Plasma membrane calcium ATPase plays a role in reducing Ca2+−mediated cytotoxicity in PC12 cells

ARTICLE *in* JOURNAL OF NEUROSCIENCE RESEARCH · JUNE 2001

Impact Factor: 2.59 · DOI: 10.1002/jnr.1120

5 AUTHORS, INCLUDING:

Emanuel E Strehler
Mayo Clinic - Rochester
158 PUBLICATIONS **5,301** CITATIONS

SEE PROFILE

Journal of Neuroscience Research 64:661–669 (2001)

Plasma Membrane Calcium ATPase Plays A Role in Reducing Ca^{2+}-Mediated Cytotoxicity in PC12 Cells

Michael L. Garcia,[1] Yuriy M. Usachev,[4] Stanley A. Thayer,[4] Emanuel E. Strehler,[1,2] and Anthony J. Windebank[1,3]★

[1]Program in Molecular Neuroscience, Mayo Graduate School, Rochester, Minnesota
[2]Department of Biochemistry and Molecular Biology, Mayo Graduate School, Rochester, Minnesota
[3]Department of Neurology, Mayo Medical School, Rochester, Minnesota
[4]Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota

In many cell types, cell death induced by a variety of insults is accompanied by an increase in intracellular calcium. The Ca^{2+} homeostatic mechanisms affected by such insults, however, have not been fully determined. Recent evidence indicates that kainic acid-induced seizures alter plasma membrane calcium ATPase mRNA expression within vulnerable hippocampal cell populations before the onset of cell death. We examined the effects of altering plasma membrane calcium ATPase expression on cell vulnerability in rat pheochromocytoma 12 cells. Pheochromocytoma 12 cells are vulnerable to Ca^{2+} overload induced by the Ca^{2+} ionophore A23187. Reverse transcriptase-PCR and Western blot data indicated that plasma membrane calcium ATPase isoform 4b constitutes a major calcium pump isoform in the pheochromocytoma 12 cells. Therefore, permanently transfected pheochromocytoma 12-derived cell lines were established that either over-expressed plasma membrane calcium ATPase isoform 4b, or suppressed the expression of the endogenous plasma membrane calcium ATPase isoform 4. Over-expressing clones were less vulnerable to Ca^{2+}-mediated cell death induced by A23187 whereas "antisense" clones were considerably more susceptible. These data indicate that regulation of plasma membrane calcium ATPase expression may be critical to cellular survival when cells are exposed to pathological increases in intracellular calcium. J. Neurosci. Res. 64:661–669, 2001.

Key words: apoptosis; calcium ATPase; cell death; PC12 cells

Glutamate toxicity may be the final common pathway of various neuronal injuries, such as seizures and traumatic brain injury. Ultimately, cell death results from over-stimulation of glutamate receptors leading to toxic intracellular calcium concentrations (Choi, 1995). It is not well established, however, which of the available calcium homeostatic mechanisms in the cell are most affected by high levels of intracellular calcium.

The plasma membrane calcium ATPases (PMCAs) are ubiquitously expressed, transmembrane proteins that are particularly abundant in excitable tissue (Carafoli and Stauffer, 1994). They range in molecular weight from 130–150 kDa. The PMCAs are activated by Ca^{2+}/calmodulin, exposure to acidic phospholipids and phosphorylation by protein kinases (Carafoli, 1991; Monteith and Roufogalis, 1995). In the rat, four major PMCA isoforms are generated as distinct gene products, denoted PMCA isoforms 1–4 (Shull and Greeb, 1988; Greeb and Shull, 1989; Keeton and Shull, 1995). Further isoform variability is achieved by RNA splicing that occurs mainly at two locations, denoted splice site A and C (Strehler, 1991). The four major isoforms are expressed in a tissue specific manner. PMCA1 has been found in all tissues examined to date (Shull and Greeb, 1988; Brandt et al., 1992; Keeton et al., 1993), whereas PMCA4 has been detected in all tissues examined except liver (Keeton and Shull, 1995). PMCA2 is expressed mainly in brain and heart, and PMCA3 is essentially confined to brain and skeletal muscle (Shull and Greeb, 1988; Greeb and Shull, 1989; Brandt et al., 1992; Keeton et al., 1993).

Due to their high affinity for calcium, the PMCAs are believed to play a major role in maintaining a basal level of intracellular calcium ($[Ca^{2+}]_i$) (Carafoli, 1994).

Contract grant sponsor: NIH; Contract grant number: F31 NS10436, GM58710, DC04200, NS40471, NS39769, DA07304, DA11806; Contract grant sponsor: National Science Foundation; Contract grant number: IBN9723796; Contract grant sponsor: Mayo Foundation; Contract grant sponsor: NIDA; Contract grant number: DA07097.

Michael L. Garcia's current address is: Ludwig Institute for Cancer Research, UCSD/CMM-E Room 3072, 9500 Gilman Drive, La Jolla, CA 92093-0660.

★Correspondence to: Anthony J. Windebank, MD, Mayo Clinic, 1501 Guggenheim Building, 200 1st Street SW, Rochester, MN 55905. E-mail: windebank.anthony@mayo.edu

Received 10 December 2000; Revised 16 March 2001; Accepted 22 March 2001

Recent evidence also suggests a more active role for the PMCAs in returning $[Ca^{2+}]_i$ to basal levels after cellular depolarization (Tepikin et al., 1992; Monteith and Roufogalis, 1995; Toescu and Petersen, 1995; Penniston and Enyedi, 1998). This role is further strengthened by the observation that in the stereocilia of bullfrog hair cells, the PMCAs are the only form of calcium extrusion mechanism available (Yamoah et al., 1998). Although a more active role in Ca^{2+} homeostasis has been assigned the PMCAs, the importance of PMCAs to the proper functioning of neuronal systems and to the survival of cells during pathological events has only recently begun to be elucidated. For example, disruption of energy metabolism may impair PMCA function. While analyzing the effects of anoxia on ATP levels, Kass and Lipton (1982, 1986) hypothesized that the decrease in ATP levels within the cells would affect the functioning of the PMCAs during anoxic insults thus leading to a loss in Ca^{2+} homeostasis. The inability of cells to recover ion homeostasis was shown to be the cause for cell loss during inhibition of oxidative phosphorylation (Riepe et al., 1995). Ion motive ATPases have been shown to be targets of free radical damage (Rohn et al., 1993, 1996), and during seizure activity, the expression of PMCA mRNA was shown to be decreased at times preceding the onset of cell death within vulnerable cell populations of the hippocampus (Garcia et al., 1997). These data indicate a crucial role for the PMCA in Ca^{2+} homeostasis during normal and pathological conditions. We have, therefore, attempted to directly ascertain the contribution offered to Ca^{2+} homeostasis by the PMCAs during pathological increases in intracellular calcium. We have established that altering the expression levels of PMCA4 alters the vulnerability of pheochromocytoma 12 (PC12) cells to Ca^{2+}-mediated cell death. Cells that over-express PMCA4 are less vulnerable whereas suppression of endogenous PMCA4 rendered the cells more susceptible to Ca^{2+}-mediated cell death.

