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# The Effects of Halothane on Voltage-dependent Calcium Channels in Isolated Langendorff-perfused Rat Heart

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*Background:* **Halothane has been previously shown *in vitro* to decrease both the inward calcium current in isolated cells and the density of calcium antagonist binding sites in cardiac sarcolemmal membranes prepared from several species, including humans, presumably contributing to the negative inotropic effects seen with volatile anesthetics. In this study we examined whether halothane produced similar changes in calcium channel antagonist binding characteristics *ex vivo* in an intact perfused heart by using isradipine, a dihydropyridine calcium channel blocker that binds specifically to the $\alpha_1$ subunit of the L-type voltage-dependent calcium channel.**

*Methods:* **The rat hearts were perfused by the Langendorff method in the presence of halothane and unlabeled isradipine. After the hearts were homogenized and prepared into membranes, a radioligand binding assay was performed and binding curves obtained. Data were analyzed by nonlinear regression analysis of a one-site binding equation and were evaluated by a paired *t* test.**

*Results:* **Halothane protected or inhibited the binding of unlabeled isradipine to calcium channels in a dose-dependent manner such that as the halothane is removed during the membrane preparation process, previously obscured sites were then available for specific binding of the radioligand. The sites that were protected by halothane had a lower affinity for [$^3$H]-isradipine than controls.**

*Conclusions:* **In both isolated membranes and the intact heart, halothane changes the availability of calcium channel antagonist binding sites, indicating a change in conformation of the voltage-dependent calcium channel in the presence of anesthetic. This change may result from a direct effect on the protein or from an indirect effect mediated through the membrane lipid bilayer. It also is demonstrated that halothane "protected" channels are probably a modified class of channels compared to those in control tissues as exemplified by the much lower affinity that the protected channels have for [$^3$H]-isradipine. We conclude that a major mechanism by which halothane depresses contractility is mediated through the voltage-dependent calcium channel, and this process results from a conformational change in the channel. (Key words: Anesthetics, volatile: halothane. Heart, myocardium: calcium channels. Measurement techniques: Langendorff perfusion; radioligand binding.)**

HOW volatile anesthetics produce their effects remains unknown. In addition to their hypnotic, amnesic, and analgesic qualities, they directly depress cardiac contractility through interference with calcium regulation and homeostasis.[1–3] One potential site at which this occurs is the voltage-dependent calcium channel (VDCC), where these agents *in vitro* decrease the inward calcium current in isolated cells[4,5] and the density ($B_{max}$) of calcium antagonist binding sites in cardiac sarcolemmal membranes.[6,7] In this study we equate $B_{max}$, defined by the specific binding of [$^3$H]-isradipine, with the density of VDCC. We present new data demonstrating that the latter of these effects also occurs *ex vivo* in intact Langendorff-perfused rat hearts. The volatile anesthetic halothane decreases both the $B_{max}$ of antagonist-binding sites and the affinity ($K_d$) of the channel for the calcium channel antagonist (CCA) isradipine. This reversible phenomenon is probably important in understanding the mechanism of halothane-induced depression of cardiac contractility.

Because of the tremendous structural dissimilarities among anesthetic agents, a single receptor site ex-

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plaining the comprehensive effects of anesthetics on both the central nervous and cardiovascular systems seems unlikely.[8] However, it is known that volatile anesthetics produce a dose-dependent depression in myocardial contractility *via* interference with intracellular myocardial calcium regulation.[1] Myocardial contractility is dependent on sarcolemmal calcium influx, sarcoplasmic reticulum calcium uptake and release, and the binding of calcium to myofibrils, and all have been shown to be sites of action for volatile anesthetics.[4,5,9] Contractile activity, and its depression by volatile anesthetics, appears to be mediated in part through the L-type VDCC,[6,7] which is also sensitive to the effects of organic CCAs[10,11] that interact with the $\alpha_1$ subunit of the VDCC.[12,13] Radiolabeled analogues of the dihydropyridine and phenylalkylamine classes of CCA serve as useful markers in studying the binding characteristics of calcium channels,[14,15] and volatile anesthetics have been shown to depress the binding of these agents to the L-type VDCC. This has subsequently been correlated with changes in calcium current and consequent changes in contractile forces (*i.e.,* negative inotropic effects).[6,16]

