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## Halothane Modifies Ischemia-associated Injury to the Voltage-sensitive Calcium Channels in Canine Heart Sarcolemma

Benjamin Drenger, M.D.,\* Yehuda Ginosar, B.Sc., M.B.B.S.,† Mucul Chandra, M.B.B.S., M.S.,‡  
Ayelet Reches, B.Ed.,§ Yaacov Gozal, M.D.¶

**Background:** Recent experimental data suggest that functional and metabolic changes in the myocardium caused during ischemia and subsequent reperfusion may be attenuated by the volatile anesthetics through the prevention of intracellular calcium accumulation. The main purpose of the current research is to identify a mechanism responsible for the alterations of ischemia-associated injury to the voltage-sensitive  $Ca^{2+}$  channels (VSCC) in the sarcolemma during halothane anesthesia.

**Methods:** The effect of 10 min myocardial ischemia in canine heart and 20 min reperfusion on the function of the VSCC in the sarcolemma was examined in the presence or absence of 1.6 vol% halothane administered *in vivo*. The membranes were isolated through differential centrifugation/filtration from the ischemic (left anterior descending territory) and normally perfused myocardium. Comparison of binding characteristics in the ischemic and nonischemic zones was made using equilibrium-binding studies of a dihydropyridine calcium channel

blocker, [ $^3H$ ]isradipine (0.05–1.0 nM), to the VSCC in the sarcolemma. Control studies were performed on membranes prepared from the same perfusion zones, but from hearts who were not exposed to ischemia.

**Results:** The control studies ( $n = 5$ ) showed no difference in binding kinetics between the different zones in the heart. After 10 min of ischemia, a 50 to 95% increase in specific [ $^3H$ ]isradipine binding to the sarcolemmal membranes was observed as compared to control membranes ( $P < 0.001$ ). The maximal binding capacity ( $B_{max}$ ) increased by 85%, whereas the dissociation constant ( $K_d$ ) remained unchanged. In the reperfusion experiments, a moderately increased binding (of 32%) was observed with a 40% increase in  $B_{max}$  ( $P = NS$ ). In the presence of 1.6% inhaled halothane, the effect of ischemia was attenuated. A decrease of 32.1% to 41.8% in equilibrium binding was observed (31% decrease in  $B_{max}$ ;  $P < 0.03$  and 0.02, respectively).

**Conclusions:** Even a brief period of myocardial ischemia produces a marked increase in the available high-affinity binding sites in the VSCC, a finding that is well correlated with previous experimental observation of increased calcium ion influx to the myocardial cell. On reperfusion, some recovery of the ischemic changes in the VSCC was evident. The binding kinetics which characterize this early phase of cell injury were reversed by halothane anesthesia, indicating a possible reduction in calcium entry, which may represent one of the beneficial effects of the anesthetic in the ischemic heart. (Key words: Anesthetics, volatile: halothane. Animal: dog. Heart, coronary circulation: occlusion; regional ischemia. Heart, stunned myocardium: postischemic; reperfusion. Ions: calcium influx. Muscle, cardiac: sarcolemma. Pharmacology, calcium channel blocking drug: isradipine.)

OCCLUSION of a coronary artery for a brief duration produces on reperfusion the phenomenon of postischemic "stunning," during which the ventricular contractile function in that region virtually ceases.<sup>1</sup> The cellular events involved in the pathogenesis of stunned myocardium result mainly from depletion of high-energy phosphates, the action of oxygen-derived free radicals, and alterations in intracellular calcium homeostasis.<sup>2,3</sup>

Recent studies indicate that, in such circumstances, the volatile anesthetics may have cardiac protective

\* Senior lecturer in Anesthesiology, Department of Anesthesiology and Critical Care Medicine.

† Instructor in Anesthesiology, Department of Anesthesiology and Critical Care Medicine.

‡ Research Fellow, J. Lunenfeld Cardiac Surgery Research Center.

§ Research Assistant, Department of Anesthesiology and Critical Care Medicine.

¶ Lecturer in Anesthesiology, Department of Anesthesiology and Critical Care Medicine.

