

Halothane Depresses D600 Binding to Bovine Heart Sarcolemma

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Volatile anesthetics exert their negative inotropic effects by interfering with Ca^{2+} homeostasis in the myocardial cell. The mechanism of this dose-dependent action is uncertain. ^3H -D600 (^3H -Gallopamil), a Ca^{2+} -channel antagonist, binds to the voltage-dependent Ca^{2+} channels (VDCC) in a specific, saturable, and reversible manner. We used this ligand to study the effect of halothane on the binding characteristics of the VDCC in purified bovine heart sarcolemma. Cardiac sarcolemmal vesicles were isolated from fresh bovine heart by differential centrifugation and filtration. ^3H -D600 equilibrium binding assays were performed in the presence or absence of 1.0 mM unlabeled D600 to determine total and nonspecific binding in room air and at 0.7, 1.3, and 2.5% (vol/vol) halothane. Halothane produced a significant dose-dependent and reversible depression of ^3H -D600 specific binding in bovine heart sarcolemma. Depression was completely reversed when halothane had evaporated from the samples prior to filtration. Halothane 1.3% (vol/vol) produced a 40% reduction in the maximum binding capacity. The dissociation constant was not affected by any concentration of halothane. One mechanism by which the volatile anesthetics may induce negative inotropism is through the reduction of functional VDCCs in the heart, leading to reduction of Ca^{2+} entry. The results of this study support this hypothesis. (Key words: Anesthetics, volatile; halothane. Ions, calcium; calcium-channel blocking drugs. Muscle, cardiac; sarcolemma. Pharmacology, calcium-channel blocking drugs: D600; Gallopamil.)

RECENT STUDIES have shown that the Ca^{2+} current resulting from depolarization of the myocardium is reduced by halothane.¹⁻³ This current is due to the flow of Ca^{2+} through the long-lasting (L)-type voltage-dependent Ca^{2+} channel (VDCC) on the sarcolemmal membrane. L-type VDCCs are sensitive to the organic Ca^{2+} -channel blockers, which have no effect on two other well-defined types of Ca^{2+} channels, designated high-threshold (N) and transient (T) channels. Recent work in our laboratory has implicated the L-type VDCC as a likely site of alteration by the volatile anesthetic halothane: halothane inhibited the binding of the dihydropyridine ^3H -nitrendipine to the L-type VDCC in crude membrane preparations from both

rabbit and rat myocardium⁴ and from purified bovine cardiac sarcolemma.⁵ In both of these studies the inhibition of binding was dose-dependent and reversible, consistent with any proposed anesthetic mechanism of action.

The VDCC is a multi-subunit membrane-bound protein with at least five covalently associated subunits. The α_1 subunit is a 175-kD protein that contains the Ca^{2+} antagonist binding sites and cyclic adenosine monophosphate-dependent phosphorylation sites, and is proposed to be the central ion channel-forming component of the complex.⁶ It has been shown that the organic Ca^{2+} channel blockers, the dihydropyridines and the phenylalkylamines, both bind to the α_1 subunit.⁷ Although diverse in structure, these two classes of drugs share several fundamental characteristics. They block not only the slow inward Ca^{2+} current but also the outward currents through Ca^{2+} channels⁸ and leave the activation of unblocked channels essentially unchanged, suggesting that these drugs act *via* true channel blockade. The inhibitory effects of these drugs show competition with extracellular Ca^{2+} ,⁹ and therefore act as true Ca^{2+} antagonists. Furthermore, the blockade of Ca^{2+} channel currents in functional cells parallel radioligand binding in isolated membranes.⁸ The dihydropyridines and phenylalkylamines bind at allosterically coupled but distinctly different sites on the α_1 subunit; *i.e.*, they lack classical competitive inhibition characteristics.⁷

We therefore reasoned that halothane, which is a small molecule of molecular weight 197 d, might decrease the Ca^{2+} current in three possible ways: 1) it could locate in the channel and directly obstruct the movement of Ca^{2+} through the channel; 2) it could bind to the protein at a site distinct from the channel, resulting in a dysfunction of channel kinetics and/or conformation; or 3) it could permeate the membrane lipid bilayer and alter the mobility, environment, and possibly the exposure of the channel within the membrane. We hypothesized that since halothane is a small molecule relative to the size of the α_1 subunit, and since D600 and nitrendipine bind to separate sites on the α_1 subunit, inhibition of binding of these two Ca^{2+} channel blockers would occur only if halothane were interfering with the lipid environment surrounding the protein or were acting on the protein at a site distinct from the channel, resulting in a global alteration of the protein.

