Volatile Anesthetics Inhibit Dihydropyridine Binding to Malignant Hyperthermia-susceptible and Normal Pig Skeletal Muscle Membranes

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Background: Surface membrane dihydropyridine receptor Ca²⁺ channels may play a role in the response of malignant hyperthermia–susceptible skeletal muscle to volatile anesthetics.

Methods: We determined the effect of halothane, enflurane, and isoflurane on the binding of the Ca²⁺ channel blocker PN200-110 to skeletal muscle membranes isolated from malignant hyperthermia–susceptible and normal pigs.

Results: In the presence of 0.4 mM halothane, the maximal [³H]PN200-110 binding to both normal and malignant hyperthermia membranes was reduced by 37–43% (P < 0.05). There was no difference in the equilibrium constant for the halothane-dependent inhibition of [³H]PN200-110 binding to these two types of membranes. There also was no significant difference among halothane, enflurane, or isoflurane in their ability to inhibit [³H]PN200-110 binding to either normal or malignant hyperthermia membranes.

Conclusions: Volatile anesthetics inhibit the binding of PN200-110 to skeletal muscle membranes by decreasing the number of functionally active dihydropyridine receptor proteins. This inhibition is similar for membranes isolated from both normal and malignant hyperthermia–susceptible muscle, thus providing no evidence for a halothane-induced functional defect in this protein in malignant hyperthermia–susceptible muscle. However, the results of this study also indicate that the mechanism by which volatile anesthetics decrease surface membrane Ca²⁺ currents in skeletal muscle is by reducing the number of functional dihydropyridine receptor Ca²⁺ channels.

(Key words: Anesthesics, volatile; enflurane; halothane; isoflurane. Muscle, skeletal: calcium channels. Pharmacology: dihydropyridines. Hyperthermia: malignant.)

DIHYDROPYRIDINES such as PN200-110 and nitrendipine bind to the α₁ subunit of skeletal muscle T-tubule L-type Ca²⁺ channels in a specific, saturable and reversible manner.¹ The binding of these pharmacologic agents to the L-type Ca²⁺ channel has led to the naming of this protein the dihydropyridine receptor. In skeletal muscle excitation–contraction coupling, depolarization of the skeletal muscle surface membrane activates an asymmetric charge movement in the T-tubule membranes,² which through a yet to be identified mechanism, opens the Ca²⁺-release channels in the sarcoplasmic reticulum.³ Dihydropyridine binding to its T-tubule receptor not only inhibits Ca²⁺ channel activity, but also is associated with the suppression of asymmetric charge movement, and the inhibition of subsequent Ca²⁺ release from the sarcoplasmic reticulum.⁴,⁵

It has been demonstrated in pigs and certain human families that the inherited disorder malignant hyperthermia is almost certainly due to a mutation in the skeletal muscle sarcoplasmic reticulum Ca²⁺-release channel.⁶,⁷ However, several studies suggest that surface membrane Ca²⁺ channels may also play a role in the skeletal muscle response to volatile anesthetics in malignant hyperthermia.⁸,⁹ For example, the anesthetic-induced contracture of malignant hyperthermia–susceptible muscle was reduced by the Ca²⁺ channel blockers nifedipine and diltiazem,⁹,¹⁰ and potentiated by the Ca²⁺ agonist BAY K 8644.¹¹,¹² Furthermore, Lamb et al. have demonstrated that halothane reduced both the Ca²⁺ current and asymmetric charge movement of pig skeletal muscle fibers by approximately 50%.¹² We have previously reported that the binding of Ca²⁺ channel blockers to purified T-tubules is reduced in malignant hyperthermia–susceptible muscle.¹³ Therefore, some of the altered responses of malignant hyperthermia–susceptible muscle to volatile anesthetics may be due to secondary changes in the L-type Ca²⁺ channel resulting from the primary defect in the sar-
cortical reticulum Ca$$^{2+}$$-release channel. In this study, we have investigated whether the decreased binding of Ca$$^{2+}$$ channel blockers to malignant hyperthermia membranes is associated with an altered response of these receptors to volatile anesthetics. Our results demonstrate that while their action on malignant hyperthermia–susceptible and normal membranes is indistinguishable, volatile anesthetics do significantly inhibit ligand binding to L-type Ca$$^{2+}$$ channels in these preparations.

