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Halothane, Isoflurane, Xenon, and Nitrous Oxide Inhibit Calcium ATPase Pump Activity in Rat Brain Synaptic Plasma Membranes

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Background: **Perturbation of neuronal calcium homeostasis may alter neurotransmission in the brain, a phenomenon postulated to characterize the anesthetic state. Because of the central role of plasma membrane Ca^{2+}-ATPase (PMCA) in maintaining Ca^{2+} homeostasis, the authors examined the effect of several inhalational anesthetics on PMCA function in synaptic plasma membranes (SPM) prepared from rat brain.**

Methods: **Ca^{2+}-ATPase pumping activity was assessed by measurement of ATP-dependent uptake of Ca^{2+} by SPM vesicles. ATPase hydrolytic activity was assessed by spectrophotometric measurement of inorganic phosphate (Pi) released from ATP. For studies of anesthetic effects on PMCA activity, Ca^{2+} uptake or Pi release was measured in SPM exposed to halothane, isoflurane, xenon, and nitrous oxide at partial pressures ranging from 0 to 1.6 MAC equivalents. Halothane and isoflurane exposures were carried out under a gassing hood. For xenon and nitrous oxide exposures, samples were incubated in a pressure chamber at total pressures sufficient to provide anesthetizing partial pressures for each agent.**

Results: **Dose-related inhibition of Ca^{2+}-ATPase pumping activity was observed in SPM exposed to increasing concentrations of halothane and isoflurane, confirmed by ANOVA and multiple comparison testing ($P < 0.05$). Concentrations of halothane and isoflurane equivalent to one minimum effective dose (MED) depressed PMCA pumping approximately 30%. Xenon and nitrous oxide also inhibited Ca^{2+} uptake by SPM vesicles. At partial pressures of these two gases equivalent to 1.3 MAC, PMCA was inhibited approximately 20%. Hydrolysis of ATP by SPM fractions was also inhibited in a dose-related fashion. An additive effect occurred when 1 vol% of halothane was added to xenon or nitrous oxide at partial pressures equivalent to 0–1.6 MAC for the latter two agents.**

Conclusions: **Plasma membranes Ca^{2+}-ATPase is significantly inhibited, in a dose-related manner, by clinically relevant partial pressures of halothane, isoflurane, xenon, and nitrous oxide. Furthermore, these anesthetics inhibit PMCA activity in accordance with their known potencies, and an additive effect was observed. How inhalational anesthetics inhibit the PMCA pump is not known at this time. It is noteworthy that the only shared characteristic of this group of agents of widely different structure is anesthetic action. The relevance of this dual commonality, anesthetic action and PMCA inhibition, to actual production of the anesthetic state remains to be determined. (Key words: Anesthetics, gases: nitrous oxide; xenon. Anesthetics, volatile: halothane; isoflurane. Mechanism of anesthesia: plasma membrane Ca^{2+}-ATPase (PMCA); synaptic plasma membranes.)**

A low cytosolic concentration facilitates the action of free calcium as an intracellular regulator, and eukaryocytes, including neurons, maintain several classes of Ca^{2+} transporting systems that control intracellular calcium concentration ($[Ca^{2+}]$).[1–3] A P-type pump, plasma membrane Ca^{2+}-ATPase (PMCA), plays a major role in maintaining low cytosolic $[Ca^{2+}]$ by ejecting Ca^{2+} from the cell.[1–3] Because general anesthesia may result from interference with information transfer at the synaptic level of brain organization and because signaling between nerve cells ensues with Ca^{2+}-dependent transmitter secretion by the distal axon, inhalational anesthetics may act by perturbing Ca^{2+} flux and concentration within the nerve terminal.[4] These considerations have evoked interest in the PMCA response to anesthetics in neuronal and other cell membranes. Effects on Ca^{2+} pumping and ATP hydrolysis by brain synaptic plasma membrane Ca^{2+}-ATPase and on ATP hydrolysis by purified erythrocytic Ca^{2+}-ATPase have been noted.[5–8] Inhibition by halothane, isoflurane, and enflurane of Ca^{2+}-ATPase isolated from erythrocytes or in red cell ghosts has recently been described in a definitive re-

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port.[9] We describe herein studies of the response of PMCA in isolated brain synaptic plasma membranes (SPM) exposed to several anesthetics of widely different structure. We have focused primarily on ion transport rather than ATP hydrolysis as a measure of anesthetic response. We report that halothane, isoflurane, xenon, and nitrous oxide inhibit PMCA pumping in a dose-related fashion at clinically relevant partial pressures.

Materials and Methods

Preparation of Synaptic Plasma Membranes

All experimental protocols were approved by the Animal Care Committee of Vanderbilt University. Male Sprague-Dawley rats (243–338 g) were allowed food and water *ad libitum* until the morning of the experiment. Animals were killed by decapitation. Whole brains were dissected on ice, and brain areas (cerebrum, cerebellum, midbrain, and medulla) were weighed and pooled in ice-cold 0.32 M sucrose (*p*H 7.4). Brain fractions were pooled from 3 to 12 rats, as determined by the amount of synaptic plasma membrane (SPM) required for each experiment. Synaptosomes were prepared by gradient ultracentrifugation.[10] Synaptic plasma membranes 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 activity. Assays of Ca^{2+}-dependent ATP hydrolysis were done either immediately or within a few days. Protein content in SPM pellets was estimated by the Bradford method.

Measurement of Ca^{2+} Pumping Across Synaptic Plasma Membranes

Ca^{2+} uptake by everted rat SPM vesicles, *i.e.*, transport from the cytosolic to the plasmic surface, was performed as described by Moore *et al.* with several modifications.[11] The incubation mixture (total volume 4 ml) consisted 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). The reaction was started by adding 70-μg aliquots of SPM protein to each tube. Temperature was maintained at 37°C. Aliquots of 0.5 ml were removed after 5, 10, 20, 30, and 60 min. For assays of PMCA pumping under hyperbaric conditions, incubation tubes were placed in a Parr Cell Disruption Bomb (PCDB; Parr Instrument Corp., Moline, IL) as described below and incubated for 30 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 0.25M KCl and 10 ml of deionized water. After vesicle collection, the filters were washed with 2 ml of 0.25M 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.

