Different Anesthetic Sensitivities of Skeletal and Cardiac Isoforms of the Ca-ATPase[†]

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ABSTRACT: We have previously shown that low levels of the volatile anesthetic halothane activate the Ca-ATPase in skeletal sarcoplasmic reticulum (SR), but inhibit the Ca-ATPase in cardiac SR. In this study, we ask whether the differential inhibition is due to (a) the presence of the regulatory protein phospholamban in cardiac SR, (b) different lipid environments in skeletal and cardiac SR, or (c) the different Ca-ATPase isoforms present in the two tissues. By expressing skeletal (SERCA 1) and cardiac (SERCA 2a) isoforms of the Ca-ATPase in Sf21 insect cell organelles, we found that differential anesthetic effects in skeletal and cardiac SR are due to differential sensitivities of the SERCA 1 and SERCA 2a isoforms to anesthetics. Low levels of halothane inhibit the SERCA 2a isoform of the Ca-ATPase, and have little effect on the SERCA 1 isoform. The biochemical mechanism of halothane inhibition involves stabilization of E2 conformations of the Ca-ATPase, suggesting direct anesthetic interaction with the ATPase. This study establishes a biochemical model for the mechanism of action of an anesthetic on a membrane protein, and should lead to the identification of anesthetic binding sites on the SERCA 1 and SERCA 2a isoforms of the Ca-ATPase.

Despite many years of investigation, the mechanism by which general anesthetics act on biological membranes remains unknown. Recently, many studies have focused on the direct interaction of general anesthetics with proteins. However, few studies have been able to clearly demonstrate direct interaction between general anesthetics and membrane proteins, and the biochemical mechanisms of anesthetic action are poorly understood (*1−5*).

The Ca-ATPases of skeletal and cardiac sarcoplasmic reticulum (SR)[1] have provided excellent models for the study of membrane proteins, in part because of the great deal of information that is available about their structure, function, and biophysical regulation. Investigation of cardiac SR has also been motivated by the well-known depressant effects of general anesthetics on myocardial contractility, which may be due to a defect in SR Ca regulation (*6*). Previous studies have found that general anesthetics have opposite effects on the Ca-ATPase in skeletal and cardiac SR, with anesthetics at lower concentrations activating the enzyme and dissociating ATPase oligomers in skeletal SR while inhibiting the enzyme and promoting oligomer formation in cardiac SR (*7−13*). The two SR systems are nearly identical except for the presence of the intrinsic regulatory protein phospholamban (PLB) in cardiac but not skeletal SR. PLB−ATPase association (which occurs at sub-micromolar Ca concentrations in the absence of PLB phosphorylation) results in inhibition of enzymatic activity. Upon PLB phosphorylation or addition of micromolar Ca concentrations, the inhibition of the Ca-ATPase is relieved (the enzyme is activated) (*14*, *15*). The opposite effects of general anesthetics in skeletal and cardiac SR may be due to either (a) the presence of the regulatory protein phospholamban (PLB) in cardiac but not skeletal SR, (b) different lipid environments in skeletal and cardiac SR, or (c) differential interaction of anesthetics with the SERCA 1 (skeletal SR) and SERCA 2a (cardiac SR) isoforms of the Ca-ATPase (*13*).

To determine whether halothane interacts differentially with the SERCA 1 and SERCA 2a isoforms of the Ca-ATPase, we expressed SERCA 1 and SERCA 2a isoforms in Sf21 insect cell organelles, to provide similar membrane environments (in the absence of PLB) for the two isoforms. The two Ca-ATPase isoforms are 85% homologous in their primary amino acid sequences and undergo nearly identical discrete conformational transitions to couple ATP hydrolysis to Ca transport (Scheme 1) (*14−16*). Thus, identifying differential anesthetic effects on the Ca-ATPase isoforms will

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[1] Abbreviations: SR, sarcoplasmic reticulum; MOPS, 3-(*N*-morpholino)propanesulfonic acid; ATP, adenosine triphosphate; EGTA, ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid; FITC, fluorescein 5-isothiocyanate; P_i, inorganic phosphate; CPA, cyclopiazonic acid.

