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Stable Inhibition of Brain Synaptic Plasma Membrane Calcium ATPase in Rats Anesthetized with Halothane

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Background: **The authors recently showed that plasma membrane Ca^{2+}-ATPase (PMCA) activity in cerebral synaptic plasma membrane (SPM) is diminished in a dose-related fashion during exposure *in vitro* to halothane, isoflurane, xenon, and nitrous oxide at clinically relevant partial pressures. They have now extended their work to *in vivo* studies, examining PMCA pumping in SPM obtained from control rats decapitated without anesthetic exposure, from rats decapitated during halothane anesthesia, and from rats decapitated after recovery from halothane anesthesia.**

Methods: **Three treatment groups were studied: 1) C, control rats that were decapitated without anesthetic exposure, 2) A, anesthetized rats exposed to 1 minimum effective dose (MED) for 20 min and then decapitated, and 3) R, rats exposed to 1 MED for 20 min and then decapitated after recovery from anesthesia, defined as beginning to groom. Plasma membrane Ca^{2+}-ATPase pumping and Ca^{2+}-dependent ATPase hydrolytic activity, as well as sodium-calcium exchanger activity and Na^+-K^+-ATPase hydrolytic activity, were assessed in cerebral SPM. In addition, halothane effect on smooth endoplasmic reticulum Ca^{2+}-ATPase (SERCA) was examined.**

Results: **Plasma membrane Ca^{2+}-ATPase transport of Ca^{2+} into SPM vesicles from anesthetized rats was reduced to 71% of control ($P < 0.01$) compared with 113% of control for the recovered group (NS). No depression by halothane of SERCA activity, sodium-calcium exchanger, or Na^+-K^+-ATPase activity was noted among the CAR treatment groups.**

Conclusions: **Plasma membrane Ca^{2+}-ATPase is selectively and stably inhibited in cerebral SPM from rats killed while anesthetized with halothane, compared with rats killed without anesthesia or after recovery from anesthesia. The studies described in this report, in conjunction with previously reported inhibition of PMCA activity *in vitro* by a wide range of anesthetic agents, indicate a relationship between inhibition of PMCA and action of inhalational anesthetics. (Key words: Anesthetics, volatile: halothane. Brain: synaptic plasma membranes. Enzymes: plasma membrane Ca^{2+}-ATPase. Mechanism of anesthesia.)**

CENTRAL nervous system lipid or protein moieties undergoing conformational change in response to inhalational anesthetics are generally believed to revert to a preexposure state with elution of anesthetic from the brain. However, we have shown that at least one process, conversion by methyltransferase I of phosphatidylethanolamine to phosphatidyl-N-monomethylethanolamine, remained enhanced twofold in neural membranes of rats killed while anesthetized with halothane or isoflurane.[1] This stimulation of methylation persisted despite elution of anesthetic from the synaptosomal fraction during the long separation process. In contrast, phospholipid methylation was found to be normal in rats killed after emergence from halothane or isoflurane anesthesia. We now report persistent anesthetic alteration of another enzyme system found in synaptosomes, the ubiquitous plasma membrane Ca^{2+}-ATPase pump (PMCA). Free calcium is an important intracellular regulator, and its cytosolic activity, $[Ca^{2+}]_i$, is controlled by at least five major classes of pumps, exchangers, and channels.[2–5] Plasma membrane Ca^{2+}-ATPase plays a critical role in this regulatory process by ejecting Ca^{2+} from the cell. We have shown that Ca^{2+} pumping in synaptic plasma membrane (SPM) vesicles is diminished in a dose-related fashion during exposure *in vitro* to halothane, isoflurane, xenon, and nitrous oxide at clinically relevant partial pressures.[6–9] Moreover, fluorinated anesthetics inhibit ATP hydrolysis by erythrocytic PMCA,[10] a process recently described in a definitive report.[11] We have extended our work to examine PMCA pumping activity in SPM obtained from rats killed during and after halothane anesthesia. We found persistent inhibition of Ca^{2+} pumping

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by PMCA in SPM obtained from rats killed while anesthetized, compared with control animals killed while awake. We also found return of PMCA function to normal in SPM obtained from animals allowed to recover from anesthesia before being killed.

Materials and Methods

Anesthetic Procedure

***In Vivo* Exposure.** Animal use was approved by the Animal Care Committee of Vanderbilt University. Male Sprague-Dawley rats (250–320 g) were allowed access to food and water until the morning of the experiment. Animals were anesthetized with halothane (Halocarbon Laboratories, River Edge, NJ) delivered in warmed, humidified air and oxygen ($F_{I_{O_2}} = 0.3$) from a dedicated, calibrated vaporizer. Halothane concentrations were confirmed by gas chromatography. Induction of anesthesia was carried out with each rat breathing 3% halothane in a 21 chamber. When immobilized, the rat was removed from the chamber and placed on a thermal pad with a heating lamp nearby. A Tygon cylinder attached to a T-piece was fitted snugly over the muzzle for continued delivery of anesthetic as the animal breathed spontaneously.[1] Temperature was monitored with a rectal probe, and warming devices were used as needed. Halothane concentration was adjusted gradually, and anesthetic effect was monitored by clamping the tail every 30–60 s. After 6–8 min, the lowest concentration that suppressed withdrawal with tail clamping was attained, thus defining the minimum effective dose (MED).[1] Anesthesia was continued, excluding induction time in the chamber, for 20 min. Three treatment groups were studied: 1) C, control rats that were decapitated without anesthetic exposure, 2) A, anesthetized rats exposed to 1 MED for 20 min and then decapitated, and 3) R, "recovered" rats exposed to 1 MED for 20 min and then decapitated after recovery from anesthesia, defined as beginning to groom.

***In Vitro* Exposure.** Synaptic plasma membranes were prepared and processed, as described below, from rats decapitated without anesthetic treatment. Synaptic plasma membranes were then exposed in incubation vials placed in a Dubnoff shaker under a gassing hood.[1,9] Halothane, in a warmed, humidified air/oxygen mixture ($F_{I_{O_2}} = 0.3$), was delivered in concentrations up to 1.5 vol% from a dedicated, calibrated vaporizer. Anesthetic concentrations were confirmed by gas chromatography.