**Methods and Materials**

**Experimental Animals**

All pigs (30–40 kg) were obtained from the University of Minnesota Experimental Farm, where they were part of a swine genetics herd maintained by Rempel and coworkers for studies of the inheritance of the malignant hyperthermia gene. The two breeds of pigs used in these studies were a Pietrain herd that is homozygous for the malignant hyperthermia–susceptibility gene on the basis of its ryr-1 genotype (i.e., cytosine at base pair 1843 determined by the PCR diagnostic procedure of Fuji et al.), and a Yorkshire herd that is homozygous normal (i.e., thymidine at base pair 1843) for the malignant hyperthermia–susceptibility gene on the basis of its ryr-1 genotype. Animals were killed by intravenous administration of sodium thiopental (Biotal) (15 mg/kg) followed by T61 euthanasia solution. Longissimus dorsi muscle was immediately removed and placed in ice. This protocol was approved by the Institutional Animal Care Committee of the University of Minnesota. Halothane was obtained from Halocarbon Laboratories (Hackensack, NJ). Nifedipine was obtained from Sigma Chemical (St. Louis, MO), and [3H]PN200-110 (Isradipine) was obtained from Amersham Life Science (Arlington Heights, IL).

**Membrane Preparation**

A crude membrane preparation that retained most of the muscle surface and sarcoplasmic reticulum membranes was isolated from pig longissimus dorsi muscle. Muscle was homogenized in 10% sucrose, 0.5 mM EDTA, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mm iodoacetamide, 0.5 mg/ml pepstatin, 0.2 mm phenyl methyl sulfonyl fluoride, 0.8 mm benzamidine, 20 mm Tris-maleate buffer (pH 7.0) (5:1 vol: wt of tissue) at 4°C. The homogenate was centrifuged at 2,600 g for 30 min, the supernatant retained, and the pellet rehomogenized in 5 volumes of the above buffer. This second homogenate was centrifuged at 2,600 g for 30 min, after which both supernatant were combined and centrifuged at 140,000 g for 60 min. The resulting pellets were resuspended in 0.6 M KCl, 10% sucrose, 0.1 mm phenyl methyl sulfonyl fluoride, 0.8 mm benzamidine, 50 mm Tris buffer (pH 7.0) and gently agitated for 30 min at 4°C. After centrifugation at 140,000 g for 60 min, the resulting crude membrane pellet was resuspended in 1 ml 10% sucrose, 0.1 mm phenyl methyl sulfonyl fluoride, 0.8 mm benzamidine, 20 mm Tris buffer (pH 7.4), frozen in liquid nitrogen, and stored at −70°C. Protein concentration was determined by the method of Lowry et al.

**Ligand Binding Assays**

Saturated aqueous solutions of anesthetic in Teflon-sealed conical glass vials (Reacti-vials, Pierce Chemical, Rockford, IL) were prepared daily by 2–4-h equilibration at room temperature. Saturated aqueous solutions of anesthetics under our conditions were calculated to be 20 mm halothane, 15 mm isoflurane, and 15 mm enflurane. Anesthetic in the aqueous layer of this vial was added directly to the [3H]PN200-110 binding medium contained in another Reacti-vial (1 ml) by injection through the septum (anesthetic contained in a volume < 5% of the binding medium volume). The actual concentration of anesthetic in the binding media were derived as described previously from the known volume of the Reacti-vials (1 ml), the liquid volume, the remaining gas volume, and the published values for the liquid–gas partition coefficients for these anesthetics. The concentrations of the anesthetics in the liquid phase were determined using the temperature-appropriate distribution coefficients, as described by Nelson and Swen.