Halothane 0.4–2.0% produced *in vitro* a dose-dependent decrease in specific [$^3$H]-nitrendipine binding to the VDCC of as great as 35% in rat and rabbit cardiac sarcolemma, and 59–66% in bovine cardiac sarcolemma,[6,7] and a dose-dependent decrease in specific [$^3$H]-gallopamil ([$^3$H]-D600) binding to the VDCC of as great as 40% in bovine cardiac sarcolemma.[17] Meanwhile, halothane 1–2% produced a 19–31% decrease in specific [$^3$H]-isradipine binding to the VDCC in human ventricular sarcolemma.[18] The inhibition of binding by the volatile agent is fully reversible, lending support to the hypothesis that halothane and other volatile anesthetics may induce their negative inotropic effects by altering channel mediated calcium influx into cardiac myocytes. The primary aim in this study is to determine whether the previous *in vitro* observations that volatile anesthetics decrease calcium channel number in cardiac sarcolemma also occurs *ex vivo* in an intact perfused heart.

## Materials and Methods

Our experimental protocol is depicted in figure 1. With approval from the Animal Care and Use Committee of Cornell University Medical College, Sprague-Dawley rats of either sex (300–350 g) were divided into three groups. After the hearts were excised and the aortas rapidly cannulated, they were suspended in a Langendorff apparatus and perfused at a constant infusion pressure of 85 cm$H_2O$ at 37°C with a modified Krebs-Ringer buffer solution (117 mM NaCl, 6 mM KCl, 3 mM $CaCl_2$, 1 mM $MgSO_4$, 16.7 mM glucose, and 0.55 mM ethylenediamine tetraacetic acid, *p*H 7.4; J. T. Baker, Phillipsburg, NJ) through which a 95% $O_2$–5% $CO_2$ gas mixture was bubbled. The hearts were paced at a standardized rate of 270 beats/min and a fluid-filled balloon tipped catheter (PE 190 tubing) was introduced into the left ventricle *via* the left atrium to allow for continuous hemodynamic measurements of left intraventricular pressure and contractility ($\delta p/\delta t$, the rate of change in pressure). Hemodynamic changes from the effects of volatile anesthetics or calcium channels antagonists were recorded as percent reduction of baseline values. After an initial 30-min equilibration period for each heart, in the control group (n = 6), 2.5% halothane (Ayerst Laboratories, New York, NY) was bubbled into the buffer reservoir for 20 min followed by a 30-min washout period. (This includes an independent control group (n = 3), perfused in a similar manner for the same time duration but not exposed to halothane, where no significant difference between the two control groups could be noted.) In the isradipine group (n = 5), after an additional 20 min of buffer perfusion, 100 nM isradipine (gift from Sandoz Pharmaceuticals, East Hanover, NJ) was added to the buffer reservoir for 30 min. In the halothane group (n = 6), 2.5% halothane was bubbled into the buffer reservoir for 20 min followed by the addition of 100 nM isradipine for 30 min. To demonstrate dose dependence, a series of parallel experiments using 1.5% halothane (n = 3) were completed. At the conclusion of each experiment, the hearts were placed in iced 50 mM Tris HCl buffer (*p*H = 7.7) and the sarcolemmal membranes prepared on ice using a modification of the methods described by Endoh *et al.*[19]

In the design of the experiments and the interpretation of the data, several key assumptions are made. The premise of our experiments is that halothane in the intact heart prevents or inhibits binding of L-type VDCC by an unlabeled CCA present in the perfusate (in this case 100 nM isradipine), such that as the halothane is removed during the membrane preparation process, these channels are subsequently available for binding by the radiolabeled CCA. However, in order for this method of indirect binding to be successful, two conditions must be met: complete evaporation of halothane away from the channels during the membrane prepa-

