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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)[1] (also called the plasma membrane Ca^{2+} pump) shares with a Na^+/Ca^{2+} exchanger 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[2] has much lower calmodulin affinity than the full-length PMCA4CI (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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[1] The abbreviations used are: PMCA, plasma membrane Ca^{2+} ATPase; CnA, calcineurin A; ΔCnA, truncated CnA; CREB, cAMP-response element-binding protein; G3PDH, 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.

[2] Isoforms PMCA4CI and PMCA4CII (46) correspond to isoforms PMCA4a and PMCA4b in the nomenclature used by others.

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 MGW-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*-aminobenzoic acid, 100 μg/ml pyruvate, 100 microunits/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, 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 MTT (17). The extent of contaminating astrocytes was estimated by immunocytochemistry using a glial fibrillary acidic protein-specific monoclonal antibody (Roche Molecular Biochemicals). Immunocytochemistry was performed as described earlier (18).

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 freeze and thaw. The particulate fraction was sedimented at 15,000 $\times$ *g* for 15 min. The resulting protein pellet was resuspended in 4 mM Tris-HCl, pH 8.0, 10% sucrose and frozen at −70 °C.

Western Blotting—Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose or polyvinylidene difluoride membranes (19). The membranes were incubated with affinity-purified isoform-specific polyclonal antibodies against the PMCA pump (7). The JA9 monoclonal antibody specific for the PMCA4 isoform was kindly donated by Dr. J. Penniston (Mayo Clinic, Rochester, MN) (8). The blots were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. The reaction was stopped after 5 min for the affinity-purified serum against PMCA1, after 10 min for the affinity-purified antibodies specific for PMCA2 and PMCA3 and for the monoclonal 5F10 antibody that recognizes all isoforms, and after 15–25 min for antibody JA9 or the rat4N polyclonal antibody specific for the rat PMCA4. When the blots were incubated with CDP-Star[a] (Tropix, Bedford, MA), they were exposed to a chemiluminescence-sensitive photographic film. If needed, Western blots were scanned with Adobe PhotoShop software and quantified with the help of National Institutes of Health Image 1.59.

Isolation of RNA and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Total RNA was prepared from granule cells according to the method of Chomczynski and Sacchi (20). 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 Guerini *et al.* (12) for alternative splicing site C. The oligonucleotide specific for the PMCA4CII isoform was 5′ GAG GAG GTG TAA CGG CAG AAG (rat PMCA4CII, 3622–3647) (13). The conditions for the PCRs were as those suggested by Perkin-Elmer for the *Taq* Gold polymerase. The identity of the PCR-generated fragments was verified by Southern blotting and by sequencing. Northern blotting was performed as described by Sambrook *et al.* (21). The PMCA4CI-specific fragment was 3456–3727 (4, 13), and that of G3PDH was 371–1015 (22). The Northern and Southern blots were scanned with Adobe PhotoShop and quantified with the help of the National Institutes of Health Image 1.59 software package.

Preparation of the Antibody Specific for Rat PMCA4—A cDNA fragment encompassing the first 98 amino acids (Pro-4 to Leu-102) of rat PMCA4 was amplified from total RNA of granule cells. The oligonucleotides used were 5′-ATGACGGATCCATCAGGACATAAC-3′ (nucleotides 168–194; the mutated sequences resulting in the generation of a novel *Bam*HI restriction site is underlined) and 5′-GTGTGAATTC**CTA**-CAGGGCTTCCCACA-3′ (nucleotides 453–483; the inserted *Eco*RI site is underlined, and the inserted stop codon is in boldface). The numbering of the oligonucleotides is according to Keeton and Shull (13). The amplified cDNA was verified by sequencing and inserted in pRESET B vector (Invitrogen Corp., San Diego, CA). The peptide was expressed in *Escherichia coli* JM109 and purified according to the procedure recommended by the provider (InVitrogen Corp., Carlsbad, CA). The peptide was then used to raise polyclonal antibodies in rabbits (7). The antibodies (named rat 4N) were affinity-purified from the serum as described earlier (7). Because the serum also reacted weakly with the human PMCA4, the isoform specificity was verified on full-length human isoforms expressed in insect cells, as described earlier (7). The rat 4N serum showed a strong reaction for PMCA4, a very weak cross-reactivity with human PMCA1, and almost no reactivity with PMCA2 and PMCA3.