[3H]PN200-110 binding to crude muscle membranes (5 μg) in the presence or absence of anesthetic was determined in 0.5 ml 50 mm Tris-Cl buffer (pH 7.4) at 22°C. Nonspecific binding was determined in the presence of (1 μM) nifedipine. Binding was assayed after filtration of the complete sample through Whatman GF/B filters that had been pretreated for 20 min with 0.5% polyethyleneimine. Filters were washed with 20 ml ice-cold 200 mm choline chloride, 20 mm Tris-Cl buffer (pH 7.4), and the [3H]PN200-110 remaining on the filter was determined in a liquid scintillation spectrometer.
Statistical Analysis
All experiments were performed on crude muscle membrane preparations obtained from at least three different animals with each measurement performed in duplicate. Sample means were compared by use of Student’s t test and unless indicated, were considered significantly different at \( P < 0.05 \).

Results
Effect of Halothane on PN200-110 Binding to Muscle Membranes
The membrane preparation used in this study was chosen to reflect the total content of both sarcoplasmic reticulum and surface (i.e., sarcolemma and transverse tubule) membranes present in the original muscle sample. The majority of the dihydropyridine receptor-containing T-tubule membranes are likely not associated with sarcoplasmic reticulum membranes in this preparation as the high salt wash has been shown to disrupt associations between these two membranes.\(^2\)
Thus, any effects of anesthetics on the dihydropyridine receptor are unlikely to be due to anesthetic-induced changes in ryanoide receptor activity. The typical yield of membrane protein from normal and malignant hyperthermia–susceptible muscle were not different (4.1 ± 0.1 mg/g wet weight of muscle).

We have demonstrated previously a decreased level of \[^3\text{H}]\text{nitrendipine binding to purified T-tubule membranes isolated from malignant hyperthermia–susceptible muscle.}^{15}\) In the study reported here, the binding of \[^3\text{H}]\text{PN200-110} \) rather \[^3\text{H}]\text{nitrendipine was examined. PN200-110 has an approximately tenfold greater affinity for the dihydropyridine receptor than does nitrendipine,}^{15}\) which results in a significantly lower nonspecific binding for PN200-110. To examine the effect of the volatile anesthetic halothane on the equilibrium binding of \[^3\text{H}]\text{PN200-110} \) to this crude membrane fraction, it was essential to ensure that the anesthetics were incubated with the muscle membranes in a sealed environment. This was achieved by injecting the desired volume of a saturated aqueous solution of anesthetic into the reaction medium that itself was contained in a 1 ml React-vial scaled with a Teflon gasket.

Binding of \[^3\text{H}]\text{PN200-110} \) to normal muscle membranes in the presence or absence of 0.4 mm halothane (fig. 1) increased rapidly during the first 10 min and reached steady state by 20 min incubation. The average \[^3\text{H}]\text{PN200-110} \) binding to membranes between 20 and 60 min in the presence of halothane was significantly (\( P < 0.005 \)) decreased compared to control binding over this same time period (control 1.97 ± 0.31 and halothane 0.84 ± 0.11 pmol/mg protein; mean ± standard deviation). Thus, the equilibrium binding of \[^3\text{H}]\text{PN200-110} \) to muscle membranes was saturable and was significantly inhibited in the presence of 0.4 mm halothane. Unless indicated, the incubation period in all further experiments was 45 min.

Equilibrium Binding of \[^3\text{H}]\text{PN200-110} \) to Muscle Membranes Isolated from Malignant Hyperthermia–Susceptible and Normal Muscle
Ligand binding to either normal or malignant hyperthermia–susceptible membranes was determined at varying \[^3\text{H}]\text{PN200-110 concentrations (figs. 2A and 2C) and converted to Scatchard plots (figs. 2B and 2D).} \) These data indicate a single binding site for the ligand with a dissociation constant (\( K_d \)) of approximately 0.25 nm that was not significantly different for malignant hyperthermia–susceptible and normal membranes (table 1).

