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Halothane and Isoflurane Alter the Ca^{2+} Binding Properties of Calmodulin

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Background: **Ca^{2+} plays an important role in signal transduction and anesthetic mechanisms. To date, no one has observed a direct effect of volatile anesthetics on a Ca^{2+}-binding protein. We therefore examined the effects of halothane and isoflurane on the Ca^{2+}-binding properties of bovine brain calmodulin.**

Methods: **The fluorescence emission of calmodulin was obtained over a range of Ca^{2+} concentrations ($10^{-7} - 10^{-4}$ M) in the presence and absence of halothane and isoflurane. The intrinsic tyrosine fluorescence of calmodulin was measured at an excitation wavelength of 280 nm and an emission wavelength of 320 nm. Fluorescence measurements were carried out in 50 mM hydroxyethylpiperazineethane sulfonic acid, 100 mM KCl, and 2 mM ethyleneglycol-bis-(β-aminoethyl ether) tetraacetic acid at *p*H 7.0 and 37°C. Experiments were performed in polytetrafluorethylene-sealed cuvettes so that the volatile anesthetic concentrations remained constant. The titration data were analyzed in two ways. The data were fit to the Hill equation by using nonlinear regression analysis to derive the Hill coefficient and the dissociation constant. The data were also analyzed by two-way analysis of variance with multiple comparisons to determine statistically significant effects. Volatile anesthetic concentrations were measured by gas chromatography.**

Results: **The presence of volatile anesthetics altered the Ca^{2+}-binding affinity of calmodulin in a dose-dependent fashion. At 0.57% (0.25 mM) halothane and 1.7% (0.66 mM) isoflurane, the affinity of calmodulin for Ca^{2+} relative to control was decreased. However, at higher concentrations of both anesthetics, the affinity for Ca^{2+} was increased. When the volatile anesthetics were allowed to evaporate from the experimental solutions, the observed rightward shift of the calmodulin–Ca^{2+} binding curve for Ca^{2+} at low concentrations of the anesthetics returned to the control position. The leftward shift seen at high concentrations of the anesthetics was irreversible after evaporation of 8.7% (3.3 mM) isoflurane and 5.7% (2.5 mM) halothane.**

Conclusions: **These data demonstrate a complex interaction of two hydrophobic volatile anesthetics with calmodulin. A biphasic effect was observed both for halothane and for isoflurane. Calmodulin, an EF-hand Ca^{2+}-binding protein, undergoes a conformational shift when binding Ca^{2+}, exposing several hydrophobic residues. These residues may be sites at which the anesthetics act.** **(Key words: Anesthetics, volatile: halothane; isoflurane. Calcium-binding proteins: calmodulin. Ions: calcium. Measurement techniques: fluorescence emission.)**

THE mechanisms through which the volatile anesthetics alter perceptions of pain, levels of consciousness, myocardial contractility, vascular resistance, and alveolar ventilation remain unknown. Several clues, however, suggest that these anesthetics may accomplish their varied and complex tasks by affecting Ca^{2+} homeostasis.[1]

Evidence indicates that halothane depresses myocardial contractility by altering voltage-dependent Ca^{2+} channels and by changing Ca^{2+} release from the sarcoplasmic reticulum.[2–5] Further studies suggest that volatile anesthetics may affect signal transduction as well, by interfering with the Ca^{2+}-binding protein calmodulin.[6,7] Stepwise and sequential binding of Ca^{2+}[8,9] to calmodulin selectively activates a host of enzymes, including phosphodiesterase, myosin light-chain kinase, calmodulin-dependent kinase, calcineurin, erythrocyte Ca^{2+}–adenosine triphosphatase, brain adenylyl cyclase, phosphorylase kinase, and nitric oxide synthase.[10]

Halothane may inhibit some of these enzymes by specific interactions with calmodulin. Nosaka and Wong found that in the presence of calmodulin, volatile anesthetics weakly reduced the activity of myosin light-chain kinase.[6] Moreover, Rudnick *et al.* suggested that halothane may potentiate antitumor activity of γ interferon by binding to calmodulin and altering its interaction with Ca^{2+}-dependent kinases.[7]

Although the volatile anesthetics have been implicated in derangements of calmodulin–Ca^{2+} signaling

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pathways, there are no data that show direct effects of these anesthetics on the Ca^{2+}-binding properties of calmodulin. In this study we monitored the intrinsic tyrosine fluorescence of calmodulin to measure the effects of halothane and isoflurane on Ca^{2+} binding.