Scheme 1: A Widely Accepted Model for the Enzymatic Cycle of the Ca-ATPase[a]
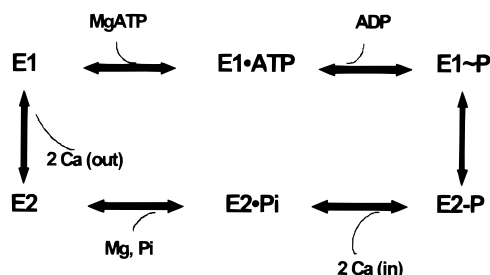
[a] In the presence of micromolar extraluminal (cytoplasmic) Ca, the conformational equilibrium is shifted strongly toward E1, which binds ATP to form a phosphorylated intermediate (E1P). Ca translocation across the SR membrane involves a conformational change of the enzyme from a state with high Ca affinity and weak phosphate bond (E1P) to a state with low Ca affinity and stronger phosphate bond (E2P). Ca is released into the SR lumen followed by hydrolysis of the bound phosphate (E2·P) and release of phosphate (forming the E2 conformation).

lead to a greater understanding of anesthetic interaction with membrane proteins, and will provide more information about the biochemical and physical regulation of the Ca-ATPase.

MATERIALS AND METHODS

Reagents and Solutions. Fluorescein 5-isothiocyanate (FITC) was obtained from Molecular Probes, Inc. (Eugene, OR), and stored in DMF under liquid nitrogen. ATP and cyclopiazonic acid were obtained from Sigma. Halothane (99%) was obtained from Aldrich. Enzyme-linked ATPase assays were carried out in a standard buffer containing 60 mM KCl, 50 mM MOPS, 2 mM $MgCl_2$, and either $CaCl_2$ alone or 5 mM EGTA and $CaCl_2$ so the desired concentration of free ionized Ca could be reached at pH 7.0. Unless otherwise indicated, [Ca] will be used to indicate the concentration of free ionized Ca, calculated as described previously (*15*).

Preparations and Assays. Light SR (LSR) vesicles were prepared from the fast-twitch skeletal muscle of New Zealand white rabbits as described previously (*17*). Cardiac SR vesicles (CSR) prepared from canine ventricular tissue were kindly provided by J. J. Feher (*18*). LSR consisted of approximately 80% Ca-ATPase, or 7.3 nmol of Ca-ATPase/ mg of SR protein. CSR consisted of approximately 30% Ca-ATPase, or 2.7 nmol of Ca-ATPase/mg of SR protein.

Recombinant Ca-ATPase, either the skeletal (SERCA 1) (*19*) or cardiac (SERCA 2a) isoform (*20*), was expressed in insect cells and purified as follows (*20*); 750×10^6 *Spodopter fugiperda* (Sf21) cells were infected for 1 h with recombinant baculovirus expressing the appropriate gene or with wild-type *Autographica californica* Nuclear Polyhedrosis Virus (AcNPV, Invitrogen) at a multiplicity of infection (MOI) of 5−10. Infected Sf21 cells were cultured in an orbital shaker (120 rpm) at 27 °C in 500 mL of Grace's insect Cell Media (Gibco) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 0.1% Pluronic F68 (Gibco), 50 μg/mL gentamicin (Sigma), and 2.5 μg/mL Amphotericin B (Sigma). At 48−72 h postinfection, Sf21 cells were harvested by centrifugation ($1000g_{max}$ for 10 min) and washed twice with 500 mL of phosphate-buffered saline (PBS).

Microsomal membranes were isolated from infected Sf21 cells by resuspending washed cells at a concentration of 10

mg/mL in a medium containing 10 mM $NaHCO_3$, 0.2 mM $CaCl_2$, and the following protease inhibitors: leupeptin (2 μg/mL, Sigma), pepstatin A (1 μg/mL, Sigma), aprotinin (10 μg/mL, Sigma), and Pefabloc (0.1 mM, Boehringer Mannheim). All solutions subsequently employed for membrane isolation contained the same protease inhibitors. Sf21 cell suspensions were homogenized by explosive decompression using Parr Cell Disruption Bomb 4635 (Parr Instruments, Moline, IL) charged with nitrogen at 600 psi. After 5 min, the pressure was released and the cellular homogenate was diluted into an equal volume of ice-cold medium containing 500 mM sucrose, 300 mM KCl, 6 mM $MgCl_2$, and 60 mM histidine (pH 7.4). Next, Sf21 homogenates were centrifuged at $1000g_{max}$ for 20 min, and supernatants were recovered and sedimented at $100000g_{max}$ for 30 min. The resulting pellets were washed once in 250 mM sucrose, 600 mM KCl, 3 mM $MgCl_2$, and 30 mM histidine (pH 7.4) and recentrifuged as described before. The final salt-washed pellets (microsomal fractions) were resuspended at concentrations of 5−10 mg/ mL in 250 mM sucrose and 30 mM histidine (pH 7.4) and stored in small aliquots at −40 °C.