Since we had already characterized halothane interaction at the dihydropyridine site, we studied the effects

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Received from the Department of Anesthesiology and Critical Care Medicine, Division of Cardiac Anesthesia, The Johns Hopkins Medical Institutions, Baltimore, Maryland. Accepted for publication August 12, 1991. Supported by grant GM 30799 from the National Institutes of Health. Presented in part at the annual meeting of the American Society of Anesthesiologists, Las Vegas, Nevada, October 1990 and at the Baltimore Muscle Physiology Symposium, Baltimore, Maryland, September, 1990.

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of halothane on the binding of the phenylalkylamine ligand ^3H -D600 (^3H -Gallopamil) to purified bovine cardiac sarcolemma to see if volatile anesthetics can alter the VDCC at a second distinct site. We quantified the effect of halothane on the maximum binding capacity (B_{max}) and dissociation constant (K_d) of ^3H -D600 to purified bovine sarcolemma. The effect of halothane on the time course for dissociation and association of ^3H -D600 to purified bovine sarcolemma was also studied.

Materials and Methods

PREPARATION OF SARCOLEMMMA

Bovine heart was obtained from a local slaughterhouse and was put on ice within 5 min of the time the animal was killed. The entire procedure was carried out on ice using 0–4° C cold buffers. The isolation of the sarcolemmal membranes was performed by differential centrifugation and filtration using a modification of the method of Jones *et al.*¹⁰ and Caroni *et al.*¹¹ and described in detail elsewhere.⁵ Final sarcolemmal vesicles were suspended in 0.25 M sucrose and 10 mM histidine and stored in a –80° C freezer.

Protein concentration was determined by the Coomassie binding method¹² using bovine serum albumin as the standard. In order to evaluate the purity of the sarcolemmal preparation and to ascertain absence of contamination by sarcoplasmic reticulum, the final four stages of the sarcolemmal isolation procedure were evaluated by parallel $^{45}\text{Ca}^{2+}$ uptake in the presence of oxalate, as previously described,¹³ and by binding of the dihydropyridine, ^3H -isradipine (^3H -PN200-110). Electron microscopy of the sarcolemmal preparation was performed using the immersion-fixation staining method.

EQUILIBRIUM BINDING ASSAYS

The binding assays were carried out in 5-ml glass vials, sealed with airtight plastic caps to maintain constant anesthetic concentration. Independent measurements demonstrated that anesthetic concentrations could be held constant for at least 60 min. Fifty to one hundred micrograms of membrane protein were incubated at a constant temperature of 25° C rather than 37° C in order to slow membrane degradation. ^3H -D600 5–100 nM in 50 mM Tris HCl buffer (pH 7.5) in a final volume of 1 ml was added to the membrane preparation in the presence or absence of 1.0 mM unlabeled D600 to determine total and nonspecific binding. Sixty-minute incubations were carried out in control samples and with the addition of 0.2, 0.4, and 0.8 μl thymol-free halothane shielded from light. Halothane was added in the liquid phase using a Hamilton microsyringe. This was equivalent to halothane concentrations of 0.7, 1.3, and 2.5% (vol/vol) in the

vapor phase, respectively, as measured by ultraviolet spectroscopy³ and did not change over the course of the experiment. The measured concentrations were within 10% of calculated theoretical maximal concentrations. In a fourth set of samples, 0.8 μl halothane was added, and the vial caps were removed 30 min prior to termination of the reaction (60 min total reaction time with label) to allow evaporation of the halothane. Total evaporation was verified by ultraviolet spectroscopy. The reaction was terminated by filtering the samples under vacuum onto Whatman GF/C glass fiber filters pretreated with 0.5% polyethylenimine, 10 μM unlabelled D600, and 20 mM Tris HCl buffer, pH 7.5 in order to reduce the nonspecific binding of ^3H -D600 to the filter itself.