Calmodulin, a 16,700-Da monomer, is a well-characterized member of the EF-hand family of Ca^{2+}-binding proteins.[8] It contains two tyrosine molecules, located within two of the four Ca^{2+}-binding loops in the protein.[8] When calmodulin binds Ca^{2+}, a conformational change occurs and enhancement of tyrosine fluorescence results.[11–13]

Analysis of fluorescence emission over a range of Ca^{2+} concentrations allowed us to estimate dissociation constants (K_d) and Hill coefficients (n) for calmodulin's Ca^{2+}-binding sites in the presence and absence of volatile anesthetics and to compare them with known Ca^{2+}-binding data. We took advantage of the volatility of these anesthetics to determine whether their effects on calmodulin were reversible or irreversible.

Materials and Methods

Bovine brain calmodulin was obtained from Sigma Chemical (St. Louis, MO) and Calbiochem (La Jolla, CA) and purity was confirmed by gel electrophoresis. The fluorescence emission of calmodulin at 320 nm was measured over a range of Ca^{2+} concentrations (10^{-7}–10^{-4} M) in the presence and absence of halothane and isoflurane. Solutions of fixed Ca^{2+} concentrations were formulated according to calculations based on the program of Fabiato and Fabiato[14] as modified by Berman.‡ Halothane and isoflurane concentrations from 0.5–8.7% (0.25–5 mM) were generated by adding aliquots of anesthetic-saturated buffer to the experimental solutions. Anesthetic concentrations were verified by gas chromatography.

The intrinsic tyrosine fluorescence of calmodulin was measured in a spectrofluorometer (SLM-Aminco 8000, Rochester, NY) at an excitation wavelength of 280 nm and an emission wavelength of 320 nm. The fluorescence of calmodulin is known to increase on incubation with Ca^{2+}. In control titration experiments, small aliquots of $CaCl_2$ were added to a fluorescence cuvette containing 1 μM calmodulin, 50 mM hydroxyethylpiperazineethane sulfonic acid, 100 mM KCl, and 2 mM ethyleneglycol-bis-(β-aminoethyl ether) tetraacetic acid, at 37°C and *p*H 7.0. Over the range of the titration, *p*H varied less than 0.25 units. This variation has been shown not to significantly alter the affinity of calmodulin for Ca^{2+}.[8] Titrations in the presence of anesthetic were carried out with known concentrations of halothane or isoflurane added from saturated solutions to cuvettes containing 2 ml buffer; the cuvettes were sealed with polytetrafluorethylene to maintain constant volatile anesthetic concentrations. Aliquots of $CaCl_2$ were added with a Hamilton syringe through the seal. In the reversibility experiments, the control and anesthetic additions were performed as described above, except that samples were kept in the dark after 15 min of exposure, and the cuvettes were unsealed and stirred for 1 h in a N_2 atmosphere before $CaCl_2$ aliquots were added. This procedure allowed time for the anesthetics to evaporate from the calmodulin-containing solutions. The absence of anesthetic was verified by gas chromatography. The cuvettes were then resealed, and Ca^{2+} titrations were carried out. Final concentrations of anesthetic were confirmed by gas chromatography.

The fluorescence data were normalized as follows. The fluorescence of calmodulin before $CaCl_2$ additions (F_0) was subtracted from intrinsic tyrosine fluorescence of calmodulin (F), and the difference $F - F_0$ was divided by the maximum fluorescence (F_{max}) achieved after saturation with Ca^{2+}. The result, F_n, equal to $(F - F_0)/F_{max}$, was plotted *versus* Ca^{2+} concentration. All titration data were fit by nonlinear regression to the Hill equation, $F_n = [Ca^{2+}]^n/([Ca^{2+}]^n + K_d^n)$. Parameters derived from nonlinear regression analyses were used with the Enzfitter program (R. J. Leatherbarrow, Elsevier Biosoft, Cambridge, UK) to generate the curves shown in figures 1–4. The statistical significance for the differences between anesthetic treatment and control were determined by two-way analysis of variance with multiple comparisons by using the Student-Neuman-Keuls method.[15] *P* values less than 0.05 were considered statistically significant.