MATERIALS AND METHODS

Cell Culture and Cell Survival

PC12 cells were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) with 10% horse serum and 10% newborn calf serum at 37°C and 7% CO_2. PC12 cells were plated in serum containing medium at a density of 5×10^5 cells per 3.5 cm dish, and were allowed to adhere overnight. Immediately before the addition of ionophore, the cells were placed in fresh media. The calcium ionophore, A23187, was added to a final concentration of 5 μM. The cells were cultured for 24 hr. Subsequently the cells were harvested via agitation, spun at 1,000 rpm for 5 min and the medium was removed. Propidium iodide (1 mg/ml) dissolved in 1× phosphate buffered saline (PBS) was added, and the cells were incubated for 2 hr at 4°C. Percent labeling of cells was determined via flow cytometric analysis utilizing a FacScan (Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ). All survival data represent the average of at least 15 independent 3.5 cm dishes.

RT-PCR

Medium was removed from the cells and cells were washed twice with 1 × PBS. Total RNA was isolated from control PC12 cells utilizing the Trizol reagent (Gibco). Briefly, cells (one confluent 10 cm dish; procedure carried out on four independent dishes) were lysed by adding 5 ml of Trizol reagent. The lysates were allowed to incubate at room temperature for 5 min. Chloroform (1.2 ml) was added to the lysates followed by brief vortexing. Samples were incubated for 10 min at room temperature and then centrifuged for 15 min at 12,000 rpm at 4°C. The aqueous phase was removed, and 3 ml of isopropanol were added. Samples were incubated at room temperature for 10 min and then centrifuged for 10 min at 12,000 rpm at 4°C. RNA pellets were washed with 5 ml of 75% ethanol and centrifuged for 5 min at 9,000 rpm at 4°C. RNA pellets were resuspended in diethyl pyrocarbonate (DEPC)-treated water and stored at −70°C. Reverse transcription was carried out utilizing the SuperScript Preamplification System (Gibco). PCR and primer design were carried out as described previously (Hammes et al., 1994).

Western Blots

PC12 cells were harvested and prepared for total protein isolation by removing media, washing twice with cold 1 × PBS, and adding 1 ml of lysis buffer (50 mM HEPES pH 7.5, 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, and 50 mM NaF). The cells were passed three times through tuberculin syringes, and then rocked for 30 min at 4°C. Cell lysates were centrifuged for 15 min at 13,000 rpm at 4°C, and then stored at −70°C. Protein concentrations were determined utilizing the Bio-Rad Protein Assay (Bio-Rad Lab, Richmond, CA). 40 μg of protein per lane were run on denaturing 4–15% acrylamide/polyacrylamide gradient gels (Bio-Rad) for 2 hr at 80V. Protein was then transferred onto a polyvinylidene difluoride (PVDF) membrane for 45 min at 60V. The membrane was rinsed twice in 1 × PBS for 10 min at room temperature, and then blocked overnight in 10% (w/v) dry milk in PBS at 4°C. The membrane was incubated with primary antibody in 0.1% BSA in PBST (PBS + 0.1% Triton X-100) for 1–2 hr at room temperature or for 1 hr at 4°C. Primary antibodies were as follows: for detection of PMCA4, mouse monoclonal JA9 (Caride et al., 1996) at 1:2,000; for detection of PMCA1, rabbit polyclonal NR1-1 (Filoteo et al., 1997) at 1:200; for detection of Trk A, mouse monoclonal MCTrks (Santa Cruz Biotechnology, Santa Cruz, CA) at 1–2 μg/ml. Antibodies JA9 and NR1-1 were gifts from A.G. Filoteo and J.T. Penniston (Mayo Clinic, Rochester). Following three 10 min washes at room temperature, the membrane was incubated in anti-mouse IgG at 1:5,000 in 0.1% BSA in PBST for 1 hr at room temperature or in anti-rabbit IgG at 1:5,000 for 1 hr at room temperature. Antibody visualization was accomplished utilizing the "Renaissance" detection kit (Dupont-NEN, Boston, MA).

Expression Constructs

A plasmid encoding full-length hPMCA4b was obtained from Dr. J.T. Penniston (Mayo Clinic). The plasmid was initially digested with Kpn I, blunted, and then digested with Sal I to liberate the hPMCA4b fragment. The insert was subcloned into Sal I and Sma I digested pCI-neo mammalian expression plasmid (Promega, Madison, WI) utilizing standard techniques (Ausubel et al., 1996), and the resulting expression construct was termed hPMCA4b. Correct insertion of the PMCA4b cDNA was verified via agarose gel electrophoresis and DNA sequence analysis. Antisense plasmids were constructed by ligating a 373 bp RT-PCR fragment corresponding to bases 71–443 of the 5′ region of PMCA4 (Keeton and Shull, 1995) into pCI-neo. The PCR product was first TA-cloned into PCRII (Invitrogen, San Diego, CA). Sequence confirmation was obtained via DNA sequence analysis. The PMCA4 fragment was removed from PCRII via EcoRI digest and then ligated into the EcoRI site of pCI-neo. Sequence confirmation was obtained via DNA sequence analysis and the final construct was termed antisense4.

Transfections and Clone Selection

PC12 cells were transfected with either hPMCA4b or antisense4 via the modified bovine serum albumin transfection kit (Stratagene, La Jolla, CA). Briefly, PC12 cells were plated at a density of 2×10^5 cells per 6 cm dish and allowed to adhere overnight in maintenance media. DNA was prepared for transfection by adding 10 μg of plasmid DNA in a volume of 500 μl to 50 μl of 2.5 M $CaCl_2$ and 500 μl of 2 × *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid buffered saline and allowed to incubate for 20 min at room temperature. During the incubation, the cells were prepared by removing the media and washing twice in cold, sterile filtered 1× PBS. Cells were then placed in DMEM containing 6% modified bovine serum albumin reagent and incubated at 37°C in 5% CO_2. DNA was resuspended and 500 μl of the DNA suspension added per dish. Cells were returned to the 37°C incubator for 3 hr. Following this incubation, media were removed, cells were washed three times in cold, sterile filtered 1× PBS, and placed in maintenance media. Cells were cultured for 48 hr before the selection of stable clones. For clone selection, cell cultures were split 1:4 into maintenance media containing 500 μg/ml G418. The cells were incubated in these conditions for 7 days with the addition of fresh media every 3 days. Following 7 days of culture, clones were selected for expansion. The clones were expanded until sufficient cells were available for storage and for analysis of PMCA4 expression via Western blot analysis.

