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Anesthetic-induced Alteration of Ca^{2+} Homeostasis in Neural Cells

A Temperature-sensitive Process That Is Enhanced by Blockade of Plasma Membrane Ca^{2+}-ATPase Isoforms

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Background: **Many inhalation anesthetics at clinically relevant concentrations inhibit plasma membrane Ca^{2+}-adenosine triphosphatase (PMCA) ion pumping in brain synaptic membranes and in cultured cells of neural origin. In this study, the authors investigated the effect of inhalation anesthetics on cytosolic calcium homeostasis in cortical neurons maintained at physiologic and room temperatures and on cortical neurons and pheochromocytoma cells with antisense blockade of specific PMCA isoforms.**

Methods: **Using Ca^{2+}-specific confocal microfluorimetry, the anesthetic effects on Ca^{2+} dynamics were examined in mouse embryonic cortical neurons in association with ligand-stimulated Ca^{2+} influx. Studies were done at 21°C and 37°C. Mouse embryonic cortical neurons with oligodeoxyribonucleotide blockade of PMCA2 expression and transfected rat pheochromocytoma cells with blocked expression of PMCA1 were also examined.**

Results: **Baseline and poststimulation peak cytosolic calcium concentrations ($[Ca^{2+}]_i$) were increased, and Ca^{2+} clearance was delayed in cells exposed at 37°C, but not at 21°C, to concentrations ≤1 minimum alveolar concentration (MAC)-equivalent of halothane, isoflurane, and sevoflurane. Neurons exposed to xenon solutions ≤0.4, 0.6, and 0.8 MAC showed dose-related perturbations of cytosolic Ca^{2+}. Calcium dynamics were altered in neural cells with blocked PMCA isoform production, but at much lower halothane concentrations: 0.5 MAC for cortical neurons and 0.1 MAC for pheochromocytoma cells.**

Conclusions: **By extruding Ca^{2+} through the plasma membrane, PMCA maintains resting neuronal $[Ca^{2+}]_i$ at low levels and clears physiologic loads of Ca^{2+} after influx through calcium channels. Inhalation anesthetics perturb this process and thus may interfere with neurotransmitter release, altering interneuronal signaling. (Key words: Ions and ion channels; mechanisms of anesthesia).**

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VARIOUS mechanisms of action for inhalation anesthetics have been postulated, and often the explanations center on alterations of central synaptic transmission, but proof remains elusive. It is, of course, a fundamental tenet of modern biology that small diffusible second messengers act within the cell to provide a link between stimulus and output, with cyclic nucleotides and their phosphorylation systems serving as classic examples. Second messenger functions for Ca^{2+} are well established,[1] and the role of intracellular Ca^{2+} in coupling neuronal excitation to the release of neurotransmitters into the synaptic space[2] makes anesthetic modulation of calcium homeostasis an attractive, although disputed, potential mechanism for anesthetic action.

Recently we observed that many inhalation anesthetics, in clinically relevant concentrations, inhibit plasma membrane Ca^{2+}-adenosine triphosphatase (ATPase; PMCA) ion pumping in brain synaptic membranes[3–5] and in isolated membranes of cultured cells of neural

origin.[6] Parallel decreases or increases in requirements for anesthetics have been demonstrated in rat models with incidentally decreased[7-10] or increased[11] PMCA pumping activity. In addition, electrophysiologic and microfluorimetric studies of cultured mouse central neurons and dorsal root ganglion sensory cells revealed delayed repolarization and delayed restoration of intracellular calcium ($[Ca^{2+}]_i$) to basal levels with exposure to halothane at 37°C,[12] changes that are consistent with anesthetic inhibition of PMCA.

In this study, we offer further evidence that anesthetics inhibit the plasma membrane calcium pump in cultured neural cells and thereby perturb Ca^{2+} homeostasis. First, we report the effects of pharmacologically relevant concentrations of halothane, isoflurane, and sevoflurane on cytosolic Ca^{2+} dynamics in cultured neurons and pheochromocytoma cells at physiologic and room temperatures. Second, we note halothane's effects on Ca^{2+} dynamics in cells of neural origin with antisense blockade of specific PMCA isoform expression. Third, we report the effects of xenon, an inert element that serves as a clinically effective anesthetic,[13-14] on neuronal Ca^{2+} homeostasis.

Materials and Methods

Mouse Neuron Preparation and Culture

Cerebral cortices were removed from mouse embryos[12] on gestational day 13 or 14. Tissue from 8-12 embryos was pooled, minced, and mechanically dissociated in a Ca^{2+}- and Mg^{2+}-free balanced salt solution. Dissociated cells were suspended in plating medium consisting of equal parts (vol/vol) of Eagle's minimal essential medium (supplemented with 1.5 g/l sodium bicarbonate and 5.5 g/l glucose) and Hank's balanced salt solution to which the following was added: 5% (vol/vol) fetal calf serum, 10% horse serum, 10 ng/ml nerve growth factor, and 1 ml/l Mito Serum Extender (Life Technologies, Grand Island, NY). Aliquots of the suspension were placed in sterile collagen-coated, 35-mm, glass-bottom plastic dishes and kept at 37°C in an incubator with an atmosphere of 90% room air and 10% carbon dioxide to maintain *p*H near 7.4.[15] Growth of rapidly dividing non-neuronal cells in cortical cell cultures was suppressed by adding 5-fluoro-2′deoxyuridine and uridine (Sigma Chemical Co., St. Louis, MO; 50 μg/ml each) to the culture medium. Subsequently, the medium was changed two or three times a week. Cultures were maintained for 4-20 weeks before the experiments.

Blockade of PMCA2 Expression in Cultured Mouse Embryonic Cortical Cells by Antisense PMCA2 Oligodeoxyribonucleotide

Cortical neurons were removed from mouse embryos on gestational day 13, before expression of PMCA2 occurs,[16] and cultured as just described here. Added to the medium was 10 μg/ml of either a 21 base antisense PMCA2 oligodeoxyribonucleotide, 5′-CAT ATC ACC CAT GTT TGC TGA-3′, or a 21 base oligodeoxyribonucleotide comprised of the same bases arranged in random order (scrambled bases), 5′-TCA GAC TTC GAT CCT TAA GCT-3′, or neither. Media containing freshly thawed oligodeoxyribonucleotides were replaced daily. These oligonucleotides were prepared by the University of Kentucky Macromolecular Structure Analysis Facility using the solid-phase phosphoramidite method with incorporation of a phosphorothioate backbone. Each oligonucleotide preparation was purified chromatographically on either PD-10 gel filtration columns or C18 Sep-Pak column cartridges (Waters, Marlboro, MA). (Neither preparation showed detectable cytotoxicity in pheochromocytoma cells at concentrations up to 50 μg/ml.) After cortical embryonic neurons had been cultured in the presence of oligonucleotides for 8-14 days, by which time PMCA2 is ordinarily expressed, immunocytochemical analysis was done with PMCA2-specific antiserum and a fluorescent second antibody.

Rat Pheochromocytoma Cells with Blockade of PMCA1 Expression

The rat pheochromocytoma cell line, PC6, was used to prepare stably transfected cell lines in which all PMCA1 isoforms were blocked by constitutively expressed PMCA1-specific antisense RNA. The details of preparation and maintenance of these cell lines has been described.[17] Briefly, cDNA encoding the first 446 nucleotides of the human PMCA1 mRNA was inserted behind the RSV promoter in an orientation to produce antisense PMCA1 RNA or a short-sense control RNA and then transfected into PC6 cells. Cell lines stably expressing the antisense RNA with no detectable PMCA1 protein were selected and propagated in Dulbecco's minimum essential medium (Life Technologies) containing 10% horse serum and 5% fetal bovine serum. Cell lines were periodically tested for expression of the antisense RNA. It is noteworthy that cells with blocked PMCA1 production express 30% less total PMCA compared with controls, the remaining 70% coming from expression of PMCA2 and PMCA4 gene products.[17]

Immunocytochemical Analysis

A modification of the method of Preiano *et al.*[18] was used. The medium was aspirated from culture plates, and cold fixative was added (3% paraformaldehyde [Sigma Chemical Co.] in phosphate-buffered saline [PBS]). After 30 min at room temperature, cells were washed twice and quickly frozen on dry ice. For staining, thawed cells were washed with PBS and then 0.1 M glycine (Sigma Chemical Co.) was added. After 20 min at room temperature and two additional washes, the cells were incubated for 1 h in a blocking solution containing 5% (vol/vol) goat serum (Sigma Chemical Co.), 0.1% bovine serum albumin (Sigma Chemical Co.), and 5% (vol/vol) glycerol (Sigma Chemical Co.) in PBS. After aspiration of the blocking solution, the appropriate dilution of the primary antibody serum in PBS (1:200–1:300) was added. The primary antibodies used in this procedure (and subsequent Western blot studies) were polyclonal antibodies raised in rabbits against recombinant human PMCA isoform fragments (first 80 amino acids from N terminal) and designated 1N, 2N, and 3N for human PMCA1, PMCA2, and PMCA3, respectively.[19] (Antiserum 4N, raised against PMCA4, was not used in this study because it does not react with rodent PMCA4.) After 1 h at room temperature, the cells were washed twice in PBS and the second, fluorescent antibody (1:200–1:300 of fluorescein isothiocyanate-conjugated polyclonal goat anti-rabbit antiserum; Sigma Chemical Co) in PBS was added. After 1 h, the cells were washed twice in PBS and placed on glass slides with Sigma mounting medium. Immunofluorescence was evaluated under an Olympus BHA microscope equipped with a fluorescence vertical illuminator (model BH-RFL, Melville, NY). Selected areas were photographed on ISO 400 Kodak color print film using a 1:400 objective. Exposure time for cell cultures was 45–60 s. The film images were scanned with a Kodak PhotoCD laser scanner (Rochester, NY) with a resolution of 2048 × 3742 pixels, and image files were stored on CD-ROM discs.