Generation of Recombinant Vaccinia Virus for the Rat Wild Type Calcineurin A and the Truncated Calcineurin A (ΔCnA)—cDNAs for the wild type and truncated rat calcineurin A were amplified by PCR from rat granule cell total RNA. The oligonucleotides were derived from the rat αCnA (23) and were as follows: CnA forward, AAGAATTCCCAAG-GCGATGATCCCAAG (nucleotides 208–237; the *Eco*RI restriction site is underlined); CnA reverse, AACTCGAGTGAACGAGAAGTGGTCA-CTG (nucleotides 1372–1399; the *Xho*I restriction site is underlined); ΔCnA reverse, AACTCGAGACCTC**CTA**CCGGGCTGCAGC (nucleotides 1768–1795; the *Xho*I restriction site is underlined, and the stop codon is in boldface). The oligonucleotide for ΔCnA inserted a stop codon at the position corresponding to Lys-392, located at the beginning of the calmodulin binding domain. The ΔCnA lacked all the sequences downstream of the calmodulin binding domain and hence was Ca^{2+}-independent (24). The PCR fragments were purified and subcloned in the pGEM-T vector (Promega). The clones that contained the desired sequences were digested with *Eco*RI and with *Xho*I, which released the full-length CnA and ΔCnA cDNAs and inserted them in the *Eco*RI and *Xho*I sites of the pTM3 vector. The pTM3 vector, a kind gift of Dr. Moss (25), allows the generation of recombinant vaccinia viruses by homologous recombination. Recombinant vaccinia viruses were prepared according to the method of Boyle and Coupar (26). Briefly, 5 μg of pTM3-CnA or pTM3-ΔCnA were used to transfect COS cells that were previously incubated for 120 min (at a multiplicity of infection of 0.01) with wild type vaccinia virus. After 8–12 incubation, fresh medium (Dulbecco's modified Eagle's medium, 10% fetal calf serum, and 50 μg of gentamicin) supplemented with 2.5 μl/ml mycophenolic acid, 250 μg/ml xanthine and 1:50 HAT, was added to the cells. Three days after the start of the infection, the virus was released from the cells by three cycles of freeze and thaw. The crude virus was plated on COS cells in the presence of mycophenolic acid and plaque-purified. The recombinant viruses were further purified on thymidine kinase-deficient cells in the presence of bromo-uridine (27). HeLa cells were plated at a density of 10^4 cells/cm^2 and infected for 30 min at 37 °C with the recombinant T7 polymerase helper virus (25) and the CnA or ΔCnA recombinant viruses (10–20 viruses per cell). After 12–16 h of infection, the cells were labeled with 150 μCi/ml [^{35}S]Met for 1 h. Granule cells were infected as indicated for HeLa cells. In some cases, different ratios of virus/cell were used (see Figure legends). The infected neurons were processed normally 2–3 days after infection. The survival of the granule cells was estimated by the MTT method.

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. 1*A*). The behavior of the PMCA4 isoform was similar to that of PMCA1CI, 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. 1*A, lane 4*). In sharp contrast to this, if the cells were cultured for a few days in the presence of low (*i.e.* nondepolarizing) concentrations of KCl (5–10 mM), higher amounts of the PMCA4 isoform became expressed (Fig. 1*B*). 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. 1*B, lanes 2* and *4*). Consistent with this, L-type Ca^{2+} channel inhibitors prevented the down-regulation (Fig. 1*B, lanes 3* and *5*). The