Isolation of Microsomes From PC12 Cells and Measurement of Ca^{2+} Uptake

Crude microsomal membranes were prepared from control or stably transfected PC12 cells essentially as described (Enyedi et al., 1993, 1996). After hypotonic lysis in 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 4 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM EGTA, 4 mM dithiothreitol (10 min on ice) and homogenization with 40 strokes in a Dounce homogenizer, the lysates were diluted with an equal volume of 0.5 M sucrose, 0.3 M KCl, 10 mM Tris-HCl, pH 7.5, 4 μg/ml aprotinin, 1 μg/ml leupeptin, 4 mM dithiothreitol, homogenized again with 20 strokes in the Dounce homogenizer, and then centrifuged at 4,000 × *g* for 15 min at 4°C. The supernatants were made 0.6 M in KCl and 2.5 mM in EDTA to strip calmodulin from the membranes, and the mixture centrifuged at 100,000 *g* for 40 min at 4°C to sediment the microsomal fraction. The final pellets were resuspended in a solution of 0.25 M sucrose, 0.15 M KCl, 10 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 20 μM $CaCl_2$, 4 μg/ml aprotinin, 1 μg/ml leupeptin at a protein concentration of 1–5 mg/ml. Aliquots of the suspension were stored in liquid nitrogen until needed. Ca^{2+} uptake by microsomal vesicles was carried out at 37°C for 5 min by rapid filtration through Millipore membranes (HA type, 0.45 μm pore size, Millipore Corp., Bedford, MA) as described (Enyedi et al., 1993, 1996). The uptake medium contained 140 mM KCl, 25 mM TES-triethanolamine, pH 7.2, 40 mM KH_2PO_4/K_2HPO_4, pH 7.2, 400 nM thapsigargin, 5 mM NaN_3, 5 μg/ml oligomycin, 7 mM $MgCl_2$, 100 μM $CaCl_2$ (labeled with ^{45}Ca, specific activity 150,000–200,000 cpm/nmol) and 0.11 mM EGTA to obtain a free Ca^{2+} concentration of 1 μM. Calmodulin was added to a final concentration of 540 nM. Microsomes at 40–70 μg/ml were preincubated at 37°C for 3 min before initiating calcium uptake by the addition of 60 mM ATP to a final concentration of 6 mM. Incubations were carried out at 37°C for 5 min and background activity was determined in the absence of ATP.

$[Ca^{2+}]_i$ Measurements

Experimental procedure. For this series of experiments, PC12 cells were plated onto 25 mm coverslips and cultured for 2–4 days under the same conditions described above (Cell Culture and Cell Survival). PC12 cells were loaded with indicator by incubation in HEPES-buffered Hank's salt solution containing 0.02% pluronic F-127 and 5 μM fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) for 30 min at room temperature. The cells from different clones demonstrated similar levels of fura-2 fluorescence, indicating that genetic alteration of the cells did not affect dye loading. HEPES-Hank's solution was composed of the following (in mM): HEPES, 10; NaCl, 137; $CaCl_2$, 1.3; $MgSO_4$, 0.4; $MgCl_2$, 0.5; KCl, 5.0; KH_2PO_4, 0.4; Na_2HPO_4, 0.6; $NaHCO_3$, 3.0; and glucose, 10. Incubating in HEPES-Hank's buffer for an additional 20 min terminated loading. The coverslip was then placed in a flow-through chamber (Thayer et al., 1988) that was superfused at a rate of 2–2.5 ml/min. Cells were depolarized by switching a multiport valve coupled to a reservoir containing HEPES-Hank's buffer to one with buffer containing 70 mM K^+ in which K^+ was substituted reciprocally for sodium. Cyclopiazonic acid (CPA) was added to HEPES-Hank's buffer from a 30 mM stock in DMSO to a final concentration of 5 μM.

Digital imaging. Fura-2-based digital imaging was carried out as described previously (Jin et al., 1994). The chamber containing the fura-2-loaded cells was mounted on the stage of an inverted microscope (Nikon Diaphot) and alternately excited at 340 or 380 nm by rapidly switching optical filters (10 nm band pass) mounted on a computer-controlled wheel (Sutter Instrument, Company, Novato, CA) placed between a 75 W Xenon arc lamp and the epifluorescence port of the microscope. Excitation light was reflected from a dichroic mirror (400 nm)

through a 70 × objective (Leitz; N.A. 1.15). Fluorescent images (510, 40 nm band pass) were projected (0.5 ×) onto a cooled charge-coupled device camera (Photometrics, Tucson, AZ; 384 × 576 binned to 192 × 288 pixels) controlled by an 80/486 computer. Image pairs were collected every 6 sec; exposure to excitation light was between 120 and 200 msec per image and the interval between paired images was 385 msec.

$[Ca^{2+}]_i$ was calculated from the ratio of the two background subtracted digital images. Cells were delimited by producing a mask that contained pixel values above a threshold applied to the 380 nm image. Background images were collected at the conclusion of each experiment after removing cells from the coverslip. Autofluorescence from cells not loaded with the dye was less than 5% and thus not corrected. Ratio values were converted to free $[Ca^{2+}]_i$ by the equation $[Ca^{2+}]_i = K_d\beta (R - R_{min})/(R_{max} - R)$, where R is the 340:380 nm fluorescence emission ratio and K_d = 224 nM, the dissociation constant for fura-2 (Grynkiewicz et al., 1985). The maximum ratio (R_{max} = 3.3) the minimum ratio (R_{min} = 0.21), and the constant β (the ratio of the fluorescence measured at 380 nm in Ca^{2+}-free and saturating solution; 6.7) were determined by treating cells with 10 μM ionomycin in Ca^{2+}-free (1 mM EGTA) and saturating (5 mM Ca^{2+}) solution.

Statistical Analysis

Flow cytometric data, for cell survival, were analyzed utilizing Cell Quest Software (Becton Dickinson Immunocytometry Systems). This calculated the percentage of labeled cells. Percent values were transferred into InStat (GraphPad Software, San Diego, CA) for statistical analysis. Overall statistical significance was determined by analysis of variance (ANOVA). Post-hoc analyses were carried out on individual group means using Bonferroni Multiple Comparisons Test (GraphPad). For Western blots, relative optical densities were acquired using the software Microcomputer Imaging Device (Imaging Research Inc.). Relative optical densities were then analyzed for statistical significance using InStat (GraphPad). Unpaired *t*-tests were carried out on average measurements from control, antisense, and overexpressing clones. A *P*-value of less than 0.05 was required to reject the null hypothesis. All $[Ca^{2+}]_i$ measurements are presented as mean ± SEM.

RESULTS

PMCA Expression

PC12 cells were initially screened for isoform expression utilizing RT-PCR analysis. Of the four PMCA isoforms in rat (Keeton et al., 1993), mRNA expression for PMCA1 and PMCA4 was detected (Fig. 1A). This analysis also revealed that only the "b" alternative splice variant of each of these PMCA isoforms is expressed, consistent with earlier findings in undifferentiated PC12 cells (Hammes et al., 1994). In contrast to a previous report (Hammes et al., 1994), no mRNA expression was detectable under our conditions for either PMCA2 or PMCA3 (data not shown). Western blot analysis confirmed the translation of the mRNA species into the corresponding PMCA protein products. The expression of