Effect of Halothane Anesthesia on Arterial Blood Gas Partial Pressures and pH

In parallel experiments, a series of six rats was anesthetized with halothane as described above, and their femoral arteries were surgically exposed. After 20 min of anesthesia, arterial blood samples (300 μl) were withdrawn sequentially through a 27.5-G needle in duplicate or triplicate. Syringes were capped after evacuation of air, placed on ice, and directly analyzed on a Ciba Corning 238 *p*H/Blood Gas Analyzer (Ciba Corning Diag. Ltd, Halstead, England). Partial pressures of O_2 and CO_2 were measured, *p*H was determined, and base excess and hemoglobin oxygen saturation were calculated. Rats were then killed with an anesthetic overdose; SPM were not prepared from these animals.

Preparation of Synaptic Plasma Membranes

Evaluation of SPM preparative techniques has been detailed elsewhere.[1,9,12] Brains were dissected on ice. Cerebra from three to eight rats were weighed and pooled in ice-cold 0.32 M sucrose (*p*H 7.4). Synaptosomes were prepared by gradient ultracentrifugation, and SPM were prepared by osmotic shock of synaptosomes followed by differential ultracentrifugation on a discontinuous sucrose gradient. Final pellets were suspended in isosmotic sucrose (0.32 M, *p*H 7.4) and used immediately for assay of PMCA Ca^{2+} pumping, sodium-calcium exchanger activity, or Na^+-K^+-ATPase hydrolysis of ATP. Assays of Ca^{2+}-dependent ATP hydrolysis were done either immediately or within a few days. Microsomes were prepared by ultracentrifugation of the supernatant of the S2 fraction at 100,000*g* for 20 min. Pellets were suspended in isosmotic sucrose (0.32 M, *p*H 7.4) and used for measurement of Ca^{2+} accumulation studies. Protein content in SPM pellets was estimated by the Bradford method.

Measurement of Ca^{2+} Pumping Across SPM Membranes

Ca^{2+} uptake by everted SPM vesicles, *i.e.,* transport from the intracellular to extracellular surface (and by smooth endoplasmic reticulum, *i.e.,* microsomes), was performed as described by Moore *et al.* with several modifications.[13] The incubation mixture (total volume 4 ml) was comprised of 30 mM imidazole-histidine (*p*H 6.8), 200 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 5 mM sodium azide, 5 mM ammonium oxalate, and 20 μM $CaCl_2$ containing (final concentration) 0.1 μCi/ml of $^{45}CaCl_2$ (NEN Products, Boston, MA; specific activity

30.7 mCi · mg^{-1}). The reaction was started by adding 70-μg aliquots of SPM protein to each tube and maintained at 37°C. Aliquots of 0.5 ml were removed after 20, 30, and 60 min. Synaptic plasma membrane vesicles were collected on 25-mm cellulose nitrate filters (0.45 μm pore size, Gelman Sciences, Ann Arbor, MI) that had been prewashed with 2 ml of 0.25 M KCl and 10 ml of deionized water. After vesicle collection, the filters were washed with 2 ml of 0.25 M sucrose and dried. They were placed in vials containing CytoScint (ICN Costa Mesa, CA), and $^{45}Ca^{2+}$ activity was assessed in a Beckman LS3801 beta counter. Results were expressed as nmoles of Ca^{2+} accumulated per milligram of SPM protein per minute of incubation time. To confirm that Ca^{2+} uptake in these experiments reflected PMCA activity, sensitivity to orthovanadate was tested by measuring Ca^{2+} uptake in SPM obtained from C, A, and R rats in the presence and absence of 0.1 mM orthovanadate.[14]

Determination of Ca^{2+}-Dependent ATPase Hydrolytic Activity

ATPase hydrolytic activity in the SPM preparations was assessed by measurement of inorganic phosphate (Pi) released from ATP during incubation with the enzyme source.[15,16] Synaptic plasma membrane aliquots (2 μg per tube) were suspended in 25 mM Tris-HCl buffer (*p*H 7.4), 50 mM KCl, 2 mM $MgCl_2$, and 1 μM $CaCl_2$. The reaction was started by adding ATP (2 mM final concentration) in a total reaction volume of 1 ml, and samples were incubated for 30 min at 37°C in a Dubnoff shaker. The reaction was stopped by adding 1 ml of a solution (cooled to 4°C) containing perchloric acid (1.1 M), ammonium molybdate (809.1 mM), ammonium hydroxide (285.3 mM), ammonium metavanadate (20.09 mM), and nitric acid (99.1 μM). Tubes were vortexed and optical densities of reaction mixtures and KH_2PO_4 standards were measured in a UV spectrophotometer at 350 nm. Results were expressed in μmoles of liberated Pi per mg protein per hour.

Effect of Halothane on the Sodium-Calcium Exchanger

Sodium-calcium exchanger activity was estimated by measuring Ca^{2+} accumulation in SPM vesicles preloaded with sodium ions.[17] Synaptic plasma membranes were obtained from rats treated according to the anesthetic protocol described above (*in vivo* group) or from untreated animals (*in vitro* group). Synaptic plasma membranes (0.7 mg protein) in 0.9 ml of 0.32 M sucrose (*p*H 7.4) plus 0.1 ml of 1 M NaCl were preincubated for 15 min at 37°C. One-tenth milliliter of the mixture containing 70 μg protein was then added to 3.9 ml of 50 mM Tris HCl buffer (*p*H 7.4) comprised of 200 mM KCl, 5 mM $MgCl_2$, and 20 μM $CaCl_2$ including (final concentration) 0.1 μCi · ml^{-1} of $^{45}CaCl_2$ (NEN Products, Boston, MA; specific activity 30.7 mCi · mg^{-1}). Aliquots of 0.5 ml were removed after 2.5, 5, 10, 20, and 30 min. Membranes prepared from rats in the *in vitro* group were exposed to desired concentrations of halothane throughout the preincubation and incubation periods, as described above. Synaptic plasma membrane vesicles were collected on 25-mm cellulose nitrate filters, which were processed as described for measurement of PMCA pumping activity. Results were expressed as nmoles of Ca^{2+} accumulated per milligram of SPM protein per minute of incubation time.