Results

Ca^{2+} binding was measured by the change in intrinsic tyrosine fluorescence of calmodulin. The change in fluorescence was plotted as a function of Ca^{2+} and fit by nonlinear regression analysis to the Hill equation to yield a value for K_d and a value for n. Calmodulin from two sources was used in the experiments: calmodulin purchased from Sigma Chemical (CaM_S) and calmodulin purchased from Calbiochem (CaM_C). These two

‡ Personal communication.

preparations were characterized by different K_d and n values (table 1), a difference that we attribute to greater Ca^{2+} binding to CaM_S than to CaM_C before the Ca^{2+} titration and fluorescence measurement. Despite this quantitative difference, the qualitative changes in Ca^{2+} binding observed on exposure to halothane and isoflurane were essentially identical. The fluorescence spectra of Ca^{2+}-bound calmodulin at low and high anesthetic concentrations were identical to the spectra of control samples.

Volatile anesthetics altered the Ca^{2+}-binding affinity of CaM_S in a dose-dependent, biphasic fashion. Titrations were carried out at halothane concentrations of 0.57% (0.25 mM), 1.14% (0.5 mM), 2.27% (1.0 mM), and 5.68% (2.5 mM) and at isoflurane concentrations of 1.74% (0.66 mM) and 8.68% (3.3 mM). At low concentrations of halothane and isoflurane, the affinity of calmodulin for Ca^{2+} was decreased. At 1.74% isoflurane, for example, K_d was increased to 6.99×10^{-7} from 5.89×10^{-7} M, indicating lower affinity. However, at higher concentrations of both anesthetics, the affinity for Ca^{2+} was increased. In the presence of 8.68% isoflurane, the K_d for Ca^{2+} decreased from 5.89×10^{-7} to 5.09×10^{-7} M (figs. 1 and 2 and table 1).

When Ca^{2+} titrations of CaM_S were carried out in the presence of 0.57% halothane and 1.74% isoflurane, the Ca^{2+}–calmodulin binding curve shifted to the right. These changes were statistically significantly different from control at the $P < 0.05$ level, as determined by two-way analysis of variance. After exposure of calmodulin to anesthetic for 15 min followed by evaporation for 1 h to remove the anesthetic, the rightward shift was completely reversed, and Ca^{2+} binding reverted to control values. At higher concentrations of isoflurane (8.7%) and halothane (5.7%), the leftward shift of the Ca^{2+}–calmodulin binding curve induced by the anesthetics also was statistically significantly different from control. The leftward shift persisted even after the concentration of anesthetic in solution was

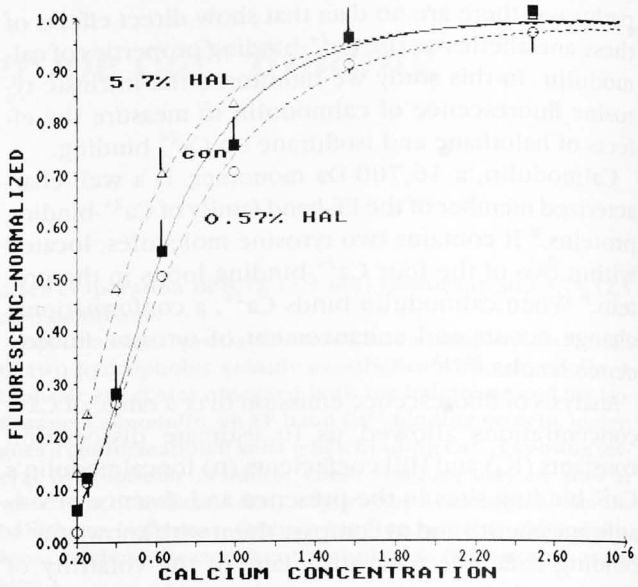

Fig. 1. The effect of halothane on the fluorescence response of calmodulin from Sigma Chemical (CaM_S) to Ca^{2+}. The intrinsic tyrosine fluorescence of CaM_S was measured ($\lambda_{excitation}$ = 280 nm and $\lambda_{emission}$ = 320 nm) in the presence of 0 (squares), 0.57% (circles), and 5.7% (triangles) halothane. Normalized fluorescence is plotted as a function of free Ca^{2+} concentration (molarity). Each data point is the mean ± SD of three independent determinations.