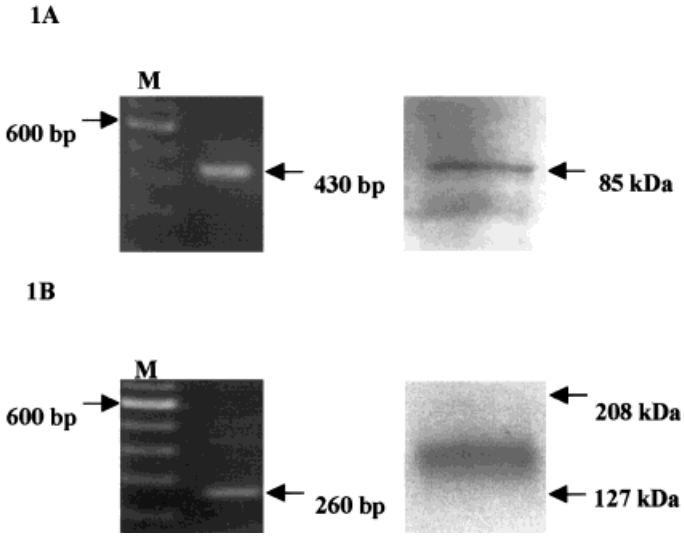

Fig. 1. Expression of PMCA1b and 4b in naïve PC12 cells. **A:** RT-PCR was utilized to screen PC12 cells for PMCA expression. mRNA was detected for PMCA 1b (430 bp, left panel) and 4b (260 bp, right panel). M, DNA molecular size standards. An arrow indicates the position of 600 bp. **B:** Western blot analysis utilizing the PMCA4-specific antibody JA9 to confirm the translation of PMCA4 mRNA into full-length protein (about 135 kDa). Arrows indicate approximate positions of protein molecular weight standards.

a full-length version of PMCA4 was detected utilizing the isoform-specific antibody JA9 (Fig. 1B), whereas only a faint band for the full-length PMCA1 and a major truncated form of about 85 kDa was detected using PMCA1-specific antibody NR1 (not shown). The reason why PMCA1 was mainly present as a truncated form is not clear, given that the cells were harvested fresh, immediately lysed and protein aliquots stored frozen. In addition, the PMCA4 showed no degradation. The truncated form of PMCA1 could also be detected with the pan-PMCA antibody 5F10, demonstrating that it is unlikely to be an unspecific cross-reacting protein (not shown). The specificity of the two antibodies JA9 and NR-1 for their respective PMCA isoform has been demonstrated previously (Caride et al., 1996; Filoteo et al., 1997), although it was noted that NR-1 is not as sensitive as the other isoform-specific antibodies or the pan-PMCA antibody 5F10 (Filoteo et al., 1997). Taken together, however, the RT-PCR and Western blot data presented above indicate that PMCA1 and PMCA4 are the major PMCA isoforms expressed in undifferentiated PC12 cells.

Cell Death

In order to cause pathological rises in intracellular calcium, the calcium ionophore, A23187, was utilized. PC12 cells were incubated in 5 μM A23187 for 24 hr. Cells were harvested and stained with propidium iodide. Percent labeling was determined using flow cytometric analysis. After 24 hr of incubation in A23187, PC12 cells

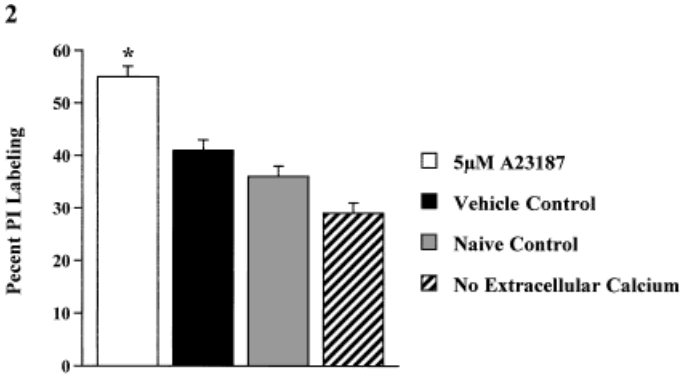

Fig. 2. PC12 cells are vulnerable to calcium ionophore. PC12 cells were incubated with 5 μM A23187 for 24 hr in standard medium or in medium with no extracellular calcium. After 24 hr, the cells were harvested and labeled with propidium iodide and analyzed via flow cytometry. PC12 cells were vulnerable to A23187 mediated increases in intracellular calcium. PC12 cells treated with A23187 labeled at 55% (±2%) compared with vehicle-only (DMSO) treated (41% ± 2%) and naïve cells (36% ± 2%). The effects of A23187 are dependent upon the presence of extracellular calcium. Removing extracellular calcium reduced the percentage of cells labeled to 29% (±2%). Group means were initially analyzed for overall statistical significance via ANOVA. Post-hoc analysis between group means was made via Bonferroni multiple comparisons test. $\star P < 0.001$ vs. no extracellular calcium, DMSO and naïve control cells.

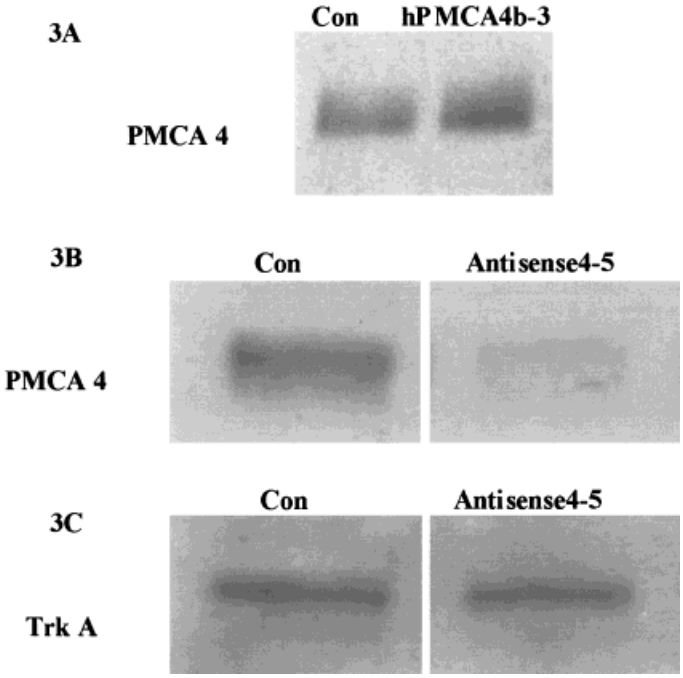

Fig. 3. Altered expression of hPMCA4 in a stably transfected PC12 cell clone. PC12 cells were transfected with either hPMCA4b or antisense4, and stable clones were selected via G418 resistance. Total protein was isolated and equal amounts of protein per lane were separated by SDS-polyacrylamide gel electrophoresis. PMCA4 was subsequently detected via Western blot analysis utilizing the isoform-specific antibody JA9. A single clone (4b-clone3) was obtained that over-expressed PMCA4 to approximately 169% of control (**A**). A clone (AS4-clone5) stably transfected with the antisense construct showed reduced expression of endogenous PMCA4 to 32% of control values (**B**). By contrast, utilizing the pan-Trk antibody MCTrks, no significant alteration in Trk A expression was detected in AS4-clone5 compared with control cells, confirming the specificity of the antisense construct (**C**).

exhibited significant vulnerability to the resultant rise in intracellular calcium, with 55% of the cells being labeled (Fig. 2). These effects of A23187 were dependent upon the presence of extracellular calcium. Incubating cells with A23187 in media that contained no extracellular calcium reduced the percentage of cells labeled to 29% (Fig. 2). Untreated or vehicle-only (DMSO) treated control cells displayed a relatively high basal death rate (41% and 36%, respectively) under our assay conditions. Media shock could have contributed to this high percentage of cell death. Because complete media changes were necessary for certain experiments (e.g., when lowering extracellular calcium), and to remain consistent throughout, media were changed for all experimental conditions.