The filters were washed three times with 10 ml cold 20 mM Tris HCl pH 7.5 buffer and allowed to air-dry for at least 3 h. The filters were then placed in scintillation vials with 5 ml 3a70 complete scintillation cocktail (Research Products International) and counted in a Beckman LS2800 scintillation counter. Counting efficiency was at least 55%. Three experiments were performed in triplicate on each of four different sarcolemmal preparations. Specific binding was determined by subtracting nonspecific binding from total binding.

KINETIC STUDIES

Rate constants for association (k_1) and dissociation (k_{-1}) were determined to verify further the effect or lack of effect of halothane on the K_d of L-channels for ^3H -D600 as determined by equilibrium binding.

Association time course studies were initiated with the addition of the sarcolemma membranes to the reaction mixture containing 100 nM ^3H -D600 in 50 mM Tris HCl buffer (pH 7.5) with or without 1.0 mM unlabeled D600 and with or without 2.5% (vol/vol) halothane. The reaction was terminated after incubation time intervals of 2, 5, 15, 30, 60, and 90 min. The specific binding was calculated as described above.

The dissociation time course experiments were performed after 1 h incubation of the sarcolemmal membranes with 100 nM ^3H -D600 in 50 mM Tris HCl buffer (pH 7.5) in a total volume of 1 ml. Three samples were used as control values of total binding at time 0, followed by addition of 1.0 mM unlabeled D600 as a displacer. The total binding after the addition of D600 for various time intervals (5, 10, 15, 20, 25, and 30 s and 1, 2, 5, 15, 30, and 60 min) was then measured. Two experiments were performed in triplicate on each of two different sarcolemmal membrane preparations.

DATA ANALYSIS

Equilibrium binding data were analyzed by using an Enzfitter software package (Robin J. Leatherbarrow, El-

sevier Science Publishers, Amsterdam) which yields a explicitly weighted, nonlinear, least-squares fit of the data to the following equation:

$$\text{D600 bound} = (B_{\max}) \frac{[\text{D600}]_{\text{free}}}{K_d + [\text{D600}]_{\text{free}}}$$

where B_{\max} = the maximum binding capacity; K_d = the dissociation constant; and $[\text{D600}]_{\text{free}}$ = the concentration of D600 in the reaction mixture. Data was linearized by a Scatchard transformation of the above equation:

$$\frac{\text{D600 bound}}{[\text{D600}]_{\text{free}}} = -\frac{1}{K_d} (\text{D600 bound}) + \frac{B_{\max}}{K_d}$$

Kinetic binding studies were also analyzed with the Enzfitter software package using an explicitly weighted, nonlinear, least-squares fit of the data to a first-order rate equation.

Statistical analysis of the binding and kinetic studies was performed using one-way analysis of variance and paired *t* test for comparing individual experiments. Values were considered significantly different at $P < 0.05$. All data are reported as the mean \pm standard error of the mean of independent experiments.

MATERIALS

^3H -D600, specific activity 80 Ci/mmol, was purchased from New England Nuclear. Thymol-free halothane was a gift from Halocarbon Laboratories (Hackensack, NJ).

Results

SARCOLEMMA PREPARATION

The sarcolemmal preparation yielded approximately 4–5 mg purified sarcolemmal membranes per 100 g ventricular tissue. Electron microscopic evaluation revealed almost pure membrane vesicles with very few mitochondria, lysosomes, or glycogen droplets present. $^{45}\text{Ca}^{2+}$ uptake studies in the final stages of membrane preparation revealed a marked decrease in adenosine triphosphate-dependent uptake (126.37 ± 8.00 to 27.18 ± 1.24 nmol/mg protein; fig. 1). In the final stage, Ca^{2+} uptake values were not significantly different from background values. ^3H -PN200-110 specific binding was gradually increased from stage 1 to stage 4, with B_{\max} in the final preparation stage (from 4.35 ± 7.40 to 208.08 ± 37.97 fmol/mg protein; fig. 1) indicating a 50-fold purification.