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.[12,13] Synaptic plasma membrane aliquots (2 μg/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. For assays of ATP hydrolysis under hyperbaric conditions, incubation tubes were placed in a PCDB. 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 30 min.

Evaluation of Synaptic Plasma Membrane Preparation

Electron Microscopy. Samples of the crude synaptosomal fraction (P2), synaptosomes, and synaptic plasma membranes were fixed in 2% glutaraldehyde and evaluated by electron microscopy.[10]

Enzymatic Markers. Purification of SPM was assessed by measuring the specific activity of plasma membrane enzyme markers in the initial brain homogenates and in subsequent fractions obtained during separation. Enzyme markers included acetylcholinesterase, alkaline phosphatase (Sigma Diagnostic Kit 245,

St. Louis, MO), gamma-glutamyl-transpeptidase (Sigma Diagnostic Kit 419), and 5′-nucleotidase.[14–17]

Extraneous Structures. The degree of contaminating mitochondria or endoplasmic reticulum in SPM preparations was assessed by adding 5 mM sodium azide or up to 15 μg/ml of oligomycin to the incubation medium to inhibit mitochondrial or, alternatively, by preincubating SPM with 50–300 nM thapsigargin to inhibit microsomal uptake of Ca^{2+}.[18]

Orientation of Vesicles. Inside/outside orientation of SPM vesicles was examined by a method[19] using specific cleavage by trypsin of Na^+-K^+-ATPase from the cytoplasmic surface of everted vesicles. Remaining ouabain-inhibitable ATPase activity was measured in membranes from trypsin-treated vesicles and compared with activity in membranes from untreated vesicles. Inside surfaces were exposed by rupture of vesicles with sodium dodecylsulfate before enzymatic assay.

Specificity of Ca^{2+} Transport. We used several criteria to demonstrate that active Ca^{2+} transport in our SPM preparation shared properties consistent with those known for PMCA from other sources. The PMCA pump is substrate (ATP) specific, requires Mg^{2+}, and is inhibited by orthovanadate.[20] Nucleotide specificity was examined by comparing 5 mM ATP, ADP, CTP, UDP, or no nucleotide substrate in parallel assays of Ca^{2+} uptake. In another series of experiments, Ca^{2+} uptake was measured in the presence of 0.005–1 mM sodium orthovanadate. Requirement for Mg^{2+} was also examined. To evaluate the relationship of Ca^{2+} uptake to its concentration, experiments were carried out with medium Ca^{2+} concentration varying from 0.1 to 1000 μM.

Anesthetic Treatment

Synaptic plasma membrane incubation mixtures were exposed to various partial pressures of halothane, isoflurane, xenon, or nitrous oxide as Ca^{2+} uptake (transport) or ATP hydrolytic reactions proceeded. For studies of the effects of potent anesthetics, incubation vials were placed in a Dubnoff shaker under a gassing hood and shaken gently at 37°C, as described previously.[21] Halothane (Halocarbon Labs, River Edge, NJ) or isoflurane (Abbott Laboratories, North Chicago, IL), in a warmed, humidified air/oxygen mixture ($F_{I_{O_2}} = 0.3$), was delivered under the hood in the desired concentrations from a dedicated, calibrated vaporizer. Delivered concentrations of potent anesthetics were always confirmed by gas chromatography. For experiments with xenon or nitrous oxide, SPM incubation mixtures were exposed to anesthetic gases in a PCDB placed in a water bath at 37°C and used as a pressure chamber. The PCDB was modified by placement of a low-pressure gauge, 0–50 psi, on the lid. The PCDB was flushed for 2 min at 6 l/min either with helium alone (A-L Compressed Gases Inc., Nashville, TN) or, for interaction studies, with helium mixed with 1 vol% halothane before and after placement of the incubation tubes in the PCDB. These tubes remained in a 100-ml beaker filled with water at 37°C throughout the incubation period; constancy of water temperature was confirmed by measurement after incubation was completed. Xenon (Research Grade from Alphagas; Morrisville, PA) or nitrous oxide (A-L Compressed Gases Inc.) was then added to the PCDB to achieve a partial pressure based on the MAC of each anesthetic gas. Finally, supplemental helium was added as needed to insure constant total pressure despite varying xenon or nitrous oxide partial pressures. After 30 min of exposure, pressure in the PCDB was released slowly to avoid disruption of SPM vesicles, and the degree of Ca^{2+} uptake or ATP hydrolysis was assayed as described above. Minimum alveolar concentration equivalents of 0, 1, and 1.6 were used for both xenon (0, 0.95, and 1.55 atm) and nitrous oxide (0, 1.55, and 2.46 atm). These delivered partial pressures were based on reported MAC values of 0.95 atmospheres or 14 psi for xenon in the mouse[22] and 1.54 atm or 23 psi for nitrous oxide in the rat.[23] The total pressure (above ambient) to which SPM incubations were exposed was 1.54 atm for xenon and 2.46 atm for nitrous oxide.

Statistical Analysis

Data were examined by multifactorial ANOVA, multiple comparison (Student–Newman–Keuls procedure), linear regression, and, when appropriate, *t* tests. Statistical significance was inferred if $P < 0.05$.

Results

Synaptic Plasma Membrane Characterization

Evidence for Isolation of a Synaptic Plasma Membrane-Rich Fraction. Synaptic plasma membrane enrichment of successive subcellular fractions isolated during the separation process was indicated by increases in specific activity in the SPM fraction of four plasma membrane-associated enzymes (table 1). Electron microscopy confirmed these biochemical findings, with results comparable with Cotman's.[10]

Table 1. Activity of Plasma Membrane Enzymatic Markers at Various Stages of Isolation of Synaptic Plasma Membranes

Preparation	AChE (μmol/mg protein per min)	AP (U/mg protein per min)	5′-N (nmol/mg protein per min)	GGTP (U/mg protein per min)
Homogenate	18.48 ± 0.84	0.59 ± 0.04	0.0271 ± 0.001	0.034 ± 0.001
Synaptosomes	45.39 ± 2.38	0.39 ± 0.05	0.019 ± 0.0003	0.033 ± 0.005
Synaptic plasma membranes	112.94 ± 1.7	2.98 ± 0.24	0.035 ± 0.0004	0.25 ± 0.02

Values are mean ± SEM (n = 3).

