 25 mM KCl (lane 4), or 25 mM KCl and 10 μM nifedipine (lane 5). C, the down-regulation of PMCA4 is prevented by FK506. 30 μg of crude membrane proteins from cells cultured for 5 days in the presence of 25 mM KCl (lanes 1 and 3) or 25 mM KCl plus 0.5 μM FK506 (lane 2) were processed as in B. D, the down-regulation of PMCA4 is controlled by immunosuppressants that inhibit calcineurin. 30 μg of total membrane proteins of granule cells cultured for 5 days in the presence of 5.3 mM KCl (lane 1), 25 mM KCl (lane 2), and 25 mM KCl plus either 3 nM FK506 (lane 3), 1 μM rapamycin (lane 4) or 3 μM FK506 plus 1 μM rapamycin (lane 5) were immunoblotted and incubated with the affinity-purified rat PMCA4 antibodies. E, a summary of 5–9 independent experiments identical to those shown in C and D. The Western blots were scanned with a cannon gel scanner and processed with Adobe PhotoShop 3.01 software. The intensity of the band specific for the PMCA4 isoform was determined with the help of the National Institutes of Health Image 1.59 program. Bars represent S.D. The intensity of the bands is given in arbitrary units. Columns 1–5 were processed as in B. The intensity of the bands specific for PMCA4 was measured in the absence of FK506 (column 1), 30 mM FK506 (column 2), 30 mM FK506 and 1 μM nifedipine (column 3), 30 mM FK506 and 1 μM rapamycin (column 4), or 30 mM FK506 and 1 μM rapamycin (column 5).

l-channel blocker nifedipine prevented the opening of NMDA channels because it prevented the penetration of small amounts of Ca\(^{2+}\) (through l-type Ca\(^{2+}\)-channels) to remove the Mg\(^{2+}\) block of the NMDA channel. The down-regulation was also prevented by the NMDA-specific inhibitors MK801 (not shown), PMCA2 and PMCA3 (Fig. 1B, panels 2N and 3N) showed an opposite behavior, i.e. they were up-regulated by the treatments that induced the sustained increased of intracellular Ca\(^{2+}\).

The Down-regulation of PMCA4 Is Dependent on Calcineurin—One of the effects of the increase of intracellular Ca\(^{2+}\) is to activate the Ca\(^{2+}\)-calmodulin-dependent protein phosphatase calcineurin, which is crucial to the regulation of the expression at several genes (28). To test whether the phosphatase also played a role in the expression of PMCA pump isoforms, granule cells were cultured under depolarizing conditions in the presence of the immunosuppressant FK506, an inhibitor of calcineurin (29), or of the inhibitors of protein phosphatases 1 and 2A, calyculin A, and okadaic acid (30). FK506 prevented the down-regulation of the expression of PMCA4 (Fig. 1C, lane 2). Calyculin A and okadaic acid turned out to be very toxic to the neurons and could not be further investigated: measurements of the activity of mitochondrial dehydrogenases with MTT (17) demonstrated that more than 90% of the cells died after 6–8 h incubation with concentrations of okadaic acid calyculin A as low as 20 and 2 nM, respectively (not shown). Attempts were made to inhibit calmodulin kinases to verify their involvement in the down-regulation of PMCA4CII or other pump isoforms. Unfortunately, the commonly used inhibitors KN-92 and KN-93 proved toxic to the granule cells: only 10–20% of them survived after a 6–8 h incubation period, a time that was too short to permit measurements of specific effects on PMCA4CII.

The results in Fig. 1, A–C, were obtained with the monoclonal antibody JA9, which generated rather weak signals in rat granule cell membranes. Stronger signals were obtained with an antiserum prepared using a recombinant peptide encompassing the N-terminal portion of the rat PMCA4 pump. The antiserum confirmed the observations made with the monoclonal antibody (Fig. 1D), in particular that FK506 prevented the depolarization-mediated down-regulation of the PMCA4. The lack of effect by rapamycin, an immunosuppressant that binds to the FK506-binding protein but fails to inhibit the activity of calcineurin, supported the conclusion that the down-regulation of PMCA4 required the activation of calcineurin. The effect of FK506 was partially reversed by high concentrations of rapamycin (a 300-fold excess was required (Fig. 1D, lane 5, see also Fig. 1E)), indicating that binding to the FK506-binding immunophilin was necessary for the effects on PMCA4 expression. As indicated in Fig. 1E, the effect of FK506 was observed down to a concentration of 3 nM. Culturing the cells for 5–6 days in the presence of up to 1 μM FK506 and in the presence of 1 μM rapamycin had no effects on cells survival estimated using MTT tests (results not shown). A summary of the results obtained in 5–9 different experiments is shown in Fig. 1E.