Effect of Halothane on Sodium-Potassium-ATPase in Synaptic Plasma Membranes

Sodium-potassium ATPase (Na^+-K^+-ATPase) hydrolytic activity in the SPM preparation was assessed by measurement of Pi release from ATP during incubation in the presence and absence of the specific Na^+-K^+-ATPase inhibitor, ouabain (2 mM final concentration).[18] Synaptic plasma membranes were obtained from rats treated according to the anesthetic protocol described above (*in vivo* group) or from untreated animals (*in vitro* group). Synaptic plasma membrane aliquots (2 μg) were suspended in (final concentrations) 0.1 M Tris-HCl buffer (*p*H 7.0), 20 mM KCl, 5 mM $MgCl_2$, 0.1 M NaCl, and 1 mM EDTA. The reaction was started by adding ATP (2 mM final concentration) in a total reaction volume of 1 ml, and samples were incubated for 30 min at 37°C in a Dubnoff shaker. Membranes prepared from rats in the *in vitro* group were exposed to desired concentrations of halothane throughout the incubation period, as described above. The reaction was stopped by adding 1 ml of a solution (cooled to 4°C) containing perchloric acid, ammonium molybdate, ammonium hydroxide, and ammonium vanadate. Tubes were vortexed, and optical densities of reaction mixtures and KH_2PO_4 standards were measured in a UV spectrophotometer at 350 nm. Results were calculated as the difference in Pi liberation in the absence and

Table 1. Arterial Blood Gases in Rats after 20 min of Halothane Anesthesia Adjusted to 1 Minimum Effective Dose with $Fi_{O_2} = 0.3$

Arterial Blood Gas	Rate
*p*H	7.42 ± 0.02
P_{CO_2}	37.64 ± 1.54
P_{O_2}	154.6 ± 14.1
BE	1.92 ± 0.66
Sa_{O_2}	98.85 ± 0.19

Values are mean ± SEM (n = 6).

P_{CO_2} = arterial carbon dioxide partial pressure (mmHg); P_{O_2} = arterial oxygen partial pressure (mmHg); BE = base excess (mEq); Sa_{O_2} = (oxyhemoglobin/total hemoglobin) × 100 (%).

presence of ouabain and expressed in μmoles of liberated Pi per mg protein per hour.

Effect of Method of Killing on Ca^{2+}-ATPase Activity

Four groups of seven rats in three separate experiments were killed by decapitation or by exposure to lethal concentrations of carbon dioxide, nitrogen, or helium. Each rat was placed in a 21 container equipped with inlet and outlet ports. Euthanasia was carried out by rapid replacement of air in the container with CO_2, N_2, or He delivered at $>10\ l \cdot min^{-1}$. Death, as judged by respiratory arrest preceded by loss of muscle tone, occurred within 30–40 s and was followed by decapitation. Control animals were exposed to air delivered at $10\ l \cdot min^{-1}$ for 40 s before decapitation. Synaptic plasma membranes were prepared from rats in each treatment group for measurement of PMCA activity.

Effect of Discontinuation of In Vitro *Halothane Exposure on Plasma Membrane Ca^{2+}-ATPase Activity*

Cerebral SPM from untreated rats were incubated and exposed as described above to an air/oxygen mixture or to 1.5 vol% halothane for 30 min. In one group of experiments, ATP was added to the incubation mixture before exposure began, as usual. In a second group, ATP was added at the conclusion of the 30-min exposure period. All incubation vials were exposed to air/oxygen for another 60 min, for a total incubation time of 90 min. Aliquots were removed from all vials at 30, 60, and 90 min for assay of Ca^{2+} uptake. Halothane concentration was measured in representative aliquots at each sampling time by high-performance liquid chromatography. Twenty microliters of the incubation solution were mixed with 20 μl of 0.05 nM toluene (serving as an internal standard) and injected directly into a Beckman System Gold HPLC equipped with a Waters C18 Novapack HPLC column (Millipore, Marlborough, MA). The eluent was methanol:water (1:1). The chromatogram was monitored for UV absorption at 210 nm.

Statistical Analysis

Data were examined by multifactorial ANOVA and multiple comparison (Student–Newman–Keuls procedure). Statistical significance was inferred if $P < 0.05$.

Results

Estimation of the Minimum Effective Dose of Halothane

Minimum effective dose (MED) for halothane was determined in 96 male rats used in this study. A mean MED of 1.49 vol% was obtained, with a standard deviation of 0.12 and a standard error of the mean of 0.012.

Arterial Blood Gas Partial Pressures and p*H in Anesthetized Rats*

Table 1 shows arterial oxygen and carbon dioxide partial pressure, *p*H, base excess, and hemoglobin oxygen saturation measured (or calculated) in duplicate or triplicate, in six rats anesthetized for 20 min. Halothane concentrations were adjusted to deliver 1 MED. Partial pressures of O_2 reflected an Fi_{O_2} of 0.3. All other values were within normal limits.

Plasma Membrane Ca^{2+}-ATPase Pump Activity in Synaptic Plasma Membranes from Control, Anesthetized, and Recovered Rats

Figure 1 shows Ca^{2+} uptake, plotted against incubation time, in SPM obtained from control (C), anesthetized (A), and recovered (R) rats in six separate experiments, with membranes pooled from three to eight rats in each treatment group and incubated in quadruplicate. Plasma membrane Ca^{2+}-ATPase inhibition is evident at all sampling times with A treatment compared with C, with a return to normal with R treatment. Orthovanadate (0.1 mM), an inhibitor of PMCA, markedly reduced uptake in all treatment groups. Figure 2 shows results from these six experiments with PMCA