AChE = acetylcholinesterase; AP = alkaline phosphatase; 5′ = N = 5′ = nucleotidase; GGTP = gamma-glutamyltranspeptidase.

Vesicle Orientation. Ca^{2+} uptake can be measured only in everted SPM vesicles. In experiments based on the method of Marin *et al.*,[19] we found that the total activity of available Na^+-K^+-ATPase in disrupted vesicles was 25.74 ± 0.34 μmoles Pi · mg protein^{-1} · 20 min^{-1}, n = 3. When SPMs were treated with trypsin and then disrupted, Na^+-K^+-ATPase activity was decreased by approximately 44% to 11.36 ± 0.23 μmoles Pi · mg protein^{-1} · 20 min^{-1}, indicating that about one-half of the SPM vesicles were everted. Freezing and thawing of vesicles may reduce the number of intact inside-out vesicles, because PMCA pumping activity in preparations thawed after 1 day of storage was decreased by about 30%. Therefore, only freshly prepared SPM were used for PMCA pumping measurements. No changes in ATP hydrolytic activity were observed after freezing and thawing.

Specificity of Ca^{2+}-ATPase in the Synaptic Plasma Membrane Fraction

Figure 1a shows Ca^{2+} uptake by SPM vesicles plotted against incubation time in the presence of several Ca^{2+}-ATPase substrates. It is evident from the regression slopes that pumping is effective only with ATP as the nucleotide substrate. In other experiments (not shown) uptake did not occur in the absence of ATP or Mg^{2+}. In addition to a requirement for ATP and Mg^{2+}, inhibition by low concentrations of orthovanadate is characteristic and specific for P-type pumps, such as PMCA. Figure 1b shows Ca^{2+} uptake with varying amounts of orthovanadate added to the medium. The inhibitory constant, Ki, (concentration of orthovanadate producing 50% inhibition of Ca^{2+} uptake) was calculated for each of several incubation times, with $Ki_{5min} > 50$, $Ki_{10min} > 50$, $Ki_{20min} = 50$, $Ki_{30min} = 41$, and $Ki_{60min} = 16$ μM. Therefore, only data derived from 20-, 30-, and 60-min aliquots of incubation medium were used for determination of Ca^{2+} uptake, thereby maximizing the contribution of P-type pump activity (*e.g.*, PMCA) to the assay. Preincubation of SPM with thapsigargin (50–300 nM), an inhibitor of smooth endoplasmic reticulum Ca^{2+}-ATPase (SERCA), did not alter SPM Ca^{2+} uptake, indicating minimal contamination by this intracellular calcium pump.[18] Some contamination of the SPM preparation with mitochondria was indicated by experiments showing a 15–30% reduction of uptake with addition of azide (5 mM) or oligomycin (5 μg/ml), but one or the other of these inhibitors of mitochondrial transport of Ca^{2+} was always added to the incubation medium in studies of the anesthetic response. Synaptic plasma membrane uptake of Ca^{2+} varies with the concentration of this ion in the medium. Studies using concentrations ranging from 0.1 to 1000 μM indicated that anesthetic effects could best be demonstrated between 10 and 50 μM Ca^{2+} in the medium.

Anesthetic Effects on PMCA Pump Activity

Halothane and Isoflurane. Data summarized in figure 2 illustrate the effects of increasing concentrations of halothane (fig. 2a) or isoflurane (fig. 2b) on PMCA pumping activity in SPM from the cerebrum. Each treatment group was comprised of three independent experiments in which membranes pooled from three to six rats were exposed, in replicates of six, to six different concentrations of halothane or isoflurane. Mean values of Ca^{2+} uptake from each experiment are shown for each anesthetic concentration, with error bars indicating 95% confidence limits from multifactor ANOVA. Multiple comparison testing confirmed dose effect. Significant differences ($P < 0.05$) in Ca^{2+} uptake were noted among the anesthetic concentrations indicated in figure 2. Orthovanadate markedly suppressed Ca^{2+} uptake by SPM vesicles, with no further inhibition by halothane at concentrations ranging from 0.5 to 1.5 vol%. Of particular interest, illustrated in figure 2, are the effects on PMCA of exposure to concentrations approximately equivalent to 1 minimum effective dose (MED) of either halothane or isoflurane. One MED, de-

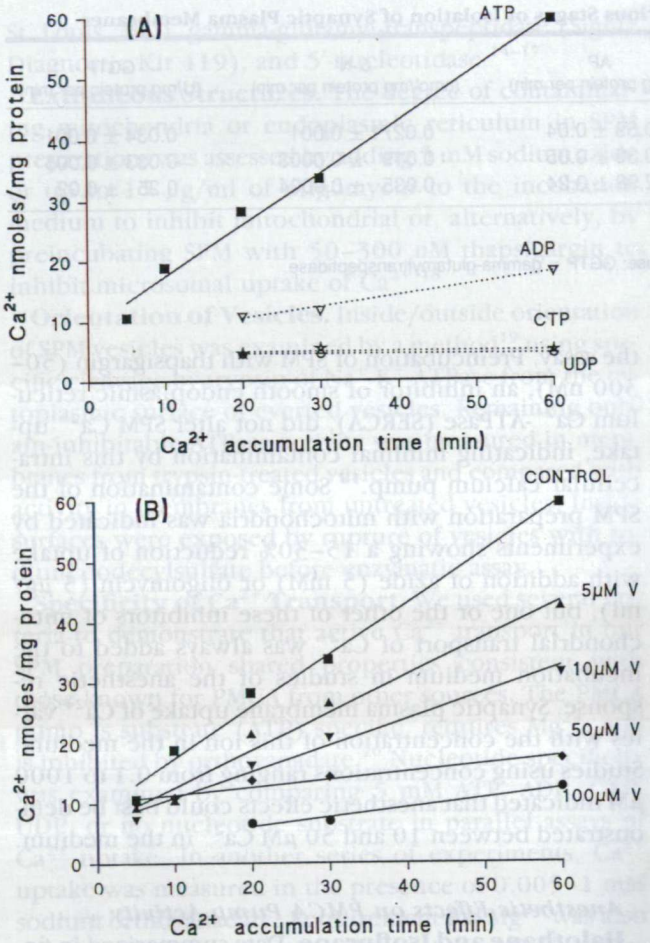

Fig. 1. Specificity of the Ca^{2+}-ATPase pump in synaptic plasma membranes (SPM) prepared from rat cerebral cortex. Ca^{2+} uptake was assayed as described in the text. The vertical axes indicate Ca^{2+} uptake (nmoles · mg protein^{-1}) and the horizontal axes denote incubation time in minutes. (*A*) Ca^{2+} uptake proceeded with ATP (5 mM) as the nucleotide substrate (solid line), but decreased substantially when ATP was replaced with 5mM ADP, CTP, or UDP (dotted lines). (*B*) Effect of varying concentrations of orthovanadate (V) on Ca^{2+} uptake by SPM vesicles. Values represent the means of four to six measurements from two independent experiments.