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Address reprint requests to Dr. Drenger: Director, Cardiothoracic Anesthesia Unit, Department of Anesthesiology and Critical Care Medicine, Hadassah University Hospital, P.O. Box 12000, Jerusalem 91120, Israel.

properties, which may prevent intracellular calcium accumulation<sup>4</sup> and reduce infarct size.<sup>5</sup> The volatile anesthetics also prevent free radical mediated reduction in coronary blood flow and contractility, as demonstrated in the isolated rabbit heart,<sup>6</sup> and inhibit superoxide anion production, as observed in human neutrophils.<sup>7</sup>

The mechanisms underlying myocardial depression after a short ischemic event are a combination of depletion of metabolic energy due to hypoxia and a change in surface charge and/or conformational changes of the calcium channel proteins.<sup>8</sup> Excessive uptake of calcium ions and oxygen molecules into the mitochondria adds further to the rapid loss of energy.<sup>1</sup>

The commonly used volatile anesthetics produce, in addition to anesthesia, a dose-dependent, negative inotropic effect on the cardiac muscle and heart. Our experimental approach is based on the hypothesis that the volatile anesthetics interfere with the normal handling (*i.e.*, recognition) and availability of calcium for the contractile process.<sup>9-12</sup> This hypothesis was examined in myocardium exposed *in vivo* for a short period to ischemia and to subsequent reperfusion.

Previous studies showed that the calcium current through the voltage-sensitive calcium channels (VSCC) in the sarcolemma, the outer membrane of the myocyte, is inhibited by the volatile anesthetics, leading to a decrease in contractile force.<sup>10,13,14</sup>

The aim of the current study is to identify at a subcellular level, specifically in the sarcolemma, the functional derangements that occur in the VSCC during ischemia. The preinfarction phase of ischemia and the associated myocardial "stunning" are of particular interest, as the assessment of the degree of the histologic changes due to this type of injury is often not feasible. Investigations to identify specific biochemical markers of reversible myocardial organelle dysfunction are therefore indicated.

Dihydropyridine calcium channel blockers, such as nitrendipine and isradipine (PN200-110), bind to the VSCC in a specific, saturable, and reversible manner<sup>15</sup> and can be used as probes of these channels. Previous studies demonstrated that decreased binding of nitrendipine was correlated with a reduction of Ca<sup>2+</sup> influx, associated with a decrease in the contractile force.<sup>16,17</sup>

Using radioligand-binding studies on isolated sarcolemmal membranes, the current experiments were designed to evaluate the effect of a short period of ischemia and reperfusion on the VSCC. The primary goal was to assess the changes occurring in binding capacity,

both during the ischemic injury and subsequently during reperfusion. The contribution of the volatile anesthetics during ischemia toward modifying the changes previously observed in binding capacity was characterized.

## Materials and Methods

### *Experimental Preparation*

To examine the injurious effect of a short period of ischemia followed by reperfusion on the VSCC in the myocardial cell, a canine "region-at-risk" model was chosen. The ischemia and reperfusion experiments were carried out on 11 mongrel dogs weighing 20–28 kg, after approval by the institutional committee on animal experimentation. Equilibrium-binding studies of [<sup>3</sup>H]isradipine, a dihydropyridine calcium channel blocker, were applied to sarcolemmal membranes that were isolated from the ischemic and normally perfused (control) myocardium. The ischemia experiments were performed in the presence or absence of halothane, which was administered *in vivo* in the inhalation mixture during the coronary occlusion. In three additional dogs, control experiments were performed on sarcolemmal membranes prepared from nonischemic hearts. The binding characteristics of the VSCC in the left anterior descending coronary artery (LAD) perfusion zone were compared to those in the rest of the ventricular muscle.

Anesthesia was induced with 25 mg/kg intravenous pentobarbital, 30 min after intramuscular injection of 0.5 mg/kg 1% propionylpromazine. During the experiment, the animal was paralyzed with pancuronium, the trachea was intubated, and ventilation was mechanically controlled using 60% O<sub>2</sub> in air. Before skin incision, 5 mg/kg intravenous pentobarbital was administered, and subsequently throughout the experiment, additional doses of pentobarbital were administered according to the experimental protocol.

