Anesthesiology
74:155–165, 1991

Volatile Anesthetics Depress Calcium Channel Blocker Binding to Bovine Cardiac Sarcolemma

Benjamin Drenger, M.D., Mary Quigg, B.Sc.,† Thomas J. J. Blanck, M.D., Ph.D.‡*

Volatile anesthetics produce their negative inotropic effect on the heart mainly by interference with calcium homeostasis in the myocardial cell. In order to elucidate the mechanism of the depression, we have evaluated the effect of the volatile anesthetics on the binding of the calcium channel blocker [^{3}H]nitrendipine to purified bovine cardiac sarcolemma. The radioligand binding studies were carried out at 25° C, with increasing concentrations of [^{3}H]nitrendipine (0.01–1 nM), in the presence or absence of unlabeled nitrendipine to determine specific binding, and with or without 1.9% halothane, 2.3% isoflurane, and 4.8% enflurane. Separately, [^{3}H]nitrendipine was measured in the presence of increasing doses of halothane (0.78, 1.33, 1.90, and 2.57%). Kinetic studies of association and dissociation rate were performed with 1.90% halothane and 1 nM [^{3}H]nitrendipine at different time intervals. All three volatile anesthetics produced depression of [^{3}H]nitrendipine binding to the isolated cardiac sarcolemma. Only halothane produced a significant depression in binding, ranging between 59 and 66% ($P < 0.05$), depending on the concentration of [^{3}H]nitrendipine used. Isoflurane produced 29–38% depression, and enflurane produced 5–22% depression. Halothane also produced a significant ($P < 0.01$) dose-dependent decrease in [^{3}H]nitrendipine-specific binding. The kinetic binding experiments demonstrated that the time course for halothane's effect on association and dissociation of [^{3}H]nitrendipine was 5 min for the half-maximum effect; the maximal reduction in binding capacity was at 15–30 min ($P < 0.05$). Scatchard analysis revealed that all three volatile anesthetics produced reduction in the maximal number of binding sites; however, they varied in their effect on binding affinity. Only halothane produced a homogenous increase in the dissociation constant, signifying reduced affinity of the Ca^{2+} blocker to the channel. We suggest that the volatile anesthetics produce conformational changes in these channels consistent with their ability to depress channel-mediated Ca^{2+} influx into myocytes. (Key words: Anesthetics, volatile: halothane; isoflurane; enflurane. Ions: calcium. Muscle, cardiac: sarcolemma. Pharmacology: calcium channel blocking drug: nitrendipine.)

VOLATILE ANESTHETICS produce a dose-dependent depression of myocardial contractility, mainly by interfering with the normal control and availability of Ca^{2+} ions for the contractile process. Contractility of cardiac cells depends on sarcolemmal Ca^{2+} influx, sarcoplasmic reticulum Ca^{2+} uptake and release, and myofibril Ca^{2+} sensitivity. The respective contribution of each volatile anesthetic on each of these sites is not well defined.[1–3]

Ca^{2+} channels in the sarcolemma are sensitive to changes in membrane potential. When the channels open during depolarization, Ca^{2+} ions move into the cell according to their electrochemical gradient, resulting in an inward membrane current. A number of studies[1,3,4] have shown that the Ca^{2+} current through the voltage-sensitive Ca^{2+} channels is inhibited by the volatile anesthetics, leading to a decrease in contractile force. The effect of the volatile anesthetics on myocardial contractility and their prevention of ischemia induced dysrhythmias are similar in some respects to the effects of the Ca^{2+} blockers, and the combination of these two types of drugs has been shown to be at least additive.[3,5,6]

The organic Ca^{2+} channel blockers are effective on one type of voltage-sensitive Ca^{2+} channel, designated the L channel.[7,8] These drugs have no effect on the other types of Ca^{2+} channels, designated N and T channels. It appears that the L channel is actively involved in Ca^{2+} transport for excitation–contraction coupling. This channel is the focus of our current study of the effect of volatile anesthetics on cardiac membranes.[9] The dihydropyridines, such as nifedipine and nitrendipine, bind to L channels in a highly specific, saturable, and reversible manner.[10] [^{3}H]nitrendipine has been used in radioligand binding studies as a specific probe for investigating the L-type Ca^{2+} channel, and these studies demonstrated that a decrease in the binding of this radioligand was correlated with a reduction of Ca^{2+} influx, followed by a decrease in the contractile force.[11]

The information available in the literature on the effect of volatile anesthetics on the Ca^{2+} channels and on the modulation of Ca^{2+} influx through the myocardial cell membrane, the sarcolemma, has mainly involved electrophysiologic studies or Ca^{2+}-transient experiments. Radioligand binding studies complement these previous studies by determining whether the anesthetics sufficiently perturb Ca^{2+} channels to alter antagonist binding. Our initial investigation[12] demonstrated that halothane produced a dose-dependent depression of binding of [^{3}H]nitrendipine to partially purified rat and rabbit cardiac membranes, an effect that was found to be reversible. In the current study, equilibrium radioligand binding of [^{3}H]nitrendipine was studied in purified bovine cardiac sarco-

* Lecturer.
† Research Assistant.
‡ Associate Professor.

Received from the Department of Anesthesiology and Critical Care Medicine, Division of Cardiac Anesthesia, Johns Hopkins Hospital, Baltimore, Maryland. Accepted for publication September 18, 1990. Supported in part by grant GM 30799 from the National Institutes of Health and in part by a grant from the Joint Research Fund of the Hebrew University and Hadassah. Preliminary reports of portions of this work were presented at the American Society of Anesthesiologists meeting, San Francisco, California, October 1988 and at the Federation of the American Society for Experimental Biology (FASEB) Meeting, New Orleans, Louisiana, March 1989.

Address correspondence to Dr. Drenger: Director, Cardiothoracic Anesthesia, Department of Anesthesiology, Hadassah University Hospital, P. O. Box 12000, Jerusalem, Israel 91120.

lemma, and the effect of equipotent doses of the three volatile anesthetics, halothane, enflurane and isoflurane, on the binding of [^{3}H]nitrendipine to the sarcolemma was tested. We quantified the effect of the volatile anesthetics on the density of the binding sites and on the dissociation constant. The dose-dependent halothane effect on the specific binding of [^{3}H]nitrendipine to sarcolemma was also examined. To further elucidate anesthetic effects, we also evaluated the time course for association and dissociation of [^{3}H]nitrendipine to purified bovine sarcolemma.

Materials and Methods

Isolation of Sarcolemma-Enriched Preparation

Bovine heart obtained from a local slaughterhouse was placed on ice within 15 min of the time the animal was killed. All of the following procedures were carried out on ice and with cold (0–4° C) buffers. The isolation of the sarcolemmal membranes was performed using a modification of the method of Jones *et al.*[13] and Caroni *et al.*[14] Ventricular muscle was trimmed of its epicardial and endocardial membranes, fat, and major vessels, and then ground in a cooled meat grinder.

The ground ventricle was then homogenized in a Waring blender, in two to three volumes 0.75 M NaCl, 10 mM histidine, *p*H 7.5 (medium 1) for 30 s, at 60% maximal speed (fig. 1). The suspension was centrifuged at 10,400 × g for 20 min. This step, which removed nuclei, cell debris, and mitochondria, was then repeated. The pellet of the second spin was resuspended in two volumes 10 mM $NaHCO_3$ and 5 mM histidine (medium 2), homogenized for 10 s, and centrifuged at 10,400 × g for 20 min. This process was repeated with a 90-s homogenization and 20-min centrifugation. The supernatant at this stage was relatively free of contractile proteins, and was spun again at 17,000 × g for 40 min. The pellet was Dounce-homogenized in 0.25 M sucrose and 10 mM histidine (medium 3), and after a 10-min centrifugation, the supernatant was used for the next step, which was ultracentrifugation at 105,000 × g for 30 min.