The monoclonal anti-PLB antibody 2D12 (PLB Ab) was prepared as described previously (*14*), and experiments in the presence of PLB Ab were performed by incubating PLB Ab (1:2 Ab:Ca-ATPase by weight) at 25 °C for 20 min prior to the start of the ATPase assay, FITC fluorescence, or enzyme phosphorylation experiments.

SR Ca-ATPase activity was measured at 25 °C using an enzyme-linked, continuous ATPase assay, in the standard buffer with the addition of 5−50 μg/mL SR (or Sf21 cell organelles), 0.42 mM phosphoenolpyruvate, 0.15 mM NADH, 7.5 IU of pyruvate kinase, and 18 IU of lactate dehydrogenase. MgATP (1 mM) was added to start the assay, and the absorbance of NADH was monitored at 340 nm to determine the rate of ATP hydrolysis. All activity measurements were performed in the presence of 1 μg/mL ionophore, A23187, which was added prior to the start of the assays (*17*). Halothane, 30−50% v/v in DMF, was added with a gastight Hamilton syringe, by injecting a small amount (less than 1% of the total sample volume) of halothane through a septum in the cuvette. After the addition of halothane, samples were allowed to incubate for 5 min at 25 °C. The total assay volume was adjusted to minimize the vapor space above the SR so that the amount of halothane that partitioned into the vapor phase was negligible. The final volume of DMF added, up to 1% v/v, did not affect Ca-ATPase activity. Simultaneous measurement of ATPase activity and Ca uptake was accomplished by measuring phosphate-facilitated Ca uptake using Fura Red as a Ca indicator in the presence of NADH, as described previously (*21*).

Optical Labeling and Sample Preparation. For fluorescence experiments, the Ca-ATPase in cardiac SR was specifically labeled with FITC as described previously (*22*). The fluorescence intensity of 0.5 μM FITC−CSR was measured with a SPEX Fluorolog II fluorimeter ($\lambda_{ex} = 495$ nm, $\lambda_{em} = 520$ nm) by summing the total fluorescence emission over 20 s. This was repeated four times per sample, and the average represents the intensity value for one experiment. The experiments were performed in the standard buffer containing 100 μM Ca (high calcium) or a buffer containing 60 mM KCl, 50 mM MOPS, 2 mM $MgCl_2$, and 1 mM EGTA (pH 7.0) (low calcium). Fluorescence changes

upon addition of Ca, EGTA, halothane, or CPA, corrected for the effects of sample dilution, were calculated as described previously (*17*).

Enzyme Phosphorylation from Inorganic Phosphate. Prior to phosphorylation, SR vesicles (0.5 mg/mL) were suspended in a buffer containing 10 mM $MgCl_2$, 2 mM EGTA, and 100 mM MOPS (pH 6.5). To initiate the phosphorylation reaction, an equal volume of 8 mM $[^{32}P]Na_2HPO_4$ in the same buffer was added to the samples and the samples were vortexed. Halothane was added to the full vials immediately after the phosphorylation buffer, and the vials were sealed for 10−15 min. After 10−15 min, the samples were transferred to a quenching solution containing 3% perchloric acid and 2 mM H_3PO_4 (final concentrations). The quenched samples were pelleted in a tabletop centrifuge and then washed three times with a solution of 5% trichloroacetic acid, 4 mM H_3PO_4, 6 mM polyphosphate, and 5 mM cold ATP. The final pellets were dissolved in 2 mL of 1 N NaOH overnight, and the ^{32}P-labeled phosphoenzyme was assayed by counting the Cerenkov radiation.

RESULTS

We previously established that clinical levels of halothane, 0.25−0.4 mM (*4, 6*), activate skeletal SR but inhibit cardiac SR Ca-ATPase function. Halothane effects in cardiac SR are dependent upon [Ca] and the regulatory state of PLB in cardiac SR such that the greatest inhibitory effects of halothane occur at low [Ca] after PLB has been specifically phosphorylated (or addition of PLB Ab, which mimics PLB phosphorylation) (*13*).

In this study, we expressed SERCA 1 and SERCA 2a Ca-ATPase isoforms in Sf21 insect cell organelles to determine whether differential effects of halothane on skeletal and cardiac SR are due to differential effects on the ATPase isoforms. The effects of halothane on SERCA 1 expressed in Sf21 cells were nearly identical to the effects of halothane on skeletal SR. The effects of halothane on SERCA 2a expressed in Sf21 cells were nearly identical to the effects of halothane in cardiac SR (Figure 1). The experiments on cardiac SR at low [Ca] were performed in the presence of PLB Ab, as PLB protects against halothane inhibition under these conditions (*13*).