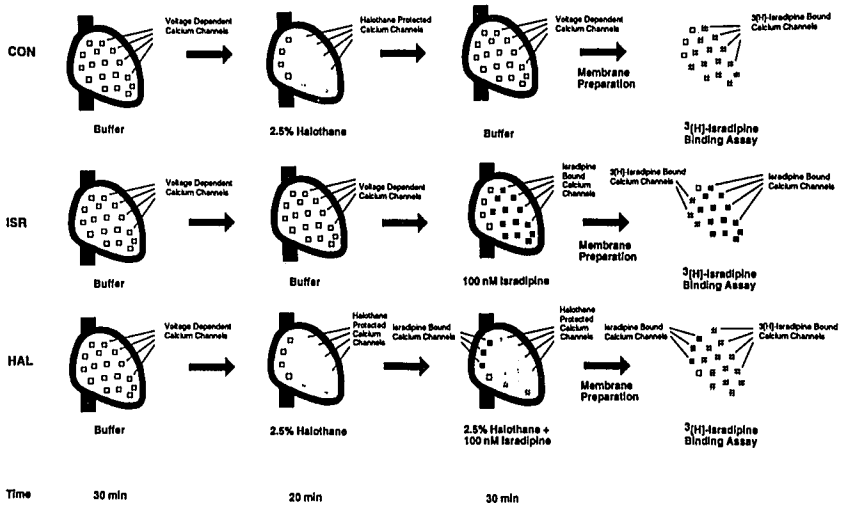


**Fig. 1. Sprague-Dawley rats of either sex were divided into three groups. After the hearts were excised, they were perfused in a Langendorff apparatus at a constant infusion pressure of 85 $cmH_2O$ with a modified Krebs-Ringer buffer solution ($pH$ = 7.4) through which a 95% $O_2$–5% $CO_2$ gas mixture was bubbled at 37°C. The hearts were paced at a standardized rate of 270 beats/min and a fluid-filled balloon tipped catheter allowed for continuous hemodynamic measurements of left intraventricular pressure and contractility (dP/dt), which were recorded as percent reduction of baseline values. After an initial 30-min equilibration period for each heart, in the control (CON) group, 2.5% halothane (HAL) was bubbled into the buffer reservoir for 20 min followed by a 30-min washout period. In the isradipine (ISR) group, after an additional 20 min of buffer perfusion, 100 nM ISR was added to the buffer reservoir for 30 min. In the HAL group, 2.5% HAL was bubbled into the buffer reservoir for 20 min followed by the addition of 100 nM ISR for 30 min. To demonstrate dose dependence, parallel experiments using 1.5% HAL were also done. At the conclusion of each experiment, the hearts were prepared into sarcolemmal membranes on ice as described elsewhere.[19]**

ration process necessary to reveal the "protected" sites; and unlabeled isradipine bound to calcium channels in the intact heart remains bound during the membrane preparation process such that they cannot be subsequently bound by the radioligand. Thus, isradipine, a new member of the dihydropyridine class of CCA, was selected for these experiments because of its extremely high affinity for the VDCC and very slow rate of dissociation from the channel at 4°C, as has been demonstrated previously by Maan and Hosey.[20] Moreover, we also assume that the unlabeled isradipine bound to the channels in the intact heart continues to remain bound during the radiolabeled binding assay, so as not to be displaced by the radioligand. In data not shown, we have demonstrated in control sarcolemmal membranes that the degree of displacement of radiolabeled isradipine from the VDCC by increasing amounts of unlabeled isradipine over a 5-h period at 25°C is negligible.

*Binding Studies*

The radioligand binding assay was carried out in a reaction medium containing [$^3$H]-isradipine (0.1–1.0 nM; Amersham, Arlington Heights, IL) in 50 mM Tris HCl, $pH$ 7.7 with 100 $\mu$g sarcolemmal membranes in a total volume of 1 ml. Protein analysis of the sarcolemma was determined by the method of Bradford.[21] Unlabeled nitrendipine, 500 nM (gift of Miles Pharmaceuticals, West Haven, CT) was added to measure nonspecific binding. All incubations were performed at 25°C for 90 min in the dark to minimize photochemical decomposition of the nitrendipine. The reaction was terminated by the addition of 10 ml cold 20 mM Tris buffer to an 800-$\mu$l aliquot of the reaction mixture and vacuum filtration (GF/C filters, Whatman, Hillsboro, OR). The filters were rinsed twice more with 10 ml cold Tris buffer, placed in 5 ml scintillation fluid and counted (LS 2800 scintillation counter, Beckman, Fullerton, CA) Specific binding was defined as the difference between the total radioligand binding and the nonspecific binding. A $K_d$ and $B_{max}$ value was calculated by nonlinear regression analysis for each binding curve based on a one-site ligand binding model using an Enzfitter software package (Robin J. Leatherbarrow, Elsevier Science Publishers, Amsterdam, The Netherlands). Statistical analysis between the various groups was made using a paired Student's $t$ test.