EQUILIBRIUM BINDING ASSAYS

^3H -D600 binds in a reversible and saturable manner to the sarcolemmal membranes. Scatchard analysis of ^3H -D600 binding revealed a linear plot supporting the assumption of a single binding site for the ligand. Control K_d and B_{\max} were 35.22 ± 6.06 nM and 263.46 ± 17.85

fmol/mg protein respectively. Figure 2 shows the effects of increasing concentrations of $[\text{H}]\text{D600}$ on specific binding to the bovine sarcolemmal preparation under conditions of increasing concentrations of halothane. These data demonstrate a significant inhibition of binding by halothane. Halothane 0.7% (vol/vol) produced a 34% reduction in B_{\max} (173.99 ± 19.73 fmol/mg protein); 1.3% (vol/vol) produced a 40% reduction in B_{\max} (159.93 ± 20.07 fmol/mg protein), and 2.5% (vol/vol) produced a 60% reduction in B_{\max} (102.20 ± 5.89 fmol/mg protein; table 1). When the halothane was allowed to evaporate prior to membrane filtration, there was a complete recovery of B_{\max} to control values (fig. 2 and table 1). There was no significant difference in K_d at any halothane concentration (table 1).

^3H -D600 BINDING KINETICS

The binding of ^3H -D600 to the sarcolemmal membranes was measured as a function of time, in the presence and absence of 2.5% (vol/vol) halothane (fig. 3). Binding increased rapidly during the first 10 min and plateaued by 15 min. The rate constant, k_1 , was not significantly different for the halothane-treated *versus* control groups (control 0.004 ± 0.001 s $^{-1}$ · nM $^{-1}$; halothane 0.005 ± 0.001 s $^{-1}$ · nM $^{-1}$). The binding limit for the halothane-treated group was significantly decreased compared to control (control 155.41 ± 5.41 fmol/mg protein; halothane 118.64 ± 7.30 fmol/mg protein).

The dissociation rate of ^3H -D600 from the sarcolemma, when displaced by unlabeled D600, was also studied (fig.

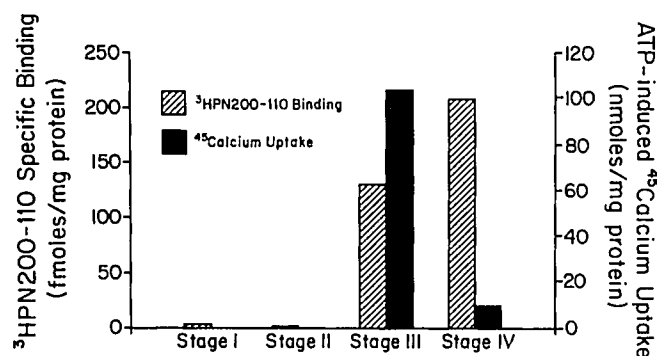
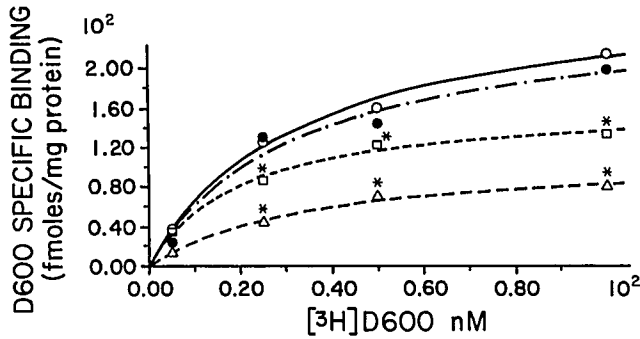
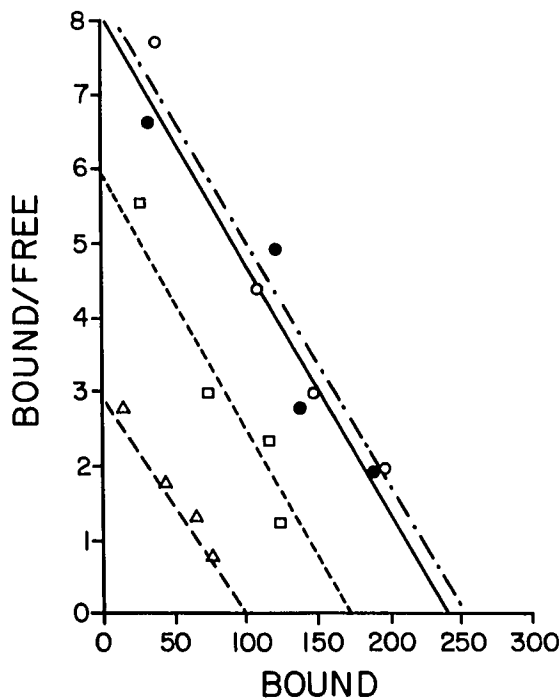