Analysis of PC12 Clones With Altered PMCA4 Expression Levels

PC12 cells were transfected with constructs that either expressed full-length human PMCA4b or a portion of the 5′ sequence of PMCA4 cloned in the antisense direction. Cells that had stably integrated the plasmid were selected via G418 resistance. Total protein was isolated from the expanded cells and expression levels were determined by Western blot analysis. Cell lines were established that over-expressed PMCA4 (4b-clone3; Fig. 3A), and that displayed suppressed expression of endogenous PMCA4 (AS4-clone5; Fig. 3B). 4b-Clone3 expressed PMCA4 at 169% of the value found in control PC12 cells (Fig. 3A). The antisense construct reduced endogenous levels of PMCA4 to 32% of control values (Fig. 3B). The expression of the high affinity NGF receptor, Trk A, was analyzed to determine the specificity of the reduction afforded by the antisense construct. No reduction in the expression of Trk A was detected (Fig. 3C). The endogenous levels of PMCA1 were also unaffected in AS4-clone5 supporting the specificity of the antisense construct (data not shown).

To determine if the alteration of PMCA expression in the antisense-treated and over-expressing PC12 cells leads to measurable changes in PMCA function, microsomal membrane vesicles were prepared and analyzed for their calcium uptake properties in the absence and presence of 0.5 μM calmodulin. As has been found for other cell lines (e.g., COS cells), a modest rate of endogenous PMCA-mediated Ca^{2+} uptake was observed into vesicles prepared from control PC12 cells (Fig. 4A). This is due to the low expression levels of endogenous PMCAs in most cell types. Ca^{2+} uptake in the control cells was stimulated only 1.1-fold in the presence of calmodulin (from 0.69

nmol Ca^{2+} × mg protein^{-1} minute^{-1} to 0.75 nmol × mg protein^{-1} minute^{-1}). This low level of calmodulin stimulation may be due to incomplete removal of calmodulin from the membranes as well as to the fact that some PMCA isoforms (particularly the "b" splice variant of isoforms 2 and likely of isoforms 1 and 3) show a high calmodulin-independent basal activity. Interestingly, uptake of $^{45}Ca^{2+}$ into vesicles from AS4-clone5 was significantly decreased (to 33% in the presence of calmodulin) compared with control untransfected PC12 cells (Fig. 4A), in agreement with the Western data showing a significant decrease in PMCA4 expression in these cells. The calcium uptake values in these cells in the absence and presence of calmodulin were 0.17 and 0.24 nmol Ca^{2+} × mg protein^{-1} minute^{--1}, respectively. By contrast, we were unable to detect an increase in $^{45}Ca^{2+}$ uptake in vesicles prepared from 4b-clone3 (not shown) although these cells exhibited an increase in PMCA4 expression as determined by Western blot (Fig. 3A). The reason for this result is not clear but may be linked to a change (increase) in total microsomal protein content leading to an altered ratio of PMCA activity per mg of membrane protein, or to the inability of the overexpressed recombinant (human) PMCA4b to functionally insert in the rat PC12 cell membranes.

As an independent means to determine if overexpression or downregulation of PMCA4 has a direct effect on important Ca^{2+} regulatory properties, we determined the kinetics of single-cell Ca^{2+} transients in the different PC12 cell lines. PC12 cells were preloaded with the Ca^{2+} indicator fura-2 and $[Ca^{2+}]_i$ determined with a digital imaging system as described in Methods. A 20 sec application of 70 mM K^+ elicited Ca^{2+} influx via voltage-gated Ca^{2+} channels elevating $[Ca^{2+}]_i$ from a basal level of 44 ± 7 nM to peak at 1,160 ± 97 nM (n = 29) (Fig. 4B). The $[Ca^{2+}]_i$ returned to basal levels by a process that was well described by a monoexponential function. Thus, the time constant provided a quantitative measure of the cell's ability to remove Ca^{2+} from the cytoplasm. A 30-min pretreatment of the cells with cyclopiazonic acid (5 μM), an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Thomas and Hanley, 1994), did not affect the $[Ca^{2+}]_i$ recovery rate in any of the clones tested suggesting a negligible role of SERCA in this process. $[Ca^{2+}]_i$ recovered with virtually the same rate in cells over-