Low levels of halothane (0−2 mM) activate both SERCA 1 and skeletal SR, while these levels of halothane inhibit both SERCA 2a and cardiac SR. Higher levels of halothane (5−10 mM) inhibit both SERCA 1 and skeletal SR, as observed previously for skeletal SR (*10, 11*). SERCA 1 expressed in Sf21 microsomes had a lower Ca-ATPase activity, due primarily to lower expression levels (judging from SDS−PAGE), than SERCA 2a (0.10 IU compared to 0.34 IU at saturating Ca concentrations). Therefore, at low Ca concentrations, the Ca-dependent activity of SERCA 1 was substantially lower than the Ca-independent background activity, so signal/noise was not sufficient to obtain reliable data.

In cardiac SR, halothane inhibition is clearly Ca-dependent, with increasing [Ca] protecting against halothane inhibition (Figure 1). To determine whether halothane inhibited Ca-ATPase activity by uncoupling Ca transport from ATP hydrolysis, we used a technique describe previously (*21*) to simultaneously measure the Ca uptake and Ca-ATPase
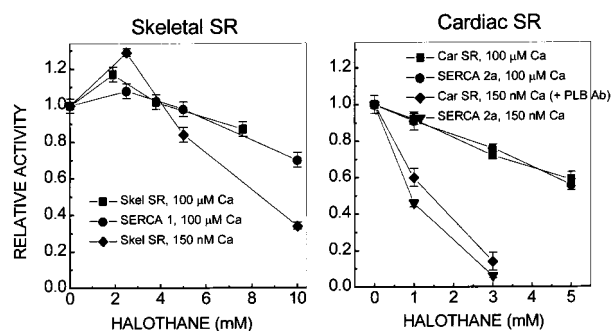

FIGURE 1: Ca-dependent Ca-ATPase activity, normalized to the activity of the sample with no halothane added, at 25 °C. (Left) Skeletal SR at 100 μM Ca (■) and 150 nM Ca (◆), compared to SERCA 1 expressed in Sf21 cell organelles at 100 μM Ca (●). (Right) Cardiac SR at 100 μM Ca (■) and 150 nM Ca with PLB Ab (◆), compared to SERCA 2a expressed in Sf21 cell organelles at 100 μM Ca (●) and 150 nM Ca (▼). Data represent the average of four experiments ± the standard deviation. Ca-ATPase activity (micromoles of ATP hydrolyzed per milligram of protein per minute) in the absence of halothane at 100 nM Ca was 7.2 ± 0.1, 1.2 ± 0.1, 0.34 ± 0.1, and 0.10 ± 0.01 for skeletal SR, cardiac SR, SERCA 2a, and SERCA 1, respectively, and at 150 nM Ca was 2.2 ± 0.1, 7.2 ± 0.1, and 0.16 ± 0.01 for skeletal SR, cardiac SR with PLB Ab, and SERCA 2a, respectively.

activity of SERCA 2a expressed in Sf21 cells. At 150 nM Ca, the Ca-dependent ATPase activity of SERCA2a expressed in Sf21 was 0.16 ± 0.01 mmol of ATP hydrolyzed mg of protein^{-1} min^{-1}, while the Ca uptake activity was 0.10 ± 0.02 mmol of Ca mg of protein^{-1} min^{-1} ($n = 3 \pm$ standard deviation). In the presence of 5 mM halothane, no Ca-dependent ATPase activity or Ca uptake activity was detected beyond that of nontransfected Sf21 cells (used as a control). Thus, halothane did not inhibit Ca-ATPase activity by decoupling Ca transport from ATP hydrolysis.

To further investigate halothane inhibition of SERCA 2a in cardiac SR, we measured the inhibitory effects of halothane on cardiac SR, in the presence of PLB Ab, at 150, 330, and 560 nM Ca. A Dixon plot (1/*V* vs [inhibitor], Figure 2) shows that halothane inhibition of the Ca-ATPase appears to be competitive with respect to [Ca], with clinical levels of halothane (0.25−0.4 mM) inhibiting the Ca-ATPase at 150 nM Ca. This is consistent with a previous study which found that halothane inhibition of calcium uptake in cardiac SR was Ca-dependent, with the anesthetic changing the K_m for Ca but not the V_{max} of the Ca-ATPase (*23*). We calculated an apparent K_I for halothane inhibition of 0.5 ± 0.1 mM, near the clinical range of anesthetic action. Because it was not possible to measure the extent of binding of halothane to the Ca-ATPase, the apparent K_I represents an upper limit for the interaction of halothane with the Ca-ATPase.