Electrocardiogram, temperature, and femoral arterial blood pressure were monitored continuously. End-tidal halothane concentration was maintained at a steady concentration of 1.6 vol% using anesthetic gas analyzer (Drager Iris, Lubeck, Germany). The administration of halothane from a calibrated vaporizer (Fluotec 3, Cyprane Keighley, England) was started 10 min before the coronary occlusion and continued during the 10-min ischemia period. Arterial blood samples confirmed adequate ventilation and oxygenation and normal met-

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abolic state during the period of ischemia and reperfusion.

The heart was exposed by a left thoracotomy; in the ischemia group, the LAD was sutured distal to the first diagonal artery, and in the ischemia/reperfusion group, an atraumatic elastic vascular ligature was applied to the LAD, distal to the first diagonal artery for 10 min, thus causing reversible regional ischemia. In each animal, the rest of the myocardial muscle perfused by the left circumflex artery and by the right coronary artery was used as the control tissue. The accurate landmarks of the ischemic zone were determined by a simultaneous injection of 20 ml 1% brilliant blue dye in normal saline into the LAD and normal saline into the aortic root, immediately after aortic cross-clamping and removal of the heart. Simultaneous application of equal pressures to both injection sites prevents the flow of dye across the collateral vessels, creating a clearly delineated ischemic myocardium stained blue.<sup>18</sup> The ventricular tissue was excised immediately according to the color landmarks, weighed, and placed into separate ice containers.

Before coronary occlusion, each animal received, in incremental doses, 25 mg/kg procainamide to prevent the risk of ischemia-induced ventricular arrhythmia.

### *Isolation of Sarcolemma Enriched Preparation*

Sarcolemmal membranes were isolated from the myocardial tissue in a series of centrifugation and extraction steps that were carried out on ice and using ice-cold (0–4°C) buffer.<sup>11,19</sup> Ventricular muscle was trimmed of its epicardial membranes, cordae tendinae, fat, and major vessels, minced into small pieces, and ground in a cooled meat grinder.

The ground ventricle was diluted in five volumes of 10 mM histidine and 0.75 M NaCl, pH 7.5, and homogenized in a Waring blender at 60% maximal speed for 90 s. The suspension was filtered through one layer of cheesecloth and centrifuged at 10,400 × g for 30 min. The supernatant was discarded, the pellet was resuspended in three volumes of 10 mM NaHCO<sub>3</sub> and 5 mM histidine, pH 7.5, homogenized again for 60 s, and centrifuged at 10,400 × g for 20 min. After this step, the pellet containing contractile proteins, nuclear debris, and mitochondria was discarded, and the supernatant was centrifuged at 17,000 × g for 40 min. The pellet containing the isolated sarcolemmal membranes was Dounce homogenized in 0.25 M sucrose and 10 mM histidine, pH 7.5, and stored in a –80°C freezer.

Protein concentration was determined by the Lowry method using bovine serum albumin as the standard.

### *Reaction Conditions*

[<sup>3</sup>H]isradipine (PN200-110), a dihydropyridine calcium channel blocker (84 Ci/mmol; Amersham, Aylesbury, England), was used to label the VSCC. Sarcolemmal membranes (80–100 μg) were added to 0.05–1.0 nM [<sup>3</sup>H]isradipine in 50 mM Tris HCl (pH 7.5) to a final volume of 1 ml, in the absence or presence of 1 μM unlabeled nitrendipine (Bayer, Wuppertal, Germany), to define total and nonspecific binding, respectively. Specific binding, which indicates binding only to the VSCC, was calculated from the difference between total binding and nonspecific binding.<sup>11,12</sup> The experiments were carried out in triplicate, and the membranes were incubated for 60 min at 25°C in the dark to minimize photochemical decomposition. The reaction was terminated when an 800-μl aliquot was filtered through a Whatman GF/C filter under vacuum and rinsed three times with 10 ml of cold 20 mM Tris buffer (pH 7.5, 4°C). The binding of the radioligand was quantitated by scintillation counting. In each experiment, the control [<sup>3</sup>H]isradipine-binding values were compared to those achieved from the ischemic area (LAD territory).

### *Study Groups*

The animal experiments consisted of a control non-ischemic group (n = 3; five binding studies) and three regional myocardial ischemia study groups, which were divided into (1) ischemia of 10 min (n = 4; eight binding studies), (2) ischemia of 10 min and reperfusion of 20 min (n = 3; six binding studies), and (3) ischemia of 10 min with concomitant *in vivo* administration of halothane (n = 4; seven binding studies). The [<sup>3</sup>H]isradipine-binding studies were performed on the sarcolemmal membranes isolated from the ischemic and from the normal muscle, to evaluate changes that occurred in the VSCC.