Western Immunoblotting

Proteins were separated by electrophoresis on 7.5% polyacrylamide gel by the method of Laemmli.[20] Resolved proteins were transferred to 0.45 μm Trans-Blot nitrocellulose filters (Bio-Rad, Rockville Center, NY) by electroblotting according to the method of Towbin *et al.*[21] These nitrocellulose filters were treated with blocking buffer (10 mM Tris-HCl, *p*H 7.8, 150 mM NaCl, and 0.1% Tween-20 with 1% bovine serum albumin) overnight at 4°C, washed twice at room temperature with wash buffer (blocking buffer with albumin reduced to 0.1%), and treated consecutively with 1N, 2N, and 3N antisera for 30 min. After three additional washes in wash buffer, the nitrocellulose filters were incubated for 30 min with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Sigma Chemical Co.; final dilution, 1:2,000), followed by incubation for 2 or 3 min with a solution of diaminobenzidine–hydrogen peroxide as the chromogen.

Confocal Laser Scanning Microfluorimetry

Calcium imaging was done in the Vanderbilt University Cell Imaging Resource. A laser scanning confocal microscope (Zeiss LSM410, Seiles Instruments, St. Louis, MO), with diffraction-limited focusing of a laser beam, coupled with a special filter placed "confocal" to the sample, provides rejection of the out-of-focus background that is a thousand times better than a conventional microscope. The system is equipped with an argon–krypton laser for excitation. Neurons in dissociated monolayer cultures were loaded with fluo-3-AM (Molecular Probes, Eugene, OR) 40 min before the study. Culture dishes, 35 mm with glass bottoms for transmission of laser beams (MatTek, Ashland, MA), were placed in a microincubation system (Medical Systems Corp., Greenvale, NY) on the stage of the confocal microscope. The microincubation system allowed precise control of media temperature and was able to change the media temperature from 21°C to 37°C, and (in conjunction with superfusion) back to 21°C in <3 min. Dulbecco's modified PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5.5 mM D-glucose served as control superfusate, delivered through Tygon (Norton Performance Plastics Corp., Akron, OH) tubing *via* a peristaltic pump[12] at a rate of 1 ml/min. Inhalation anesthetic solutions in the buffer in a 250-ml plastic bag (designed for intravenous infusions; Baxter, Deerfield, IL) were delivered in a similar way. Buffer volume in the dish was maintained at 1.5 ml by appropriate positioning of the outflow tubing. This buffer volume and superfusion rate, in conjunction with the microincubation system, allowed expeditious wash in and wash out of anesthetics in 3 or 4 min while the medium was maintained at the specified temperatures of 21°C or 37°C. For preparation of volatile anesthetic solutions, appropriate volumes were injected from a glass syringe into buffer in plastic bags from which the air had been expelled. The bags containing the anesthetic solutions were equili-

brated for 2 h on a rocking device at 4°C and then allowed to warm to room temperature. Anesthetic concentrations were measured by high-performance liquid chromatography, gas chromatography, or both.[22-23] Concentrations were measured in samples taken from the bag (room temperature) and from the center of the microincubation dish (37°C). Losses from solutions in plastic bags could not be detected during a 4-h period after initial equilibration. Consistent, reproducible volatile anesthetic losses of about 50% were observed during passage of the solution through the delivery tubing and into the incubation dish. Initial volatile anesthetic solutions thus could be prepared so that the measured dish concentrations referred to throughout this report were equal to or just less than concentrations equivalent to 1 minimum alveolar concentration (MAC), except in experiments wherein significantly lower concentrations of anesthetic were needed. The MAC-equivalent concentrations at 37°C were 0.30 mM for halothane and 0.35 mM for isoflurane.[24] The MAC-equivalent for sevoflurane (MAC reported as 2.7% for young rats[25]) was estimated conservatively as 0.50 mM based on a series of experiments. Sevoflurane, 2.5% in air, was bubbled through Dulbecco's modified phosphate buffer at 37°C for 1 h, and concentrations were compared with standard solutions. Buffer concentrations averaged 0.533 ± 0.062 (SD) mM (n = 10).

Preparation of superfusion buffer containing xenon was done as follows. Dulbecco's modified buffer (100 ml) was placed in a 250-ml intravenous infusion bag. Air was expelled and replaced with 60 ml of a gas mixture composed of 20% oxygen and xenon - nitrogen in ratios providing 20%, 40%, 60%, and 80% xenon. (These gas volumes would appear to be adequate for solution preparation, because the relative solubilities of nitrogen, oxygen, and xenon are approximately 18, 32, and 119 ml per liter of water at room temperature and ambient pressure.[26]) Thus all xenon concentrations used in these experiments were less than the MAC, which was reported as 95% in mice.[27] Bags containing the buffer and gas mixtures were placed on a rocking device, maintained at 4°C overnight, and used the next day for microfluorimetric analysis. Bags were warmed to 37°C before use to avoid the possibility of xenon effervescence when the superfusate was transferred to the incubation dish. Taking into account the vapor pressure of water, xenon concentrations in the superfusate would have been equivalent to 0.2, 0.4, 0.6, and 0.8 MAC if there was no loss in transit. We could not measure dish concentrations of xenon, but the observed effects of xenon superfusates on neuronal $[Ca^{2+}]_i$ suggest that loss rates during delivery to the cells were smaller than those seen with volatile anesthetics.

Influx of Ca^{2+} into cells was induced by application of a ligand *via* a patch-clamp pipette within 3 s of starting the laser beams. Fluorescence intensity in sequential images (1 or 2 per second) of selected microscopic fields was digitally recorded with a resolution of 256 × 256 pixels and stored on zip-drive cartridges. (Control experiments done at 21°C and 37°C without ligand application showed no artifactual bleaching with this procedure. Nor was there any significant change in Ca^{2+}-related fluorescence in the absence of ligand and anesthetic.) Microscopic fields in three or more culture dishes were selected so that imaged cells lay within 100 μm of the tip of the patch-clamp pipette. The time-courses of Ca^{2+}-associated fluorescence in magnified images of randomly selected individual cells in a field were subsequently analyzed and converted to scalar values of fluorescence with the LSM410-associated software. Measurement of fluorescence in a fixed field containing a single cell allowed accurate measurement of relative changes in $[Ca^{2+}]_i$ consequent to ligand stimulation and anesthetic exposure.

Figure 1 illustrates this process of analysis. Sixty-four sequential images of a microscopic field of cells were captured just before and after stimulation of calcium influx by N-methyl-D-aspartate (NMDA). Shown are two time courses of Ca^{2+}-associated fluorescence, the lower obtained from an untreated mouse embryonic cortical neuron and the upper from that same neuron after a 10-min anesthetic exposure. Each curve is derived from computer conversion of fluorescence intensity of 64 sequential images to arbitrary units of fluorescence. Selected images are shown in the figure to illustrate how points on the graph were obtained. The abscissa shows time in seconds, and the ordinate indicates fluorescence in arbitrary units. As is clear in both sequences of images, Ca^{2+}-associated fluorescence increased after NMDA application, and there is subsequent clearance of calcium back to prestimulation levels. This analog output derived from digitized images of Ca^{2+}-associated fluorescence provides three parameters of interest: baseline fluorescence, observed before each application of NMDA or other ligand; peak fluorescence achieved by ligand-stimulated calcium influx; and Ca^{2+} clearance time (*i.e.*, the time required for return of Ca^{2+}-associated fluorescence to the prestimulus level). For clarity in illustrating anesthetic effects on $[Ca^{2+}]_i$, fluorescence