At the end of this step a soft white–beige pellet was found lying over a firm pellet; this soft microsomal pellet was collected by aspiration and placed over 0.6 M sucrose buffer, 10 mM Tris, 300 mM NaCl, 50 mM and Na pyrophosphate, *p*H 7.1 (medium 4) and centrifuged at 170,000 × g for 90 min. The final sarcolemmal vesicles were collected from the interphase, washed with 10 mM

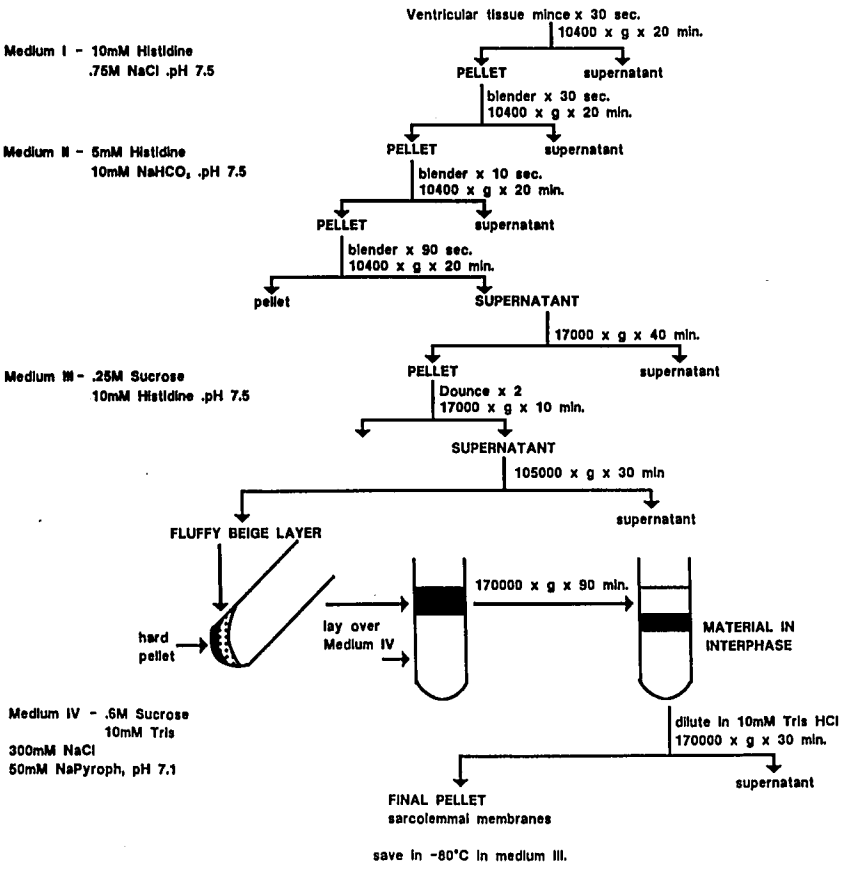

FIG. 1. Procedure for isolation of sarcolemma-enriched preparation from bovine heart ventricular muscle. The entire procedure was performed with ice-cold buffers.

Tris for 30 min, spun at 170,000 × g, and then suspended in medium 3 and stored in a −80° C freezer.

Protein concentration was determined by the Coomassie binding method using bovine serum albumin as the standard. In order to evaluate the purity of the sarcolemmal preparation, the final four stages of the sarcolemma isolation procedure were evaluated by $^{45}Ca^{2+}$ uptake studies, in the presence of oxalate. As sarcolemmal $^{45}Ca^{2+}$ uptake is not amplified by oxalate, we used the diminution of oxalate supported $^{45}Ca^{2+}$ uptake as a marker for the decrease in sarcoplasmic reticulum contamination of our sarcolemmal preparations. In parallel we performed [^{3}H]nitrendipine binding studies in each of the final four stages of the preparations. Electron microscopy of the sarcolemmal preparation was performed using the immersion-fixation staining method. Electron microscopy is particularly helpful in determining mitochondrial contamination, but it is more difficult to distinguish between sarcolemma and sarcoplasmic reticulum vesicles.

Binding Assays

Binding assays were carried out in 31-ml glass vials, sealed with Teflon® liners to maintain constant anesthetic concentration. Anesthetic was added to the solution in liquid form, with a Hamilton microliter syringe. Anesthetic concentration was measured by gas chromatography.[15] Blanck[16] showed that under identical conditions, a stable partial pressure is maintained for more than 30 min. Measurements performed on samples taken during the current study produced a similar conclusion. Addition of 3 μl halothane, 5 μl isoflurane, and 8 μl enflurane produced anesthetic partial pressures equivalent to 1.9, 2.3, and 4.8 vol%, respectively. Equipotent doses of the volatile anesthetics usually are calculated in animal or human studies at 37° C. However, there is a large uncertainty in the extrapolation of these values in isolated membrane experiments performed at 25° C. It is the number of anesthetic molecules at the site of anesthetic action that has to be considered, and this number remains roughly constant despite the change in temperature.[17] Data on equipotent anesthetic concentration for *in vitro* or *in vivo* experiments in the cow are not available.

In light of these considerations, for the current study we used the volatile anesthetics in concentrations close to those published in the anesthetic literature for human equipotent ratios. Halothane was used in its thymol-free form (Halocarbon Laboratories, Hackensack, NJ), even though our preliminary studies with thymol, in final concentration of 1–10 μM, produced no effect on [^{3}H]nitrendipine binding to the membranes.

The membranes were incubated during the experiment in a shaking water bath at a constant temperature of 25° C. This temperature was used in order to slow membrane degradation. Equilibrium binding data are complete after 1 h of incubation, a period of time that might be too long for achievement of reproducible results at 37° C. It has been shown[18] that radioligand experiments at 25° C correlate well with patch clamp experiments on the effect of nitrendipine on inhibition of cardiac calcium currents. A decrease in nitrendipine binding affinity at 37° C has been reported previously and is related to an increase in the dissociation rate constant.[19,20] However, the effect of a temperature increase on the capacity of the binding sites has not been determined with certainty.[20,21]

In the equilibrium binding studies, the sarcolemmal membranes (40–90 μg) were added to 0.01–1 nM [^{3}H]nitrendipine (specific activity 74–79 Ci/mmol; New England Nuclear, Boston, MA) in 50 mM Tris buffer (*p*H 7.5) in a final volume of 1 ml in the absence or presence of 500 nM unlabeled nitrendipine to define total and nonspecific binding. All measurements at all concentrations were made in triplicate. In each experiment the control [^{3}H]nitrendipine binding values were compared to those achieved when 1.9% halothane or 2.3% isoflurane or 4.8% enflurane was added. In another set of six experiments, the effect of increasing concentration of halothane (0.78, 1.33, 1.90, 2.57%) on the binding of 1 nM [^{3}H]nitrendipine was evaluated.