### *Data Analysis*

The data were assessed by nonlinear regression analysis with explicit weighting, using the Enzfitter program (Elsevier, Amsterdam, The Netherlands) to obtain estimates of K<sub>d</sub> and density of binding sites (B<sub>max</sub>).

For each experimental setting (ischemia, ischemia and reperfusion, ischemia and halothane), the difference in binding between the control and experimental regions in each [<sup>3</sup>H]isradipine concentration was statistically compared by Student's paired *t* test, and be-

tween the study groups, by analysis of variance. Significance was considered to be less than 0.05. Data are reported as mean  $\pm$  SEM.

## Results

Five control experiments which compared nonischemic sarcolemmal membranes from the LAD territory to membranes from the residual ventricular muscle, showed similar binding properties. Mean [ $^3$ H]isradipine-specific binding values at 1-nM concentration were 90.5 fm/mg  $\pm$  19 and 92.4  $\pm$  21, respectively ( $P = \text{NS}$ ).

Myocardial ischemia was observed to profoundly affect the binding properties of sarcolemmal VSCC in each of the eight binding experiments performed. Equilibrium-binding studies of [ $^3$ H]isradipine to sarcolemmal membranes showed a mean increase of 58% in specific binding to the ischemic membranes, as compared to control membranes ( $P < 0.001$ ; fig. 1). Reaching equilibrium, at [ $^3$ H]isradipine concentrations of 0.5, 0.75, and 1.0 nM, ischemia produced a significant increase in binding of 50–95% ( $P < 0.0075$ ).

Evaluation of binding kinetics by Scatchard analysis revealed a linear plot (fig. 2), which implies a saturable and specific, single class of high-affinity binding sites to the sarcolemmal membranes.<sup>20</sup> The maximal binding capacity ( $B_{\text{max}}$ ) was calculated from the intercept of this Scatchard plot with x-axis.

The mean binding capacity of the VSCC in the control membranes was 98.1 fm/mg protein  $\pm$  15 (mean  $\pm$  SE) compared to 181.1  $\pm$  34 in the ischemic (LAD) membranes ( $P = 0.08$ , NS; table 1). The dissociation constant of the calcium channel blocker from its binding sites on the VSCC was derived from the slope of the Scatchard plot and was found to be unchanged, *i.e.*, mean of 0.84 nM  $\pm$  0.1 for control and 0.99 nM  $\pm$  0.2 for the ischemic membranes.

In the six ischemia and reperfusion experiments, [ $^3$ H]isradipine-specific binding tended to increase, but the effect did not reach significance. The 32% change in mean specific binding in the reperfused membranes was equivalent to 40% nonsignificant increase in binding capacity ( $B_{\text{max}}$ ) compared to control membranes, with no change in Kd.

During the regional ischemia experiments, control membranes prepared from nonischemic muscle showed some animal variability in specific binding. However, comparing the controls of the three study groups confirmed the absence of statistical differences between their binding kinetics.

In the presence of 1.6 vol% halothane in the inhalation mixture, the effect of ischemia on the function of the VSCC was attenuated. Ischemia did not produce the previously described changes in VSCC kinetics. On the contrary, over seven experiments, an overall decrease of 26.3% in specific [ $^3$ H]isradipine binding to the ischemic membranes was observed as compared to control ( $P < 0.05$ ; fig. 1). When equilibrium was reached at 0.75–1.0 nM, the decrease in binding was of 32.3–43.4% ( $P < 0.02$  and 0.01, respectively). Scatchard analysis revealed a 31% nonsignificant decrease in binding capacity, from 146  $\pm$  69 to 100.3  $\pm$  22 fm/mg with no change in Kd (table 1).

## Discussion

Short regional myocardial ischemia due to a temporary occlusion of a coronary artery sets into motion a complex series of events in several subcellular systems involved in calcium homeostasis. The structural and functional changes impair the ability of the cell to regulate ion exchange and, upon the restitution of blood flow, massive intracellular accumulation of calcium ions is established.<sup>1–3,8</sup> The cellular basis for this marked augmentation in calcium content is not completely understood.