## Results

The results of our experiments are illustrated in figure 2 and summarized in table 1. In the control group, the addition of 2.5% halothane produced an 80 ± 7% decrease in contractility as measured by the rate of change in pressure that returned to baseline during the 30-min washout period. The binding data after halothane washout revealed a $B_{max}$ of 475 ± 26 fmol/mg protein and a $K_d$ of 0.15 ± 0.02 nM. In the isradipine group, the addition of 100 nM isradipine produced a 61 ± 8% decrease in contractility, and resulted in a marked reduction of $B_{max}$ to 44 ± 8 fmol/mg protein with a $K_d$ of 0.34 ± 0.11 nM. This 91% decrease in $B_{max}$ compared to controls is what we might expect based on our hypothesis that the available calcium channels are occupied by the unlabeled ligand in the intact heart such that they cannot be bound by the radioligand in the subsequent membrane assay. In the 2.5% halothane group, the addition of 2.5% halothane produced an 82 ± 5% decrease in contractility, similar to that seen in the control group, and the addition of 100 nM isradipine resulted in a total reduction in contractility of 96 ± 1% compared to baseline. The binding data produced a $B_{max}$ of 264 ± 51 fmol/mg protein and a $K_d$ of 1.07 ± 0.31 nM. The increase in $B_{max}$ relative to the isradipine group indicates that halothane was able to "protect" some of the channel sites from unlabeled isradipine in the intact heart so that eventually they could be radiolabeled after halothane removal during the membrane preparation process. In the 1.5% halothane group, the addition of 1.5% halothane again produced a 68 ± 2% decrease in contractility, and the addition of 100 nM isradipine resulted in a total reduction in contractility of 93 ± 1% compared to baseline. The binding data produced a $B_{max}$ of 163 ± 7 fmol/mg protein and a $K_d$ of 1.05 ± 0.07 nM, and this increase in $B_{max}$ relative to the isradipine group illustrates the dose-dependent effect of halothane in that the decreased concentration of halothane protects fewer channel sites allowing more of the unlabeled substrate to bind in the intact heart leaving less available for radiolabeled binding. figure 3 shows the same data presented in Scatchard format emphasizing the marked change in $B_{max}$ and $K_d$ with the addition of halothane to isradipine.