After 60 min incubation, during which the vials were protected from light to minimize the photochemical decomposition of nitrendipine, the reaction was terminated when an aliquot of 800 μl was filtered under vacuum through 2.5-cm Whatman GF/C glass fiber filters placed in a 30-well Millipore filtration apparatus and washed three times with 10 ml cold 20 mM Tris buffer. Flushing the filters ensured that any unbound drug was washed away. Prior to use the filters were pretreated with 5 nM unlabeled nitrendipine in 20 mM Tris in order to reduce the nonspecific binding of [^{3}H]nitrendipine to the filter itself. The filters were placed in scintillation vials with 5 ml 3a70 complete scintillation cocktail (Research Products International, Mount Prospect, IL), and samples were counted in a Beckman LS2800 scintillation counter. Counting efficiency was approximately 55%.

Nonspecific binding is defined as binding of [^{3}H]nitrendipine that occurs in the presence of a high concentration of unlabeled ligand (nitrendipine, in this study). Specific binding, which indicates binding only to the voltage-sensitive Ca^{2+} channels, is calculated from the difference between total binding and nonspecific binding (fig. 2). In the current study, 500 nM unlabeled nitrendipine (Miles Laboratories, West Haven, CT) was used for displacement of the [^{3}H]nitrendipine from its high-affinity binding sites. Other studies[22,23] also used nitrendipine in the same concentration range to define nonspecific binding. Nonspecific binding represents the labeled ligand

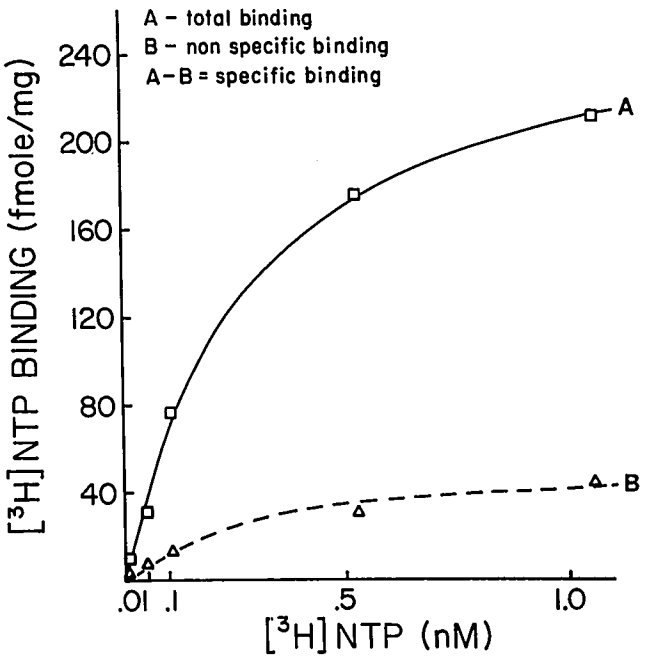

FIG. 2. Equilibrium binding of [^{3}H]-nitrendipine to bovine cardiac sarcolemma. Each point on the total binding (A) and the nonspecific binding curves (B) was determined in triplicate for each of the five [^{3}H]-nitrendipine concentrations. Nonspecific binding was obtained in the presence of 500 nM unlabeled nitrendipine. Specific binding is calculated from the difference between total and nonspecific binding (A − B).

trapped to irrelevant lipoidal regions or other charged sites, and since the chance of both drugs to bind to those sites is small, the ability of the unlabeled nitrendipine to displace the labeled drug is also small.[24] The cold (unlabeled) nitrendipine was prepared fresh daily in absolute ethanol, and appropriate dilutions were made with 50 mM Tris to a final concentration of 1 μM ethanol. We have found in preliminary experiments that in this dilution the ethanol did not affect the [^{3}H]nitrendipine binding to the sarcolemma.

KINETIC STUDIES

Association time course studies were initiated with the addition of the sarcolemmal membranes to the reaction mixture containing 1 nM [^{3}H]nitrendipine in 50 mM Tris buffer (*p*H 7.5), in the presence or absence of unlabeled nitrendipine and with and without 3 μl halothane. The reaction was terminated after incubation time intervals of 1, 5, 15, 30, and 60 min. The specific binding was calculated as described above.

Dissociation time course experiments were performed after 1 h incubation of the sarcolemmal membranes with 1 nM [^{3}H]nitrendipine. Three 800-μl aliquots were used as control values of total binding at time 0, after which 500 nM unlabeled nitrendipine was added as a displacer. The [^{3}H]nitrendipine binding after the addition of cold nitrendipine, with and without the addition of 3 μl halothane, was then measured at the various time intervals (0 and 30 s and 1, 5, 15, 30, and 60 min). In all experiments samples were taken in triplicate 800-μl aliquots. The dissociation rate constants (k_{-1}) for control and halothane studies were then calculated with linear regression analysis.

DATA ANALYSIS

Equilibrium binding data were analyzed by the method of Scatchard using the Enzfitter program,* which yields a nonlinear, least-squares fit of the data and estimates of the maximum number of binding sites (B_{max}) and the dissociation constant (K_D) for nitrendipine binding to the L channel. In the lowest concentration (0.01 nM) of [^{3}H]nitrendipine used in these experiments, a Scatchard plot could not be computed because the counts were too close to the range of the nonspecific binding and were out of the effective range for valid Scatchard analysis. Therefore, another set of experiments was added, in which concentrations were chosen closer to the expected K_D.[25] The Scatchard analysis in these experiments showed a better fit of the individual points to a straight line.

Statistical analysis of the equilibrium binding studies was performed using a paired *t* test, in which the effect of each volatile anesthetic was compared to the control binding values for each concentration. The effect of the three volatile anesthetics was compared by one-way analysis of variance. The evaluation of the anesthetic effect on Scatchard analysis also was compared by one-way analysis of variance. The effect of halothane on kinetic binding values was compared for significance by the paired *t* test for each time interval. Values were considered significantly different at $P < 0.05$. All data are given as the mean ± SEM of independent experiments.

Results

EVALUATION OF THE SARCOLEMMAL PREPARATION

The final yield of sarcolemmal membranes was approximately 4–5 mg per 100 g crude ventricular tissue. Evaluation of the preparation by electron microscopy (fig. 3) revealed that almost the entire preparation was enriched in sarcolemmal vesicles. Very few mitochondria, lysosomes, and glycogen droplets were observed. Oxalate-supported $^{45}Ca^{2+}$ uptake, which is a marker of the calcium pump of the sarcoplasmic reticulum, was examined in the four final steps of preparation, using the same amount of

* Leatherbarrow RJ: Enzfitter. Elsevier Science Publishers, Amsterdam, 1987.