The current study shows that even a short period of ischemia causes a dramatic increase in the number of high-affinity binding sites available to the calcium channel blocker [ $^3$ H]isradipine in the sarcolemma. The linear Scatchard plot (fig. 2) suggests the existence of a single class of binding sites that is associated with the VSCC.

Because the dihydropyridines are used as specific markers of the VSCC, the current findings indicate a growth in the number of available VSCC in the sarcolemma during ischemia, a phenomenon that may explain the increase in calcium ions influx on reperfusion. However, extrapolation from *in vitro* binding studies to the situation during myocardial ischemia must be made cautiously. At present, only indirect evidence supports a linkage between increase in VSCC-binding capacity and increase in calcium ion flux to the myocardial cell. In a study performed on human nonischemic ventricular muscle strips, Schmidt *et al.*<sup>17</sup> demonstrated a direct correlation between a decrease in dihydropyridine binding and a parallel decrease in the force of contraction. Another group investigated neural calcium channels under ischemic conditions and showed a parallel increase in [ $^3$ H]isradipine-specific

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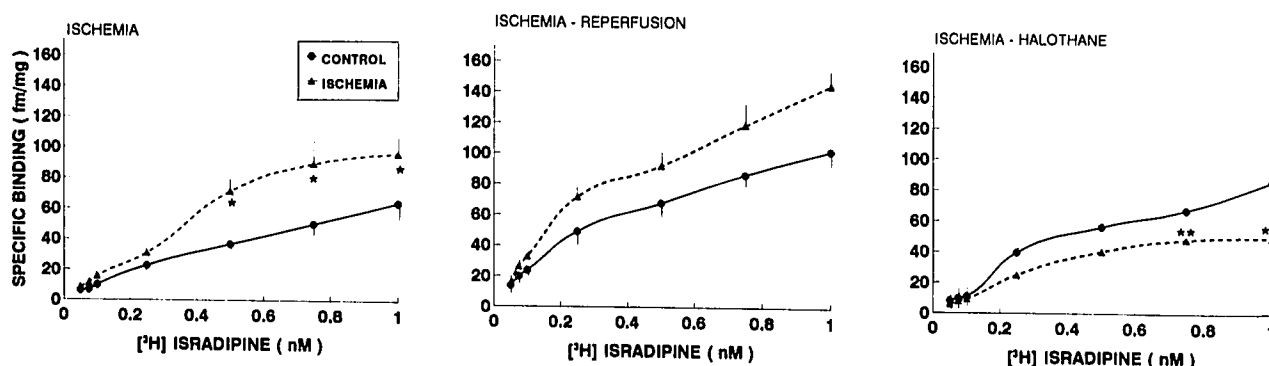


Fig. 1. Effect of 10 min of ischemia ( $n = 8$ ), 10 min of ischemia and 20 min of reperfusion ( $n = 6$ ), and 10 min of ischemia with concomitant *in vivo* administration of 1.6 vol% halothane ( $n = 7$ ) on [ $^3\text{H}$ ]isradipine-specific binding to canine heart sarcolemma. The data are presented for [ $^3\text{H}$ ]isradipine concentrations of 0.05–1.0 nM in control (straight line) and study (dashed line) groups. All measurements were performed in triplicate and presented as mean results  $\pm$  SEM. \* $P < 0.0075$ . \*\* $P < 0.02$ .

binding to striatal membranes and in dopamine release from striatum brain slices, a cellular function that appears to be mediated by an increase in inward calcium current.<sup>21</sup> We suggest that the changes in myocardial VSCC-binding capacity may be an early and specific biochemical marker of cell injury, the time dependence of which is similar to that of the development of myocardial infarction, and may be used as an alternative measure of the existence of ischemia.

It may be argued that acidosis that develops during the coronary occlusion may lead to protonation of cell membrane and blocks inward calcium current to the myocyte.<sup>22</sup> Although the actual intracellular pH at the time of ischemia is unknown, precaution was taken to maintain normal blood pH during the animal experiment, and all binding studies were performed in neutral environment (pH 7.50). It seems that acidotic conditions cause increased sensitivity of the calcium channels to nifedipine and verapamil, and these changes were expressed as reduction in the dissociation constant.<sup>23</sup> In the current *in vitro* studies, Kd values were not altered, suggesting that changes observed in the VSCC-binding capacity were directly related to the effect of ischemia and not to alteration in acid-base status.