The Down-regulation of PMCA4 Is Controlled at the Transcriptional Level—The down-regulation of the PMCA4 isoform was also studied at the transcriptional level. A Northern blot with total RNA isolated from cells cultured for 5 days in 25 mM KCl showed higher amounts of PMCA4 mRNA when FK506 was present in the culturing medium (Fig. 2A). To further analyze the effect, RT-PCR was performed using oligonucleotides specific for PMCA4 (Fig. 2B). The intensity of the PMCA4-specific bands was decreased by the depolarizing treatment (Fig. 2B, lane 2), the effect being prevented by low concentrations of FK506 (Fig. 2B, lane 3) but not by rapamycin (Fig. 2B, lane 5). If rapamycin was present in the culturing medium in a 300-fold molar excess to FK506, it competed with it (Fig. 2B,
FK506 and rapamycin did not seem to affect the expression of PMCA4 in the presence of nondepolarizing concentration of KCl (not shown). No effects of FK506 were observed on the mRNAs for the PMCA1, 2, and 3 isoforms (Fig. 2C). In the case of PMCA4, oligonucleotides specific for both splice site A and C were used (Fig. 2B, top panel). Two bands were amplified for site C (Fig. 2B, middle panel). Whereas that of higher molecular mass (420 bp, 4CII) had a strong sensitivity to depolarization and was protected by FK506, the lower band (270 bp, 4CI) was almost insensitive to these treatments. This band, which corresponds to the CI splice variant of isoform 4, is likely to be contributed mostly by the PMCA4 isoform of the glia cells contaminating the neuronal culture. Even if glia cells represented less than 5% of the total cell population in the culture, they contain large amounts of PMCA4CI mRNA (31), which is much better amplified than that of the PMCA4CII isoform.

The down-regulation of the PMCA4 mRNA by depolarization became evident 2–4 h after the initiation of the treatment (Fig. 3) and was thus slower than the up-regulation of the c-fos mRNA (15–20 min (32), not shown). It was, however, much faster than the up-regulation of the PMCA1, 2, and 3 isoforms.

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more than 85–90% of the granule cells in the culture, as detected by immunocytochemistry (not shown). Recombinant viruses for CnA and ΔCnA were generated by homologous recombination in COS cells as described under “Materials and Methods.” When co-transfected with the t7 polymerase, these viruses were able to drive high level of expression of the CnA and ΔCnA proteins in HeLa cells (Fig. 4A). Strong signals were visible at 59 (Fig. 4A, lane 2) and 42 kDa (Fig. 4A, lane 3), corresponding to the molecular masses of CnA and ΔCnA, respectively, when HeLa cells were labeled with [35S]Met and total proteins were separated by SDS-polyacrylamide gel electrophoresis. Preliminary experiments showed that granule cells survived relatively well the exposure to the virus, i.e. the infection increased the proportion of dead cells by no more than 5–10%, provided that the infection was performed not later than 3–4 days after plating and that it did not last more than 3 days (not shown). As shown in Fig. 4B, the overexpression of wild type Ca2+-calmodulin-dependent CnA (Fig. 4A, lane 2) in cells cultured in low potassium failed to induce the down-regulation of PMCA4, whereas the expression of the Ca2+-independent, constitutively active calcineurin (Fig. 4B, lane 3) resulted in the marked down-regulation of the isoform. Infection with the t7 polymerase virus had no effect (Fig. 4B, lane 1). The samples in Fig. 4B, lanes 1–3, were probed with a monoclonal antibody that recognized calcineurin (Fig. 4B, lanes 4–6), confirming that the viruses were able to drive a 5–6-fold overexpression of the recombinant proteins as compared with endogenous CnA. Cells were treated with different amounts of virus to evaluate whether low (2–3-fold) levels of expression of the truncated phosphatase would have been sufficient for the down-regulation of PMCA4: expression of a 3–4-fold excess of the truncated calcineurin as compared with the endogenous phosphatase was required to observe an effect on the PMCA pump. No effect of the overexpression of CnA and ΔCnA was observed on the expression profile of the other pump isoforms (not shown).