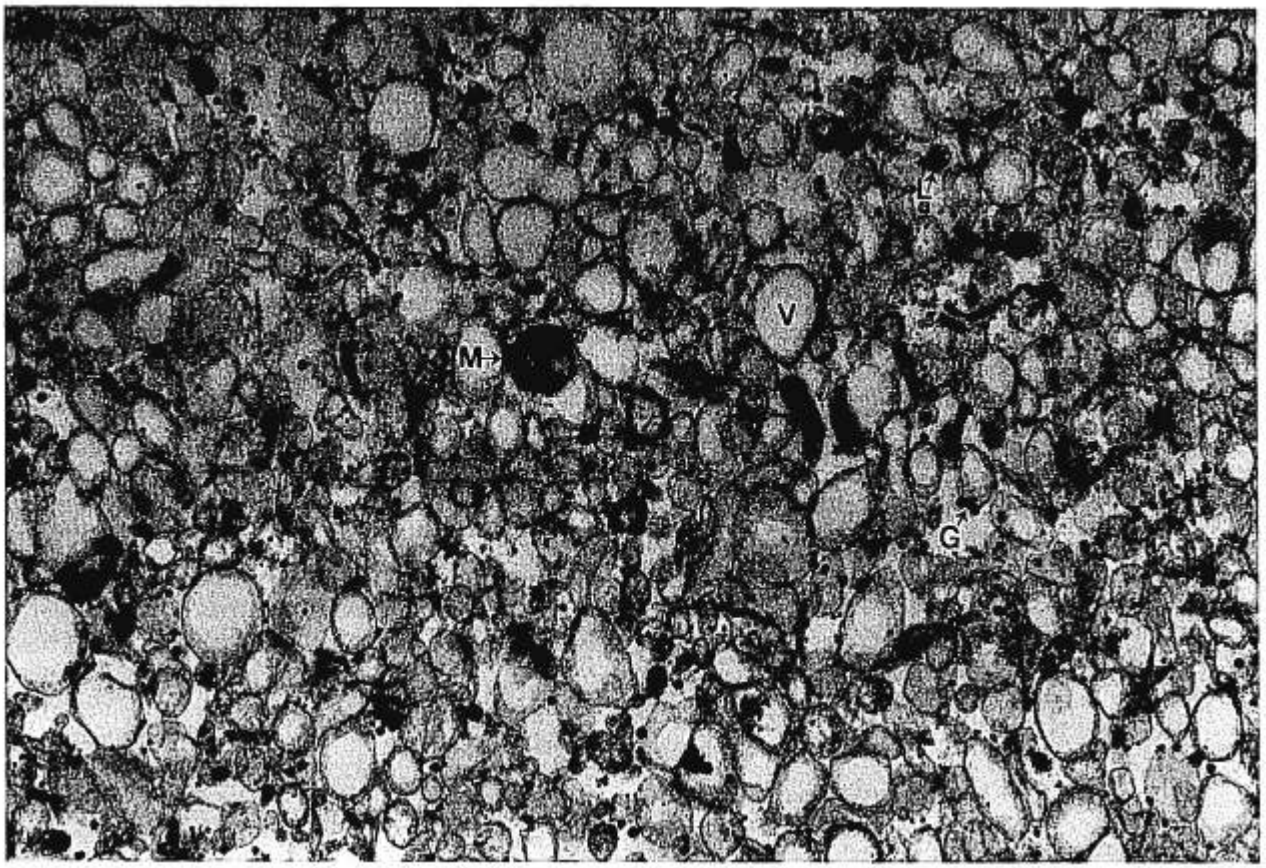

FIG. 3. Sarcolemmal vesicles isolated from bovine heart by immersion fixation technique. The following cellular details are apparent: V = membrane vesicle; the differentiation between sarcolemmal or sarcoplasmic reticulum vesicles is impossible. M = mitochondria with cristae and a double membrane; G = glycogen droplets; L = lysosome. (Magnification ×25,000).

protein; a gradual decrease in uptake (from 8,530 to 403 μmol/mg) was observed. In the final step, uptake values were almost as low as the background readings, indicating little sarcoplasmic reticulum contamination. [^{3}H]nitrendipine binding was gradually increased, with maximal binding capacity in the final preparation step (from 50 to 722 fmol/mg). These binding and uptake studies are also in agreement with those of Brandt,[26] who demonstrated that cardiac sarcoplasmic reticulum does not contain nitrendipine binding sites, and that any binding of [^{3}H]nitrendipine results from the presence of sarcolemmal vesicles.

EQUILIBRIUM BINDING EXPERIMENTS

[^{3}H]nitrendipine binds in a reversible and saturable manner to the sarcolemmal membranes. Table 1 shows that in seven experiments with each volatile anesthetic on seven different sarcolemmal preparations, halothane, isoflurane, and enflurane decreased [^{3}H]nitrendipine-specific binding to the sarcolemma. At a [^{3}H]nitrendipine concentration of 0.01 nM, the total binding values were low and approximated the nonspecific binding. At a [^{3}H]nitrendipine concentration of 0.05–1 nM, halothane produced in each individual experiment a significant depression of binding, in the range of 58–66% ($P < 0.05$ for 0.05 nM and 0.1 nM and $P < 0.025$ for 0.5 and 1 nM) (fig. 4). Isoflurane produced 29–38% depression and enflurane 5–22% depression; neither was statistically significant. Depression of binding did not always occur with

TABLE 1. Effect of Volatile Anesthetics on [^{3}H]-Nitrendipine-specific Binding

[^{3}H]NTP (nM)	Control	Halothane	Isoflurane	Enflurane
0.01	8.6 ± 2.4	7.7 ± 3.3	7.3 ± 2.0	8.0 ± 5.0
0.05	21.4 ± 5.5	8.2 ± 3.0*	21.0 ± 10.0	14.2 ± 5.0
0.10	43.0 ± 12.0	18.0 ± 6.5*	28.0 ± 8.0	41.0 ± 16.0
0.50	116.5 ± 22.0	39.0 ± 10.0†	72.0 ± 28.0	96.0 ± 34.0
1.00	199.0 ± 33.0	82.0 ± 14.0†	141.0 ± 42.0	155.0 ± 62.0

Mean ± standard error.
n = 7 experiments with each anesthetic.
* $P < 0.05$; † $P < 0.025$ compared to control.

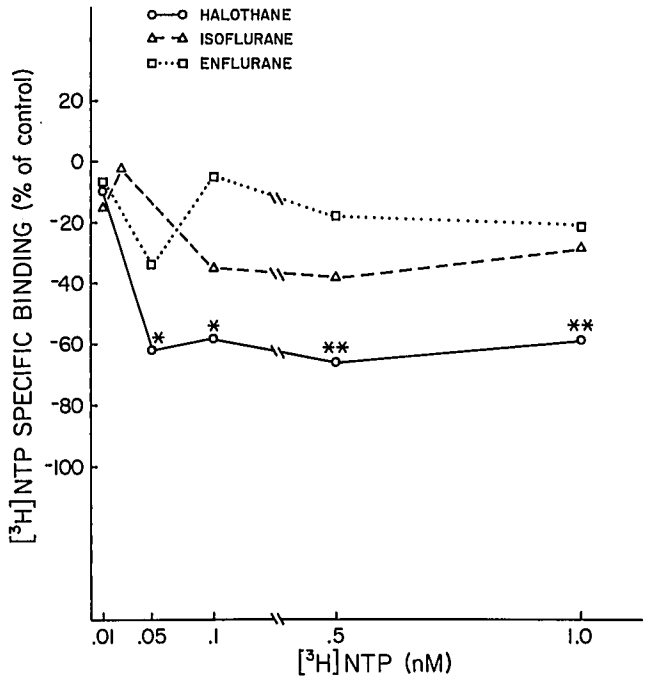

FIG. 4. Volatile anesthetics depression of [^{3}H]-nitrendipine-specific binding to bovine cardiac sarcolemma, as a function of [^{3}H]-nitrendipine concentration. The data are expressed as percentage of control (without the anesthetics). Each point represents a triplicate measurement of seven experiments with each of the volatile anesthetics, 1.9% halothane, 2.3% isoflurane and 4.8% enflurane.

isoflurane and enflurane, but when it did occur it was less than that with halothane. Comparison of the effect of the three volatile anesthetics showed no significant difference among them, probably because of the variability in binding capacity of the different sarcolemmal preparations and the inconsistent effect shown by isoflurane and enflurane.