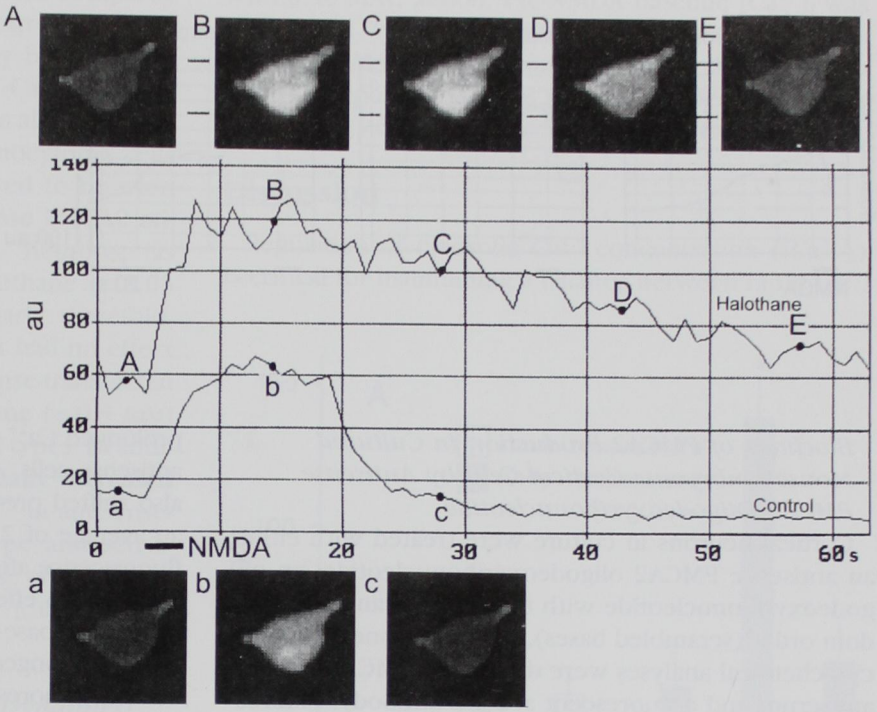

Fig. 1. Analysis of the time course of Ca^{2+}-related fluorescence in a mouse embryonic cortical neuron. Sequential digitized images of a microscopic field of cells previously loaded with Flu3-AM (a calcium-sensitive dye) were captured just before and after stimulation of calcium influx by N-methyl-D-aspartate (NMDA; 10^{-4} M) and stored on a zip drive. Subsequently, single cells in the field were chosen at random and magnified for analysis. Shown are two time courses of Ca^{2+}-associated fluorescence, the lower obtained from an untreated (control) neuron and the upper from that same cell after a 10-min superfusion with an inhalation anesthetic solution (0.3 mM halothane). Each curve is derived from a computer conversion of fluorescence intensity of 64 sequential images to scalar values of fluorescence. Typical images are shown in the figure to illustrate how points on the graph are obtained. The abscissa shows time in seconds (s), and the ordinate indicates arbitrary units (au) of fluorescence provided by the LSM410-associated software. As is clear in both sequences of images, Ca^{2+}-associated fluorescence is increased after NMDA application, and calcium clearance subsequently returns to prestimulation levels.

shown in summary figures in the Results section has been normalized:

relative fluorescence = (fluorescence in arbitrary units at time t)/(mean initial baseline fluorescence in arbitrary units at time 0).

Thus initial, control baseline fluorescence is normalized to unity, and subsequent measures of fluorescence, whether baseline or peak, are given as fractional increases above control baseline.

All chemicals and reagents used were of the highest grade available and supplied by Sigma Chemical Company unless otherwise specified.

Statistical Analysis

Data were examined by (1) multifactorial analysis of variance and the Student-Newman-Keuls procedure for multiple comparison of means and (2) the Kruskal-Wallis nonparametric procedure, with box-and-whiskers plots to determine which medians differed significantly.

Results

Temperature-specific Anesthetic Effects on Neuronal Calcium Dynamics

Figure 2 illustrates the effect of a clinically relevant concentration of halothane at 37°C on the Ca^{2+} homeostatic response of a cultured embryonic mouse cortical neuron when Ca^{2+} influx was induced by application of NMDA. Figure 3 summarizes these experiments and gives results obtained when cortical neurons were exposed to halothane at 21°C. As indicated in both figures and reported previously,[12] halothane, at concentrations at the lower end of the usual pharmacologic dose, prolonged the clearance time of intracellular Ca^{2+} threefold compared with pre-exposure control responses (fig. 3A). In addition, these experiments showed that exposure to halothane for 10 min produced a 2.5-fold upward shift of baseline Ca^{2+}-associated fluorescence before a second application of NMDA and an increase in the peak fluorescence attained after NMDA application (fig. 3B). These parameters returned to normal after a 10-min halothane washout. In contrast, no significant changes in clearance time or in baseline and peak $[Ca^{2+}]_i$ occurred with halothane exposure at 21°C. Figure 4 shows the effects of isoflurane and sevoflurane on Ca^{2+} dynamics in cortical neurons. As with halothane, prolongation of elapsed time to baseline (fig. 4A) and elevation of baseline and peak fluorescence (fig. 4B) occurred with exposure of neurons at 37°C. No significant isoflurane (n = 10) or sevoflurane (n = 8) effects on Ca^{2+} dynamics were seen in cells maintained at 21°C.

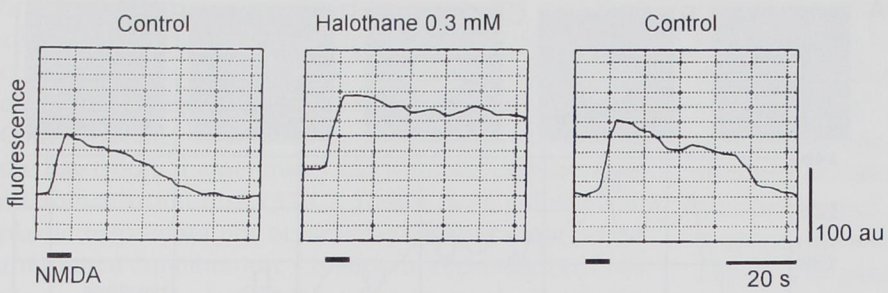

Fig. 2. Halothane alteration of Ca^{2+} homeostasis in cortical neurons. Shown are confocal microfluorimetric tracings of the N-methyl-D-aspartate (NMDA) response of a representative cultured mouse embryonic cortical neuron before, during, and after exposure to 0.30 mM halothane. The abscissa shows seconds (s), and the ordinate shows Ca^{2+}-related fluorescence in arbitrary units (au). NMDA (10^{-4} M) was applied for 3 s, indicated by horizontal bars, after 10 min of superfusion with Dulbecco's modified buffer (see text), buffer with halothane, or buffer alone. Cells were maintained at 37°C.

Blockade of PMCA2 Production in Cultured Mouse Embryonic Cortical Cells by Antisense PMCA2 Oligodeoxyribonucleotide

Cortical neurons in culture were treated with either an antisense PMCA2 oligodeoxyribonucleotide, an oligodeoxyribonucleotide with the bases arranged in random order (scrambled bases), or neither one. Immunocytochemical analyses were done with PMCA2-specific antiserum and a fluorescent second antibody. Fluorescence of scrambled base-treated and -untreated (not shown) cells indicated the presence of PMCA2, whereas only background fluorescence was observed in antisense-treated neurons (fig. 5).

Increased Halothane Sensitivity of Cultured Mouse Embryonic Cortical Cells with Blocked PMCA2 Production

When examined by confocal microfluorimetry, antisense-treated neurons manifested significantly increased sensitivity to halothane, compared with wild-type and scrambled base-treated cells. Figure 6 shows the results from a typical experiment. Each row shows the Ca^{2+}-dependent fluorescence response in cells of each type when subjected to different treatments. The recordings in the first column indicate that application of NMDA resulted in a brisk influx of Ca^{2+} in all cell types. Low-dose halothane (≤0.15 mM, recordings in the second column) had little effect on Ca^{2+} dynamics in wild-type and scrambled base-treated cells, in contrast to antisense-treated cells. Moderate doses of halothane (≤0.30 mM, recordings in the fourth column) affected all three cell types. Figure 7 summarizes the results in these cells. On the average, Ca^{2+} clearance time in antisense-treated neurons was doubled by exposure to low-dose halothane, whereas no effect was seen in wild-type and scrambled base-treated cells (fig. 7A). Moderate doses of halothane prolonged Ca^{2+} clearance in all three types, especially in antisense cells. A 10-min exposure to 0.15 mM halothane also shifted pre-NMDA baseline fluorescence upward by an average of 2.5 times and significantly increased peak fluorescence after NMDA (fig. 7B). This lower concentration had no effect on these parameters in wild-type and scrambled base-treated cells. In contrast, 0.30 mM halothane prolonged clearance time and increased baseline and peak fluorescence in all three cell types. No significant halothane effects on Ca^{2+} dynamics were observed in antisense-treated cells maintained at 21°C (n = 10).