Halothane inhibition of the SERCA 2a was Ca-dependent, and halothane has been reported to inhibit Ca-induced conformational changes of the plasma membrane Ca-ATPase (*5*). Therefore, we expected that halothane might prevent Ca-induced changes in the enzyme conformation of the SR Ca-ATPase. Transitions between the E1 and E2 conformations (Scheme 1) can be monitored using FITC-labeled SR, with the fluorescence intensity of FITC−SR decreasing as the enzyme undergoes the E2 to E1 transition, and increasing as the enzyme undergoes the E1 to E2 transition (*17*). To better understand the effects of halothane on ATPase conformation, we compared halothane-induced changes in
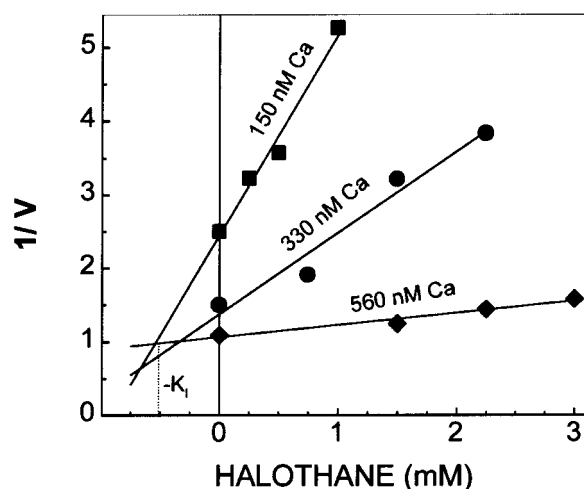
FIGURE 2: Dixon plot ($1/V$, where V is activity in micromoles per milligram per minute) showing halothane competitive inhibition of the Ca-ATPase in cardiac SR with respect to Ca, at 25 °C. The abscissa gives the total concentration of halothane added. Each point represents the average of three experiments. The lines are the best fit (by least-squares analysis) to the data at each [Ca], and K_I was determined by averaging the intersections of the three lines ± the standard deviation. Halothane inhibition is shown at 150 (■), 330 (●), and 560 nM Ca (◆).
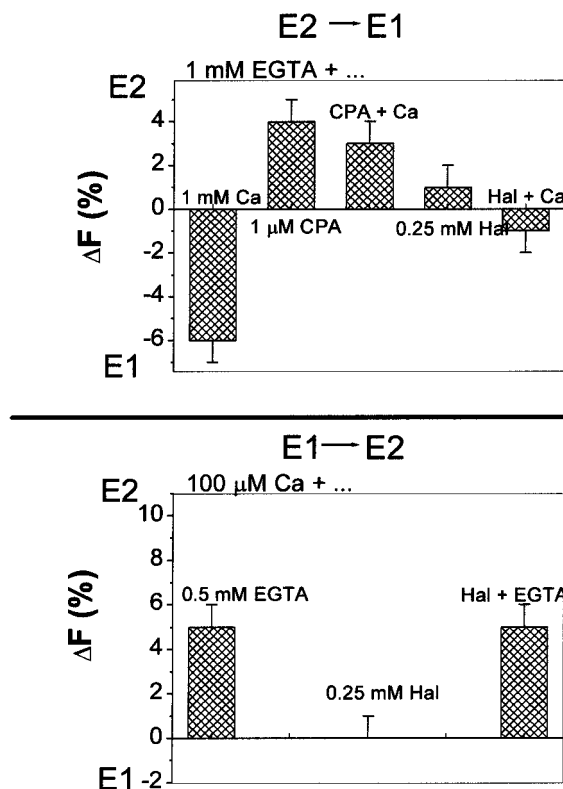

FIGURE 3: Fluorescence changes of FITC−CSR at 25 °C upon addition of 0.25 mM halothane, 1 μM CPA, and/or 1 mM Ca to FITC−CSR in 1 mM EGTA buffer (top) or addition of 0.25 mM halothane and/or 0.5 mM EGTA to FITC−CSR in 100 μM Ca buffer (bottom), as described in Materials and Methods. Halothane at 0.25 mM was chosen because greater levels of halothane directly stabilized FITC fluorescence (see the text), whereas 1 μM CPA was used because it is sufficient to completely inhibit Ca-ATPase activity in cardiac SR (data not shown). Data represent the average of three experiments ± the standard deviation.