Several studies previously showed that an ischemic interval of 10 min is too short to cause irreversible damage to the myocyte.<sup>24</sup> Therefore, in the current study, the described alterations in calcium channel kinetics represent functional changes that may be reversible with time. Indeed, in the set of experiments in which reperfusion followed the period of ischemia, the change in binding capacity of [ $^3\text{H}$ ]isradipine was smaller when compared to that observed in the isch-

emia experiments without reperfusion. The characteristic reperfusion injury would not be expressed if the experiment was terminated after 20 min of reperfusion and the excised myocardium was immediately placed on ice.

Calcium accumulation during the immediate period of reperfusion usually is related to calcium influx from the extracellular space due to increased sarcolemmal permeability.<sup>25</sup> A continuing rise in intracellular calcium stores after the first 20 min, however, is attributed mainly to both functions of the sarcoplasmic reticulum, *i.e.*, a decrease in calcium uptake<sup>26,27</sup> and enhanced release of calcium from the sarcoplasmic reticulum.<sup>4</sup>

Under normal conditions, the volatile anesthetics, and particularly halothane, cause a decrease in calcium availability to the myocyte, leading to a dose-dependent reduction in contractile force. This effect of the volatile anesthetics was demonstrated in electrophysiologic studies that clarified that a major site of volatile anesthetics' effect is the sarcolemma, where they cause a decrease in slow inward current carried by  $\text{Ca}^{2+}$  ions.<sup>9,10</sup> These findings were supported by biochemical studies of the VSCC in the sarcolemma, in the presence of volatile anesthetics, which confirmed the reduction in available calcium channels as part of the mechanism of reduction in myocardial contractility.<sup>11</sup>

The beneficial role of the volatile anesthetics during myocardial ischemia was well described as early as 1969 by Spieckermann *et al.*,<sup>28</sup> who observed a prolongation of the tolerance of global ischemia in canine myocardium exposed to halothane. This effect was related to an enhanced preservation of the high-energy phosphate compounds.