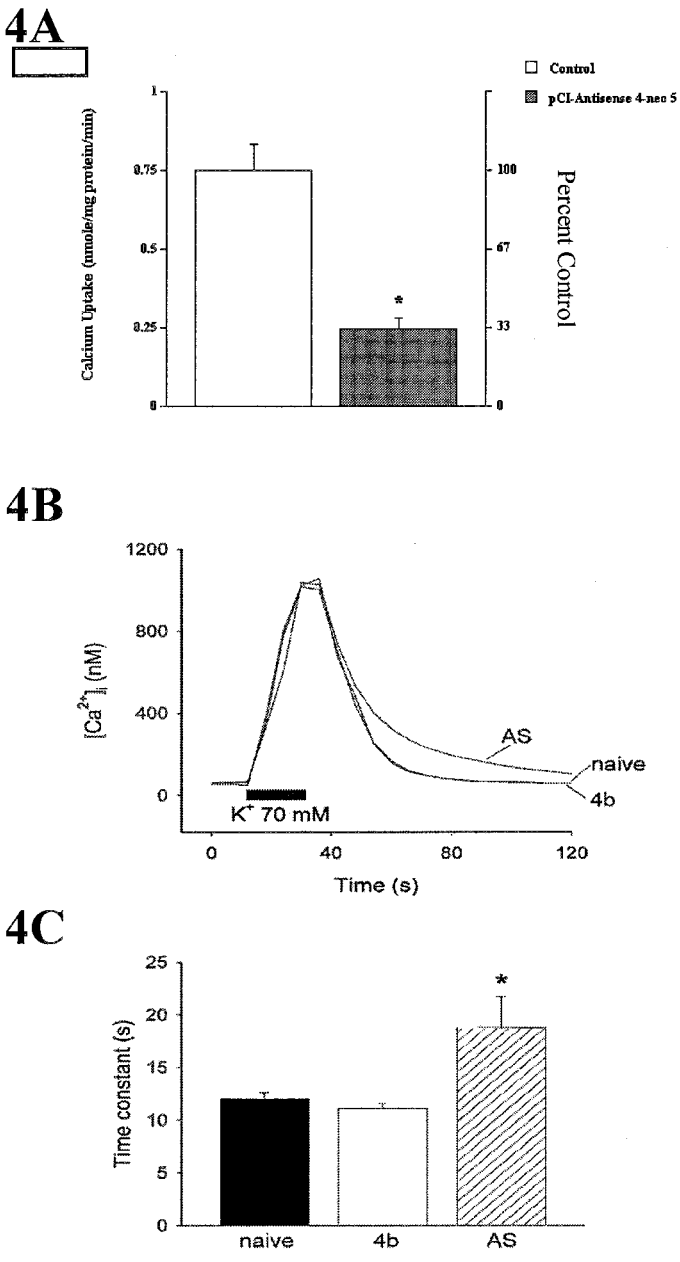

Fig. 4. Calcium uptake (**A**) and recovery kinetics from calcium transients (**B**,**C**) in PC12 cells. A: $^{45}Ca^{2+}$ uptake in the presence of 540 nM calmodulin was measured into microsomal vesicles from control PC12 cells and from AS4-clone5 cells as described in Materials and Methods. The average from two independent measurements ± SD is displayed in nmol Ca^{2+} × mg protein^{-1} min^{-1} as well as percent of control (set at 100%). B: Representative traces from single cells show that $[Ca^{2+}]_i$ recovery kinetics are slowed in PMCA4-knockdown clones. $[Ca^{2+}]_i$ concentration was measured in fura-2/AM loaded naïve PC12 cells and in cells from 4b-clone3 (4b) and AS4-clone5 (AS). Seventy mM KCl was applied at the time indicated by the horizontal bar. C: Histogram summarizing recovery kinetics from multiple single cell recordings such as that shown in (B). $[Ca^{2+}]_i$ recovery kinetics were fit with a monoexponential function and a corresponding time constant was calculated by using a non-linear, least-squares curve fitting algorithm (Origin 4.0 software); the multiple correlation coefficient was higher than 0.99 in experiments from all three clones. Recovery kinetics were determined for 20, 19 and 20 cells from wild type, "AS" and "4b" clones, respectively. At least two coverslips were studied for each clone. The time constant for AS4-clone5 (AS) was significantly greater than that for the naïve PC12 cells and cell clone 4b-clone3 (4b) ($^\star P < 0.05$, one-way ANOVA, Bonferroni post-hoc test). The data are presented as mean ± SEM.

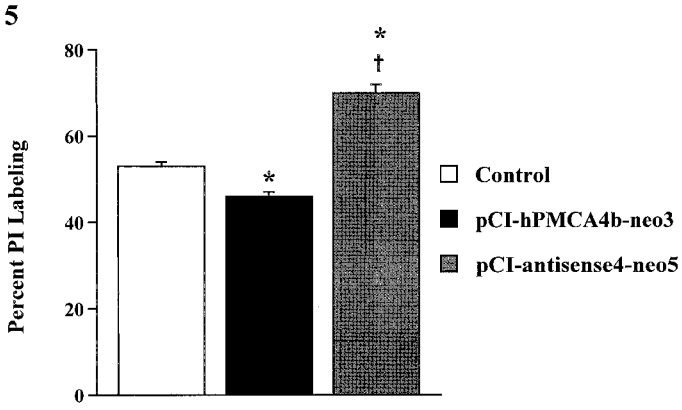

Fig. 5. Altering the expression level of PMCA4 alters the vulnerability of PC12 cells to A23187. PC12 cells were incubated with 5 μM A23187 for 24 hr and then harvested and labeled with propidium iodide. The percentage of labeled cells was moderately though significantly reduced in PC12 cells over-expressing PMCA4. Cells of 4b-clone3 labeled at 46% (±1%) whereas control cells labeled at 53% (±1%). Suppressing the expression of endogenous PMCA4 enhanced PC12 cell vulnerability. AS4-clone5 cells labeled at 70% (±2%) compared with the control cells that labeled at 53% (±1%). Group means were initially analyzed for overall statistical significance via ANOVA. Post-hoc analysis between group means was made via Bonferroni Multiple Comparisons Test. *$P < 0.01$ 4b-clone3 vs. control and $P < 0.001$ for AS4-clone5 vs. control; †$P < 0.001$ vs. 4b-clone3.

expressing hPMCA4b ($\tau = 11 \pm 1$ s, n = 20) and naïve cells ($\tau = 12 \pm 1$ s, n = 20). Thus, the 1.7-fold increase in hPMCA4 expression (as determined by Western blot) did not alter the regulation of $[Ca^{2+}]_i$ in this model, in agreement with our inability to detect an increase in calcium uptake into microsomal vesicles in the 4b-clone3 cells. In contrast, Ca^{2+} removal from the cells expressing PMCA4-antisense occurred with significantly slower kinetics ($\tau = 18 \pm 2$ s, n = 19) (Fig. 4C). Thus, a decrease in endogenous PMCA4 expression to about 30% of control expression levels results in a significant loss of Ca^{2+} removal capacity as indicated by a 58% slowing in $[Ca^{2+}]_i$ recovery kinetics.

Effects of Altering PMCA Expression Levels on Cell Survival

To determine the effects of altered PMCA4 expression on PC12 cell vulnerability, survival studies were carried out on stable cell lines. Cells were incubated in A23187 for 24 hr and then labeled with propidium iodide. Flow cytometric analysis of the labeled cells revealed marked differences in the vulnerability of the cell lines to A23187. The over-expressing clone (4b-clone3) displayed a small but significant decrease in the percent of labeled cells versus controls (Fig. 5), yielding 46% labeled cells versus 53% for control cells (Fig. 5). Antisense4-clone5 cells, which expressed PMCA4 at 32% of control cells, displayed greater vulnerability to A23187 than the controls. Seventy percent of cells from antisense4-clone5 labeled compared with 53% of control cells (Fig. 5).

DISCUSSION

Several lines of evidence indicate a role for PMCAs in the regulation of calcium in normal and pathological conditions. Recent data obtained on knockout and "deaf-waddler" mice indicate that the developmental loss of PMCA2 functioning can lead to pathologic alterations of neuronal systems (Kozel et al., 1998; Street et al., 1998). The results presented in this contribution show that the PMCA may play a role in protection of PC12 cells against Ca^{2+} overload.

PC12 cells were determined by RT-PCR and Western blotting to express PMCA isoforms 1 and 4 with isoform 4 being predominantly expressed. Although an earlier study using RT-PCR and Southern blotting indicated that all four PMCA genes may be expressed in PC12 cells (Hammes et al., 1994), the expression of the PMCA isoforms was not addressed at the protein level, and no quantitative comparisons between different RT-PCR products was made. Our data on PMCA isoform expression in PC12 cells are in agreement with the findings of others that almost all tissues and cell types express significant amounts of PMCA1 and PMCA4, whereas PMCA2 and PMCA3 are either undetectable or expressed at comparatively low levels in most cell types (Stauffer et al., 1993, 1995).

In order to analyze the contribution offered by PMCA4, permanently altering PMCA4 expression was required. For this, stable cell lines were established that either overexpressed or suppressed the endogenous levels of PMCA4. If PMCA functioning was required for the survival of cells during pathologic changes in intracellular calcium, then the over-expressing cells should display enhanced survival rates, and antisense cells decreased survival rates in response to A23187. This seemed to be the case, although the data on the overexpressing cells must be interpreted with caution due to the discrepancy between the PMCA4 protein levels detected by Western blotting (Fig. 3A) and the lack of a corresponding increase in microsomal calcium uptake and a faster single cell calcium recovery in these cells (Fig. 4). The calcium uptake and recovery experiments indicate that even though the over-expressing cells contained 1.7-fold higher levels of PMCA4 than the controls, most of that pump seems to be non-functional. Therefore, a more pronounced decrease in cell vulnerability to calcium overload could probably not occur in the 4b-clone3 cells. The reason for the apparent functional incompetence of the overexpressed PMCA is not clear but may be related to the fact that the human isoform 4b was expressed in the rat cells. Of note, the human and rat PMCA4 proteins are much more divergent (87% identity on the amino acid level) than the human and rat PMCA1, 2 and 3 isoforms that share over 98% identity (Strehler, 1991). It would be interesting to see if the protection against calcium-induced cell death in the PC12 cells could be more substantially increased if more of the PMCA were present in a functionally competent state. Compensatory changes in the calcium entry

and handling systems, however, may also play a role in the overall effect of PMCA overexpression on cell death. For example, increased PMCA expression may lead to a concomitant upregulation of capacitative Ca^{2+} entry pathways that would potentially dampen any protective effect of the PMCAs on Ca^{2+} overload.