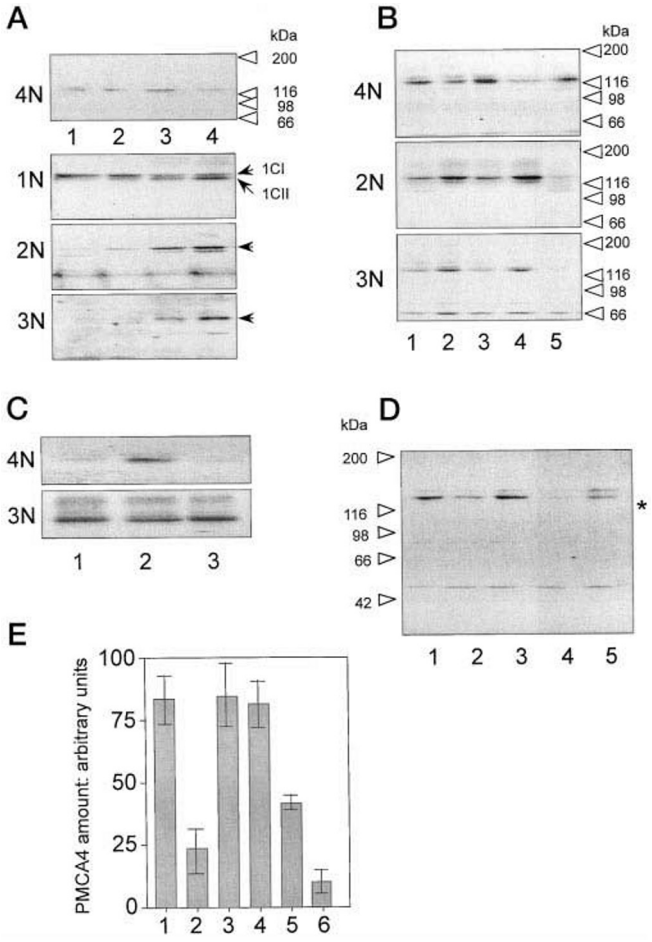

FIG. 1. **The expression of the PMCA4 protein in granule cells is controlled by Ca^{2+} and calcineurin.** *A,* expression of PMCA4 during the maturation of the cells *in vitro*. 30 μg of crude membrane proteins from granule cells cultured for 2 (*lane 1*), 3 (*lane 2*) 5 (*lane 3*), and 7 (*lane 4*) 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 4N*) 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 nM 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. Granule cells were cultured in the presence of 5.3 mM KCl (*column 1*); 25 mM KCl (*column 2*); 25 mM KCl and 3 nM FK506 (*column 3*); 25 mM KCl and 1 μM FK506 (*column 4*); 25 mM KCl, 3 nM FK506, and 1 μM rapamycin (*column 5*); or 25 mM KCl and 1 μM rapamycin (*column 6*).

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. 1*B, 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. 1*C, 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. 1*D*), 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. 1*D, lane 5,* see also Fig. 1*E*), indicating that binding to the FK506-binding immunophilin was necessary for the effects on PMCA4 expression. As indicated in Fig. 1*E,* 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. 1*E*.

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. 2*A*). To further analyze the effect, RT-PCR was performed using oligonucleotides specific for PMCA4 (Fig. 2*B*). The intensity of the PMCA4-specific bands was decreased by the depolarizing treatment (Fig. 2*B, lane 2*), the effect being prevented by low concentrations of FK506 (Fig. 2*B, lane 3*) but not by rapamycin (Fig. 2*B, lane 5*). If rapamycin was present in the culturing medium in a 300-fold molar excess to FK506, it competed with it (Fig. 2*B,*