PMCA1 Isoform Expression in Pheochromocytoma Cells Stably Transfected with Sense- and Antisense-producing Constructs

Figure 8 shows the results of Western immunoblotting with PMCA isoform-specific antisera of (1) purified human erythrocytic PMCA (isoforms 1 and 4; provided by Dr. John Penniston), (2) rat brain synaptic plasma membrane, (3) crude plasma membrane fractions of sense-transfected (RSV14) pheochromocytoma cells, (4) antisense-transfected (RSV9) pheochromocytoma cells, and (5) wild-type (PC6) pheochromocytoma cells. Figure 8 indicates that isoform 1 was present in all samples except antisense-transfected pheochromocytoma cells, whereas isoform 2 was present in all samples except erythrocytic PMCA, as expected. Isoform 3, confined in the brain to the choroid plexus, was not found in any of these samples. As noted before, antiserum against rodent PMCA4 was not available. Immunocytochemical analysis (not shown) confirmed the absence of PMCA1 in antisense-transfected cells.

Increased Halothane Sensitivity of Transfected Pheochromocytoma Cells with Blocked Production of PMCA1

Using confocal laser scanning microfluorimetric analysis, we examined the effect of halothane on cytosolic

Ca^{2+} dynamics in antisense-transfected cells compared with wild-type and sense-transfected controls. Ca^{2+} influx into the cytoplasm was induced by bradykinin.[17] Because bradykinin mobilizes internal Ca^{2+}, applications were separated by at least 20 min to allow restoration of internal Ca^{2+} stores. Pheochromocytoma cells transfected with PMCA1 antisense proved to be even more sensitive to halothane than antisense PMCA2 oligodeoxyribonucleotide-treated cortical neurons, as shown in figure 9. Very low dose halothane (≤0.03 mM) increased Ca^{2+} clearance time nearly threefold, compared with unexposed controls, but had no effect on Ca^{2+} clearance in wild-type and sense-transfected cells (fig. 9A). A moderate dose halothane (≤0.3 mM) prolonged Ca^{2+} clearance in all three cell types. In addition, very low dose halothane substantially increased resting baseline and postligand peak Ca^{2+} in antisense-transfected cells, in contrast to wild-type and sense-transfected cells (fig. 9B). A moderate dose of halothane increased pre- and poststimulation $[Ca^{2+}]_i$ in all three pheochromocytoma cell types.

Xenon Effects on Neuronal Calcium Dynamics

Exposure to xenon produced striking alterations in Ca^{2+} homeostasis in cultured cortical neurons. Figure 10 shows these effects on a cell exposed successively to xenon solutions that were, at the maximum, equivalent to 0.2, 0.4, 0.6, and 0.8 MAC, and figure 11 summarizes the results from 10 such studies. Significant differences in Ca^{2+} responses were seen with exposure to 0.6 and 0.8 MAC. Ca^{2+} clearance time was prolonged more than two and five times, respectively, compared with the prexenon treatment period (fig. 11A). A small but significant prolongation of clearance time occurred with 0.40 MAC xenon. Pre-NMDA baseline $[Ca^{2+}]_i$ was increased 1.7 times with 0.60 and more than two times with 0.80 MAC xenon, compared with the prexenon control response, whereas post-NMDA peak $[Ca^{2+}]_i$ increased to 1.5 and 1.8 times that of control (fig. 11B).

Discussion

Regulation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is critical for maintaining a balance between rapid Ca^{2+}

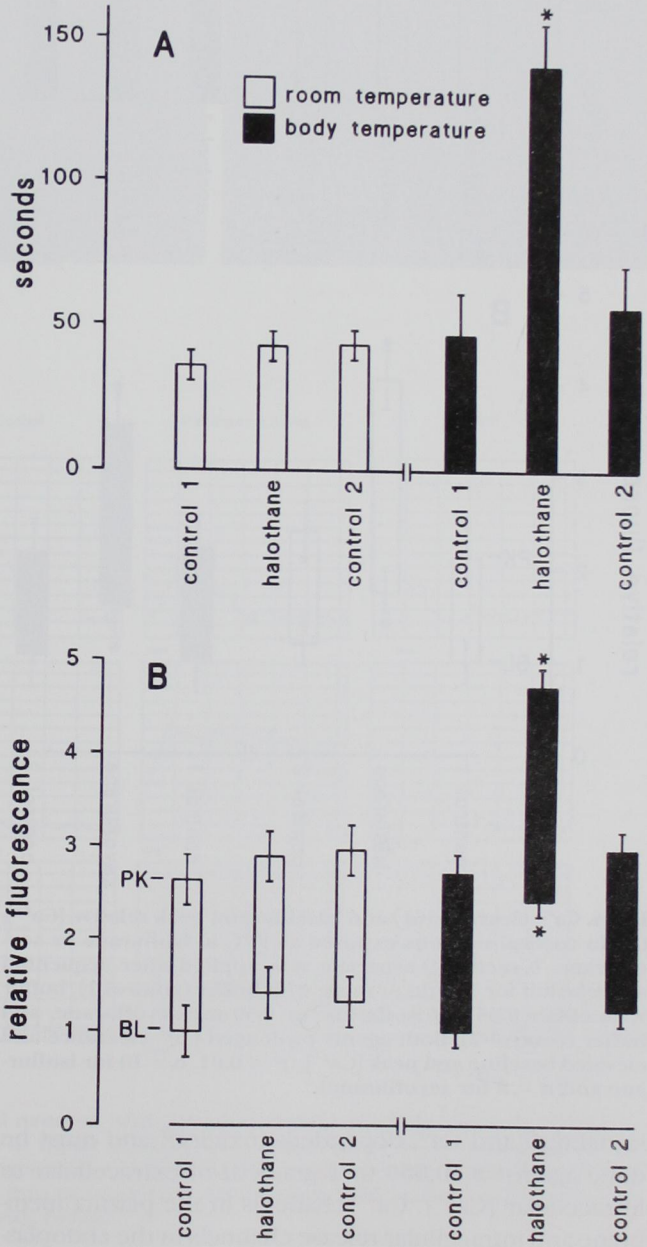

Fig. 3. Summary of the temperature-dependent effects of halothane on Ca^{2+} clearance (*A*) and on baseline and peak Ca^{2+}-related fluorescence (*B*) in cortical neurons. Each cell was stimulated by N-methyl-D-aspartate (NMDA) after sequential superfusion for 10 min with buffer (control 1), buffer with 0.30 mM halothane, and buffer (control 2). Experimental conditions were as described in figure 2. The ordinates give time and relative Ca^{2+}-associated fluorescence, the latter normalized so that initial, prehalothane baseline is unity. Subsequent pre-NMDA baseline (BL) and post-NMDA peak (PK) fluorescence levels are shown as fractional increases above initial baseline. Columns show mean values with 99% confidence limits. Asterisks denote significant prolongation of Ca^{2+} clearance time and elevation of baseline and peak $[Ca^{2+}]_i$ in cells exposed to halothane at 37°C ($P < 0.01$, n = 22). No halothane effects were seen at 21°C (n = 8).

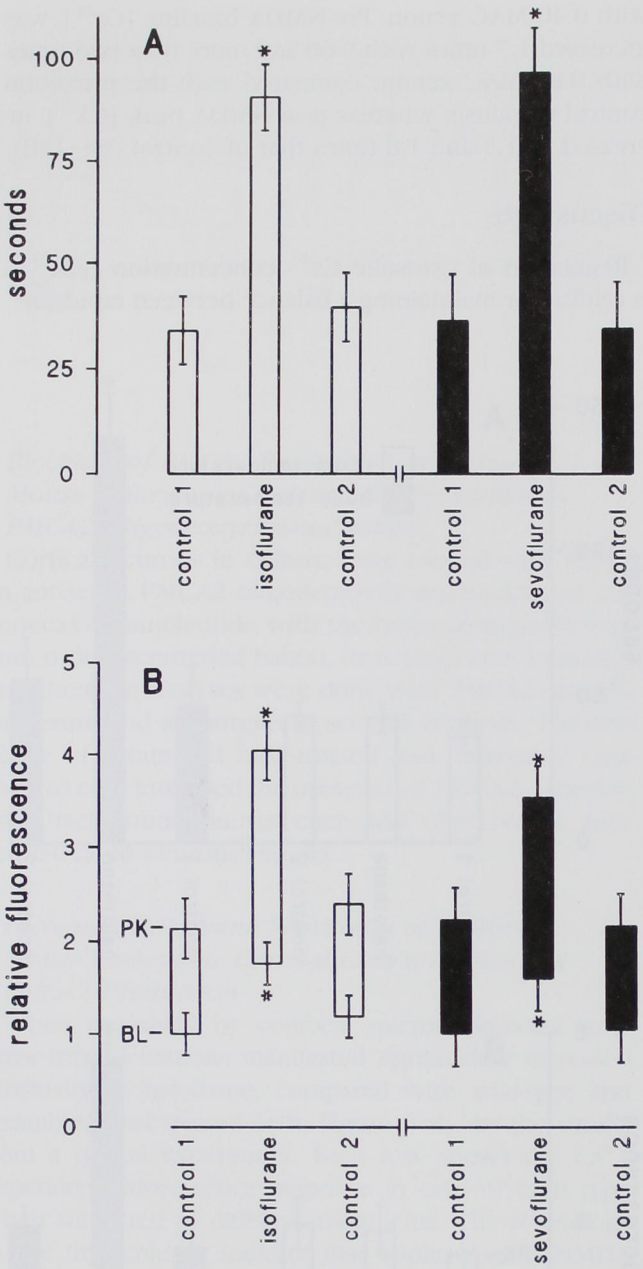

Fig. 4. Ca^{2+} clearance (*A*) and baseline and peak relative $[Ca^{2+}]_i$ (*B*) in cortical neurons exposed at 37°C to isoflurane or sevoflurane. N-methyl-D-aspartate was applied after sequential superfusion for 10 min or more with buffer (control 1), buffer with either 0.35 mM isoflurane or 0.50 mM sevoflurane, and buffer (control 2). Both agents prolonged Ca^{2+} clearance and elevated baseline and peak $[Ca^{2+}]_i$ ($P < 0.01$, n = 10 for isoflurane and n = 8 for sevoflurane).