On the other hand, our results show that the partial loss of PMCA4 (to 30% of control) led to a significant change in the ability of PC12 cells to withstand Ca^{2+} overload. These data support the proposal that the PMCA has a critical role in the regulation of intracellular Ca^{2+}. Taken together with a previous study on PMCA expression in the hippocampus after seizures (Garcia et al., 1997), PMCA expression levels may begin to explain differential vulnerabilities to specific insults involving Ca^{2+} dyshomeostasis within neuronal populations. For example, assuming that the decrease in mRNA correlates with a decrease in the expression of the corresponding protein, protein products for two isoforms of the PMCA may be decreased within vulnerable cell populations of the hippocampus at times preceding the onset of cell death (Garcia et al., 1997). Following seizures, expression of PMCA isoforms 1 and 2 was decreased within the pyramidal cells of the hippocampus. This decrease occurred maximally at 12 hr after seizure induction; a time that preceded the onset of cell death within this population. Within the same anatomical structure, the cells of the dentate gyrus were not vulnerable to seizures. During seizures, the dentate gyrus experienced a decrease in only one isoform of the PMCA at 4 hr. The expression of this isoform however had begun to return to control values by 12 hr (Garcia et al., 1997). Studies on PMCA2 knockout and deafwaddler mice confirm that loss of functional PMCA alone can lead to cell loss within some specific neuronal systems (Kozel et al., 1998; Street et al., 1998). The ability to regulate the expression of Ca^{2+} homeostatic mechanisms in response to Ca^{2+} loads may determine a cell's fate when faced with different pathological stimuli. Evidence suggests that Ca^{2+} influx may regulate the expression of the PMCA genes in cerebellar granule cells (Guerini et al., 1999). In addition, increases in intracellular calcium can change the splicing pattern of PMCA mRNA in a rapid manner (Zacharias and Strehler, 1996). It may, therefore, be possible that certain cells are less vulnerable to pathological changes in intracellular Ca^{2+} due to their ability to adapt the pattern of expression of calcium homeostatic mechanisms in response to an insult.

Additionally, data on the PMCA2 knockout mice (Kozel et al., 1998) as well as that on PC6 cells transfected with antisense DNA to PMCA1 (Brandt et al., 1996) lend independent support to our findings that the loss of one isoform does not lead to compensatory changes in the remaining isoforms. Furthermore, although alterations of PMCA expression (both downregulation and overexpression) can lead to compensatory changes in the SERCA pumps as well as other Ca^{2+} handling systems (Guerini et al., 1995; Liu et al., 1996; Kuo et al., 1997), all available data demonstrate that these changes are not sufficient to compensate for the specific effects of the loss of the PMCA. Although there is cross-talk among the different systems that participate in intracellular Ca^{2+} handling, these systems can only be partially redundant. For example, even if the SERCA pump were upregulated in PC12 cells with reduced levels of the PMCA, it could only compensate for a certain length of time. Eventually, the intracellular stores would become saturated leading to a rise in intracellular Ca^{2+}. This would eventually lead to a large increase in intracellular Ca^{2+} that would kill cells. It seems, therefore, that changes in the levels of PMCAs can profoundly influence the survival rate of PC12 cells exposed to pathological increases in intracellular calcium.

ACKNOWLEDGMENTS

This work was supported by a NIH National Research Fellowship Award (F31 NS10436) to M.L.G., by grants from the NIH (GM58710, DC04200 to E.E.S., NS40471, NS39769 to A.J.W., and DA07304, DA11806 to S.A.T.), National Science Foundation (IBN9723796 to S.A.T.) and the Mayo Foundation; Y.M.U. was supported by NIDA Training Grant DA07097. We are grateful to Ms. Adelaida G. Filoteo and Dr. John T. Penniston for the generous gifts of antibodies JA9 and NR1-1 as well as for the hPMCA4b cDNA plasmid. We thank Ms. Billie Jo Brown for assistance with the preparation of microsomal membranes and for performing the calcium uptake assays. The flow cytometric work was carried out in the Mayo Clinic Flow Cytometry Core Facility with the assistance of Mr. James E. Tarara, Ms. Holly B. Lamb, Ms. Teresa J. Halsey and Ms. Colleen A. Moe. We would also like to thank Dr. Jagjit S. Gill and Ms. LouAnn Gross for valuable advice. We thank Ms. Linda A. Goldbeck for her expert secretarial assistance.

REFERENCES

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. (Eds.) 1996. Enzymatic manipulation of DNA and RNA. In: Current protocols in molecular biology. New York: John Wiley & Sons, Inc. p 3.0.1–3.19.8.

Brandt P, Neve RL, Kammesheidt A, Rhoads RE, Vanaman TC. 1992. Analysis of the tissue-specific distribution of mRNAs encoding the plasma membrane calcium-pumping ATPases and characterization of an alternately spliced form of PMCA4 at the cDNA and genomic levels. J Biol Chem 267:4376–4385.

Brandt PC, Sisken JE, Neve RL, Vanaman TC. 1996. Blockade of plasma membrane calcium pumping ATPase isoform I impairs nerve growth factor-induced neurite extension in pheochromocytoma cells. Proc Natl Acad Sci USA 93:13843–13848.

Carafoli E. 1991. Calcium pump of the plasma membrane. Physiol Rev 71:129–153.

Carafoli E. 1994. Biogenesis. Plasma membrane calcium ATPase: 15 years of work on the purified enzyme. FASEB J 8:993–1002.

Carafoli E, Stauffer T. 1994. The plasma membrane calcium pump: functional domains, regulation of the activity, and tissue specificity of isoform expression. J Neurobiol 25:312–324.

Caride AJ, Filoteo AG, Enyedi A, Verma AK, Penniston JT. 1996. Detection of isoform 4 of the plasma membrane calcium pump in human tissues by using isoform-specific monoclonal antibodies. Biochem J 316:353–359.

Choi DW. 1995. Calcium: still center-stage in hypoxic-ischemic neuronal death. Trends Neurosci 18:58–60.

Enyedi A, Verma AK, Filoteo AG, Penniston JT. 1993. A highly active 120-kDa truncated mutant of the plasma membrane Ca^{2+} pump. J Biol Chem 268:10621–10626.

Enyedi A, Verma AK, Filoteo AG, Penniston JT. 1996. Protein kinase C activates the plasma membrane Ca^{2+} pump isoform 4b by phosphorylation of an inhibitory region downstream of the calmodulin-binding domain. J Biol Chem 271:32461–32467.

Filoteo AG, Elwess NL, Enyedi A, Caride A, Aung HH, Penniston JT. 1997. Plasma membrane Ca^{2+} pump in rat brain. Patterns of alternative splices seen by isoform-specific antibodies. J Biol Chem 272:23741–23747.