FITC−CSR fluorescence with changes in fluorescence intensity induced by cyclopiazonic acid (CPA). CPA is an ATPase inhibitor which specifically stabilizes the E2 conformation with respect to both the E1 and E2P conformations, and prevents Ca-induced changes in FITC−LSR fluorescence (*17*).

In the presence of 1 mM EGTA, addition of 1 mM Ca (change in [Ca] from 0 to 20 μM) decreased the FITC−CSR intensity. Addition of 1 μM CPA increased the fluorescence intensity, indicating greater stabilization of the E2 conformation. Addition of 1 mM Ca after 1 μM CPA did not decrease the fluorescence beyond the level of the control (EGTA alone), indicating that CPA locks the enzyme in E2, preventing the Ca-induced transition to the E1 conformation (Figure 3, top panel). These results are nearly identical to the effects of CPA on FITC-labeled skeletal SR (*17*), indicating that FITC−CSR fluorescence can be used to determine the effects of SR perturbants on ATPase conformation.

Addition of 0.25 mM halothane to FITC−CSR did not significantly increase the fluorescence intensity, but did prevent Ca-induced decreases in FITC fluorescence (Figure 3, top panel). Addition of higher levels of halothane resulted in direct stabilization of FITC fluorescence, as measured by increased fluorescence of FITC−BSA (used as a control). Thus, it was not possible to use more than 0.25 mM halothane in FITC experiments. These low levels of halothane had no effect on skeletal SR (data not shown). Thus, halothane prevented the E2 to E1 transition in cardiac SR, but did not stabilize the E2 conformation to the same extent as CPA.

The E1 to E2 transition is examined by measuring changes in the FITC−CSR fluorescence in the presence of 100 μM Ca. Addition of 0.5 mM EGTA to FITC−CSR in the presence of 100 μM Ca (change in [Ca] from 100 μM to 100 nM) increased the FITC fluorescence. Halothane (0.25 mM) had no effect on either the fluorescence of FITC−CSR

in Ca buffer or EGTA-induced fluorescence changes, indicating that halothane had no effect on the E1 to E2 transition (Figure 2, bottom panel).

Many agents that inhibit the Ca-ATPase by stabilizing the E2 conformation, such as CPA, thapsigargin, and melittin, also prevent phosphorylation of the ATPase from inorganic phosphate (Scheme 1) at very low [Ca] (*17*, *24*, *25*). Therefore, we measured the effects of halothane on E2P formation from inorganic phosphate in both skeletal and cardiac SR (Scheme 1). In skeletal SR, levels of halothane that activate enzymatic activity (0−4 mM) had no significant effect on E2P formation. Higher levels of halothane, which inhibit enzymatic activity in skeletal SR, did result in a greater level of E2P formation (Figure 4). In cardiac SR, halothane stabilized E2P with respect to E2 at lower levels than in skeletal SR, demonstrating that E2P stabilization correlates well with the inhibitory effects of halothane in both skeletal and cardiac SR. The presence of PLB Ab did not affect the halothane-induced stabilization of E2P (Figure 4).

DISCUSSION

Differential Effects of Halothane. We previously demonstrated differential effects of halothane, activating the Ca-ATPase in skeletal SR and inhibiting the Ca-ATPase in cardiac SR. This may be due to either (a) the presence of
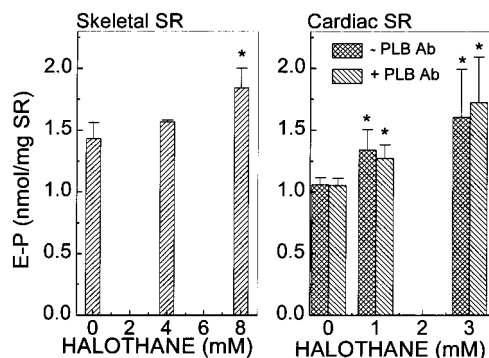
FIGURE 4: Steady-state Ca-ATPase phosphorylation by inorganic phosphate at very low Ca concentrations (2 mM EGTA, no added Ca) and 25 °C in skeletal (left) and cardiac (right) SR, as described in Materials and Methods. Halothane at 0, 4, and 8 mM was added to skeletal SR; 0, 1, and 3 mM halothane, in the presence and absence of PLB Ab, was added to cardiac SR. Data represent the average of three to six experiments ± the standard deviation. Asterisks denote a statistically significant difference from the control (no halothane added) sample ($p < 0.01$).