signaling[28] and Ca^{2+}-dependent toxicity[29] and must be done against a 10,000 to 1 gradient of extracellular to intracellular $[Ca^{2+}]$. Ca^{2+} channels in the plasma membrane and intracellular release channels in the endoplasmic reticulum can increase $[Ca^{2+}]_i$ rapidly with appropriate stimulation. Three principal systems serve to reduce or maintain $[Ca^{2+}]_i$ at low levels: a plasma membrane Na^+/Ca^{2+} exchanger, a plasma membrane Ca^{2+}-ATPase pump (PMCA), and a smooth endoplasmic reticulum Ca^{2+}-ATPase pump. Mitochondrial uptake and release functions are thought to operate principally at high $[Ca^{2+}]_i$.[30]

The development in the last decade of powerful methods for recording changes in $[Ca^{2+}]_i$ in single cells, based on electrophysiologic and Ca^{2+}-specific microfluorimetric techniques, has led to a substantial advance in knowledge of the complexities of neuronal $[Ca^{2+}]_i$ regulation and signaling. Ca^{2+} influx *via* channels and release from intracellular stores have been studied extensively.[31] So also, although to a lesser extent, have been the modulation of processes that remove Ca^{2+} from neuronal cytosol[32] and their relative importance in neuronal function. The consensus has been that the high capacity and low affinity of the Na^+/Ca^{2+} exchanger relegates its role to removal of Ca^{2+} when concentrations are 1 μM or more, whereas high-affinity PMCA serves as a precise regulator that maintains $[Ca^{2+}]_i$ at low resting levels.[33]

Several interesting studies have shed light on just how important PMCA is in removing Ca^{2+} loads in neurons. Benham *et al.*,[34] using patch-clamp and microfluorimetric techniques in cultured rat dorsal root ganglion cells, showed that whereas Na^+/Ca^{2+} exchange played only a small part in removing physiologic Ca^{2+} loads (approximately 500 nM) and no apparent part in maintaining resting $[Ca^{2+}]_i$, inactivation of PMCA with orthovanadate or high *p*H dramatically slowed Ca^{2+} removal and significantly increased baseline $[Ca^{2+}]_i$. They concluded that PMCA is critical for the removal of Ca^{2+} loads of similar amplitude to those generated by the firing of action potentials. Using similar techniques, Bleakman *et al.*[35] showed that mitochondria, endoplasmic reticulum stores, and the Na^+/Ca^{2+} exchanger have little effect on short-term clearance of modest Ca^{2+} loads in cultured rat septal neurons. More recently, Werth *et al.*,[36] in an extensive study, evaluated Ca^{2+} efflux systems in rat dorsal root ganglion cells and confirmed that PMCA-mediated Ca^{2+} extrusion is the primary process responsible for recovery to basal $[Ca^{2+}]_i$ after stimulation. They showed that sequestration of Ca^{2+} in intracellular stores or extrusion of Ca^{2+} from the cell *via* the Na^+/Ca^{2+} exchanger contributed minimally to recovery of $[Ca^{2+}]_i$ to baseline. On the other hand, treatment of neurons with C28R2, a synthetic peptide representing the au-

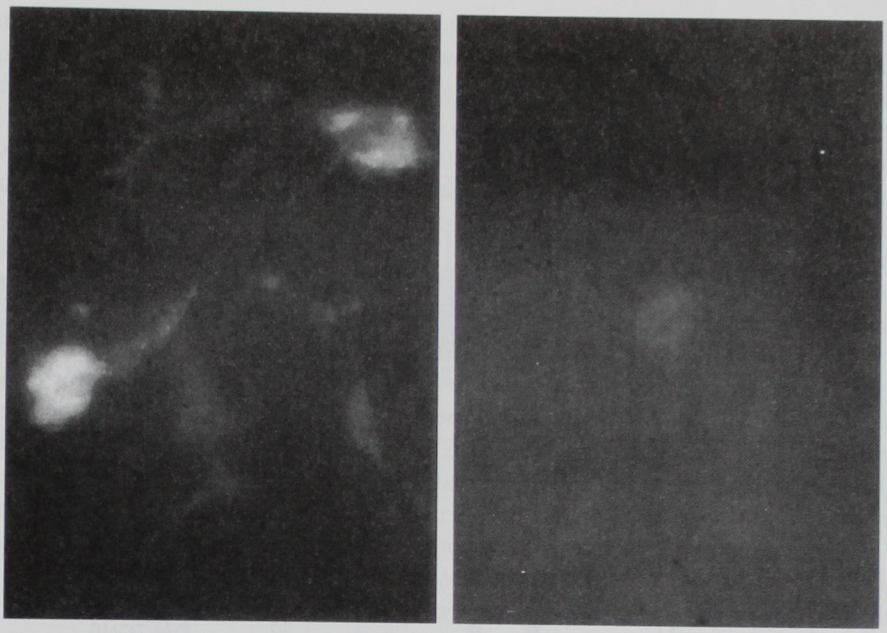

Fig. 5. Immunofluorescent staining of embryonic mouse cortical neurons with oligodeoxyribonucleotide blockade of PMCA2 expression. Cells were immunostained with isoform-specific rabbit antibody, which reacts only with PMCA2. (*Left*) Control neurons showing PMCA2 specific immunostaining. (*Right*) Reduced immunostaining intensity resulting from deficiency in PMCA2 (×1,200).