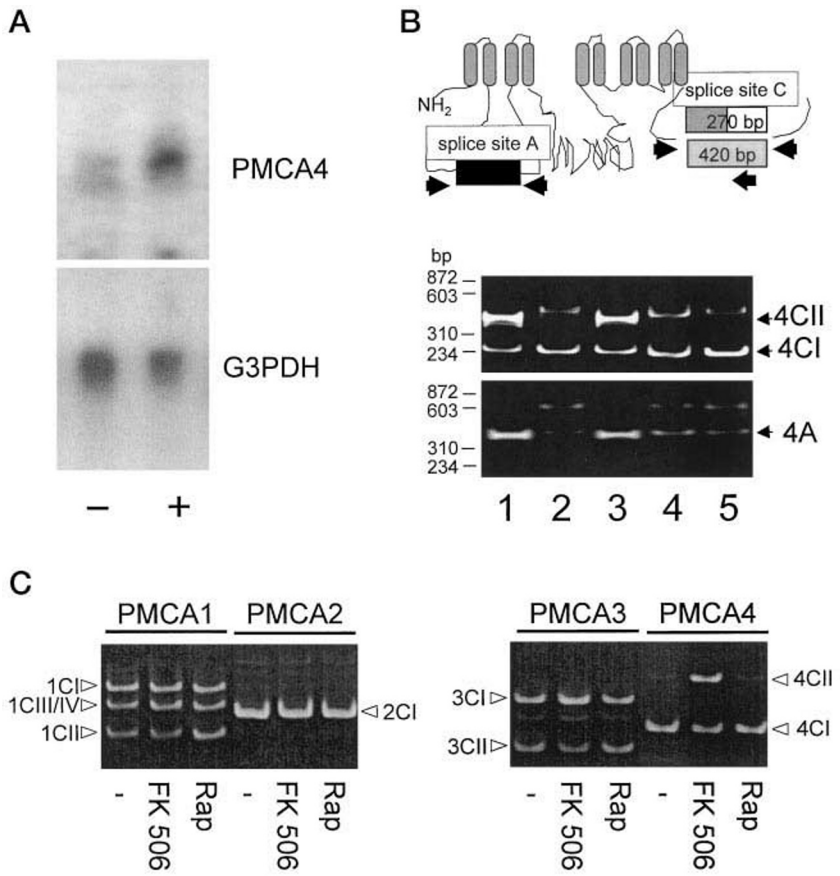

FIG. 2. **Effects of depolarization, Ca^{2+}, and calcineurin on PMCA4 transcripts in granule cells.** *A,* Northern blots. 15 μg of total RNA isolated from granule cells cultured for 5 days in the presence of 25 mM KCl and in the absence (−) or presence (+) of 300 nM FK506. The blots were incubated with ^{32}P-labeled PMCA4 and G3PDH probes. The G3PDH signal (the band at 1700 nucleotides) was obtained after 2 days of autoradiographic exposure and was used as a control for the integrity and concentration of mRNA. The signal for PMCA4 (band at 5000–6000 nucleotides) was obtained after 10 days of exposure. *B,* RT-PCR. Total RNA isolated from granule cells cultured for 5 days 5.3 mM KCl (*lane 1*), 25 mM KCl (*lane 2*), 25 mM KCl in the presence of 3 nM FK506 (*lane 3*), 25 mM KCl in the presence of 1 μM rapamycin plus 3 nM FK506 (*lane 4*), or 25 mM KCl in the presence of 1 μM rapamycin (*lane 5*). The PCR was performed with PMCA4 oligonucleotides specific for the A-splice site (*4A*) or for the C-splice site (*4CI* and *4CII*). The locations of the primers are shown in the topographic model of the pump (*black arrowheads*). The PCR products were separated on an 8% polyacrylamide gel electrophoresis gel and stained with ethidium bromide. The two fragments (*4CI* and *4CII*) indicated by the *arrows* resulted from the amplifications from two alternatively spliced transcripts. *C,* the effect of FK506 is specific for the PMCA4 isoform. RT-PCR was performed using oligonucleotides specific for isoforms (splice site C) 1, 2, 3, and 4 on RNA isolated from granule cells cultured in 25 mM KCl (−) or in 25 mM KCl plus 1 μM FK506 (*FK 506*) or 1 μM rapamycin (*Rap*).

lane 4). 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. 2*C*). In the case of PMCA4, oligonucleotides specific for both splice site A and C were used (Fig. 2*B, top panel*). Two bands were amplified for site C (Fig. 2*B, 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 the 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.[3]

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 (12). To establish whether *de novo* protein synthesis was required for the down-regulation of PMCA4, cells were incubated in the presence of cycloheximide, a classical inhibitor of protein synthesis. Cycloheximide prevented the decrease of the PMCA4 mRNA occurring when cell cultures were switched from 5.3 to 25 mM KCl (Fig. 3). Under the same conditions, cycloheximide failed to block the up-regulation of c-*fos* (not shown), in line with the observation that the up-regulation of c-*fos*, like that of all other immediate early genes, does not require *de novo* protein synthesis.