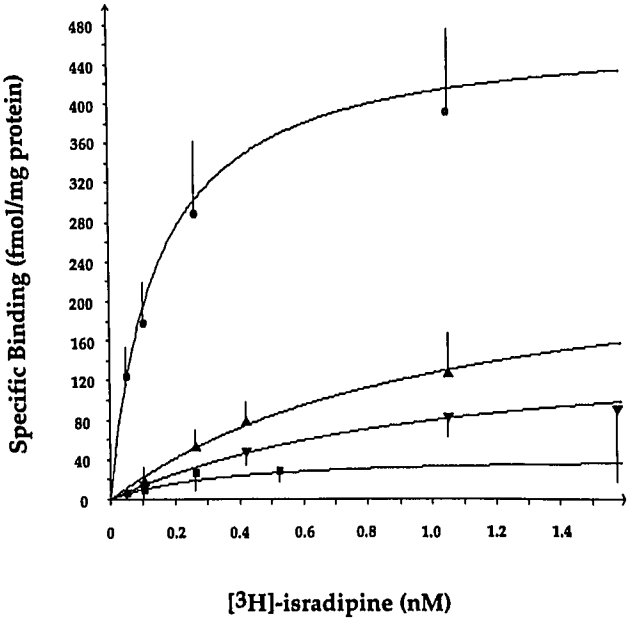


**Fig. 2. The composite specific binding data of our experimental groups is illustrated above: control (CON) (circles), 100 nM isradipine-treated (ISR) (squares), 1.5% halothane + 100 nM isradipine-treated (HAL 1.5%) (reverse triangles), and 2.5% halothane + 100 nM isradipine-treated (HAL 2.5%) (triangles). The radioligand binding assay was carried out in a reaction medium containing [$^3$H]-ISR (0.1–1.0 nM) in 50 mM Tris HCl, *p*H 7.7 with 100 μg sarcolemma membranes. 500 nM unlabeled nitrendipine was added to measure nonspecific binding, and all incubations were performed at 25°C for 90 min in the dark to minimize photochemical decomposition of the nitrendipine. All experiments were conducted in triplicate, and specific binding was defined as the difference between the total radioligand binding and the nonspecific binding. Each point on a curve represents the mean of the specific binding for all animals in that group at a particular [$^3$H]-ISR concentration, with the error bars indicating plus or minus the standard error of the mean. The points were then fitted by nonlinear regression analysis to computer generated curves based on a one-site ligand binding model using the Enzfitter program, which directly determines the affinity ($K_d$) and density ($B_{max}$) for each group. It is important to note that even with the ISR group, which had a very low signal to noise ratio (total binding relative to nonspecific binding), a weighted composite curve of specific binding could be fit to the data.**

## Discussion

Previous studies in our laboratory examining the effects of halothane and other volatile anesthetics on the binding of radiolabeled CCA to VDCC *in vitro* in cardiac sarcolemmal membranes have demonstrated a markedly decreased binding of CCA to receptors of the L-type VDCC.[6,7] This suggests that halothane changes or alters these binding sites on the channel. The sites that remained in the presence of halothane had the same affinity as control sites, and the sites that reappear after halothane evaporates also had the same affinity.[17] This suggests that no apparent permanent modification

**Table 1. Effects of Halothane on Contractility and Radioligand Binding of [$^3$H]Isradipine to Voltage-dependent Calcium Channels in Langendorff-perfused Rat Heart**

| | % Decrease ($\delta p/\delta t$) | $B_{max}$ (fmol/mg protein) | % Decrease ($B_{max}$) | $K_d$ (nM) |
|---|---|---|---|---|
| Control | 80 ± 7* | 475 ± 26 | | 0.15 ± 0.02 |
| Isradipine | 61 ± 8 | 44 ± 8† | 91 | 0.34 ± 0.11† |
| 1.5% Halothane | 68 ± 2 | | | |
| + Isradipine | 93 ± 1 | 163 ± 7† | 66 | 1.05 ± 0.07† |
| 2.5% Halothane | 82 ± 5 | | | |
| + Isradipine | 96 ± 1 | 264 ± 51† | 44 | 1.07 ± 0.31† |

Values are the results of the *in vitro* radioligand binding studies. The various experimental groups were compared with the control group by a paired Student's *t* test.

* Returns to baseline after removal of 2.5% halothane.

† $P < 0.05$ *versus* control.

of the VDCC or the surrounding membrane occurs in the presence of halothane. With these findings, we sought to investigate whether similar changes occur in a preparation that more closely resembles the clinical situation, and our current report indicates that this phenomenon only partially occurs in the intact heart.

Using a "receptor protection" assay, we have shown that halothane can "protect" the VDCC from being bound by unlabeled isradipine in the intact heart. So as occurs *in vitro,* the total number of channels available to be bound by isradipine is reduced by halothane and the number of VDCC "protected" from CCA binding increases with increasing halothane concentration. However, unlike the earlier *in vitro* experiments, the VDCC "protected" by halothane in the intact heart had a sevenfold lower affinity for [$^3$H]-isradipine.

One explanation for this change in $K_d$ is that the calcium channels in isolated membranes used in the previous *in vitro* studies are markedly depolarized relative to the intact organ, and under these conditions, where the channels are presumably in the inactivated state, the affinity of dihydropyridines for these sites is significantly enhanced, as has been demonstrated.[22–24] However, this does not completely account for the changes noted in our current experiments because all binding was conducted using isolated membranes prepared from intact hearts in the same manner, and we have no evidence that the membranes prepared for the control group were depolarized to any greater extent than in the isradipine or either halothane group.