In another series of six dose–response studies, with increasing halothane concentrations of 0.78, 1.33, 1.90, and 2.57 vol%, the anesthetic produced increasing depression of [^{3}H]nitrendipine-specific binding (fig. 5). A mean decrease of 25–55% in binding was observed ($P < 0.05$ for 1.33% and $P < 0.01$ for 1.90 and 2.57% halothane).

We investigated the mechanism of the depression of the volatile anesthetics on [^{3}H]nitrendipine binding. Figures 6A and 6B illustrate typical Scatchard analyses plotted from one of the four additional experiments performed with each anesthetic using a [^{3}H]nitrendipine concentration range of 0.075 to 2.0 nM. [^{3}H]nitrendipine binding shows a single component of high- affinity binding, represented by the straight line. Values for pooled results (table 2) in the control group (n = 10) were B_{max} = 476 ± 69 fmol · mg^{-1} protein and Kd = 0.66 ± 0.2 nM. A trend toward overall reduction in the number of the binding sites was observed under the volatile anesthetic effect. Increase in K_D, which is equivalent to reduced affinity, was observed only with halothane and isoflurane; enflurane produced a net decrease in Kd values. However, both isoflurane and enflurane showed a large variability in calculated B_{max} and K_D values. Only with halothane were we able to demonstrate in each experiment a nonsignificant but consistent decrease in the number of binding sites and in affinity.

[^{3}H]Nitrendipine Binding Kinetics

Association Binding

The binding of [^{3}H]nitrendipine to the sarcolemmal membranes was measured as a function of time, in the presence and absence of halothane (fig. 7). The nonspecific binding was identical over the whole time range. Binding increased rapidly during the first 15 min, then approached a plateau by 60 min. In the presence of halothane the rate was significantly reduced within 5 min; the mean decrease was approximately 38% in the specific binding at plateau. The half-life for the development of anesthetic-induced depression in binding was close to 5 min. These results are in accordance with the findings of the equilibrium binding studies in which halothane reduced the affinity of binding.

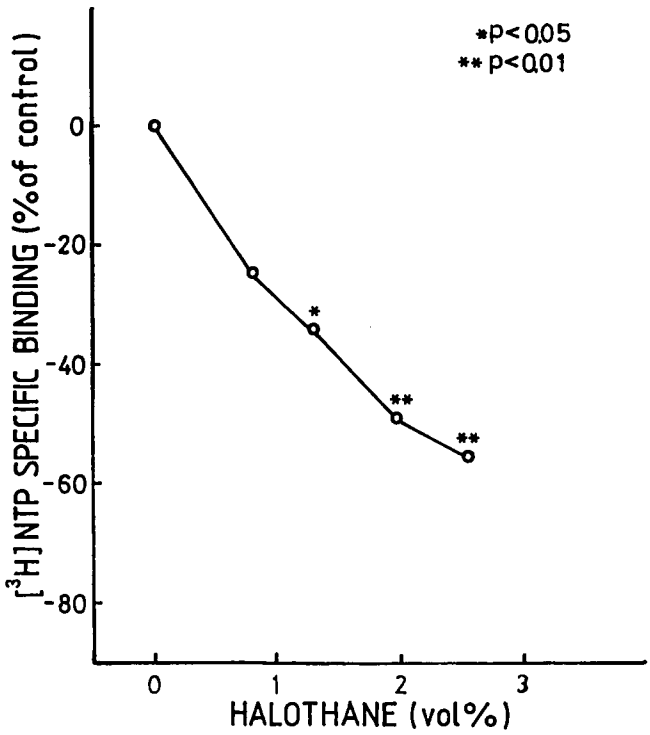

FIG. 5. Dose-dependent depression of [^{3}H]-nitrendipine-specific binding by increasing concentrations of halothane. The data are expressed as percentage of control. Each point represents a triplicate measurement of six experiments.

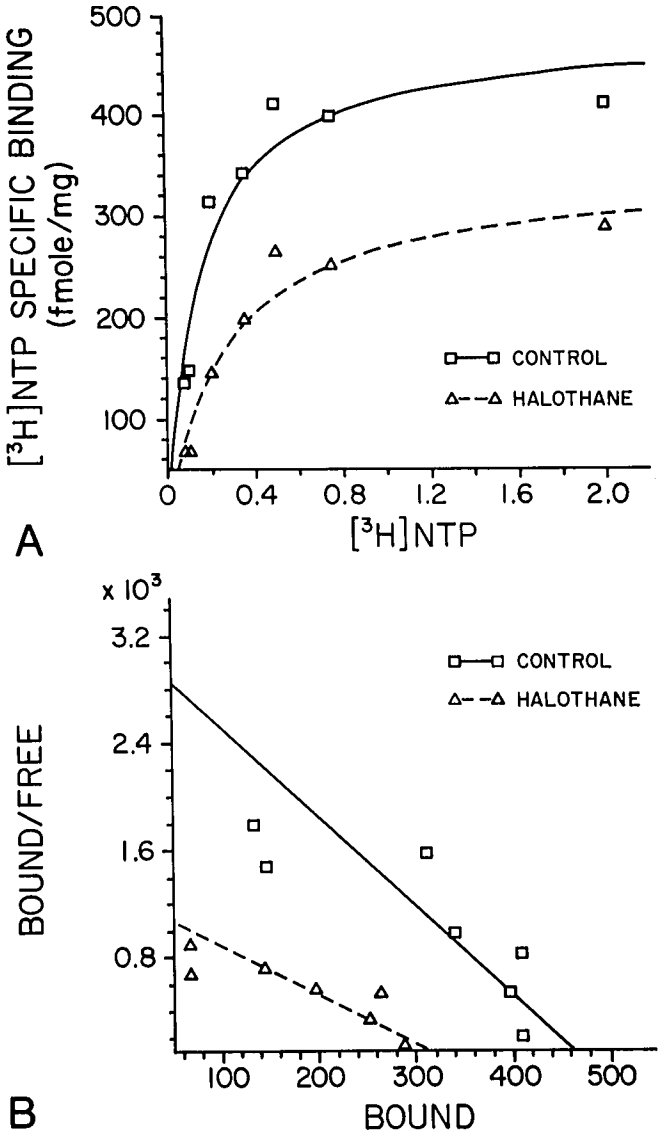

FIG. 6. (*A*) Saturation of binding of [^{3}H]-nitrendipine to bovine cardiac sarcolemma. Specific binding is plotted in the presence or absence of 1.9% halothane. (*B*) Scatchard analysis of [^{3}H]-nitrendipine binding with and without halothane. Free concentrations of [^{3}H]-nitrendipine varied from 0.075 to 2 nM. The plot was obtained by linear regression. The dissociation constant (Kd) was derived from the slope of the curve. The maximal binding capacity (B_{max}) was calculated from the intercept of the plot with x-axis. For this experiment Kd was 0.15 nM for control and 0.27 nM with halothane, and B_{max} was 478 and 342 fmol/mg, respectively.

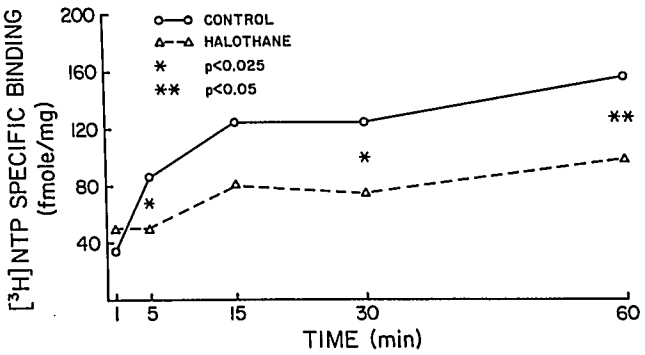

FIG. 7. Time course for association of 1 nM [^{3}H]-nitrendipine to the sarcolemma, with and without 1.9% halothane. In each data point the specific binding was calculated as explained in the text and represents the mean of triplicate measurements in each of the experiments. The association reaction is markedly depressed by halothane within the first 5 min.