Table 1. The Effect of Halothane and Isoflurane on Calcium Binding to Calmodulin

Halothane (%)	0	0.57	1.14	2.27	5.68
CaM_s					
K_d (M)	5.89×10^{-7}	6.84×10^{-7}	5.77×10^{-7}	5.08×10^{-7}	4.39×10^{-7}
n	2.6	2.5	2.4	2.2	2.1
CaM_c					
K_d (M)	1.76×10^{-6}	3.1×10^{-6}			4.00×10^{-7}
n	1.8	1.3			2.2
Isoflurane (%)	**0**	**1.74**	**8.68**		
CaM_s					
K_d (M)	5.89×10^{-7}	6.99×10^{-7}	5.09×10^{-7}		
n	2.6	2.6	2.7		

Data show the effect of halothane and isoflurane on the binding of Ca^{2+} to calmodulin. Ca^{2+}/calmodulin titration data were fit to the Hill equation by nonlinear regression analysis. K_d and n were derived from nonlinear regression analysis.

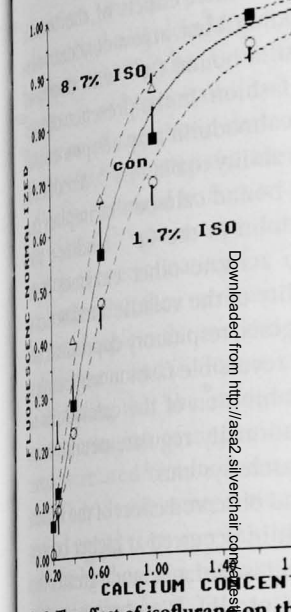

Fig. 2. The effect of isofluran on th
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Titrations also were carried ou
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isoflurane. Figure 4A demonstrat
at 0.57% (0.25 mM) halothane a
the left at 5.7% (2.5 mM) haloth
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no reversibility (fig. 4B). The re
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Discussion

We have shown, by using intr
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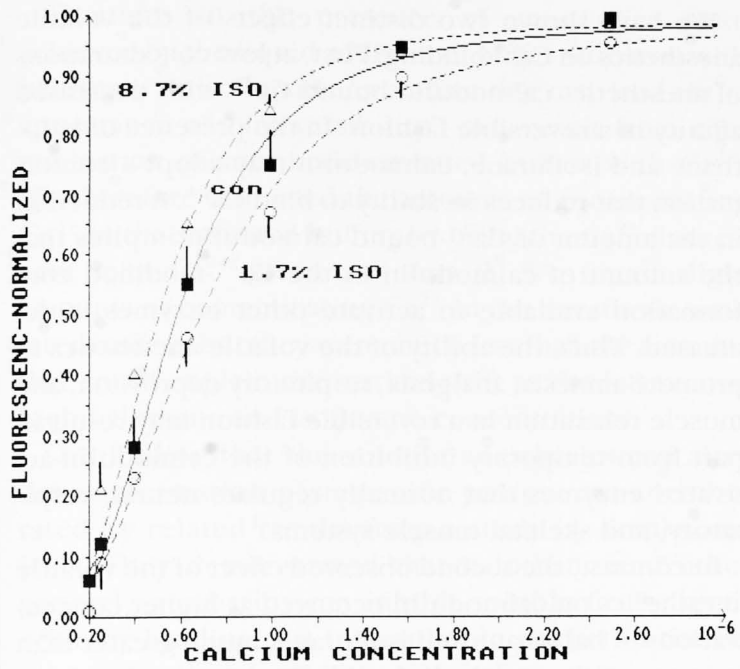

Fig. 2. The effect of isoflurane on the intrinsic tyrosine fluorescence response of calmodulin from Sigma Chemical (CaM_S) to Ca^{2+} in the presence of 0 (squares), 1.7% (circles), and 8.7% (triangles) isoflurane. Each data point is the mean ± SD of three independent determinations.

measured to be zero, both for high halothane and for high isoflurane concentrations (fig. 3).

Titrations also were carried out with CaM_C. The control titration resulted in a K_d of $1.76 \pm 0.66 \times 10^{-6}$ M and $n = 1.81 \pm 0.095$. The same effect was observed for CaM_C as for CaM_S when exposed to halothane and isoflurane. Figure 4A demonstrates the shift to the right at 0.57% (0.25 mM) halothane and the marked shift to the left at 5.7% (2.5 mM) halothane. Furthermore, with evaporation of halothane (5.7%, 2.5 mM), there was no reversibility (fig. 4B). The response to isoflurane of CaM_C was qualitatively similar to that observed with CaM_S (data not shown).