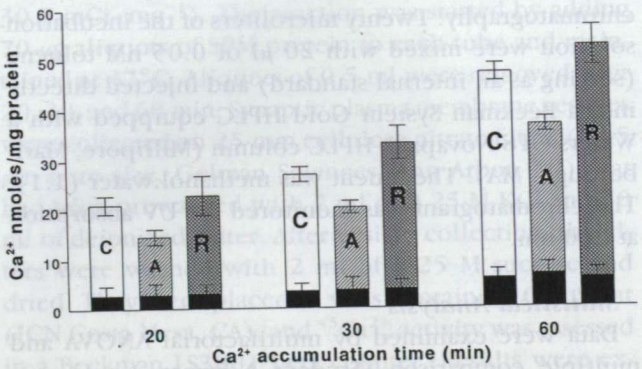

Fig. 1. Time course of plasma membrane Ca^{2+}-ATPase (PMCA) transport of Ca^{2+} into synaptic plasma membrane (SPM) vesicles prepared from cerebra of control (unanesthetized) rats (C), rats anesthetized with halothane 1 MED (minimum effective dose) for 20 min (A), and rats recovered (R) from anesthesia. Data (means) were derived from six separate experiments, with membranes pooled from three to eight rats and incubated in quadruplicate. The vertical axis denotes Ca^{2+} uptake (nmoles · mg protein^{-1}) and the horizontal axis incubation time (minutes). Treatment groups are indicated by open columns (C), hatched columns (A), and cross-hatched columns (R). Solid columns in the foreground demonstrate the inhibitory effect of 0.1 mM orthovanadate on PMCA pumping activity for all treatment groups. Error bars indicate 95% confidence limits for the mean derived from ANOVA. Methods are described in the text.

pump activity expressed as nmoles of transported Ca^{2+} per mg of protein per minute for all treatment groups. Multifactor ANOVA and multiple comparison testing indicate that Ca^{2+} transport by PMCA was significantly inhibited ($P < 0.01$) in SPM from animals killed while anesthetized. Transport in membranes from animals recovered from anesthesia did not differ significantly from the unanesthetized control group.

Ca^{2+}-Dependent ATPase Hydrolytic Activity in Synaptic Plasma Membranes from Control, Anesthetized, and Recovered Rats

Figure 3 shows that hydrolysis of ATP by Ca^{2+} dependent ATPase in SPM was not altered by prior anesthesia, *i.e.*, C, A, and R groups do not differ significantly. Results were derived from four experiments in which hydrolysis was assayed, out of the six studies described in the preceding section in which Ca^{2+} pumping was measured.

Effect of In Vivo *Halothane Exposure on Ca^{2+} Uptake by Microsomes*

Figure 4 shows the effect of prior *in vivo* halothane treatment (1 MED) on Ca^{2+} transport by smooth endoplasmic reticulum Ca^{2+}-ATPase (SERCA) in the microsomal fraction of rat cerebral homogenates in two separate experiments (eight rats in each). No differences in SERCA activity were noted among the C, A, and R treatment groups.

Effect of In Vivo *and* In Vitro *Halothane Exposure on Sodium-Calcium Exchanger Transport of Ca^{2+} and Na^+-K^+-ATPase Activity in Synaptic Plasma Membranes*

Figures 5a and b show the effects of prior *in vivo* halothane treatment and concurrent *in vitro* treatment with different halothane concentrations on sodium-calcium exchange in SPM obtained from seven rats. No discernible inhibition with halothane exposure was noted in either circumstance. Figures 5c and d show the effects of prior *in vivo* halothane treatment and concurrent *in vitro* treatment at different halothane concentrations on Na^+-K^+-ATPase activity in SPM obtained from seven rats. Again, no discernible depression with halothane was noted.

Plasma Membrane Ca^{2+}-ATPase Activity in Synaptic Plasma Membranes Obtained from Rats Killed by Decapitation or by Exposure to Carbon Dioxide, Nitrogen, or Helium

Figure 6 indicates PMCA transport of Ca^{2+} in SPM obtained from rats killed by decapitation or by exposure to one of several gases. No significant differences in PMCA activity, as measured by Ca^{2+} accumulation by SPM vesicles at 20, 30, and 60 min, were observed between the rats killed while awake or by exposure to lethal concentrations of CO_2, N_2, or He.

Effect of Discontinuing In Vitro *Halothane on Plasma Membrane Ca^{2+}-ATPase Pump Activity*

Figure 7 shows the effects of interrupting *in vitro* halothane exposure during measurement of Ca^{2+} uptake in cerebral SPM. Membranes were prepared from previously unexposed rats. Each indicated value is the mean of triplicate measurements with SPM pooled from ten rats in two experiments. When ATP was added initially, before a 30-min exposure to halothane (air/oxygen for controls), Ca^{2+} uptake continued linearly after halothane was replaced with air/oxygen. Pumping was depressed to approximately 80% of control ($P < 0.01$). Similar results were obtained when ATP was added immediately after 30 min of halothane exposure. Despite replacement with air/oxygen during the remainder of the incubation period, Ca^{2+} uptake continued linearly

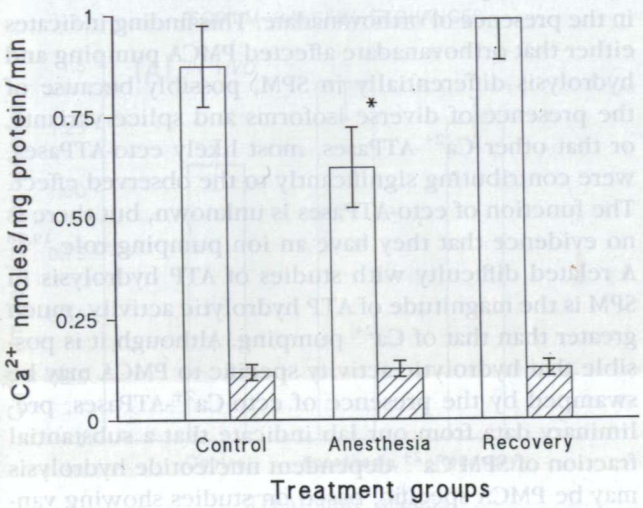

Fig. 2. Plasma membrane Ca^{2+}-ATPase pumping activity in cerebral SPM vesicles from rats in three treatment groups: control (C), anesthetized (A), and recovered (R). Ca^{2+} transport rates (nmoles · mg protein^{-1} · min^{-1}) were derived from time-course data displayed in figure 1. Plasma membrane Ca^{2+}-ATPase pumping (open columns) was significantly reduced to 71% of control ($P < 0.01$) in rats killed while anesthetized, compared with C and R treatments. Orthovanadate (hatched columns) inhibited Ca^{2+} uptake by SPM vesicles in all treatment groups. Error bars indicate 95% confidence limits derived from ANOVA.

and was depressed to 85% of control ($P < 0.01$). Halothane concentration in the incubation medium was 0.18 mM at 30 min, reduced to 0.050 mM at 60 min and to 0 at 90 min.