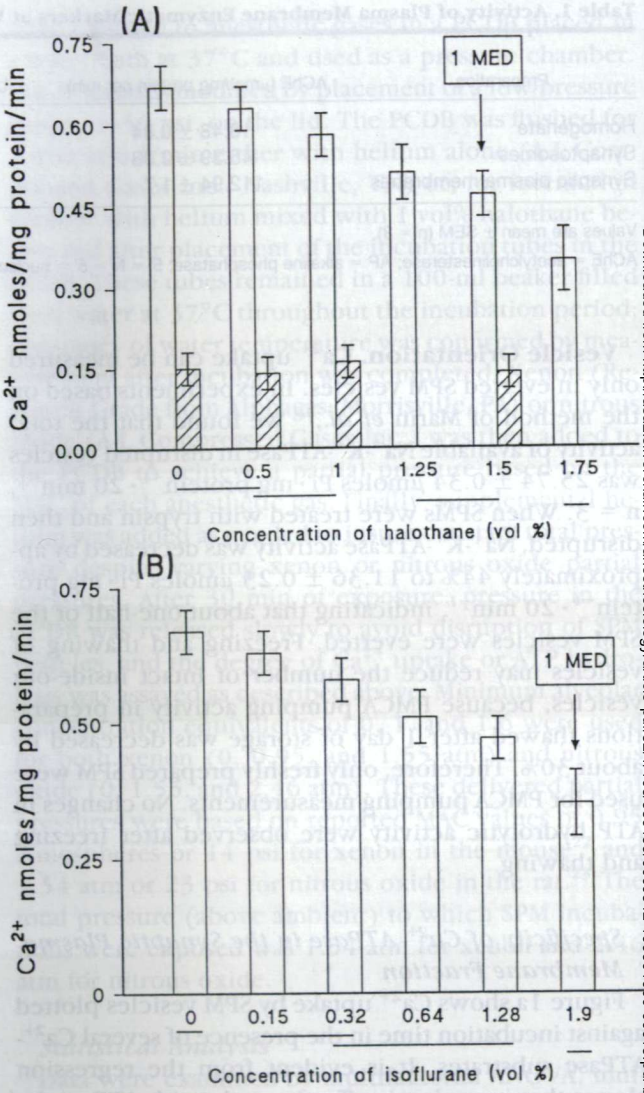

Fig. 2. Inhibition of Ca^{2+}-ATPase (PMCA) pump activity in SPM by halothane (*A*) and isoflurane (*B*). Vertical axes indicate Ca^{2+} uptake in nmoles · mg protein^{-1} · min^{-1}, derived as described in the text. Horizontal axes indicate anesthetic concentration (vol%). Inhibition of PMCA by 0.1 mM orthovanadate, unaffected by varying concentrations of halothane, is also shown (hatched columns, *A*). Values in the halothane and isoflurane treatment groups (open columns) represent means of six replicate measurements from three separate experiments in which membranes pooled from three to six rats were used. For the orthovanadate study, n = 3. Error bars indicate 95% confidence limits for means, derived from ANOVA. Differences among various anesthetic exposure levels are defined by line segments below each horizontal axis. Statistical significance ($P < 0.05$) is indicated when segments are discontinuous and do not overlap. This figure also demonstrates comparable inhibition of PMCA pumping, 29.2% and 31.7%, respectively, by equipotent concentrations, equivalent to 1 MED, of halothane (1.5 vol%) and isoflurane (1.9 vol%). One MED, defined in intact rats, is the lowest deliverable anesthetic concentration that suppresses movement in response to tail clamping.

termined in intact rats,[21] is the lowest deliverable anesthetic concentration that suppresses movement in response to tail clamping. Concentrations of halothane (1.5%) and isoflurane (1.9%), approximating 1 MED in intact animals, produced PMCA inhibition *in vitro* of similar degree, 29.2% and 31.7%, respectively.

Xenon and Nitrous Oxide. Figure 3a shows the effect of xenon on PMCA pumping in SPM isolated from four different rat brain areas. Cerebra, midbrains, cerebella, and medullae were obtained from 12 rats and