Constitutively Active Calcineurin Down-regulates PMCA4 in the Absence of Depolarization—To establish whether the down-regulation of PMCA4 could be induced by calcineurin even in the absence of the depolarizing treatment, *i.e.* in the absence of cellular Ca^{2+} increases, experiments were performed using a truncated, Ca^{2+}-independent calcineurin catalytic subunit (ΔCnA) (33). To detect effects on the down-regulation of the PMCA4 in granule cells, it would have been necessary to achieve an efficiency of transfection of recombinant calcineurin approaching 80–90%. Because this is unrealistic in neurons, which are normally very difficult to transfect, it was decided to use a viral expression system. Preliminary experiments had shown that the t7 polymerase-based vaccinia virus system (25) was able to drive the expression of recombinant proteins in

[3] Guerini, D., unpublished observations.

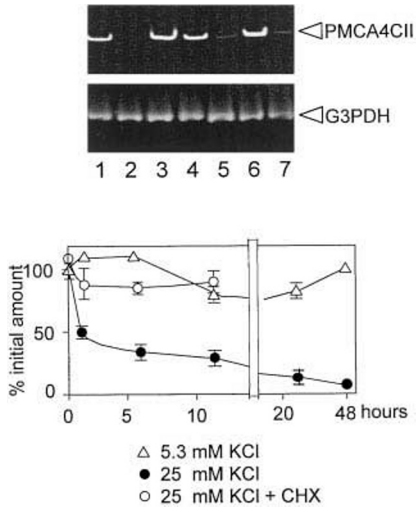

FIG. 3. **Cycloheximide prevents the down-regulation of the PMCA4 mRNA.** RT-PCR on total cell RNA. Granule cells were cultured for 3–4 days in low potassium (5.3 mM) and then kept for 12 (*lane 1*), 24 (*lane 4*), or 48 (*lane 6*) h in low potassium or switched to 25 mM KCl for 12 (*lane 2*), 24 (*lane 5*), or 48 (*lane 7*) h or switched to 25 mM KCl in the presence of 10 μg of cycloheximide for 12 h (*lane 3*). Incubation times in excess of 12–15 h could not be explored because cycloheximide became toxic. In this experiment, an oligonucleotide pair specific for the PMCA4CII isoform (Fig. 2*B, white arrowhead*) was used. Amplification of G3PDH RNA was used as a control for the integrity of the mRNA-cDNA and for the presence of similar amounts of mRNA in the different reactions. The *bottom panel* summarizes the results of 2–5 independent experiments. *Bars* represent S.D.