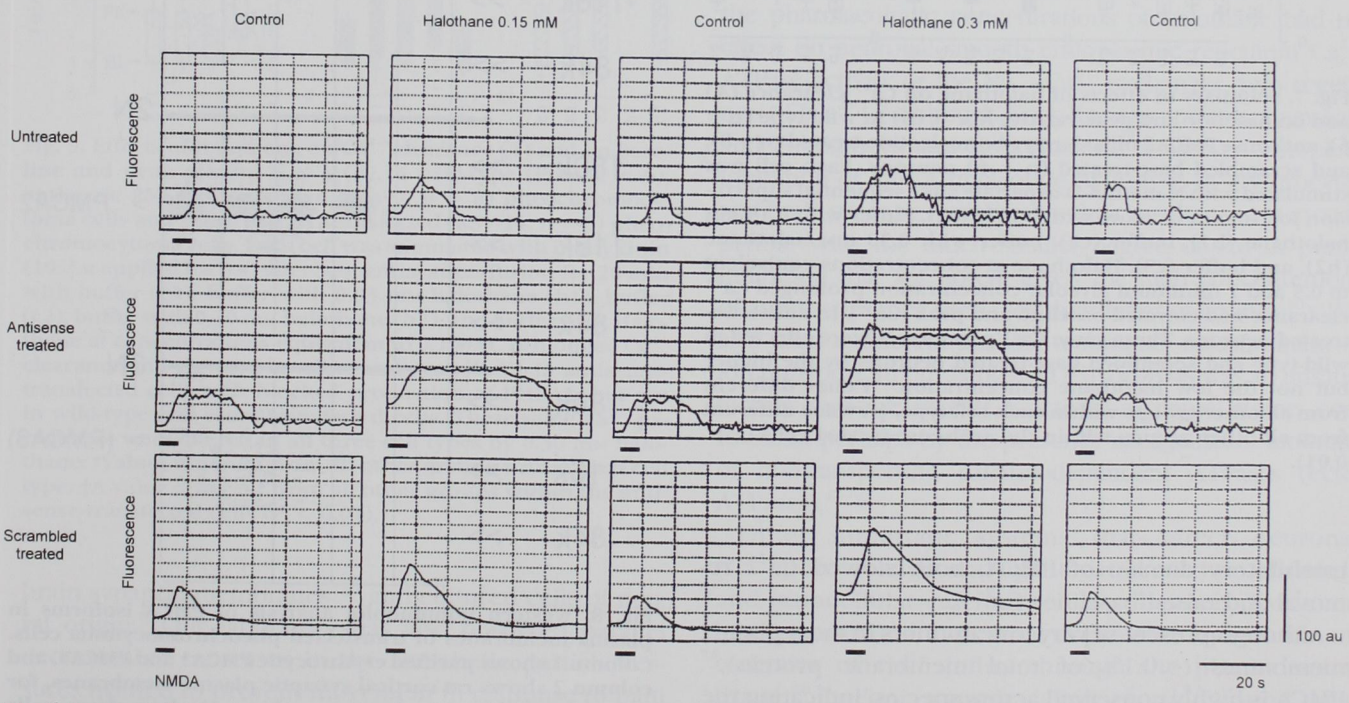

Fig. 6. Increased anesthetic sensitivity of embryonic mouse cortical neurons with oligodeoxyribonucleotide blockade of PMCA2 expression. Experimental conditions were the same as described in figure 2. Shown are microfluorimetric recordings of the Ca^{2+} response in wild-type (*top row*), antisense oligodeoxyribonucleotide-treated (*middle row*), and scrambled base-treated (*bottom row*) neurons. N-methyl-D-aspartate was applied after 10 min of sequential superfusion with buffer, buffer with 0.15 mM halothane, buffer, buffer with 0.30 mM halothane, and again with buffer.

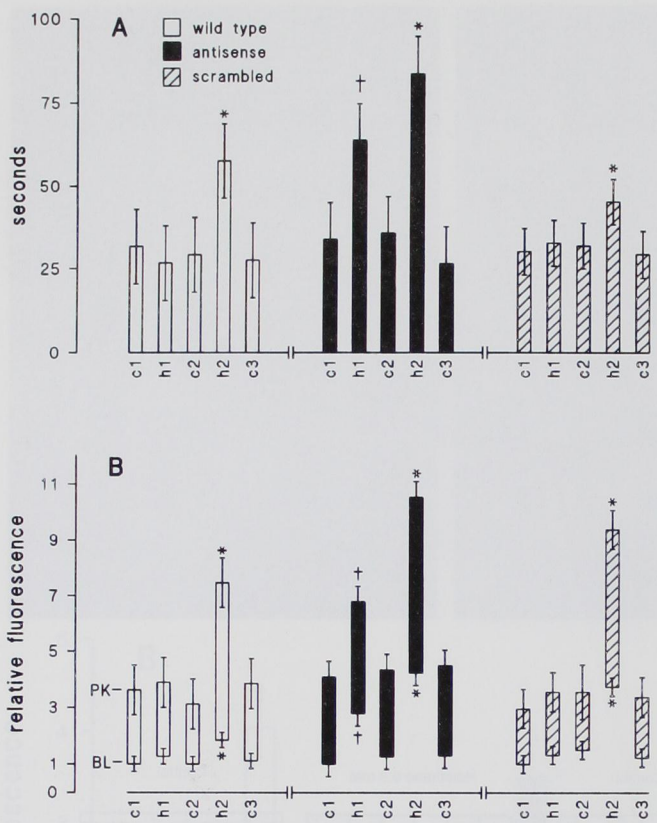

Fig. 7. Summary of effects of halothane on Ca^{2+} clearance (*A*) and on baseline and peak relative $[Ca^{2+}]_i$ (*B*) in wild-type (n = 6), antisense PMCA2 oligodeoxyribonucleotide-treated (n = 8), and scrambled base-treated (n = 6) neurons. Each cell was stimulated with N-methyl-D-aspartate after sequential superfusion for 10 min or more with buffer (c1), buffer with 0.15 mM halothane (h1), buffer (c2), buffer with 0.30 mM halothane (h2), and buffer (c3). Halothane at concentrations equivalent to 0.5 and 1 minimum alveolar concentration prolonged Ca^{2+} clearance and elevated baseline and peak $[Ca^{2+}]_i$ in antisense-treated neurons. These same Ca^{2+} parameters were affected in wild-type and scrambled base-treated neurons by the higher but not the low halothane concentration. *Values different from all other groups within each cell type. †A value different from all other groups within the antisense-treated cells ($P < 0.01$).

toinhibitory domain of PMCA, both slowed Ca^{2+} removal and raised baseline $[Ca^{2+}]_i$.

Although present in very low amounts in most plasma membranes (<0.1% of total membrane protein),[37] PMCA is highly conserved across species, indicating the essential role this pump plays in eukaryocytic function. Comparison of PMCA2 human and rat protein sequences, for example, shows >98% homology.[38] PMCA isomer[19] and splice variant expression[39] is strikingly regional in the rat brain. Splicing options in different isoforms change the affinities for Ca^{2+} and calmodulin[40] and present unique sites for phosphorylation. Alternate splicing thus may be a tool for regional regulation of the Ca^{2+} pump. Interestingly, the two splice variants of PMCA2 show a very high affinity for Ca^{2+} and thus tend to maintain a lower free cytosolic Ca^{2+} level in cells where they are expressed.[40] This isomer, PMCA2, is confined to the central nervous system where it is strategically located in nerve terminals and synapses.[41,42] These findings suggest specific functions for enzyme isoforms and splice variants in neurons and may even augur isoform - splice variant differences in the susceptibility to anesthetic depression that may be enhanced by strategic location within the brain.

These observations may be relevant to the pharmacodynamics of inhalation anesthetics, because both gaseous and volatile agents specifically inhibit PMCA in

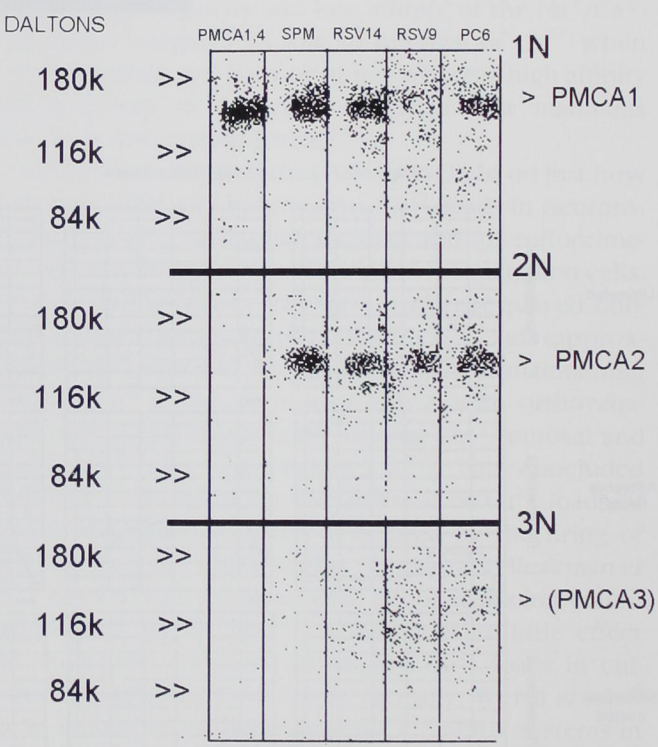

Fig. 8. Western immunoblot analysis of PMCA isoforms in plasma membranes of transfected pheochromocytoma cells. Column 1 shows purified erythrocytic PMCA1 and PMCA4, and column 2 shows rat cortical synaptic plasma membranes, for reference. Column 3 shows membrane isolates from cells transfected with PMCA1 sense cDNA (RSV14), column 4 shows isolates from cells transfected with PMCA1 antisense cDNA (RSV9), and column 5 shows isolates from wild-type (PC6). Rabbit antisera 1N, 2N, and 3N react with PMCA isoforms 1, 2, and 3. Marker protein positions are indicated along the left margin.