Dissociation Kinetics

In these experiments we evaluated the dissociation rate constant of the [^{3}H]nitrendipine from the sarcolemma, when displaced by unlabeled nitrendipine (fig. 8). Cold nitrendipine was added, with and without halothane, to membranes that already had been equilibrated for 60 min with [^{3}H]nitrendipine alone. Immediately after addition of the unlabeled nitrendipine, 38% of the [^{3}H]nitrendipine was displaced; we were unable to resolve this phase of displacement because of its rapidity. In the control studies, without the anesthetic, a further mean displacement of 35% occurred over the next 30 min, compared to 54% in the presence of halothane ($P < 0.05$ after 15 min). The calculated mean k_{-1} values were $1.5 \cdot 10^{-2}$ min^{-1} in the control studies and $2.5 \cdot 10^{-2}$ min^{-1} with the halothane. These calculations excluded the data at 60 min, in which an unexpected change in dissociation kinetics occurred. This change might be explained by membrane degradation from the long incubation period of 120 min. However, even when the 60-min data are included, the effect of halothane remains the same, a 60–70% increase in the dissociation rate constant, consistent with the reduced affinity demonstrated in the equilibrium studies in the presence of halothane.

Both types of kinetic studies demonstrate the revers-

TABLE 2. Binding Properties of [^{3}H]-Nitrendipine

Anesthetic	B_{max} (fmol/mg)			Kd (nM)		
	Control	Anesthetic	Change (%)	Control	Anesthetic	Change (%)
Halothane	498 ± 71	355 ± 76	−29	0.40 ± 0.10	0.52 ± 0.10	+30
Isoflurane	578 ± 102	438 ± 124	−24	0.39 ± 0.01	0.53 ± 0.10	+36
Enflurane	720 ± 60	327 ± 149	−55	0.92 ± 0.12	0.42 ± 0.10	−54

Mean ± standard error.
n = 4 experiments with each anesthetic.

B_{max} = maximal number of binding sites; Kd = dissociation constant.
P value not significant.

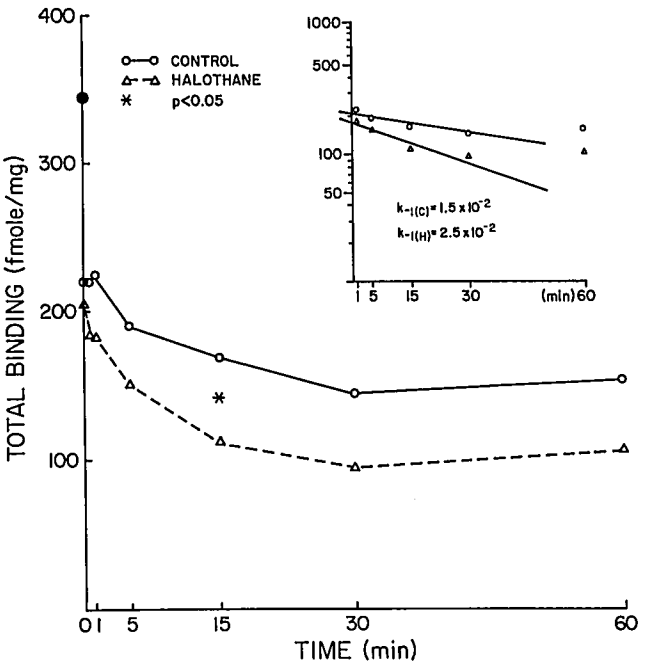

FIG. 8. Time course of dissociation of 1 nM [^{3}H]-nitrendipine from bovine heart sarcolemmal membranes. Dissociation was initiated in equilibrium (after 60 min incubation at 25° C) by addition of 500 nM unlabeled nitrendipine, with or without the addition of 1.9% halothane. Data shown are mean values of four experiments. The time 0 point (filled circle) represents triplicate measurements in each experiment of total [^{3}H]-nitrendipine binding prior to its displacement. (*Insert*) The linear transformation of the kinetic data. The lines were fitted by linear regression analysis of the 1—30-min point. The data show the increase in the dissociation rate constant in 1.9% halothane ($k_{-1(H)}$) compared to control ($k_{-1(C)}$). The maximum depression of halothane on nitrendipine binding was observed by the 15-min point, and half-maximum effect was observed within 5 min.

ibility and saturability of [^{3}H]nitrendipine binding to the purified sarcolemmal membranes.

Discussion

During the normal, rhythmic action potentials of the myocardial cell, the voltage-sensitive Ca^{2+} channels continuously oscillate among the resting, open, and inactivated states, to which the Ca^{2+} channel blockers show different binding affinities.[11] The 1,4-dihydropyridines bind preferentially to channels in the inactivated state, in which the channel is closed and unavailable for stimulation.[11,27,28] In the absence of depolarizing activity, as in isolated muscle membrane preparations, the bulk of the channels are in the inactivated state, unlike the situation in intact cells, which contain more channels in the open or resting states. The absence of membrane potential and protein phosphorylation may convert the channel into a high affinity binding state.[11,27] Thus, isolated muscle membranes tend to have single-site high-affinity binding characteristics, whereas intact cells may reveal low-affinity and/or high-affinity binding sites, depending upon the state of the channel and membrane potential.[11,29] Furthermore, the various Ca^{2+} channel blockers show different preferences to the different states of the channel. Nitrendipine, for example, produces maximal blockade with the first current, which might represent binding of the drug to the low-affinity, activated Ca^{2+} channels (open and resting states).[30]

In the current study, using Scatchard analysis, we have shown that [^{3}H]nitrendipine binding produces a linear plot, which implies a single class of high-affinity binding sites in the sarcolemmal membranes. A mean binding capacity of 476 ± 69 fmol · mg^{-1} and a dissociation constant of 0.66 ± 0.2 nM both are similar to those described in other studies for high-affinity binding sites in isolated cardiac sarcolemma.[22]

Equipotent doses of the volatile anesthetics produced depression of nitrendipine binding to the isolated cardiac sarcolemma. These equilibrium binding studies, using increasing concentrations of [^{3}H]nitrendipine, enabled quantification of the anesthetic-induced changes in binding properties. The decrease in Ca^{2+} channel blocker binding, which might be associated with reduction in inward Ca^{2+} influx, showed dose dependence to increasing concentrations of halothane. Similar findings were reported previously[12] at a lower concentration of halothane (0.39 vol%), compared to concentrations used in the current study (0.78–2.57 vol%). These results signify that in isolated membranes (as in the current study), alterations produced by the volatile anesthetics in the high-affinity binding state correlate with the anesthetic-induced decrease in Ca^{2+} influx through the channel. However, at the current time, the low affinity binding state of the channel cannot be as readily studied in a biochemical system.[31] The possibility that the channels have reverted to a resting state by volatile anesthetic exposure cannot be excluded. However, this assumption seems unlikely, since both Ca^{2+} influx and Ca^{2+} transients are decreased by halothane, suggesting that fewer open channels are available.