The effect of low and high concentrations of halothane on CaM_C were compared by two-way analysis of variance. The data sets obtained from Ca^{2+} titrations during exposure to 0.57% and 5.68% halothane were statistically significantly different from the control data set ($P < 0.05$).

Discussion

We have shown, by using intrinsic tyrosine fluorescence emission data, that the volatile anesthetics halothane and isoflurane alter calmodulin's Ca^{2+}-binding properties. At low concentrations of anesthetic these effects appear reversible, whereas at higher concentrations they are irreversible for both isoflurane and halothane.

Our observations are based on the phenomenon that calmodulin in the presence of Ca^{2+} undergoes a conformational shift with a corresponding enhancement of fluorescence.[9] Several sources of evidence support this finding. Spectroscopy studies have shown that Ca^{2+} induces structural changes in the third and fourth Ca^{2+}-binding domains of calmodulin, where the two tyrosine residues lie.[16] Proteolysis studies have corroborated these findings by noting that calmodulin's third domain undergoes folding in the presence of Ca^{2+}, rendering the protein resistant to trypsin digestion.[17] Moreover, pK_a analysis has shown that the environment of tyrosine 138, which resides in calmodulin's fourth domain, is altered in the presence of Ca^{2+}.[18,19]

The conformational change that occurs in the presence of Ca^{2+}, in addition to increasing fluorescence emission, exposes hydrophobic regions in calmodulin.[20] These hydrophobic surfaces have been implicated as necessary for calmodulin's stimulatory effects on

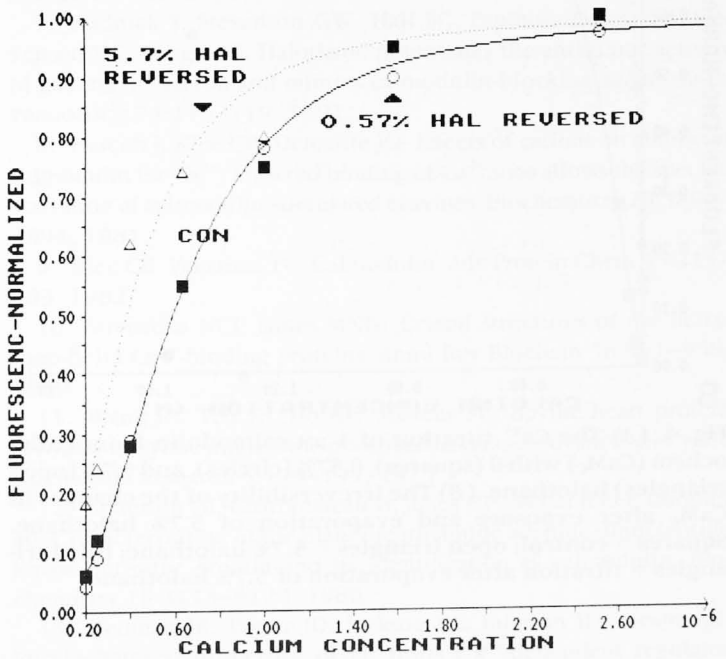

Fig. 3. The reversibility of halothane's effects on the Ca^{2+}-binding affinity of calmodulin from Sigma Chemical (CaM_S). CaM_S was exposed to 0.57% and 5.7% halothane, which then was allowed to evaporate. Curves for titrations with CaM_S exposed to 0.57% (circles) and 5.7% (triangles) halothane that had been allowed to evaporate are compared with control data (squares). The 0.57% halothane reversed curve is coincident with the control curve. Similar results were found with isoflurane.