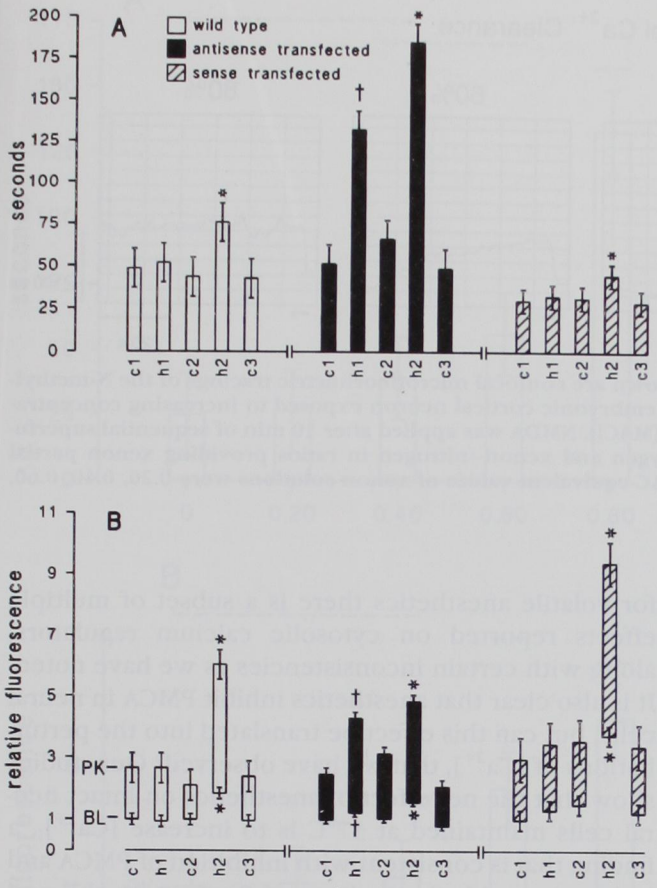

Fig. 9. Effects of halothane on Ca^{2+} clearance (*A*) and on baseline and peak relative $[Ca^{2+}]_i$ (*B*) in wild-type (PC6, n = 6), antisense PMCA1-transfected (RSV-9, n = 8) pheochromocytoma cells and sense PMCA1-transfected (RSV-14, n = 6) pheochromocytoma cells. Each cell was stimulated with bradykinin (10^{-3} M applied for 3 s) after sequential superfusion for 20 min with buffer (c1), buffer with 0.03 mM halothane (h1), buffer (c2), buffer with 0.30 mM halothane (h2), and buffer (c3). Halothane at concentrations equivalent to 0.1 MAC prolonged Ca^{2+} clearance and elevated baseline and peak $[Ca^{2+}]_i$ in antisense-transfected cells with blocked production of PMCA1, but not in wild-type and sense-transfected cells. These same Ca^{2+} parameters were altered in all three cell types by 0.30 mM halothane. *Values different from all other groups within each cell type. †A value different from all other groups within the antisense-transfected cells ($P < 0.01$).

brain synaptic membranes[3-5] and cultured cells of neural origin.[6] Our laboratory has also observed parallel decreases or increases in the anesthetic partial pressures needed to prevent movement in response to pain in animal models with synaptic PMCA pumping activity that is incidentally decreased (in diabetic, spontaneously hypertensive, and aged rats)[7-10] or increased (in obese Zucker rats).[11] To determine if inhalation anesthetics actually interfere with neuronal Ca^{2+} homeostasis, we did electrophysiologic and microfluorimetric studies in cultured mouse central neurons (cortical, spinal, and dorsal root ganglion).[12] We observed delayed repolarization and delayed restoration of $[Ca^{2+}]_i$ to basal levels after NMDA stimulation with exposure to halothane, ≤1 MAC equivalent, at 37°C. In contrast, Bleakman *et al.*[43] reported that Ca^{2+} dynamics were unchanged in septal neurons exposed to halothane, isoflurane, enflurane, or desflurane. The substantive difference between their study and ours was the experimental temperature (room *vs.* physiologic). Our finding reported here that effects on intracellular Ca^{2+} dynamics induced by halothane, isoflurane, and sevoflurane occurred at 37°C but not at 21°C raises the issue of the importance of physiologic temperature in the study of anesthetic effects. It is interesting that other treatments (orthovanadate, high *p*H, and C28R2) do inhibit PMCA at 21°C.[34]

Given the now-established role of PMCA in fine tuning resting $[Ca^{2+}]_i$ and in removing physiologic Ca^{2+} loads in neurons,[34-36] it seems likely that the increases in resting and postligand $[Ca^{2+}]_i$ and the delay in Ca^{2+} clearance that we observed with anesthetic exposure were in fact due to PMCA inhibition. Therefore it is of interest that the pharmacologic concentrations of halothane had no effect on neuronal smooth endoplasmic reticulum Ca^{2+}-ATPase activity or the Na^+-Ca^{2+} exchanger, two regulators of $[Ca^{2+}]_i$.[4] Although Kosk-Kosicka and Roszczynska[44] and Fomitcheva and Kosk-Kosicka[45] observed complete inhibition of PMCA nucleotide hydrolysis in erythrocytes and brain synaptic membranes, their results are not incompatible with the much smaller degree of PMCA pump inhibition that we noted, given the high concentrations of anesthetics they used (see their letter of erratum[46]). We also used a different analytic technique, measurement of ion transport. In our hands, ATP hydrolysis was an unsatisfactory measure of PMCA activity in neural tissue, confounded perhaps by the presence of significant amounts of nontransporting calcium-dependent ATPases (ecto-ATPases).[47-49]

Other important systems that effect neuronal $[Ca^{2+}]_i$ are voltage- and ligand-gated calcium channels and intracellular calcium release channels. Evidence has accumulated indicating depression by volatile anesthetics of Ca^{2+} influx through the plasma membrane.[50,51] For example, Puil *et al.*[52] found that isoflurane reduced the increase in $[Ca^{2+}]_i$ resulting from glutamate-linked Ca^{2+} influx, and Daniell[53] observed that halothane and enflurane (at 2 or 3 MAC concentrations) inhibited hippocampal NMDA-receptor responses. Takenoshita and Steinbach[54] noted re-

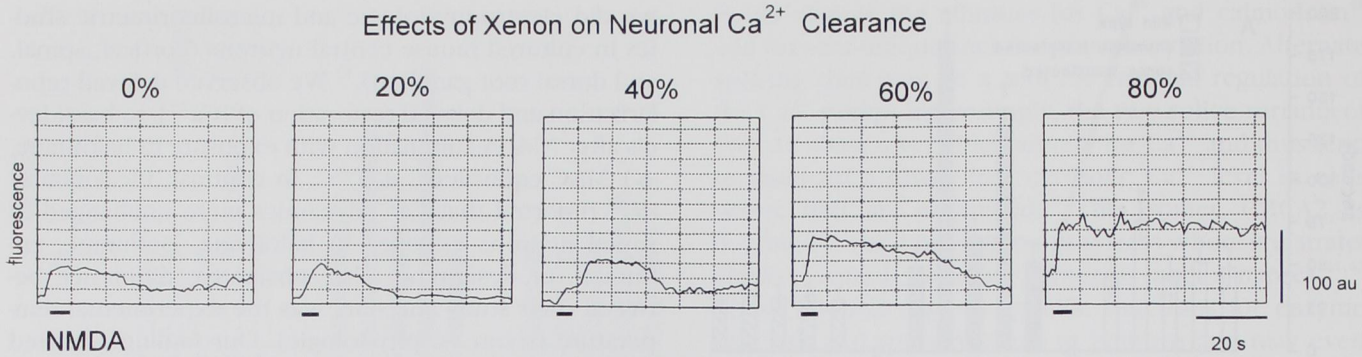

Fig. 10. Xenon effects on Ca^{2+} homeostasis in cortical neurons. Shown are confocal microfluorimetric tracings of the N-methyl-D-aspartate (NMDA) response in a representative cultured mouse embryonic cortical neuron exposed to increasing concentrations of xenon (all less than the minimum alveolar concentration [MAC]). NMDA was applied after 10 min of sequential superfusion with buffers equilibrated with dry gas mixtures of 20% oxygen and xenon–nitrogen in ratios providing xenon partial pressures ranging from 0–80% of 1 atm. Calculated maximum MAC-equivalent values of xenon solutions were 0.20, 0.40, 0.60, and 0.80, as described in the text.

duction by halothane of low-voltage-activated Ca^{2+} currents in rat sensory neurons, and Study[55] reported inhibition by isoflurane of multiple voltage-gated Ca^{2+} currents in hippocampal neurons. Miao *et al.*[56] found that isoflurane, enflurane, and halothane diminished Ca^{2+} transients and calcium concentration in isolated brain synaptosomes, effects they concluded were compatible with depression of voltage-gated channels. MacIver *et al.*[57] observed temperature-independent anesthetic depression of glutamate-mediated excitatory postsynaptic potential amplitudes in hippocampal slices and proposed depression of Ca^{2+} entry *via* voltage-gated channels as a likely mechanism of this effect. On the other hand, Pearce[58] provided evidence that halothane did not block voltage-sensitive channels in hippocampal slices. A general finding of studies of Ca^{2+} channels is that anesthetics reduce Ca^{2+} currents and Ca^{2+} influx into cells. Thus a predicted effect would be reduction of $[Ca^{2+}]_i$,[24] a change contrary to that seen by Daniell.[53] Similarly, Mody *et al.*[59] provided evidence that halothane increases $[Ca^{2+}]_i$ in hippocampal brain slices, possibly by stimulating release of intraneuronally stored calcium. Bickler *et al.*[60] studied isoflurane effects on Ca^{2+} dynamics in hippocampal slices and noted depression of NMDA-mediated Ca^{2+} influx at temperatures ranging from 28–39°C. They also noted, however, that isoflurane increased basal $[Ca^{2+}]_i$ and did so to an increasing degree at higher brain slice temperatures, with mean basal $[Ca^{2+}]_i$ more than three times greater at 37°C than at 28°C.