FIG. 1. PN200-110 binding and $^{45}\text{Ca}^{2+}$ uptake: serial stages of membrane preparation. Stages 1–4 represent the final four separations of the membrane preparation and demonstrate increasing purification of the SL membranes. ATP-induced $^{45}\text{Ca}^{2+}$ uptake in the presence of oxalate was used as a marker for sarcoplasmic reticulum contamination in the final stage of the sarcolemmal preparation. In the final stage (stage 4) there was a significant decrease in ATP-induced $^{45}\text{Ca}^{2+}$ uptake, which was not different from background. ^3H -PN200-110-specific binding was used as a marker for sarcolemmal purification in the final stage of the sarcolemmal preparation. Specific binding of ^3H -PN200-110 increased significantly by stage 4 of the preparation, indicating a 50-fold purification. Values represent means of triplicate measurements of three experimental preparations.



—○— Control -△- Halothane 2.5 v/v %
 -□- Halothane 1.7 v/v % -●- Halothane evaporated
 *p<0.05 compared to control



—○— Control -△- Halothane 2.5 v/v %
 -□- Halothane 1.7 v/v % -●- Halothane evaporated

FIG. 2. A: Halothane depression of ^3H -D600-specific binding to bovine cardiac sarcolemma as a function of ^3H -D600 concentration. Each point represents the mean of a triplicate measurement of four experimental preparations. B: Scatchard analysis of ^3H -D600 binding to bovine cardiac sarcolemma with and without halothane. The plot was obtained by linear regression analysis. The dissociation constant (K_d) can be derived from the slope of the curve. The maximal binding capacity (B_{\max}) can be calculated from the intercept of the plot with the x-axis. Each point represents the mean of a triplicate measurement of four experimental preparations.

4). The calculated mean dissociation constant, k_{-1} , was not significantly different between control ($0.102 \pm 0.019 \text{ s}^{-1}$) and halothane-treated samples ($0.132 \pm 0.008 \text{ s}^{-1}$). Halothane 2.5% (vol/vol) did produce a significant 35% decrease in the limit of specific binding (control 188.00

TABLE 1. K_d and B_{\max} of ^3H -D600 Binding to Bovine Cardiac Sarcolemma as a Function of Halothane Concentration

| | K_d (nM) | B_{\max} (fmol/mg protein) |
|-------------------------------|-------------------|------------------------------|
| Control | 35.22 ± 6.06 | 263.46 ± 17.85 |
| Halothane 0.7 v/v% | 35.57 ± 10.37 | $173.99 \pm 19.73^*$ |
| Halothane 1.3 v/v% | 24.42 ± 9.12 | $159.93 \pm 20.07^*$ |
| Halothane 2.5 v/v% | 30.99 ± 4.79 | $102.20 \pm 5.89^*$ |
| Halothane 2.5 v/v% evaporated | 27.57 ± 9.70 | 236.19 ± 29.42 |

B_{\max} decreased significantly ($P < 0.05$) in a reversible manner. K_d did not change significantly. Data are presented as mean \pm SEM; $N = 4$ (triplicate measurement of four experimental preparations).

* Different from control, $P < 0.05$.

$\pm 4.74 \text{ fmol/mg protein}$; halothane $121.90 \pm 1.90 \text{ fmol/mg protein}$). These results are consistent with the equilibrium binding results in which there was no change in the binding affinity and a decrease in binding capacity. Furthermore, since $K_d = k_{-1}/k_1$, the calculated K_d obtained from these kinetic studies (control 25.5 nM, halothane 26.4 nM) is in close agreement to that obtained by the equilibrium binding studies.

Discussion

We have demonstrated that ^3H -D600 binding produces a linear Scatchard plot, which implies a single class of high-affinity binding sites for D600 in the isolated sarcolemmal membranes. Control B_{\max} of $263.46 \pm 17.85 \text{ fmol/mg protein}$ and K_d of $35.22 \pm 6.06 \text{ nM}$ are similar to those of other studies¹⁴ and are reinforced by our kinetic data ($K_d = 25.5 \text{ nM}$). Halothane was shown to decrease the binding of D600 to the VDCC in bovine cardiac sarcolemma as indicated by a marked decrease in B_{\max} . This decrease in binding was dose-dependent and completely reversible.