In order to correlate the volatile anesthetic effect on binding properties to the negative inotropic effect, we first have to define what type of preparation is used. When the transmembrane potential gradient is minimized, as in the isolated membranes, the shift to the inactivated state produces a 100-fold higher sensitivity to the blocking activity of the dihydropyridines[29] compared to intact cells. Some investigators suggest that in the intact myocyte, the low-affinity site, representing primarily the resting channel, is more closely associated with the pharmacologic action of the drug.[30,32] In one study on embryonic chick ventricular cells,[32] the investigators used a correlation between the time course of association and dissociation of

nitrendipine and the time course of development of its negative inotropic effect, in order to show that low-affinity binding sites in the intact cell were related to the negative inotropic effect of the dihydropyridine.

In the current study we have also shown that the time required for the half-maximum halothane effect on association and dissociation of [^{3}H]nitrendipine was about 5 min. This time interval approximates that shown in other experiments, in which a depression of aequorin light intensity due to decrease in intracellular Ca^{2+} transients was demonstrated. However, these studies were performed at 37° C on cat papillary muscle exposed to halothane.[3] In order to correlate binding studies to clinically relevant situations, further studies should be performed, in which the effect of the volatile anesthetics on dihydropyridine binding to intact depolarized myocytes is tested.

We hypothesize that the mechanism of the electrophysiologic changes in the Ca^{2+} channel under the influence of volatile anesthetics may well be the same as that which causes reduction in binding of [^{3}H]nitrendipine. These changes are probably also responsible for the decrease in Ca^{2+} influx into the myocyte and the parallel reduction in the force of contraction. All three volatile anesthetics—halothane, isoflurane and enflurane—seem to produce a reduction in the number of available binding sites, but the influence on binding affinity was varied among the anesthetics. No data are available to distinguish between alteration in the conformation of the protein molecules of the channel and the changes occurring in the membrane lipid bilayer itself. On the basis of the current knowledge, we cannot determine if the volatile anesthetics have a direct action on the channel protein itself, resulting in altered drug binding and prevention of Ca^{2+} influx, or if their action is mediated *via* the membrane lipid, resulting in an indirect alteration of the channel structure and behavior.

Despite the uncertainty in the site of action, the result of sarcolemmal membrane exposure to halothane appears to be an alteration in the high-affinity, inactivated state of the channel, such that it is still inactivated but that the binding of [^{3}H]nitrendipine becomes less favorable. As we stated above, this does not appear to be a reversion to the open or resting state but rather an alteration in the [^{3}H]nitrendipine binding site.

Interpreting the results with Scatchard plots is also not free of pitfalls since any small shift in the consistency of the data is widely magnified by this type of analysis.[25] Furthermore, any experimental artifact, such as contamination of bound ligand by free ligand, or ligand or receptor degradation, will produce a curvilinear plot, which might be incorrectly interpreted as receptor heterogeneity.[33] In addition, because of the high sensitivity, this method requires a high level of purification of the binding sites.

Electrophysiologic studies have clarified some aspects of the volatile anesthetic effect on the Ca^{2+} channels in the sarcolemma, as part of the overall mechanism for negative inotropism. In a series of studies on guinea pig papillary muscle,[1,34,35] Lynch and co-workers showed that halothane caused significant depression of the maximum rate of rise ($\dot{V}_{max}$) of the slow action potentials (the potentials elicited by slow inward calcium current), whereas enflurane and isoflurane depressed the $\dot{V}_{max}$ to a lesser extent. These findings are in agreement with our results, in which we demonstrated a significant dose-dependent depression in nitrendipine binding with halothane, while isoflurane and enflurane produced depression, albeit smaller and not always consistent, on the Ca^{2+} channels. Another recent study[36] confirms our current results. However, because of the absence of statistical analysis, or comparison of binding capacity and affinity of the three volatile anesthetics, the latter study does not demonstrate quantitatively the differences that exist between the various anesthetics in their effect on the sarcolemma.

Studies of intact ventricular muscle suggest that the volatile anesthetics produce a decrease in myofibrillar responsiveness to calcium.[37] However, two studies of the effect of the volatile anesthetics on skinned fibers have resulted in differing conclusions. Su and Kerrick[38] noted a minimal reduction in tension and slight decrease in calcium sensitivity in fibers obtained from mechanically disrupted myocardium, whereas Murat *et al.*[39] noted a substantial halothane-induced decrease in calcium sensitivity and in maximum activated force in chemically skinned fibers.

Shibata *et al.*[40] showed that in barium-induced dynamic stiffness, the volatile anesthetics do not alter the normal actin–myosin crossbridge cycling kinetics. However, the high-frequency dynamic stiffness was reduced, indicating a decrease in the number of cycling crossbridges involved in tension generation. A very similar effect to that produced by the volatile anesthetics has been observed with the calcium channel blocker, nifedipine.[41] By demonstrating that the changes induced in dynamic stiffness are not related to reuptake of barium into the sarcoplasmic reticulum[42] and that both the effects of the volatile anesthetics and nifedipine can be reversed by increasing extracellular barium concentration, the authors suggest that derangement of sarcolemmal Ca^{2+} transport may well be one mechanism by which the volatile anesthetics depress myocardial contractility.

This effect on the Ca^{2+} channels by the volatile anesthetics is not limited to the heart. In a series of studies on rat brain membranes,[43] we have demonstrated a very similar effect on the L-type Ca^{2+} channels in the brain; this suggests that volatile anesthetics might also interfere with Ca^{2+} regulation of neuronal function as part of their overall pharmacologic effect.

In conclusion, many electrophysiologic studies have emphasized the importance of sarcolemmal mechanisms in the negative inotropism induced by the volatile anesthetics. In the current study we have demonstrated the depressant effect of the volatile anesthetics on the binding of dihydropyridines to the voltage-sensitive Ca^{2+} channels and on the kinetics of such binding as part of the mechanism of reduction in myocardial contractility. The inhibitory effect of the volatile anesthetics on the Ca^{2+} channels in various excitable cells deserves further study on functionally reconstructed Ca^{2+} channels. This kind of study will broaden our understanding of the molecular basis of myocardial depression induced by volatile anesthetics and of the mechanism of anesthesia in general.

The authors wish to thank Dr. D. Wheeler for helpful discussion and review of the manuscript; Dr. R. Royall, Professor of Biostatistics, for counsel on statistical analysis; Ms. J. Fisher for expert editorial assistance; Ms. C. Sheeler for secretarial assistance; and Halocarbon Laboratories (Hackensack, NJ) for donating the thymol-free halothane.