PLB in cardiac but not skeletal SR, (b) different effects of halothane on SR lipids in skeletal and cardiac SR, or (c) differential interaction of halothane with the SERCA 1 (skeletal SR) and SERCA 2a (cardiac SR) isoforms of the Ca-ATPase (*13*). The Sf21 insect cell organelle system employed for these experiments allowed us to express SERCA 1 and SERCA 2a isoforms of the Ca-ATPase in similar lipid environments in the absence of PLB. These experiments demonstrate that the SERCA 1 and SERCA 2a isoforms of the Ca-ATPase have differential sensitivities to halothane, with only the SERCA 2a (cardiac) isoform inhibited by low levels of halothane (Figure 1).

In this study, we have also shown that halothane inhibition of the SERCA 2a is competitive with respect to Ca. Neither differential anesthetic sensitivity of the two isoforms nor Ca dependence of halothane inhibition is due to the presence of PLB in cardiac SR, as both anesthetic sensitivity and Ca dependence were identical for expressed SERCA 2a (containing no PLB) and cardiac SR (Figure 1). We conclude that halothane differentially inhibits SERCA 1 and SERCA 2a isoforms of the Ca-ATPase, and halothane inhibition of SERCA 2a is dependent upon both [Ca] and the regulatory state of PLB.

In a recent study, high-affinity halothane binding to skeletal SR ($K_I = 0.6$ mM) was demonstrated, though only 38−56% of the halothane was bound to the SERCA 1 (*26*). The K_I determined for halothane binding to skeletal SR membranes is similar to the estimated K_I for halothane interaction with SERCA 2a (0.5 mM, Figure 2). There are at least three possible explanations for the apparently equal binding of halothane by SERCA 1 and SERCA 2a isoforms with opposite effects on skeletal and cardiac SR: (1) halothane could bind with high affinity to different sites on the SERCA 1 and SERCA 2a isoforms, resulting in activation (skeletal SR) or inhibition (cardiac SR); (2) halothane could bind to the same site on both isoforms, resulting in activation (SERCA 1) of one and inhibition (SERCA 2a) of the other; and (3) halothane binding to skeletal SR could represent high-affinity binding to sites which have no functional effect, with activation of skeletal SR resulting from alteration of lipid−protein interactions caused by halothane partitioning into the lipid membrane, as suggested previously (*11*). Further studies

are necessary to determine the mechanism of halothane activation of the Ca-ATPase in skeletal SR.

Because the mechanism of halothane activation of skeletal Ca-ATPase is not understood, it is not possible to determine whether binding of halothane to the SERCA 1 and SERCA 2a isoforms produces opposite effects. However, it is clear that low levels of halothane have different effects on the two isoforms. Low levels of halothane inhibit the SERCA 2a isoform. These same levels of halothane either (a) have no direct effect on the SERCA 1 isoform (if activation is due to alteration of lipid−protein interactions) or (b) activate the SERCA 1 isoform. The finding that anesthetic interaction with enzyme isoforms that are 85% homologous produces different effects is very remarkable. The SERCA 1 and SERCA 2a isoforms of the Ca-ATPase represent an ideal system for studying anesthetic effects on membrane proteins. Further study should lead to identification of halothane binding sites on SERCA 1 and SERCA 2a, and a greater understanding of both anesthetic effects on membrane proteins and Ca-ATPase regulation in SR.

Biochemical and Biophysical Mechanisms of Halothane Inhibition. Because halothane inhibition of the SERCA 2a is Ca-dependent, we wondered whether halothane might inhibit the ATPase by preventing Ca-induced changes in enzyme conformation, such as the E2 to E1 transition (i.e., stabilize the E2 conformation). Other agents that inhibit the ATPase by stabilizing the E2 conformation (or at least an E2-like conformation), such as CPA, thapsigargin, and melittin, increase the fluorescence of FITC−LSR and prevent Ca-induced decreases in FITC fluorescence (*17*, *25*; thapsigargin data not shown). CPA increased the fluorescence of FITC−CSR in EGTA buffer, and prevented Ca-induced decreases in the FITC fluorescence. This is consistent with stabilization of the E2 conformation, as observed in skeletal SR (*17*). Halothane, however, did not increase the fluorescence of FITC−CSR, but did prevent Ca-induced changes in FITC fluorescence (Figure 3). Thus, halothane interferes with the E2 to E1 transition, but does not stabilize the E2 conformation to the same extent as CPA.