Discussion

We recently showed that PMCA activity in SPM from rat brain is diminished in a dose-related fashion during exposure *in vitro* to halothane, isoflurane, xenon, or nitrous oxide at clinically relevant partial pressures.[1,6–9] We have now described studies of PMCA activity in SPM from rats treated in three different ways before killing: no anesthetic exposure, exposure to halothane for 20 min (1 MED), or exposure to halothane for 20 min followed by a recovery period. Animals in the last group were considered to be recovered from anesthesia when they began grooming. One MED was defined as the lowest anesthetic concentration delivered to a rat that suppressed withdrawal with tail clamping. Minimum effective dose levels are highly reproducible in individual rats and are clustered closely in groups of rats. We previously reported MED values of 1.43 vol% (about 1.3 MAC) for halothane in eight rats.[1] Minimum effective dose determined in 96 rats in the course of the current study averaged 1.49 vol%. At this "surgical" level of anesthesia, rats breathed spontaneously. Adequacy of ventilation in this setting was assessed by arterial blood gas analysis in six similarly anesthetized rats. Table 1 shows that these animals were able to maintain a normal *p*H and partial pressure of CO_2, as well as a partial pressure of O_2 consistent with breathing 30% oxygen.

As indicated in figure 1, Ca^{2+} accumulation at three sampling times during SPM incubation (20, 30, and 60 min) was depressed in animals killed while anesthetized ($P < 0.01$). Plasma membrane Ca^{2+}-ATPase returned to levels that did not differ significantly from control values in SPM prepared from brains of animals allowed to recover from anesthesia before decapitation. Inhibition by low concentrations of orthovanadate is characteristic and specific for P-type pumps, such as PMCA. Orthovanadate inhibited Ca^{2+} uptake in all treatment groups, with no difference in residual Ca^{2+} accumulation among treatment groups, and thus is indicative of a specific effect of halothane on the PMCA

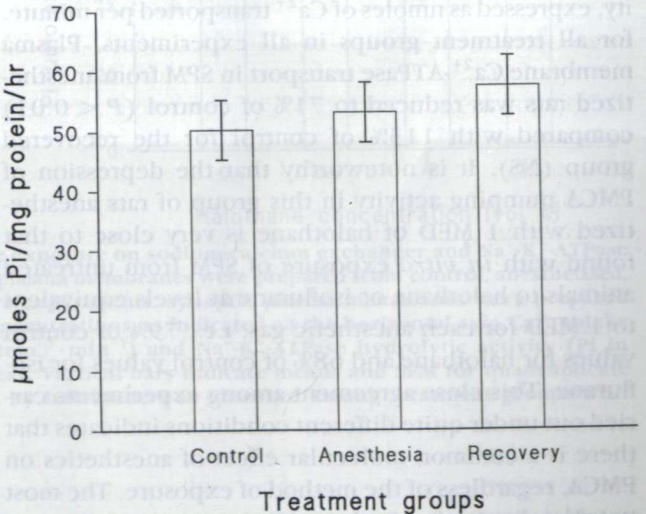

Fig. 3. Ca^{2+}-ATPase hydrolytic activity in cerebral synaptic plasma membranes obtained from control and anesthetized rats and from rats recovered from anesthesia. Ca^{2+}-ATPase hydrolysis was measured as described in the text. The vertical axis indicates the amount of inorganic phosphate (Pi) liberated from ATP in μmoles · mg protein^{-1} · h^{-1}. Columns represent means from four experiments in which ATP hydrolysis was measured out of the six experiments illustrated in figures 1 and 2. No significant differences in Ca^{2+}-dependent ATP hydrolysis were found among the treatment groups. Error bars indicate 95% confidence limits for the mean.

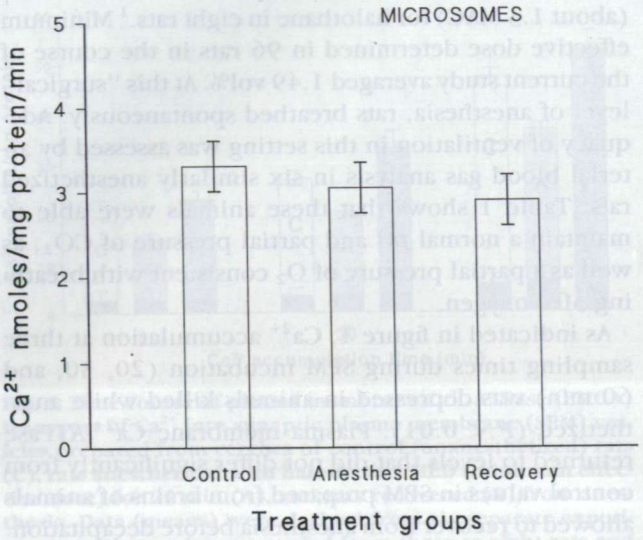

Fig. 4. Ca^{2+} uptake by cerebral microsomes, a measure of smooth endoplasmic reticulum Ca^{2+} ATPase (SERCA) activity, in rats in the control, anesthetized, and recovered treatment groups. Uptake was assayed as described in the text. Columns represent means of two experiments comprised of eight rats each. No significant differences in SERCA activity were noted among the treatment groups. Error bars indicate 95% confidence limits for the mean.

pump. Figure 2 compares mean PMCA pumping activity, expressed as nmoles of Ca^{2+} transported per minute, for all treatment groups in all experiments. Plasma membrane Ca^{2+}-ATPase transport in SPM from anesthetized rats was reduced to 71% of control ($P < 0.01$) compared with 113% of control for the recovered group (NS). It is noteworthy that the depression of PMCA pumping activity in this group of rats anesthetized with 1 MED of halothane is very close to that found with *in vitro* exposure of SPM from untreated animals to halothane or isoflurane at levels equivalent to 1 MED for each anesthetic gas, *i.e.,* 79% of control values for halothane and 68% of control values for isoflurane. This close agreement among experiments carried out under quite different conditions indicates that there is a common molecular effect of anesthetics on PMCA, regardless of the method of exposure. The most notable observation in these *in vivo* studies, however, is the persistence of PMCA inhibition in the absence of the precipitating anesthetic agent.