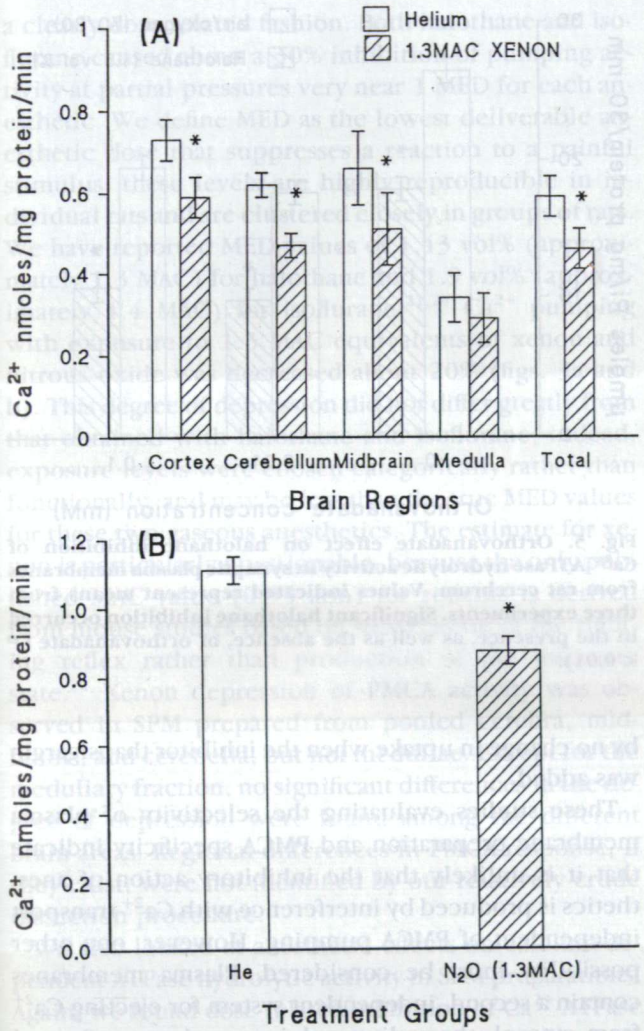

Fig. 3. Inhibition by xenon and nitrous oxide (1.3 MAC equivalents) of Ca^{2+}-ATPase pump activity in synaptic plasma membranes from rat brain. Minimum alveolar concentration was taken as 0.95 atmospheres for xenon[22] and 1.54 atmospheres for nitrous oxide.[23] Total pressure in the incubation chamber for both treatment groups was 1.5 atmospheres for xenon and 2.5 atmospheres for nitrous oxide. Open columns represent Ca^{2+}-ATPase pump activity in membranes exposed to helium alone, and hatched columns indicate activity in membranes exposed to a mixture of helium and xenon (*A*) or nitrous oxide (*B*). (*A*) The effect of xenon on membranes from four different brain regions: cerebrum, cerebellum, midbrain, and medulla. Mean values for all brain regions (total) are shown on the right of the figure. Values shown for each brain region are means from five separate experiments, each carried out with membranes pooled from 12 rats. Error bars indicate 95% confidence limits. An asterisk indicates significant differences between treatment groups for each brain region ($P < 0.05$), as determined by ANOVA. (*B*) The effect of nitrous oxide on membranes from rat cerebrum. Values represent the mean of six separate experiments. Nitrous oxide depressed Ca^{2+} uptake significantly ($P < 0.01$).

pooled for SPM preparation in five separate experiments. Incubation mixtures were exposed for 30 min to either helium or to helium plus xenon at a partial pressure equivalent to 1.3 MAC. Mean values for Ca^{2+} uptake for SPM from each brain fraction are depicted, with error bars indicating 95% confidence limits. Xenon depressed PMCA pumping activity significantly ($P < 0.05$) in all brain fractions except the medulla, and to approximately the same degree (20–25%). Nitrous oxide also affected Ca^{2+} transport (fig. 3b). In experiments with SPM prepared from cerebra pooled from three rats, Ca^{2+} uptake was depressed 21% ($P < 0.05$) by a partial pressure of nitrous oxide equivalent to 1.3 MAC.

Anesthetic Effects on Ca^{2+}-Dependent ATPase Hydrolytic Activity

Another measure of Ca^{2+}-ATPase activity, one that also has been used to assess PMCA function, is release of inorganic phosphate (P_i) by hydrolysis of the nucleotide substrate. All of the anesthetic agents we have examined inhibit ATP hydrolysis in SPM. Figure 4 illus-

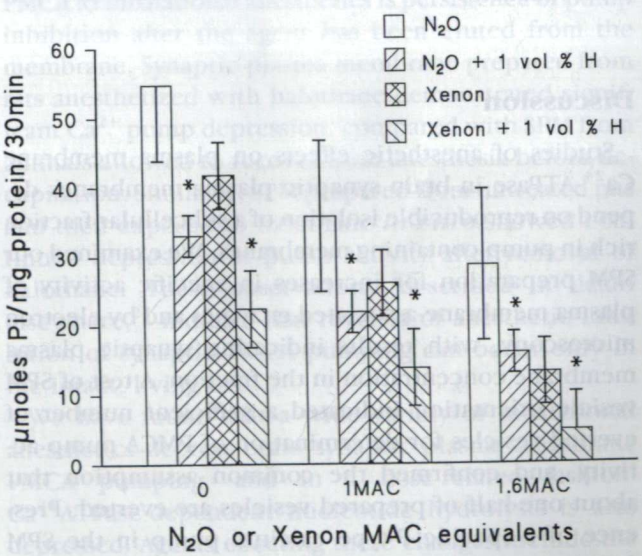

Fig. 4. Effect of different levels of nitrous oxide and xenon (0, 1, and 1.6 MAC), with and without 1 vol% of halothane (H), on Ca^{2+}-ATPase hydrolytic activity in synaptic plasma membranes from rat cerebrum. Indicated values represent means for four separate experiments. Error bars indicate 95% confidence limits. An asterisk indicates significant differences caused by halothane treatment at each nitrous oxide or xenon treatment level ($P < 0.05$). A dose-related response at different treatment levels of nitrous oxide or xenon, with and without halothane, was also demonstrated by ANOVA ($P < 0.05$).

trates additive and dose-related anesthetic inhibition of hydrolysis in membranes isolated from pooled, cerebral homogenates. One treatment group was comprised of four experiments in which membranes pooled from three to six rats were exposed for 30 min, in replicates of four, to 0, 1, or 1.6 MAC equivalents of nitrous oxide with and without 1 vol% of halothane. In the second treatment group, xenon was substituted for nitrous oxide. Mean values of ATP hydrolysis from all experiments are plotted against gaseous anesthetic (nitrous oxide or xenon) concentrations, with error bars indicating 95% confidence limits from ANOVA. Multiple comparison testing confirmed a dose-related response. Significant differences ($P < 0.05$) in ATP hydrolysis were noted, with a single exception, among different MAC equivalent values for nitrous oxide and for xenon. When 1 vol% of halothane was added to the gaseous anesthetics, further inhibition of ATP hydrolysis was noted in both treatment groups at all gaseous anesthetic MAC values, and significant differences were maintained among these MAC equivalents. Figure 5 shows that, although halothane significantly depressed Ca^{2+}-dependent ATP hydrolysis, halothane inhibition persisted in the presence of the specific PMCA inhibitor, orthovanadate.