It is clear that among the multiple effects reported for volatile anesthetics there is a subset of multiple effects reported on cytosolic calcium regulators, along with certain inconsistencies as we have noted. It is also clear that anesthetics inhibit PMCA in neural cells, but can this effect be translated into the perturbations of $[Ca^{2+}]_i$ that we have observed? Our studies show that the net effect of anesthetics on intact neural cells maintained at 37°C is to increase $[Ca^{2+}]_i$, a finding that is consistent with inhibition of PMCA and in keeping with the findings of Benham *et al.*[34] and Werth *et al.*[36]

What about reports indicating that anesthetics inhibit some Ca^{2+} channels, sometimes with the expected associated result of a decrease in $[Ca^{2+}]_i$? Our studies support the idea that in circumstances wherein the anesthetic effects on PMCA are operational (available energy source, appropriate ionic constituents and physiologic temperature), effects on PMCA predominate, and $[Ca^{2+}]_i$ will increase rather than decrease. In the experiments of Miao *et al.*,[56] PMCA was not a factor because no ATP was added. On the other hand, Winlow *et al.*,[61] in a study of cultured molluscan neurons, found that although halothane and isoflurane depressed calcium currents, the unexpected net effect on $[Ca^{2+}]_i$ was an increase, and these results are compatible with our observations. Franks and Lieb,[24] noting that the literature indicates that nearly all systems studied are affected by anesthetics, emphasized the need for attention to pharmacologic concentrations and physiologic temperatures. We would add two other conditions, noted already: available energy source and ionic constituents.

If inhalation anesthetic interference with Ca^{2+} dynam-

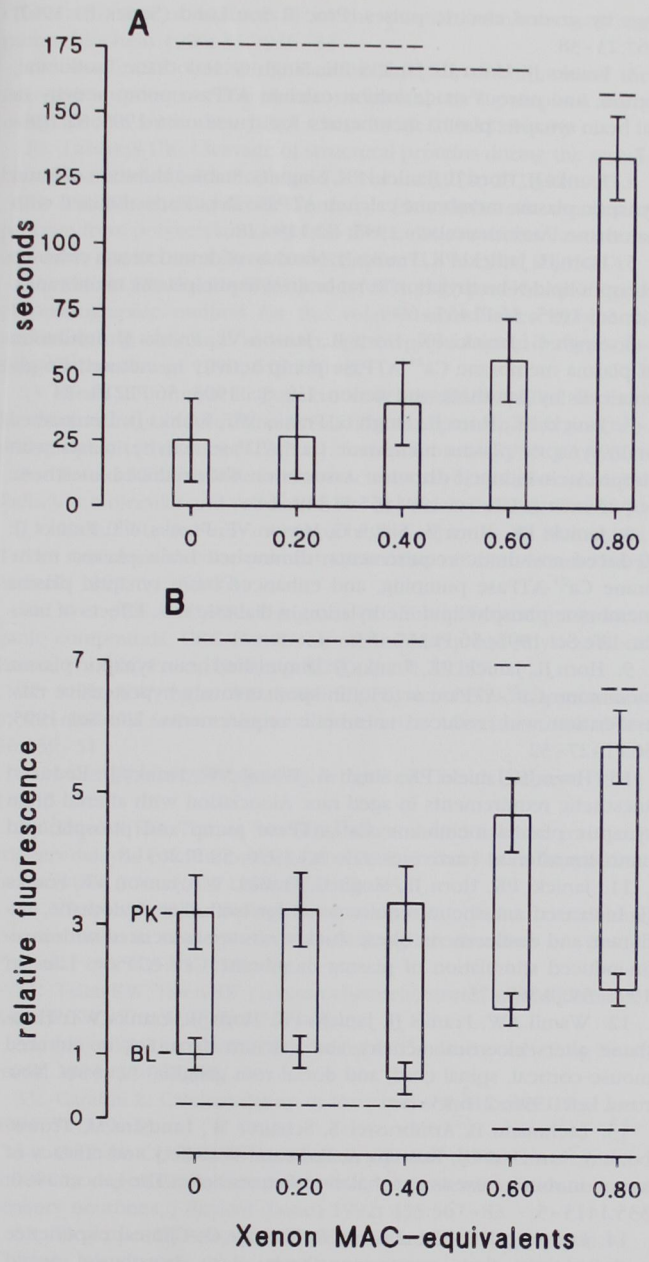

Fig. 11. Summary of effects of different concentrations of xenon on Ca^{2+} clearance (*A*) and on baseline and peak Ca^{2+}-related fluorescence (*B*) in cultured cortical neurons. Exposure conditions and procedures are described in figure 10. Columns in part A give 95% confidence limits, whereas those in part B give 99% confidence limits. Differences among responses to varying xenon concentrations are defined by line segments adjacent to each response parameter. Statistical significance, based on multifactor analysis of variance, is indicated when segments are discontinuous and do not overlap ($P < 0.05$ for panel A and $P < 0.01$ for panel B, n = 10). Nonparametric testing (Kruskal-Wallis) confirmed the differences observed in Ca^{2+}-associated fluorescence (*panel B*) and indicated that median clearance times in cells exposed to xenon minimum alveolar concentration-equivalent values of 0.40, 0.60, and 0.80 differed significantly from each other and from 0 and 0.20 ($P < 0.01$). Thus a clear pattern of increased perturbation of Ca^{2+} dynamics with increasing xenon concentrations was observed.

ics in cultured primary neurons is a result of PMCA inhibition, a similar effect of anesthetics and a specific PMCA antagonist on Ca^{2+} homeostasis should be demonstrable. Eosin may be such an antagonist,[62,63] and we have observed that eosin and halothane both prolong capsaicin-mediated Ca^{2+}-dependent depolarization in mouse adult dorsal root ganglion neurons.[12] Further, we found that infusion of eosin into the cerebroventricular system of rats produced a significant, reversible decrease in anesthetic requirements.[64] However, eosin has intrinsic fluorescence that interferes with microfluorimetric analyses. Because of this problem and a desire for greater specificity, we studied cells with specific blockade of PMCA isoform expression.

Cultured mouse embryonic cortical neurons maintained in media containing a 21 base antisense PMCA2 oligodeoxyribonucleotide, and with histochemical evidence of suppression of PMCA2 production, showed perturbation of Ca^{2+} dynamics at one half the concentration of halothane required to produce a similar effect in control cells. (No halothane effect was observed at 21°C, however, even in these supersensitive neurons.) Rat pheochromocytoma cells transfected with an antisense-producing cDNA construct that blocked PMCA1 production manifested intracellular Ca^{2+} perturbation when exposed to one tenth the concentration of halothane required to affect control cells. The fact that neural crest cells that are (1) of different tissue origin, (2) deficient in a different isoform of PMCA, (3) made deficient in isoform expression by different techniques, and (4) stimulated by a different ligand showed increased anesthetic sensitivity is especially noteworthy. These differences underscore the likelihood of a common site of anesthetic disturbance of calcium homeostasis in pheochromocytoma cells and neurons that is independent of these factors. It is interesting that pheochromocytoma cells transfected with antisense PMCA1 were particularly sensitive to halothane, compared with neurons with blocked PMCA2 production. Although extrapolation from one cell type to another is problematic, it is possible that the so-called housekeeping isoform,

PMCA1,[19] may be more resistant to anesthetics than PMCA2, an isoform confined to the central nervous system. It is clear, however, that the results we obtained with these two cell models provide further evidence that anesthetic perturbation of intracellular Ca^{2+} homeostasis is a result of effects on PMCA.

Finally, a fundamental test of any proposed site of general anesthetic action is its response to inhalation agents of widely different structure. For this reason, xenon, an inert element, is very important in the construction of any theory of the mechanism of anesthetics. Previously we reported that xenon inhibited PMCA activity in isolated neural membranes.[3,5,6] We have now examined the effect of this unusual anesthetic on Ca^{2+} homeostasis in cultured neurons. If our postulate is correct, that inhibition of PMCA is an important mechanism of inhalation anesthetic action, we would expect to see perturbation of neuronal Ca^{2+} dynamics with xenon exposure that is similar to that observed with volatile agents. Figures 10 and 11 show such perturbation by xenon. This common Ca^{2+} response of neurons to xenon and to potent anesthetics, along with the findings of Benham *et al.*[34] and Werth *et al.*,[36] would also appear to dismiss a systematic, nonspecific, toxic effect of volatile agents on cell membranes as the cause of $[Ca^{2+}]_i$ changes.

Together the observations reported in this and preceding publications, along with recent information regarding the function and location of PMCA in the central nervous system, provide consistent evidence for an important role for this calcium pump in the production of the anesthetic state. Proposing a single molecule as a principal anesthetic target may appear simplistic given current knowledge of the complexity of the central nervous system and the multiple effects of anesthetics. We offer our experimental evidence fully aware that the complete story is still untold.

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