In *in vitro* studies previously reported, we observed depression of both calcium pumping and ATP hydrolysis in SPM with anesthetic exposure.[6–9] We questioned the precision of ATP hydrolysis as a guide to anesthetic effects on SPM PMCA because anesthetic inhibition of ATP hydrolysis occurred, to some degree, in the presence of orthovanadate. This finding indicates either that orthovanadate affected PMCA pumping and hydrolysis differentially in SPM, possibly because of the presence of diverse isoforms and splice variants, or that other Ca^{2+}-ATPases, most likely ecto-ATPases, were contributing significantly to the observed effect. The function of ecto-ATPases is unknown, but there is no evidence that they have an ion pumping role.[19,20] A related difficulty with studies of ATP hydrolysis in SPM is the magnitude of ATP hydrolytic activity, much greater than that of Ca^{2+} pumping. Although it is possible that hydrolytic activity specific to PMCA may be swamped by the presence of ecto-Ca^{2+}-ATPases, preliminary data from our lab indicate that a substantial fraction of SPM Ca^{2+}-dependent nucleotide hydrolysis may be PMCA specific, based on studies showing vanadate inhibition and calmodulin stimulation of one-third of the hydrolytic activity. Why, then, the excessive ratio of Pi release to Ca^{2+} transport? One contributing factor may be artifact caused by vesicular leakiness, causing a degree, perhaps a large degree, of repumping of "lost" Ca^{2+} back into the interior with associated excess ATP utilization. However, our work and the work of others,[21,22] who also find comparatively high levels of Pi release, indicates uncoupling of a large fraction of nucleotide hydrolytic activity from Ca^{2+} transport. Transport requires an intact PMCA molecule properly situated in the plasma membrane, whereas hydrolytic action may proceed under less stringent requirements. Sola-Penna *et al.*[23] reported that the simple sugar, trehalose, inhibited PMCA pumping in kidney tubules and yet left PMCA hydrolytic activity unaffected. In a similar vein, the Ca^{2+} transport/ATP hydrolysis coupling ratio of sarcoplasmic reticulum Ca^{2+}-ATPase was affected by the lipid composition of the membrane.[24] A polypeptide antibiotic, duramycin, that modifies phospholipid-protein interactions in sarcoplasmic reticulum vesicles inhibited ATP-dependent Ca^{2+} uptake without altering ATP hydrolysis.[25] It is interesting that we did not note stable inhibition of Ca^{2+}-ATPase-dependent nucleotide hydrolysis in SPM obtained from anesthetized rats (fig. 3), in contrast to persistent inhibition of Ca^{2+} pumping. This observation indicates differential inhalational anesthetic effects on the hydrolytic and pumping domains of PMCA, with stable anesthetic inhibition of the physiologically relevant and pharmacologically specific Ca^{2+} transport moiety. Alternatively, failure of persistent inhibition of ATP hydrolysis may reflect the presence and reversible inhibition of other, nonpumping Ca^{2+}-ATPases.