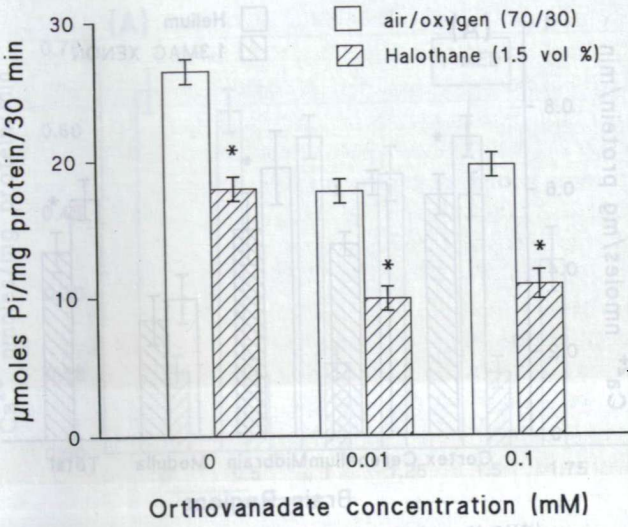

Fig. 5. Orthovanadate effect on halothane inhibition of Ca^{2+}-ATPase hydrolytic activity in synaptic plasma membranes from rat cerebrum. Values indicated represent means from three experiments. Significant halothane inhibition occurred in the presence, as well as the absence, of orthovanadate ($P < 0.01$).

Discussion

Studies of anesthetic effects on plasma membrane Ca^{2+}-ATPase in brain synaptic plasma membranes depend on reproducible isolation of a subcellular fraction rich in pump-containing membranes. We examined our SPM preparation for increases in specific activity of plasma membrane-associated enzymes and by electron microscopy, with results indicating synaptic plasma membrane concentration in the fraction. A test of SPM vesicle orientation indicated a sufficient number of everted vesicles for determination of PMCA pump activity, and confirmed the common assumption that about one-half of prepared vesicles are everted. Presence of a specific P-type calcium pump in the SPM fraction was further supported by a variety of identifying characteristics: requirement for ATP as substrate, magnesium dependence, and orthovanadate sensitivity. Synaptic plasma membrane fraction contaminants did not appear to distort our measures of Ca^{2+} uptake. Mitochondrial uptake was minimized by including azide or oligomycin in the incubation mixture, and SERCA contribution to uptake was essentially zero as indicated by no change in uptake when the inhibitor thapsigargin was added.

These studies evaluating the selectivity of plasma membrane preparation and PMCA specificity indicate that it is unlikely that the inhibitory action of anesthetics is produced by interference with Ca^{2+} transport independent of PMCA pumping. However, one other possibility must be considered. Plasma membranes contain a second, independent system for ejecting Ca^{2+} from cytosol, the sodium-calcium exchanger. Several considerations make exchanger participation unlikely: low intravesicular Na^+ concentration, minimal uptake of Ca^{2+} by SPM vesicles in the absence of ATP, and inhibition of Ca^{2+} uptake by orthovanadate. Continued Ca^{2+} accumulation for 60 min also argues against an exchanger effect. Uptake from this high-capacity system occurs rapidly, usually within 10 min. Finally, we recently showed that Ca^{2+} uptake into SPM vesicles is unaffected by halothane under conditions designed to maximize sodium-calcium exchange.[24]

One feature of PMCA pumping in membranes exposed or not exposed to anesthetics is reproducibility. Studies reported herein have been carried out over several years and with different lots of experimental animals, yet results have proved consistent, as indicated in figure 2. Inhibition of PMCA occurred within a clinically useful range of anesthetic concentrations, and in

a clearly dose-related fashion. Both halothane and isoflurane caused about a 30% inhibition of pumping activity at partial pressures very near 1 MED for each anesthetic. We define MED as the lowest deliverable anesthetic dose that suppresses a reaction to a painful stimulus; these levels are highly reproducible in individual rats and are clustered closely in groups of rats. We have reported MED values of 1.43 vol% (approximately 1.3 MAC) for halothane and 1.9 vol% (approximately 1.4 MAC) for isoflurane.[21,24] Ca^{2+} pumping with exposure to 1.3 MAC equivalents of xenon and nitrous oxide was decreased about 20% (figs. 3a and b). This degree of depression did not differ greatly from that obtained with halothane and isoflurane; indeed, exposure levels were chosen categorically rather than functionally, and may be less than the true MED values for these two gaseous anesthetics. The estimate for xenon is particularly questionable, because the only published MAC value (0.95 atm) for rodents is derived from mouse studies based on suppression of the righting reflex rather than production of the insensate state.[22] Xenon depression of PMCA activity was observed in SPM prepared from pooled cerebra, midbrains, and cerebella, but not medullae. Except for the medullary fraction, no significant differences in the degree of depression were found among the different brain areas. Regional differences in PMCA response, if they exist, were not identified by our relatively crude dissection procedure.

We also looked at anesthetic effects on calcium-dependent ATPase hydrolytic activity in SPM preparations. Again, we found dose-related inhibition of Ca^{2+}-ATPase activity and an additive effect with mixtures of 1% halothane and either xenon or nitrous oxide (fig. 4). It is noteworthy that most reports of anesthetic or related drug effects on PMCA rely on ATP hydrolysis as a measure of enzyme activity, *e.g.*, in erythrocytic[7,9] and brain synaptic membranes.[25,26] Our findings with the hydrolytic method are certainly consistent with these reports. However, experiments illustrated in figure 5 indicate that inhibition of release of inorganic phosphorous is a consequence of anesthetic effects not only on the P-type pump, PMCA, but also on calcium-dependent enzyme(s) that are not inhibited by orthovanadate. Thus, ATP hydrolysis, at least in neural membranes, may be only partially associated with Ca^{2+} pumping by PMCA. In recent years, there has been considerable interest in the finding that there are calcium-dependent ATPases on the outer surface of plasma membranes.[27,28] These ecto-ATPases appear to be of several varieties. Their function is unknown, but there is no evidence that they have an ion-pumping role. Ecto-ATPases can use substrates other than ATP, and they are not inhibited by 0.01 mM orthovanadate. Anesthetic depression of calcium-dependent ATPase(s) other than PMCA is of interest. As noted above and in an additional report,[29] we found that anesthetics do not depress Na^+-K^+-ATPase, SERCA, or sodium-calcium exchanger activity in SPM. On the other hand, if anesthetics inhibit ecto-ATPases as well as PMCA, it is important to note this in the literature in view of the wide reliance on an assay that may not be specific for PMCA. There is a possibility that orthovanadate primarily inhibits PMCA pumping and that substrate hydrolysis by PMCA remains partially intact in its presence. This phenomenon must be demonstrated before substrate hydrolysis can truly be relied on as a measure of PMCA activity. Such evidence would be welcome in view of the labor intensity of pumping assays, with their requirement for fresh SPM. Although assays of nucleotide hydrolysis probably provide useful approximations, we have placed primary reliance on measurement of Ca^{2+} transport across SPM.