Figure 2 also shows the effect of 0.4 mm halothane on the specific ligand binding to muscle membranes.
at various concentrations of [3H]PN200-110. These data indicate a single ligand binding site, with a reduction in maximal [3H]PN200-110 binding to both normal and malignant hyperthermia–susceptible membranes in the presence of halothane (table 1). The reduction of maximal [3H]PN200-110 binding (37–43%) was similar for malignant hyperthermia–susceptible and normal membranes. There was no significant effect of 0.4 mM halothane on the Kd for [3H]PN200-110. The reduction in maximal [3H]PN200-110 binding with no effect on Kd was true for all halothane concentrations examined (data not shown).

Halothane Dependence of [3H]PN200-110 Binding to Muscle Membranes

The data in figure 2 indicate that binding of [3H]PN200-110 to muscle membranes was inhibited by approximately 40% in the presence of 0.4 mM halothane, which is close to the MAC value for this anesthetic (0.54 mM aqueous concentration).19 To examine the concentration dependence of halothane inhibition of [3H]PN200-110 binding, various concentrations of halothane were added to muscle membranes incubated with either 0.2 nM (fig. 3A) or 2.5 nM (fig. 3B) [3H]PN200-110. These two concentrations were chosen to approximate 1× and 10× the Kd for [3H]PN200-110 binding to muscle membranes so an effect of halothane on either the Kd or maximal [3H]PN200-110 binding would be detectable. The binding of [3H]PN200-110 to both normal and malignant hyperthermia–susceptible membranes was inhibited by similar concentr-

Table 1. Effect of Halothane on the Binding Properties of [3H]PN200-110 to Normal and Malignant Hyperthermia Susceptible Muscle Membranes

<table>
<thead>
<tr>
<th>Condition</th>
<th>Kd (nM)</th>
<th>Bmax (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.22 ± 0.03</td>
<td>6.45 ± 1.05*</td>
</tr>
<tr>
<td>Normal + 0.4 mM halothane</td>
<td>0.19 ± 0.03</td>
<td>3.72 ± 0.23*</td>
</tr>
<tr>
<td>Malignant hyperthermia</td>
<td>0.27 ± 0.05</td>
<td>4.58 ± 0.28†</td>
</tr>
<tr>
<td>Malignant hyperthermia + 0.4 mM halothane</td>
<td>0.41 ± 0.08</td>
<td>2.91 ± 0.29†</td>
</tr>
</tbody>
</table>

Data are derived from figure 2 and reflect mean values ± SE. Means within a pair with common superscript symbols were significantly different.

* P < 0.005.
† P < 0.05.

Fig. 2. Saturation binding of [3H]PN200-110 to normal and malignant hyperthermia–susceptible muscle membranes. Specific binding of [3H]PN200-110 to normal (A) or malignant hyperthermia–susceptible (C) membranes was determined in the presence (filled circles) or absence (open circles) of 0.4 mM halothane. (B) Scatchard analysis of the data in A. (D) Scatchard analysis of the data in C. Data reflect the mean ± standard error for four normal and five malignant hyperthermia–susceptible preparations.
Fig. 3. Dose-dependent inhibition by halothane of \(^{3}^{\text{H}}\)PN200-110 binding to normal and malignant hyperthermia-susceptible muscle membranes. Specific binding of \(^{3}^{\text{H}}\)PN200-110 to normal (open circles) or malignant hyperthermia-susceptible (filled circles) membranes was determined in the presence of either 0.2 nm (A) (n = 4 for both normal and malignant hyperthermia preparations) or 2.5 nm \(^{3}^{\text{H}}\)PN200-110 (C) (n = 3 for both normal and malignant hyperthermia preparations). (B) Hill plot analysis of the data in A. (D) Hill plot analysis of the data in C. Data reflect the mean ± standard error.