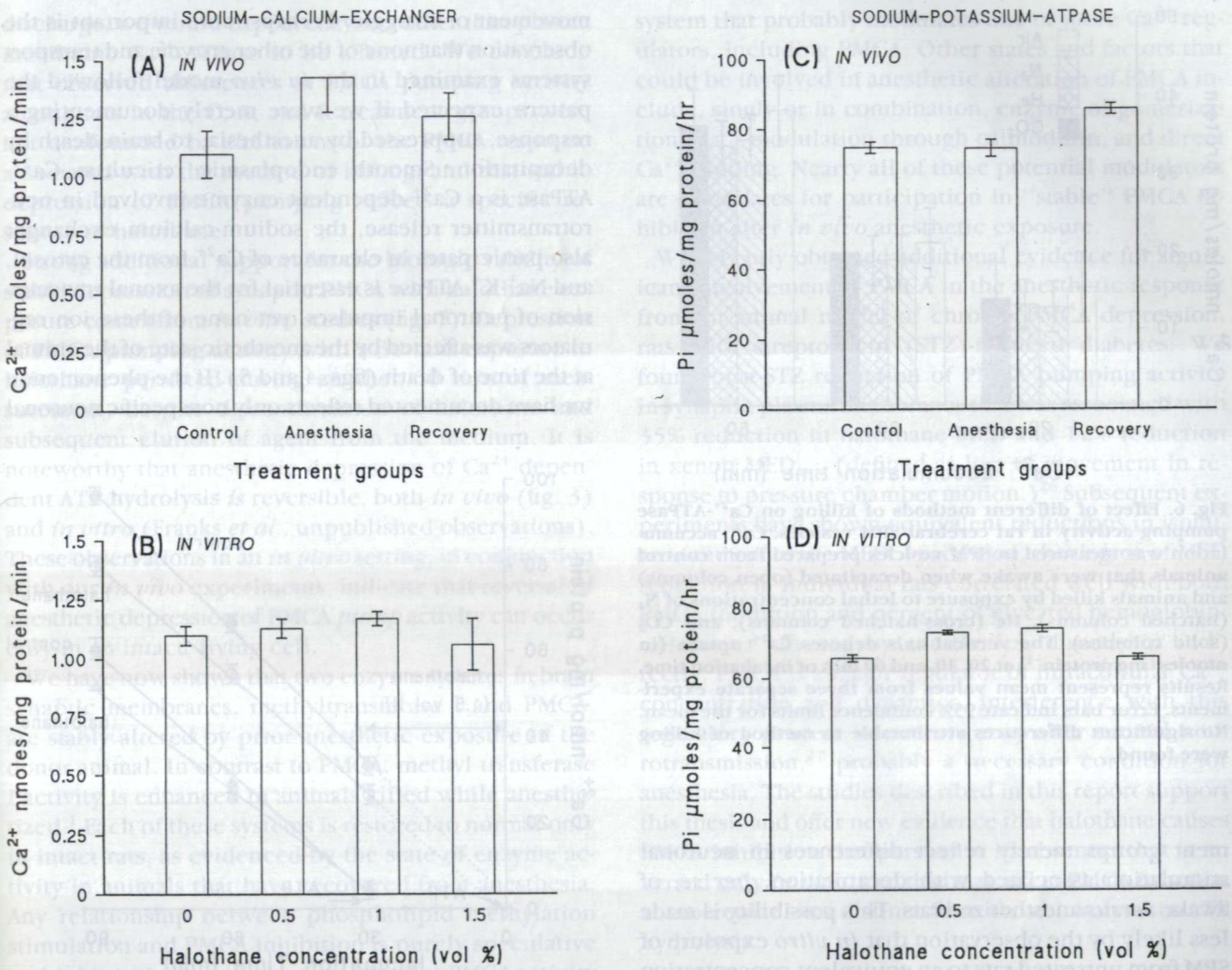

Fig. 5. Effect of *in vivo* (*A* and *C*) and *in vitro* (*B* and *D*) halothane exposure on sodium-calcium exchanger and Na^+-K^+-ATPase activity in cerebral SPM vesicles. For *in vivo* experiments, synaptic plasma membranes were prepared from control, anesthetized, and recovered rats (seven rats for each experiment). For *in vitro* experiments, synaptic plasma membranes were prepared from seven untreated rats and exposed to a range of halothane concentrations, as indicated on the horizontal axis. Ca^{2+} uptake *via* the sodium-calcium exchange mechanism (nmoles · mg protein^{-1} · min^{-1}) and Na^+-K^+-ATPase hydrolytic activity (Pi in µmoles · mg protein^{-1} · min^{-1}) were assayed as described in the text. Vertical bars indicate means and SEM for quadruplicate measurements made for each treatment category. No inhibition by halothane was observed among treatment groups with either system.

We also investigated three other subcellular systems involved in ion transport with respect to the CAR model. Figure 4 shows studies of smooth endoplasmic reticulum Ca^{2+}-ATPase (SERCA) in microsomes prepared from rat brain homogenates. This active intracellular Ca^{2+} transport system did not appear to be affected by previous anesthetic treatment of the donor rat. We have previously shown that there is very little contamination of the SPM fraction itself with SERCA.[9]

Figure 5 indicates the effects of prior *in vivo* halothane treatment and concurrent *in vitro* treatment on the sodium-calcium exchanger in SPM (fig. 5a and b) and on ATP hydrolysis by Na^+-K^+-ATPase in SPM (fig. 5c and d). No discernible inhibition with halothane exposure was noted in either circumstance.

An important question to be considered in the experimental design presented herein is the possibility that observed differences in PMCA activity in the treat-

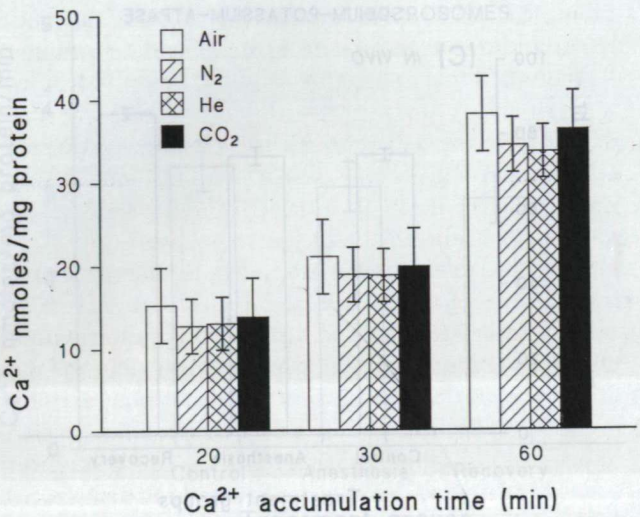

Fig. 6. Effect of different methods of killing on Ca^{2+}-ATPase pumping activity in rat cerebral SPM vesicles. Ca^{2+} accumulation was measured in SPM vesicles prepared from control animals that were awake when decapitated (open columns) and animals killed by exposure to lethal concentrations of N_2 (hatched columns), He (cross-hatched columns), and CO_2 (solid columns). The vertical axis denotes Ca^{2+} uptake (in nmoles · mg protein^{-1}) at 20, 30, and 60 min of incubation time. Results represent mean values from three separate experiments. Error bars indicate 95% confidence limits for the mean. No significant differences attributable to method of killing were found.

ment groups merely reflect differences in neuronal stimulation associated with decapitation, *per se,* of awake *versus* anesthetized rats. This possibility is made less likely by the observation that *in vitro* exposure of SPM from untreated rats to an equivalent concentration of halothane (1 MED) resulted in the same degree of PMCA inhibition, *i.e.,* about 30%. To examine this question further, we conducted a series of experiments in which rats were killed by exposure to high concentrations (virtually 100%) of He, N_2, or CO_2. These results, summarized in figure 6, demonstrate that death from exposure to N_2, He, or CO_2 did not significantly modify PMCA activity compared with that in animals decapitated while awake. Therefore, decapitation, with associated massive discharge from the severed cord, is an unlikely cause of nonspecific PMCA alteration.