One interesting feature of the response of synaptic PMCA to inhalational anesthetics is persistence of pump inhibition after the agent has been eluted from the membrane. Synaptic plasma membrane prepared from rats anesthetized with halothane demonstrated significant Ca^{2+} pump depression, compared with SPM from animals allowed to recover from anesthesia before decapitation. Similarly, SPM prepared from untreated rats and then exposed to halothane *in vitro* showed continued depression of pump activity after removal of halothane. These observations, described in detail elsewhere,[24] indicate that reversal of anesthetic inhibition of synaptic PMCA pumping can occur only in the intact, living cell.

We have found that a wide variety of inhalational anesthetics depress brain synaptic plasma membrane PMCA pumping, and in a dose-related fashion. Ca^{2+}ATPase-dependent nucleotide hydrolysis is also depressed. Agents effecting these changes include the elemental noble gas, xenon; a simple inorganic compound, nitrous oxide; and two widely used fluorinated organic compounds, halothane and isoflurane. How these anesthetics inhibit the pump is unknown at this time, but it is noteworthy that their only shared characteristic is anesthetic action. The relevance of this commonality to actual production of the anesthetic state remains to be determined. As always when looking

at physiologic processes altered by anesthetics, the question of nonspecific, even toxic, side effects unrelated to the anesthesia arises. Two considerations make this implausible in this group of experiments. First, PMCA inhibition occurs at agent partial pressures within pharmacologic ranges, it begins at low pressures, and it increases as anesthetizing pressures are attained. Many side effects occur only at high anesthetic concentrations. Second, it appears highly unlikely that PMCA inhibition is a nonspecific toxic effect of fluorinated anesthetics when it is so clearly reproduced with exposure of membranes to xenon, an agent with few pharmacologic effects other than the production of anesthesia. If an unrelated side effect can be excluded, another possibility is that PMCA serves as a reporter molecule, *i.e.,* PMCA inhibition is an epiphenomenon, indicating, but not participating in, the anesthetic state. Alternatively, PMCA inhibition may follow, as a secondary corrective response, perturbation of cytosolic Ca^{2+} dynamics that results from effects on otherwise unrelated processes more fundamental to the production of anesthesia. However, the observation of Kosk-Kosicka *et al.*[9] on the direct, inhibiting effects of potent, fluorinated anesthetics on purified erythrocytic Ca^{2+}-ATPase, in concert with our findings in neural membranes, lends credence to the possibility that PMCA itself, as a fine tuner of $[Ca^{2+}]$, may play a fundamental role in the processes that lead to the anesthetic state.

The link between rapid shifts in intracellular ($[Ca^{2+}]$) and intercellular signaling has been recognized for many years.[4] Homeostatic mechanisms controlling intracellular $[Ca^{2+}]$ and dynamics are complex, and regulation is linked with cytosolic ion compartmentalization to a degree that is only now becoming evident.[30] Despite its relatively low transport capacity compared with other cellular Ca^{2+} regulators, PMCA may be strategically located within the cell so as to alter $[Ca^{2+}]$ in critical submembranal areas, perhaps especially in synapses. PMCA effects on Ca^{2+} decay curves and on $[Ca^{2+}]$ oscillatory dynamics may predominate, with profound effects emerging consequent to anesthetic inhibition.[31] Benham *et al.* have provided particularly important information on the effect of PMCA on Ca^{2+} decay curves with cultured rat dorsal root ganglion sensory neurons.[32] By a combination of microfluorimetric and patch-clamp techniques, they showed that the PMCA is significantly more important than either sodium-calcium exchange or caffeine-sensitive intracellular storage for the removal of cytosolic Ca^{2+} loads generated by actional potentials. It is apparent that the complexity of intracellular Ca^{2+} dynamics and regulation confounds the construction of a simple model explaining these global effects of PMCA inhibition. Further complexity may derive from the presence in the brain, unlike the erythrocyte, of several nonhomogeneously distributed PMCA isoforms and, perhaps, splice variants that vary in their regulatory arm structure and, possibly, in their anesthetic response. A relatively crude look at differential PMCA responses in several brain areas (fig. 3) revealed no apparent differences. A precise analysis of the question presents a formidable task, but may offer important new information. It seems likely that other Ca^{2+} regulators, such as the intracellular calcium release channels (ICRC), may also be affected, and these too offer the potential of varying anesthetic susceptibility among nonhomogeneously distributed isoforms.[33] Perhaps most intriguing is the possibility that anesthetics alter neuronal Ca^{2+} homeostasis by direct effects on specific protein kinases, phosphatases, or both, which, in turn, serve as common modulators of several controllers of cytosolic $[Ca^{2+}]$, including PMCA and ICRC.[34] Defining anesthetic effects on one or more of these Ca^{2+} regulatory systems has great potential for furthering our understanding of how anesthetics alter central neurotransmission.