The preparation used in this study was highly enriched sarcolemmal vesicles as observed by electron microscopy.

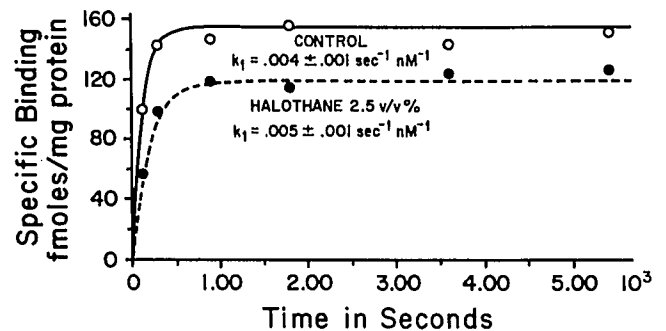


FIG. 3. Association time course of ^3H -D600 binding to bovine cardiac sarcolemma in the presence or absence of 2.5% halothane. Although the maximal binding was lower with halothane, the rate constant (k_1) was not significantly different. Each point represents the mean of a triplicate measurement of two experiments in each of two different sarcolemmal preparations.

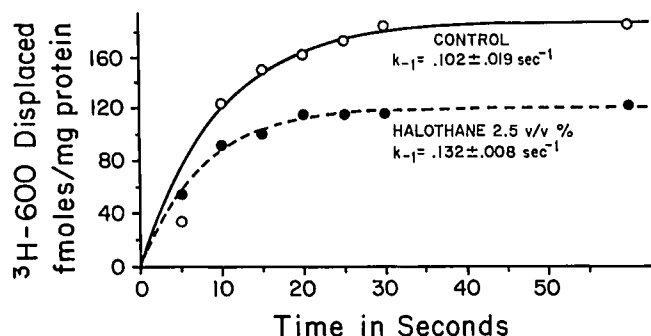


FIG. 4. Dissociation time course of ^3H -D600 binding to bovine cardiac sarcolemma by cold D600 in the presence or absence of 2.5% halothane. Although maximal binding was lower with halothane, the dissociation rate constant (k_1) was not significantly different. Each point represents the mean of a triplicate measurement of two experiments in each of two different sarcolemmal preparations.

Electron microscopy is particularly helpful in determining mitochondrial contamination, but it is harder to distinguish between sarcolemma and sarcoplasmic reticulum vesicles. Because sarcolemmal $^{45}\text{Ca}^{2+}$ uptake is not amplified by oxalate and sarcoplasmic $^{45}\text{Ca}^{2+}$ uptake is, we used the decrease of oxalate supported adenosine triphosphate-dependent $^{45}\text{Ca}^{2+}$ uptake as a marker for the decrease in sarcoplasmic reticulum contamination of our sarcolemmal preparations. Brandt¹⁵ has demonstrated that cardiac sarcoplasmic reticulum does not contain dihydropyridine binding sites. The continuous increase in ^3H -PN200-110-specific binding in parallel with a decrease in $^{45}\text{Ca}^{2+}$ uptake further supports the high purity of our preparation and is in agreement with the previous report by Drenger *et al.*⁵

The membrane vesicles were incubated during these experiments at a constant temperature of 25° C. This temperature was used to slow membrane degradation over the 1-h incubation period used in these experiments. It is known that a decrease in nitrendipine binding affinity, due to an increase in the dissociation rate constant, occurs at 37° C^{16,17}; however the effects of increased temperature on binding capacity is not certain.^{17,18} It has been shown that radioligand experiments at 25° C correlate well with patch-clamp experiments on the effect of nitrendipine on inhibition of cardiac Ca^{2+} currents.¹⁹

During normal function, the VDCCs of the myocardial cell are continuously alternating among the resting, open, and inactivated states. Ca^{2+} channel blockers show different binding affinities to the different states of the channel. The phenylalkylamines, such as D600, show high-affinity binding to the open or inactivated states. The VDCCs in isolated sarcolemma are in the inactivated state with high-affinity binding characteristics for the phenylalkylamines.