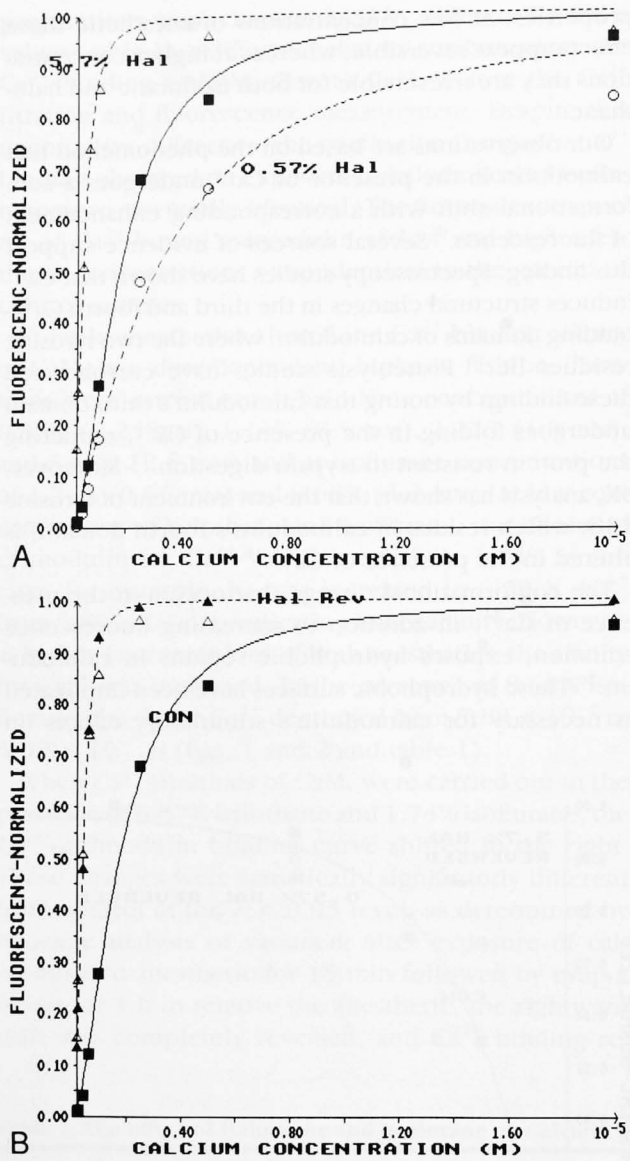

Fig. 4. (*A*) The Ca^{2+} titration of 1 μM calmodulin from Calbiochem (CaM_C) with 0 (squares), 0.57% (circles), and 5.7% (open triangles) halothane. (*B*) The irreversibility of the changes in CaM_C after exposure and evaporation of 5.7% halothane. Squares = control; open triangles = 5.7% halothane; filled triangles = titration after evaporation of 5.7% halothane.

other enzymes.[10] The same hydrophobic residues are also thought to be the sites where a host of lipophilic molecules, including the phenothiazines, interact with calmodulin to block its enzyme-activating steps.[20–25] Perhaps by binding to these hydrophobic sites the lipophilic volatile anesthetics alter calmodulin's Ca^{2+}-binding properties.

We have shown two distinct effects of the volatile anesthetics on Ca^{2+} binding. First, at low concentrations of anesthetic, calmodulin bound Ca^{2+} with decreased affinity in a reversible fashion. In the presence of halothane and isoflurane, calmodulin may adopt a conformation that reduces its ability to bind Ca^{2+}. A reduction in the amount of Ca^{2+}-bound calmodulin implies that the amount of calmodulin in the Ca^{2+}-modified conformation available to activate other enzymes is decreased. Thus, the ability of the volatile anesthetics to promote amnesia, analgesia, respiratory depression, and muscle relaxation in a reversible fashion may result in part from temporary inhibition of the calmodulin-activated enzymes that normally regulate neural, respiratory, and skeletal muscle systems.[1]

In contrast, the second observed effect of the volatile anesthetics on calmodulin occurred at higher concentrations of halothane and isoflurane, much greater than concentrations used clinically. We observed a dose-dependent increase in affinity for Ca^{2+}. It is conceivable that at concentrations greater than some critical level, the hydrophobic anesthetics alter calmodulin's folding so that Ca^{2+} binding is favored. At high concentrations of both anesthetics, the change in Ca^{2+} binding was not reversed, even after the anesthetic concentrations were measured to be zero by gas chromatography. These studies were performed in the dark to prevent the possibility that photoactivation of the anesthetics would lead to a covalent interaction.

One factor in our experimental system that may account for an irreversible alteration in calmodulin structure and Ca^{2+} binding is *p*H change. We observed a decrease in *p*H in our experimental solutions from 7.00 to 6.75 over the course of Ca^{2+} additions in all titration experiments. Three observations, however, make it doubtful that *p*H shifts explain our results. First, data from Haiech *et al.* suggest that the *p*H decrease we encountered is not sufficient to alter Ca^{2+} binding. Only at *p*H less than 6.0 is the affinity of calmodulin for Ca^{2+} significantly altered.[8] Second, as noted by Haiech *et al.*, a *p*H decrease confers a concomitant decrease in Ca^{2+} affinity.[8] At high concentrations of anesthetics, we saw an increase in Ca^{2+} affinity despite a slight *p*H decrement. Third, although we did observe a decrease in Ca^{2+}-binding affinity at low concentrations of halothane and isoflurane, this decrease cannot be ascribed to a *p*H change, because although the *p*H change was irreversible, the decrease in Ca^{2+} affinity that we observed reversed as the anesthetic evaporated.