Table 2. Effect of \(^{3}^{\text{H}}\)PN200-110 Concentration on the \(K_i\) for the Halothane-Dependent Inhibition of \(^{3}^{\text{H}}\)PN200-110 Binding to Normal and Malignant Hyperthermia Susceptible Muscle Membranes

<table>
<thead>
<tr>
<th></th>
<th>0.2 nm (^{3}^{\text{H}})PN200-110</th>
<th>2.5 nm (^{3}^{\text{H}})PN200-110</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.47 ± 0.08 (n = 4)</td>
<td>0.53 ± 0.29 (n = 4)</td>
</tr>
<tr>
<td>Malignant hyperthermia</td>
<td>0.51 ± 0.07 (n = 3)</td>
<td>0.89 ± 0.19 (n = 3)</td>
</tr>
</tbody>
</table>

Data are derived from figure 3 and reflect mean values ± SE. There were no significant differences between any of these values.

Effect of Different Anesthetics on \(^{3}^{\text{H}}\)PN200-110 Binding to Muscle Membranes

The effect of three different volatile anesthetics, each at a concentration of 0.4 mm, on \(^{3}^{\text{H}}\)PN200-110 binding to muscle membranes was compared at both 0.2 nm and 2.5 nm.

Table 3. Effect of Halothane, Enflurane, and Isoflurane on the Percent Inhibition of \(^{3}^{\text{H}}\)PN200-110 Binding to Normal Muscle Membranes

<table>
<thead>
<tr>
<th></th>
<th>0.2 nm (^{3}^{\text{H}})PN200-110</th>
<th>2.5 nm (^{3}^{\text{H}})PN200-110</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.4 mm</td>
<td>68 ± 6</td>
<td>65 ± 4</td>
</tr>
<tr>
<td>halothane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4 mm</td>
<td>57 ± 6</td>
<td>66 ± 5</td>
</tr>
<tr>
<td>enflurane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4 mm</td>
<td>60 ± 7</td>
<td>61 ± 3</td>
</tr>
<tr>
<td>isoflurane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4 mm</td>
<td>73 ± 9</td>
<td>60 ± 6</td>
</tr>
</tbody>
</table>

Effect of anesthetic on \(^{3}^{\text{H}}\)PN200-110 binding is expressed as percentage of control for three normal and three malignant hyperthermia susceptible skeletal muscle membrane preparations. Data reflect the mean values ± SE.

* The effect of anesthetic on the percent inhibition of specific binding was determined in the presence of either 0.2 nm or 2.5 nm \(^{3}^{\text{H}}\)PN200-110.
and 2.5 nM [3H]PN200-110 (table 3). There was no statistically significant difference among halothane, enflurane, and isoflurane in their ability to inhibit malignant hyperthermia or normal membrane [3H]PN200-110 binding at either concentration of [3H]PN200-110.

Discussion

The goal of this study was to determine whether clinical concentrations of volatile anesthetics differentially affected the binding of an L-type voltage-dependent Ca2+ channel blocker to skeletal muscle membranes isolated from normal and malignant hyperthermia–susceptible pigs. Our data demonstrate that halogenated volatile anesthetics inhibit the binding of the dihydropyridine receptor ligand [3H]PN200-110 to skeletal muscle membranes at clinical concentrations of anesthetic (0.47–0.89 mM halothane, with 1 MAC = 0.54 mM19,20; i.e., 1.2–2.3% halothane). However, there was no difference in the anesthetic sensitivity of normal and malignant hyperthermia–susceptible membranes.