Electrophysiologic studies have confirmed that alterations in Ca^{2+} entry across the sarcolemma play an impor-

tant role in the overall mechanism of negative inotropic effect of volatile anesthetics. Lynch *et al.*^{1,20} have shown in guinea pig papillary muscle that halothane causes a significant depression of the maximum rate of rise of the slow action potentials. These potentials are generated by the slow inward Ca^{2+} current *via* the VDCCs. In single canine cardiac Purkinje cells, volatile anesthetics (isoflurane, enflurane, and halothane) depressed both L- and T-type Ca^{2+} channel currents measured by voltage clamp techniques.²¹ These results suggest that the volatile anesthetics interact with both the L- and T-type channels to a similar extent. Our results are consistent with these electrophysiologic studies.

Drenger *et al.* have previously demonstrated dose-dependent halothane inhibition of the binding of dihydropyridine ^3H -nitrendipine to purified sarcolemmal vesicles,⁵ and Blanck *et al.* have obtained similar results with crude membranes from rabbit and rat hearts.⁴ However, from these previous data we are not able to distinguish whether the halothane inhibition of dihydropyridine binding is due to an alteration in the conformation of the protein molecules of the channel or due to changes occurring in the membrane lipid bilayer itself. That two separate binding sites, the dihydropyridine and the phenylalkylamine sites, on the α_1 subunit of the VDCC could be equally affected by halothane suggests a rather significant alteration in the conformation or environment of the channel. Since halothane is a small molecule and yet can interfere with the binding of two distinct ligands that have separate binding sites on the VDCC, the effect of halothane is most likely an alteration of the sarcolemmal lipid environment surrounding the VDCC or a lipophilic site or sites on the VDCC (perhaps access to which is gained through the membrane lipid), rather than a direct effect on one or both of the binding sites. Support for the existence of a non-receptor-linked general membrane effect of volatile anesthetics comes from data showing the lack of specificity of volatile anesthetic affects on different types of ionic channels. For example, Eskinder *et al.*²¹ have shown that volatile anesthetics equally depress L- and T-type Ca^{2+} channel currents in single canine cardiac Purkinje cells. Halothane has also been shown to depress the fast Na^+ current in isolated rat ventricle cells²² and the delayed rectifier K^+ current in guinea pig atrial and ventricular myocytes.²³

The dihydropyridine and phenylalkylamine binding sites are allosterically coupled, phenylalkylamines being able to displace dihydropyridine binding in rat myocardium.⁷ The interaction, however, does not exhibit classical competitive inhibition between the two classes of Ca^{2+} channel blockers, suggesting that they are located at separate sites on the α_1 subunit. Since the VDCC contains several α_1 subunits, the binding of one ligand to a single α_1 monomer may influence binding (negatively or positively) of a separate or identical ligand to a different

monomeric α_1 subunit (heterotropic or homotropic cooperativity, respectively). One study has failed to demonstrate heterotropic cooperativity between the dihydropyridine and phenylalkylamine binding sites,⁷ giving further support to the hypothesis that the binding of a halothane molecule to a single site could not interfere with both the dihydropyridine and phenylalkylamine sites. Other studies have, however, demonstrated negative heterotropic cooperativity between the two sites.²⁴

The question arises as to where the binding sites have gone during halothane exposure. We have shown that upon the removal of halothane the specific binding returns to control levels, suggesting that the binding sites are in some way obscured but can readily reappear. The questions that these data prompt are why the number of binding sites decrease and whether the decrease in VDCC binding sites actually occurs *in vivo* during anesthetic exposure. The problem with proving whether the decrease in VDCCs upon anesthetic exposure is an actual *in vivo* mechanism is compounded by the volatility of halothane and the reversibility of this process.

In summary, we have described the inhibition by halothane of the binding of the phenylalkylamine D600 to high-affinity binding sites on bovine sarcolemmal VDCCs. This inhibition is dose-dependent and reversible and occurs at clinically relevant concentrations. The results of this study support the hypothesis that one mechanism by which the volatile anesthetics may induce negative inotropism is the reduction of functional VDCCs in the heart, leading to reduction of Ca^{2+} entry. Furthermore, our results of halothane depression of both phenylalkylamine and dihydropyridine binding argue that volatile anesthetics do not interact with the VDCC as a competitive inhibitor but may interact in a general way on the structural status of the protein in the membrane, whether at a lipophilic region of the protein or through the lipid bilayer surrounding the channel.

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