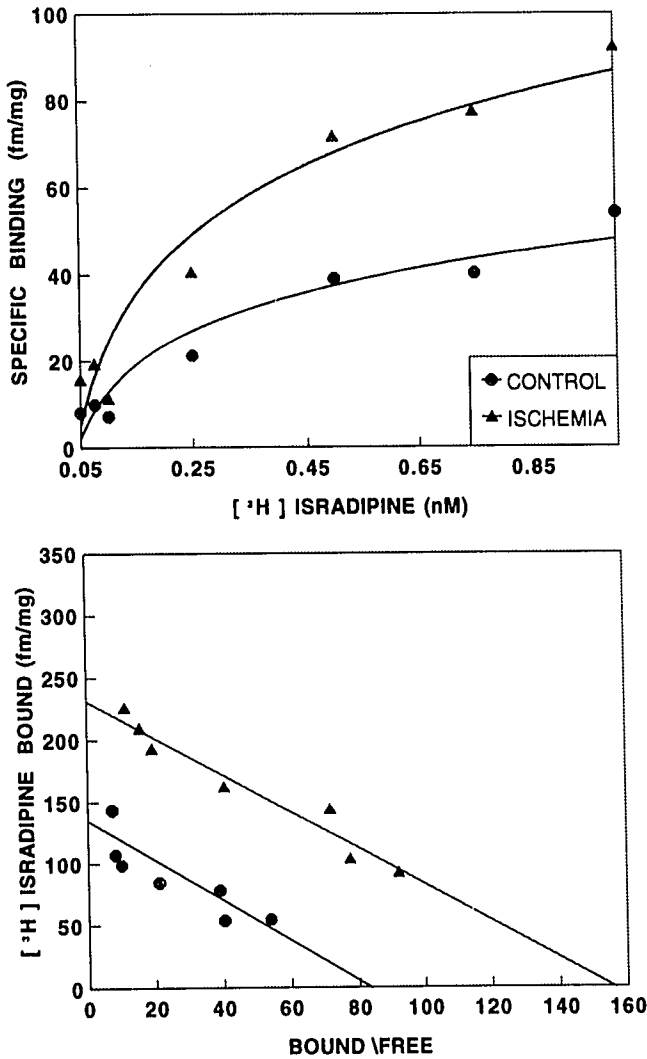


Fig. 2. Demonstrative equilibrium-binding curve of [<sup>3</sup>H]isradipine to the cardiac sarcolemma isolated from ischemic myocardium (left anterior descending coronary artery territory) and from normally perfused control myocardium. Scatchard plots are shown in the lower figure. The plot was obtained by linear regression. The dissociation constant (K<sub>d</sub>) was derived from the slope of the curve. The maximal binding capacity (B<sub>max</sub>) was calculated from the intercept of the plot with the x-axis.

Davis *et al.*<sup>5</sup> described reduction in infarct size after coronary ligation in dogs anesthetized with halothane. Kroll and Knight<sup>29</sup> showed a reduced incidence of ventricular fibrillation in a canine ischemia/reperfusion model. Warltier *et al.*<sup>30</sup> also demonstrated better recovery of contractile function of stunned canine myocardium if halothane was administered during acute

ischemia. Most of the beneficial effects are explained by the action of anesthesia on the sympathoadrenergic activity and on the myocardial oxygen demand, rather than by any intrinsic property of the volatile anesthetics.

In the current study, halothane administration to the anesthetized animal before and during regional myocardial ischemia dramatically changed the basic state of the VSCC, rendering them less responsive to the effect of ischemia. A significant decrease in the available number of VSCC in the sarcolemma was observed, as shown by [<sup>3</sup>H]isradipine-binding studies. These changes in binding kinetics are identical to those described in experiments that used *in vitro* halothane administration to nonischemic sarcolemmal membranes.<sup>11</sup> It seems, therefore, that the decrease in binding observed is explained by the direct effect of halothane on calcium channel constituents and is more than just a diminished consumption of high-energy stores during cessation of oxygen supply.

The mechanism responsible for the increase in B<sub>max</sub>, *i.e.*, in the available calcium channels, is uncertain in the literature. Reversible changes in channel kinetics produced by short ischemic episodes or during administration of volatile anesthetics may be explained as general conformational changes in the channel structure. During ischemia, the increase in available VSCC is explained by a mechanism of differential unmasking of latent channels in the cell membrane and was related to a methylation process of the membrane phospholipids.<sup>31,32</sup>

Conversely, halothane causes a reduction in the number of available VSCC, probably by indirect alterations in the high-affinity, inactivated state of the channel, thus reducing its favorable binding capacity to the calcium channel blocker.<sup>33,34</sup>

Table 1. Regional Ischemia Experiments: Scatchard Analysis

	Ischemia	Ischemia + Reperfusion	Ischemia + Halothane
n	8	6	7
B <sub>max</sub>			
Control	98.1 ± 15	208.7 ± 32	146.0 ± 31
LAD	181.1 ± 34	287.8 ± 46	100.3 ± 22
K <sub>d</sub>			
Control	0.84 ± 0.10	0.92 ± 0.30	0.93 ± 0.40
LAD	0.99 ± 0.20	0.93 ± 0.30	0.89 ± 0.35

Values are mean ± SEM.

B<sub>max</sub> = maximal number of binding sites (fmol/mg protein units); LAD = left anterior descending artery; K<sub>d</sub> = dissociation constant (nM).

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The current findings support the results of the study of Hoka *et al.*<sup>4</sup> on isolated guinea pig hearts in which 1% halothane was given to reverse changes in cellular calcium content produced by 10 min of ischemia. Halothane significantly decreased <sup>45</sup>Ca<sup>2+</sup> accumulation associated with myocardial ischemia and calcium paradox, signifying a potentially beneficial effect of the anesthetic to an ischemic heart.

The clinical use of volatile anesthetics for their beneficial role in preventing myocardial ischemia- and reperfusion-related injury is controversial, particularly because the anesthetics should be administered before and during potential ischemic conditions. However, as such a condition exists during open heart surgery when using continuous oxygenated warm blood cardioplegia, concomitant administration of volatile anesthetics may be indicated.

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