Titrations in the presence
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We therefore estimated the am
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Although we now report signi
atile anesthetics on calmodulin
Blanck *et al.* were unable to s
halothane on troponin C.[2] Trop
both members of the EF-hand
proteins, share as much as 76%
the considerable similarity betw
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In summary, by measuring the
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Titrations in the presence of anesthetics were achieved by the liquid addition of buffer saturated with halothane or isoflurane. An artifact that may lead to the appearance of "irreversibility" may result from contamination of anesthetic-saturated buffers with Ca^{2+}. We therefore estimated the amounts of contaminating Ca^{2+} in the saturated buffers by using the Ca^{2+}-fluorescent dyes Ca^{2+} green and fura 2. We found slight amounts of contaminating Ca^{2+} in the anesthetic-saturated buffers, but they were much less than the amounts that could explain the marked shift to the left seen at high halothane and isoflurane concentrations.

Our finding that the volatile anesthetics can alter the conformation of a specific protein are corroborated by related results from a study by Franks and Lieb. They demonstrated that halothane and other general anesthetics can inhibit soluble firefly luciferase by binding at a hydrophobic pocket in the luciferase molecule.[26] Taken together, these experiments lend further credence to the theory that general anesthetics can act by specific interactions with target protein molecules and that nonspecific associations with the lipid bilayer component of cell membranes may not be required.

Although we now report significant effects of the volatile anesthetics on calmodulin's ability to bind Ca^{2+}, Blanck *et al.* were unable to show similar effects of halothane on troponin C.[27] Troponin C and calmodulin, both members of the EF-hand family of Ca^{2+}-binding proteins, share as much as 76% homology. In light of the considerable similarity between these two proteins, it is reasonable to expect that the volatile anesthetics may affect both similarly. However, given the dose-dependent, biphasic nature of the effects of the volatile anesthetics on calmodulin, we suggest that a significant alteration in Ca^{2+} binding was not observed in the case of troponin C because of the concentration of halothane used in that experiment. In the troponin experiment, Ca^{2+} binding was measured in the presence of 1.0 and 0.9 mM halothane. These concentrations are between the lower and higher concentrations of anesthetic that alternately yield a decrease or an increase in Ca^{2+} binding in the case of calmodulin and may be pertinent to the lack of an observed alteration on Ca^{2+} binding to troponin C.

In summary, by measuring the Ca^{2+}-dependent fluorescence emission of calmodulin, we found that low concentrations of halothane and isoflurane reversibly decreased calmodulin's Ca^{2+} affinity. At higher anesthetic concentration, however, Ca^{2+} binding was increased in an irreversible fashion both by halothane and by isoflurane.

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Amrinone Is Su
Bupivacaine-ind
Sevoflurane-ane

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Background: Bupivacaine-induced
is known to be difficult to treat, and t
for treatment of bupivacaine-induc
sion is in doubt. We compared the
that of epinephrine for the treatme
cardiovascular depression in anesth

Methods: In dogs receiving 1.5–2
0.5% bupivacaine was infused at a
intravenously until mean arterial
to 40 mmHg or less. In the amrinon
(4 mg · kg^{-1}, intravenously) was giv
diovascular depression, followed b
a rate of 0.1 mg · kg^{-1} · min^{-1}. In the
9), epinephrine (0.01 mg · kg^{-1}, intr
bolus, and the same dose was given

Results: All nine dogs that receive
the nine dogs that received epinep
cardiovascular depression develop
animals ($P < 0.05$). Only one anim
showed tachyrhythmia with wide
suscitation, whereas all nine animals
showed tachyrhythmia with wide
suscitation.

Conclusions: Amrinone is superio
treatment of bupivacaine-induced
in sevoflurane-anesthetized dogs.
local: bupivacaine. Anesthetics, vol
cardiovascular depression. Pharma
pathetic nervous system, catechola

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