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References

1. Penniston JT, Enyedi A: Plasma membrane Ca^{2+} pump: Recent developments. Cell Physiol Biochem 4:148–159, 1994

2. Carafoli E, Guerini D: Molecular and cellular biology of plasma membrane calcium ATPase. Trends Cardiovasc Med 5:177–184, 1993

3. Wang KKW, Villalobo A, Roufogalis BD: The plasma membrane calcium pump: A multiregulated transporter. Trends Cell Biol 2:46–52, 1992

4. Kandel ER: Transmitter release, Principles of Neural Sciences. Edited by Kandel ER, Schwartz JH, Jessell TM. Norwalk, Appleton & Lange, 1991, pp 194–212

5. Horn JL, Franks JJ, Sastry BVR, Singh G: Xenon reduces Ca^{2+} adenosine triphosphatase pump activity in synaptic plasma membranes from the rat brain (abstract). ANESTHESIOLOGY 77(suppl):A417, 1992

6. Horn JL, Franks JJ, Sastry BVR, Singh G: Halothane inhibition of Ca^{2+}-adenosine triphosphatase pump activity in the brain is linked to phospholipid N-methylation (abstract). ANESTHESIOLOGY 77(suppl): A441, 1992

7. Roszczynska G, Kosk-Kosicka D: Specific inhibition of Ca^{2+}-ATPase activity by volatile anesthetics (abstract). ANESTHESIOLOGY 77(suppl):A775, 1992

8. Singh G, Franks JJ, Catlin RW, Janson V, Sastry BVR: Depression of Ca^{2+}-ATPase pump activity of the rat cerebral synaptosomal plasma membrane by isoflurane (abstract). ANESTHESIOLOGY 79:A410, 1993

9. Kosk-Kosicka D, Roszczynska G: Inhibition of plasma membrane Ca^{2+}-ATPase activity by volatile anesthetics. ANESTHESIOLOGY 79:774–780, 1993

10. Cotman CW: Isolation of synaptosomal and synaptic plasma membrane fractions, Methods in Enzymology, vol. 31. Edited by Fleischer S, Packer L. New York, Academic Press, 1974, pp 445–452

11. Moore L, Chen T, Knapp HR, Landon EJ: Energy-dependent sequestration activity in rat liver microsomes. J Biol Chem 250:4562–4568, 1975

12. Lecocq J, Inesi G: Determination of inorganic phosphate in the presence of adenosine triphosphate by the molybdo-vanadate method. Anal Biochem 15:160–163, 1966

13. Michaelis EK, Michaelis ML, Chang HH, Kitos TE: High affinity Ca^{2+}-stimulated Mg^{2+} dependent ATPase in rat brain synaptosomes, synaptic membranes, and microsomes. J Biol Chem 258:6101–6108, 1983

14. Ellman GL, Courtney KD, Andres V Jr., Featherstone RM: A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95, 1961

15. Bowers Jr GN, McComb RB: A continuous spectrophotometric method of measuring the activity of serum alkaline phosphatase. Clin Chem 12:70–89, 1966

16. Rosalki SB, Rau D, Lehman D, Prentice M: Determination of gamma-glutamyltranspeptidase activity and its clinical applications. Ann Clin Biochem 7:143–148, 1970

17. Avruch J, Wallach DFH: Preparation and properties of plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. Biochim Biophys Acta 233:334–347, 1971

18. Kijima Y, Ogunbunmi E, Fleischer S: Drug action of thapsigargin on the Ca^{2+} pump protein of sarcoplasmic reticulum. J Biol Chem 266:22912–22918, 1991

19. Marin R, Proverbio T, Proverbio F: Inside-out basolateral plasma membrane vesicles from rat kidney proximal tubular cells. Biochim Biophys Acta 858:195–201, 1986

20. Enyedi A, Minami J, Caride AJ, Penniston JT: Characteristics of the Ca^{2+} pump and Ca^{2+}-ATPase in the plasma membrane of rat myometrium. Biochem J 252:215–220, 1988

21. Franks JJ, Sastry BVR, Surber MJ, England RE: Halothane and isoflurane alter phospholipid transmethylation in rat brain synaptosomes. ANESTHESIOLOGY 73:984–989, 1990

22. Miller KW, Paton WDM, Smith EB, Smith RA: Physico-chemical approaches to the mode of action of general anesthetics. ANESTHESIOLOGY 36:339–351, 1972

23. Russell GB, Graybeal JM: Direct measurement of nitrous oxide MAC and neurologic monitoring in rats during anesthesia under hyperbaric conditions. Anesth Analg 75:995–999, 1992

24. Franks JJ, Horn JL, Janicki PK, Singh G: Stable inhibition of brain synaptic plasma membrane calcium ATPase in rats anesthetized with halothane. ANESTHESIOLOGY 82:118–128, 1995

25. Iqbal Z, Sze PY: Ethanol modulates calmodulin-dependent Ca^{2+}-activated ATPase in synaptic plasma membranes. Neurochem Res 19:475–482, 1994

26. Roszczynska G, Zylinska L, Kosk-Kosicka D: Similar inhibitory effect of halothane on the function of the Ca^{2+}-ATPase from cerebellum and erythrocytes (abstract). Biophys J 66:A119, 1994

27. Nagy AK, Shuster TA, Delgado-Escueta AVD: Ecto-ATPase of mammalian synaptosomes: Identification and enzymatic characterization. J Neurochem 47:976–986, 1986

28. Hohmann J, Kowalewski H, Vogel M, Zimmermann H: Isolation of Ca^{2+} or Mg^{2+}-activated ATPase (ecto-ATPase) from bovine brain synaptic membranes. Biochem Biophys Acta 1152:146–154, 1993

29. Singh G, Horn JL, Janson V, Franks JJ, Janicki PK: Halothane depression of Ca^{2+}-ATPase pump, but not Na^{+}-Ca^{2+}-exchanger or Na^{+}-K^{+}-ATPase in rat cerebral synaptosomal plasma membranes (abstract). ANESTHESIOLOGY 81:31A, 1994

30. Muallem S, Loessberg P, Sachs G, Wheeler LA: Agonist-sensitive and -insensitive intracellular Ca^{2+} pools. Separate Ca^{2+}-releasing mechanisms revealed by manoalide and benzohydroquinone. Biochem J 279:367–375, 1991

31. Foder B, Scharff O: Solitary calcium spike dependent on calmodulin and plasma membrane Ca^{2+} pump. Cell Calcium 13:581–591, 1992

32. Benham CD, Evans ML, McBrain CJ: Ca^{2+} efflux mechanisms following depolarization evoked calcium transcients in cultured rat sensory neurones. J Physiol 455:567–583, 1992

33. Ross CA, Danoff SK, Shell MJ, Snyder SH, Ullrich A: Three additional inositol 1,4,5-triphosphate receptors: Molecular cloning and differential localization in brain and peripheral tissues. Neurobiology 89:4265–4269, 1992

34. Slater SJ, Cox KJ, Lombardi JV, Ho C, Kelly MB, Rubin E, Stubbs CD: Inhibition of protein kinase C by alcohols and anaesthetics. Nature 364:82–84, 1993