Alternatively, others have found that isolated membranes tend to have single-site high-affinity binding characteristics, whereas intact cells may reveal low- or high-affinity binding sites dependent on the state of the channel (open *vs.* resting) and the membrane potential at a given time.[16,24] This suggests that in the intact organ or cell, halothane potentially modifies the VDCC by a different mechanism. For example, one form of modification may be a change in the phosphorylation state of the VDCC, leading to a site with a lower affinity. This is a possibility because in the intact heart, the phosphorylation and dephosphorylation mechanisms are intact and could be enhanced or inhibited by halothane. Other covalent modifications of the membrane that could occur are glycosylation or myristylation, although at present there is no evidence to support either of these particular mechanisms. Other possibilities include a direct conformational change of the VDCC attributable to interaction of halothane with hydrophobic domains of the protein or with the surrounding lipid bilayer. However, one would expect these latter effects to be reversible on the removal of the halothane, and the affinity to return to control values during the homogenization and binding assay, as we have found in the *in vitro* studies. One might argue then that perhaps the halothane was incompletely vaporized away from the channels during the membrane preparation process. That, however, seems unlikely given the fact that the control group was subject to a similar membrane preparation conditions. In addition, in data not shown, control hearts in which the halothane was not washed out before preparation into sarcolemmal membranes exhibited no change in $K_d$ compared to the actual control group where the halothane was washed out.

Another possibility is that a separate population of VDCC exists and is selectively "protected" by halothane.[25] This appears unlikely because the *in vitro* binding data has always fit quite well to a single site