These experiments do not rule out nonspecific responses to anoxia resulting from either decapitation or lethal gas exposure in the absence of "protective" general anesthesia. It is noteworthy that animals exposed to CO_2 appeared to pass through an anesthetic stage before death. Death occurred very quickly with no movement or struggle. Perhaps more important is the observation that none of the other enzyme and transport systems examined in the *in vivo* model followed the pattern expected if we were merely documenting a response, suppressed by anesthesia, to brain death by decapitation. Smooth endoplasmic reticulum Ca^{2+}-ATPase is a Ca^{2+}-dependent enzyme involved in neurotransmitter release, the sodium-calcium exchanger also participates in clearance of Ca^{2+} from the cytosol, and Na^+-K^+-ATPase is essential for the axonal transmission of neuronal impulses, yet none of these ion regulators was affected by the anesthetic state of the animal at the time of death (figs. 4 and 5). If the phenomenon we have documented reflects only nonspecific neuronal

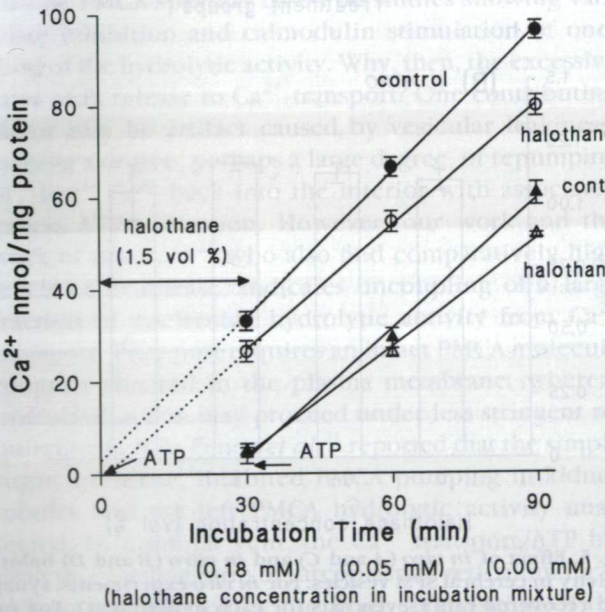

Fig. 7. Effect of discontinuing *in vitro* halothane exposure on PMCA pump activity. Ca^{2+} transport was measured in cerebral SPM from previously unexposed rats, as described in the text. The vertical axis indicates Ca^{2+} uptake (nmoles · mg protein^{-1}) and the horizontal axis denotes incubation time in minutes and halothane concentration (mM) in treated incubation mixtures at 30, 60, and 90 min. Treated vials (open circles or triangles) were exposed to 1.5% halothane, delivered as described in the text, for 30 min, followed by air/oxygen for the remainder of the incubation period. Control vials (solid circles or triangles) were exposed to air/oxygen only; ATP was added at t = 0 (circles) or at t = 30 min (triangles). Aliquots were obtained at 30, 60, and 90 min for assay of Ca^{2+} uptake and for measurement of halothane concentration. Indicated values represent the mean of triplicate measurements on SPM pooled from ten rats in two experiments. Error bars indicate 95% confidence limits from ANOVA. Halothane reduced Ca^{2+} uptake to 81% of control when ATP was added before halothane exposure and to 85% of control when ATP was added after halothane was discontinued ($P < 0.01$).

discharge, we would expect enzymes other than plasma membrane Ca^{2+}-ATPase to be affected. We conclude that observed differences in PMCA pumping activity among rats in the C, A, and R treatment groups were not confounded by alterations of the PMCA response associated with the method of killing, and that stable depression of PMCA pumping reflects a specific response to halothane.

Strong additional support for the biologic validity of stable depression of synaptic PMCA with halothane exposure comes from *in vitro* studies (fig. 7). Depression of PMCA pumping activity with a 30-min exposure to halothane persisted during another 60 min of measurement, despite discontinuation of halothane and subsequent elution of agent from the medium. It is noteworthy that anesthetic depression of Ca^{2+}-dependent ATP hydrolysis *is* reversible, both *in vivo* (fig. 3) and *in vitro* (Franks *et al.,* unpublished observations). These observations in an *in vitro* setting, in conjunction with our *in vivo* experiments, indicate that reversal of anesthetic depression of PMCA *pump* activity can occur only in an intact, living cell.

We have now shown that two enzyme systems in brain synaptic membranes, methyltransferase I and PMCA, are stably altered by prior anesthetic exposure of the donor animal. In contrast to PMCA, methyl transferase I activity is enhanced in animals killed while anesthetized.[1] Each of these systems is restored to normal only in intact rats, as evidenced by the state of enzyme activity in animals that have recovered from anesthesia. Any relationship between phospholipid methylation stimulation and PMCA inhibition is purely speculative at this juncture, but it is noteworthy that PMCA activity is very sensitive to the lipid environment in which it is situated.[3] One could even postulate that anesthetic-induced diversion of phosphatidylethanolamine to synthesis of phosphatidyl-N-monomethylethanolamine and, ultimately, phosphatidylcholine could occur only at the expense of diverting phosphatidylethanolamine from conversion to acid phospholipids, known to enhance PMCA activity.[2–5] Whether phospholipid methylation with associated PMCA inhibition relates to the anesthetic effect of inhalation agents remains an open question at this time, but one worth continued exploration.

Anesthetic inhibition of PMCA may also derive from a quite different series of molecular events. Inhalational anesthetics may affect one or more elements of select and specific intracellular signal transduction pathways, *e.g.,* protein phosphorylation/dephosphorylation, a system that probably modulates one or more Ca^{2+} regulators, including PMCA. Other states and factors that could be involved in anesthetic alteration of PMCA include, singly or in combination, enzyme oligomerization, Ca^{2+} modulation through calmodulin, and direct Ca^{2+} binding. Nearly all of these potential modulators are candidates for participation in "stable" PMCA inhibition after *in vivo* anesthetic exposure.

We recently obtained additional evidence for significant involvement of PMCA in the anesthetic response from an animal model of chronic PMCA depression, rats with streptozocin (STZ)-induced diabetes. We found post-STZ reduction of PMCA pumping activity in synaptic plasma membranes that was associated with 35% reduction in halothane MED and 12% reduction in xenon MED_{mov} (defined as loss of movement in response to pressure chamber motion.)[26] Subsequent experiments have shown equivalent reductions in isoflurane and enflurane MED. In addition, the degree of MED reduction in individual rats correlated well with both PMCA inhibition and percent of glycated hemoglobin.

However affected by anesthetics, directly or indirectly, PMCA is a major regulator of intracellular Ca^{2+} concentration and dynamics. Interference with this regulatory process in neurons may alter central neurotransmission,[27] probably a necessary condition for anesthesia. The studies described in this report support this thesis and offer new evidence that halothane causes PMCA inhibition that persists in its absence, that is reversed only *in vivo,* and that may affect PMCA function at molecular sites distinct from those involved in ATP hydrolysis.

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