Other SR perturbants that stabilize the E2 conformation, such as CPA, thapsigargin, and melittin, all prevent enzyme phosphorylation from inorganic phosphate at low Ca levels (*17*, *24*, *25*). Halothane, however, increased the amount of E2P formed from inorganic phosphate at low Ca levels (stabilized E2P with respect to E2, Scheme 1) (Figure 4). Stabilization of the E2P conformation correlates well with enzyme inhibition; i.e., low levels of halothane stabilize E2P and inhibit ATPase activity in cardiac SR, while higher levels of halothane are necessary to stabilize E2P and inhibit enzymatic activity in skeletal SR (Figures 1 and 4). This demonstrates that inhibition of both skeletal and cardiac SR is likely due to direct interaction of halothane with the Ca-ATPase. Activation of skeletal SR by halothane may be the result of changes in lipid−protein or protein−protein interactions, or may be due to conformational changes induced by halothane that were not detected in these experiments.

Several well-understood SR perturbants, such as CPA and thapsigargin, inhibit Ca-ATPase activity by interfering with the E2 to E1 transition. These agents act at nanomolar to micromolar concentrations, and stabilize the E2 conformation with respect to both the E1 and E2P conformations (*17*, *24*). In contrast, halothane acts at millimolar concentrations and

stabilizes both the E2 and E2P conformations. Thus, halothane may not bind with high affinity to a single site on the Ca-ATPase to interfere with a specific conformational change, as is the case for CPA and thapsigargin. However, it is clear that halothane interacts preferentially with E2 conformations of the Ca-ATPase, as inhibition appears to be competitive with respect to Ca (Figure 2) and halothane has no effect on the E1 to E2 transition (Figure 3). These results suggest that halothane binds to a hydrophobic site or sites on the Ca-ATPase present in E2 but not E1 conformations. Binding of halothane to this site(s) interferes with multiple conformational changes, until the E1 conformation is formed which does not interact with halothane.

In cardiac SR, inhibition of the Ca-ATPase by halothane correlates well with formation of large oligomers of the ATPase (*13*). This is consistent with previous studies of skeletal SR which have shown that the formation of larger ATPase oligomers is associated with inhibition by CPA, thapsigargin, melittin, and lidocaine (*12*, *17*, *25*, *27*). The role that oligomer formation plays in halothane-induced enzyme inhibition and E2P stabilization remains to be elucidated.

Relationship to Other Studies. Anesthetics are known to depress myocardial contractility in vivo, and this may be due to a defect in SR calcium regulation (*6*). Though halothane induces Ca release from SR in both skeletal and cardiac SR, cytoplasmic Ca levels are affected by halothane in cardiac but not skeletal SR (*6*, *28*). Differential inhibition of the SERCA 1 and SERCA 2a isoforms may explain why Ca levels are affected by clinical levels of anesthetics in cardiac but not skeletal SR. Inhibition of the Ca-ATPase by general anesthetics may play a role in anesthetic depression of myocardial contractility.

High-affinity inhibition of the plasma membrane Ca-ATPase by halothane has also been demonstrated previously, and this was shown to be due to halothane inhibition of Ca-induced conformational changes (*5*, *29*). Further investigation is needed to determine whether anesthetic inhibition of the plasma membrane Ca-ATPase occurs via a mechanism similar to that of inhibition of the SERCA 2a.

Previous studies of general anesthetic interaction with firefly luciferase have led to a proposal that general anesthetics interact with a hydrophobic binding site on the enzyme (*31*). Anesthetic sensitivity of luciferase is modulated by ATP, suggesting that conformational changes may make proteins more sensitive to anesthetic action (*31*). On the basis of these and other results, it has been predicted that anesthetics interact with hydrophobic pockets on membrane proteins to produce their physiological effects (*4*). It was concluded that general anesthetics interact directly with the regulatory region of PKC to inhibit that enzyme, suggesting that anesthetic interaction with regulatory regions of membrane proteins may be important (*3*).

In this study, we have demonstrated that halothane interacts directly with the SERCA 2a isoforms of the Ca-ATPase to inhibit the enzyme. Inhibition occurs via stabilization of E2 conformations. As observed for firefly luciferase, conformational transitions (to E2 states) make the enzyme more sensitive to anesthetic action. PLB protection against halothane inhibition remains poorly understood, but it is possible that halothane either interacts with a regulatory domain on the ATPase associated with PLB binding or

interacts with a conformation of the Ca-ATPase that is destabilized by PLB. Because the two isoforms are 85% homologous, further investigation should allow identification of halothane binding sites on the SERCA 1 and SERCA 2a isoforms, and lead to a greater understanding of anesthetic interaction with membrane proteins and regulation of the Ca-ATPase in SR.

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