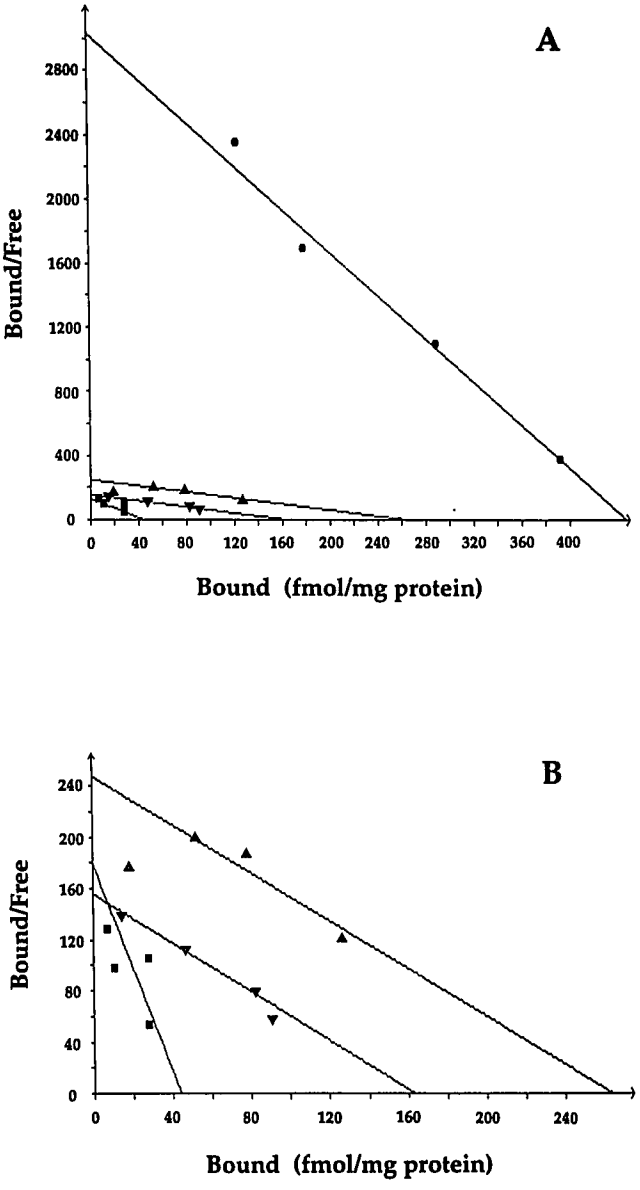


**Fig. 3. Panel A represents the Scatchard plot of the transformed composite binding data illustrated in Figure 2: control (CON) (circles), 100 nM isradipine-treated (ISR) (squares), 1.5% halothane + 100 nM isradipine-treated (HAL 1.5%) (reverse triangles), and 2.5% halothane + 100 nM isradipine-treated (HAL 2.5%) (trinagles). Panel B represents the latter three plots enlarged to show greater detail. The HAL 1.5% and HAL 2.5% groups exhibit a marked decrease of their slope, indicative of a decrease in affinity ($K_d$) of ISR for the voltage-dependent calcium channels, relative to those of the CON and ISR groups, which were quite similar. Also, a linear relation exists for each plot, indicative of a single population of high- or low-affinity binding sites. In addition, HAL in the concentrations chosen clearly does not protect all the available channel sites, as is illustrated by the large difference in the x-intercepts (density [$B_{max}$]) of both HAL groups relative to the CON group.**

binding model and did not demonstrate two linear portions on Scatchard plots. On the current experimental plots seen in figure 3, the transformed binding data again demonstrates a linear relation indicative of a single class of high- or low-affinity binding sites in the sarcolemmal membranes for the control *versus* halothane groups, respectively. Unfortunately, interpreting data by Scatchard plots is often fraught with error as experimental artifact can often be magnified by this type of analysis.[26,27] Thus to avoid this pitfall, the explicitly weighted composite binding curves for each experimental group were fit by nonlinear regression analysis to a one-site model in order to determine $K_d$ and $B_{max}$. As such, the Scatchard plots merely serve to confirm the aforementioned binding parameters as opposed to determine them.

Regardless, it is apparent from the binding data that halothane at the concentrations used in these experiments does not completely protect all the channels. Otherwise, one would have expected the $B_{max}$ values in the halothane groups to be closer to the control group. Attempts to address this issue by further increasing the concentration of halothane added were unsuccessful, as the degree of depression of myocardial contractility reached a point where the heart spontaneously fibrillated. However, because the size of the "protected" population is dependent on the concentration of halothane used (*i.e.*, as the concentration of halothane increased, the size of the low-affinity CCA binding sites increased), this suggests the possibility that channel modification in the intact heart is a halothane dependent process leading to a decrease in the affinity of CCA binding sites for [$^3$H]-isradipine and a decrease in the number of functional VDCC involved in the contractile activity of the heart.

The contractility data supports the belief that depression of contractility involves more than one mechanism. The perfusion of the heart with isradipine alone at a concentration that blocks over 90% of the VDCC, resulted in only a 61 ± 8% decrease in contractility. The addition of halothane at 1.5% or 2.5% led to a further depression of contractility to >90%. These data suggest that halothane not only contributes to blocking calcium channels, but may also work on mechanisms beyond the sarcolemma, such as calcium release by the sarcoplasmic reticulum and/or sensitivity of the contractile proteins to calcium.[28]

To understand the relevance of these data, it is useful to think of anesthetic effects on calcium channels from a quantitative as well as a qualitative perspec-

tive. We have stated that halothane reduces VDCC number as measured by antagonist binding and this is presumably a common pathway by which changes in voltage-gated slow inward calcium current produce changes in contractility.[29] Thus one could rationalize that a decrease in the number of available VDCC, which translates into a decrease in inward calcium current, may in part explain the negative inotropic effects seen clinically with volatile anesthetics and CCA therapy. However, as suggested by the change in $K_d$ for isradipine, there also seems to be qualitative changes present in the channels. Here one might postulate the total number of measurable VDCC is irrelevant, rather the decrease in inward calcium current and contractility is attributable to a change in the affinity or conformation of the channels secondary to the effects of halothane. In fact, others have implicated a low-affinity VDCC as important in the regulation of contractility.[30] Unfortunately the influence of the low-affinity channel population on the magnitude of inward calcium current is unknown, but this may also play a part in our overall understanding of how halothane and other volatile anesthetics produce their effects on the heart.

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