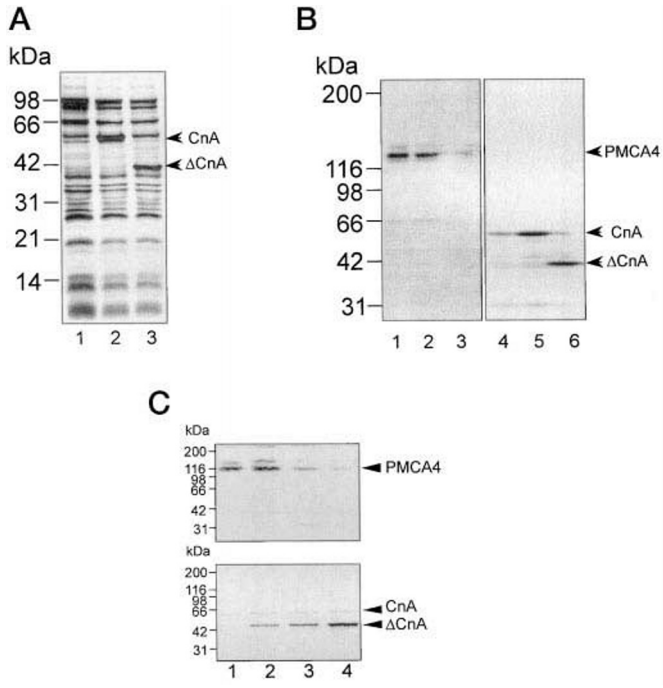

FIG. 4. **Overexpression of a constitutively active, Ca^{2+}-independent calcineurin induces depolarization-independent down-regulation of PMCA4.** *A,* characterization of the recombinant vaccinia virus. HeLa cells were infected with the recombinant T7 polymerase helper virus (*lanes 1–3*) and coinfected with newly produced recombinant viruses encoding the catalytic subunit of calcineurin (*CnA, lane 2*) or encoding a truncated, Ca^{2+}-independent catalytic subunit of calcineurin (Δ*CnA, lane 3*). After 15 h of infection, the cells were labeled with [^{35}S]Met for 1 h and collected. Aliquots corresponding to 250,000 cpm-labeled protein were loaded on a SDS gel. After drying, the gel was exposed overnight with x-ray film. The positions of the recombinant proteins are indicated. *B,* effect of overexpression of CnA and ΔCnA on PMCA4. Granule cells cultured in low potassium (5.3 mM) for 4 days, were infected with recombinant vaccinia virus coding for the t7 polymerase (*lanes 1* and *4*), co-infected with the t7 virus and a recombinant virus encoding the catalytic subunit of CnA (*lanes 2* and *5*) or co-infected with the t7 polymerase vaccinia virus and a recombinant virus encoding a truncated, Ca^{2+}-independent catalytic subunit of calcineurin (ΔCnA) (*lanes 3* and *6*). The infection lasted 2 days. Finally, 30 μg of crude membrane proteins were analyzed by Western blotting with a monoclonal antibody specific for CnA (*lanes 4–6*) or the monoclonal antibody JA9 specific for PMCA4 (*lanes 1–3*). *C,* the down-regulation of PMCA4 is proportional to the amounts of overexpressed ΔCnA. Granule cells cultured in low potassium (5.3 mM) for 4 days were infected with the t7 polymerase virus (*lanes 1–4*) and co-infected with increasing amounts (at a multiplicity of infection of 2, 4, and 10 for *lanes 2, 3,* and *4,* respectively) of recombinant virus encoding the truncated catalytic subunit of calcineurin (ΔCnA). The amount of the t7 polymerase virus was changed so that the total multiplicity of infection was 20 in all four experiments. The infection lasted 2 days. Finally, 30 μg of crude membrane proteins were analyzed by Western blotting with a monoclonal antibody specific for CnA (*lower panel*) or the monoclonal antibody JA9 specific for PMCA4 (*upper panel*).

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. 4*A*). Strong signals were visible at 59 (Fig. 4*A, lane 2*) and 42 kDa (Fig. 4*A, lane 3*), corresponding to the molecular masses of CnA and ΔCnA, respectively, when HeLa cells were labeled with [^{35}S]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. 4*B,* the overexpression of wild type Ca^{2+}-calmodulin-dependent CnA (Fig. 4*A, lane 2*) in cells cultured in low potassium failed to induce the down-regulation of PMCA4, whereas the expression of the Ca^{2+}-independent, constitutively active calcineurin (Fig. 4*B, lane 3*) resulted in the marked down-regulation of the isoform. Infection with the t7 polymerase virus had no effect (Fig. 4*B*, *lane 1*). The samples in Fig. 4*B, lanes 1–3,* were probed with a monoclonal antibody that recognized calcineurin (Fig. 4*B, 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 Ca^{2+} 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 PMCA4CI isoform, which is the other splice variant present in granule cells, was not affected by the Ca^{2+} 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

principle, the experiments do not rigorously demonstrate that calcineurin regulated the PMCA4CII expression at the transcriptional level: it could have influenced the processing of the transcript to yield the mature mRNA or reduced the stability of the latter. However, the PCR experiments showing the complete disappearance of the PMCA4CII transcript upon depolarization would be more consistent with the shutting off of a specific promoter. PCR would have amplified even a very minute remaining amount of transcript.

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 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 after 5–10 min of depolarization and was short-lived, *i.e.* it was barely detectable after 30 min. Under those conditions, at variance with those used in hippocampal neurons (37), phosphorylated CREB was not affected by FK506: 50 mM KCl had to be used to detect phosphorylated CREB, because the usual depolarizing concentration (25 mM) was less effective in inducing its phosphorylation. In summary, then, although other indirect mechanisms, *e.g.* the activation of NO synthase, cannot be excluded, calcineurin is more likely to directly control the transcription of the gene for PMCA4. It could influence the activity of an unknown transcription factor in analogy with what described for the yeast *Saccharomyces cerevisiae* (38), or, which is more likely in the light of the recent findings on hippocampal neurons (36), it could operate through an NFAT-linked mechanism.