Garcia ML, Murray KD, Garcia VB, Strehler EE, Isackson PJ. 1997. Seizure-induced alterations of plasma membrane calcium ATPase isoforms 1, 2 and 3 mRNA and protein in rat hippocampus. Mol Brain Res 45:230–238.

Greeb J, Shull GE. 1989. Molecular cloning of a third isoform of the calmodulin-sensitive plasma membrane Ca^{2+}-transporting ATPase that is expressed predominantly in brain and skeletal muscle. J Biol Chem 264:18569–18576.

Grynkiewicz G, Poenie M, Tsien RY. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Guerini D, Schröder S, Foletti D, Carafoli E. 1995. Isolation and characterization of a stable Chinese hamster ovary cell line overexpressing the plasma membrane Ca^{2+}-ATPase. J Biol Chem 270:14643–14650.

Guerini D, García-Martin E, Gerber A, Volbracht C, Leist M, Merino CG, Carafoli E. 1999. The expression of plasma membrane Ca^{2+} pump isoforms in cerebellar granule neurons is modulated by Ca^{2+}. J Biol Chem 274:1667–1676.

Hammes A, Oberdorf S, Strehler EE, Stauffer T, Carafoli E, Vetter H, Neyses L. 1994. Differentiation-specific isoform mRNA expression of the calmodulin-dependent plasma membrane Ca^{2+}-ATPase. FASEB J 8:428–435.

Jin W, Lee NM, Loh HH, Thayer SA. 1994. Opioids mobilize calcium from inositol 1,4,5-trisphosphate-sensitive stores in NG108-15 cells. J Neurosci 14:1920–1929.

Kass IS, Lipton P. 1982. Mechanisms involved in irreversible anoxic damage to the in vitro rat hippocampal slice. J Physiol (London) 332:459–472.

Kass IS, Lipton P. 1986. Calcium and long-term transmission damage after anoxia in dentate gyrus and CA1 regions of the rat hippocampal slice. J Physiol (London) 378:313–334.

Keeton TP, Shull GE. 1995. Primary structure of rat plasma membrane Ca^{2+}-ATPase isoform 4 and analysis of alternative splicing patterns at splice site A. Biochem J 306:779–785.

Keeton TP, Burk SE, Shull GE. 1993. Alternative splicing of exons encoding the calmodulin-binding domains and C termini of plasma membrane Ca^{2+}-ATPase isoforms 1, 2, 3, and 4. J Biol Chem 268:2740–2748.

Kozel PJ, Friedman RA, Erway LC, Yamoah EN, Liu LH, Riddle T, Duffy JJ, Doetschman T, Miller ML, Cardell EL, Shull GE. 1998. Balance and hearing deficits in mice with a null mutation in the gene encoding plasma membrane Ca^{2+}-ATPase isoform 2. J Biol Chem 273:18693–18696.

Kuo TH, Liu B-F, Yu Y, Wuytack F, Raeymaekers L, Tsang W. 1997. Coordinated regulation of the plasma membrane calcium pump and the sarco(endo)plasmic reticular calcium pump gene expression by Ca^{2+}. Cell Calcium 21:399–408.

Liu B-F, Xu X, Fridman R, Muallem S, Kuo TH. 1996. Consequences of functional expression of the plasma membrane Ca^{2+} pump isoform 1a. J Biol Chem 271:5536–5544.

Monteith GR, Roufogalis BD. 1995. The plasma membrane calcium pump-a physiological perspective on its regulation. Cell Calcium 18:459–470.

Penniston JT, Enyedi A.1998. Comparison of ATP-powered Ca^{2+} pumps. In: Bittar EE, editor. Advances in molecular and cell biology, vol 23B. Greenwich: JAI Press, Inc. p 249–274.

Riepe MW, Hori N, Ludolph AC, Carpenter DO. 1995. Failure of neuronal ion exchange, not potentiated excitation, causes excitotoxicity after inhibition of oxidative phosphorylation. Neuroscience 64:91–97.

Rohn TT, Hinds TR, Vincenzi FF. 1993. Ion transport ATPases as targets for free radical damage. Protection by an aminosteroid of the Ca^{2+} pump ATPase and Na^+/K^+ pump ATPase of human red blood cell membranes. Biochem Pharmacol 46:525–534.

Rohn TT, Hinds TR, Vincenzi FF. 1996. Inhibition of Ca^{2+}-pump ATPase and the Na^+/K^+-pump ATPase by iron-generated free radicals. Protection by 6,7-dimethyl-2,4-DI-1-pyrrolidinyl-7H-pyrrolo[2,3-d] pyrimidine sulfate (U-89843D), a potent, novel, antioxidant/free radical scavenger. Biochem Pharmacol 51:471–476.

Shull GE, Greeb J. 1988. Molecular cloning of two isoforms of the plasma membrane Ca^{2+}-transporting ATPase from rat brain. Structural and functional domains exhibit similarity to Na^+,K^+- and other cation transport ATPases. J Biol Chem 263:8646–8657.

Stauffer TP, Guerini D, Carafoli E. 1995. Tissue distribution of the four gene products of the plasma membrane Ca^{2+} pump. A study using specific antibodies. J Biol Chem 270:12184–12190.

Stauffer TP, Hilfiker H, Carafoli E, Strehler EE. 1993. Quantitative analysis of alternative splicing options of human plasma membrane calcium pump genes. J Biol Chem 268:25993–26003.

Street VA, McKee-Johnson JW, Fonseca RC, Tempel BL, Noben-Trauth K. 1998. Mutations in a plasma membrane Ca^{2+}-ATPase gene cause deafness in deafwaddler mice. Nat Genet 19:390–394.

Strehler EE. 1991. Recent advances in the molecular characterization of plasma membrane Ca^{2+} pumps. J Membr Biol 120:1–15.

Tepikin AV, Voronina SG, Gallacher DV, Petersen OH. 1992. Pulsatile Ca^{2+} extrusion from single pancreatic acinar cells during receptor-activated cytosolic Ca^{2+} spiking. J Biol Chem 267:14073–14076.

Thayer SA, Sturek M, Miller RJ. 1988. Measurement of neuronal Ca^{2+} transients using simultaneous microfluorometry and electrophysiology. Pflügers Arch Eur J Physiol 412:216–223.

Thomas D, Hanley MR. 1994. Pharmacological tools for perturbing intracellular calcium storage. Methods Cell Biol 40:65–89.

Toescu EC, Petersen OH. 1995. Region-specific activity of the plasma membrane Ca^{2+} pump and delayed activation of Ca^{2+} entry characterize the polarized, agonist-evoked Ca^{2+} signals in exocrine cells. J Biol Chem 270:8528–8535.

Yamoah EN, Lumpkin EA, Dumont RA, Smith PJS, Hudspeth AJ, Gillespie PG. 1998. Plasma membrane Ca^{2+}-ATPase extrudes Ca^{2+} from hair cell stereocilia. J Neurosci 18:610–624.

Zacharias DA, Strehler EE. 1996. Change in plasma membrane Ca^{2+}-ATPase splice-variant expression in response to a rise in intracellular Ca^{2+}. Curr Biol 6:1642–1652.