Our data also demonstrate that the effects of volatile anesthetics on T-tubule dihydropyridine binding are different from their previously described stimulatory effect on Ca2+ release from sarcoplasmic reticulum vesicles.18 In sarcoplasmic reticulum membranes containing identical Ca2+-release channel content, there was a significantly greater stimulation of Ca2+ release from malignant hyperthermia–susceptible sarcoplasmic reticulum vesicles than from normal vesicles indicating an inherent abnormality in the malignant hyperthermia–susceptible sarcoplasmic reticulum Ca2+ release channel protein.18

Several different approaches have been used previously to demonstrate that halogenated volatile anesthetics affect L-type Ca2+ channels in cardiac muscle. Thus, Blanck and colleagues have demonstrated that these anesthetics reduced the number of nitrendipine or D600 high affinity binding sites in isolated bovine heart sarcolemma membranes.22–24 It is noteworthy that these authors were unable to demonstrate a consistent depression of nitrendipine binding by isoflurane and enflurane, which contrasts with the results we report here with PN200-110 binding to skeletal muscle membranes.23 This may reflect differences in both the L-type Ca2+ channel isoforms and membrane compositions of these two different muscle types. These studies measuring volatile anesthetic-induced inhibition dihydropyridine binding to cardiac muscle membranes complement a number of other studies demonstrating that halothane caused a significant depression of the maximum rate of rise of the slow action potentials in guinea pig papillary muscle.25–29 Thus, these different studies in cardiac muscle indicate that anesthetic inhibition of dihydropyridine binding correlates with the anesthetic inhibition of L-type Ca2+ channel activity.

In contrast to the several different studies of the effect of volatile anesthetics on cardiac muscle L-type Ca2+ channels, there have been few comparable studies of skeletal muscle. Adnet et al.11 have shown that the magnitude of contracture response of muscle bundles isolated from malignant hyperthermia–susceptible human patients produced by incremental concentrations of halothane, is significantly inhibited by verapamil or nifedipine. Furthermore, Lamb et al.12 have demonstrated that 1% halothane caused an almost 50% reduction in both the Ca2+ current and asymmetric charge movement of pig skeletal muscle fibers. Thus, as observed in cardiac muscle, inhibition of skeletal muscle L-type Ca2+ channel function by halothane correlates with the inhibition of [3H]PN200-110 binding, and occurs at similar concentrations of anesthetic to those reported here.

Our results suggest that the mechanism by which halothane decreases L-type Ca2+ channel activity in intact muscle fibers may be very similar to that which causes the reduction in [3H]PN200-110 binding to the Ca2+ channel dihydropyridine receptor in skeletal muscle membranes. In both cases there is an apparent decrease in the number of functional L-type Ca2+ channel proteins. We are unable to conclude from our data whether halothane has a direct effect on the channel protein itself, or instead interacts with the lipid bilayer that in turn exerts an effect on the channel protein. However, the results reported in this study now allow us to compare the halothane-sensitivity of [3H]PN200-110 binding to skeletal muscle membranes (equilibrium constant for the halothane-dependent inhibition of [3H]PN200-110 binding = 0.47–0.89 mM halothane) with sarcoplasmic reticulum Ca2+ release (50% stimulation at 0.97 mM halothane) and Ca2+-ATPase activity (50% inhibition > 10 mM halothane).18 Thus, at clinical concentrations of halothane (1 MAC = 0.54 mM), decreased L-type Ca2+ channel activity or charge movement would decrease excitation–contraction coupling and thus reduce contractility. However, this would be compensated for by a halothane-dependent stimulation of
sarcoplasmic reticulum Ca\(^{2+}\) release, which would enhance muscle contractility.

We conclude that clinical concentrations of halothane inhibit the binding of the Ca\(^{2+}\) channel blocker PN200-110 to skeletal muscle membranes by decreasing the number of functionally active receptor proteins. Furthermore, this inhibition is similar for membranes isolated from both malignant hyperthermia–susceptible and normal muscle, thus providing no evidence for a functional defect in this protein in malignant hyperthermia–susceptible muscle. The results of this study also indicate that one mechanism by which volatile anesthetics decrease asymmetric charge movement and Ca\(^{2+}\) currents in skeletal muscle is by reducing the number of functional L-type Ca\(^{2+}\) channels. The mechanism by which this reduction in charge movement and Ca\(^{2+}\) currents occurs awaits a fuller understanding of the mechanism of action of volatile anesthetics.

References