References

1. Lynch C III: Differential depression of myocardial contractility by halothane and isoflurane in vitro. ANESTHESIOLOGY 64:620–631, 1986
2. Rusy BF, Komai H: Anesthetic depression of myocardial contractility: A review of possible mechanisms. ANESTHESIOLOGY 67: 745–766, 1987
3. Bosnjak ZJ, Kampine JP: Effects of halothane on transmembrane potentials, Ca^{2+} transients, and papillary muscle tension in the cat. Am J Physiol 251:H374–H381, 1986
4. Terrar DA, Victory JGG: Isoflurane depresses membrane currents associated with contraction in myocytes isolated from guinea pig ventricle. ANESTHESIOLOGY 69:742–749, 1988
5. Lynch C III: Combined depressant effects of diltiazem and volatile anesthetics on contractility in isolated ventricular myocardium. Anesth Analg 67:1036–1046, 1988
6. Chelly JE, Rogers K, Hysing ES, Taylor A, Hartley C, Merin RG: Cardiovascular effects of and interaction between calcium blocking drugs and anesthetics in chronically instrumented dogs: I. Verapamil and halothane. ANESTHESIOLOGY 64:560–567, 1986
7. Snyder SH: Drug and neurotransmitter receptors: New perspectives with clinical relevance. JAMA 261:3126–3129, 1989
8. Nilius B, Hes P, Lansman JB, Tsien RW: A novel type of cardiac calcium channel in ventricular cells. Nature 316:443–446, 1985
9. Reiter M: Calcium mobilization and cardiac inotropic mechanisms. Pharmacol Rev 40:189–207, 1988
10. Miller RJ: Multiple calcium channels and neuronal function. Science 235:46–52, 1987
11. Janis RA, Scriabine A: Sites of action of Ca^{2+} channel inhibitors. Biochem Pharmacol 32:3499–3507, 1983
12. Blanck TJJ, Runge S, Stevenson RL: Halothane decreases calcium channel antagonist binding to cardiac membranes. Anesth Analg 67:1032–1035, 1988
13. Jones LR, Besch HR, Fleming JW, McConnaughey MM, Watanabe AM: Separation of vesicles of cardiac sarcolemma from vesicles of cardiac sarcoplasmic reticulum. J Biol Chem 254:530–539, 1979
14. Caroni P, Zurini M, Clark A, Carafoli E: Further characterization and reconstitution of the purified Ca^{2+} pumping ATPase of heart sarcolemma. J Biol Chem 258:7305–7310, 1983
15. Miller MS, Gandolfi AJ: A rapid sensitive method for quantifying enflurane in whole blood. ANESTHESIOLOGY 51:542–544, 1979
16. Blanck TJJ: A simple closed system for performing biochemical experiments at clinical concentrations of volatile anesthetics. Anesth Analg 60:435–436, 1981
17. Eger EI, II, Saidman LJ, Brandstater B: Temperature dependence of halothane and cyclopropane anesthesia in dogs: Correlation with some theories of anesthetic action. ANESTHESIOLOGY 26: 764–770, 1965
18. Bean BF: Nitrendipine block of cardiac calcium channels: High-affinity binding to the inactivated state. Proc Natl Acad Sci USA 81:6388–6392, 1984
19. Boles RG, Yamamura HI, Schoemaker H, Roeske WR: Temperature-dependent modulation of [^{3}H]nitrendipine binding by the calcium channel antagonists verapamil and diltiazem in rat brain synaptosomes. J Pharmacol Exp Ther 229:333–339, 1984
20. Schilling WP, Drewe JA: Voltage-sensitive nitrendipine binding in an isolated sarcolemma preparation. J Biol Chem 261:2750–2758, 1986
21. Schoemaker H, Itoga E, Boles RG, Roeske WR, Ehlert FJ, Kito S, Yamamura HI: Temperature dependence and kinetics of [^{3}H]nitrendipine binding in the rat brain, Nitrendipine. Edited by Scriabine A, Vanov S, Deck K. Baltimore, Urban and Schwarzenberg, 1984, pp 196–204
22. Sarmiento JG, Janis RA, Colvin RA, Triggle DJ, Katz AM: Binding of the calcium channel blocker nitrendipine to its receptor in purified sarcolemma from canine cardiac ventricle. J Mol Cell Cardiol 15:135–137, 1983
23. Gould RJ, Murphy KMM, Snyder SH: Tissue heterogeneity of calcium channel antagonist binding sites labeled by [^{3}H]nitrendipine. Mol Pharmacol 25:235–241, 1984
24. Waud DR: A primer on "Grind on Bind". Anesth Analg 67:1025–1031, 1988
25. Munson PJ, Rodbard D: Number of receptor sites from Scatchard and Klotz graphs: A constructive critique. Science 220:979–981, 1983
26. Brandt N: Identification of two populations of cardiac microsomes with nitrendipine receptors: Correlation of the distribution of dihydropyridine receptors with organelle specific markers. Arch Biochem Biophys 242:306–319, 1985
27. Reuter H: Calcium channel modulation by neurotransmitters, enzymes and drugs. Nature 301:569–574, 1983
28. Sanguinetti MC, Kass RS: Voltage-dependent block of calcium channel current in the calf cardiac purkinje fiber by dihydropyridine calcium channel antagonists. Circ Res 55:336–348, 1984
29. Janis RA, Sarmiento JG, Maurer SC, Bolger GT, Triggle DJ: Characteristics of the binding of [^{3}H]nitrendipine to rabbit ventricular membranes: Modification by other Ca^{2+} channel antagonists and by the Ca^{2+} channel agonist Bay K 8644. J Pharmacol Exp Ther 231:8–15, 1984
30. Lee KS, Tsien RW: Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells. Nature 302:790–794, 1983
31. Brush KL, Perez M, Hawkes MJ, Pratt DR, Hamilton SL: Low affinity binding sites for 1,4-dihydropyridines in mitochondria and in guinea pig ventricular membranes. Biochem Pharmacol 36:4153–4161, 1987
32. March JD, Loh E, Lachance D, Barry WH, Smith TW: Relationship of binding of a calcium channel blocker to inhibition of con-

traction in intact cultured embryonic chick ventricular cells. Circ Res 53:539–543, 1983

33. Kermode JC: The curvilinear Scatchard plot: Experimental artifact or receptor heterogeneity? Biochem Pharmacol 38:2053–2060, 1989
34. Lynch C, III, Vogel S, Sperelakis N: Halothane depression of myocardial slow action potentials. ANESTHESIOLOGY 55:360–368, 1981
35. Lynch C, III, Vogel S, Pratila G, Sperelakis N: Enflurane depression of myocardial slow action potentials. J Pharmacol Exp Ther 222:405–409, 1982
36. Nakao S, Hirata H, Kagawa Y: Effects of volatile anesthetics on cardiac calcium channels. Acta Anaesthesiol Scand 33:326–330, 1989
37. Housmans PR, Murat I: Comparative effects of halothane, enflurane, and isoflurane at equipotent anesthetic concentrations on isolated ventricular myocardium of the ferret: I. Contractility. ANESTHESIOLOGY 69:451–463, 1988
38. Su JY, Kerrick WGL: Effects of halothane on Ca^{2+}-activated tension development in mechanically disrupted rabbit myocardial fibers. Pfluegers Arch 375:111–117, 1978
39. Murat I, Ventura-Clapier R, Vassort G: Halothane, enflurane, and isoflurane decrease calcium sensitivity and maximal force in detergent-treated rat cardiac fibers. ANESTHESIOLOGY 69: 892–899, 1988
40. Shibata T, Blanck TJJ, Sagawa K, Hunter W: The effect of halothane, enflurane, and isoflurane on the dynamic stiffness of rabbit papillary muscle. ANESTHESIOLOGY 70:496–502, 1989
41. Chung OY, Blanck TJJ, Berman MR: Depression of myocardial force and stiffness without change in crossbridge kinetics: Effects of volatile anesthetics reproduced by nifedipine. ANESTHESIOLOGY 71:444–448, 1989
42. Drenger B, Blanck TJJ: The effect of Ba^{2+} on rabbit cardiac sarcoplasmic reticulum. J Mol Cell Cardiol 20:S 18, 1988
43. Drenger B, Casella ES, Tierney M, Blanck TJJ: Volatile anesthetic depression of calcium channels in brain membranes. (abstract). ANESTHESIOLOGY 71:A591, 1989