Whereas the PMCA4CII splice variant was down-regulated during the maturation, PMCA4CI, the other isoform 4 variant found in brain (7), was not. Possibly, the CII variant could be the only PMCA4 isoform expressed in granule cells, the PMCA4CI variant being contributed by the 3–5% glial cells in the cultures. If so, specific transcription factors must exist in neurons that are responsible for the expression of PMCA4 CII isoform. Alternatively, both CI and CII transcripts could be present in neurons, in which case the transcription of the two splice variants would be regulated independently. A role of the splicing machinery (spliceosome) in the transcriptional control of specific splice variants has been described (39, 40); calcineurin, therefore, could control the activity of the spliceosome. The experiments with cycloheximide have indicated that, at variance of the transcription of c-*fos*, *de novo* protein synthesis was required for the down-regulation of PMCA4CII. The expression of the transcription factor(s) involved may be, therefore, directly controlled by calcineurin.

The increase of Ca^{2+} in the cultured granules was not essential for their maturation. Previous work (12) has shown that they developed to mature neurons equally well when cultured in low KCl. However, under these conditions, they underwent early apoptosis, whereas cells incubated in depolarizing KCl concentrations survived much longer. Thus, the relatively modest increase in cytosolic Ca^{2+} induced by depolarization protected the cells against apoptosis. Very likely, this was due to the activation of the protein kinase B pathway, which is controlled by the calmodulin-dependent protein kinase-kinase (41). Activation of this pathway is essential for the neutralization of the preapoptotic protein BAD, a member of the BCL-2 family of proteins, which becomes phosphorylated and sequestered to protein 14-3-3. The important point, here, is that the Ca^{2+} increase that protects against apoptosis must be kept within modest limits: a prolonged and uncontrolled Ca^{2+} overload would evidently be incompatible with cell survival. Very likely, the rationale for the changed pattern of Ca^{2+} pump expression is the necessity to cope with the increased influx of Ca^{2+} and to modulate its levels in the cell, within the limits necessary to prevent apoptosis and to ensure survival. In principle, one should expect an increase in the total Ca^{2+} pumping capacity of the cultured cells, and in fact, during their maturation in the presence of high KCl, their total PMCA pump protein and activity have been found to increase 4–5-fold (12). Thus, the finding that one of the pump isoforms, PMCA4CII, was instead down-regulated was rather surprising. Because the amounts of PMCA4CII 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 PMCA4CI 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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REFERENCES