**DISCUSSION**

The sustained increase of cytosolic Ca2+ induced by the partial depolarization of the plasma membrane during the maturation of granule cells was accompanied by the down-regulation of PMCA4CII and by the up-regulation of PMCA1CII, 2, and 3. The PMCA4CII isoform, which is the other splice variant present in granule cells, was not affected by the Ca2+ increase. Remarkably, the kinetics of the down-regulation of the PMCA4CII isoform was much faster than that of the up-regulation of the other isoforms. 2 h of exposure to the depolarizing concentrations of KCl were sufficient to induce the disappearance of its mRNA. Equally remarkable was the finding that calcineurin regulated the disappearance of PMCA4CII but not the up-regulation of the other PMCA isoforms. In
Calcineurin has been shown to regulate the transcription of the genes involved in the antigen-mediated activation of T-cells (14) by dephosphorylating the cytosolic form of the transcription factor NFAT and by allowing its translocation to the nucleus (34, 35). This mechanism has now been shown to be functional also in other cells, e.g., heart, in which recent experiments have indicated that calcineurin may be involved in the genesis of cardiac hypertrophy (15), and hippocampal neurons, in which calcineurin translocated a variant of NFAT (NFATc4) to the nucleus of cells activated by depolarization (36). The possible involvement of NFAT in the control by calcineurin of PMCA4 levels in granule cells is thus an attractive possibility. It was therefore decided to explore the presence of NFAT-like transcription factors in granule cells. Due to the lack of specific antibodies, RT-PCR experiments using degenerate primers were performed. They have permitted the cloning and sequencing of a fragment of NFAT (NFATc4) in granule cells.

Calcineurin in hippocampal neurons has also been shown to increase the fraction of activated protein phosphatase 1 (PP1), i.e., its inhibition by immunosuppressants resulted in the reduction of active PP1 (37). Because PP1 is crucial to the phosphorylation level of the transcription factor CREB, calcineurin indirectly influenced gene expression. However, the involvement of PP1 (or of PP2a) in the down-regulation of PMCA4CII seems unlikely, because all experiments with inhibitors of phosphatases 1 and 2A (okadaic acid and calyculin A, respectively) resulted in the death of the neurons, whereas the cells survived up to 10 days of incubation with FK506 without evident signs of toxicity. The matter of CREB phosphorylation was further explored by treating cells depolarized with 50 mM KCl with FK506, and by testing them for the presence of phosphorylated CREB. Phosphorylated CREB was detected with specific antibodies in the down-regulation of PMCA4CII (38). The C-terminal portion may thus play a role in the proper localization of some PMCA isoforms. PDZ and similar binding domains are responsible for the high concentration of PMCA1CII in adult cerebellum (but also in other regions of rat brain) are much lower than those of the PMCA4CI variant (8), the 4CII splice variant may have a specific function only during the early phases of the maturation of neurons. Later on, granule cells would compensate the down-regulation of PMCA4CII with the up-regulation of the PMCA1CII isoform. Functional studies have shown that the human PMCA4CII protein has much lower affinity for calmodulin and higher Ca$^{2+}$-calmodulin-independent ATPase activity than the 4CI splice variant (10). Although the functional properties of the rat PMCA1CII isoform are not known, sequence similarities suggest that they should be very similar to those of PMCA4CII.

One important aspect that ought to be also considered is the spatial distribution of the PMCA isoforms, i.e., their up and down-regulation could specifically concern the cell soma, the dendrites, or the axon in response to local demands for altered Ca$^{2+}$ homeostasis. Domains located at the C-terminal portion of PMCA4CII bind to membrane-associated guanylate cyclase kinase through PDZ domains that are absent in PMCA4CII (42). The C-terminal portion may thus play a role in the proper cellular localization of some PMCA isoforms. PDZ and similar binding domains are responsible for the high concentration of NMDA channels in postsynaptic membranes (43–45); the down-regulation of the PMCA4CII isoform may thus have the aim of removing the PMCA isoforms that do not carry signals for proper membrane localization in mature granule cells.

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