1. Carafoli, E. (1987) *Annu. Rev. Biochem.* **56,** 395–433
2. Carafoli, E., and Stauffer, T. (1994) *J. Neurobiol.* **25,** 312–324
3. Carafoli, E., and Guerini, D. (1993) *Trends. Cardiovasc. Med.* **3,** 177–184
4. Keeton, T. P., Burk, S. E., and Shull, G. E. (1993) *J. Biol. Chem.* **268,** 2740–2748
5. Stauffer, T., Hilfiker, H., Carafoli, E., and Strehler, E. E. (1993) *J. Biol. Chem.* **268,** 25993–26003
6. Guerini, D. (1998) *Cell Tissue Res.* **292,** 191–197
7. Stauffer, T., Guerini, D., and Carafoli, E. (1995) *J. Biol. Chem.* **270,** 12184–12190
8. Filoteo, A., Elwess, N., Enyedi, A., Caride, A., Aung, H., and Penniston, J. (1997) *J. Biol. Chem.* **272,** 23741–23747
9. Hilfiker, H., Guerini, D., and Carafoli, E. (1994) *J. Biol. Chem.* **269,** 26178–26183
10. Seiz-Preiano, B., Guerini, D., and Carafoli, E. (1996) *Biochemistry* **35,** 7946–7953
11. Kuo, T. H., Wang, K. K. W., Carlock, L., Diglio, C., and Tsang, W. (1991) *J. Biol. Chem.* **266,** 2520–2525
12. Guerini, D., Garcia-Martin, E., Gerber, A., Volbracht, C., Leist, M., Merino, C. G., and Carafoli, E. (1999) *J. Biol. Chem.* **274,** 1667–1676
13. Keeton, T. P., and Shull, G. E. (1995) *Biochem. J.* **306,** 779–785
14. Clipstone, N. A., and Crabtree, G. R. (1992) *Nature* **357,** 695–697
15. Molkentin, J. D., Lu, J. R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. N. (1998) *Cell* **93,** 215–228
16. Gallo, V., Kingsbury, A., Balázs, R., and Jörgensen, O. S. (1987) *J. Neurosci.* **7,** 2203–2213
17. Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D., and Mitchell, J. B. (1987) *Cancer Res* **47,** 943–946
18. Foletti, D., Guerini, D., and Carafoli, E. (1995) *FASEB J.* **9,** 670–680
19. Towbin, H., Staehlin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
20. Chomczynski, P., and Sacchi, N. (1897) *Anal. Biochem.* **162,** 156–159
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Tso, J. Y., Sun, X. H., Kao, T. H., Reece, K. S., and Wu, R. (1985) *Nucleic Acids Res.* **13,** 2485–2502
23. Ito, A., Hashimoto, T., Hirai, M., Takeda, T., Shuntoh, H., Kuno, T., and Tanaka, C. (1989) *Biochem. Biophys. Res. Commun.* **163,** 1492–1497
24. Klee, C. B., Ren, H., and Wang, X. (1998) *J. Biol. Chem.* **273,** 13367–13370
25. Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8122–8126
26. Boyle, D. B., and Coupar, B. E. (1988) *Gene* **65,** 123–128
27. Drillien, R., and Spehner, D. (1983) *Virology* **131,** 385–393
28. Guerini, D. (1997) *Biochem. Biophys. Res. Commun.* **235,** 271–275
29. Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991) *Cell* **66,** 807–815
30. Ishihara, H., Martin, B. L., Brautigan, D. L., Karaki, H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimoto, K., Uemura, D., and Hartshorne, D. J. (1989) *Biochem. Biophys. Res. Commun.* **159,** 871–877
31. Fresu, G., Dehpour, A., Genazzani, A. A., Carafoli, E., and Guerini, D. (1999) *Glia* **28,** 150–155
32. Thompson, M. A., Ginty, D. D., Bonni, A., and Greenberg, M. E. (1995) *J. Biol. Chem.* **270,** 4224–4235
33. Clipstone, N. A., Fiorentino, D. F., and Crabtree, G. R. (1994) *J. Biol. Chem* **269,** 26431–26437
34. Luo, C., Shaw, K. T., Raghavan, A., Aramburu, J., Garcia Cozar, F., Perrino, B. A., Hogan, P. G., and Rao, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 8907–8912
35. Shibasaki, F., Price, E. R., Milan, D., and McKeon, F. (1996) *Nature* **382,** 370–373
36. Graef, I. A., Mermelstein, P. G., Stankunas, K., Nellson, J. R., Deisseroth, K., Tsien, R. W., and Crabtree, G. R. (1999) *Nature* **401,** 703–708
37. Bito, H., Deisseroth, K., and Tsien, R. W. (1996) *Cell* **87,** 1203–1214
38. Matheos, D. P., Kingsbury, T. J., Ahsan, U. S., and Cunningham, K. W. (1997) *Genes Dev.* **11,** 3445–3458
39. Ashiya, M., and Grabowski, P. J. (1997) *RNA* **3,** 996–1015
40. Wang, H. Y., Lin, W., Dyck, J. A., Yeakley, J. M., Songyang, Z., Cantley, L. C., and Fu, X. D. (1998) *J. Cell Biol.* **140,** 737–750
41. Yano, S., Tokumitsu, H., and Soderling, T. R. (1998) *Nature* **396,** 584–587
42. Kim, E., DeMarco, S. J., Marfatia, S. M., Chishti, A. H., Sheng, M., and Strehler, E. E. (1998) *J. Biol. Chem.* **273,** 1591–1595
43. Ehlers, M. D., Tingley, W. G., and Huganir, R. L. (1995) *Science* **269,** 1734–1737
44. Kornau, H. C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995) *Science* **269,** 1737–1740
45. O'Brien, R. J., Lau, L. F., and Huganir, R. L. (1998) *Curr. Opin. Neurobiol.* **8,** 364–369
46. Carafoli, E. (1994) *FASEB J.* **8,** 993–1002